DISSERTATION

THE ACQUISITION OF DENDRITIC CELL TOLERANCE DURING MALARIA INFECTION RESULTS IN DIFFERENTIAL T-CELL ACTIVATION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JAMES A. PERRY TITLED THE ACQUISISTION OF DENDRITIC CELL TOLERANCE DURING MALARIA INFECTION RESULTS IN DIFFERENTIAL T-CELL ACTIVATION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE ACQUISITION OF DENDRITIC CELL TOLERANCE DURING MALARIA INFECTION RESULTS IN DIFFERENTIAL T-CELL ACTIVATION

Malaria is caused by intracellular protozoan parasites belonging to the genus *Plasmodia.* These single cell eukaryotes have a complex life cycle requiring both mammalian (and in certain *Plasmodium* species, avian) and mosquito hosts. Clinical malaria in humans and other animals is the result of red blood cell (RBC) infection. Although infection directly destroys erythrocytes, causing anemia, a significant degree of anemia and morbidity is the result of the host immune response. Inflammatory cytokines have been implicated in the pathogenesis of severe malaria anemia (SMA) and cerebral malaria (CM), two diseases that are responsible for most malaria-related morbidity. Therefore, understanding the regulation of host immunity and inflammatory cytokine production during malaria infection will improve our understanding of malaria related illness.

Malaria is one of the greatest medical, social and economical problems facing the majority of the world's population. The consequences of malaria infection for the host immune response continue to be a profound topic of interest and research. This dissertation is focused on several aspects of the host immune response to *Plasmodium yoelii*, a murine malaria parasite. The studies described herein suggest that in situations where host and parasite are well adapted, as in our model using *P. yoelii* and B6/B10D2 mice, malaria

infection guides the immune system to preferentially produce anti-inflammatory cytokines as infection progresses. When anti-inflammatory cytokines are not produced, such as in IL-10 knockout (KO) mice, severe pathology is seen.

Initial studies, as described in chapters 2 and 3 of this dissertation, demonstrated that CD4 T cell responses to an exogenous antigen (Ova) were diminished in infected mice. Subsequent work linked this immune dysfunction to a suppressive/inhibitory population of splenic macrophages. We then showed that the capacity of purified splenic dendritic cells (DCs) to stimulate IL-2 production and T cell proliferation was equivalent to that of DCs isolated from spleens of uninfected mice, and that inhibition of IL-2 production was recapitulated when splenic macrophages from infected mice were added back to cultures of purified DCs. The specific mechanism by which macrophages inhibit IL-2 production has yet to be identified but appears to be independent of IL-10, TGF-β, nitric oxide, PGE_2 and tryptophan catabolism.

When looking further at T cell responses induced by DCs from naïve versus infected mice, we found that while IL-2 levels are comparable, the expression and secretion of inflammatory cytokines varied dramatically. In chapter 4, we show that the cytokine profiles obtained from T cells cultured with DCs from infected mice also varied significantly with stage of infection. Specifically, T cells activated by DCs from mice 3 days post infection (p.i.) produced high levels of IFN-γ, TNF-α and little IL-10, whereas T cells stimulated by DCs from day 17 p.i. mice predominantly produced IL-10 with little accompanying IFN-γ and TNF-α.

We then determined how the phenotype of DCs shapes T cell responses as a function of time post infection. An extensive series of cell sorting experiments indicated that DCs isolated from mice during the acute phase of infection (day 3 p.i.) produced much larger quantities of IL-12p40 and TNF-α in response to innate immune stimuli such as LPS and CpG DNA. As the infection progressed to day 17 p.i. however, purified DCs produced statistically smaller amounts of IL-12p40 and TNF-α while secreting larger amounts of IL-10 as measured both by *in vitro* stimulation assays as well as *ex vivo* mRNA analysis. These data are consistent with the previously described T cell stimulation assays where DCs isolated early during infection activate IFN-γ producing T cells whereas DCs isolated late during infection induce IL-10 producing T cells. Thus malaria infection results in down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines.

In chapter 5 we attempted to determine the mechanism of regulation. We found that IL-10 is necessary for IL-12 downregulation, but not TNF-α. Failure to down-regulate IL-12 in IL-10 KO mice was especially evident *in vivo* following administration of LPS to naïve and infected mice. Again, lack of IL-10 had little effect on the down regulation of TNF-α with *in vivo* LPS stimulation. The disparate regulation of IL-12 and TNF-α is currently being explored and appears independent of NF-kB p50 as well as the MAP kinases p38 and ERK 1/2.

These changes in cytokine production a malaria infection progresses correlates to clinical severity of disease. Downregulation of IL-12 (and IFN-γ indirectly) by IL-10 protects the host from inflammation-induced pathology. Mice lacking IL-10 develop significantly more

hepatic necrosis and greater anemia despite a lower parasite burden compared with wild type mice. Our findings are consistent with epidemiological data from human malaria infections where increased pro- to anti-inflammatory cytokine ratios are correlated with an increased severity of malaria syndromes such as severe malarial anemia, cerebral malaria and placental malaria.

The observation that DCs play a dominant role in guiding the activation of IL-10 producing anti-inflammatory T cells and that IL-10 is responsible for mitigating pathology during murine malaria infection provides a basis for the development of potential therapeutics utilizing this phenomenon. One hypothetical use for such information would be the production of an "anti-disease" vaccine where IL-10 producing DCs would be used to specifically activate an anti-inflammatory adaptive immune response. Clearly, more research will be necessary before such trials become feasible as there is a tight balance between too much inflammation and too little inflammation in the context of malaria infection both in humans and animals, alike.

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List of Abbreviations:

Ag: Antigen
APC: Antigen presenting cell
DC: Dendritic Cell
i.v.: Intravenous
i.p.: Intraperitoneal
IFN-γ: Interferon-gamma
IL-10: Interleukin-10
IL-12: Interleukin-12
KO: knockout
KJ1: Cells surface marker present on DO11.10 transgenic T cells
LPS: Lipopolysaccharide
MHC: Major histocompatability complex
MAPK: Mitogen activator protein-kinase
MyD88: Adaptor molecule associated with TLR signallilng
NF-kB: Nuclear factor-kappa B
Ova: Ovalbumin
p.i.: Post infection
T cell: CD4+ T lymphocyte
TCR: T cell receptor
Tg: Transgenic
TLR: Toll like receptor
TNF-α: Tumor necrosis factor-alpha
WT: Wild type

CHAPTER 1: BACKGROUND AND SIGNIFICANCE-LITERATURE REVIEW

1.1 Plasmodium Historical Perspective

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1.1 PLASMODIUM: HISTORICAL PERSPECTIVE

While the discovery of *Plasmodium* parasites was not made until the late 1800's, descriptions of signs characteristic of malarial disease have been observed in writings dating back to 2700 BC. Until the publication of Luis Pastures' "Germ Theory", scientists postulated that the causative agent of malaria was "bad air". For this reason, malaria received its name— "mala aria" is the translation of "bad air" in Italian. This dogma was discredited in 1880 with the discovery of intra-erythrocytic single celled organisms, *Plasmodia*, within blood samples of affected patients. This discovery was made by the French army surgeon, Charles Louis Alphonse Laveran, at a military base in Constantine, Algeria, who later was awarded the Nobel Prize for his discovery. Less than ten years later, two scientists, Giovanni Batista Grassi and Ronald Ross simultaneously discovered that *Plasmodia* species are vector born parasites being transmitted from host to host by female *Anopheles* mosquitoes. Interestingly, twenty years prior to making the association between malaria and mosquitoes, Ross was the first scientist to demonstrate that a parasite known to infect humans could also be transmitted to mosquitoes--in this case, he was working with the filarial worm, *Wuchereria*, responsible for elephantiasis. Grassi also went on to further characterize the *Plasmodia* lifecycle within their invertebrate mosquito host (Humphreys 2001; Spielman 2001)(NIH NIAID publication #07-7139) .

The first known treatment was learned from native South Americans who found that feeding of the quinine-containing bark of Cinchona trees could be used to reduce fevers associated with malaria. It is important to note that this, now proven use of quinine in the treatment of malaria, was discovered prior to any knowledge of the spread, infectious

etiology or contents of the Cinchona trees. The subsequent use of gin and tonic to prevent severe malarial disease amongst occupying British forces demonstrates that malaria has not only shaped human genetics, but has also influenced human social interactions!

Malaria is one of the most detrimental diseases to plague humans and animals alike. Over 50 percent of the worlds population is at risk of malaria infection (Figure 1.1.1) and it is estimated that this disease occurs in greater than 500 million individuals annually, with death occurring in 1-2 million of these individuals (Murphy and Breman 2001).

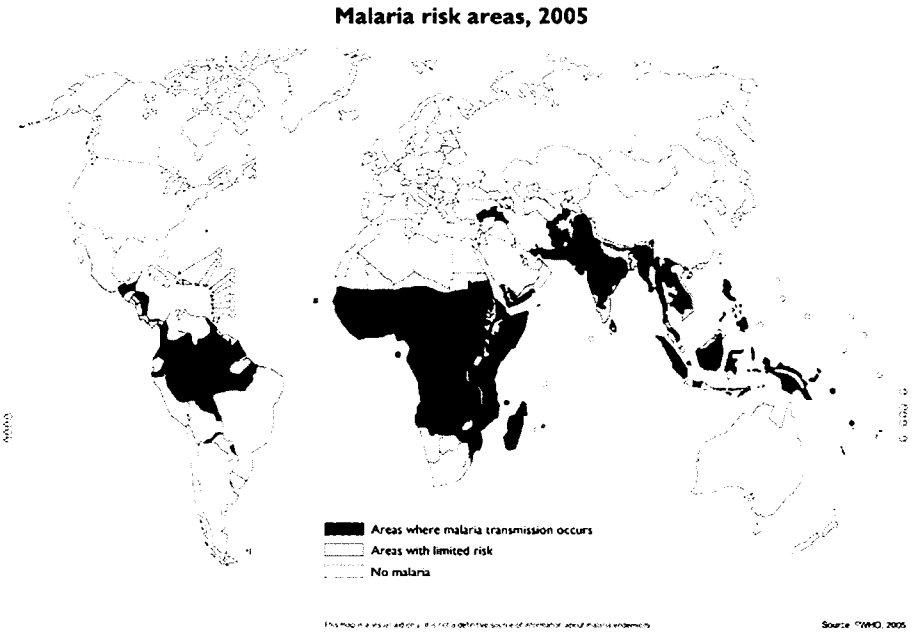


Figure 1.1.1: World-wide distribution of human malaria. Reproduced from *WHO 2005*.

Of the people infected with malaria, children, pregnant women (particularly those who are primiparous) and those experiencing the infection for the first time are most adversely affected (Murphy and Breman 2001). In fact, 40 percent of child admissions to hospitals in sub-Sahara Africa are due to malaria and over 20 percent of childhood deaths in this region can be attributed to this disease—90 percent of the worlds malaria burden resides in Africa (WHO and UNICEF, 2003). Up to 20 percent of women infected with malaria while pregnant show signs of severe anemia which has been strongly associated with subsequent low birth weights in their newborns—the single greatest risk factor associated with infant morbidity and mortality (Murphy and Breman 2001).

With the exception of Mycobacterium tuberculosis, few other infectious diseases have had such a persistent and detrimental impact on the history of human civilization—not to mention remaining obstinate with our attempts to create an effective vaccine against malaria. In past history, malaria has been blamed for the deaths of more soldiers during the civil war than combat itself as well as responsible for the deaths of one percent of workers involved in the construction of the Panama Canal. Perhaps the most detrimental association with malaria is the suspected result that malaria may have been one of the major factors that led to the fall of the Roman Empire in the late 1700's (Humphreys 2001).

Malaria has had a profound influence on the human genome (Miller 1999; Fortin, Stevenson et al. 2002). The best known of these genetic polymorphisms is the selection of the sickle cell gene in African and African-American populations. It is the most

common polymorphism described in the United States affecting 72,000 Americans or 1 in 500 African Americans. This well studied polymorphism is associated with a point mutation leading to the replacement of glutamic acid with valine in the β-chain of hemoglobin found on chromosome 11p15.4. Carrier frequency of this mutation in the hemoglobin gene varies significantly around the world, with higher rates occurring regions of high malaria incidence secondary to natural selective pressures (NCI 2006). While those individuals homozygous for the sickle cell trait suffer significant anemia secondary to erythrocyte fragility, heterozygous individuals have few adverse signs associated with sickle cell disease. It is not clear if individuals with the polymorphism are truly less susceptible to infection with malaria. For example, does the parasite grow less well because it cannot metabolize the hemoglobin or are these heterozygous individuals more likely to combat and clear the infection before pathology results. Several other genetic polymorphisms, including those associated with TNF-α, G6PD, and the Duffy chemokine receptor occur and appear to play a role in transmission and severity of infection. Taken in whole, it is speculated that that malaria has had the most significant evolutionary force with respect to host-pathogen interactions in human history.

In addition to the direct consequences of parasitemia, malaria infection renders its hosts more susceptible to severe disease caused by other pathogens, including Salmonella (Mabey DC 1987), hepatitis B virus (Thursz MR 1995), herpes zoster (Cook 1985), Epstein-Barr virus (Whittle HC 1984), as well as nematodes (Phillips RS 1974). There is also a well established association between the development of Burkitt's lymphoma and malaria infection (Kafuko GW 1970). Malaria infection has also been shown to reduce

the efficacy of vaccines if given during infection (Williamson WA 1978). Little is known, however, about the mechanisms by which *Plasmodium* suppresses the immune system.

The societal strain malaria imposes on productivity, the economy, and growth of communities within endemic regions is devastating. It has been estimated that highly endemic countries suffer a greater than one percentage point reduction in economic growth per year due to illness caused by the parasite (Teklehaimanot 2005). The negative effects of malaria on productivity are especially evident in communities in which agriculture makes up the majority of their gross domestic product. Because planting season often coincides with the spring hatch of mosquitoes, and therefore peak malaria transmission, illness during this time is commonplace thereby resulting in severe ramifications on the producer's ability to plant his crops.

With the emergence of drug resistant strains of *Plasmodia*—chloroquine resistant *P. falciparum* malaria in Africa (Wongsrichanalai, Pickard et al. 2002) and sulfadoxine-pyrimethamine (SP) resistant species in Southeast Asia, Southern China, the Amazon basin, western Oceania, and parts of Africa (Guerin, Olliaro et al. 2002) malaria is encroaching upon regions formerly free or eradicated of these parasites (Baird 2000). According to the Copenhagen Consensus, malaria control is ranked among the four greatest global challenges along with HIV/AIDs, yet, funding towards malaria research is only a fraction of that allocated HIV control and research—such a small fraction in fact, that moneys going toward malaria research represents merely "rounding error" with respect to that allocated to the fight against HIV/AIDs (Copenhagen Consensus 2004; Sacks 2002-Keystone keynote address).

1.2 SPREAD OF PLASMODIUM PARASITES AND LIFE CYCLE

Like many other infectious protozoa such as *Trypanosoma* and *Leishmania*, *Plasmodium* species are transmitted from host to host by insect vectors. In the case of malaria, female *Anopheles* mosquitoes are the definitive host and provide the primary means of spread. The link between *Plasmodium* spread and *Anopheles* mosquitoes, as mentioned earlier, was first made by Major Ronald Ross in August of 1897 which later earned him the Nobel Prize for Medicine in 1902. Once the mechanism of spread was learned, implementation of mosquito control measures greatly reduced the incidence of malaria. The use of bed nets, spraying, and mosquito environment destruction saved thousands of lives during the construction of the Panama Canal. It is estimated that a 90 percent reduction in morbidity and mortality associated with malaria between the years of 1906 and 1909 occurred as direct result of these efforts. (Marshall 1913). Additionally, with the use of mosquito habitat destruction as well as powerful insecticides ultimately led to the eradication of *Anopheles* mosquitoes and malaria itself from the United States by 1951. It is these measures (those focusing on prevention of the spread of infection through mosquito control) even with the advent of new drugs, vaccines and other control measures used against malaria, remain the most effective. This is especially true in the poorest countries where access to drug and hospital resources is not available.

All *Plasmodium spp.* undergo two stages of replication: sexual and asexual. The *Plasmodium* lifecycle "begins" with the ingestion of both male and female gametocytes by a female *Anopheles* mosquito feeding from an infected individual. Male *Anopheles* mosquitoes do not feed on mammalian hosts and therefore do not play a role in transmitting malaria.

Within the mosquito, the haploid male (microgametocytes) and female (macrogametocytes) gametocytes fuse to form a diploid zygote that eventually elongates and differentiates into an ookinete. This stage then penetrates the mosquito's midgut where it developes into an oocyst. The oocyst produces thousands of sporozoites (Despommier 2005). From the midgut, sporozoites enter into the hemocoel of the mosquito where they subsequently migrate to the salivary glands where it undergoes a series of further maturation steps. The final product is an infectious, hepatotropic, sporozoite stage. Upon taking a subsequent blood meal on a naïve host, the mosquito injects these sporozoites intravascularly into the new host. Once circulating in the blood stream, the sporozoites sequester to the liver where they infect hepatocytes. This stage of the *Plasmodia* lifecycle is nonpathogenic. In the liver, the parasites undergo asexual proliferation and develop into exoerythrocytic, merozoite containing, schizonts. Each infected hepatocyte can support the development thousands of merozoites (~30,000 for *P. falciparum* (Good, Xu et al. 2005) that upon its rupture showers the bloodstream with erythrocytotropic merozoite stage. The average time from hepatocyte invasion to lysis is roughly 7 days, however, in the case of *P. vivax* and *P. ovale*, formation of latent, 'hypnozoites' can form and remain quiescent within the hepatocyte for several years (Despommier 2005). Merozoites then invade erythrocytes using specific receptors on the red cell surface. In the red cell, the parasites develop into a trophozoite or ring stage, then undergo rapid replication forming an erythrocytic schizont. This ultimately leads to the lysis of the infected erythrocyte and an exponential release of more infectious merozoites. At the same time, a select number of merozoites undergo gametogenesis and develop into new micro- and macrogametocytes within the intracellular compartment of the erythrocyte. The formation of new gametocytes allows for the completion of the

Plasmodium lifecycle assuming an *Anopheles* mosquito is available to take a blood meal from the infected individual (Figure 1.1.2).

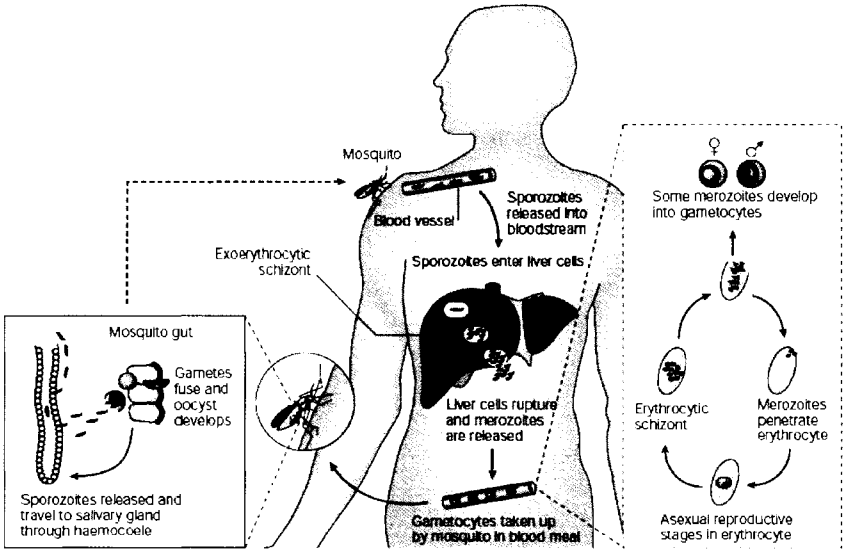


Figure 1.1.2: Plasmodium Lifecycle. From Stevenson 2005

Measures used to reduce the spread of malaria have included mosquito control (destruction of suitable mosquito habitats, employment of widespread insecticide spraying, use of bed nets, etc), prophylactic and post-exposure use of malariacidal drugs, and various vaccination strategies. Because of its complex lifecycle, ability to adapt resistance to antimalarial drugs, and high antigenic variability/shifting makes control of Plasmodium exquisitely difficult. As discovered empirically and through practice, reducing the burden of malaria requires aggressive, multimodal strategies combining mosquito control, drug therapy and vaccination. Alone, these modalities, as has been

observed in the past, have little long term efficacy and often select for more robust parasite strains.

1.3 MALARIA, THE DISEASE

The diseases associated with malaria infection can have clinical manifestations ranging from mild (asymptomatic/flu-like symptoms) to life-threatening disease (respiratory distress/coma). The most severe clinical syndromes often involve multiple organs leading and sepsis-like/systemic inflammatory response (SIRs) disease. The most commonly affected body systems include the neurologic/neurovascular, reproductive and hematopoietic systems. The disease entities associated with these includes cerebral malaria (CM), placental malaria (PM) and severe malarial anemia (SMA), respectively. Severe clinical disease from any one of these syndromes can progress to SIRs (diagnosed when two of the following criteria are met: tachycardia (>90 bpm); tachypnea (>20 resp/min); hypo or hyperthermia (36 > x >38 degrees C); and/or neutropenia or neutrophilia or (4000 cells/ul > x > 12000 cells/ul with a left shift (ACCCM 1992). Such severe manifestations can often be associated with dysregulation of proinflammatory cytokine production, electrolyte imbalances (hyponatremia, hypoglycemia), insulin resistance, metabolic acidosis, coagulopathy, thrombocytopenia and decreased red cell deformability (Clark, Budd et al. 2006). Overall, mortality associated with severe malaria reaches 30 percent with the highest percentage of deaths occurring as a complication of *P. falciparum* induced CM (Ho and White 1999).

While many of these pathologic occurrences can be directly attributed to the parasite; for example, parasite mediated erythrocyte lysis, decreased RBC deformability and microvascular thrombosis secondary to aberrant erythrocyte binding to vascular endothelium, a large component of the pathology associated with severe malaria is immune mediated. Excessive production of inflammatory mediators (TNF-α, IL-12 and IFN-γ), immune complex formation, and anti-erythrocyte antibodies are all likely to contribute to the immune mediated pathology.

1.3.1 Placental Malaria:

Significant maternal and especially fetal morbidity and mortality (75,000-200,000 deaths/year) is attributable to pregnancy-associated malaria (Steketee, Nahlen et al. 2001). Placental malaria is characterized by the aberrant accumulation of *P. falciparum* parasitized erythrocytes bound within the blood spaces of the placenta. Adhesion to vascular beds allows for sequestration of parasites from the splenic and hepatic reticuloendothelial system, thereby preventing their removal from the circulation. *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP-1) binds to chondroitin sulfate A (CSA) on the placental endothelium resulting in vascular occlusion inflammation (Duffy and Fried 2003). *Pf*EMP1 is a large, highly variable protein encoded by the parasites vast set (~60 copies) of *var* genes. Interestingly, only a single copy of these genes is expressed in any given parasite at any given time. The formation of a robust immune response leading to the infiltration of immune cells and local production of pro-inflammatory cytokines, particularly TNF-α, are associated with maternal anemia, maternal mortality, low birth weight and fetal loss (Fried, Muga et al. 1998; Rogerson, Brown et al. 2003; Beeson 2005; Rogerson, Hviid et al. 2007).

1.3.2 Cerebral Malaria:

The most acute and severe manifestation of the malarial syndromes in humans is cerebral malaria (CM), also caused by *P. falciparum*. This disease primarily occurs in 'naïve' individuals experiencing infection for the first time or in children from endemic regions following the waning of maternal antibody protection. The pathogenesis of CM is not completely understood, however, as in placental malaria, sequestration of infected erythrocytes within the vasculature (cerebral vasculature in the case of CM) appears to be an essential component. Interestingly, it appears specific subtypes of *P. falciparum*, depending on the tropism of their *Pf*EMP1, are more likely to cause CM relative to PM and vice versa (Duffy and Fried 2003). One of the earliest clinical pathologic abnormalities observed associated with CM is a disruption in the blood-brain protein barrier and subsequent encephalitis (Hunt, Golenser et al. 2006). Clinical signs of CM in children often include a 1-3 day history of fever, vomiting and anorexia with the main neurologic features being seizures, coma and nonspecific brainstem signs. Encephalography of seizure patients reveals that many of the seizures originate over the temporoparietal regions suggesting an ischemic/hypoxic pathogenesis (Idro 2005). In support of this pathogenesis, seizure patients are often concurrently hypoxemic and hypercapnic, and at autopsy, many of these individuals present with microscopic vascular occlusions within the cerebral vasculature likely exacerbating the hypoxia. Brainstem signs include, but are not limited to, changes in pupillary size and poor pupillary light reflex reactions, aberrations in respiratory rate and pattern, and postural abnormalities (decerebrate, decorticate, or opisthotonic positioning). These nonspecific signs of brainstem disease are most likely secondary effects of increased intracranial pressures

due to hypercapnia rather than generalized hypoglycemia or other electrolyte abnormalities (Idro 2005).

Cerebral malaria occurring in adult individuals is often concomitant with multi-organ disease. Adult patients with CM often present with fever, malaise, joint and generalized body aches, delirium, and anorexia (Idro 2005). Encephalitic disease manifests in upper motor neuron signs such as hyper-reflexia, increased extensor muscle tone and decorticate and decerebrate rigidity. Additionally, infection with *P. falciparum* skews the hemostatic balance in favor of a hypercoagulative state. This imbalance makes these patients prone to thromboembolic disease such as cortical infarcts, dural sinus thrombosis, and/or cerebral venous thrombosis (Krishnan A 2004). While any or all of these signs may be suggestive of CM, they are nonspecific. Other diseases that commonly manifest in such clinical signs include viral encephalitis, acute hepatic necrosis/encephalopathy, or Reye's syndrome. For this reason, diagnosis of CM is often made by the process of elimination. However, the one consistent finding that is relatively specific for CM is the presence of malarial retinopathy (Idro 2005) (Figure 1.3.1).

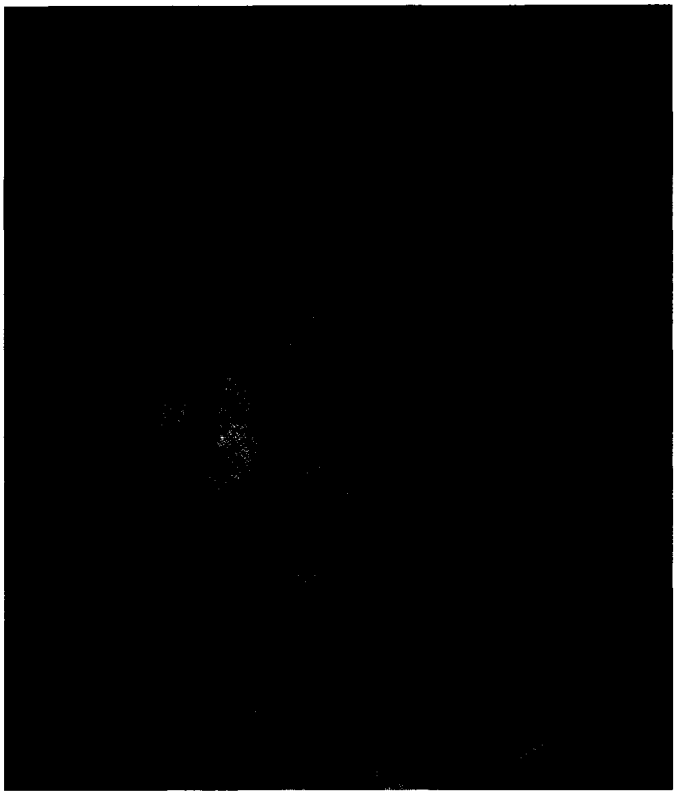


Figure 1.3.1: *P. falciparum* induce retinopathy associated with cerebral malaria. Characteristic lesions include multifocal retinal petichiation and hemorrhage (arrows).

Mortality among children diagnosed with *P. falciparum* CM reaches upwards of 20 percent. Those individuals that survive the acute stage often recover without experiencing subsequent sequelae of CM with the exception of neurocognitive deficits which are being recognized with an increasing frequency in recovered children (Idro 2005). The ultimate cause of death varies among individuals, especially between children and adults. The most common causes of death include respiratory arrest due to brainstem dysfunction (as a result of severe acidosis or transtentoral herniation of the brainstem) and cerebral edema in children with CM, and pulmonary edema and renal failure in adults.

A consistent histopathologic finding associated with *P. falciparum*, particularly in cases of CM, is marginal sequestration of both parasitized and uninfected erythrocytes to the vascular endothelium within the cerebrum (Figure 1.3.2). This is a common feature seen in both adults and children, alike. While the mechanism of sequestration and adhesion to the cerebral vascular endothelium is not completely understood, several logical hypotheses have been proposed and studied. The first of these includes upregulated expression of adhesion molecules such as CD36, P-selectin, vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on the surface of the cerebral endothelial cells (Ho and White 1999).

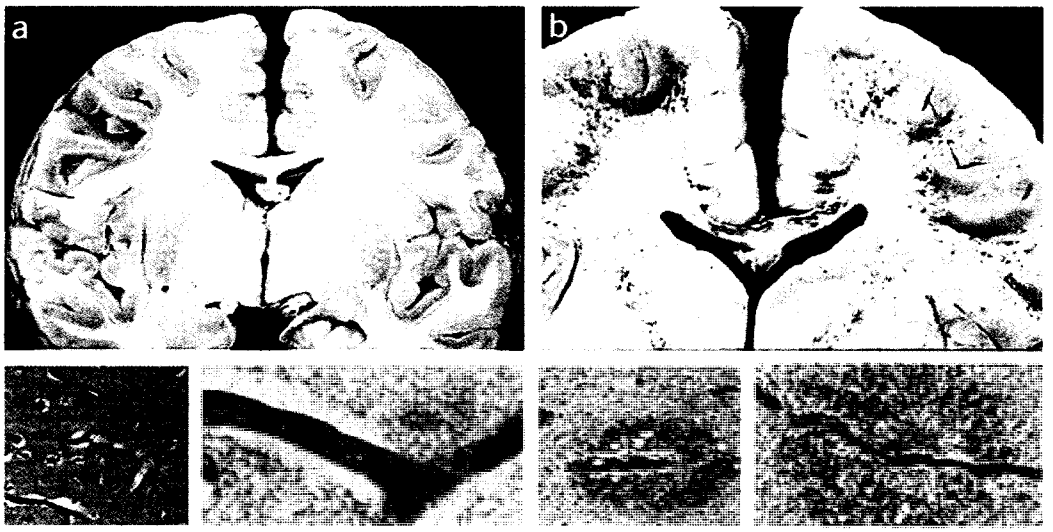


Figure 1.3.2: Pathological features in patients who died of clinically defined cerebral malaria. (**a**) Example of sequestration. Fresh coronal section of brain is swollen and slightly gray in color, and has no visible hemorrhages. Bottom photomicrographs of cortical vessels containing pRBCs. (**b**) Pattern of sequestration and microvascular pathology. Fixed coronal section of brain is swollen petechial hemorrhages in the cortical white matter. Bottom photomicrographs of cortex showing hemorrhage and necrosis surrounding a parasitized vessel.

Recent studies suggest that binding and sequestration of erythrocytes to cerebral vascular endothelium directly affects the selective permeability of the blood brain barrier. One such study showed that binding of infected erythrocytes to endothelial ICAM-1 results in the activation of intracellular signaling cascades ultimately leading to disruption of intercellular junctions within the cerebral microvasculature (Adams, Brown et al. 2002). Pro-inflammatory cytokines and other mediators also function to selectively increase vascular permeability and likely play a role in this process during malaria infection. The consequences of such changes in intercellular junctions have yet to be definitively determined, but it is likely that this leads to increased leakage of plasma proteins into the extravascular space raising the oncotic pressure thereby drawing fluid into the CNS compartment. Abnormal amounts of fluid within the confinements of this region is defined as cerebral edema, and can contribute to seizures, coma and other signs associated with CM and generalized encephalopathy.

Control of infection is by far the best method of limiting deaths due to CM. However, when the disease does occur, treatment is often focused on correction of inflammatory, metabolic and fluid abnormalities followed by antimalarial therapy. Because most deaths occur within the 24 hours of presentation, a time frame too short for antimalarials to take effect, immediate supportive therapy is critical to improving clinical outcome.

1.3.3 Severe Malarial Anemia:

While the development of CM often results in a poorer prognosis than other malarial syndromes, severe malarial anemia (SMA), because of its relatively high incidence, is arguably the most frequent life threatening complication of malaria infection (Evans,

Hansen et al. 2006). This condition is associated with greater than half of all malaria-related morbidity and mortality in African children under the age of 5 years old. In these children, SMA primarily affects them between the ages of six months (associated with the waning of maternal antibodies) to three years, with the mean occurring at 1.8 years of age. This is in contrast to the median age of 3.6 years with respect to CM (Murphy and Breman 2001). The underlying mechanism of this age difference has yet to be definitively resolved but may be explaned as a result of differences in immune status as well as differences in parasite strains that cause SMA verses CM (Sanchez, McWilliams et al. 2007). Like CM, SMA often manifests as an acute syndrome as noted by Lackritz et al. who found that greater than 50 percent of SMA deaths occur within 24 hours post hospitalization that also likely contains a significant immune mediated component (Lackritz EM 1997).

Severe malarial anemia is defined by a hemoglobin concentration or hematocrit of less than 5g/dl or 15%, respectively, with a parasitemia of greater than 10,000 parasites/ul and a normocytic blood film (WHO). As stated above, it is most commonly seen in young children but pregnant women in malaria endemic/high transmission regions and naïve individuals are also highly susceptible (Greenwood 1997). Both in humans and in murine models, extravascular hemolysis within the splenic and hepatic reticuloendothelial system is the primary mode of RBC destruction, with the exception of a rare syndrome referred to as "Blackwater Fever". This syndrome has been associated with treatment with quinine and is characterized by severe intravascular hemolysis and associated hemoglobinuria resulting in severe anemia and acute renal failure (Ghosh 2007). Again, as with CM, SMA is most commonly associated with *P.*

falciparum infection, and the pathogenesis of the anemia likely contains both parasite mediated and immune mediated components. Severe anemia can also occur as a result of *P. vivax* and *P. ovale* infection, however this is much less common since these species of human malaria primarily infect immature RBCs (reticulocytes) and thereby rarely induces the destruction of mature RBCs (normocytes) which is necessary for the manifestation of acute severe anemia (Galinski, Medina et al. 1992).

Anemia in humans and animals can occur as the result of three broad mechanisms: erythrocyte loss (bleeding-internally and/or externally), erythrocyte destruction (intravascular and/or extravascular), and/or decreased erythrocyte production/dyserythropoiesis (ineffective erythropoiesis). *Plasmodia* cause direct erythrocyte destruction during schizogony, when the parasite lyses the red blood cell in order to free merozoites. Antibody and complement also destroy infected red blood cells by lysis or opsonization. Other, less well understood mechanisms of erythrocyte destruction include changes in membrane stability and/or decreased deformability secondary to oxidative damage or alterations in erythrocyte permeability leading to osmotic lysis (Wagner, Andemariam et al. 2003). As mentioned previously, treatment with quinine (i.e. Blackwater fever) as well as mefloquine has also been associated with intravascular hemolysis (Price, van Vugt et al. 1999). Decreased deformability of red cells may lead to increased clearance by the spleen likely secondary to delayed transit through the sinusoids. One study looking at adult patients with severe *P. falciparum* malaria, the degree of reduced red cell deformability as demonstrated by laser diffraction under low shear stress correlated with the severity of clinical signs and prognosis (Dondorp, Angus et al. 1997; Dondorp, Kager et al. 2000). The causes of the decreased RBC deformability

during malaria infection are not known, however, it is likely at least in part mediated by factors within the serum of infected patients. This is based on experiments showing that when RBCs from naïve individuals are cultured with plasma from patients with severe malaria, they showed reduced deformability (Dondorp, Angus et al. 1999). These factors may include, but are not limited to, compliment and antibodies against normal RBC antigens.

Malaria infection can result in massive red cell turn-over even in the face of low parasitemia. Evans *et al.* found that in Balb/c mice infected with *P. berghei*, nearly the entire animal's RBC compartment is turned over within a week of infection. This is compared to a normal erythrocyte lifespan of 35-45 days in a mouse. Interestingly, this group also found that RBCs transferred from animals with severe malarial anemia showed no decrease in survival in naïve recipients relative to transferred naïve red cells. By contrast, the life span of naïve RBCs transferred to infected and then drug cured mice is significantly decreased suggesting that significant RBC destruction is mediated by host factors rather than directly mediated by the parasite (Evans, Hansen et al. 2006). In their system, the peak parasitemia to peak anemia (maximum change in hemoglobin) was significantly lower in semi-immune mice relative to naïve mice, where semi immune mice are those that have been repeatedly infected then drug cured.

The reticulocyte response associated with severe malarial anemia was similar to that in response to phenylhydrazine (PHZ) induced anemia suggesting that erythropoietic responses are fully functional in these mice. They also found that the majority of the red cell destruction occurs via extravascular clearance versus intravascular hemolysis as seen

associated with PHZ treatment. However, SMA animals took several days longer to recapitulate their Hct relative to PHZ treated animals which could be the result of continued RBC destruction.

The studies by Evans *et al.* suggest that the majority of RBC lysis is a result of the surrounding milieu during infection rather than directly mediated by the parasite itself. Also based on comparisons of regenerative responses between SMA and PHZ treated mice, the primarily mechanism of anemia is likely RBC destruction rather than lack of regeneration. As suggested by several other recent studies, the production of pro-inflammatory cytokines and the associated activation of innate and adaptive immune cells likely plays a major role in this 'immune mediated' RBC destruction. The role of pro- versus anti-inflammatory cytokines in this process will be further addressed in detail in subsequent sections of this dissertation.

Contrary to the findings just described (Evans, Hansen et al. 2006) there is growing evidence using other models, that the severe anemia associated with malaria infection is not only the result of parasite and immune mediated RBC lysis, but also due to diminished regenerative responses within the bone marrow and extramedullary compartments of secondary hematopoietic tissues. This is based on both experimental evidence as well as observations that most acute malarial anemias are relatively non-regenerative (normocytic/normochromic) in the face of ongoing hemolysis (Newton CR 1997; Roberts DJ 2005). This is often in the face of acutely hypercellular bone marrows with erythroid hyperplasia (Wickramasinghe and Abdalla 2000) which would suggest that there is targeted destruction of immature erythrocytes as they leave the bone marrow

compartment. The dichotomy of a normocytic/normochromic anemia in the face of erythroid hyperplasia within the bone marrow could also be the result of dyserythropoiesis (Abdalla S 2004).

Anemia associated with chronic malaria infection, however, is often characterized by erythroid hypoplasia with dyserythropoiesis and disturbances in iron metabolism. The observations of decreased erythropoiesis during chronic, often occult, malaria infection were made over half a century ago when it was noted that parasitemic individuals were relatively reticulocytopenic compared to anemic individuals that have cleared the infection (Vryonis 1939). A more recent study showed chronically infected and anemic individuals, in addition to their peripheral reticulocytopenia, had significant morphologic abnormalities associated with their erythroid precursors, indicative of dyserythropoiesis. These abnormalities included fragmentation, basophilic stippling, cytoplasmic vacuolization, nuclear fragmentation, and intercellular bridging (Abdalla S 1990). Interestingly, in bone marrow sections from children who died of SMA, there was a correlation between the amount of the malaria derived byproduct of hemoglobin, hemozoin (present within erythroid precursors and macrophages) and the number of abnormal erythroid cells in the section (Casals-Pascual, Kai et al. 2006). This finding is especially important in the face of new findings that show hemazoin can induce the production of cytokines known to inhibit hematopoiesis (Schofield, Hewitt et al. 2002).

Proinflammatory cytokines such a TNF-α and IFN-γ which are produced in abundant amounts during acute malaria infection have been associated with inhibition of erythropoietic responses (Means and Krantz 1992). However, Yap et al. failed to see an

improvement in the erythroid response in *P. berghei* infected mice in which TNF-α and IFN-γ were neutralized *in vitro* (Yap and Stevenson 1994). In addition to studies looking at the effects of TNF-α on erythropoiesis alone, several others have added to these by showing that high TNF-α to the anti-inflammatory cytokine, interleukin-10 (IL-10) ratios in the serum of infected children correlates with severe anemia in these individuals (Kurtzhals, Adabayeri et al. 1998; Othoro, Lal et al. 1999). In support of the role of IL-10 in the protection against anemia, Linke et al found that *P. chabaudi* infected IL-10 KO mice had more severe anemia presumably as a result of decreased erythropoiesis (Linke, Kuhn et al. 1996). The effects of the lack of IL-10 are reversed following TNF-α blockade (Li, Sanni et al. 2003). An alternative hypothesis is that the absence of IL-10 may lead to more severe anemia during the acute phase of malaria infection secondary to an increased immune mediated RBC destruction due to excessive pro-inflammatory cytokine production as well as decreased erythropoiesis. In addition to IL-10, the anti-inflammatory cytokine TGF-β has also been implicated in protection from SMA (Li, Sanni et al. 2003). To date no studies have described a specific mechanism of action for TGF-β. The role of other inflammatory cytokines in malarial anemia is less well understood. High IL-12, the primary mediator of IFN-γ production in T_H1 T cells and NK cells, levels have been associated with increased, decreased or no effect on anemia depending on the report (Chaiyaroj, Rutta et al. 2004; Lyke, Burges et al. 2004). Further studies are necessary to delineate the specific role of IL-12 in malarial induced anemia.

Few diseases or syndromes recapitulate the pathogenesis of malarial anemia in such a way as malaria with its ability to cause decreased functional erythropoiesis in the face of

red cell destruction. A comparison can be made to patients infected with bone marrow suppressing viruses, such as parvovirus B19, who have an underlying anemia such as sickle cell disease. In cases such as these, acute suppression of erythropoiesis in the face of hemolytic anemia has a severely detrimental effect on the patient (Pagliuca A 1993).

As one can see, the pathogenesis of cerebral malaria, placental malaria, and severe malarial anemia associated with *Plasmodium* infection is very complex and involves both parasite induced damage to host tissues, as well as immune mediated pathology following perhaps too robust an activation of the immune system. In Section 1.5 of this introduction, the cellular and molecular biology of the immune system and its role during malaria infection will be discussed in detail.

1.4 PLASMODIUM SPECIES:

While greater the 400 species of *Plasmodia* have been described, malaria disease in humans is often caused by one of four species of parasites within the *Plasmodium* genus. These include *P. vivax, P. malariae, P. falciparum*, and *P. ovale*, with *P. falciparum* being responsible for the majority of severe disease and death observed in sub-Saharan Africa and the tropics. *Plasmodium vivax* infection, while rarely fatal, is also responsible for significant morbidity—primarily anemia and fever, in parts of Asia and South America. Pathology associated with *P. malariae* and *P. ovale* infection is rarely observed in immunocompetent hosts.

While no murine malaria model exactly recapitulates the pathogenesis of human malaria, several murine specific *Plasmodium* species are used in malaria research. As a whole, these

mouse models have been an invaluable resource in studying specific components of human malaria such as resistance and immunity to infection as well as improving our ability to treat and prevent this disease in human populations. The four most commonly used species of Plasmodia to study malaria in mice are *P. chabaudi, P. berghei, P. yoelii,* and *P. vinckei.*

The majority of these parasite-mouse models demonstrate an acute malaria infection with parasitemias often exceeding 20%, which is in contrast to severe malaria in humans where acute malaria frequently occurs with a lower parasitemia. Additionally, most murine infections lead to sterile immunity following natural clearance of the primary infection, whereas repeated frequent infections are necessary to induce an immune state in humans.

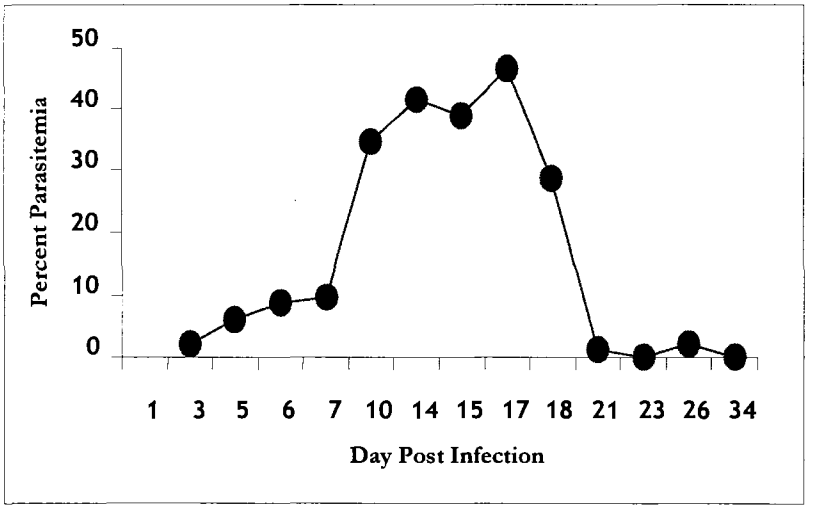


Figure 1.3.1: Graph showing average parasitemia relative to time post inocculation with 10^6 pRBCs i.p. associated with non lethal *P. yoelii* 17X in C57B/6 mice. This figure was generated from greater than three experiments in our laboratory.

Plasmodium berghei, because of its ability to induce clinical features similar to cerebral malaria (CM) in humans, has been used primarily to model the pathophysiology of malaria induced encephalopathy. In susceptible strains of mice, *Plasmodium berghei* ANKA produces pathologic features similar to that of CM in humans that are dependent on pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), lymphotoxin-α (LT-α), interferon-γ (IFN-γ), which are thought to play an important role in *P. falciparum* induced CM (Grau, Piguet et al. 1986; Grau, Fajardo et al. 1987; Grau, Heremans et al. 1989; Grau, Taylor et al. 1989; Engwerda, Mynott et al. 2002).

Infection in susceptible mice with *P. yoelii*, like *P. chabaudi* and *P. vinckei*, exhibits other features of malarial disease in humans such as severe anemia (Clark IA 1988; Cross and Langhorne 1998; Mohan K 1998). The exact involvement of pro-inflammatory cytokines in these pathogenic processes is not clear. *Plasmodium yoelii* 17XNL is non lethal for B6 and B10 background mice, but is variably lethal for BALB/c mice. This species and strain of *Plasmodium* has been used to extensively study immune mechanisms and pathogenesis of malaria infection and is the primary strain of parasite used in the studies described in this dissertation. *Plasmodium yoelii* shares many genetic similarities to *P. falciparum*. Both genomes contain 14 chromosomes rich in G + C DNA (20 percent), with the number of genes encoded by *P. yoelii* and *P. falciparum* are 5,875 and 5,268 respectively (Carlton, Angiuoli et al. 2002). Many of the candidate vaccine antigens encoded by the genes identified in *P. falciparum* are also present in *P. yoelii*, including (MSP-1, AMA-1, RAP-1 and RAP-2). Specifically, the usefulness of this model has been exemplified by the availability of a recombinant version of merizoite surface protein-1 (MSP-1) which has improved the ability of researches to study antigen specific T and B

cell responses during infection. Utilization of this protein has also allowed for the identification of the primary antigen presenting cells responsible for activation of such adaptive cellular and humoral immune responses.

Unlike *P. falciparum* however, *P. yoelii* infections in mice do not lead to signs associated with CM. Rather, *P. yoelii* has been primarily employed to study immune responses and severe anemia associated with malaria infection. Another difference between *P. falciparum* and *P. yoelii* is that non-lethal strains of *P. yoelii* have a tropism for immature red blood cells (reticulocytes). In this regard, *P. yoelii* resembles the human parasite *P. vivax* (Chotivanich, Udomsangpetch et al. 2000; Swardson-Olver, Dawson et al. 2002).

Figure 1.3.2: Plasmodium species that cause malaria. Reproduced from *Stevenson et al. 2004*

In humans

- *Plasmodium falciparum*: causes the most severe form of malaria and can be fatal. Can cause chronic infections (up to 2–3 years), but does not form hypnozoites (dormant stages that persist in hepatocytes) and does not relapse.
- *Plasmodium vivax*: a major cause of clinical malaria, but is rarely fatal. Distribution is restricted by the absence of Duffy antigen (which determines entry into red blood cells) in African populations. This parasite forms hypnozoites and might relapse many years after apparent cure.
- *Plasmodium malariae*: infrequent cause of clinical malaria, especially in Africa. Untreated infections can persist as low-grade parasitaemia for several decades.
- *Plasmodium ovale*: infrequent cause of mild–moderate clinical malaria, but might be found in mixed infections with other species. Forms hypnozoites and might relapse.

In mice

- *Plasmodium chabaudi* (*P. chabaudi chabaudi* AS and *P. chabaudi adami*): used to study immune mechanisms and immunoregulation by cytokines, to identify susceptibility loci and to study the immune basis of pathology. *P. chabaudi chabaudi* AS causes non-lethal infection in resistant mouse strains and lethal infection in susceptible mouse strains. *P. chabaudi adami* causes a mild, non-lethal infection.
- *Plasmodium berghei* (*P. berghei* ANKA and *P. berghei* K173): widely used to study pathogenesis. *P. berghei* ANKA serves as a model of experimental cerebral malaria (ECM); there is genetic variation in the development of ECM between inbred mouse strains, which correlates with the production of pro-inflammatory cytokines.
- *Plasmodium yoelii* (*P. yoelii* 17XL, *P. yoelii* 17XNL and *P. yoelii* YM): used to study immune mechanisms and pathogenesis, including ECM, as recombinant merozoite surface protein 1 (MSP1) is available. *P. yoelii* 17XL is widely used to identify vaccine-induced immune responses.
- *Plasmodium vinckei*: *P. vinckei vinckei*, which causes a lethal infection, is used to study pathogenesis and for chemotherapy studies; *P. vinckei petteri*, which causes a non-lethal infection, is used to study immune mechanisms.

In addition to the mammalian *Plasmodium* species (Figure 1.3.2), avian malaria has been associated with severe mortality in wild birds, fowl and penguins. Our understanding and ability to control infections in the wild, as with human malaria, needs significant improvement.

1.5 IMMUNITY TO MALARIA:

Malaria poses a unique challenge for the mammalian immune system, and immunity to *Plasmodia* is often both stage and species specific. Because of *Plasmodium's* complex life cycle in addition to antigenic variation, it is critical for all 'arms' of the immune system to function properly in order to successfully eliminate the infection. It is highly important to do this without inducing excessive immune mediated pathology. The specific arms just mentioned include the innate immune system, the cellular and humoral components of the adaptive immune system as well as the regulatory arm. The absence of any of these systems results in a detrimental phenotype. Before we can explore in detail the mechanisms by which malaria species affect the host's immune system, it is necessary to review the pertinent aspects of the mammalian immune system in the context of malaria infection.

1.5.1 INNATE IMMUNE SYSTEM:

The innate immune system is the body's front line defense against pathogens. This complex system is comprised of a number of specific cell types with natural killer (NK) cells, γδ T cells, macrophages and dendritic cells (DCs) receiving the most attention with respect to malaria infection. In general, NK and γδ T cells are associated with cytotoxic

activities and inflammatory cytokine production; macrophages are primarily involved in receptor (i.e. complement and Fc) mediated uptake of infected cells and pathogens themselves; and DCs function as professional antigen presenting cells thereby coordinating the subsequent adaptive and regulatory immune response (Stevenson and Riley 2004).

NK cells and γδ T cells

NK and γδ T cells are both responsible for early production of the pro-inflammatory cytokines IFN-γ and TNF-α allowing for subsequent control of parasitemia during non-lethal *P. yoelii* infection, and this innate production of IFN-γ was required for the initiation of a protective T_H1 response (Chaudhury 2000). Interestingly, this innate production of IFN-γ by NK cells was not observed during infection with the lethal strain of *P. yoelii*, *P yoelii* XL, or *P. berghei* infection which may be a correlate as to why these infections are either lethal or more chronic, respectively (Chaudhury 2000). In another study looking at NK cell function during *P. chabaudi* infection, it was observed that early NK cell derived IFN-γ played a beneficial role in the clearance of blood stage parasitemia (Mohan, Moulin et al. 1997). γδ T cell activity has been implicated in immunity to *P. yoelii* sporozoites (Tsuji, Mombaerts et al. 1994) Natural killer cells also aid in the control acute parasitemia in wild type mice and chronic parasitemia in a B cell deficient model of *P. Chabaudi* infection (Kopacz and Kumar 1999; Seixas and Langhorne 1999).

Regardless of the innate immune cells involved during acute malaria infection, ultimately, IFN-γ production is of utmost importance in early parasite control as IFN-γ knockout mice succumb to acute infections (Figure 1.5.1) (Urban 2005). One of the best characterized functions of IFN-γ is associated with the activation of macrophages thereby improving their microbial killing capabilities (respiratory burst capacity, phagocytic activity, NO and further IL-12 production (Trinchieri 1995; Boehm, Klamp et al. 1997). This activation is associated with increased clearance of intraeythrocytic and free merizoites (Stevenson and Riley 2004). Based on the necessity of early IFN-γ production, it is imperative to understand how IFN-γ production is regulated. Based on immunologic studies in the context of other infectious disease models, as well as stimulation with pathogen associated molecules/adjuvants, it appears that interleukin-12 (IL-12), previously described as NK cell growth factor and activator (Kobayashi M 1989), plays a major role in stimulating the production of IFN-γ from NK cells as well as αβ T cells of the adaptive immune system. Interleukin-12 is a heterodimeric protein consisting of a p35 subunit (α chain) and a p40 subunit (β chain) to form biologically active IL-12p70. The IL-12p35 subunit is constitutively secreted in most cell types whereas the IL-12p40 subunit is restricted to those cells capable of secreting biologically active IL-12p70, such as dendritic cells (D'Andrea A 1992; Trinchieri, Rengaraju et al. 1993). The transcriptional and translational control of these subunits is still under investigation, but it appears that IL-12p35 and IL-12p40 are regulated by distinct mechanisms (Carra, Gerosa et al. 2000).

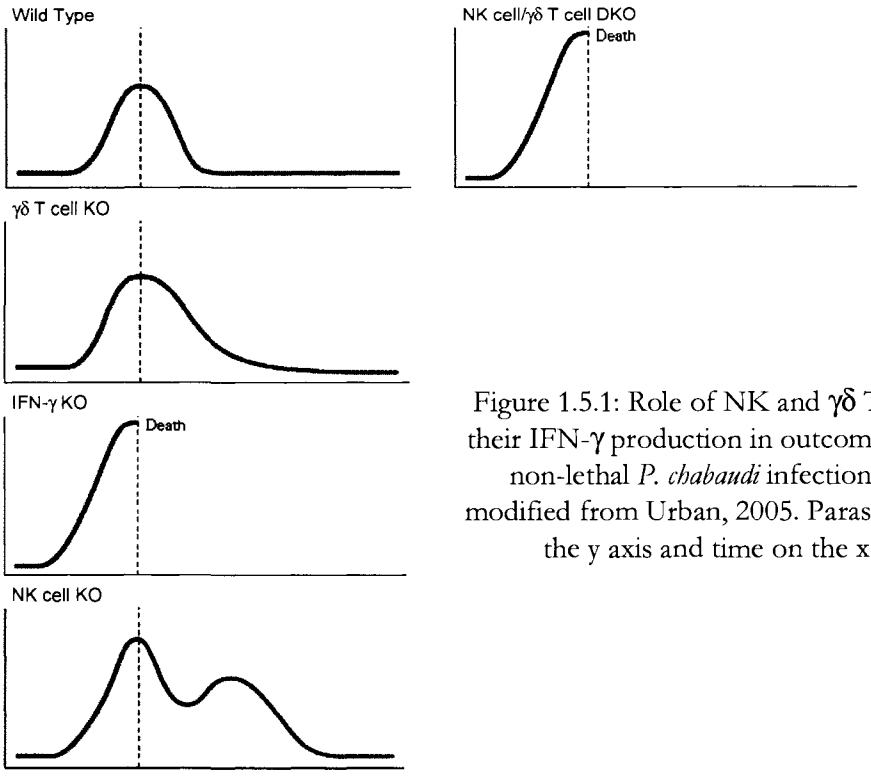


Figure 1.5.1: Role of NK and γδ T cells, and their IFN-γ production in outcome following non-lethal *P. chabaudi* infection. Figure modified from Urban, 2005. Parasitemia is on the y axis and time on the x axis.

Originally thought to be the product of macrophages (Trinchieri 1995), it is becoming more apparent that dendritic cells (DCs) produce the majority of IL-12p70 *in vivo*. This makes biological sense since DCs are especially important in bridging the innate immune response with that of the adaptive and regulatory immune systems through their professional antigen presenting capabilities, and IL-12 plays a role within each of these systems in the face of infection, including malaria.

Dendritic Cells

Until recently, very few studies have addressed the role of DCs in malaria infection. With the emergence of studies pertaining to this subject, we are beginning to gain a better understanding of the function of these cells in response to malaria. However, prior to going into depth about their role specific to malaria infection, as these cells are the

primary focus of the works described herein, it is necessary to provide general background on their basic cellular biology.

Dendritic Cell Subsets:

Accumulating evidence suggests that DCs are the most potent antigen presenting cells within the immune system. In the mouse, there are at least five distinct DC subsets based primarily on surface marker phenotype. While all murine DC subsets express CD11c, they vary in expression of CD4, CD8, CD11b and DEC205. The five subsets include: $CD11b^{+}CD8^{-}CD4^{-}DEC205^{-}$, $CD11b^{-}CD8^{+}CD4^{-}DEC205^{+}$, $CD11b^{+}CD8^{-}CD4^{+}DEC205^{-}$, $CD11b^{+}CD4^{-}CD8^{lo}DEC205^{hi}$ and $CD11b^{+}CD4^{-}CD8^{-}DEC205^{+}$. These various subsets populate tissues differentially, as well as respond to stimuli in a subset specific manner. For example, $CD11c^{+}CD11b^{+}CD4^{+}$ DCs are most numerous in the spleen, and relatively sparse in the thymus and draining lymph nodes, whereas $CD11c^{+}CD11b^{+}CD8^{+}DEC205^{hi}$ cells are only present in the thymus. (Figure 1.5.2A) (Shortman and Liu 2002). For the purposes of the studies described here in, we have either focused on $CD11c^{+}$ DCs as a whole or sorted $CD11c^{+}$ DC based on their expression of CD8 and CD11b allowing for the differentiation of lymphoid versus myeloid, respectively.

A

	'Lymphoid' DCs CD4+CD8α+ CD205+CD11b-	'Myeloid' DCs CD4+CD8- CD205-CD11b+	'Myeloid' DCs CD4-CD8- CD205-CD11b+	'Myeloid' DCs CD4-CD8- CD205+CD11b+	'Langerhans' DCs CD4-CD8+ CD205+CD11b+
Percentage total DCs in					
Spleen	23	56	19	< 4	< 1
Thymus	70				
Mesenteric lymph nodes	19	4	37	26	< 4
Skin-draining lymph nodes	17	4	17	20	33

B

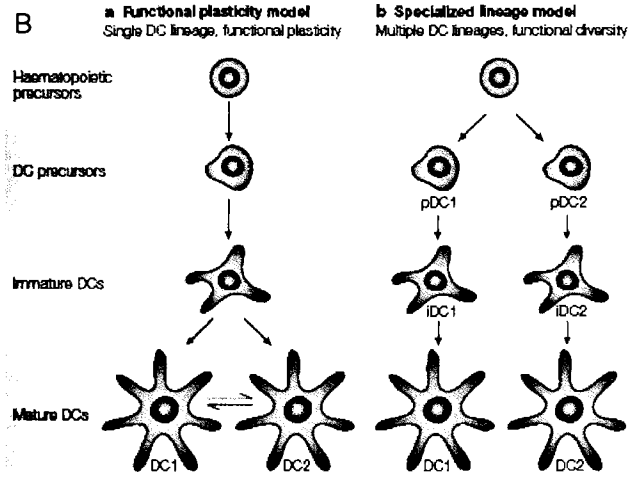


Figure 1.5.2: A) Tissue specific distribution of DC subsets in mice. B) Proposed models of DC origin and maturation. From Shortman et al. 2002

Originally, it was hypothesized that the different DC subsets were derived from distinct precursor cells such as "myeloid" versus "lymphoid" progenitors. However, recent work has led to the creation of a new model suggesting plasticity within and between DC subsets, depending upon the stimuli to which they are exposed. In support of this more current model, d'Ostiani *et al.* found that the unicellular yeast form of *Candida albicans* stimulates IL-12 production in DCs and priming of T_H1 associated cytokines in T cells whereas the hyphal stage of *C. albicans* specifically inhibited IL-12 production and the T_H1 response while stimulating the production of IL-4, a T_H2 cytokine in T cells stimulated by similarly derived DCs exposed to hyphae (d'Ostiani, Del Sero et al. 2000). Experiments such as these suggest a "functional plasticity model" of DC development rather than the previously hypothesized "specialized lineage model" where specific DC

subsets arose from their respective progenitors. This "specialized lineage model" (Figure 1.5.2B) resulted in the myeloid and lymphoid nomenclature that is still used today, but no longer implies the hematopoietic origin of cells that phenotypically fall within one of these categories (Shortman and Liu 2002).

In reality, DCs probably reach their specific subset phenotype via a combination of these two models. For example, myeloid precursors favor the development of a specific subset under steady state conditions, but these cells can shift to a different subset if stimuli (cytokines or microbial products) warrant such a shift. Regardless of the DC subset, it is apparent that all DCs are capable of recognizing and responding to the presence of specific pathogens as well as presenting antigen in the context of MHC on their surface. With the emergence of new knowledge of innate pathogen recognition and the importance of DCs in linking the innate and adaptive immune responses, we are beginning to understand the mechanisms associated with immune initiation and subsequent regulation.

Toll Like Receptors:

Many pathogens, including protozoa, directly stimulate DCs and other cells of the innate immune system via various surface and intracellular receptors, most notably, toll like receptors or TLRs (Takeda 2003 (Gazzinelli, Ropert et al. 2004). To date, thirteen mammalian TLRs have been described; ten of which are present in humans (TLR 1-10) and twelve in mice (TLR 1-9 and 11-13). Each of these TLRs are germline-encoded type I transmembrane proteins of the interleukin-1 receptor (IL-1R) family that respond to distinct sets of ligands, ranging from DNA complexes to lipid/carbohydrate moieties.

Although much is known about the ligands associated with TLRs 1-9 and 11, little is known about TLRs 10, 12 and 13. For this reason, the information corresponding to TLRs presented in this dissertation will be referring to TLRs 1-9 and 11.

Dendritic Cell Maturation:

Following engagement of TLRs with their ligands on naïve DCs, these cells mature by increasing MHC class II (MHCII) and co-stimulatory molecule expression (CD40, CD80 and CD86), secreting of pro-inflammatory cytokines (TNF-α, IL-12 and IL-6) and migrating to secondary lymphoid tissues (Janeway and Medzhitov 2002). Ultimately, these changes produce DCs engineered for efficient antigen presentation and activation of naive T cells. However, as with many biological processes, maturation of DCs is not an all or nothing event, and depending on the maturation status of the DC as well as the cytokine milieu at the time of antigen presentation to naïve T cells, the subsequent T cell response can vary significantly (Qian, Jiang et al. 2006; Qian, An et al. 2007). The original dogma was that DCs had two maturation states; immature (naïve) and mature—where immature $CD40^{low}$ $CD80/86^{low}$ $MHCII^{low}$ $IL\text{-}12^{-}$ DCs favor the production of anergic or apoptotic T-cells and mature $CD40^{hi}$ $CD80/86^{hi}$ $MHCII^{hi}$ $IL\text{-}12^{+}$ DCs activate T_H1 effector cells (Schwartz 2003). More recent data is emerging in support of the hypothesis that many maturation states within these subsets of DCs exist. Lutz *et al.* recently described what he referred to as semi-mature, now often referred to as tolerant or regulatory $CD11c^{mid}CD45RB^{hi}CD40^{hi}CD80/86^{hi}MHCII^{hi}$, $IL\text{-}12^{-}$, $IL\text{-}10^{+}$ DCs that have the capacity to activate IL-10 secreting regulatory T cells (Lutz, Schnare et al. 2002; Svensson, Maroof et al. 2004; Svensson and Kaye 2006).

The most well understood TLR:TLR-ligand interactions are as follows: TLR2/6: peptidoglycan, zymozan, GPI, Pam3Cys; TLR3:PolyI:C; TLR4/MD2: LPS; TLR5: flagellin; TLR7/8: ssRNA; TLR9: CpG DNA motifs; and TLR11: prophilin, a class of biotin binding proteins (Figure 1.5.3). Diverse recognition of pathogen associated molecular patterns PAMPs through TLR is made possible by conserved leucine rich regions that repeat (LRRs) throughout the extracellular domain of the TLR. The LRR is a tandem repeat of a 20-29 residue sequence motif L(X2)LXL(X2)NXL(X2)L(X7)L(X2), where the X is any amino acid which in turn determines the receptor specificity (Akashi-Takamura 2006).

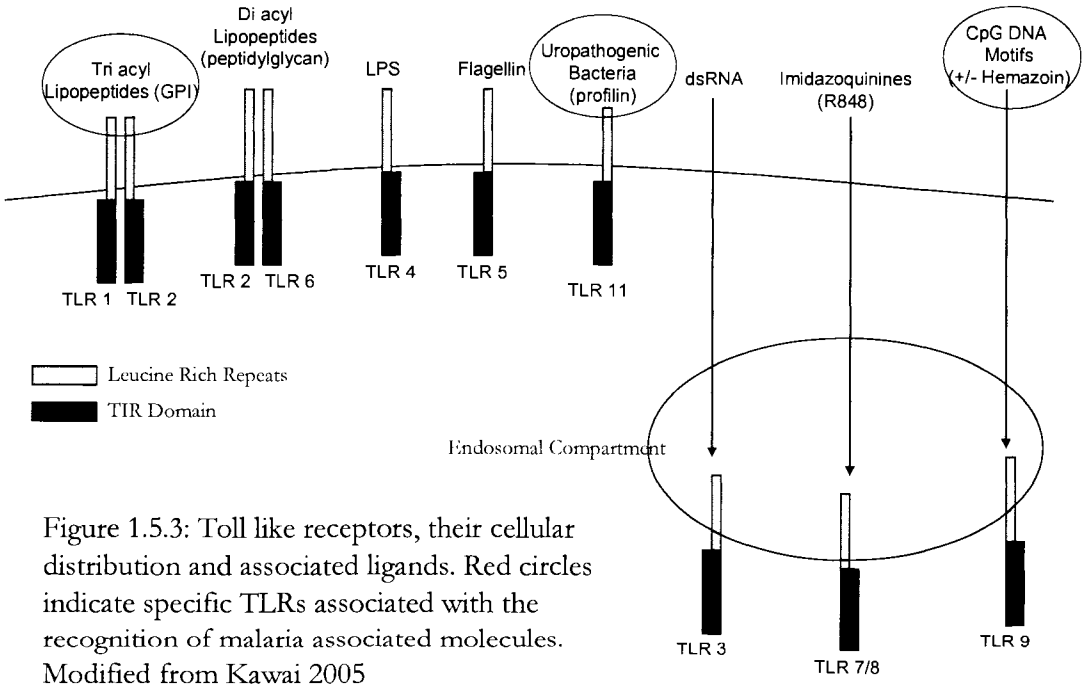


Figure 1.5.3: Toll like receptors, their cellular distribution and associated ligands. Red circles indicate specific TLRs associated with the recognition of malaria associated molecules. Modified from Kawai 2005

TLR Signaling Pathways:

With the exception of TLR 3, all other TLRs signal through the adaptor molecule MyD88, ultimately leading to the activation of NF-κB and transcription factors associated with the MAP kinase pathway (Figure 1.5.4) (Dong, Davis et al. 2002; Janeway and Medzhitov 2002). Toll like receptor 3, which does not associate with MyD88 has been shown to activate NF-κB and downstream transcription factors via an MyD88 independent pathway involving the receptor associated molecule, TRIF (Figure 1.5.5). It was subsequently determined that other TLRs, specifically TLR4, posseses the ability to activate the TRIF pathway as well, albeit, with slower kinetics with respect to the canonical MyD88 dependent pathway (Figure1.5.4). Activation of the MyD88 independent pathway appears to lead to similar downstream activation as the MyD88 dependent pathway, including induction of maturation and activation of IFN-inducible genes, MAP kinases, and NF-κB, thereby leading to the production of cytokines, chemokines, type 1 interferons (IFN-α/β), in addition to numerous other products (West, Koblansky et al. 2006).

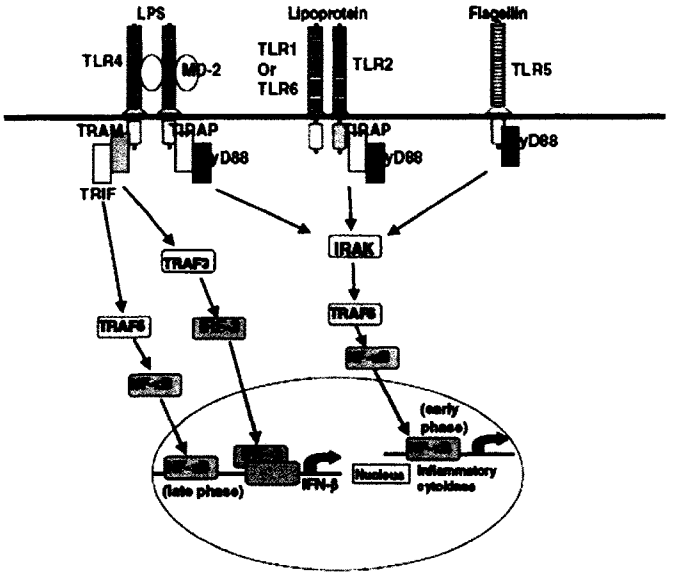


Figure 1.5.4: Downstream TLR signaling pathways involving the MyD88 adaptor molecule. Also shown is the TRIF dependant MyD88 independent pathway associated with TLR4 (and TLR3). Modified from Dong, 2002

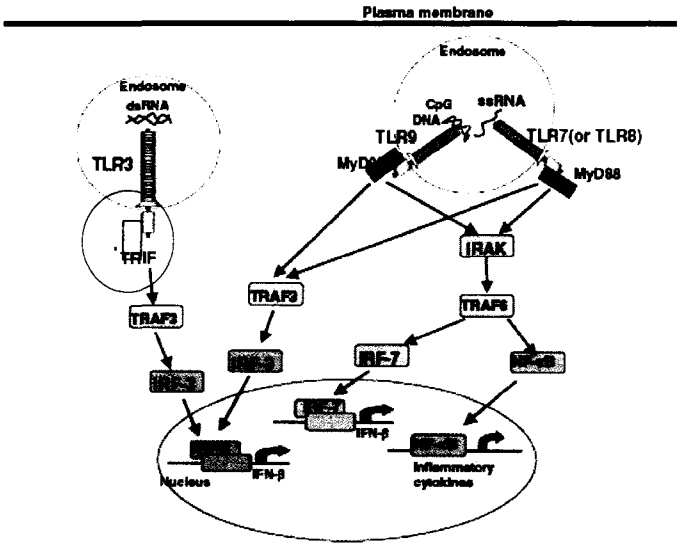


Figure 1.5.5: TRIF dependent, MyD88 independent signaling associated with TLR3. Modified from Dong 2002.

The role of TLR signaling in parasite recognition was first described looking at apicomplexan parasites *Toxoplasma gondii* and *Plasmodium berghei* (Adachi, Tsutsui et al. 2001; Scanga, Aliberti et al. 2002). Aliberti *et al.* subsequently revealed that soluble tachyzoite antigen (STA), a *T. gondii* derived molecule, also stimulated IL-12 production through a non-TLR receptor, CCR5, in a partially MyD88 independent manner (Huang, Aliberti et al. 2003). However, from these studies, it was clear that signaling through CCR5 was not the sole factor mediating DC activation to *T. gondii.* More recently, several groups have further characterized signaling of *T. gondii* through TLR 2, 4 and 11 via parasite derived GPI linked proteins, parasite derived heat shock proteins (HSPs) and profilin, respectively (Mun, Aosai et al. 2005; Yarovinsky, Zhang et al. 2005). Other protozoan parasites that have been shown to activate TLR signaling include *Leishmania spp.* (Hawn, Ozinsky et al. 2002; De Trez, Brait et al. 2004) and *Trypanosoma cruzi* (Gazzinelli, Ropert et al. 2004).

To date, only a few studies have evaluated the role of TLR signaling during malaria infection, but the available data suggests that *Plasmodium spp.* contain ligands for TLRs 2, 4, 9 and 11. Adachi *et al* showed that the absence of MyD88 resulted in a decrease in IL-12 dependent liver damage caused by blood stage *P. berghei* (Adachi, Tsutsui et al. 2001). Neither a specific TLR nor parasite derived ligand was identified in these studies. None the less, a role for TLR signaling in immune mediated pathology during malaria infection was established. In a more recent study, Pichyangkul determined that *P. falciparum* schizonts, the most mature RBC stage, subsequently determined to be hemozoin (Coban, Ishii et al. 2005), activated DCs in a MyD88, TLR9 dependent manner. Specifically, DCs from MyD88$^{-/-}$ and TLR9$^{-/-}$ mice failed to upregulate IL-12, CD40 and

CD86 expression in response to a schizont-soluble fraction whereas expression of these proteins in WT DCs was significantly increased (Pichyangkul, Yongvanitchit et al. 2004). Interestingly, neither of these studies found a significant difference in mortality between infected wild type and MyD88$^{-/-}$ mice infected with *P. berghei*. A more recent study by Hisaeda *et al.* did find significant differences in mortality when TLR9$^{-/-}$ and WT mice were infected with the lethal strain of *P. yoelii* (Hisaeda, Tetsutani et al. 2008) .

The mechanism by which hemazoin activates TLR9 has been a subject of debate. It is becoming apparent that hemazoin's ability to activate TLR9 requires the presence of parasite DNA on its surface. This stems from the observation that hemozoin pretreated with DNase fails to activate TLR9 (Parroche, Lauw et al. 2007). The contrary is also true. Purified malarial DNA, in the absence of hemozoin fails to activate DCs through TLR9. Together, these data show that hemozoin and malarial DNA act together in order to activate TLR9. The authors of this report hypothesize that hemozoin acts to traffic malarial DNA, the active unit of the complex, into TLR9-expressing endosomes. This idea was supported by the observation that coupling of malarial DNA, in the absence of hemozoin, to the monocationic transfection reagent DOTAP (which has been used to target nucleotides into endosomal compartments) results in strong activation of several genes that are down stream of TLR9 (Parroche, Lauw et al. 2007).

Prior to the discovery of TLR receptors, Tachado *et al.* showed that purified glycophosphatidyl inositol (GPI) moieties, now known to be a general class of TLR2 agonists, from *Plasmodium spp.* elicit TNF-α and IL-1 production from macrophages *in vitro*, and cause fever and hypoglycemia *in vivo* (Riley, Wahl et al. 2006). More recently,

Krishnegowda *et al.* showed *P. falciparum* derived GPI molecules specifically activate DCs through TLR2 (Krishnegowda, Hajjar et al. 2005), a similar finding to that observed with GPIs derived from *T. cruzi* several years prior (Campos, Almeida et al. 2001). By contrast, Adachi and Pichyangkul found little to no role for malaria GPIs mediated TLR2 activation. Specifically, the absence of TLR2 appeared to have no observable affect in terms of reducing liver pathology during malaria infection (Adachi, Tsutsui et al. 2001; Pichyangkul, Yongvanitchit et al. 2004). These discrepancies could be the result of TLR2 having a recessive phenotype relative to a functioning TLR9, differences between parasite strains, or simply differences in the sensitivities of that assays utilized between these various laboratories. It would be valuable to test these parameters in TLR9/TLR2 double knockout mice.

Irrespective of the specific TLRs or other receptors utilized during malaria infection, we anticipate that, like *T. gondii, Leishmania spp.* and *T. cruzi*; *Plasmodium spp.* are recognized through multiple TLRs (and possibly other receptors such as CCR5 (Aliberti, Reis e Sousa et al. 2000) and therefore multiple signaling pathways are likely to be involved in DC activation. For the purpose of this dissertation, however, we will focus on TLR signaling.

In addition to signaling through TLR receptors and other pathogen recognition receptors (i.e. CCR5 and previously unmentioned NOD-like receptors), DCs also require signals provided by T cells to undergo full maturation (Sanchez, McWilliams et al. 2007). Ligation of CD40 with CD154 (CD40-Ligand) on T cells has been shown to mature naïve DCs and stimulate cytokine production often in synergy with TLR ligation

(Sanchez, McWilliams et al. 2007). It is hypothesized that the cytoplasmic domain of CD40 interacts with TRAF6, a factor also in common with the MyD88 dependent TLR pathway (Figure 1.5.6) (O'Sullivan and Thomas 2002). This pathway is likely to have more significant effects on DC function during the adaptive phase of infection as well as during subsequent infections.

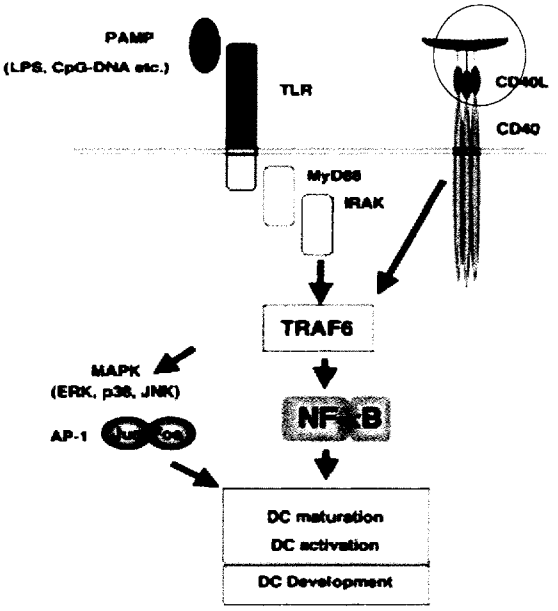


Figure 1.5.6: CD40 signaling activates signaling pathways in common with the TLR MyD88 dependent pathway. Modified from Dong, 2002

1.5.2 ADAPTIVE IMMUNITY:

The adaptive immune system is critical for the control of many infectious agents, and provides the foundation for the mechanism by which vaccination is efficacious against such infectious agents. Additionally, the adaptive immune system is implicated in the acquisition of anti-disease immunity associated with malaria infection which is defined as

resistance to clinical signs, but still susceptible to infection itself. B and T lymphocytes are the primary effector cells that make up the adaptive immune system, and it's their ability to maintain immunologic memory over extended periods of time even in the absence of continued stimulation that allows an individual to become immune to specific pathogens after infection or vaccination. These two cell types can be further subdivided into numerous subsets including but not limited to naïve, mature effector, and memory. The central dogma associated with the generation and maintenance of the adaptive immune system begins with the interaction of naïve, antigen specific, $CD4^+$ T cells with professional antigen presenting cells (APC) bearing the T cell's corresponding antigen. Following this interaction with the APC and antigen, the now activated $CD4^+$ T cells undergo clonal expansion, and depending on the nature of the pathogen, the phenotype of the APC, and the surrounding milieu at the time of presentation, mature into either T_H1 or T_H2 cells which then interact with cytotoxic $CD8^+$ T cells or B cells leading to their activation. While the majority of these $CD8^+$ T cells and B cells differentiate into effector cells, a small percentage of them will differentiate into long lived memory cells that continue to circulate as sentinels for subsequent infection with the same or antigenically similar pathogen.

The necessity for both cellular and humoral immunity in malaria infection has been demonstrated using mouse models. Specifically, it appears that the cellular arm of the adaptive immune system functions primarily during the pre-erythrocytic, intrahepatic stage of malaria infection, whereas both the cellular and humoral arms are exquisitely important for the control of the erythrocyte stage of infection (Vinetz, Kumar et al. 1990; von der Weid T 1993; von der Weid, Honarvar et al. 1996; Urban 2005). In

addition to the effector mechanisms that mediate control of parasitemia early, B cells are required for resolution of the chronic phase of infection (Urban 2005). Specifically, mice lacking B cells do not have the capacity to completely clear malaria parasites from the circulation during the chronic phase of *P. Chabaudi* infection (von der Weid, Honarvar et al. 1996). The necessity for the humoral system is not limited to antibody production only, however, as immune serum transferred to B cell KO mice is not protective against infection (Hirunpetcharat, Vukovic et al. 1999). Langhorne et al. found that B cells are also involved in the downregulation of IFN-γ dominated T cell responses as infection progresses (Langhorne, Cross et al. 1998) which, as mentioned above, may play a role in limiting immunopathology and therefore anti-disease immunity during subsequent malaria infections in people.

Although the adaptive immune response has been shown to effectively reduce parasite proliferation and aid in their clearance from the host, it is also clear that this response also contributes to significant immunopathology (Good, Xu et al. 2005). Mechanisms by which this immunopathology is controlled with respect to the adaptive immune system are discussed below.

1.5.3 Tolerogenic Immunity

Of the millions of individuals infected with *Plasmodia* annually, children and those experiencing the infection for the first time are most adversely affected. In addition to lacking memory T cells and B cells specific for malaria antigens, naïve individuals tend to mount excessively strong innate pro-inflammatory responses to primary malaria infection. The most notable clinical signs associated with severe malarial disease in these

individuals include cerebral malaria and severe anemia. Both of these pathological processes are linked to excessive pro-inflammatory cytokine production by the innate immune system in response to infection. Specifically, production of TNF-α and IFN-γ has been shown to contribute to cerebral malaria and likely severe anemia. TNF-α production contributes to this at least in part through the induction of ICAM expression on cerebral vascular endothelium (McGuire, Hill et al. 1994). This increased expression results in the sequestration of erythrocytes and leukocytes to the brain often leading to diminished blood flow, ischemia and encephalopathy (Gimenez F 2003). Likewise, TNF-α has been shown to play a role in the occurrence of anemia through epidemiological studies linking high TNF-α levels, or low IL-10/TNF-α ratios, to the occurrence of anemia in human malaria infection (Othoro, Lal et al. 1999; May, Lell et al. 2000). The mechanism of IFN-γ's role in cerebral malaria has yet to be elucidated, but its general involvement is evidenced by studies done in IFN-γ receptor knockout mice. Mice lacking IFN-γ signaling are resistant to cerebral malaria, although they die of overwhelming parasitemia (Nakagawa, Naka et al. 2002).

Ideally, these proinflammatory cytokines will be produced during the acute phase of infection then downregulated once the adaptive immune system is activated. Recent evidence suggests that this does occur in the context of several infectious diseases, including malaria, and occurs as the result of the activation of a novel subset of DCs refered to as regulatory DC (diffDCs) and regulatory T cells (T regs) (Svensson, Maroof et al. 2004; Hisaeda H 2005; Svensson and Kaye 2006; Nie, Bernard et al. 2007; Wong

and Rodriguez 2008). An in depth discussion of regulatory T cells is beyond the scope of this dissertation, however, these cells likely play a significant role during malaria.

References:

Abdalla S, H. (1990). "Hematopoiesis in human malaria." Blood Cells **16**(2-3): 401-16.

Abdalla S, H., Pasvol G. (2004). Malaria: A Hematological Perspective, London: Imperial College Press.

ACCCM (1992). ""American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis"." Crit. Care Med. **20**(6): 864-74.

Adachi, K., H. Tsutsui, et al. (2001). "Plasmodiumberghei Infection in Mice Induces Liver Injury by an IL-12- and Toll-Like Receptor/Myeloid Differentiation Factor 88-Dependent Mechanism." J Immunol **167**(10): 5928-5934.

Adams, S., H. Brown, et al. (2002). "Breaking down the blood-brain barrier: signaling a path to cerebral malaria?" Trends in Parasitology **18**(8): 360-366.

Akashi-Takamura, S. a. K. M. (2006). "Toll-like receptors (TLRs) and immune disorders." Journal of Infection and Chemotherapy **12**(5): 233-240

Aliberti, J., C. Reis e Sousa, et al. (2000). "CCR5 provides a signal for microbial induced production of IL-12 by CD8[alpha]+ dendritic cells." Nat Immunol **1**(1): 83-87.

Baird, J. (2000). "Resurgent malaria at the millennium: control strategies in crisis." Drugs **59**(4): 719-43.

Beeson (2005). "The immunology and pathogenesis of malaria during pregnancy." Curr Top Microbiol Immunol. **297**: 187-227.

Boehm, U., T. Klamp, et al. (1997). "Cellular Responses to Interferon-alpha." Annual Review of Immunology **15**(1): 749-795.

Campos, M. A. S., I. C. Almeida, et al. (2001). "Activation of Toll-Like Receptor-2 by Glycosylphosphatidylinositol Anchors from a Protozoan Parasite." J Immunol **167**(1): 416-423.

Carlton, J. M., S. V. Angiuoli, et al. (2002). "Genome sequence and comparative analysis of the model rodent malaria parasite Plasmodium yoelii yoelii." Nature **419**(6906): 512-519.

Carra, G., F. Gerosa, et al. (2000). "Biosynthesis and Posttranslational Regulation of Human IL-12." J Immunol **164**(9): 4752-4761.

Casals-Pascual, C., O. Kai, et al. (2006). "Suppression of erythropoiesis in malarial anemia is associated with hemozoin in vitro and in vivo." Blood **108**(8): 2569-2577.

Chaiyaroj, S. C., A. S. M. Rutta, et al. (2004). "Reduced levels of transforming growth factor-[beta]1, interleukin-12 and increased migration inhibitory factor are associated with severe malaria." Acta Tropica **89**(3): 319-327.

Chotivanich, K., R. Udomsangpetch, et al. (2000). "Parasite Multiplication Potential and the Severity of Falciparum Malaria." The Journal of Infectious Diseases **181**(3): 1206-1209.

Clark, I., A. Budd, et al. (2006). "Human malarial disease: a consequence of inflammatory cytokine release." Malaria Journal **5**(1): 85.

Clark IA, C. G. (1988). "Tumour necrosis factor may contribute to the anaemia of malaria by causing dyserythropoiesis and erythrophagocytosis." Br J Haematol. **70**(1): 99-103.

Coban, C., K. J. Ishii, et al. (2005). "Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin." Journal of Experimental Medicine **201**(1): 19-25.

Cook, I. (1985). "Herpes zoster in children following malaria." J Trop Med Hyg **88**(4): 261-4.

Cross, C. E. and J. Langhorne (1998). "Plasmodium chabaudi chabaudi(AS): Inflammatory Cytokines and Pathology in an Erythrocytic-Stage Infection in Mice." Experimental Parasitology **90**(3): 220-229.

D'Andrea A, R. M., Valiante NM, Chehimi J, Kubin M, Aste M, Chan SH, Kobayashi M, Young D, Nickbarg E, et al. (1992). "Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells." J Exp Med. **176**(5): 1387-98.

d'Ostiani, C. F., G. Del Sero, et al. (2000). "Dendritic Cells Discriminate between Yeasts and Hyphae of the Fungus Candida albicans: Implications for Initiation of T Helper Cell Immunity In Vitro and In Vivo." J. Exp. Med. **191**(10): 1661-1674.

De Trez, C., M. Brait, et al. (2004). "Myd88-Dependent In Vivo Maturation of Splenic Dendritic Cells Induced by Leishmania donovani and Other Leishmania Species." Infect. Immun. **72**(2): 824-832.

Despommier, G., Hotez, Knirsch (2005). Parasitic Diseases, Apple Trees Productions.

Dondorp, A. M., B. J. Angus, et al. (1999). "Red blood cell deformability as a predictor of anemia in severe falciparum malaria." Am J Trop Med Hyg **60**(5): 733-737.

Dondorp, A. M., B. J. Angus, et al. (1997). "Prognostic Significance of Reduced Red Blood Cell Deformability in Severe Falciparum Malaria." Am J Trop Med Hyg **57**(5): 507-511.

Dondorp, A. M., P. A. Kager, et al. (2000). "Abnormal Blood Flow and Red Blood Cell Deformability in Severe Malaria." Parasitology Today **16**(6): 228-232.

Dong, C., R. J. Davis, et al. (2002). "MAP KINASES IN THE IMMUNE RESPONSE." Annual Review of Immunology **20**(1): 55-72.

Duffy, P. E. and M. Fried (2003). "Plasmodium falciparum adhesion in the placenta." Current Opinion in Microbiology **6**(4): 371-376.

Engwerda, C. R., T. L. Mynott, et al. (2002). "Locally Up-regulated Lymphotoxin {alpha}, Not Systemic Tumor Necrosis Factor {alpha}, Is the Principle Mediator of Murine Cerebral Malaria." J. Exp. Med. **195**(10): 1371-1377.

Evans, K. J., D. S. Hansen, et al. (2006). "Severe malarial anemia of low parasite burden in rodent models results from accelerated clearance of uninfected erythrocytes." Blood **107**(3): 1192-1199.

Fortin, A., M. M. Stevenson, et al. (2002). "Susceptibility to malaria as a complex trait: big pressure from a tiny creature." Hum. Mol. Genet. **11**(20): 2469-2478.

Fried, M., R. O. Muga, et al. (1998). "Malaria Elicits Type 1 Cytokines in the Human Placenta: IFN-{gamma} and TNF-{alpha} Associated with Pregnancy Outcomes." J Immunol **160**(5): 2523-2530.

Galinski, M. R., C. C. Medina, et al. (1992). "A reticulocyte-binding protein complex of plasmodium vivax merozoites." Cell **69**(7): 1213-1226.

Gazzinelli, R. T., C. Ropert, et al. (2004). "Role of the Toll/interleukin-1 receptor signaling pathway in host resistance and pathogenesis during infection with protozoan parasites." Immunological Reviews **201**(1): 9-25.

Ghosh, K., Gosh, K (2007). "Pathogenesis of anemia in malaria: a concise review." Parasitology Research **101**(6).

Gimenez F, B. d. L. S., Fernandez C, Pino P, Mazier D. (2003). "Tumor necrosis factor alpha in the pathogenesis of cerebral malaria." Cell Mol Life Sci. **60**(8): 1623-35.

Good, M. F., H. Xu, et al. (2005). "Development and Regulation of Cell-Mediated Immune Responses to the Blood Stages of Malaria: Implications for Vaccine Research." Annual Review of Immunology **23**(1): 69-99.

Grau, G. E., L. F. Fajardo, et al. (1987). "Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria." Science **237**(4819): 1210-1212.

Grau, G. E., H. Heremans, et al. (1989). "Monoclonal Antibody against Interferon gamma Can Prevent Experimental Cerebral Malaria and its Associated Overproduction of Tumor Necrosis Factor." Proceedings of the National Academy of Sciences **86**(14): 5572-5574.

Grau, G. E., P. F. Piguet, et al. (1986). "L3T4+ T lymphocytes play a major role in the pathogenesis of murine cerebral malaria." J Immunol **137**(7): 2348-2354.

Grau, G. E., T. E. Taylor, et al. (1989). "Tumor necrosis factor and disease severity in children with falciparum malaria." N Engl J Med **320**(24): 1586-1591.

Greenwood, B. (1997). "The epidemiology of malaria." Ann Trop Med Parasitol. **91**(7): 763-9.

Guerin, P. J., P. Olliaro, et al. (2002). "Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development." The Lancet Infectious Diseases **2**(9): 564-573.

Hawn, T. R., A. Ozinsky, et al. (2002). "Leishmania major activates IL-1[alpha] expression in macrophages through a MyD88-dependent pathway." Microbes and Infection **4**(8): 763-771.

Hirunpetcharat, C., P. Vukovic, et al. (1999). "Absolute Requirement for an Active Immune Response Involving B Cells and Th Cells in Immunity to Plasmodium yoelii Passively Acquired with Antibodies to the 19-kDa Carboxyl-Terminal Fragment of Merozoite Surface Protein-1." J Immunol **162**(12): 7309-7314.

Hisaeda H, S. H. C. M.-O. K. T. T. I. H. W. K. H. K. Y. (2005). "Resistance of regulatory T cells to glucocorticoid-viduced TNFR family-related protein (GITR) during <I>Plasmodium yoelii</I> infection." European Journal of Immunology **35**(12): 3516-3524.

Hisaeda, H., K. Tetsutani, et al. (2008). "Malaria Parasites Require TLR9 Signaling for Immune Evasion by Activating Regulatory T Cells." J Immunol **180**(4): 2496-2503.

Ho, M. and N. J. White (1999). "Molecular mechanisms of cytoadherence in malaria." Am J Physiol Cell Physiol **276**(6): C1231-1242.

Huang, L.-Y., J. Aliberti, et al. (2003). "Heat-Killed Brucella abortus Induces TNF and IL-12p40 by Distinct MyD88-Dependent Pathways: TNF, Unlike IL-12p40 Secretion, Is Toll-Like Receptor 2 Dependent." J Immunol **171**(3): 1441-1446.

Humphreys, M. (2001). Malaria: Poverty Race and Public Health in the United States, Johns Hopkins University Press.

Hunt, N. H., J. Golenser, et al. (2006). "Immunopathogenesis of cerebral malaria." International Journal for Parasitology **36**(5): 569-582.

Idro, R., Neil E Jenkins, Charles R J C Newton (2005). "Pathogenesis, clinical features, and neurological outcome of cerebral malaria." Lancet Neurology **4**: 827-41.

Janeway, C. A. and R. Medzhitov (2002). "INNATE IMMUNE RECOGNITION." Annual Review of Immunology **20**(1): 197-216.

Kafuko GW, B. D. (1970). "Burkitt's lymphoma and malaria." Int J Cancer. **6**(1): 1-9.

Kobayashi M, F. L., Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G. (1989). "Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes." J Exp Med. **170**(3): 827-45.

Kopacz, J. and N. Kumar (1999). "Murine gamma delta T Lymphocytes Elicited during Plasmodium yoelii Infection Respond to Plasmodium Heat Shock Proteins." Infect. Immun. **67**(1): 57-63.

Krishnan A, K. D., Limaye U, Siddharth W (2004). "Cerebral venous and dural sinus thrombosis in severe falciparum malaria." J Infect **48**: 86-90.

Krishnegowda, G., A. M. Hajjar, et al. (2005). "Induction of Proinflammatory Responses in Macrophages by the Glycosylphosphatidylinositols of Plasmodium falciparum: Cells Signaling Receptors, Glycosylphophatidylinositol (GPI) Structural Requirement, and Regulation of GPI Activity." J. Biol. Chem. **280**(9): 8606-8616.

Kurtzhals, J. A., V. Adabayeri, et al. (1998). "Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria." The Lancet **351**: 1768-1772.

Lackritz EM, H. A., Zucker JR, Ruebush TK 2nd, Onudi CO, Steketee RW, Were JB, Patrick E, Campbell CC (1997). "Longitudinal evaluation of severely anemic children in Kenya: the effect of transfusion on mortality and hematologic recovery." AIDS. **11**(12): 1487-94.

Langhorne, J., C. Cross, et al. (1998). "A role for B cells in the development of T cell helper function in a malaria infection in mice." PNAS **95**(4): 1730-1734.

Li, C., L. A. Sanni, et al. (2003). "Pathology of Plasmodium chabaudi chabaudi Infection and Mortality in Interleukin-10-Deficient Mice Are Ameliorated by Anti-Tumor Necrosis Factor Alpha and Exacerbated by Anti-Transforming Growth Factor {beta} Antibodies." Infect. Immun. **71**(9): 4850-4856.

Linke, A., R. Kuhn, et al. (1996). "Plasmodium chabaudi chabaudi:Differential Susceptibility of Gene-Targeted Mice Deficient in IL-10 to an Erythrocytic-Stage Infection." Experimental Parasitology **84**(2): 253-263.

Lutz, M. B., M. Schnare, et al. (2002). "Differential Functions of IL-4 Receptor Types I and II for Dendritic Cell Maturation and IL-12 Production and Their Dependency on GM-CSF." J Immunol **169**(7): 3574-3580.

Lyke, K. E., R. Burges, et al. (2004). "Serum Levels of the Proinflammatory Cytokines Interleukin-1 Beta (IL-1{beta}), IL-6, IL-8, IL-10, Tumor Necrosis Factor Alpha, and IL-12(p70) in Malian Children with Severe Plasmodium falciparum Malaria and Matched Uncomplicated Malaria or Healthy Controls." Infect. Immun. **72**(10): 5630-5637.

Mabey DC, B. A., Greenwood BM. (1987). "Plasmodium falciparum malaria and Salmonella infections in Gambian children." J Infect Dis. **155**(6): 1319-21.

May, J., B. Lell, et al. (2000). "Plasma interleukin-10:Tumor necrosis factor (TNF)-alpha ratio is associated with TNF promoter variants and predicts malarial complications." Journal of Infectious Diseases **182**(5): 1570-1573.

McGuire, W., A. V. Hill, et al. (1994). "Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria." Nature **371**(6497): 508-510.

Means, R. T., Jr. and S. B. Krantz (1992). "Progress in understanding the pathogenesis of the anemia of chronic disease [see comments]." Blood **80**(7): 1639-1647.

Miller, L. H. (1999). "Evolution of the human genome under selective pressure from malaria: applications for control." Parassitologia **41**(1-3): 77-82.

Mohan, K., P. Moulin, et al. (1997). "Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage Plasmodium chabaudi AS infection." J Immunol **159**(10): 4990-4998.

Mohan K, S. M. (1998). "Interleukin-12 corrects severe anemia during blood-stage Plasmodium chabaudi AS in susceptible A/J mice." Exp Hematol. **26**(1): 45-52.

Mun, H.-S., F. Aosai, et al. (2005). "Toll-Like Receptor 4 Mediates Tolerance in Macrophages Stimulated with Toxoplasma gondii-Derived Heat Shock Protein 70." Infect. Immun. **73**(8): 4634-4642.

Murphy, S. and J. Breman (2001). "Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy
" Am J Trop Med Hyg **64**(90010): 57-67.

Nakagawa, R., T. Naka, et al. (2002). "SOCS-1 Participates in Negative Regulation of LPS Responses." Immunity **17**(5): 677-687.

NCI (2006). Genes and Disease. Bethesda, MD, National Institutes of Health.

Newton CR, W. P., Winstanley PA, Peshu N, Snow RW, Pasvol G, Marsh K. (1997). "Severe anaemia in children living in a malaria endemic area of Kenya." Trop Med Int Health **2**(2): 165-78.

Nie, C. Q., N. J. Bernard, et al. (2007). "CD4+ CD25+ Regulatory T Cells Suppress CD4+ T-Cell Function and Inhibit the Development of Plasmodium berghei-Specific TH1 Responses Involved in Cerebral Malaria Pathogenesis." Infect. Immun. **75**(5): 2275-2282.

O'Sullivan, B. J. and R. Thomas (2002). "CD40 Ligation Conditions Dendritic Cell Antigen-Presenting Function Through Sustained Activation of NF-{kappa}B." J Immunol **168**(11): 5491-5498.

Othoro, C., A. A. Lal, et al. (1999). "A low interleukin-10 tumor necrosis factor-a ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya." Journal of Infectious Diseases **1999**: 279-282.

Pagliuca A, H. M., Layton DM. (1993). "Human parvovirus infection in sickle cell disease." Lancet **342**(8862): 49.

Parroche, P., F. N. Lauw, et al. (2007). "From the Cover: Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9." Proceedings of the National Academy of Sciences **104**(6): 1919-1924.

Phillips RS, S. G., Wakelin D. (1974). "The effect of Plasmodium berghei and Trypanosoma brucei infections on the immune expulsion of the nematode Trichuris muris from mice." Int J Parasitol 4(4): 409-15.

Pichyangkul, S., K. Yongvanitchit, et al. (2004). "Malaria Blood Stage Parasites Activate Human Plasmacytoid Dendritic Cells and Murine Dendritic Cells through a Toll-Like Receptor 9-Dependent Pathway." J Immunol **172**(8): 4926-4933.

Price, R., M. van Vugt, et al. (1999). "Adverse effects in patients with acute falciparum malaria treated with artemisinin derivatives." Am J Trop Med Hyg **60**(4): 547-555.

Qian, C., H. An, et al. (2007). "TLR agonists induce regulatory dendritic cells to recruit Th1 cells via preferential IP-10 secretion and inhibit Th1 proliferation." Blood **109**(8): 3308-3315.

Qian, C., X. Jiang, et al. (2006). "TLR agonists promote ERK-mediated preferential IL-10 production of regulatory dendritic cells (diffDCs), leading to NK-cell activation." Blood **108**(7): 2307-2315.

Riley, E. M., S. Wahl, et al. (2006). "Regulating immunity to malaria." Parasite Immunology **28**(1-2): 35-49.

Roberts DJ, C.-P. C., Weatherall DJ (2005). "The clinical and pathophysiological features of malarial anaemia." Curr Top Microbiol Immunol. **295**: 137-67.

Rogerson, S. J., H. C. Brown, et al. (2003). "Placental Tumor Necrosis Factor Alpha but Not Gamma Interferon Is Associated with Placental Malaria and Low Birth Weight in Malawian Women." Infect. Immun. **71**(1): 267-270.

Rogerson, S. J., L. Hviid, et al. (2007). "Malaria in pregnancy: pathogenesis and immunity." The Lancet Infectious Diseases **7**(2): 105-117.

Sanchez, P. J., J. A. McWilliams, et al. (2007). "Combined TLR/CD40 Stimulation Mediates Potent Cellular Immunity by Regulating Dendritic Cell Expression of CD70 In Vivo." J Immunol **178**(3): 1564-1572.

Scanga, C. A., J. Aliberti, et al. (2002). "Cutting Edge: MyD88 Is Required for Resistance to Toxoplasma gondii Infection and Regulates Parasite-Induced IL-12 Production by Dendritic Cells." J Immunol **168**(12): 5997-6001.

Schofield, L., M. C. Hewitt, et al. (2002). "Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria." Nature **418**(6899): 785-789.

Seixas, E. M. G. and J. Langhorne (1999). "{gamma}{delta}T Cells Contribute to Control of Chronic Parasitemia in Plasmodium chabaudi Infections in Mice." J Immunol **162**(5): 2837-2841.

Shortman, K. and Y.-J. Liu (2002). "Mouse and human dendritic cell subtypes." Nat Rev Immunol **2**(3): 151-161.

Spielman, A. (2001). Mosquito: A Natural History of Our Most Persistent and Deadly Foe Hyperion.

Steketee, R. W., B. L. Nahlen, et al. (2001). "The burden of malaria in pregnancy in malaria-endemic areas." Am J Trop Med Hyg **64**(1_suppl): 28-35.

Stevenson, M. M. and E. M. Riley (2004). "Innate immunity to malaria." Nat Rev Immunol **4**(3): 169-180.

Svensson, M. and P. M. Kaye (2006). "Stromal-cell regulation of dendritic-cell differentiation and function." Trends in Immunology **27**(12): 580-587.

Svensson, M., A. Maroof, et al. (2004). "Stromal Cells Direct Local Differentiation of Regulatory Dendritic Cells." Immunity **21**(6): 805-816.

Swardson-Olver, C. J., T. C. Dawson, et al. (2002). "Plasmodium yoelii uses the murine Duffy antigen receptor for chemokines as a receptor for normocyte invasion and an alternative receptor for reticulocyte invasion." Blood **99**(8): 2677-2684.

Teklehaimanot, A. (2005). "Coming to Grips with Malaria in the New Millennium." Task Force on HIV?AIDS, Malaria, TB and Access to Eessential Medicines.

Thursz MR, K. D., Torok ME, Allsopp CE, Greenwood BM, Whittle HC, Thomas HC, Hill AV (1995). "Association of hepatitis B surface antigen carriage with severe malaria in Gambian children." Nat Med **1**(4): 374-5.

Trinchieri, G. (1995). "Interleukin-12: A Proinflammatory Cytokine with Immunoregulatory Functions that Bridge Innate Resistance and Antigen-Specific Adaptive Immunity." Annual Review of Immunology **13**(1): 251-276.

Trinchieri, G., M. Rengaraju, et al. (1993). "Producer cells of interleukin 12." Parasitology Today **9**(3): 97.

Tsuji, M., P. Mombaerts, et al. (1994). "{gamma}{delta}T Cells Contribute to Immunity Against the Liver Stages of Malaria in {alpha} {beta} T-Cell-Deficient Mice." Proceedings of the National Academy of Sciences **91**(1): 345-349.

Urban, B., Ing R, Stevenson MM (2005). "Early interactions between blood-stage plasmodium parasites and the immune system." Curr Top Microbiol Immunol. **297**: 25-70.

Vinetz, J. M., S. Kumar, et al. (1990). "Adoptive transfer of CD8+ T cells from immune animals does not transfer immunity to blood stage Plasmodium yoelii malaria." J Immunol **144**(3): 1069-1074.

von der Weid, T., N. Honarvar, et al. (1996). "Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection." J Immunol **156**(7): 2510-2516.

von der Weid T, L. J. (1993). "Altered response of CD4+ T cell subsets to Plasmodium chabaudi chabaudi in B cell-deficient mice." Int Immunol. **5**(10): 1343-8.

Vryonis, G. (1939). "Observation on the Parasitization of Erythrocytes by Plasmodium vivax, with Special Reference to Reticulocytes." Am. J. Hyg (Am. J. Epidemiology) **30**: 41-48.

Wagner, M. A., B. Andemariam, et al. (2003). "A Two-Compartment Model of Osmotic Lysis in Plasmodium falciparum-Infected Erythrocytes." Biophys. J. **84**(1): 116-123.

West, A. P., A. A. Koblansky, et al. (2006). "Recognition and Signaling by Toll-Like Receptors." Annual Review of Cell and Developmental Biology **22**(1): 409-437.

Whittle HC, B. J., Marsh K, Greenwood BM, Seidelin P, Tighe H, Wedderburn L (1984). "T-cell control of Epstein-Barr virus-infected B cells is lost during P. falciparum malaria." Nature **312**(5993): 449-50.

Wickramasinghe, S. N. and S. H. Abdalla (2000). "Blood and bone marrow changes in malaria." Best Practice & Research Clinical Haematology **13**(2): 277-299.

Williamson WA, G. B. (1978). "Impairment of the immune response to vaccination after acute malaria." Lancet **1**(8078): 1328-9.

Wong, K. A. and A. Rodriguez (2008). "Plasmodium Infection and Endotoxic Shock Induce the Expansion of Regulatory Dendritic Cells." J Immunol **180**(2): 716-726.

Wongsrichanalai, C., A. L. Pickard, et al. (2002). "Epidemiology of drug-resistant malaria." The Lancet Infectious Diseases **2**(4): 209-218.

Yap, G. S. and M. M. Stevenson (1994). "Inhibition of in vitro erythropoiesis by soluble mediators in Plasmodium chabaudi AS malaria: lack of a major role for interleukin 1, tumor necrosis factor alpha, and gamma interferon." Infect. Immun. **62**(2): 357-362.

Yarovinsky, F., D. Zhang, et al. (2005). "TLR11 Activation of Dendritic Cells by a Protozoan Profilin-Like Protein." Science **308**(5728): 1626-1629.

Chapter 2: **Activation and inhibition of T cell responses are mediated by different antigen presenting cell populations during *P. yoelii* infection**

The work in this chapter was published in part in The Journal of Immunology (2004) 172: 475 – 482.

The hypothesis of this study was that T cell proliferation during malaria infection is inhibited by splenic macrophages.

The aim of this study was to:

1. Determine the extent of T cell inhibition during malaria infection *in vivo.*
2. Determine which subset(s) of splenocytes are responsible for the observed T cell inhibitory capacity.
3. Determine mechanism responsible for the observed T cell inhibition.

2.1 T cell proliferation is inhibited *in vivo* during malaria infection

2.2 Macrophages isolated late during infection inhibit IL 2 production and T cell proliferation but have little effect on IFN-γ production by T cells

2.3 IL-2 inhibition is not mediated by IL-10 production or tryptophan starvation.

Abstract:

In this chapter we show using a *P. yoelii* model of malaria infection that T cell proliferation is inhibited both *in vivo* and *in vitro*. Naïve DO11.10 transgenic T cells adoptively transferred to infected mice undergo significantly fewer divisions when exposed to ovalbumin (Ova) *in vivo* relative to naïve controls. This was measured both by CFSE dilution and by quantifying the number of KJ-1 positive cells. These findings were recapitulated *in vitro* by measuring IL-2 production and T cell proliferation following CD3 stimulation of whole spleen cells and by culturing naïve DO11.10 T cells with infected versus naïve spleen cells in the presence of Ova. This observed inhibition appears to be at least in part a result of the production of soluble inhibitory factors produced by splenocytes as infection progresses. The soluble factors are unlikely IL-10 or indoleamine 2, 3-dioxygenase (IDO), as blocking IL-10 and IDO function (using 1-methyl-tryptophan) *in vitro* had no significant effect on IL-2 production or T cell proliferation. Although we have yet to isolate the inhibitory factor involved, through the use of depletion and add back studies, we demonstrate that purified macrophages are key players in the inhibition of IL-2 production during *P. yoelii* infection. Interestingly, this inhibitory effect is specific for IL-2 in that macrophages have little to no effect on IFN–γ or TNF–α production.

Introduction:

With the exception of the most virulent strains of *Plasmodium falciparum*, human malaria parasites are extremely well adapted to their hosts. Infection can occur as a result of inoculation with as few as 10 parasites which often subsequently leads to long parasitemic phases and poor immunologic memory with immunity to severe disease occurring before immunity to infections itself (sterile immunity) (Ungureanu E 1977). This allows for efficient spread and repeated infections within an individual and throughout populations. It is likely these characteristics which make malaria one of the most insidious infectious diseases facing humans today.

Several previous studies have shown that malaria infection leads to a general state of immunosuppression. Previous work done in our lab showed that spleen cells from infected mice produce a soluble factor distinct from PGE_2, TGF-β and nitric oxide (NO) that inhibits IL-2 production without direct cell-cell contact (Luyendyk, Olivas et al. 2002). Scorza *et al.* showed similar inhibitory properties associated with peritoneal exudate cells (PECs) that also appeared independent of PGE_2 and NO activity (Scorza, Magez et al. 1999). These *in vitro* observations are consistent with our subsequent findings that T cells maturing in an infected environment *in vivo* undergo limited division *ex vivo*, but produce high levels of IFN–γ at day 7 post infection (Luyendyk, Olivas et al. 2002). While a large body of earlier literature indicates that macrophages from malaria infected individuals have immunosuppressive properties, there is a surprising paucity of studies in which macrophages ($CD11c^-CD11b^+$) are shown to directly suppress T cell proliferation (Correa, Narayanan et al. 1980; Scorza, Magez et al. 1999).

In addition to the observed immunosuppressive properties associated with splenocytes, more specifically $CD11c^{-}CD11b^{+}$ cells, several groups have suggested that malaria infection effects dendritic cell maturation and function thereby further resulting in suppressed immune responses. Ocaña-Morgner et al. showed that bone marrow derived DCs (BMDC), when matured in the presence of pRBC, fail to mature and carry an anti-inflammatory phenotype (Ocana-Morgner, Mota et al. 2003). They follow these data with experiments looking at DC (as defined by $CD11c^{+}$ expression[1]) maturation status in splenic DCs and show that these cells too, carry reduced levels of CD80, CD86 and MHCII expression. Additionally, these cells show a much poorer capacity to phenotypically mature in the presence of LPS. Ocaña-Morgner *et al.* go on to show that these pRBC exposed DCs carry a reduced T cell stimulatory capacity by measuring T cell proliferation following activation with DCs isolated from in infected mice (Ocana-Morgner, Mota et al. 2003).

Work done in our lab and by Seixas *et. al.* argue against the findings that DCs are directly responsible for the observed reduction in T cell activation. In the subsequent chapter of this dissertation, we show that DCs are fully functional APCs throughout malaria infection as measured by *in vitro* T cell activation studies, and that macrophages and B cells have little T cell stimulatory capacity in the context of presenting malaria antigens (Perry, Rush et al. 2004). In this chapter, we will focus on the general observation that T

[1] Subsequent to this work, Laouar et al. showed that splenic NK cells also express CD11c. Therefore, it should be noted that in all experiments where DCs were isolated by magnetic selection based on CD11c expression likely contain up to 25 percent NK cells Laouar, Y., F. S. Sutterwala, et al. (2005). "Transforming growth factor-[beta] controls T helper type 1 cell development through regulation of natural killer cell interferon-[gamma]." Nat Immunol **6**(6): 600-607.. These cells were gated out is sorting experiments based on CD11chi expression and later exclusion with NK1.1 antibody.

cell proliferation is inhibited during malaria infection *in vivo*, and that macrophages, at least in part, play a significant role in this inhibition *ex vivo*.

Materials and Methods:

Mice:

B10.D2 and B10.D2-DO11.10 (referred to as TCR-Tg) mice were purchased from Jackson Laboratories and used after a two week acclimation period. B10.D2-DO11.10 mice express a T cell receptor for ovalbumin (Ova) on CD4 T cells (Bluthmann, Kisielow et al. 1988). These mice were purchased from Jackson Laboratories and bred in our laboratory animal facility by crossing heterozygous males with non-transgenic females. Offspring were determined to be heterozygous if greater than 90% of the $CD4^+$ cells in the peripheral blood were positive when stained with anti-Vβ8.1/8.2. Mice were housed in the Painter Center, Colorado State University, and all experiments approved by the Institutional Animal Care and Use Committee.

Infections with P. yoelii 17X NL:

For infection with *P. yoelii* 17X NL (non-lethal) parasites (Sayles and Wassom 1988), a frozen stock of infected BALB/c red blood cells was thawed and injected intraperitoneally (i.p.) into several BALB/c mice (source mice). Four to seven days later, mice were bled from the tail and parasitemia was evaluated. Source mice with parasitemia close to 10% were used. The source mouse was sacrificed and bled from the heart. A total of 10^6 infected red blood cells diluted in PBS were given to each experimental mouse i.p. in a total volume of 0.1 ml. Female mice were used in all experiments, except

male and female TCR-Tg mice were used as a source of $CD4^+$ T cells *in vitro*. Parasitemias were determined by counting a minimum of 100 Wright-Giemsa-stained red blood cells from a tail nick on day 6 post-infection. *P. yoelii* 17X NL infection is not lethal for B10.D2 mice, and resolves by approximately day 25 (Figure 1.3.1).

In vivo T cell inhibition assay:

All mice were obtained from Jackson Laboratories (Bar Harbour, ME) and housed in the Painter Research Animal Facility (Colorado State University, Fort Collins, CO). Mice were sacrificed using CO_2 and spleens placed in HBSS + 2% FBS + DNase (5 IU/ml). Splenocytes were isolated using a stainless steel sieve and 20cc syringe plunger. The resultant cell suspension was subjected to ACK lysis buffer (0.15M NH_4Cl, 1M $KHCO_3$, 0.1mM Na_2EDTA, and 1N HCl to pH 7.4) at 5ml/spleen for 10 minutes at room temperature (RT). The remaining cells were washed once in HBSS + 2% FBS + DNase then passed over sterile gauze to remove particulate matter. The individualized cells were counted then labeled with 10 ug/ml Carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Bend, OR) dye in PBS at 200ul/$1.5x10^8$ cells for 10 minutes followed by two washes with PBS. The CFSE stained spleen cells were resuspended at $5x10^8$ cells/ml in sterile PBS then 100 ul ($2x10^7$ cells) was transferred intravenously (i.v.) into the tail vein of 5 naïve and 5 infected mice (day 5 post infection). Two hours following i.v. transfer of the stained splenocytes, mice were either given 1mg Ova (Sigma Cat# A5503) intraperitoneally (i.p.) in Ribi (Sigma Cat# M6536) (2 mice/group), 1mg Ova IP in PBS (3 mice/group), or PBS only (3 mice/group). Three days later the mice were sacrificed and their spleens removed and splenocytes isolated as previously described. $3x10^6$ cells were blocked with 10 ug/ml anti-CD16/32 for 60 min. on ice then stained

with anti-KJ-1 (specific for the DO11.10 T-cell receptor) and anti-CD4 for 30min at room temperature (Figure 2.1.1). The cells were washed twice in PBS then analyzed using a Coulter EPICS XL flow cytometer. This experiment was repeated three times with similar findings.

Antibodies:

Antibodies used for purification and staining were purchased from B-D Biosciences; anti-Vβ8.1/8.2 (clone MR5-2), anti-CD4 (clone L3T4), anti-CD8 (clone 53-6.7), anti-CD25 (clone PC61), anti-Fas (clone Jo2), Ig (BD), anti-CD11c (clone HL3), anti-CD11b (clone M1/70), anti-CD40 (clone 3/23), anti-CD80 (clone 16-10A1), anti-CD86 (clone GL1), anti-CD3 (clone 145-2C11), anti-CD19 (clone 1D3) and anti-class II MHC (clone 2G9). Anti-TCR clonotype antibody (KJ1-26) was purchased from Caltag. Biotinylated MOMA-1 (T-2021) and ERTR9 (T-2010) antibodies used for selection of marginal zone macrophage subsets were purchased from Bachem (Torrence, CA).

$CD11b^+$ Cell Selection:

Spleen cells were isolated as described above. Cells were then spun at 1600 rpm in a Sorvall RT6000 centrifuge for 10 minutes at 4 degrees and resuspended at $1x10^8$ cells/ml in sterile PBS + 0.5% BSA + 2mM EDTA pH 7.4 (Staining buffer). Cells were blocked with anti-CD16/32 for 20 minutes then stained with anti-CD11c for 1 hour on ice. The stained cells were washed with staining buffer then resuspended in 10% SA-microbeads (Miltenyi Biotech) at a concentration of 10^9 cells/ml for 30 minutes on ice. Again, cells were washed twice with staining buffer then ran over Miltenyi LS columns while collecting both the effluent and the positively selected fraction. The CD11c+ cells were

used for DC assays. The effluent fractions were subsequently stained for CD11b in a similar fashion as described for CD11c. Once isolated, $CD11b^+$ cells were resuspended in DMEM-5 and used as described.

Cell culture conditions:

Cell cultures were carried out in round-bottomed 96-well plates. $3x10^5$ APCs and $2x10^5$ T cells were used in each well, together with 1 mg/ml Ova. Lower concentrations of OVA resulted in proportionally lower production of each cytokine measured. Titration of DC numbers showed that as the numbers were decreased (to 10^4 DC with 10^5 T cells) IL-2, IFN-γ and TNF-α production decreased proportionally in the presence of both nDC and iDC (not shown). Inhibition studies were carried out by addition of the indicated inhibitor diluted in 25ul of DMEM-5.

The Ova (Sigma Cat# A5503, Grade V) was not certified endotoxin free. The addition of Ova to DC cultures without T cells resulted in mild TNF-α production (but no other cytokine) that was twice the spontaneous release. By contrast, the deliberate addition of LPS to DC cultures resulted in an 8 – 10-fold increase in TNF-α. The magnitude of both effects (Ova and LPS) was similar in DC from infected and uninfected mice. When macrophages were added to cell cultures to look for inhibitory activity, $3x10^5$ macrophages were added to DCs and T cells.

Cytokine Analysis:

IL-2, IFN–γ, IL-10, and IL-12 p40 and p70 cytokine ELISAs were carried out using reagents from B-D BioSciences as described in (Luyendyk, Olivas et al. 2002). Briefly,

purified capture antibodies against their respective cytokine were diluted in coating buffer (0.1M $NaHCO_3$ pH 8.1) plated out in MaxiSorb 96 well plates (Nunc-VWR#62409-004) and allowed to incubate overnight at 4 degrees. Plates were washed 5 times with PBS-Tween 20 (PBS-Tw) then blocked for 4 hours at 4 degrees with 200 ul PBS + 10% FBS. After blocking, 100ul of sample or standard was added to each well and allowed to incubate for 12-24 hours at 4 degrees. Plates were then washed 5 times with PBS-Tw followed by addition of secondary antibody which was allowed to incubate at RT for exactly 45 minutes. Again, plates were washed 5 time with PBS-Tw then incubated with Streptavidin-HRP (SAv-HRP) (B-D Biosciences Cat#554066) at 1:1000 as recommended by manufacturer for exactly 30 minutes at RT. Plates were subsequently developed with peroxidase substrate and H_2O_2 (Kirkegaard and Perry Cat# 50-76-00) and stopped with 1M H_3PO_4. Cytokine bead assays (CBA) to quantify IL-2, IFN–γ, TNF–α, IL-4 and IL-5 were obtained from BD BioSciences and used as described by the manufacturer. Beads were analyzed on a Coulter Epics XL, using FL2 and FL4 for acquisition. Cytokine quantities obtained by ELISA were similar to those obtained with the bead assay.

Statistical analysis:

Two tailed Student's T tests were performed to evaluate statistical differences between groups. For the *in vivo* T cell proliferation assays, three mice were used in each group, and three individual experiments were completed. For *in vitro* assays, splenocytes were pooled from individual mice prior to selection with magnetic beads then plated out in triplicate for each treatment group.

Results:

2.1 T cell proliferation is inhibited during malaria infection *in vitro* and *in vivo*

Historically, human malaria infection is often associated with a general state of immunosuppression, specifically with regard to $CD4^+$ T cell activation and function (Ho, Webster et al. 1988). In order to confirm this finding in our model system, we developed an adoptive T cell transfer system. As depicted in Figure 2.1.1, freshly isolated CFSE stained splenocytes from TCR-Tg mice were transferred intravenously (i.v.) to day 5 post infected (p.i.) B10.D2 recipient mice. Two hours post transfer; these mice were then injected intraperitoneally (i.p.) with PBS alone, Ova alone or Ova with adjuvant. Three days after stimulation, splenocytes were isolated and TCR-Tg T cells ($CD4^+KJ1^+$ cells) examined by flow cytometry for their capacity to divide *in vivo* (Figure 2.1.1). Differences in T cell proliferation were evaluated by measuring absolute number of TCR-Tg T cells recovered and correlating this with CFSE fluorescence intensity. These parameters should become inversely proportional as the cells divide. Using this analysis system also allows T cell proliferation to be distinguished from cell death.

In three separate transfer experiments, we confirmed that antigen specific T cell proliferation is significantly inhibited in *P. yoelii* infected mice. TCR-Tg T cells transferred to naïve mice showed a 5 to 20 fold increase in cell number following i.p. injection of Ova irrespective of the presence of adjuvant. Infected mice however, supported much lower T cell proliferation, ranging from 1 to 5 fold increase over PBS

controls (Figures 2.1.2). This proliferation data was supported by the presence of high levels of CFSE in TCR-Tg T cells isolated from the infected mice. The percent of CFSE positive TCR-Tg T cells in infected mice ranged from 35 to 85 percent following administration of Ova or Ova with adjuvant. Naïve recipients however, contained fewer than 20 percent CFSE positive TCR-Tg T cells, which in the face of significantly higher absolute numbers of TCR-Tg T cells supports the conclusion that these cells are dividing rather than undergoing cell death (Figure 2.1.3). The differences in T cell proliferation between naïve and infected mice were significant regardless of the presence of adjuvant ($p<0.05$). No significant difference in T cell proliferation was observed in naïve and infected mice that did not receive Ova (Figure 2.1.3B).

These studies using an *in vivo* method for measuring antigen specific T cell proliferation has allowed us to show that in our model of malaria infection, T cell proliferation is in fact inhibited. Knowledge of this allowed us to further explore the cellular and molecular players involved in this inhibition in subsequent *in vitro* studies as addressed further in this dissertation.

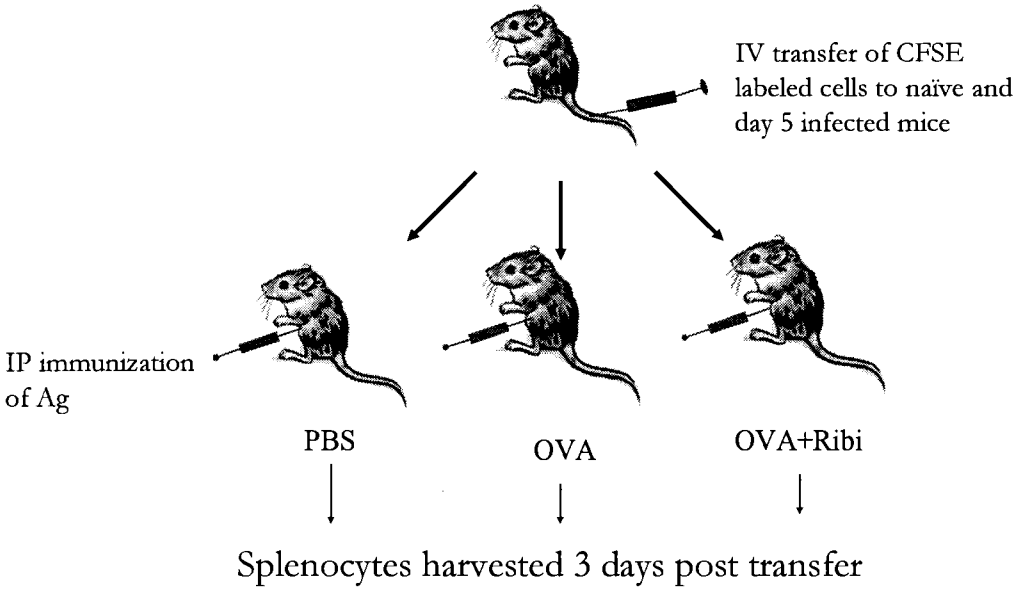


Figure 2.1.1: Schematic of *in vivo* T cell inhibition assay as described in the methods section of this chapter. Briefly, CFSE labeled splenocytes from TCR-Tg DO11.10 mice were transferred i.v. to infected or naïve recipients. These mice were then challenged with PBS, Ova alone or Ova + adjuvant (three mice for each treatment group). Splenocytes from these mice were harvested 3 days post transfer and analyzed for T cell proliferation.

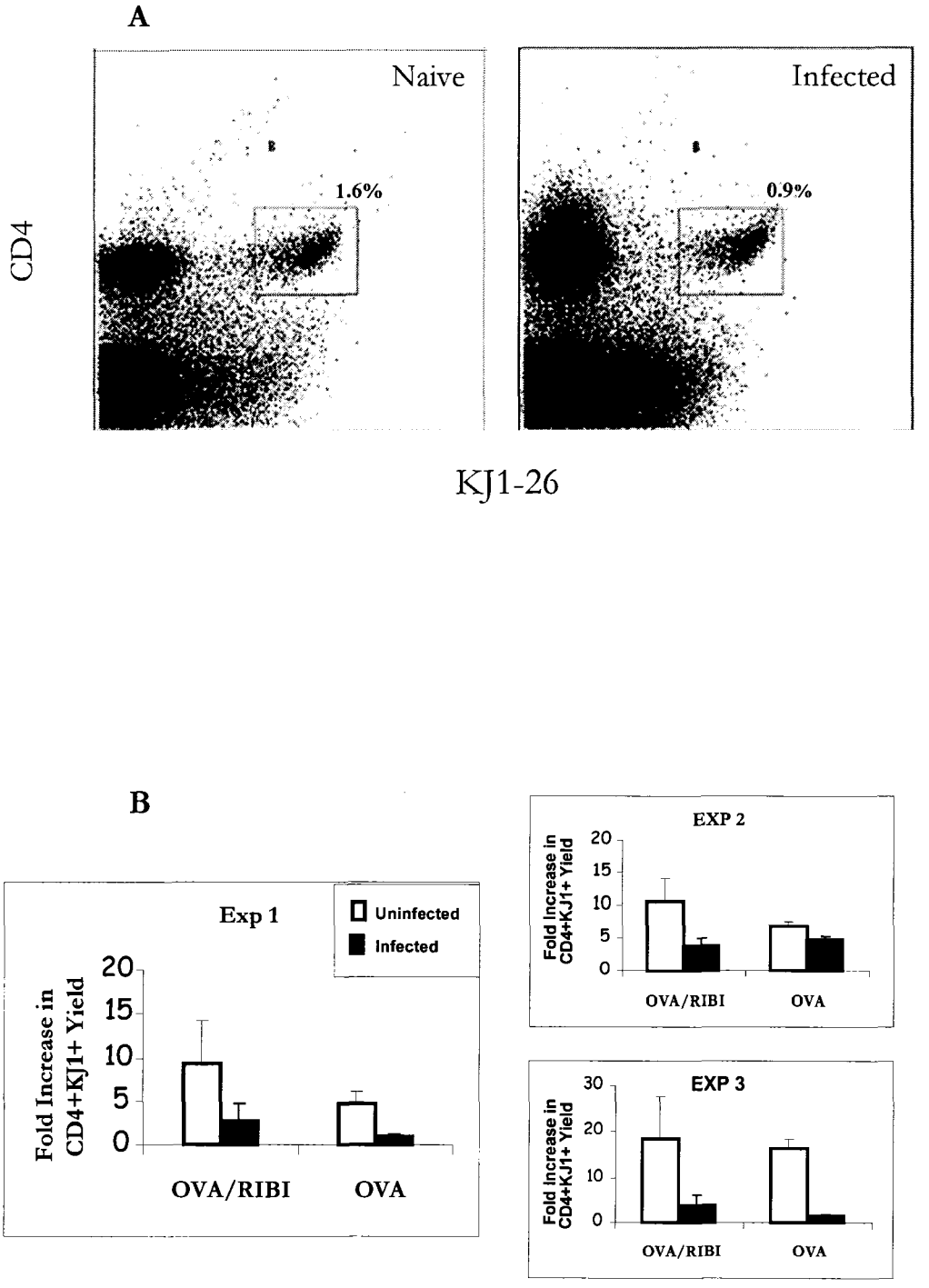
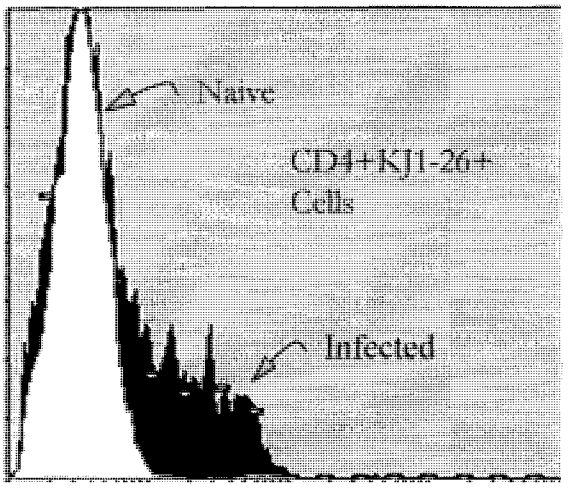
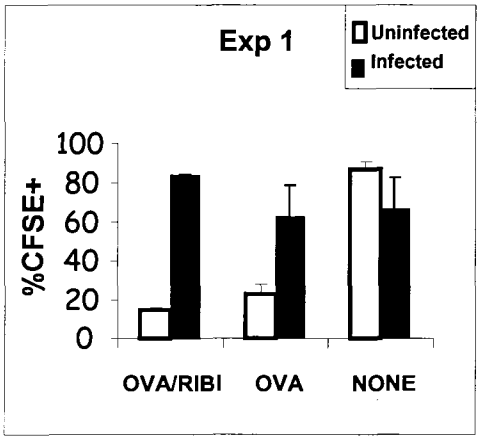


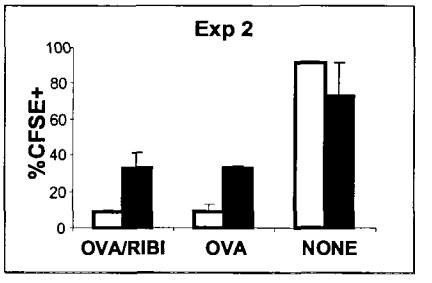
Figure 2.1.2 A) Gating criteria used for quantifying TCR-Tg T cell proliferation. The gated percentage was multiplied by the total number of spleen cells to obtain the absolute number of TCR-Tg T-cells present within each spleen. B) Fold increase in TCR-Tg T cells following administration of Ova. The PBS control group was used as a baseline.

A



B





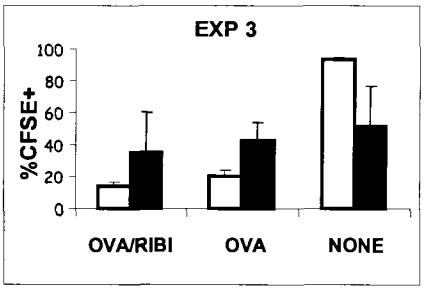


Figure 2.1.3: A) Histogram of CFSE staining in TCR-Tg T cells transferred to infected versus naïve mice. The higher level of staining in cells from infected mice is indicative of fewer divisions in vivo. B) Cell division as indicated by percent CFSE. TCR-Tg T cells were in gated then assessed for CFSE staining. The higher percentage of CFSE indicates fewer divisions.

2.2 Macrophages isolated late during infection specifically inhibit IL-2 production and T cell proliferation.

We next sought to determine which cell type within the spleen was responsible for inhibiting T cell proliferative responses. In previous work we found that naïve T cells cultured in the presence of splenocytes from malaria infected mice as antigen presenting cells exhibited reduced IL-2 production and subsequent T cell proliferation. In addition to these findings, depletion of $CD11b^+$ cells (macrophages and neutrophils) from the spleens of infected mice abrogated IL-2 inhibitory capacity (Luyendyk, Olivas et al. 2002). To directly demonstrate that $CD11c^-CD11b^+$ cells from infected mice inhibit T cell proliferation, we carried out a series of add-back experiments, in which magnetically selected $CD11c^-CD11b^+$ cells, and subpopulations of these cells were added to cultures of naïve TCR transgenic T cells and magnetically selected $CD11c^+$ DCs. Magnetic selection of $CD11c^+$ and $CD11c^-CD11b^+$ cells yielded populations of with greater than 80 percent purity in all experiments as determined by flow cytometry (data not shown).

We first showed that $CD11c^-CD11b^+$, when cultured as a purified population alone with naïve TCR-Tg T cells plus Ova antigen did not stimulate IL-2 production, whereas $CD11c^+$ cells from both infected and naïve mice did (Figure 2.2.1). The addition of these cells from both uninfected and infected mice tended diminish IL-2 production, a finding consistent with the literature (Munn, Shafizadeh et al. 1999). The macrophage enriched population from infected mice, however, suppressed IL-2 production substantially more than those from uninfected mice (Figure 2.2.2). IL-2 inhibition was statistically significant in 4 of 4 experiments ($p < 0.05$). In an attempt to attribute the IL-2

inhibitory properties to a specific subset of splenic macrophages, MOMA-1 (splenic marginal zone metallophilic macrophages) and ERTR-9$^+$ (splenic marginal zone macrophages) splenocytes were selected and cultured with DCs, TCR-Tg T cells, and Ova as was done with CD11c$^-$CD11b$^+$ cells. The addition of ERTR-9$^+$ cells led to a significantly decreased IL-2 production relative to DCs alone, 2090 pg/ml versus 2920 pg/ml IL-2, respectively ($p=0.01$). However, this reduction in IL-2 was much less significant than that seen with total CD11c$^-$CD11b$^+$ cells from infected mice; 467 pg/ml versus 2920 pg/ml IL-2, respectively ($p < 0.001$). MOMA-1$^+$ splenocytes from infected mice did not significantly affect IL-2 production from naïve T cells when cultured with naïve DCs (Figure 2.2.3).

We then analyzed the influence of CD11c$^-$CD11b$^+$ cells from infected mice on two other T cell cytokines. The effect of these cells on IFN–γ production and TNF–α production at this time of infection (day 7 p.i.) was variable, either leading to slight enhancement (two experiments) or no difference (one experiment) (Figure 2.2.4). The data shown here is from the first in the series of experiments. Of note, the degree of parasitemia was similar throughout infection in all three experiments, as was the degree of purity of the APC subsets (data not shown). These results indicate that in the presence of macrophages from infected mice, T cells are still functional with respect to production of other cytokines, albeit, produce significantly lower amounts of IL-2.

Taken together, these results confirm findings by others that CD11c$^-$CD11b$^+$ cells from infected mice inhibit T cell proliferative responses (Scorza, Magez et al. 1999; Luyendyk, Olivas et al. 2002). Furthermore, the inhibition appears to be specific for IL-2

production. The finding that these cells neither inhibited nor enhanced the production of other T cell derived cytokines, specifically IFN–γ and TNF–α, suggests that while T cell proliferation may be inhibited, these $CD11c^{-}CD11b^{+}$ cells have little effect on the production of pro-inflammatory cytokines early in malaria infection.

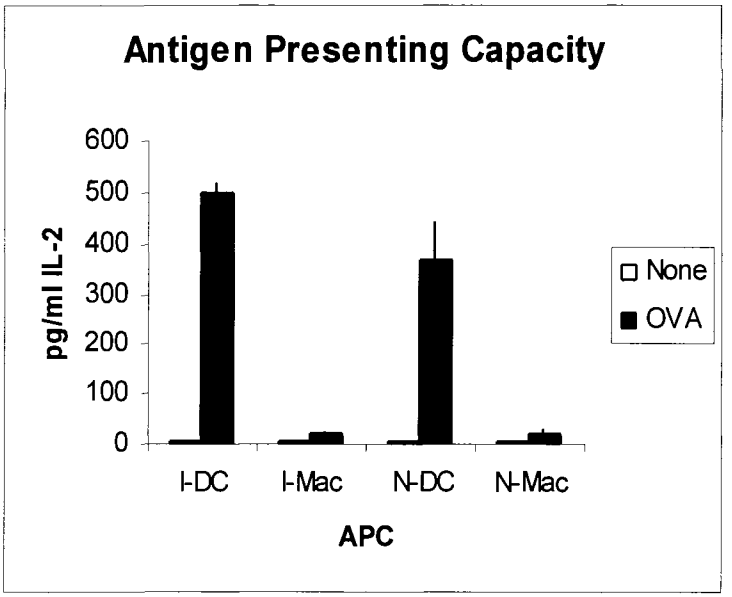


Figure 2.2.1: DCs contain the stimulatory capacity for naïve T cells in the spleen. DCs were positively selected from 3-5 pooled spleens using anti-CD11c-biotin followed by streptavidin coupled magnetic beads. Macrophages were purified from the CD11c- population using positive selection with CD11b. Cells from uninfected and infected mice were cultured with TCR-Tg T cells and 1mg/ml Ova for 24hrs before harvesting for cytokine ELISA.

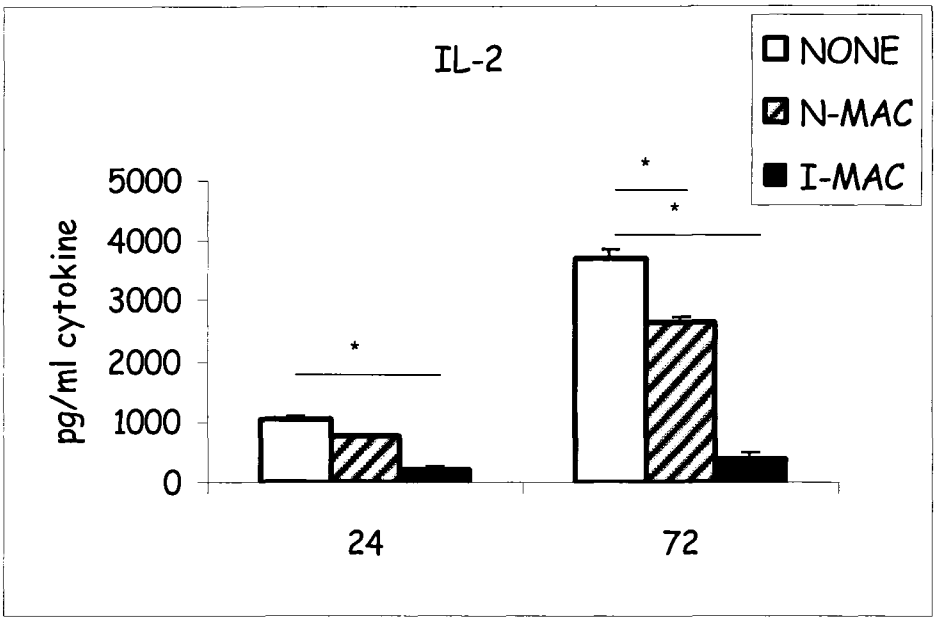


Figure 2.2.2: CD11c+ cells and TCR-Tg T cells cultured with or without naïve or infected CD11b+ cells. Addition of CD11c-CD11b+ cells from naïve and infected mice inhibit IL-2 production, however, these cells from infected mice do so significantly more than their naïve counterparts (* $p < 0.05$).

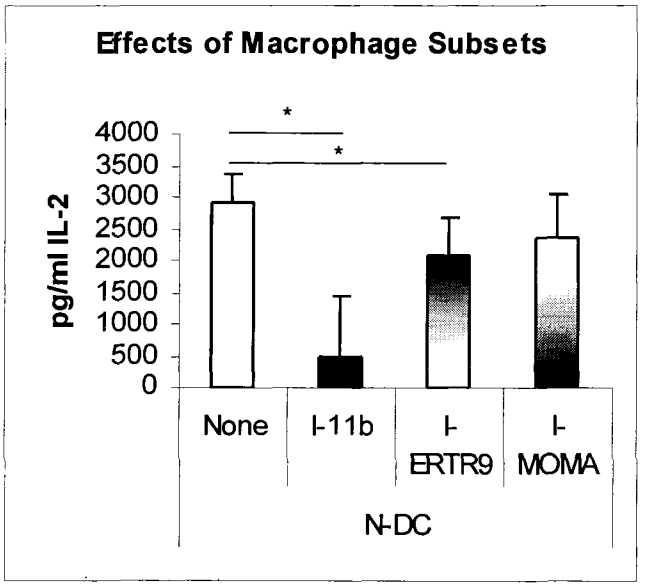


Figure 2.2.3: CD11c cells were first selected from pooled splenocytes from 5 *P. yoelii* infected mice, then the effluent was divided into three groups which were then subjected to positive selection with either CD11b, ERTR9 or MOMA. Both CD11b+ and ERTR9+ cells significantly inhibited IL-2 production, however, ERTR9+ inhibited to a much lower extent relative to CD11b+ cells.

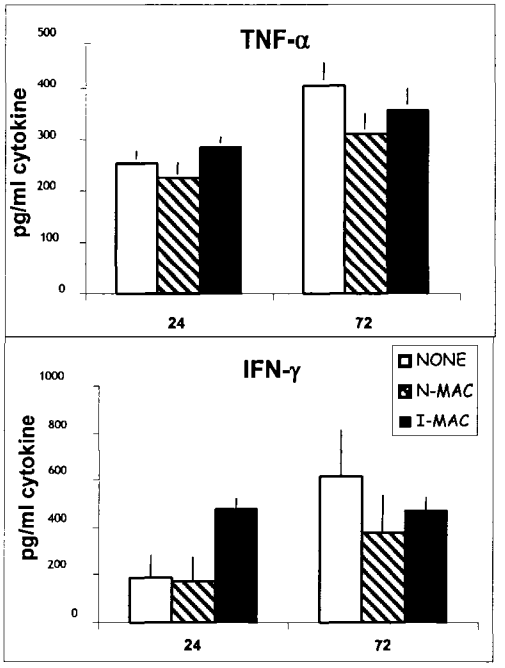


Figure 2.2.4: Pooled splenocytes from five mice from each treatment group were used to generate CD11c-CD11b+ cells from naïve (shaded) or infected (black) were added to cultures of naïve CD11c+ cells and TCR-Tg T cells with Ova. No statistically significant differences were appreciated over 3 experiments in TNF-α or IFN-γ production.

2.3 IL-2 inhibition is not mediated by IL-10 or tryptophan catabolism/deficiency.

Previous work done in the lab showed that the inhibition of IL-2 production by infected splenocytes is mediated by a soluble factor distinct from prostaglandin E_2 (PGE_2), transforming growth factor-beta (TGF-β) and nitric oxide (NO) (Luyendyk, Olivas et al. 2002). These three factors have previously been shown to inhibit IL-2 production in other model systems (Ahvazi, Jacobs et al. 1995; Sternberg 1996; Letterio and Roberts 1998). The TGF-β findings were extended in this dissertation using positively selected $CD11c^-CD11b^+$ cells cultured with $CD11c^+$ DC and naïve TCR-Tg T-cells (Figure 2.3.1). Two additional factors, IL-10 and indoleamine 2,3-dioxygenase (IDO) mediated tryptophan catabolism, were ruled out as mediators of IL-2 inhibition in infected

splenocytes. This was carried out by co-culturing splenocytes from infected mice with TCR-Tg T cells in the presence of Ova and 1-methyl-tryptophan, a competitive inhibitor for IDO and by culturing $CD11c^+$ with or without $CD11c^-CD11b^+$ cells and TCR-Tg T cells in the presence of Ova and anti-IL-10. Neither the addition of anti-IL-10 or 1-methyl-tryptophan significantly improved IL-2 production to that of DCs alone or naïve splenocytes, respectively (Figures 2.3.2 and 2.3.3). The presence of anti-IL-10 did however, significantly increase the IFN-γ production by TCR-Tg T cells at all concentrations tested (Figure 2.3.4), suggesting that an effective blocking concentration of this antibody was being used. Addition of 1-methyl-tryptophan did not significantly alter the levels of any of the cytokines measured. The concentration at which 1-methyl-tryptophan was used has been shown to be effective at competitively inhibiting tryptophan catabolism by other groups (Munn, Shafizadeh et al. 1999).

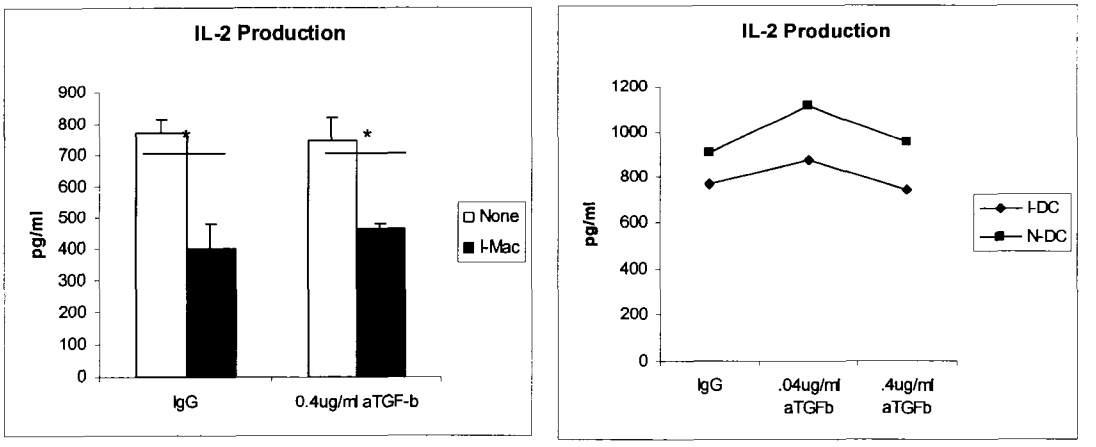


Figure 2.3.1: TGF-β inhibition has little effect on IL-2 production. A) DCs from pooled infected mice (3 mice) were cultured with or without I-Macs from these same mice in the presence of anti-TGF-β or not. (* $p < 0.05$. B) Titration of anti-TGF-β showing that at the higher concentration IL-2 is neither significantly increased or decreased.

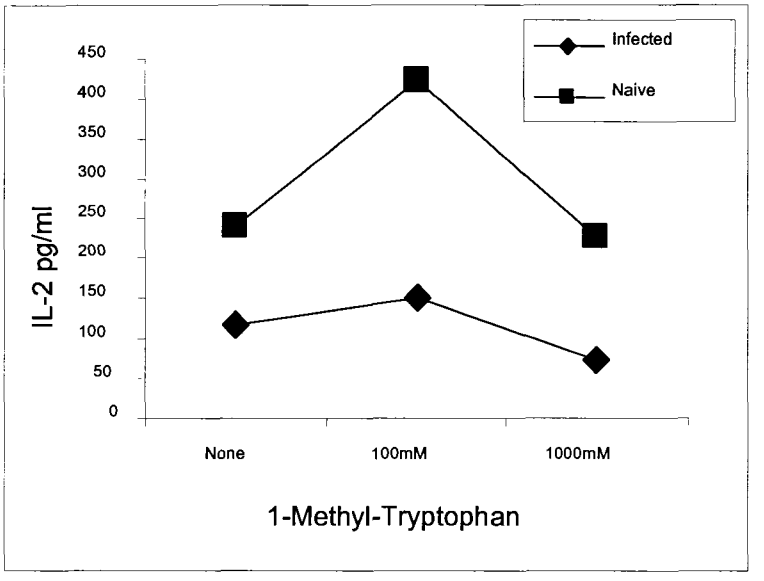


Figure 2.3.2: Splenocytes from 3 individual infected mice were cultured with TCR-Tg T cells in the presence of Ova with or without the indicated concentrations of 1-methyl-tryptophan for 24 hours then IL-2 measured by ELISA. Inhibition of IDO does not recapitulate IL-2 production from T cells activated by splenocytes from infected mice. Data shown is an average of replicates.

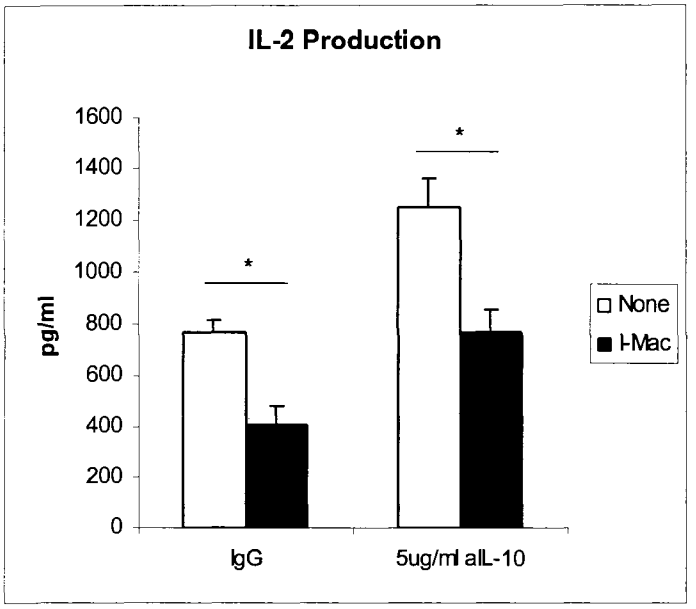


Figure 2.3.3: Infected macrophages inhibit IL-2 production in the presence of anti-IL-10. DCs from pooled spleens from 3 infected mice were cultured with or without I-Macs in the presence of anti-IL-10 or not. While anti-IL-10 did increase IL-2 production in both groups, I-Macs retained their ability to inhibit IL-2 production relative to cultures with DCs alone. * $p < 0.05$.

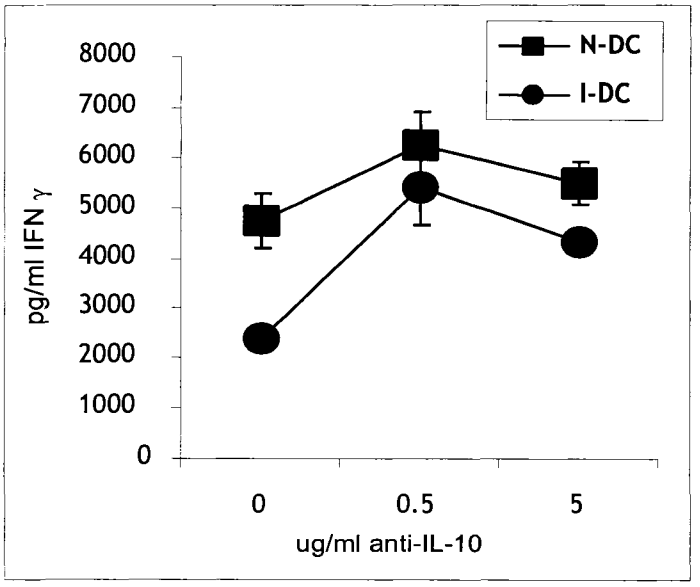


Figure 2.3.4: Anti-IL-10 increased IFN-γ production in T cells cultured with CD11c+ DCs from both naïve and infected mice. DCs from naïve and infected mice were cultured with TCR-Tg T cells and Ova with or without the indicated concentration of anti-IL-10 then IFN-γ measured by ELISA.

Discussion:

In this series of experiments we tested the hypothesis that $CD4^+$ T cell proliferative responses are inhibited during *P. yoelii* infection both *in vivo* and *in vitro*. Our studies here confirm previous observations that malaria infection specifically inhibits IL-2 production and T cell division. While the T cells in our system were responding to unrelated antigen, other studies have established that the presence of a pathogen influences the nature of the T cell response, even when the antigen is not derived from the pathogen. For example, Chen and Jenkins (Chen and Jenkins 1998) showed that TCR-Tg T cells specific for Ova (similar to those used here but on a BALB/c background) responded with high IL-2 and IFN–γ to both Ova expressed by *E. coli*, and to an Ova-containing protein injected together with *E. coli* that did not express Ova. The response however was qualitatively and quantitatively different when the Ova-containing protein was given without co-injection of *E. coli*. Thus the immune response to a pathogen can mold the response to third party antigens, and the response to third party antigens can be used to model pathogen-specific immune responses. Knowing this, we still attempted to create an Ova transgenic strain of *P. yoelii* but were unsuccessful due to poor parasite growth and viability following transfection (data not shown). The ability to produce such a transgenic parasite may become more readily possible as transfection technology improves.

The studies described here confirm a long standing observation that malaria infection leads to a reduced capacity to activate naïve T cells *in vivo*, specifically in association with *P. yoelii* infection. Using the system described above, TCR-Tg T cells stained with CFSE,

allowed us to show that T cells transferred to infected mice undergo reduced division in the face of antigenic stimulation rather than undergo apoptosis. This was evident in the observation that the majority of TCR-Tg T cells isolated post transfer to infected recipients maintained high levels of CFSE expression. Had these cells undergone division followed by activation induced cell death, one would expect to see primarily few TCR-Tg T cells remaining, of which would be CFSE negative.

Based on these findings, we moved to an *in vitro* model system in an attempt to dissect the cellular and molecular mechanisms underscoring this reduction in T cell activation. Macrophages are perhaps the most notable population of cells that have been shown to produce several T cell inhibitory mediators (Cahill and Hopper 1984; Munn, Shafizadeh et al. 1999; van der Veen, Dietlin et al. 2000), therefore we sought to determine the role of these cells in malaria induced T cell inhibition. This hypothesis was confirmed using both depletion and add-back studies with $CD11c^{-}CD11b^{+}$ cells. It is worth mentioning that on average 30% of the $CD11c^{-}CD11b^{+}$ cells were neutrophils cytologically. These cells also express CD11b on their surface which likely explains the observed contamination. We cannot exclude the possibility that these cells participate in the suppression of IL-2 production, since these cells have been shown to mediate IL-2 suppression through direct cell contact (d'Ostiani, Del Sero et al. 2000) and via the production of soluble factors (Kusmartsev, Li et al. 2000). While the specific role of these contaminating neutrophils remains to be addressed, depletion of these cells using the neutrophil specific marker Gr-1 (Ly-6G) prior to culture would likely clarify this issue. A single preliminary experiment done in our lab to address this question showed minimal inhibitory effects of $CD11c^{-}CD11b^{+}Gr\text{-}1^{+}$ cells, while $CD11c^{-}CD11b^{+}Gr\text{-}1^{-}$

cells retain the majority of the IL-2 inhibitor capacity as $CD11c^{-}CD11b^{+}$ cells as a whole (data not shown).

We attempted to isolate the specific population of splenic macrophages responsible for the majority of the IL-2 inhibition by positively selecting MOMA-1^{+} and ERTR-9^{+} cells. These markers were chosen as they appear to be specific for macrophages within the splenic marginal zones—termed marginal zone metallophilic macrophages (MZMM) and marginal zone macrophages (MZMs), respectively, a region where exposure to the vasculature is high. Additionally, previous reports have shown that these subsets of macrophages are highly phagocytic, especially with respect to taking up antigens within the bloodstream (Kraal G 1986; Geijtenbeek, Groot et al. 2002). It was hypothesized that based on their location within the spleen and phagocytic activities, that these subsets of macrophages would be good candidates for mediating IL-2 suppression as their exposure to infected RBCs and parasite is likely high. Based on the results presented here, it appears that these specific subsets have little role in $CD11c^{-}CD11b^{+}$ cell mediated inhibition. While ERTR-9^{+} cells did significantly inhibit IL-2 in our culture system relative to DCs cultured with T cells and antigen alone, the IL-2 inhibition relative to $CD11c^{-}CD11b^{+}$ cells was miniscule.

Previous work by Luyendyk et al. showed that splenocytes from *P. yoelii* infected mice secrete a soluble factor that inhibits T cell activation and proliferation in a cell-cell contact independent fashion (Luyendyk, Olivas et al. 2002). Studies described here show that $CD11c^{-}CD11b^{+}$ cells are primarily responsible for the observed IL-2 inhibition in splenocytes from infected mice and the soluble factor is distinct, or at least not mutually

exclusive, from IL-10, TGF-β, PGE_2, NO or a mediator of tryptophan catabolism. While the specific factors have yet to be identified, future studies using large scale microarray/gene expression analysis followed by functional studies using these results will likely shed light on this subject.

In addition to not knowing the specific factors produced by macrophages mediating this observed IL-2 inhibition, the processes by which macrophages are induced to produce such a factor also remain elusive. One possible mechanism could involve phosphatidylserine (PS) expression on pRBCs as a result of infection (Sherman IW 2004). Phosphatidylserine acts as a ligand to multiple receptors located within the host, specifically CD36 and thrombospondin (TSP). The most well established role of PS is to facilitate the removal of apoptotic cells by macrophages. Receptors on the surface of macrophages bind directly to PS exposed on apoptotic cells and infected erythrocytes resulting in phagocytosis and elimination of these cells. Additionally, receptors for PS are located on the surface of endothelial cells, and binding of pRBCs to these receptors has been implicated in the pathogenesis of CM (Eda S 2002). Two recent studies showed that inhibition of PS exposure on pRBCs prevented CM in a *P. berghei* model (Ponzin, Mancini et al. 1989; Combes, Coltel et al. 2005). Interestingly, patients with a mutation in CD36 show more severe clinical manifestations of CM. It was originally thought that CD36:PS interactions within the post-capillary venules was partly responsible for the pathogenesis of CM, making the finding that individuals with a CD36 mutation have exacerbated CM, surprising. Additionally, CD36 has been shown to cooperate with TLR2 in innate sensing and induction of inflammatory cytokines in response to intact bacteria and bacterial ligands (Hoebe, Georgel et al. 2005). However, there is evidence

suggesting that another major role of CD36:PS interactions is to down modulate immune response. (Ponzin, Mancini et al. 1989). Pilot studies done in our lab looking at PS exposure on pRBCs shows no significant increases during *P. yoelii* infection, nor do we observe macrophages or other CD36 expressing cells respond to *P. yoelii* infected pRBCs (data not shown). Based on these studies looking at the involvement of PS and CD36, it would be interesting to test whether these molecules play a role in macrophage mediated IL-2 inhibition and/or regulation of inflammatory cytokine production.

Our results have implications for the progression of immune responses to malaria, as well as to other antigens encountered during malaria infection, such as those derived from concurrent viral infections or vaccines. Knowing the mechanisms associated with the observed inhibition of T cell responses *in vivo* will allow us to better understand and potentially manipulate these processes in order medically manage malaria infected individuals.

References:

Ahvazi, B. C., P. Jacobs, et al. (1995). "Role of macrophage-derived nitric oxide in suppression of lymphocyte proliferation during blood-stage malaria." J Leukoc Biol **58**(1): 23-31.

Bluthmann, H., P. Kisielow, et al. (1988). "T-cell-specific deletion of T-cell receptor transgenes allows functional rearrangement of endogenous [alpha]- and [beta]-genes." Nature **334**(6178): 156-159.

Cahill, J. and K. E. Hopper (1984). "Immunoregulation by macrophages III prostaglandin E suppresses lymphocyte activation but not macrophage effector function during Salmonella enteritidis infection." International Journal of Immunopharmacology **6**(1): 9-17.

Chen, Z. and M. K. Jenkins (1998). "Revealing the in vivo behavior of CD4+ T cells specific for an antigen expressed in Escherichia coli." Journal of Immunology **160**: 3462-3470.

Combes, V., N. Coltel, et al. (2005). "ABCA1 Gene Deletion Protects against Cerebral Malaria: Potential Pathogenic Role of Microparticles in Neuropathology." Am J Pathol **166**(1): 295-302.

Correa, M., P. R. Narayanan, et al. (1980). "Suppressive activity of splenic adherent cells from *Plasmodium chabaudi*-infected mice." Journal of Immunology **125**: 749-754.

d'Ostiani, C. F., G. Del Sero, et al. (2000). "Dendritic Cells Discriminate between Yeasts and Hyphae of the Fungus Candida albicans: Implications for Initiation of T Helper Cell Immunity In Vitro and In Vivo." J. Exp. Med. **191**(10): 1661-1674.

Eda S, S. I. (2002). "Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine." Cell Physiol Biochem. **12**(5-6): 373-84.

Geijtenbeek, T. B. H., P. C. Groot, et al. (2002). "Marginal zone macrophages express a murine homologue of DC-SIGN that captures blood-borne antigens in vivo." Blood **100**(8): 2908-2916.

Ho, M., H. K. Webster, et al. (1988). "Defective production of and response to IL-2 in acute human falciparum malaria." J Immunol **141**(8): 2755-2759.

Hoebe, K., P. Georgel, et al. (2005). "CD36 is a sensor of diacylglycerides." Nature **433**(7025): 523-527.

Kraal G, J. M. (1986). "Marginal metallophilic cells of the mouse spleen identified by a monoclonal antibody." Immunology **58**(4): 665-9.

Kusmartsev, S. A., Y. Li, et al. (2000). "Gr-1+ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation." Journal of Immunology **165**: 779-785.

Laouar, Y., F. S. Sutterwala, et al. (2005). "Transforming growth factor-[beta] controls T helper type 1 cell development through regulation of natural killer cell interferon-[gamma]." Nat Immunol **6**(6): 600-607.

Letterio, J. J. and A. B. Roberts (1998). "REGULATION OF IMMUNE RESPONSES BY TGF-&#x03B2." Annual Review of Immunology **16**(1): 137-161.

Luyendyk, J., O. R. Olivas, et al. (2002). "Antigen-Presenting Cell Function during Plasmodium yoelii Infection." Infect. Immun. **70**(6): 2941-2949.

Munn, D. H., E. Shafizadeh, et al. (1999). "Inhibition of T Cell Proliferation by Macrophage Tryptophan Catabolism." J. Exp. Med. **189**(9): 1363-1372.

Ocana-Morgner, C., M. M. Mota, et al. (2003). "Malaria Blood Stage Suppression of Liver Stage Immunity by Dendritic Cells." J. Exp. Med. **197**(2): 143-151.

Perry, J. A., A. Rush, et al. (2004). "Dendritic Cells from Malaria-Infected Mice Are Fully Functional APC." J Immunol **172**(1): 475-482.

Ponzin, D., C. Mancini, et al. (1989). "Phosphatidylserine-induced modulation of the immune response in mice: effect of intravenous administration." Immunopharmacology **18**(3): 167-176.

Sayles, P. C. and D. L. Wassom (1988). "Immunoregulation in murine malaria. Susceptibility of inbred mice to infection with Plasmodium yoelii depends on the dynamic interplay of host and parasite genes." J Immunol **141**(1): 241-248.

Scorza, T., S. Magez, et al. (1999). "Hemozoin is a key factor in the induction of malaria-associated immunosuppression." Parasite Immunology **21**(11): 545-554.

Sherman IW, E. S., Winograd E (2004). "Erythrocyte aging and malaria." Cell Mol Biol **50**(2): 159-69.

Sternberg (1996). "Nitric oxide-mediated suppression of T cell responses during Trypanosoma brucei infection: soluble trypanosome products and interferon-gamma are synergistic inducers of nitric oxide synthase." European Journal of Immunology **26**(3): 539-43.

Ungureanu E, K.-K. R., Garnham PC, Branzei P, Romanescu C, Shute PG. (1977). "Prepatent periods of a tropical strain of Plasmodium vivax after inoculations of tenfold dilutions of sporozoites." Trans R Soc Trop Med Hyg **70**(5-6): 482-3.

van der Veen, R. C., T. A. Dietlin, et al. (2000). "Macrophage-Derived Nitric Oxide Inhibits the Proliferation of Activated T Helper Cells and Is Induced during Antigenic Stimulation of Resting T Cells." Cellular Immunology **199**(1): 43-49.

Chapter 3: **Dendritic cells are fully functional during murine malaria infection, and prime the adaptive immune response to *Plasmodium yoelii.***

The work in this chapter was published in part in The Journal of Immunology (2004) 172: 475 – 482.

The hypothesis of this study was that DCs are fully functional and play a role in initiating a robust T_H1 T cell response early during malaria infection.

The aim of this study was to:

1. Determine whether dendritic cells (DCs) are the primary antigen presenting cells during murine malaria infection
2. Determine how DCs shape the immune response during acute blood stage malaria infection.

3.1 Dendritic cells from infected mice contain the majority of the antigen presenting properties of spleen cells.

3.2 Dendritic cells from infected spleens expressed increased levels of CD40 and CD80, but not CD86

3.3 Day 6 PI DC stimulate IL-12, IFN-γ, and TNF-α

Abstract:

Several recent reports address the function of DCs during malaria infection but these offer a mixed picture of the function of these cells *in vivo* and *in vitro*. Here we have directly examined the ability of DCs taken directly from infected mice to activate naïve T cells using a system that allows us to mimic the initial encounter between these two cell types *in vivo*. We find that DCs from infected mice express high levels of co-stimulatory proteins and are equivalent to those of DCs from naïve mice in their ability to support IL-2 production from naïve T cells. Furthermore, DCs from day 6 p.i. mice stimulate markedly higher levels of IFN–γ and TNF-α by responding T cells than their counterparts from uninfected mice. Interleukin-12 inhibitory studies *in vitro* show that this increase in IFN-γ production is through an IL-12 dependent mechanism. Our results indicate that DCs from malaria infected mice are not impaired in their ability to activate T cells, and do not inhibit T cell responses at day 7 post infection. Additionally, DCs appear to have a central role in stimulating high levels of T helper type 1 (T_H1) pro-inflammatory cytokines during the early stages of murine malaria infection.

Introduction:

The elimination of parasites during the liver stage of malaria infection requires a strong cell mediated immune response like most intracellular infections. The importance of the cellular arm of the immune system has been well established in mice lacking a functional thymus (Cavacini, Long et al. 1986). Murine models of blood stage infection have also indicated a requirement for humoral responses in the clearance of malaria infection (von der Weid, Honarvar et al. 1996). Antibodies act both on the exoerythrocytic phase between pRBC lysis and merozoite invasion into another RBC, as well as on infected RBC, where antibodies recognize parasite-derived molecules (i.e. *Pf*EMP1 and likely yet to be described molecules from murine *Plasmodium spp.* (Mota, Brown et al. 2001; Fairhurst, Baruch et al. 2005). A strong humoral response has also been shown to be protective against disease severity through the blocking of specific pyogens such as parasite derived GPI molecules, as will be discussed further in later chapters (Krishnegowda, Hajjar et al. 2005).

There has been a long-standing observation that immune responses, in particular T cell proliferative responses, are inhibited during acute infection (Weinbaum, Weintraub et al. 1978; Morakote and Justus 1988; Schwarzer, Turrini et al. 1992; Omer, de Souza et al. 2003). Most experimental evidence supports the idea that macrophages are the host cell responsible for inhibition of T cell proliferation in malaria and other infectious disease models (Loose 1984; Wangoo, Ganguly et al. 1990; Perry, Rush et al. 2004; Bagchi, Herrup et al. 2007). Two reports however, one using *P. falciparum* (Urban, Ferguson et al. 1999) and the other using *P. yoelii* (Ocana-Morgner, Mota et al. 2003) have introduced

the idea that DCs may participate in the inhibition or failure of T cell responses during malaria infection. Both studies showed that when DCs are matured *in vitro*, in the presence of infected erythrocytes, they express lower levels of MHC class II and co-stimulatory proteins as well as provide poorer stimulation for T cells than control DCs. Additionally, DCs isolated from peripheral blood of children infected with *P. falciparum* showed reduced levels of HLA-DR relative to uninfected controls (Urban, Mwangi et al. 2001). A more recent study by Millington *et al.* showed that that hemazoin, rather than infected erythrocytes, reproduces the inhibitory effect of intact infected red blood cells on LPS induced DC maturation (Millington OR 2006). It is important to note that hemazoin, in the absence of LPS, had no observable effect on cultured DCs in this study.

Results from our lab as well as those of Li *et al.* found increased levels of MHC class II and co-stimulatory proteins on DCs during both *P. yoelii* infection and interaction with *P. chabaudi*, respectively (Li C 2001; Luyendyk, Olivas et al. 2002). Neither study however, tested the ability of DCs to activate naïve T cells. Additionally, preliminary studies done in our laboratory in collaboration with Dr. Peter Henson at National Jewish Medical Center showed no immunogenic activity associated with culturing *P. yoelii* infected pRBCs with naïve whole spleen cells, purified DCs, bone marrow derived DCs, or monocytic cell lines (RAW 267 or J776 cells) (Perry unpublished data), a similar finding too that seen by Millington *et al.* (Millington OR 2006). This is in contrast to other groups which have shown subtle effects of *P. yoelii* (Ocana-Morgner, Mota et al. 2003; Urban 2005)) and different strains of parasites on macrophages and DCs (Ian G. Scragg 1999; Ing, Segura et al. 2006).

In the following set of experiments we sought to determine if DCs from malaria infected mice are inhibitory to T cell activation, as suggested by the literature, or fully functional in their ability to support T cell activation, as suggested by preliminary data from our laboratory and the experiments described in the previous chapter.

MATERIALS AND METHODS

Mice and Plasmodium yoelii infection

Mice used in this series of infections were similarly obtained, housed and treated as described in the previous chapter.

Plasmodium yoelii 17X was used as described in the previous chapter and in (Luyendyk, Olivas et al. 2002). Briefly, at day 6-7 p.i., when the experiments described here were carried out, the average parasitemia is 13% (n = 39).

Antibodies

Antibodies used for purification and staining were purchased from BD Biosciences; anti-Vβ8.1/8.2 (clone MR5-2), anti-CD4 (clone L3T4), anti-CD11c (clone HL3), anti-CD11b (clone M1/70), anti-CD40 (clone 3/23), anti-CD80 (clone 16-10A1), anti-CD86 (clone GL1), anti-CD3 (clone 145-2C11), anti-CD19 (clone 1D3) and anti-class II MHC (clone 2G9). Anti-TCR clonotype antibody (KJ1-26) was purchased from Caltag.

Purification of cellular subsets

Dendritic cells were purified from the pooled spleens of 3 to 5 mice by positive selection with anti-CD11c. Initially we used both directly conjugated anti-CD11c beads (clone N418, Miltenyi) as well as biotinylated anti-CD11c (clone HL3) followed by streptavidin coupled to microbeads (Miltenyi). Cytokine production was similar with either purification, with slightly higher purity and yield using the indirect method. Therefore this method was used for all experiments in this dissertation. Purification was carried out according to the manufacturer. The purity of each population was routinely > 80 percent as determined by flow cytometry. Cells were kept in the separation buffer on ice until they were used, and were then put in culture together with T cells and antigen within 8 - 10 hours of sacrifice.

$CD4^+$ T cells were positively selected from the spleens of TCR-Tg mice. Spleen cells were passed first over Sephadex G10 (Coligan and Kruisbeek 1996), and the non-binding cells used for positive selection with anti-CD4 biotin (clone L3T4) and streptavidin conjugated microbeads (Miltenyi). Purity was greater than 90% in all experiments.

Cell sorting

DC subpopulations ($CD11c^+CD11b^-$ and $CD11c^+CD11b^+$) were sorted by fluorescence activated cell sorting (FACS) as follows. $CD11c^+$ cells were enriched as described above. The cells were then stained with anti-CD3 and anti-CD19 conjugated to phycoerythrin,

anti-CD11c conjugated to biotin, followed by streptavidin-PE-CY5 (BD-Biosciences) and CD11b conjugated to FITC. Cells were sorted on a MoFlo (DakoCytomation) with technical assistance kindly provided by Dako Cytomation. CD3 and CD19 positive cells were outgated, and $CD11c^+CD11b^-$ and $CD11c^+CD11b^+$ sorted into separate populations. In both uninfected and infected mice there were 4x as many $CD11c^+CD11b^+$ cells as $CD11c^+CD11b^-$ cells in the population before sorting. DC and T cells were cultured at a 1:1 ratio (10^5 of each cell type).

Cell culture conditions

Cell cultures were carried out as described in (Luyendyk, Olivas et al. 2002). Briefly, cells were cultured in round-bottomed 96-well plates. Unless otherwise specified, 3 x 10^5 DCs and 2 x 10^5 $CD4^+$ T cells were used in each well, together with 1 mg/ml ovalbumin (OVA). Lower concentrations of OVA resulted in proportionally lower production of each cytokine measured. Titration of DC numbers showed that as the numbers were decreased (to 1:10) IL-2, IFN-γ and TNF-α production decreased proportionally in the presence of both nDC and iDC (not shown).

The ovalbumin (Sigma, Grade V) was not endotoxin free. The addition of OVA to DC cultures without T cells resulted in TNF-α production (but no other cytokine) that was twice the spontaneous release. By contrast, the deliberate addition of LPS to DC cultures resulted in an 8 – 10-fold increase in TNF-α. The magnitude of both effects (OVA and LPS) was similar in DC from infected and uninfected mice. When

macrophages were added to cell cultures to look for inhibitory activity, $3x10^5$ macrophages were added to DCs and T cells.

Cytokine assays

IL-2, IFN–γ, IL-10, and IL-12p40 and p70 cytokine ELISAs were carried out using reagents from B-D BioSciences as described in (Luyendyk, Olivas et al. 2002). Cytokine bead assays (CBA) to quantify IL-2, IFN–γ, TNF–α, IL-4 and IL-5 were obtained from B-D BioSciences and used as described by the manufacturer with the exception that 15ul of sample, beads and detection reagent rather than the recommended 50ul. This change was validated by comparing results obtained by both methods (data not shown). Beads were analyzed on a Coulter Epics XL, using FL2 and FL4 for acquisition. Cytokine quantities obtained by ELISA were similar to those obtained with the bead assay.

Co-Stimulatory Molecule Staining

Whole spleen cells were isolated from either naïve or days 6-7 infected mice. Cells were plated out into 96 well plates and blocked with PBS + 10% FBS for 1hr at 4°C. $2x10^6$ Cells were added to each well. Following the block, the cells were stained with anti-CD3 and anti-CD19 conjugated to biotin, as well as anti-CD11c-APC and either anti-CD80, CD86, CD40 or an isotype control all conjugated to FITC. Streptavidin APC-Cy7 or Alexa Fluor 350 (Molecular Probes) was used to gate out the $CD3^+$ and $CD19^+$ lymphocytes. The expression of co-stimulatory proteins on the $CD11c^+$ population was then determined using a Cyan flow cytometer (Dako Cytomation).

IL-12 inhibition studies

Anti IL-12 studies were conducted in order to measure the dependence of IFN–γ production on IL-12. The IL-12 neutralizing antibody was obtained from Caltag Laboratories (clone C17.8 Cat# 9200). The antibody reacts with the p40 subunit of IL-12. Titration of the Ab showed nearly complete inhibition of IL-12 dependent IFN–γ production at 5 μg/ml and inhibition to a slightly lesser amount occurred at a one fold lower dilution (data not shown). Cells were isolated from naïve or infected mice as described above, and cultured with 5 μg/ml, 0.5 μg/ml or 0 ug/ml anti-IL-12. The 0 ug/ml group contained 5 ug/ml of isotype control.

Statistical analysis:

Two tailed Student's t-test was used to calculate p-values and to compare cytokine levels between treatment groups within an individual experiment. Individual experiments in which cytokines were measured using DCs from uninfected and infected mice generally used pooled spleen cells from 3 to 5 mice, resulting in two treatment groups. In order to analyze cytokine levels between multiple experiments, a mixed effects model was used (Wakkach, Fournier et al. 2003). Using this model, we were able to directly compare the differences in cytokine levels between treatments across multiple experiments and obtain p-values for these differences. The value of this approach was that we could measure multiple independent infections. All mixed model analyses were made using the SAS system software.

RESULTS

3.1 CD11c$^+$ cells contain the majority of stimulatory capacity for naïve T cells in the spleens of malaria infected mice

The focus of these studies was to determine how DCs guide T cell responses to malaria. Specifically, we wanted to understand how DCs influence the early phases of immunity to malaria, when the T cell response is initiated. In previous studies, we established that TCR-Tg T cells, when transferred to infected mice, responded to antigen (Ova) with high levels of IFN–γ, low levels of IL-2, and reduced cellular division (Luyendyk, Olivas et al. 2002; Perry, Rush et al. 2004) reflecting the behavior described for malaria specific T cells at early stages of infection. Therefore we chose to use this system to dissect antigen-presenting cell function *in vitro*.

In order to confirm that DCs are the cells primarily responsible for the activation of naïve T cells in our system, we purified CD11c$^+$ DCs pooled spleens of 3-5 naive and day 6-7 p.i. mice. We purified these cells directly through positive magnetic selection based upon the expression of surface markers (CD11c$^+$), rather than differential adherence and culture methods. This was done because the latter protocols are known to affect the maturation state and function of both DCs and macrophages (Aliberti, Reis e Sousa et al. 2000; Kamath, Pooley et al. 2000; Sweet, Leung et al. 2001). Macrophage enriched populations were positively selected from the CD11c negative cells using anti-CD11b. We then asked which of these populations was able to support the activation of naïve T cells.

As expected, the DC population contained the majority of the T cell accessory capacity for IL-2 and IFN–γ production in both naïve and infected mice (Figures 3.1.1 and 2.2.1). TNF-α is also significantly upregulated in these cultures with DCs as well as macrophages (Figure 3.1.2). In these experiments, DCs from naïve and days 6-7 mice were cultured with naïve TCR-Tg T cells, with and without antigen. The $CD11c^{-}CD11b^{+}$ cells alone supported small amounts of TNF–α, but no IFN–γ or IL-2 from naïve T cells (Figure 3.1.1). In addition, purified B cells from either uninfected or acutely infected mice had minimal capacity to support IL-2 production by naïve T cells (data not shown). Cytokine production in the absence of Ova was below the limits of detection (data not shown and Fig 3.1.2)

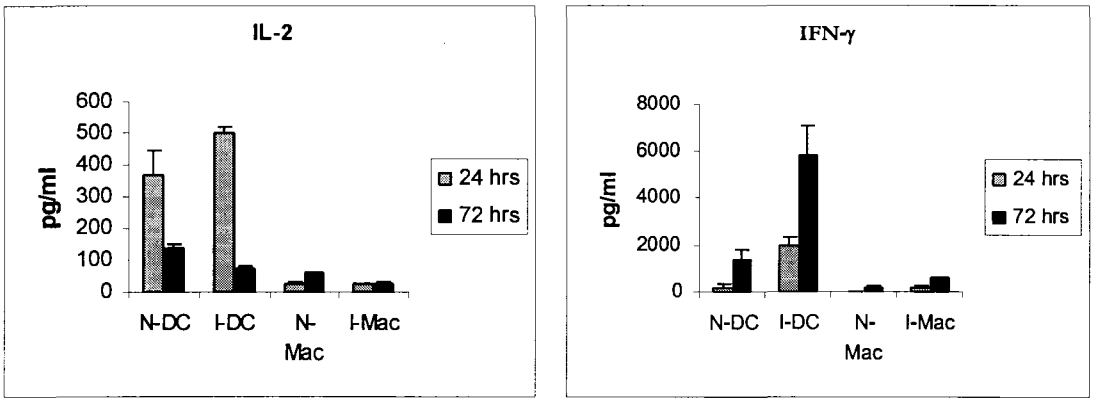


Figure 3.1.1: DC contain the majority of the stimulatory capacity for naïve T cells in the spleen. DCs from pooled spleens of 3-5 mice in each experiment were purified by positive selection using anti-CD11c-biotin followed by streptavidin coupled magnetic beads. Macrophages were purified from the CD11c- population using positive selection with CD11b. Cells from uninfected and infected mice were cultured with TCR-Tg T cells and 1mg/ml ovalbumin for the indicated times before harvesting for cytokine ELISA.

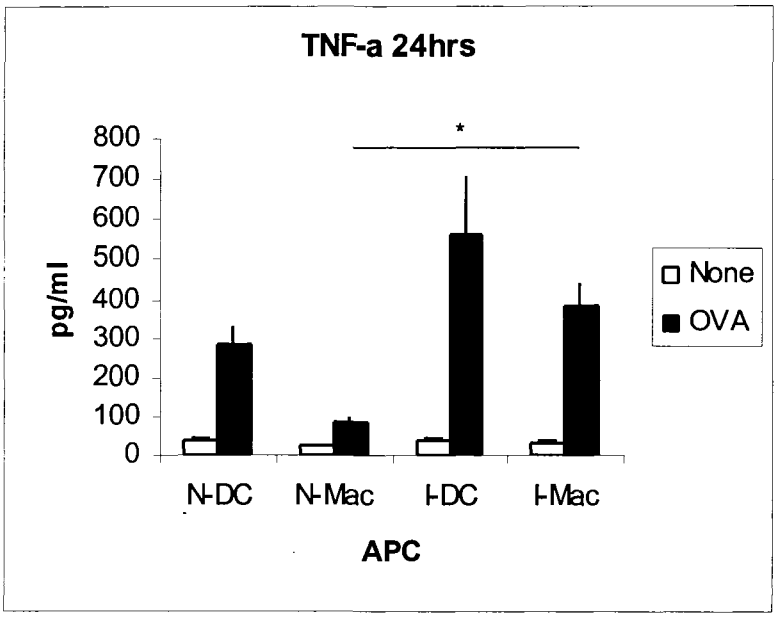


Figure 3.1.2: Macrophages support significant amounts of antigen specific TNF-α. Macrophages and DCs from day 7 p.i. or naïve mice were cultured with TCR-Tg T cells with or without Ova then TNF-α measured by CBA assay.

3.2 Dendritic cells from infected spleens expressed increased levels of CD40 and CD80.

Nominal T cell activation requires the expression of co-stimulatory proteins on the surface of DCs, and presentation of antigen by MHC Class II. We measured the expression of MHC class II, CD80, CD86 and CD40, all contributors to CD4 T cell activation, on DCs isolated from naïve and infected mice at various time points during *P. yoelii* infection. A higher percentage of DCs from day 6 p.i. mice express MHC class II, CD80 and CD40 relative to naïve controls (Figure 3.2.1). Similarly, significant differences can be seen when using mean fluorescence intensity to assess the level of CD80 expression (data not shown). In other words, not only does infection increase the

number of cells expressing these costimulatory molecules, but the majority of the cells expressing these molecules are expressing them at a higher level. Unexpectedly, expression of CD86 remained low on DC throughout infection and no significant difference between DCs from naïve and infected mice could be appreciated. (data not shown).

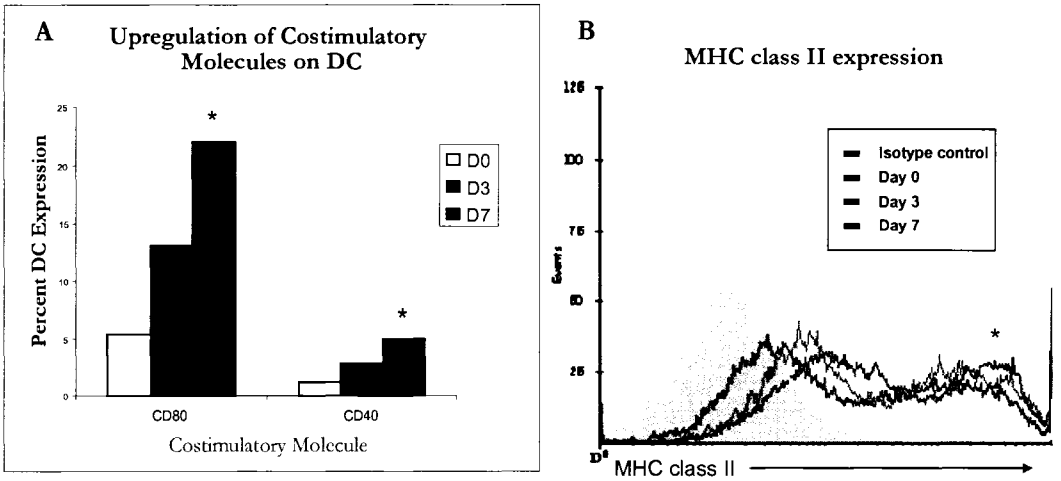


Figure 3.2.1: Splenocytes were isolated from day 3 and 7 p.i. mice and DCs were analyzed for costimulatory molecule expression by flow cytometry. A) CD11c+MHCII+ cells were analyzed for expression of CD80 and CD40. B) Total CD11c+ cells from these same mice were analyzed based on mean fluorescent intensity of MHC Class II expression (* $p < 0.05$).

3.3 Day 6 PI DC stimulate IL-12, IFN-γ, and TNF-α

In conjunction with the experiments described in 3.1 of this dissertation, we compared the ability of DCs from day 7 p.i. mice to support the production of pro-inflammatory cytokines (IFN–γ and TNF–α), and anti-inflammatory cytokines (IL-4, IL-5 and IL-10) from naïve T cells. The amounts of IL-4 and IL-5 detected were at or below the limits

of detection (approximately 15 pg/ml for each) in all assays regardless of the source of DCs. This was true even when cells were stimulated for 5 days, then restimulated with fresh antigen and APC (whole spleen cells) for two additional days (data not shown).

By contrast, DCs from day 7 p.i. mice supported high levels of IL-2, IFN–γ and TNF–α. Cytokine production in this system was dependent upon the addition and commingling of DCs, naïve T cells and antigen, since production of all cytokines was nominal in the absence of these elements (Table 1). As discussed in 3.1, and shown here, DCs from day 7 p.i. mice and naïve DCs did not differ in their ability to support IL-2 production when measured at either 24 or 72 hours of culture. By contrast, IFN–γ was consistently higher when DCs from day 7 p.i. mice were used as APC ($p < 0.05$). TNF–α also tended to be higher in the presence of DCs from day 7 p.i. mice, but this comparison did not achieve significance ($p = 0.08$). Table 2 summarizes the results of 12 experiments carried out over the course of greater than 1 year. Our findings indicate that DCs from day 7 p.i. mice are as effective at T cell activation as naïve DCs and that they support a higher level of pro-inflammatory/type 1 cytokines by T cells at this stage of infection.

The major stimulus for IFN–γ production is IL-12, although an IL-12 independent pathway for IFN–γ production has been described for *Plasmodium berghei* (Feng, Watanabe et al. 1999). Therefore we measured IL-12 p40 and p70. IL-12 p40 was significantly higher in DCs from day 7 p.i. mice than DCs from naïve mice over 3 experiments, one of which is shown in Figure 3.3.1. IL-12 p70 was undetectable by

ELISA at 24 or 72 hours ($x < 20$ pg/ml). We then determined if IFN–γ production in our system was dependent upon IL-12 by adding anti-IL-12 to cultures with iDCs plus naïve T cells. The anti p40 antibody effectively abrogated the detection of IL-12 p40, and reduced the production of IFN–γ to less than 5% of the no anti-IL-12 antibody control (Figure 3.3.2). Furthermore, IFN–γ production depended upon the presence of antigen (Table I), indicating that contaminating NK cells in the DC preparation could not account for the observed IFN–γ. This finding indicates that biologically relevant concentrations of IL-12 were present even though IL-12 p70 was not detectable.

In order to rule out the possibility that a small contaminating population of cells co-purified with DCs was influencing cytokine production, we sort purified the $CD11c^+$ subsets, $CD11c^+CD11b^-$ and $CD11c^+CD11b^+$ populations from CD3/CD19 negatively selected splenocytes (Figure 3.3.2a). These two subpopulations represent the major subsets of murine dendritic cells; the myeloid ($CD11c^+CD11b^+$) and the lymphoid ($CD11c^+CD11b^-$) (Shortman and Liu 2002).

When sorted DCs were cultured with naïve T cells and antigen, both subpopulations ($CD11c^+CD11b^-$ and $CD11c^-CD11b^+$) from infected mice supported substantially higher levels of IL-2, IFN-γ and TNF-α than the same population from uninfected mice (Figure 3.3.2b), confirming the behavior observed when DC were purified by positive selection with magnetic beads. In order to make this comparison directly, $CD11c^+CD11b^-$ and $CD11c^-CD11b^+$ cells were mixed together at their original ratios and cultured with T cells and Ova (Figure 3.3.2b). The results again confirmed that DCs

from infected mice are fully functional in their ability to support cytokine production by responding T cells. A second experiment comparing sorted $CD11c^+CD11b^+$ DCs from infected mice and naïve DCs yielded similar results. Interestingly, we found $CD11c^+CD11b^+$ cells carried higher levels of hemazoin, a parasite derived breakdown product of heme, relative to their $CD11c^+CD11b^-$ counterparts (Figure 3.3.4). As will be discussed in the subsequent chapter, it appears that this differential uptake of hemazoin correlates with a decreased production of IL-12 and TNF-α during the late stages of infection.

Table I: IL-2, IFN–γ and TNF–α production (all shown in pg/ml) by naïve T cells is dependent upon the addition of antigen. In each experiment, nDCs and iDCs were obtained from pooled splenocytes of 3-5 mice.

		T cells	-	+	+
		OVA	-	-	+
IL-2	***nDC***		1.9	1.9	532.7
	iDC		1.9	1.9	448.6
IFN-γ	***nDC***		2.2	2.0	28.1
	iDC		5.2	5.2	1697.5[a]
TNF-α	***nDC***		4.2	4.5	241.6
	iDC		10.2	9.5	288.1

[a]The difference between infected and uninfected DC was different using the t test ($p<0.05$).

Table II: Cytokine production by naïve T cells in the presence of dendritic cells derived from infected and uninfected mice. Again, in each experiment nDCs and iDCs were obtained from pooled splenocytes of 3-5 mice.

	Hour Harvest	Average ratio of uninf:inf[a]	N[b]
IL-2	*24*	1.2	8
	72	1.1	5
IFN-γ	*24*	0.4	6[c]
	72	0.3	5[c]
TNF-α	*24*	0.5	4[d]
	72	0.7	4

[a]For each experiment, the mean pg/ml of cytokine from cultures with nDCs was divided by mean pg/ml from cultures with iDCs. This ratio from all experiments was averaged.

[b]The number of experiments used to contribute to this number.

[c]Statistically significant difference between uninfected and infected, $p < 0.05$ using mixed effects model

[d]DCs from uninfected mice supported higher TNF-α production after 24 hours $p = 0.08$

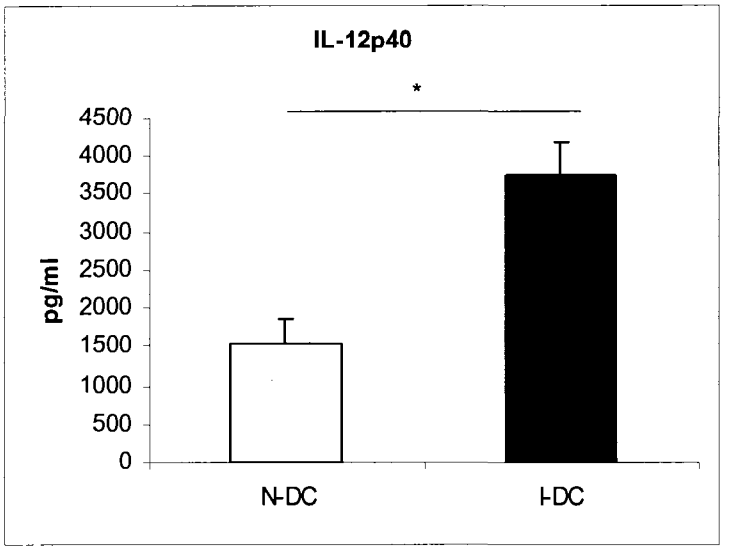


Figure 3.3.1: One of three experiments showing that IL-12p40 production was significantly higher in DCs day 7 p.i. mice than DCs from naïve mice. CD11c+ DCs from pooled spleens of 3-5 naïve or day 7 p.i. mice were cultured with naïve TCR-Tg T cells with Ova. IL-12p40 levels were measured 24hrs later by ELISA. * $p < 0.05$.

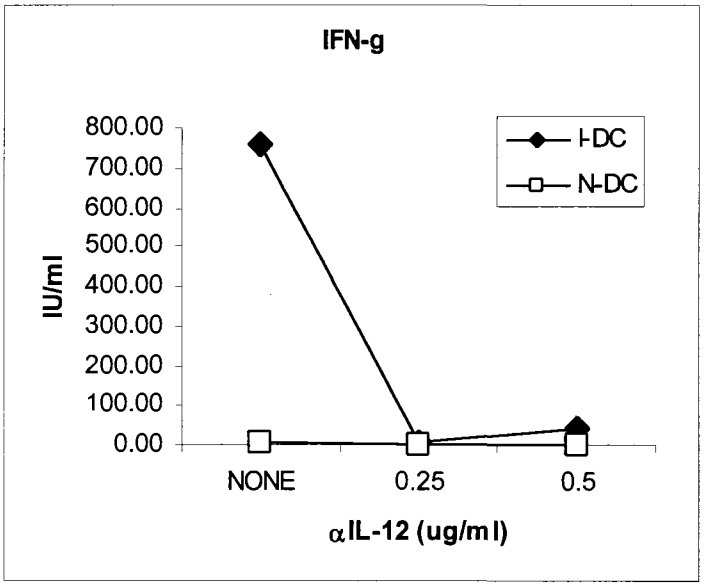


Figure 3.3.2: IFN-γ production by naïve T cells stimulated with DCs is IL-12 dependent. Dendritic cells from naïve of day 6 p.i. mice were cultured with TCR-Tg T cells in the presence of Ova with or without the addition of various concentrations of anti-IL-12. Data shown is from of a single experiment with treatments done in triplicate.

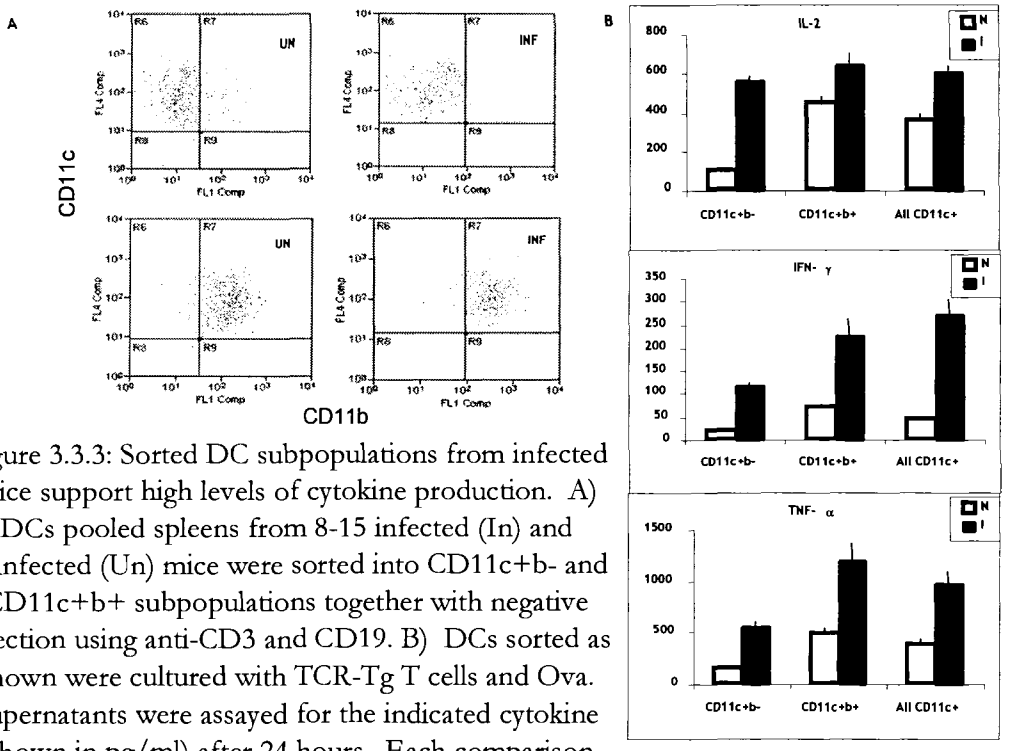


Figure 3.3.3: Sorted DC subpopulations from infected mice support high levels of cytokine production. A) DCs pooled spleens from 8-15 infected (In) and uninfected (Un) mice were sorted into CD11c+b- and CD11c+b+ subpopulations together with negative selection using anti-CD3 and CD19. B) DCs sorted as shown were cultured with TCR-Tg T cells and Ova. Supernatants were assayed for the indicated cytokine (shown in pg/ml) after 24 hours. Each comparison between triplicate cultures with nDC and iDC was statistically significant.

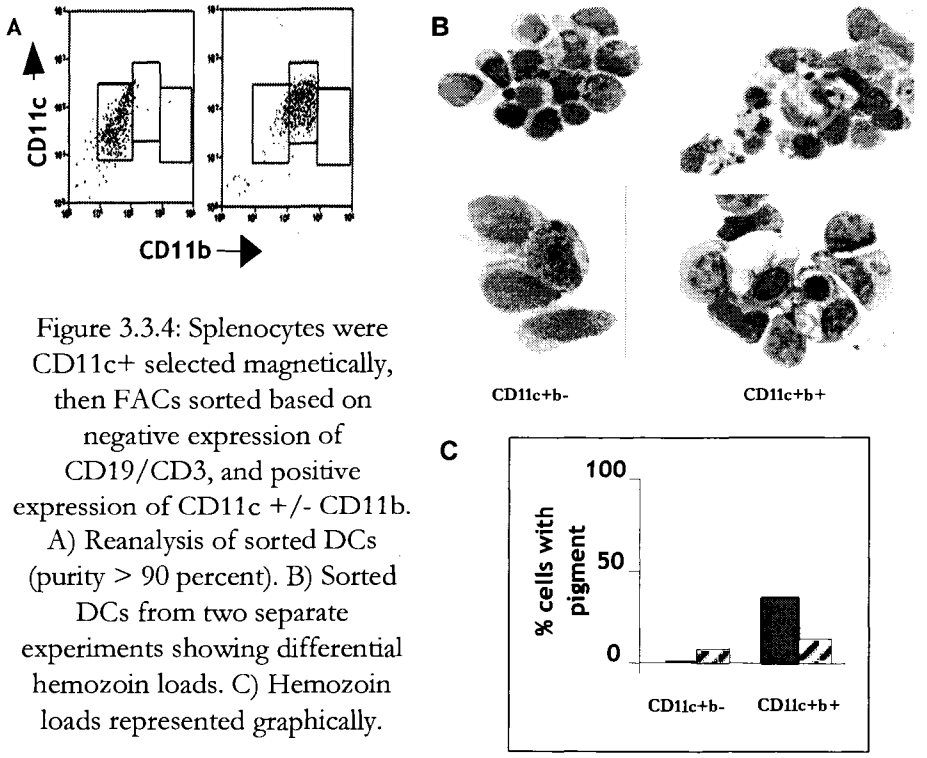


Figure 3.3.4: Splenocytes were CD11c+ selected magnetically, then FACs sorted based on negative expression of CD19/CD3, and positive expression of CD11c +/- CD11b. A) Reanalysis of sorted DCs (purity > 90 percent). B) Sorted DCs from two separate experiments showing differential hemozoin loads. C) Hemozoin loads represented graphically.

DISCUSSION

When these experiments were initiated, there was a paucity of studies regarding the function of DCs during malaria infection, and those studies that were available offered conflicting results. Ocaña-Morgner et al and Urban *et al.* demonstrate that immature bone marrow derived DCs from mice or peripheral DCs from naive people, when matured in the presence of malaria parasites fail to mature in response to subsequent LPS stimulation. It is important to note that both of these studies looked at DC maturation in immature cells isolated from bone marrow or peripheral blood, rather than specifically looking at DCs exposed to pRBCs *in situ* in the company of other cell types and factors associated with infection.

The experiments described here were performed in order to gain a better understanding of how DCs are affected by malaria infection *in vivo*, and their role in T cell activation during the acute phase of murine malaria infection, specifically days 6 and 7 post infection. We addressed several important questions about the interaction between DCs and naïve T cells during infection. First, we asked if DCs are responsible for diminished T cell responses frequently described in malaria as suggested by others (Urban, Ferguson et al. 1999; Ocana-Morgner, Mota et al. 2003), second, we asked how DCs might control the pro-inflammatory response documented early in both human and murine malaria (De Souza, Williamson et al. 1997; Othoro, Lal et al. 1999; Riley, Wahl et al. 2006) and lastly looked at the phenotypic and functional role of specific DC subsets ($CD11c^+CD11b^-$ and $CD11c^+CD11b^+$ DCs) at this time during infection.

The experimental approach that we chose was to isolate and analyze splenic DCs from infected mice, and study their function after purification by either positive selection or fluorescence activated cell sorting (FACS)—all done on ice without a period of culture. In this way their function *in vitro* would most closely reflect their function *in vivo*. The ability of these DCs to activate a heterologous homogenous population of naïve T cells was then assessed in order to re-create the initial encounter between DCs and naïve T cells. We found that DCs taken directly from infected mice, which have matured *in vivo* rather than *in vitro*, 1) express greater levels of MHC class II, CD40 and CD80 than DCs from uninfected mice 2) support proliferation and IL-2 production by naïve T cells to the same extent as DCs from uninfected mice, and 3) support substantially higher levels of IFN–γ from responding T cells in an IL-12 dependant manner as well as TNF-α. A potential limitation to our model system is that the T cells used in these studies were not specific for a malaria specific antigen, rather TCR-Tg T cells respond to exogenous Ova. While the T cells in our system were responding to unrelated antigen, other studies have established that the presence of a pathogen influences the nature of the T cell response, even when the antigen is not derived from the pathogen.

In further support of the validity of our model, and that DCs do support T cell activation efficiently during malaria infection, we showed in earlier studies that DCs purified directly from infected mice stimulated high levels of IL-2 production by a *P. yoelii* specific T cell hybridoma without the addition of additional parasite antigen, indicating that the purified DCs present parasite antigen *ex vivo* (Perry, Rush et al. 2004).

Two recent reports have suggested that human and murine DCs grown in GM-CSF and IL-4, when exposed to malaria infected erythrocytes *in vitro* or *in vivo* and then matured with LPS, have diminished T cell stimulatory capacity (Urban, Ferguson et al. 1999; Ocana-Morgner, Mota et al. 2003). In our studies, DCs taken directly from infected mice showed no impairment of their ability to activate T cells. While it is possible that differences in the responding T cell populations may account for some of the discrepancies between this report and previous ones, we favor the idea that the different signals to which the DCs are exposed during development *in vivo*, in the context of infection, explain the results.

When DCs are matured *in vitro* using LPS they mature without cross talk between T and other cell types. A recent study by Straw *et al.* found that even when DCs are exposed to infectious stimuli provided by another protozoan pathogen, *T. gondii*, *in vivo*, they fail to exhibit a mature phenotype in the absence of T cells. This group subsequently determined that CD40-CD40L interactions on DCs and T cells, respectively, provide the required costimulation (Straw, MacDonald et al. 2003). CD40 ligation on DCs has been shown to activate pathways similar to TLRs (Figure 1.5.6) as well as activation of the noncanonical NF-κB pathway (p52/RelB) (Martin, O'Sullivan et al. 2003; Quezada, Jarvinen et al. 2004; Speirs, Lieberman et al. 2004). This later pathway has been implicated in DC maturation with respect to costimulatory molecule expression (Speirs, Lieberman et al. 2004). Reis e Sousa *et al.* found that CD40 ligation was required for maximal stimulation of cytokine production by DCs, even in the presence of potent microbial stimuli (Sweet, Leung et al. 2001; Cao, Zhang et al. 2006). Thus, the phenotype and responsiveness of DCs grown *in vitro* without direct T cell signaling may

be very different than that of DCs grown *in vivo*. We hypothesize that when T cells are present during DC development and maturation, the signals they provide to DCs overcome the inhibitory effects of infected erythrocytes.

Other factors that could influence DC maturation *in vivo*, including signaling through multiple TLRs, antigen uptake through Fc or complement receptors, and interaction of DCs with aberrant host cells (i.e. apoptotic and necrotic cells) are also not recapitulated in *in vitro* culture systems. For example, DCs have been shown to respond to malarial GPI anchors via TLR2/6 (Zhu, Krishnegowda et al. 2005), hemazoin:DNA complexes via TLR9 (Pichyangkul, Yongvanitchit et al. 2004; Coban, Ishii et al. 2005; Parroche, Lauw et al. 2007) and profilin via TLR 11 (Yarovinsky, Zhang et al. 2005). Repeated stimulation TLRs 2/6, 9 and/or 11, or stimulation of these receptors in the context of infection likely affects the quality and quantity of DC activation as compared to pRBCs and LPS alone. In support of this, we show here that $CD11c^{+}CD11b^{+}$ and to a lesser amount $CD11c^{+}CD11b^{-}$ DCs appear to phagocytize and retain significant levels of hemazoin pigment, a known innate stimulus (Pichyangkul, Yongvanitchit et al. 2004). The presence of such a factor may influence *in vivo* maturation, something that is not present when pRBCs are cultured with naïve DCs. Fc and complement receptor ligation on DCs has also been shown to effect maturation in these cells (Boruchov AM 2005; Reis, Barbuto et al. 2008). Boruchov *et al.* showed that ligation of the CD32a (FcγRIIa) led to enhance maturation and T cell stimulatory capacity of DCs (Boruchov AM 2005). Reis *et al.* showed similar findings with respect to the presence of complement factor C3. Specifically, they found that in the absence of C3, DCs retain a more immature phenotype when stimulated with LPS relative to DCs cultured in the presence of C3

(Reis, Barbuto et al. 2008). Based on these differences between *in vitro* and *ex vivo* systems, we suggest that the functional studies of DCs taken directly from an infected environment more closely resemble their phenotype *in vivo* relative to activation of DCs in culture with pRBC.

Our results have implications for the progression of immune responses to malaria pathogens, as well as to other antigens encountered during malaria infection, such as those derived from concurrent secondary infections or vaccines. During the acute phase of *P. yoelii* infection, naive T cells recruited to the immune response may be stimulated to produce IFN–γ and TNF–α. As discussed in the following chapter, regulation of these cytokines can be crucial in determining the balance between protection and pathologic responses.

References:

Aliberti, J., C. Reis e Sousa, et al. (2000). "CCR5 provides a signal for microbial induced production of IL-12 by CD8[alpha]+ dendritic cells." Nat Immunol **1**(1): 83-87.

Bagchi, A., E. A. Herrup, et al. (2007). "MyD88-Dependent and MyD88-Independent Pathways in Synergy, Priming, and Tolerance between TLR Agonists." J Immunol **178**(2): 1164-1171.

Boruchov AM, H. G., Veri MC, Bonvini E, Ravetch JV, Young JW. (2005). "Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions." J Clin Invest. **115**(10): 2914-23.

Cao, S., X. Zhang, et al. (2006). "NF-{kappa}B1 (p50) Homodimers Differentially Regulate Pro- and Anti-inflammatory Cytokines in Macrophages." J. Biol. Chem. **281**(36): 26041-26050.

Cavacini, L. A., C. A. Long, et al. (1986). "T-cell immunity in murine malaria: adoptive transfer of resistance to Plasmodium chabaudi adami in nude mice with splenic T cells." Infect. Immun. **52**(3): 637-643.

Coban, C., K. J. Ishii, et al. (2005). "Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin." Journal of Experimental Medicine **201**(1): 19-25.

Coligan, J. E. and A. M. Kruisbeek, Eds. (1996). Current Protocols in Immunology, John Wiley.

De Souza, J. B., K. H. Williamson, et al. (1997). "Early gamma interferon responses in lethal and nonlethal murine blood- stage malaria." Infect. Immun. **65**(5): 1593-1598.

Fairhurst, R. M., D. I. Baruch, et al. (2005). "Abnormal display of PfEMP-1 on erythrocytes carrying haemoglobin C may protect against malaria." Nature **435**(7045): 1117-1121.

Feng, C., S. Watanabe, et al. (1999). "An alternate pathway for type 1 T cell differentiation." International Immunology **11**(8): 1185-1194.

Ian G. Scragg, M. H. C. A. W. B. D. K. (1999). "Early cytokine induction by <I>Plasmodium falciparum</I> is not a classical endotoxin-like process." European Journal of Immunology **29**(8): 2636-2644.

Ing, R., M. Segura, et al. (2006). "Interaction of Mouse Dendritic Cells and Malaria-Infected Erythrocytes: Uptake, Maturation, and Antigen Presentation." J Immunol **176**(1): 441-450.

Kamath, A. T., J. Pooley, et al. (2000). "The Development, Maturation, and Turnover Rate of Mouse Spleen Dendritic Cell Populations." J Immunol **165**(12): 6762-6770.

Krishnegowda, G., A. M. Hajjar, et al. (2005). "Induction of Proinflammatory Responses in Macrophages by the Glycosylphosphatidylinositols of Plasmodium falciparum: Cells Signaling Receptors, Glycosylphophatidylinositol (GPI) Structural Requirement, and Regulation of GPI Activity." J. Biol. Chem. **280**(9): 8606-8616.

Li C, S. E., Langhorne J. (2001). "Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite." Med Microbiol Immunol. **189**(3): 115-26.

Loose, L. D. (1984). "Characterization of macrophage dysfunction in rodent malaria." Journal of Leukocyte Biology **36**: 703-718.

Luyendyk, J., O. R. Olivas, et al. (2002). "Antigen-Presenting Cell Function during Plasmodium yoelii Infection." Infect. Immun. **70**(6): 2941-2949.

Martin, E., B. O'Sullivan, et al. (2003). "Antigen-Specific Suppression of a Primed Immune Response by Dendritic Cells Mediated by Regulatory T Cells Secreting Interleukin-10." Immunity **18**(1): 155-167.

Millington OR, D. L. C., Phillips RS, Garside P, Brewer JM. (2006). "Suppression of adaptive immunity to heterologous antigens during Plasmodium infection through hemozoin-induced failure of dendritic cell function." J Biol. **5**(2): 5.

Morakote, N. and D. E. Justus (1988). "Immunosuppression in malaria: effect of hemozoin produced by Plasmodium berghei and Plasmodium falciparum." International Archives of Allergy and Applied Immunology **86**: 28-34.

Mota, M. M., K. N. Brown, et al. (2001). "Antibody Recognition of Rodent Malaria Parasite Antigens Exposed at the Infected Erythrocyte Surface: Specificity of Immunity Generated in Hyperimmune Mice." Infect. Immun. **69**(4): 2535-2541.

Ocana-Morgner, C., M. M. Mota, et al. (2003). "Malaria Blood Stage Suppression of Liver Stage Immunity by Dendritic Cells." J. Exp. Med. **197**(2): 143-151.

Omer, F. M., J. B. de Souza, et al. (2003). "Differential Induction of TGF-{beta} Regulates Proinflammatory Cytokine Production and Determines the Outcome of Lethal and Nonlethal Plasmodium yoelii Infections." J Immunol **171**(10): 5430-5436.

Othoro, C., A. A. Lal, et al. (1999). "A low interleukin-10 tumor necrosis factor-a ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya." Journal of Infectious Diseases **1999**: 279-282.

Parroche, P., F. N. Lauw, et al. (2007). "From the Cover: Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting

malaria DNA to Toll-like receptor 9." Proceedings of the National Academy of Sciences **104**(6): 1919-1924.

Perry, J. A., A. Rush, et al. (2004). "Dendritic Cells from Malaria-Infected Mice Are Fully Functional APC." J Immunol **172**(1): 475-482.

Pichyangkul, S., K. Yongvanitchit, et al. (2004). "Malaria Blood Stage Parasites Activate Human Plasmacytoid Dendritic Cells and Murine Dendritic Cells through a Toll-Like Receptor 9-Dependent Pathway." J Immunol **172**(8): 4926-4933.

Quezada, S. A., L. Z. Jarvinen, et al. (2004). "CD40/CD154 Interactions at the Interface of Tolerance and Immunity." Annual Review of Immunology **22**(1): 307-328.

Reis, E. S., J. A. M. Barbuto, et al. (2008). "Impaired dendritic cell differentiation and maturation in the absence of C3." Molecular Immunology **In Press, Corrected Proof**.

Riley, E. M., S. Wahl, et al. (2006). "Regulating immunity to malaria." Parasite Immunology **28**(1-2): 35-49.

Schwarzer, E., F. Turrini, et al. (1992). "Impairment of macrophage function after ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment." Journal of Experimental Medicine **176**: 1033-1041.

Shortman, K. and Y.-J. Liu (2002). "Mouse and human dendritic cell subtypes." Nat Rev Immunol **2**(3): 151-161.

Speirs, K., L. Lieberman, et al. (2004). "Cutting Edge: NF-{kappa}B2 Is a Negative Regulator of Dendritic Cell Function." J Immunol **172**(2): 752-756.

Straw, A. D., A. S. MacDonald, et al. (2003). "CD154 plays a central role in regulating dendritic cell activation during infections that induce Th1 orTh2 responses." Journal of Immunology **170**: 727-734.

Sweet, M. J., B. P. Leung, et al. (2001). "A Novel Pathway Regulating Lipopolysaccharide-Induced Shock by ST2/T1 Via Inhibition of Toll-Like Receptor 4 Expression." J Immunol **166**(11): 6633-6639.

Urban, B., Ing R, Stevenson MM (2005). "Early interactions between blood-stage plasmodium parasites and the immune system." Curr Top Microbiol Immunol. **297**: 25-70.

Urban, B. C., D. J. P. Ferguson, et al. (1999). "Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells." Nature **400**(6739): 73-77.

Urban, B. C., T. Mwangi, et al. (2001). "Peripheral blood dendritic cells in children with acute Plasmodium falciparum malaria." Blood **98**(9): 2859-2861.

von der Weid, T., N. Honarvar, et al. (1996). "Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection." J Immunol **156**(7): 2510-2516.

Wakkach, A., N. Fournier, et al. (2003). "Characterization of Dendritic Cells that Induce Tolerance and T Regulatory 1 Cell Differentiation In Vivo." Immunity **18**(5): 605-617.

Wangoo, A., N. K. Ganguly, et al. (1990). "Immunosuppression in murine malaria: suppressor role of macrophages and their products during acute and chronic *Plasmodium berghei* infection." APMIS **98**: 407-414.

Weinbaum, F. I., J. Weintraub, et al. (1978). "Immunity to *Plasmodium berghei yoelii* in mice II. Specific and nonspecific cellular and humoral responses during the course of infection." Journal of Immunology **121**: 629-636.

Yarovinsky, F., D. Zhang, et al. (2005). "TLR11 Activation of Dendritic Cells by a Protozoan Profilin-Like Protein." Science **308**(5728): 1626-1629.

Zhu, J., G. Krishnegowda, et al. (2005). "Induction of Proinflammatory Responses in Macrophages by the Glycosylphosphatidylinositols of Plasmodium falciparum: The Requirement of Extracellular Signal-Regulated Kinase, p38, c-Jun N-Terminal Kinase and NF-kB Pathways for the Expression of Proinflammatory Cytokines and Nitric Oxide." J. Biol. Chem. **280**(9): 8617-8627.

Chapter 4: **Malaria infection leads to induction of an anti-inflammatory immunophenotype.**

The work in this chapter was published in part in The Journal of Immunology (2005 174: 5921–5925.

The hypothesis of this study was that DCs function to activate naïve T cells throughout malaria infection while specifically guiding the production of pro-inflammatory cytokines early then specifically downregulating these through the production of anti-inflammatory cytokines as infection progresses.

The aim of this was to:

1. To determine the APC function of DCs throughout malaria infection
2. To determine the kinetics of pro- versus anti-inflammatory cytokine production in DCs as infection progresses, and how this affects T cell and global immune responses.

4.1 DCs support activation of naïve T cells throughout infection

4.2 DCs support less IFN-γ as infection progresses and preferentially induce high levels of IL-10, and stimulate its production in responding T cells

4.3 MSP-1 specific T cells in infected and recovered mice preferentially make IL-10.

4.4 DCs isolated late during infection carry an anti-inflammatory phenotype

4.5 LPS *in vivo*

ABSTRACT:

As shown in the previous section, DCs isolated from days 6-7 post infection are fully functional with respect to expression of costimulatory molecules and ability to activate of naïve T cells. We hypothesized that DCs remain robust in their ability to activate naïve T cells throughout all stages of *P. yoelii* infection. In this chapter we extend these day 6-7 p.i. findings to time points early (3 days p.i.) and late (15-21 days p.i.) during infection. Unlike IL-2 where production remained equivalent relative to naïve DCs, IFN-γ, TNF-α and IL-10 production varied significantly as a function of the stage of infection. Similar to days 6-7 dendritic cells, DCs from day 3 p.i. mice preferentially stimulated high levels of IFN-γ and TNF-α when cultured with naïve TCR-Tg T cells and Ova, whereas DCs from day 17 p.i. mice induced IL-10 and little IFN-γ and TNF-α under these same culture conditions. When looking specifically at cytokines produced by the DCs alone in response to TLR ligation rather than DC-T cell-Ag interactions, a similar switch from pro-inflammatory to anti-inflammatory phenotype was observed. Specifically, DCs isolated at day 3 p.i. produce IL-12 and TNF-α then as infection progresses these cells preferentially produce IL-10 by day 15 p.i.. Similar trends are observed using intracellular cytokine staining and quantitative PCR on sorted DCs.

INTRODUCTION:

The ideal immune response to any pathogen is one that is robust enough to combat replication of the infectious agent while limiting bystander immune mediated pathology. Regulation of this is likely occurring at all levels within the immune system including innate, cellular and humoral. We hypothesize that such an intricate balance between immunity and immunopathology occurs during *P. yoelii* infection, and that DCs play a pivotal role in this balance. The importance of this balance is typified by the disease syndromes that occur as a result of human malaria infection. For example, placental malaria (PM), cerebral malaria (CM) and severe malarial anemia (SMA) can all be associated with exuberant inflammatory responses. Abundant pro-inflammatory cytokine (IL-12, IFN-γ, TNF-α) production is associated with the occurrence of cerebral malaria in humans and in animal models (Kwiatkowski, Hill et al. 1990; Kurtzhals, Adabayeri et al. 1998; Engwerda, Mynott et al. 2002), and may contribute to severe malarial anemia (Kurtzhals, Adabayeri et al. 1998; Othoro, Lal et al. 1999). In this chapter, we specifically hypothesize that DCs guide the immune system during malaria infection to preferentially produce pro-inflammatory cytokines during the acute phase of infection until the adaptive immune system is sufficiently activated then down regulate proinflammatory cytokine production to limit immunopathology. Figure 4.0.1 illustrates a hypothetically ideal immune response that can be used as a guide with respect to our hypotheses associated with this chapter.

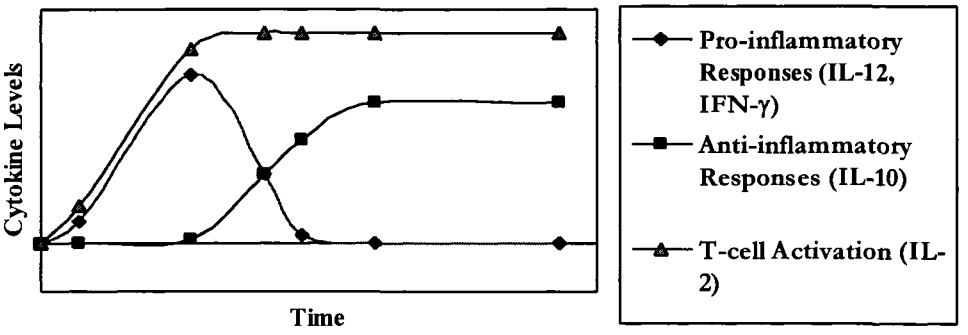


Figure 4.0.1: Schematic of a hypothetical ideal immune response to malaria as we hypothesize to be occurring in our model of *P. yoelii* infection.

- Pro-inflammatory: Increased parasite clearance, placental malaria, cerebral malaria and severe malarial anemia.
- Anti-inflammatory: Reduced Immune Mediated Pathology, High Parasitemia: Immunity to Disease.
- A healthy mixture…

Infection of mice with *P. chabaudi, P yoelii* and *P. berghei* can be used to study the control of pro-inflammatory cytokines during malaria infection (Langhorne, Albano et al. 2004). As mentioned previously, unlike human malaria infection, murine *Plasmodium* infection leads to sterile immunity following a single infection in most model systems. However, dividing the infection in mice into stages, early versus late or acute versus chronic, has been a useful method of modeling infection in non-immune versus anti-disease immune human individuals, respectively. Additionally, semi-immune mice can be generated by infecting then treating with parasitasidals prior to development of sterile immunity (Evans, Hansen et al. 2006). In the studies described herein, we define early/acute infection as that occurring between days 0 to 7 p.i. and late/chronic as that occurring from day 13 p.i. until clearance of the infection.

The literature provides strong evidence that non-lethal *Plasmodium* infection is characterized by early pro-inflammatory cytokine production including TNF-α (Ahvazi, Jacobs et al. 1995) IL-12 (Sam, Su et al. 1999) and IFN-γ (Langhorne, Gillard et al. 1989; Taylor-Robinson and Phillips 1994). There are few studies of cytokine production at later stages of infection, but those that have been conducted found increased levels of the anti-inflammatory cytokines TGF-β (Omer and Riley 1998; Li, Sanni et al. 2003) and IL-10 (Omer, de Souza et al. 2003), and decreased levels of IFN-γ (Langhorne, Gillard et al. 1989; Taylor-Robinson and Phillips 1994) and TNF-α (Ahvazi, Jacobs et al. 1995).

Interleukin-10, originally described as "cytokine synthesis inhibiting factor" (Fickenscher, Hor et al. 2002) and TGF-β clearly participate in the control of pro-inflammatory responses during malaria infection. A variety of studies demonstrate that in the absence of IL-10, malaria infected mice become sicker (greater weight loss, hypoglycemia and experience increased mortality (Linke, Kuhn et al. 1996; Hirunpetcharat, Vukovic et al. 1999; Li, Sanni et al. 2003). In addition to the context of malaria infection, the anti-inflammatory/ immunomodulatory activities, IL-10 have been shown to alleviate signs of endotoxic shock when challenged with a sublethal dose of LPS (Grutz 2005) as well as limit pathology in many infectious diseases including *Leishmania* (Miles, Conrad et al. 2005), *Toxoplasma* (Gazzinelli, Wysocka et al. 1996; Roers, Siewe et al. 2004), Vaccinia virus (Maloney, Schroder et al. 2005) and Mycobacteria (Bleharski, Li et al. 2003) as well as autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease and psoriasis (Blumberg, Saubermann et al. 1999; Driessler, Venstrom et al. 2004). While the mechanism behind this phenomenon remains obscure, it is thought that IL-10 signaling

does not directly interfere with the downstream signaling events associated with inflammatory stimuli. Rather, it appears that de novo synthesis of other factors is necessary for IL-10 to mount its anti-inflammatory effects (Aste-Amezaga, Ma et al. 1998).

Studies of the role of TGF-β have been more limited, but those that are available show anti-inflammatory functions similar to IL-10. Mutation of the *tgf-β* gene results in an embryonic lethal phenotype, therefore studies have been limited to using TGF-β neutralizing antibodies to abrogate the function of this cytokine. None the less, the complexity of the role of TGF-β in murine malaria infection was clearly demonstrated by Omer *et al.* where this tactic was employed. In their study, mice treated with anti-TGF-β presented with significantly greater parasitemias (using *P. chabaudi*, *P. bergei* and *P. yoelii*) and increased mortality (*P. chabaudi* and *P. bergei* but not *P. yoelii*) (Omer and Riley 1998). In another study using a *P. chabaudi* model as well showed opposite results when anti-TGF-β was administered relative to Omer's findings. Here, Tsutsui showed that the addition of anti-TGF-β to susceptible Balb/c mice leads to a reduction in parasitemia and improved survival (Tsutsui and Kamiyama 1999). The discrepancy between these two studies has yet to be resolved, but could include slight differences in *P. chabaudi* strain virulence as the untreated mice in Tsutsui's work appear to produce more TGF-β early as well as show an earlier rise in parasitemia relative to Omer's findings. The fact that these two apparently identical systems can result in opposite findings may exemplify the complexity associated with TGF-β during malaria and immune function in general.

The factors leading to TGF-β production during malaria infection also remain elusive. Studies have shown that phosphatidylserine (PS) exposure on the surface of apoptotic cells results in the induction of TGF-β expression in immune cells (Huynh ML 2001). This is relevant to malaria infection as it has been historically shown that parasitization of RBCs with *Plasmodium spp.* results in PS exposure on the surface of the infected RBC (Sherman IW 2004). Additionally, there appear to be parasite specific differences that regulate TGF-β production and activation (Omer, de Souza et al. 2003). The lethal strain of *P. yoelii, P. yoelii* 17XL, causes rapid increases in TGF-β within the first 24 hours following infection whereas infection with the non-lethal strain, *P. yoelii* 17XNL, does not result in TGF-β production until much later during infection (Omer, de Souza et al. 2003). This differential induction of TGF-β between these strains has been implicated in their virulence. The kinetic difference in TGF-β production between the apparently identical strains of *P. chabaudi* in the studies by Omer et al and Tsutsui also support this idea (Omer and Riley 1998; Tsutsui and Kamiyama 1999).The recent development of *tgf-βR* conditional knockout mice (Laouar, Sutterwala et al. 2005; Li, Sanjabi et al. 2006) will likely improve our knowledge of this cytokine's function.

Of the studies investigating the production of these cytokines, few have specifically determined which cells make these cytokines. IL-10 was originally isolated from mouse T_H2 cells and was thought to be specifically produced by these cells to suppress T_H1 responses. It became clear that this cytokine was also produced by a plethora of other cell types including macrophages, DCs, B cells, mast cells, intestinal epithelial cells, keratinocytes and other subsets of T cells (Roers, Siewe et al. 2004; Jankovic, Kullberg et

al. 2007; O'Garra and Vieira 2007). DCs have been shown to produce IL-12, TNF-α, and IL-10 upon interaction with *P. chabaudi* (Li C 2001). A subsequent study by Omer et al. showed that adherent cells produce IL-10, TNF-α, TGF-β in response to culture with *P. yoelii* 17XL (Omer, de Souza et al. 2003). Production of these cytokines by macrophages and DCs also effects cytokine secretion by other cell types. $CD8^{+}CD25^{-}$ T cells have been shown to secrete TGF-β in response to co-culture with *P. yoelii* 17X and 17XL (Omer, de Souza et al. 2003).

Because the initial interaction between naïve T cells, dendritic cells and pathogen associated molecules (TLR ligands and likely others as described in Chapter 1) determines the outcome of the T cell response (Moulin, Andris et al. 2000), we hypothesized that DCs isolated at different times of infection would be phenotypically distinct, resulting in differential T cells responses. Specifically, we predicted that DCs isolated from mice during the acute phase of infection would produce biologically active IL-12 in response to TLR ligation and/or T cell costimulation stimulate high levels of IFN-γ by responding T cells, whereas DCs isolated later during the more chronic phase would preferentially secrete IL-10 in response to TLR ligation and stimulate naïve T cells do produce the same. As seen in Chapter 3, we established the first part of this hypothesis by showing that DCs isolated from day 7 post infection with *P. yoelii* stimulate high levels of IFN-γ by naïve T cells in an IL-12 dependent manner (Perry, Rush et al. 2004).

In the following series of experiments we support these hypotheses by showing that DCs activate naïve T cells throughout infection, and naïve T cells activated by DCs from day 3 p.i. mice produce high levels of IFN-γ whereas T cells activated by DCs from day 17 show significantly more production of IL-10 with reduced IFN-γ and TNF-α levels. We go on to show that DCs from day 3 p.i. mice produce IL-12 in response to multiple TLR ligands while DCs from day 17 p.i. mice preferentially produce IL-10. We also show that this trend is recapitulated *in vivo* in response to LPS administration where day 3 p.i. mice produced significantly higher levels of IL-12p70 but significantly lower levels of IL-10, than day 17 p.i. mice.

MATERIALS AND METHODS

Mice

B10.D2, B6, IL-10 knockout and p50 knockout mice were purchased from Jackson Laboratories as described in previous chapters. B10.D2-DO11.10 (TCR-Tg) mice which express a T cell receptor for ovalbumin (OVA) on CD4 T cells were purchased from Jackson Laboratories then bred in house and used as heterozygotes. Parasitemia was tracked throughout infection by thin blood film preparation.

Infections with P. yoelii 17X NL:

Infections with *P. yoelii* were carried out as described in Chapter 2.

Purification of cellular subsets

DCs and T cells were purified from the pooled spleens of 3 to 5 mice by positive selection with anti-CD11c and anti-CD4 respectively, using magnetic beads as described (Perry, Rush et al. 2004). For FACS purification, spleen cells were enriched for CD11c+ cells using magnetic beads, and then stained according to the specific experiment. Initial studies where DC were sorted for culture with TLR ligands or DO11.10 T cells plus ovalbumin, CD11c+ cells were stained for CD3/CD19, CD11c and CD11b. CD3/CD19 negative $CD11c^+$ DCs were then sorted based upon differential expression of CD11b. In subsequent studies looking at cytokine RNA levels, CD11c+ cells were stained for CD3/CD19/NK1.1, CD11c, CD11b, CD8a and MHC class II (I-A/I-E clone). CD3/CD19/NK1.1 negative, CD11c/MHC class II positive cells were then sorted based on CD11b and CD8 expression (Figure 4.2.2). All FACS studies were carried out using a MoFlo cell sorter (Dako Cytomation).

Cell culture conditions

T cell stimulation assays were carried out as described as above (Luyendyk, Olivas et al. 2002; Perry, Rush et al. 2004). Briefly, $3x10^5$ DC were cultured with $2x10^5$ naïve DO11.10 T cells in DMEM-5 then stimulated or not with 1mg/ml OVA for 24 and 72hrs. Dendritic cell TLR ligation experiments were carried out by stimulating 3×10^5 DCs with 100 ng/ml LPS (InVivoGen Cat# tlrl-pelps) or 1.0 ug/ml CpG ODN 1826 (InVivoGen Cat# tlrl-modn). Total culture volumes for all experiments were 200ul in 98

well round bottom plates. Supernatants were collected at 24 and 72 hours and analyzed for cytokines. All cultures were carried out in triplicate.

Cytokine assays

TNF-α, IFN–γ, IL-10, and IL-12 p70 were analyzed by CBA (BD Biosciences Cat # 552364) as described in chapter 3 of this dissertation. Briefly, 15ul of beads, 15ul of undiluted sample and 15ul of PE detection reagent were incubated for 2hr at room temperature. The samples were then washed in 500ul wash buffer, spun for 5min at 14,000 rpm, then resuspended for analysis by flow cytometry in 200ul wash buffer. IL-12p40 was analyzed by sandwich ELISA (eBiosciences) as previously described.

Intracellular cytokine staining (ICCS)

Spleen cells were isolated in the same fashion as described previously. For intracellular cytokine analysis of DCs, 15x106 cells were stimulated in polypropylene tubes with 1ug/ml CpG with 10ug/ml BrefeldinA for 8hrs in a total volume of 1ml of DMEM 5% FBS. At the completion of 8 hours of culture, cells were washed 2x in PBS 1% BSA 0.2 mM EDTA (stain buffer) then surface stained for CD11c, CD11b, CD8, MHC class II and a combination of CD3/CD19/NK1.1. A similar antibody cocktail was used for FACs of DCs. Cells were stained for 1 hour then washed twice with stain buffer followed by a 20 minute fixation in 4% paraformaldehyde. After fixation, cells were washed twice in PBS 1% BSA 0.2 mM EDTA 0.5% Saponin (perm buffer) then stained for IL-12p40 and TNF-α or IL-10 in perm buffer for 1 hour at RT. Cells were washed

3x with perm buffer then resuspended in PBS for analysis using a CyAn flow cytometer (Dako Cytomation).

Intracellular cytokine staining and analysis was carried out in a similar fashion. Briefly, whole spleen cells were stimulated with 0.05ug/ml PMA and 0.5ug/ml Ionomycin in the presence of BFA for 4 hours then stained for CD4 and CD44. The cells were fixed, permeablized and stained for IFN-γ and IL-10 then analyzed by flow cytometry. To assure that the cytokine staining was specific, a cold competitor of each cytokine titrated to 5x the concentration of the anti-cytokine antibody was included as a control.

Q-PCR

RNA is isolated with the RNAqueous-4 PCR kit (Ambion Inc). The method prepares total RNA free of DNA. This is used with the RETROscript Kit from Ambion Inc. to prepare first-strand cDNA. Q-PCR was carried out by Dr. Robert Burnett. The SYBR Green detection method, was used to quantify cytokine (IL-12p40, TNF-α, and IL-10) message. Relative cytokine expression values are generated using the delta-delta Ct method. Amplification efficiencies of all reported genes are greater than 90%. GAPDH is used as the normalizing house-keeping gene.

LPS treatment in vivo

Mice were bled via tail nick (approximately 30 ul) and then given 0.1-1 mg *S. typhimurium* LPS (Sigma-Aldrich Cat# L6511) dissolved in PBS intraperitoneally (IP). Three hours

later they were sacrificed and bled for serum via cardiac puncture. Blood was collected in 1ml serum separator tubes then spun for 5 minutes at 14,000 rpm. The serum fraction was collected and frozen at -80 degrees until analysis.

Recombinant *P. yoelii* proteins

MSP-N (330 aa starting at aa 20 of MSP-1) and MSP-C (182 aa starting at aa 1619) recombinant proteins were generated by Dr. Robert Burnett from our laboratory strain of *P. yoelii* by cloning and expression in *E. coli* with a HIS tag, followed by purification with nickel chromatography.

Statistical analysis:

Two tailed student t-tests, assuming unequal variances, were used to calculate p-values and to compare cytokine levels between treatment groups within individual experiments. Unless otherwise indicated all experiments were carried out at least twice.

RESULTS:

4.1 DCs support activation of naïve T cells throughout infection

In previous work we demonstrated that DCs isolated from days 6-7 p.i. were as efficient at activating naïve CD4 T cells as DCs from uninfected mice (Perry, Rush et al. 2004). We extended these observations to DCs isolated at additional time points (days 3 and

day 17 p.i., Fig. 4.1.1). DCs from day 3 and day 17 p.i. were cultured with TCR-Tg T cells and Ova. DCs from both days supported as much T cell IL-2 production as control DCs in 24 hour cultures. T cells cultured with day 17 DCs however, produced significantly less IL-2 at 72 hours (65% of control, p=.007) (Figure 4.1.1). In order to further explore this question, we measured the yield of T cells after 7 days of culture with DCs from each day of infection. Cultures with DCs from uninfected mice yielded a mean of 8.5×10^5 ($\pm 3 \times 10^5$) cells, day 3 DCs yielded 9.7×10^5 cells ($\pm 2.1 \times 10^5$), and day 17 DCs yielded 6.8×10^5 ($\pm 2.5 \times 10^5$) cells. None of these differences were statistically significant. Finally, we restimulated T cells that had been cultured for 5 days with DCs from naïve or day 17 p.i. mice. IL-2 production was similar with the two different preparations of DCs (Fig. 4.1.2). From these studies we concluded that, while DCs from late in infection support slightly less IL-2 production at 72 hours, this does not appear to have significant consequences for T cell division. Additional studies also demonstrated that both $CD11c^+CD11b^-$ and $CD11c^+CD11b^+$ DC subsets maintained high levels of co-stimulatory proteins and class II MHC during the later stages of infection, consistent with their ability to activate naïve T cells (Figure 4.1.3).

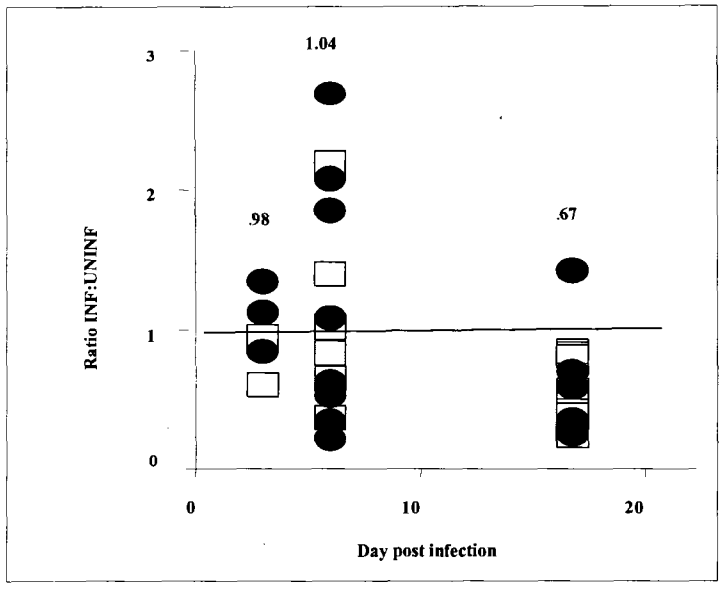


Figure 4.1.1 Naive TCR-Tg T cells were cultured with DCs isolated at the indicated day of infection and Ova. In each experiment, DCs were also isolated from pooled spleens of 3-5 uninfected or from infected mice at the indicated day p.i.. IL-2 production measured after 24 (●) or 72 (□) hours of culture. Each symbol represents a different experiment. The mean ratio of all experiments combined is shown over the column of symbols)

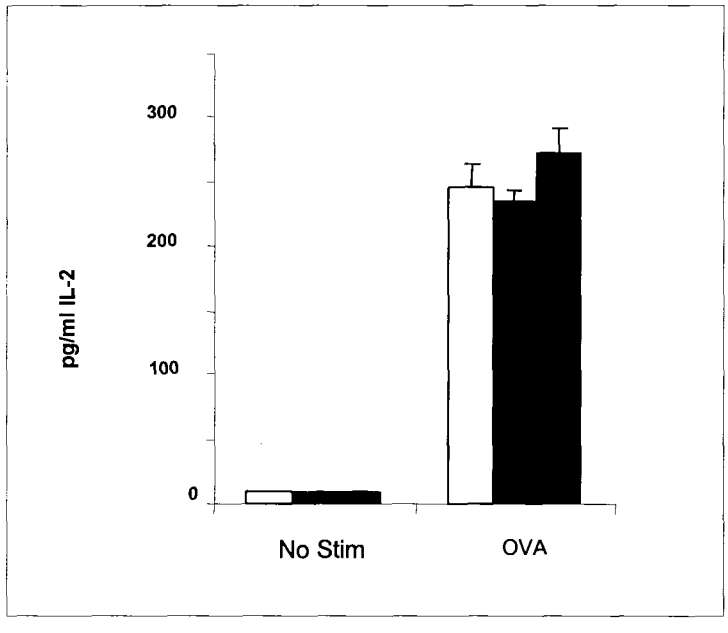


Figure 4.1.2: TCR-Tg cells cultured for 5 days with DCs from uninfected (white bars) or day 17 p.i. (black bars) were harvested and restimulated with Ova using naive spleen cells as APCs. IL-2 was then measured after 24 h of culture.

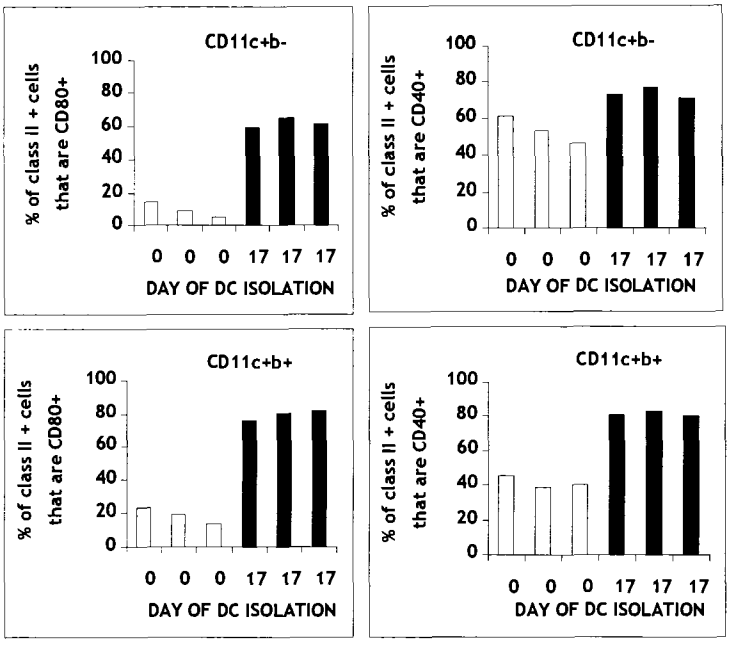


Figure 4.1.3: Flow cytometric analysis of 3 individual naïve and day 17 p.i. mice selecting for CD11c+HHCII+ cells. All comparisons between DCs from naïve and day 17 p.i. mice were statistically significant ($p < 0.05$)

4.2 DCs support less IFN-γ as infection progresses and preferentially induces high levels of IL-10 and stimulates its production in responding T cells

Although DCs retain the ability to stimulate IL-2 production and T cell division and throughout infection, their ability to elicit IFN-γ production by naïve T cells increases during the acute stages of infection, and then declines markedly by day 17 p.i. (Figure 4.2.1). Previously we demonstrated that IFN-γ production in this system is dependent upon IL-12 (Perry, Rush et al. 2004). We asked if IL-12 production paralleled that of IFN-γ, and found that, although IL-12p70 was below the limits of detection (approximately 15 pg/ml) in all in vitro experiments, production of IL-12 p40 in the presence of T cells and antigen was significantly lower in DCs from day 17 p.i. (mean IL-

12 with DCs from uninfected mice 2449 +/- 120, mean IL-12 with DCs from day 17 infected mice, 453 +/- 12, $p < 0.05$). It is important to note that at day 17 p.i. the mean parasitemia was 18%, whereas at day 3 the mean parasitemia was 0.5%. Thus IL-12 production is not diminished because the parasite stimulus was removed.

In contrast to pro-inflammatory cytokine production, DCs from late stages of infection induced high levels of IL-10 and low levels of IFN-γ in responding T cells upon restimulation (Fig. 4.2.2). Interleukin-10 was undetectable after primary stimulation and therefore restimulation was necessary for analysis (data not shown). This decrease in IFN-γ stimulation *in vitro* was at least in part mediated by IL-10 production itself as addition of anti-IL-10 resulted in a significant increase in IL-10 (Figure 4.2.3). Also as shown in this figure, blocking TGF-β, which has been implicated in the inhibition of IFN-γ during many infectious processes including malaria (Omer, de Souza et al. 2003), had little effect on IFN-γ production (Figure 4.2.3). Neither IL-4 nor IL-5 was detected in these cultures at either 24 or 72 hour time points (data not shown).

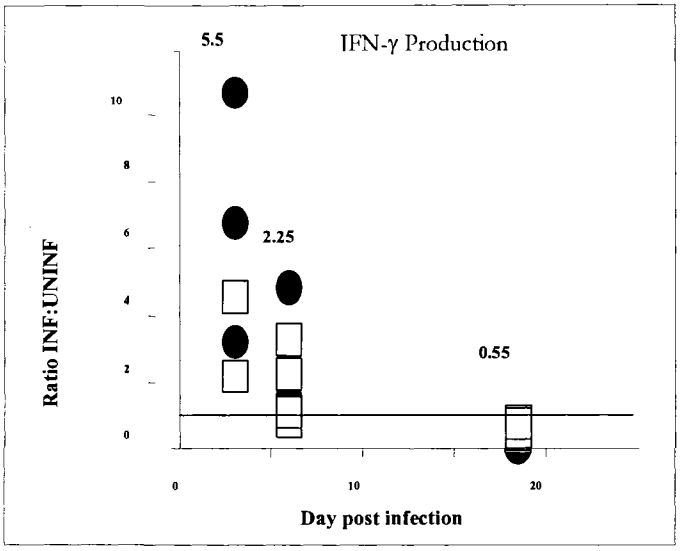


Figure 4.2.1: Cytokine production by naïve T cells cultured with DCs from pooled spleens of 3-5 mice at different days of infection. Naive TCR-Tg T cells were cultured with DCs isolated at the indicated day of infection and Ova. In each experiment, DCs were also isolated from uninfected mice. IFN-γ production measured after 24 (●) or 72 (□) hours of culture. Each symbol represents a different experiment. The mean ratio of all experiments combined is shown over the column of symbols)

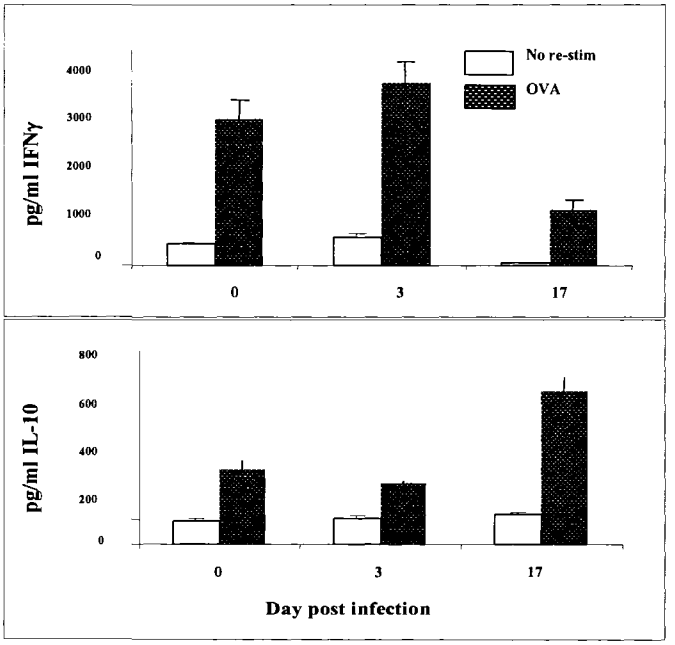


Figure 4.2.2: Naive T cells cultured with DCs from late in infection produce predominantly IL-10. TCR-Tg T cells were cultured for 7 days with DCs from the indicated day of infection and Ova. The cells were then harvested and restimulated with (shaded bars) or without (clear bars) Ova for 24 hours using naïve spleen cells as APCs.

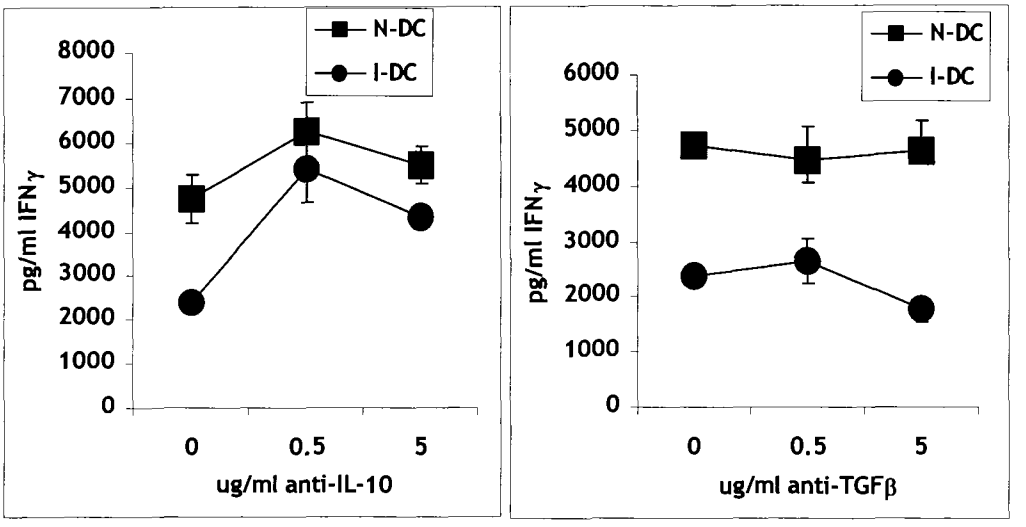


Figure 4.2.3: IL-10 and TGF-β do not restore IFN-γ production in the presence of DCs from infected mice. CD11c+ DCs from day 17 p.i. mice were cultured with TCR-Tg T cells and Ova with or without the indicated concentration of anti-cytokine antibody. After 24 hours of culture, supernatants were harvested and analyzed for IFN-γ by ELISA.

4.3 T cells isolated from day 17 infected mice make IL-10 and IFN-γ upon *ex vivo* restimulation.

Since we demonstrate that naïve, TCR-Tg T cells produce IL-10 when stimulated by day 17 p.i. DCs, we asked if such IL-10 producing T cells are generated *in vivo* during infection. We purified $CD4^+$ T cells from day 17 p.i. mice and stimulated them with two peptides representing the N- and C- termini of merozoite surface protein-1 (MSP-1) as well as anti-CD3, a non-specific T cell mitogen. These T cells produced significantly more of both IFN-γ and IL-10 (Figure 4.3.1). This mixed pro and anti-inflammatory response might be explained by the presence of a mixture of effector T cells – some activated during the acute stage of infection and others activated later during infection.

However, when stimulated with PMA and Ionomycin followed by staining for intracellular IFN-γ and IL-10, we found that there is a significant increase in splenic $CD4^+$ T cells from day 17 p.i. mice that co-express IL-10 and IFN-γ arguing against the previously mentioned 'mixed T cell hypothesis' (Figure 4.3.2 and in conjunction with Patti Kiser's dissertation). We further explored this question by looking at T cells from recovered mice. Approximately 60 days after mice had recovered from *P. yoelii* infection they were re-infected to expand the antigen specific T cell population. After 5 days of infection we purified $CD4^+$ T cells and cultured them with MSP-1 peptides using naïve spleen cells as antigen presenting cells (Figure 4.3.3). These T cells did not produce IFN-γ, IL-4 or IL-5 (data not shown), but did produce significant levels of IL-10. Together these results demonstrate that, while dendritic cells retain their capacity for T cell activation throughout malaria infection, the phenotype of responding naïve T cells changes as infection

progresses.

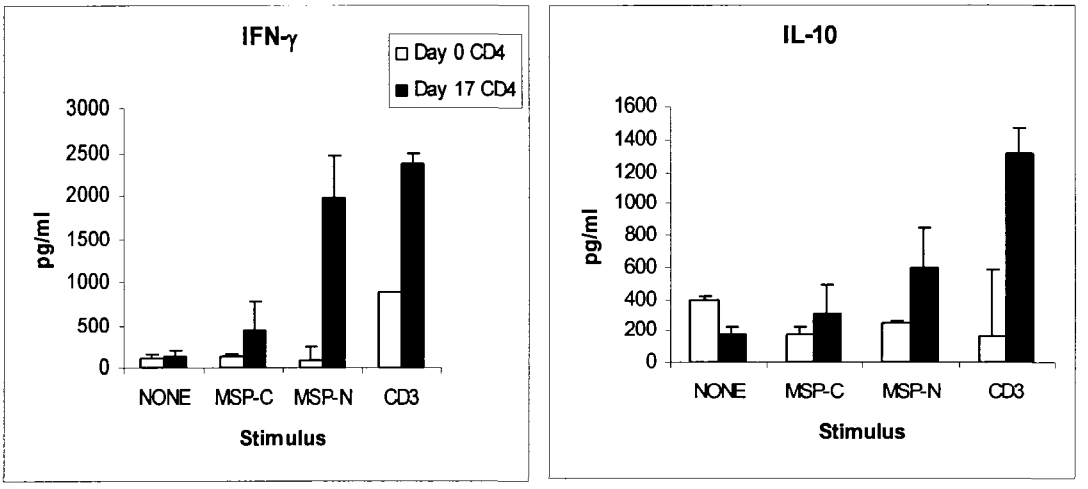


Figure 4.3.1: CD4+ T cells purified 3 individual mice at day 0 and day 17 of infection were cultured with naïve spleen and 5ug/ml MSP-N, MSP-C, anti-CD3 or no stimulus. IFN-γ and IL-10 were measured.

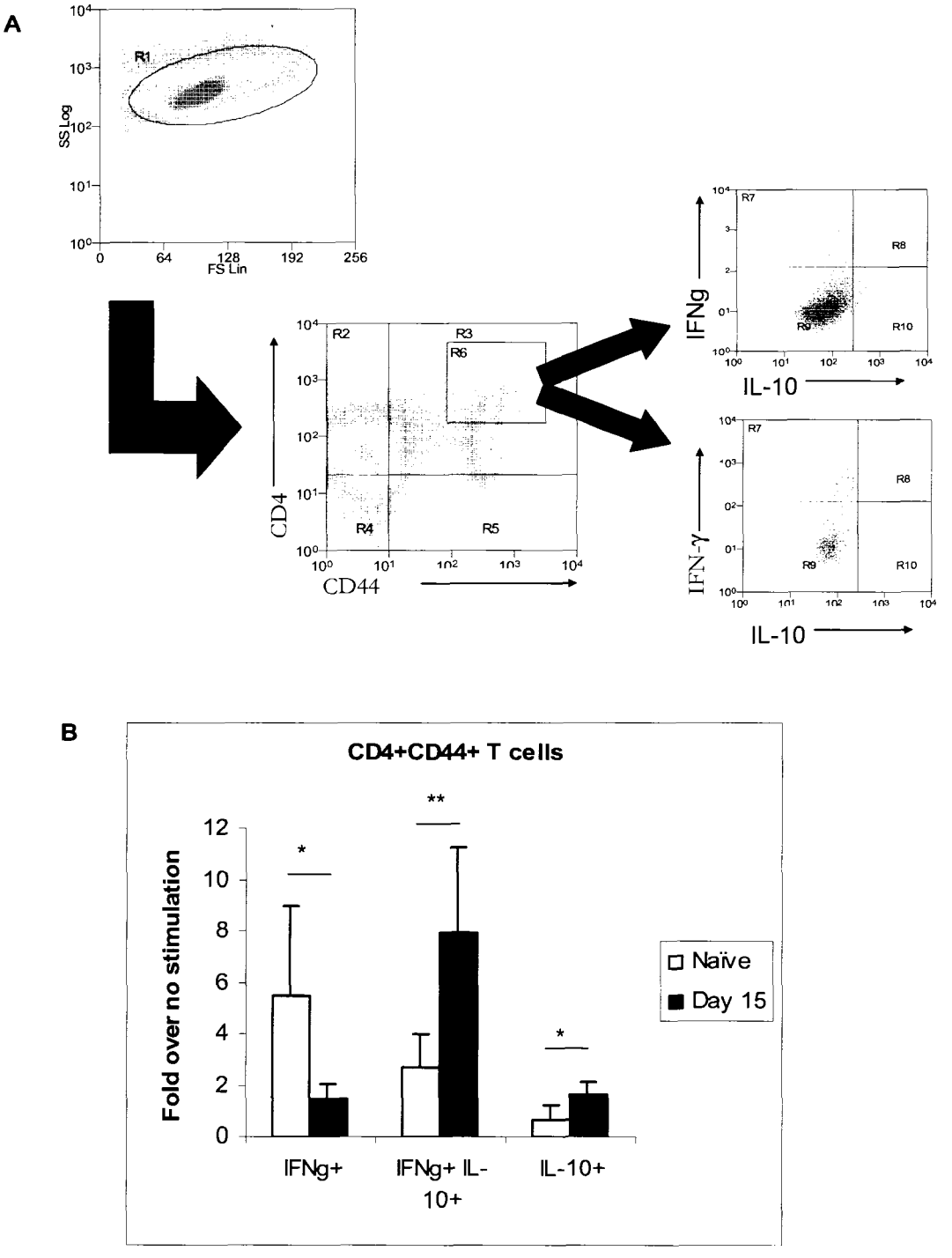


Figure 4.3.2: A) Gating criteria used for selecting activated T cells for intracellular IFN-γ and IL-10 analysis. B) Gating in on CD4+CD44hi cells, fold increase in expression following stimulation with PMA and Ionomycin was compared. $p<0.05$; ** $p=0.07$ in this experiment, however this difference is statistically significant over 3 independent experiments ($p<0.05$) in conjunction with data provided by Patti Kiser in our laboratory.

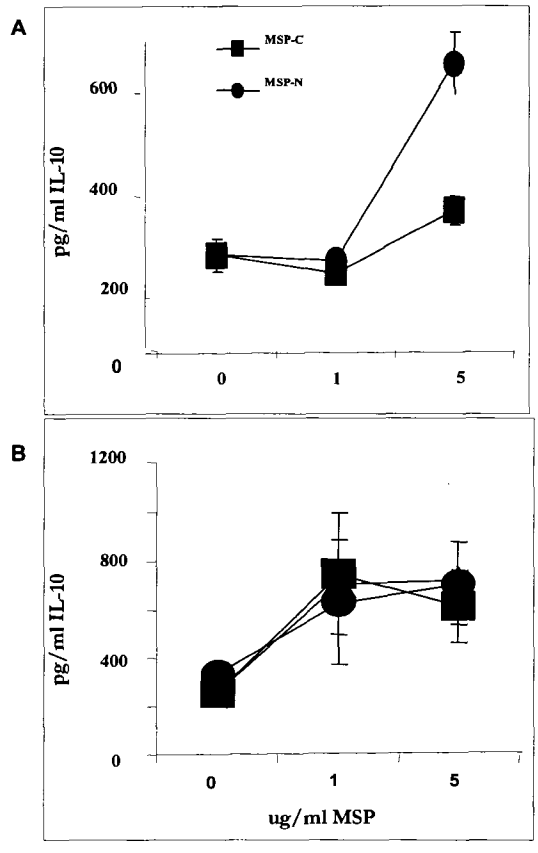


Figure 4.3.3: T cells from reinfected, previously recovered mice make IL-10 in response to parasite antigen. A) Unfractionated splenocytes were isolated from mice that had cleared infection two months previously, but reinfected 5 days before sacrifice. Cells were cultured with the indicated concentrations of MSP-1 N and C terminal peptides. B) In a separate experiment, CD4 T cells were purified from similarly treated mice and cultured with naïve spleen cells as antigen presenting cells, together with the indicated concentration of MSP-N terminal peptide. Cultures were stimulated for 24 hours before harvest for cytokine measurement. IFN-γ, IL-4 and IL-5 were below the limits of detection (not shown).

4.4 DCs isolated late during infection carry an anti-inflammatory phenotype

In the previous sections of this chapter we demonstrated that DCs from early in infection supported high levels of IFN-γ production by naïve T cells. Production of IFN-g declines when naïve T cells are cultured with DCs from later time points during infection. Therefore we sought to determine if DCs demonstrated a similar decline in production of inflammatory cytokines.

Perhaps the most crucial inducers of pro-inflammatory cytokines are signals transmitted by interaction of pathogen derived molecules with TLRs (Janeway and Medzhitov 2002).

We therefore asked if the response to TLR ligation by DCs was inhibited during the later stages of infection. Figure 4.4.1 shows that DCs isolated at day 17 p.i. and stimulated with LPS or CpG produce significantly lower levels of pro-inflammatory cytokines than DCs from day 0 or day 3. Additionally, IL-10 levels were significantly increased at this time point (Figure 4.4.1). Zymosan and R848, ligands for TLR 6 and TLR 7/8, respectively, were tested with similar results (data not shown). These results suggest significant inhibition of TLR mediated pro-inflammatory responses during the late stages of malaria infection.

Using another method to evaluate DC cytokine production in response to CpG stimulation, intracellular cytokine staining (ICCS), we show that splenic DCs ($CD11c^{+}MHCII^{hi}$) from day 17 p.i. mice are less capable of upregulating IL-12 production CpG stimulation (Figure 4.4.2). Significantly fewer DCs express IL-12p40 following CpG stimulation as expressed by total percentage of IL-12p40 positive DC, and fold increase over unstimulated controls. This is in confirmation of other studies using CD11c selected and sorted DCs in culture. To our surprise, and in contrast to culture data and message data (below), a higher percentage of IL-12p40 positive DCs were present in the unstimulated day 17 group than their naïve counter parts (mean infected = 5.2 and mean naive = 1.7; $p < 0.05$). Changes in IL-10 and TNF-α production upon CpG stimulation were undetectable by this method (data not shown).

We also measured cytokines at the transcriptional level in freshly sorted DCs, without the addition of exogenous TLR ligands. As hypothesized, DCs from day 17 p.i. mice had significantly lower mRNA levels of IL-12p40 and TNF-α, while having greatly increased

amounts of IL-10 message (Figure 4.4.3). Because these cells are taken directly from infected mice and not further manipulated, they are likely the best representation of the state of DCs *in vivo*, and serve to validate our findings with ELISA and flow cytometry. Of note, these findings are in contrast to unstimulated DCs analyzed by ICCS. Possible explanation of this could be IL-12 protein present within or on the surface of DCs from infected mice even though there is decreased production at the transcription level and secretion.

Our results show that dendritic cells lose their ability to produce pro-inflammatory cytokines as infection progresses. This change in dendritic cell phenotype is paralleled by a change in T cell phenotype. Thus T cells from late stages of infection lose their ability to produce IFN-γ, but produce high levels of IL-10.

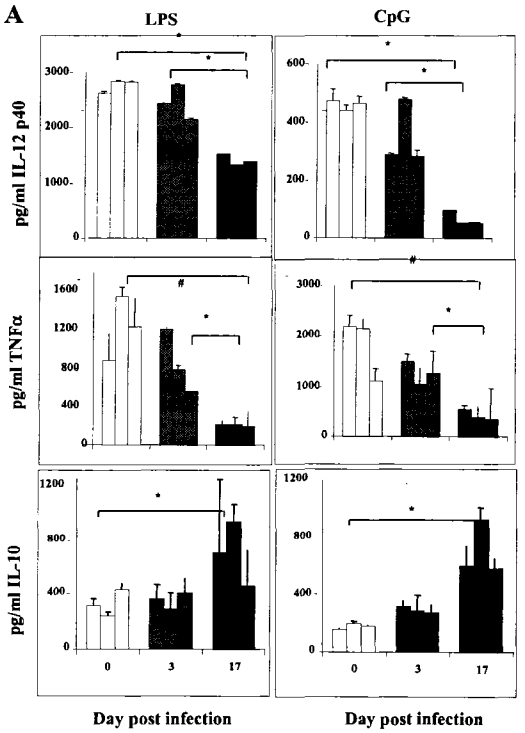
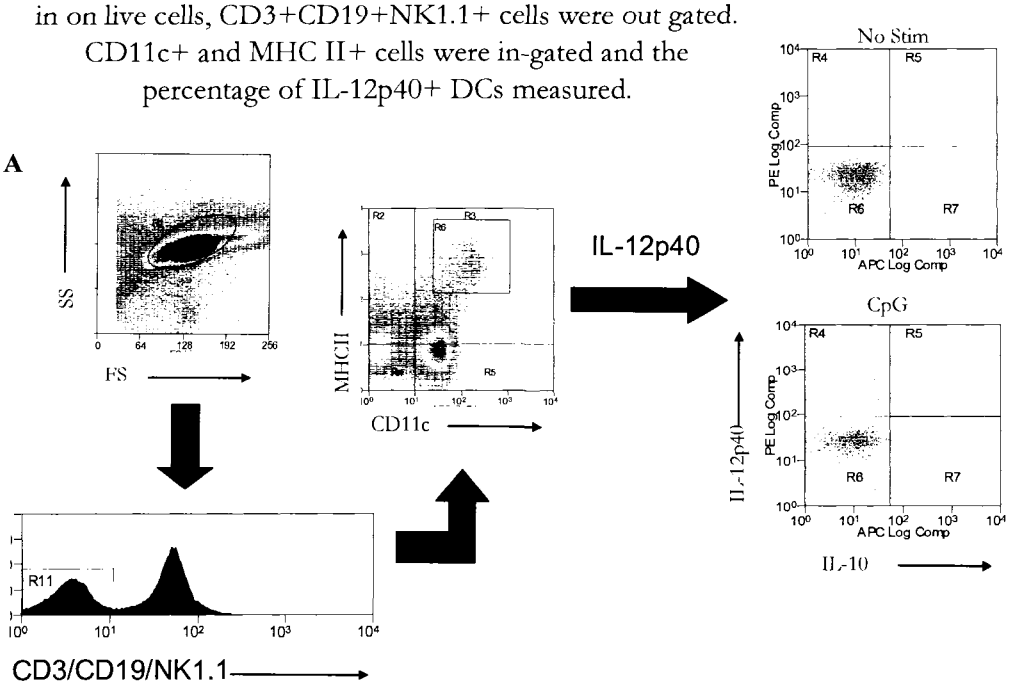


Figure 4.4.1: A) Magnetically selected CD11c+ Dendritic cells from late stages of infection produce less pro-inflammatory cytokines in response to TLR ligation, but greater amounts of IL-10. DCs were isolated at the indicated day of infection and cultured with CpG or LPS for 72 hours. Each bar represents cytokine produced by DCs isolated from an individual mouse, or pooled spleens from 2 or 3 in the case of day 0 mice. # $p \leq .05$, * $p \leq .01$ using an unpaired two tailed student t test. Similar differences were seen after 24 hours of stimulation.

Figure 4.2.2A: Gaiting scheme for DC ICCS. Briefly, gating in on live cells, CD3+CD19+NK1.1+ cells were out gated. CD11c+ and MHC II+ cells were in-gated and the percentage of IL-12p40+ DCs measured.



B

Exp 1	*Treatment*	*No Stim*	*CpG*	*Fold increase*
N=5	Naïve	1.9	15.3	8.0
N=5	D15	2.3	6.7#	2.9

Exp 2	*Treatment*	*No Stim*	*CpG*	*Fold increase*
N=2	Naïve	1	4.7	4.6
N=2	D15	4.6	3.8#	0.8

Exp 3	*Treatment*	*No Stim*	*CpG*	*Fold increase*
N=5	Naïve	2.2	12.7	5.8
N=5	D15	8.6	9.7#	1.1

C

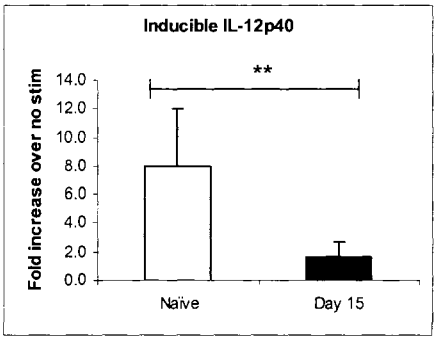


Figure 4.2.2: Whole spleen cells were cultured for 8 hours in DMEM-5 + BFA with or without 1 ug/ml CpG. B) shows data from three independent experiments. C) the mean fold increase in IL-12p40 production for Day 15 and naïve CD11c+MHCII+CD19+ (* $p < 0.05$ between naïve and infected with no stim; # $p < 0.05$ between CpG treated and no stim; ** $p < 0.05$ between fold increase in IL-12p40 after stimulation between Day 15 and infected DCs.)

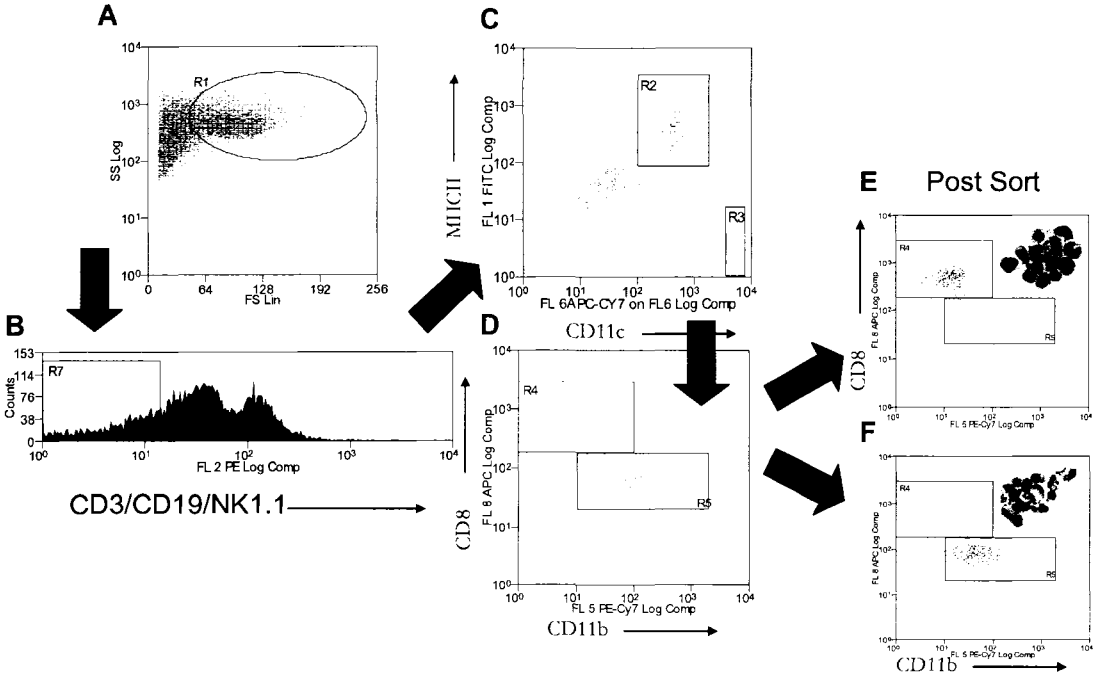


Figure 4.4.3A: Sorting criteria for splenic dendritic cells. Live cells (A) were gated in while gating out CD3, CD19 and NK1.1 (B). MHC class II high, CD11c+ cells were gated then sorted based on CD8 and CD11b (C and D). CD8+ DC and CD11b+ DC populations were analyzed for purity (E and F, respectively).

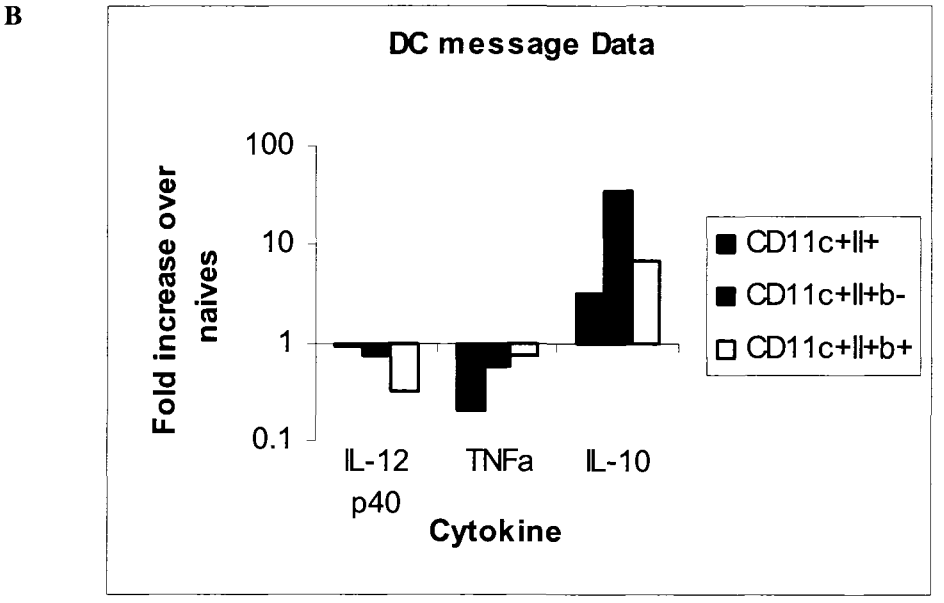


Figure 4.4.3B: Fold increase in cytokine expression, normalized to *gapdh*, in Day 15 DCs over naïve. The data here represents two experiments, one where CD3-CD19-NK1.1-CD11c+MHCII+ cells were sorted, and the second with this population further sorted based on expression of CD11b. 10-15 mice in each group were used to obtain the necessary numbers of DCs for these studies.

4.5 Infected mice show a global anti-inflammatory phenotype as infection progresses

In order to determine if the shift in cytokine production occurs at the systemic level, we treated uninfected and day 17 p.i. mice with LPS, then measured serum cytokine levels after 3 hours. We hypothesized that a similar shift from predominantly IL-12, IFN-γ and TNF-α early to IL-10 occurs on a global scale as infection progresses. At baseline, pre LPS challenge, there were no significant differences in IL-12p70, TNF-α, IFN-γ or IL-10 between infected and naive mice (data not shown). After LPS administration, however, naive mice made between 5 and 50 fold more IL-12p70, IFN-γ and TNF-α than infected mice. In contrast, LPS provoked very high levels of IL-10 in day 17 p.i. mice compared to their naïve counterparts (Figure 4.5.1). Since these mice were sacrificed at 3 hours post LPS inoculation, long term survival/resistance to LPS was not evaluated.

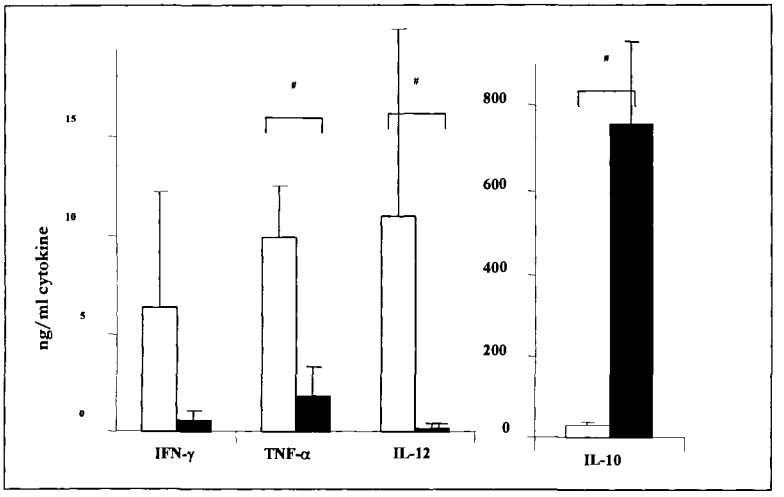


Figure 4.5.1: Uninfected (white bars n = 3) or day 17 PI mice (filled bars n = 5) were treated with 1 mg of LPS IP. Serum was taken 3 hours post treatment and assayed for the indicated cytokines. All comparisons between day 0 and day 17 were statistically significant ($p < .01$) except IFN-γ, which did not achieve significance.

DISCUSSION

Control over the the adaptive immune response begins at the level of the interaction between antigen presenting cells (APC) and T cells (Janeway and Medzhitov 2002). This is particularly important during malaria infection as outcome is highly dependent of the quality and quantity of the immune response elicited. Although an early proinflammatory response appears to be essential for the survival of a lethal infection (Stevenson, Tam et al. 1995; De Souza, Williamson et al. 1997; Su and Stevenson 2000), the complications associated with this inflammation can lead to severe malarial disease.

The studies described in this chapter offer several novel findings. First, we demonstrate that dendritic cells from infected mice maintain their ability to activate naïve T cells

throughout infection, an issue that has been controversial. While some studies show that DCs derived *in vitro* in the presence of parasitized red blood cells are dysfunctional (Urban, Ferguson et al. 1999; Ocana-Morgner, Mota et al. 2003), other work, including our own, found that DCs purified from infected mice are fully functional with respect to expression of co-stimulatory proteins and T cell activation (Leisewitz, Rockett et al. 2004; Perry, Rush et al. 2004; Pouniotis, Proudfoot et al. 2004). One possible explanation for the discrepant results is the lack of T cell-DC interaction in systems where DCs are matured *in vitro*. Such DCs do not receive T cell derived signals such as CD40-CD40L interaction, which may contribute to their antigen presenting capacity. Other possible explanations were discussed in the previous chapter.

Through an extensive series of experiments looking at the kinetics involved in pro- and anti-inflammatory cytokine production by DCs isolated from infected mice, we show that these cells preferentially produce high levels of IL-12p40 and TNF-α early during infection (days 3-7 post infection) then preferentially produce IL-10 later (days 14-21 post infection). We also show that this switch within the DC population is reflected in the phenotype of naïve T cells activated by these DCs at different stages of infection. Interesting, and somewhat unexpectedly, T cells isolated at day 17 p.i. produce both IFN-γ and IL-10 suggesting that "T_H1" cells may contain the capacity to produce IL-10 during malaria infection, a phenomenon recently described by Jankovic et al using a *T. gondii* system. (Jankovic, Kullberg et al. 2007).

This switch within the DC and T cell populations is also observed at the level of the whole animal following LPS challenge *in vivo*. This latter observation exemplifies the

global change in the inflammatory status during malaria infection, a phenomenon similar to endotoxin tolerance where repeated exposure to LPS leads to diminished pro-inflammatory responses to subsequent LPS challenges (Varma, Toliver-Kinsky et al. 2001; Sato, Takeuchi et al. 2002; Dobrovolskaia, Medvedev et al. 2003).

There are several hypotheses to explain the shift from pro to anti-inflammatory cytokine production. Extrapolation from current literature looking at other systems support the hypothesis that repeated signaling through TLRs (2 and 9 and other *Plasmodium* derived ligands in the case of malaria infection) tolerize or more specifically shunt the common MyD88 dependant TLR signaling pathway such that anti-inflammatory cytokines are preferentially produced. This alteration in the TLR signaling pathway results in the "refractory" or anti-inflammatory state we and others (Rubenstein M 1965) have observed and refer to as endotoxin-like or TLR tolerance. This has important implications with respect to secondary infections that may occur during a primary malarial episode as well as subsequent infections with malaria. While experiments specifically looking at this in our model of malaria infection have yet to be done, it would be interesting to see how malaria infected mice during the anti-inflammatory phase would respond to organisms that normally induce high levels of IL-12 and IFN-γ such as intracellular bacteria and viruses. The failure of DCs to produce IL-12p40 late in infection is particularly intriguing because at this point in infection there is a substantial parasite burden but further accentuates the phenomenon of tolerance.

The purpose of this chapter was to introduce and describe the shift from a pro- to anti-inflammatory state that occurs during malaria infection, as well as the role of DCs in this

switch. In the following chapter, Chapter 5, we expand on these observations in an attempt to elucidate the molecular mechanisms associated with this inflammatory shift.

References:

Ahvazi, B. C., P. Jacobs, et al. (1995). "Role of macrophage-derived nitric oxide in suppression of lymphocyte proliferation during blood-stage malaria." J Leukoc Biol **58**(1): 23-31.

Aste-Amezaga, M., X. Ma, et al. (1998). "Molecular Mechanisms of the Induction of IL-12 and Its Inhibition by IL-10." J Immunol **160**(12): 5936-5944.

Bleharski, J. R., H. Li, et al. (2003). "Use of Genetic Profiling in Leprosy to Discriminate Clinical Forms of the Disease." Science **301**(5639): 1527-1530.

Blumberg, R. S., L. J. Saubermann, et al. (1999). "Animal models of mucosal inflammation and their relation to human inflammatory bowel disease." Current Opinion in Immunology **11**(6): 648-656.

De Souza, J. B., K. H. Williamson, et al. (1997). "Early gamma interferon responses in lethal and nonlethal murine blood- stage malaria." Infect. Immun. **65**(5): 1593-1598.

Dobrovolskaia, M. A., A. E. Medvedev, et al. (2003). "Induction of In Vitro Reprogramming by Toll-Like Receptor (TLR)2 and TLR4 Agonists in Murine Macrophages: Effects of TLR "Homotolerance" Versus "Heterotolerance" on NF-{kappa}B Signaling Pathway Components." J Immunol **170**(1): 508-519.

Driessler, F., K. Venstrom, et al. (2004). "Molecular mechanisms of interleukin-10-mediated inhibition of NF-kB activity: a role for p50." Clin Exp Immunol **135**(1): 64-73.

Engwerda, C. R., T. L. Mynott, et al. (2002). "Locally Up-regulated Lymphotoxin {alpha}, Not Systemic Tumor Necrosis Factor {alpha}, Is the Principle Mediator of Murine Cerebral Malaria." J. Exp. Med. **195**(10): 1371-1377.

Evans, K. J., D. S. Hansen, et al. (2006). "Severe malarial anemia of low parasite burden in rodent models results from accelerated clearance of uninfected erythrocytes." Blood **107**(3): 1192-1199.

Fickenscher, H., S. Hor, et al. (2002). "The interleukin-10 family of cytokines." Trends in Immunology **23**(2): 89-96.

Gazzinelli, R. T., M. Wysocka, et al. (1996). "In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF- alpha." J Immunol **157**(2): 798-805.

Grutz, G. (2005). "New insights into the molecular mechanism of interleukin-10-mediated immunosuppression." J Leukoc Biol **77**(1): 3-15.

Hirunpetcharat, C., P. Vukovic, et al. (1999). "Absolute Requirement for an Active Immune Response Involving B Cells and Th Cells in Immunity to Plasmodium yoelii Passively Acquired with Antibodies to the 19-kDa Carboxyl-Terminal Fragment of Merozoite Surface Protein-1." J Immunol **162**(12): 7309-7314.

Huynh ML, F. V., Henson PM. (2001). "Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation." J Clin Invest. **109**(1): 41-50.

Janeway, C. A. and R. Medzhitov (2002). "INNATE IMMUNE RECOGNITION." Annual Review of Immunology **20**(1): 197-216.

Jankovic, D., M. C. Kullberg, et al. (2007). "Conventional T-bet+Foxp3- Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection." J. Exp. Med. **204**(2): 273-283.

Kurtzhals, J. A., V. Adabayeri, et al. (1998). "Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria." The Lancet **351**: 1768-1772.

Kwiatkowski, D., A. V. Hill, et al. (1990). "TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated Plasmodium falciparum malaria." Lancet **336**(8725): 1201-1204.

Langhorne, J., S. Gillard, et al. (1989). "Frequencies of CD4+ T cells reactive with *Plasmodium chabaudi chabaudi*: distinct response kinetics for cells with T_{h1} and T_{h2} characteristics during infection." International Immunology **1**: 416-424.

Langhorne, J. R., F. Albano, et al. (2004). "Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection." Immunological Reviews **201**: 35-47.

Laouar, Y., F. S. Sutterwala, et al. (2005). "Transforming growth factor-[beta] controls T helper type 1 cell development through regulation of natural killer cell interferon-[gamma]." Nat Immunol **6**(6): 600-607.

Leisewitz, A. L., K. A. Rockett, et al. (2004). "Response of the Splenic Dendritic Cell Population to Malaria Infection." Infect. Immun. **72**(7): 4233-4239.

Li, C., L. A. Sanni, et al. (2003). "Pathology of *Plasmodium chabaudi chabaudi* infection and mortality in interleukin-10 deficient mice are meliorated by anti-tumor necrosis factor alpha and exacerbated by anti-transforming growth factor β antibodies." Infection and Immunity **71**(9): 4850-4856.

Li C, S. E., Langhorne J. (2001). "Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite." Med Microbiol Immunol. **189**(3): 115-26.

Li, M. O., S. Sanjabi, et al. (2006). "Transforming Growth Factor-[beta] Controls Development, Homeostasis, and Tolerance of T Cells by Regulatory T Cell-Dependent and -Independent Mechanisms." Immunity **25**(3): 455-471.

Linke, A., R. Kuhn, et al. (1996). "Plasmodium chabaudi chabaudi:Differential Susceptibility of Gene-Targeted Mice Deficient in IL-10 to an Erythrocytic-Stage Infection." Experimental Parasitology **84**(2): 253-263.

Luyendyk, J., O. R. Olivas, et al. (2002). "Antigen-Presenting Cell Function during Plasmodium yoelii Infection." Infect. Immun. **70**(6): 2941-2949.

Maloney, G., M. Schroder, et al. (2005). "Vaccinia Virus Protein A52R Activates p38 Mitogen-activated Protein Kinase and Potentiates Lipopolysaccharide-induced Interleukin-10." J. Biol. Chem. **280**(35): 30838-30844.

Miles, S. A., S. M. Conrad, et al. (2005). "A role for IgG immune complexes during infection with the intracellular pathogen Leishmania." J. Exp. Med. **201**(5): 747-754.

Moulin, V., F. Andris, et al. (2000). "B Lymphocytes Regulate Dendritic Cell (DC) Function In Vivo: Increased Interleukin 12 Production by DCs from B Cell-deficient Mice Results in T Helper Cell Type 1 Deviation." J. Exp. Med. **192**(4): 475-482.

O'Garra, A. and P. Vieira (2007). "TH1 cells control themselves by producing interleukin-10." Nat Rev Immunol **7**(6): 425-428.

Ocana-Morgner, C., M. M. Mota, et al. (2003). "Malaria Blood Stage Suppression of Liver Stage Immunity by Dendritic Cells." J. Exp. Med. **197**(2): 143-151.

Omer, F. M., J. B. de Souza, et al. (2003). "Activation of Transforming Growth Factor {beta} by Malaria Parasite-derived Metalloproteinases and a Thrombospondin-like Molecule." J. Exp. Med. **198**(12): 1817-1827.

Omer, F. M., J. B. de Souza, et al. (2003). "Differential Induction of TGF-{beta} Regulates Proinflammatory Cytokine Production and Determines the Outcome of Lethal and Nonlethal Plasmodium yoelii Infections " J Immunol **171**(10): 5430-5436.

Omer, F. M., J. B. de Souza, et al. (2003). "Differential Induction of TGF-{beta} Regulates Proinflammatory Cytokine Production and Determines the Outcome of Lethal and Nonlethal Plasmodium yoelii Infections." J Immunol **171**(10): 5430-5436.

Omer, F. M. and E. M. Riley (1998). "Transforming Growth Factor beta Production Is Inversely Correlated with Severity of Murine Malaria Infection." J. Exp. Med. **188**(1): 39-48.

Othoro, C., A. A. Lal, et al. (1999). "A low interleukin-10 tumor necrosis factor-a ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya." Journal of Infectious Diseases **1999**: 279-282.

Perry, J. A., A. Rush, et al. (2004). "Dendritic Cells from Malaria-Infected Mice Are Fully Functional APC." J Immunol **172**(1): 475-482.

Pouniotis, D. S., O. Proudfoot, et al. (2004). "Dendritic cells induce immunity and long-lasting protection against blood-stage malaria despite an in vitro parasite-induced maturation defect." Infection & Immunity **72**(9): 5331-5339.

Roers, A., L. Siewe, et al. (2004). "T Cell-specific Inactivation of the Interleukin 10 Gene in Mice Results in Enhanced T Cell Responses but Normal Innate Responses to Lipopolysaccharide or Skin Irritation." J. Exp. Med. **200**(10): 1289-1297.

Rubenstein M, M. J., JEFFERY GM, WOLFF SM (1965). "MALARIA INDUCED ENDOTOXIN TOLERANCE." Proc Soc Exp Biol Med **118**: 283-7.

Sam, H., Z. Su, et al. (1999). "Deficiency in tumor necrosis factor alpha activity does not impair early protective Th1 responses against blood-stage malaria." Infection and Immunity **67**(5): 2660-2664.

Sato, S., O. Takeuchi, et al. (2002). "A variety of microbial components induce tolerance to lipopolysaccharide by differentially affecting MyD88-dependent and -independent pathways." International Immunology **14**(7): 783-791.

Sherman IW, E. S., Winograd E (2004). "Erythrocyte aging and malaria." Cell Mol Biol **50**(2): 159-69.

Stevenson, M. M., M. F. Tam, et al. (1995). "IL-12-induced protection against blood-stage Plasmodium chabaudi AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide- dependent mechanism." J Immunol **155**(5): 2545-2556.

Su, Z. and M. M. Stevenson (2000). "Central Role of Endogenous Gamma Interferon in Protective Immunity against Blood-Stage Plasmodium chabaudi AS Infection." Infect. Immun. **68**(8): 4399-4406.

Taylor-Robinson, A. W. and R. S. Phillips (1994). "B cells are required for the switch from Th1- to Th2-regulated immune response to *Plasmodium chabaudi chabaudi* infection." Infection and Immunity **62**: 2490-2498.

Tsutsui, N. and T. Kamiyama (1999). "Transforming Growth Factor beta -Induced Failure of Resistance to Infection with Blood-Stage Plasmodium chabaudi in Mice." Infect. Immun. **67**(5): 2306-2311.

Urban, B. C., D. J. P. Ferguson, et al. (1999). "Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells." Nature **400**(6739): 73-77.

Varma, T. K., T. E. Toliver-Kinsky, et al. (2001). "Cellular mechanisms that cause suppressed gamma interferon secretion in endotoxin-tolerant mice." Infection & Immunity **69**(9): 5249-5263.

Wysocka, M., S. Robertson, et al. (2001). "IL-12 Suppression During Experimental Endotoxin Tolerance: Dendritic Cell Loss and Macrophage Hyporesponsiveness." J Immunol **166**(12): 7504-7513.

Chapter 5: **Molecular mechanisms associated with DC and TLR tolerance during malaria infection.**

The work in this chapter was published in part in The Journal of Immunology (2005 174: 5921–5925.

The hypothesis of this study was that IL-10 is required for the downregulation of IL-12, IFN-γ and TNF-α during malaria infection. We also hypothesized that IL-10 production was mediated through the activation of NF-κB p50 and the MAP kinase pathway.

The aim of this work was to:

1. To determine the mechanism of pro-inflammatory cytokine expression
2. To gain insight into the molecular mechanisms of IL-10 upregulation late during infection.
3. To determine the importance of pro-inflammatory cytokine downregulation in vivo.

5.1 Downregulation of IL-12 and IFN-γ during infection is IL-10 mediated.

5.2 IL-10 upregulation as infection progresses is NF-κB p50 independent

5.3 In the absence of IL-10, significant anemia and liver pathology ensues.

Abstract:

Characterization of the mechanism by which the DC response is shifted from pro- to anti-inflammatory will help us to better understand circumstances in which this shift does not occur (perhaps resulting in more severe immunopathology) as well as better understand similar events in other infectious and immune mediated diseases. We developed and tested several hypotheses in an attempt to explain the down-regulation of pro-inflammatory cytokines as infection progresses.

Experiments using IL-10 knockout mice (KO) to explore the role of IL-10 in mediating this switch from a pro- to anti-inflammatory phenotype reveal a role for this cytokine in down-regulating IL-12 and IFN-γ, but not TNF-α. This suggests disparate regulatory mechanisms for these two groups of pro-inflammatory cytokines. Also apparent in studies using IL-10 KO mice is that in the absence of IL-10, mice become relatively more anemic and show significantly more liver pathology as a result of infection. This occurs in spite of lower parasitemias in these mice relative to B6 controls. While several attempts were made to elucidate the molecular mechanisms involved in the progressive upregulation of IL-10, we found no role for NF-κB p50 and the MAP kinases p38 and ERK, all of which have been implicated in IL-10 production in other systems. The mechanisms of IL-10 production in the context of malaria infection remain elusive.

Introduction:

The production of pro-inflammatory cytokines such as IL-12, IFN-γ and TNF-α are essential for successful resolution of malaria infection (Stevenson and Riley 2004). At the same time however, the requirement for such cytokine production carries its caveats, and in the absence of tight control over the quality and quantity of their production can lead to detrimental outcomes. Despite the wealth of knowledge associated with the signals and processes involved in the initiation and promotion of cellular inflammation, little is known about the mechanisms involved in regulation and inhibition of these ensuing inflammatory responses, including those occurring during chronic infections such as malaria.

One experimental model used to better understand the down-regulation of inflammatory responses is the phenomenon of endotoxin tolerance. In this model system, repeated exposure to LPS results in downregulation of pro-inflammatory responses with subsequent challenges. Additionally, this down regulation in pro-inflammatory cytokines is associated with an upregulation of anti-inflammatory mediators and a resistance to the pathological side effects of LPS (Fan H 2004).

However, even using this relatively simple model system, the studies available looking specifically at the mechanisms associated with this shift from pro- to anti-inflammatory cytokine production following repeated LPS challenges often offer conflicting results. A review directly comparing the similarities between endotoxin tolerance and the shift from pro- to anti-inflammatory cytokine production during malaria infection was

recently published (Boutlis, Yeo et al. 2006). Subsequent studies exploring this phenomenon throughout the course of malaria infection(s) have found that this resistance to disease is associated with this switch in inflammatory cytokine production (Carapau, Kruhofer et al. 2007).

In general, both during endotoxin tolerance and during malaria infection, there is an observed shift from a pro-inflammatory state to one that is anti-inflammatory in nature. As previously stated, the pro-inflammatory phase is characterized by high levels of IL-12, TNF-α and IFN-γ whereas the subsequent anti-inflammatory stage is dominated by IL-10 and likely other anti-inflammatory cytokines such as TGF-β. Numerous studies have attempted to address the cellular and molecular mechanisms associated with the transition from pro- to anti-inflammatory cytokine production, but there is little consensus about the mechanism.

While it is becoming more evident who the important cellular players are with respect to shifting from a pro- to anti-inflammatory state, specifically DCs, B cells and other cells of the innate and adaptive immune systems, the molecular processes leading up to these changes are much less well understood. In fact, it wasn't until recently that researchers have begun to address this complicated topic associated with malaria infection (Boutlis, Yeo et al. 2006; Carapau, Kruhofer et al. 2007). The molecular players receiving the most attention are members of the mitogen activated kinase (MAPK) and NF-κB families of signaling molecules and transcription factors, respectively.

The best described members of the MAP kinase family of signaling molecules involved in the activation of cytokine transcription are the ERK 1/2 and p38 (Dong, Davis et al.

2002). Several studies implicated a role of p38 and ERK ½ activation, as measured by phosphorylation, in the regulation of IL-12p40 and IL-10, respectively (Lu HT 1999; Yi, Yoon et al. 2002). Lu *et al.* show that upon stimulation of bone marrow derived DCs ($CD11c^{+}B220^{-}$ selected) with a single dose of LPS, activation of p38 leads to IL-12 production. However, upon restimulation of these cells with a subsequent dose of LPS, p38 is preferentially downregulated in the face of an upregulation of ERK 1/2. This change in activation is associated with a drop in IL-12 production, and an increase in IL-10 (Lu HT 1999). Yi et al. shows similar findings looking at DCs stimulated with CpG (Yi, Yoon et al. 2002). Additionally, U0126, a specific inhibitor of ERK 1/2, reverses this switch from IL-12p40 to IL-10, suggesting that ERK1/2 plays an important role in this process. Another more recent report showed a similar role for p38 and ERK in IL-12p40 and IL-10 production using an infectious disease model. Here, Mathur *et al* showed that macrophages stimulated with CD40-ligand in the presence of *Leishmania* promastigotes preferential activated ERK 1/2 leading to production of IL-10. Interestingly, blockade of either ERK 1/2 (with PD098059) or IL-10 led to increased activation of p38 and preferential IL-12 production. The reciprocal was also true with respect to p38 blockade (with SB203580) which resulted in enhanced ERK 1/2 activation and subsequent IL-10 production (Figure 5.0.1) (Mathur, Awasthi et al. 2004).

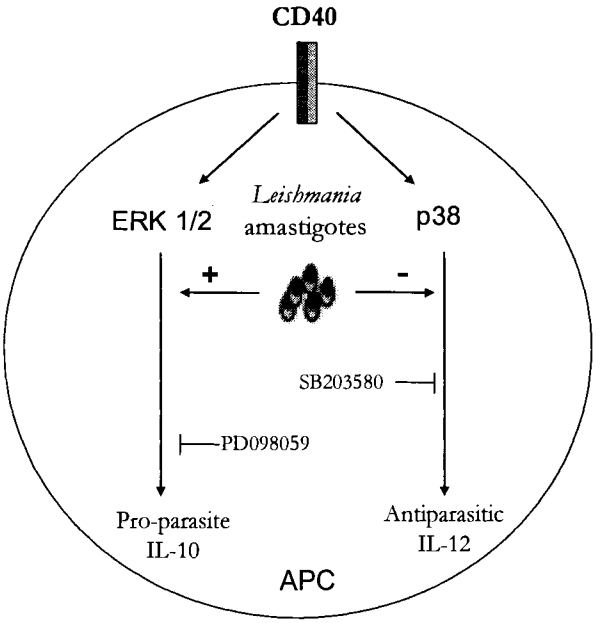


Figure 5.0.1: Schematic of the opposing roles of p38 and ERK 1/2 in the regulation of IL-12 and IL-10 in the context of *Leishmania* infection. Modified from Mathur et al. 2004

While the exact mechanism of ERK 1/2's role in IL-10 production is not completely understood, modification of chromatin in the region of the IL-10 locus has been shown in several systems to be important. Lucas *et al* showed that following ligation of FcγR on bone marrow derived macrophages as well as RAW cells resulted in phosphorylation of ERK 1/2 and subsequent chromatin modifications at the IL-10 locus (Lucas, Zhang et al. 2005). Specifically, ERK 1/2 activation lead to phosphorylation of Serine 10 on H3 at the *il-10* gene thereby making the promoter more accessible for binding of the transcription factors Sp1 and STAT3. Another study looking at ERK 1/2 induction by *L. major* also showed phosphorylation of histone H3 at the IL-10 promoter allowing for Sp1 binding (Yang, Mosser et al. 2007). This latter study did not look at STAT3 binding.

Like the MAP kinases p38 and ERK 1/2, NF-κB members have been implicated in the regulation of pro- and anti-inflammatory cytokines in DCs. NF-κB molecules have a wide range of functions including DC maturation, cytokine production and cell survival in the context of infections (Rescigno 1998; Ouaaz 2002). The first NF-κB transcription factor, p50:p65 heterodimer, was discovered for its ability to bind and activate transcription of the kappa light chain gene in B cells hence it received its name (Sen and Baltimore 1986). Since its discovery, multiple other NF-κB proteins have been described, and their function (and/or dysfunction) implicated in many biological processes including inflammation, apoptosis, cell activation, migration and repair, as well as self regulation (Ghosh, May et al. 1998).

To date, the known members of the NF-κB family include five distinct proteins: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), c-Rel and RelB (Ghosh and Karin 2002). All mammalian NF-κB family members contain a conserved DNA binding motif, the Rel homology domain (RHD), and each has the capacity to bind DNA. Based on the structure of these RH domains, NF-κB proteins can be divided into two classifications. The RHDs of p50 and p52 contain long C-terminal ankyrin repeats (protein domains containing tandem repeats of identical 33-amino acid long sequences) where as RelA, RelB and c-Rel contain C-terminal transcription activation domains (Figure 5.0.2). The long ankyrin repeats associated with p105 and p100 act to inhibit nuclear translocation and DNA binding. Upon polyUbiquitination and truncation by limited proteolysis, the active forms of p105 and p100, p50 and p52, respectively, become activated and localize to specific gene targets.

All of the NF-κB proteins dimerize to form hetero and homodimers, with the exception of RelB which can only form heterodimers. p50 and p52 are unable to activate transcription unless heterodimerized with one of the C-terminal activator domain containing NF-κB members c-Rel, RelA or RelB. In addition to being activators of transcription, specific homodimers are capable of inhibiting transcriptional activation. For example, homodimers of p50 and p52 have been shown experimentally to repress the transcription of specific target genes, including IL-12 and TNF-α (Zhong, May et al. 2002).

The composition of the NF-κB dimer dictates its DNA binding specificity and avidity as well as its efficiency at activating transcription. Classical heterodimers include p50:p65, p50:c-Rel and p52:RelB (Figure 5.0.2). In resting cells, these dimers are often retained in the cytoplasm, complexed with an inhibitor of nuclear location. In the case of p50:p65 and p50:c-Rel, inhibitor of kappaB (IκB) forms a complex with these dimers within the cytoplasm blocking their ability to translocate into the nucleus, and thereby inhibiting their activity. In the case of p52:RelB heterodimers, the inactive form of p52, p100, inhibits its nuclear translocation by the presence of the untruncated ankyrin repeats previously mentioned.

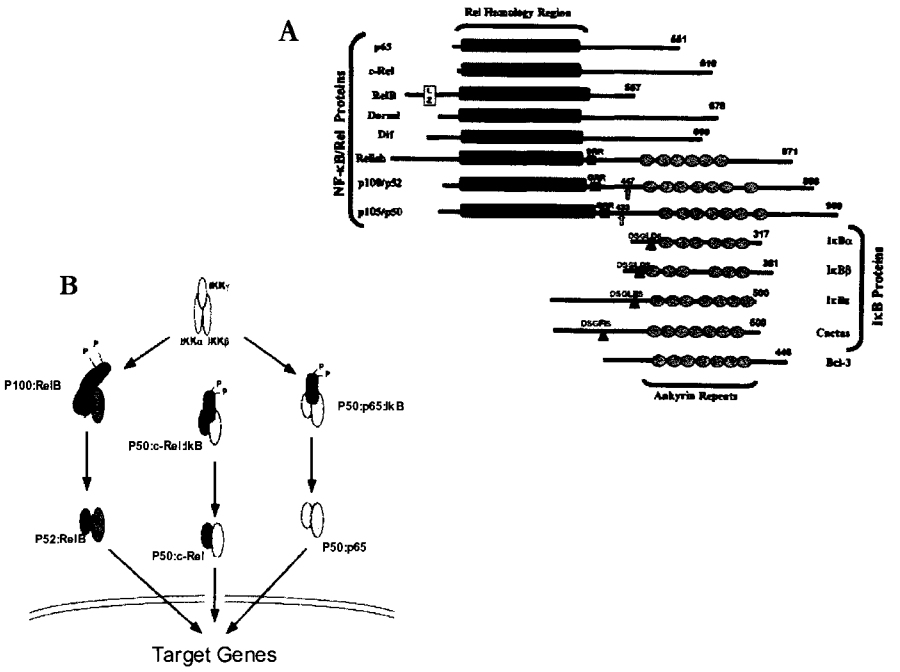


Figure 5.0.2: A) Composition of NF-kB family proteins (mammalian and *Drosophila*). In this proposal we will focus on p65, c-Rel, RelB, p52 and p50. B) Pathway leading to the translocation of different NF-kB heterodimers. From Ghosh et al. 2002

This system of preformed but inhibited NF-κB dimers within the cytoplasm allows for rapid activation of transcription in response to stimuli. Such activators of NF-κB include endogenous cytokines (IL-1β, TNF-α) and surface receptor ligands (CD40L) as well as pathogen associated molecular pattern molecules (TLR ligands such as LPS, CpG, zymozan, etc). Inhibitors of κB (or the ankyrin repeats associated with p100 in the case of p52:RelB activation) are phosphorylated by inhibitor of κB kinase (IκK). Once phosphorylated, IκB is polyUbiquitinated and degraded by the proteosome (Karin and Delhase 2000; Ghosh and Karin 2002). Additionally, it appears that phosphorylation at multiple serine sites within the p65 subunit is necessary for full transcriptional activity. The PI-3K/Akt pathway, which has been shown to become activated by CpG in a TLR9 independent mechanism (Dragoi AM 2005), has been implicated to play a role in the phosphorylation of p65 and therefore the activation of NF-κB. This tightly regulated

process ultimately leads to the exposure of the nuclear localization sequence and translocation of the dimer to the nucleus for gene activation.

Knockout mice deficient in specific members of the NF-κB protein family have been pivotal in determining the function and gene targets of many of these proteins. For example, $p50^{-/-}p65^{-/+}$ deficient DCs had profound defects in DC development. Virtually no $CD11c^{+}$ cells could be isolated from the spleens of these mice. Interestingly, the absence of p50/p65 had little affect on the maturation of macrophages (Ouaaz, Arron et al. 2002). In contrast to $p50^{-/-}p65^{-/+}$ mice, $p50^{-/-}$c-$Rel^{-/-}$ mice produce typical numbers of DCs that undergo normal development and LPS induced costimulatory and MHC class II molecule upregulation relative to wild type mice. Although these parameters of DC function remained in tact, $p50^{-/-}$c-$Rel^{-/-}$ DCs failed to produce IL-12p70 in response to LPS and CD40 ligation (Ouaaz, Arron et al. 2002). It is important to note that p50, p65 and c-Rel single knockouts showed no significant differences relative to control mice in these studies. This hints that redundancy exists in NF-κB induced transcription. Although there appears to be redundancy associated with p50, p65 and c-Rel, RelB appears to be solely responsible for LPS induced upregulation of MHC class II, CD40 and CD80 (Zanetti, Castiglioni et al. 2003), a process that appears to occur on DC throughout *P. yoelii* infection (Perry, Rush et al. 2004). Dendritic cells lacking this NF-κB member are therefore unable to activate naïve T-cells resulting in severe immune deficiency.

A recent study challenges the thought that p50 plays a pivotal role in forming dimers with p65 and c-Rel for activation of inflammatory cytokines. Cao et al show that the

absence of p50 has little effect on activation of the IL-12p40 and TNF-α promoter. Interestingly, p50 was required for transcriptional activation of the IL-10 gene in these studies. In addition to this finding, this group showed that p50 homodimers as well as heterodimers with CREB-binding protein (CBP) that are involved in IL-10 gene activation (Figure 5.0.3) (Cao, Zhang et al. 2006).

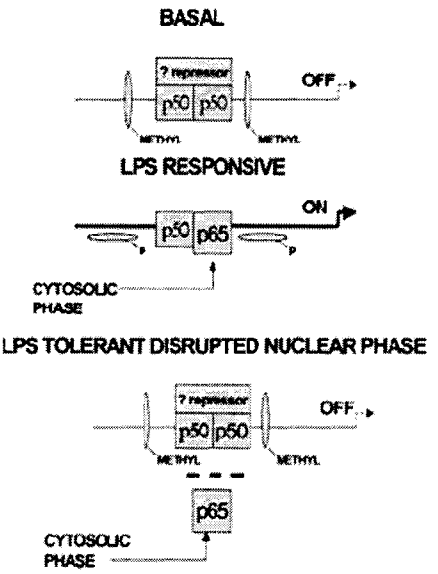


Figure 5.0.3: A model of NF-κB regulation in the basal state, LPS responsive state, and during tolerance of proinflammatory genes and the role of p50 homo and heterodimers in these processes. Here, p50 homodimers inhibit basal proinflammatory cytokine production and promote anti-inflammatory cytokine production in tolerant cells. From Chan et al. 2005

Based on works using other systems, as just described, we hypothesize that multiple mechanisms of inflammatory cytokine regulation are playing a role in our system of malaria infection. These mechanisms may include activation of the signaling molecule ERK 1/2, formation of p50 homodimers, downregulation of phospho-p38, NF-kB p65 inhibition, or quite likely, a currently undescribed mechanism allowing for the production of IL-10 and downregulation of IL-12 and TNF-α. In the studies described

herein, we first explored the role of IL-10 in the down-regulation of pro-inflammatory responses, and then tested the hypothesis that NF-kB p50 and the MAP kinases p38 and ERK 1/2 are central to IL-10 regulation.

Materials and Methods:

Mice

B10.D2, B6, IL-10 knockout and p50 knockout mice were purchased from Jackson Laboratories as previously described. Parasitemia was tracked throughout infection by thin blood film preparation as well as by flow cytometry, staining with CD71, CD45 and Hoechst, for reticulocytes, white blood cells and nucleated cells, respectively. Quantification of red cell counts during infection was done using Caltag counting beads (Caltag # PCB-100).

Purification of cellular subsets

DCs and T cells were purified from the pooled spleens of 3 to 5 mice by positive selection with anti-CD11c and anti-CD4 respectively, using magnetic beads as described (Perry, Rush et al. 2004). For FACS purification, spleen cells were enriched for CD11c+ cells using magnetic beads, and then stained according to the specific experiment. Initial studies where DC were sorted for culture with TLR ligands or DO11.10 T cells plus ovalbumin, CD11c+ cells were stained for CD3/CD19, CD11c and CD11b. CD3/CD19 negative $CD11c^+$ DCs were then sorted based upon differential expression

of CD11b. In subsequent studies looking at cytokine RNA levels, CD11c+ cells were stained for CD3/CD19/NK1.1, CD11c, CD11b, CD8a and MHC class II (I-A/I-E clone). CD3/CD19/NK1.1 negative, CD11c/MHC class II positive cells were then sorted based on CD11b and CD8 expression. All FACS studies were carried out using a MoFlo cell sorter (Dako Cytomation). Cytometric compensation was carried out using anti-Rat/Hamster Ig, k BD CompBeads (BD Cat# 51-90-9000949) with the corresponding antibodies used in these studies.

Cell culture conditions

Dendritic cell TLR ligation experiments were carried out by stimulating 3 x 10^5 DCs with 100 ng/ml LPS (InVivoGen Cat# tlrl-pelps) or 1.0 ug/ml CpG ODN 1826 (InVivoGen Cat# tlrl-modn). Total culture volumes for all experiments were 200ul in 98 well round bottom plates. Supernatants were collected at 24 and 72 hours and analyzed for cytokines. All cultures were carried out in triplicate.

Cytokine assays

TNF-α, IFN–γ, IL-10, and IL-12 p70 were analyzed by CBA (BD Biosciences Cat # 552364) as described in chapter 3 of this dissertation. Briefly, 15ul of beads, 15ul of undiluted sample and 15ul of PE detection reagent were incubated for 2hr at room temperature. The samples were then washed in 500ul wash buffer, spun for 5min at 14,000 rpm, then resuspended for analysis by flow cytometry in 200ul wash buffer. IL-

12p40 was analyzed by sandwich ELISA (R&D Biosystems Cat# MAB499 capture; BAF499 detection) as previously described.

ICCS

Spleen cells were isolated in the same fashion as described previously. For intracellular cytokine analysis of DCs, 15x106 cells were stimulated in polypropylene tubes with 1ug/ml CpG with 0.5ug/ml BrefeldinA for 8hrs in a total volume of 1ml of DMEM 5% FBS. At the completion of 8 hours of culture, cells were washed 2x in PBS 1% BSA 0.2 mM EDTA (stain buffer) then surface stained for CD11c, CD11b, CD8, MHC class II and a combination of CD3/CD19/NK1.1. A similar antibody cocktail was used for FACs of DCs. Cells were stained for 1 hour then washed twice with stain buffer followed by a 20 minute fixation in 4% paraformaldehyde. After fixation, cells were washed twice in PBS 1% BSA 0.2 mM EDTA 0.5% Saponin (perm buffer) then stained for IL-12p40 and TNF-α or IL-10 in perm buffer for 1 hour at RT. Cells were washed 3x with perm buffer then resuspended in PBS for analysis using a CyAn flow cytometer (Dako Cytomation).

Intracellular cytokine staining and analysis was carried out in a similar fashion. Briefly, whole spleen cells were stimulated with PMA and Ionomycin in the presence of BFA for 4 hours then stained for CD4 and CD44. The cells were fixed, permeablized and stained for IFN-γ and IL-10 then analyzed in a similar fashion.

Q-PCR

Q-PCR was carried out in the same fashion as described in Chapter 4.

Western blot analysis of MAPK and NF-κB activation

Dendritic cells were obtained by FACs as previously described. Cells were lysed in IP buffer ( ) at $1x10^5$ cells/100ul and frozen until analysis. Protein content within each sample was quantified using BCA (Pierce #23235) according to the manufacturer. 10ug protein was added to 1:4 running buffer (4x NuPage LDS sample buffer + 800mM DTT) and heated to 100 degrees C for 2 minutes. Samples were then run on a 4-12 percent gradient SDS-PAGE gel (InVitrogen #NP0329) for ~ 1.5 hours at 150 Amps. Bands were transferred to nitrocellulose membranes and blots probed for total and phospho- p38, ERK 1/2 and NF-kB p65 (Cell Signaling) overnight at RT. The blots were then probed with anti-rabbit HRP then developed using SuperSignal (Pierce Cat#34075) then analyzed using a Kodak chemiluminescence reader. Band intensities were quantified using Quantity One software (BioRad,)

LPS treatment in vivo

Mice were bled via tail nick (approximately 30 ul) and then given 0.1-1 mg *S. typhimurium* LPS (Sigma-Aldrich Cat# L6511) dissolved in PBS intraperitoneally (IP). Three hours later they were sacrificed and bled for serum via cardiac puncture. Blood was collected in 1ml serum separator tubes then spun for 5 minutes at 14,000 rpm. The serum fraction was collected and frozen at -80 degrees until analysis.

Preparation and analysis of liver histopathology:

Livers from infected IL-10 KO and C57B6 mice were harvested at day 17 post infection and fixed in 10% buffered formalin for >24hrs. Sections were cut, mounted and H&E stained by the Colorado State University Diagnostic Laboratory. Histopathologic scoring was based on severity of necrosis, extra-medullary hematopoiesis, and inflammation on a 0-5 scale. Sections were scored by two individuals independently, first by the author of this dissertation, and then confirmed by a diplomat of the American Collage of Veterinary Pathologists. Serum ALT levels were measure by the Colorado State University Diagnostic Laboratory.

Statistical analysis:

Two tailed student t-tests, assuming unequal variances, were used to calculate p-values and to compare cytokine levels between treatment groups within individual experiments. Unless otherwise indicated all experiments were carried out at least twice.

Results:

5.1 Suppression of IL-12 is mediated by IL-10 *in vivo*

Based on the wealth of data in the literature showing that IL-10 plays a dominant role in down regulating pro-inflammatory immune responses to infections and endotoxin, we

asked if IL-10 was responsible for the down-regulation of pro-inflammatory cytokines production in response to TLR signaling during malaria infection. While wild type mice down-regulated the production of pro-inflammatory cytokines in response to LPS administration *in vivo* LPS (Figures 4.5.1 and 5.1.1), infected IL-10 KO mice, however, failed to down regulate IL-12p70 and IFN-γ. Interestingly, TNF-α was down-regulated as in wild type mice (Figure 5.1.1).

We then determined that the behavior of dendritic cells from wild type and IL-10 KO mice reflected the *in vivo* observations. DCs from B6 and IL-10 KO mice were purified by FACS into three different subpopulations based on level of CD11b expression. The rationale for this division was that with increasing expression of CD11b, dendritic cells tend be increasingly phagocytic (Pulendran, Lingappa et al. 1997). This was indirectly confirmed by the observation that cells expressing the highest levels of CD11b had the highest percentage of pigment containing cells (Figure 3.3.4 and 5.1.2D). We reasoned that dendritic cells with greater phagocytic capacity might be more dramatically affected by malaria infection. The sorted subpopulations were cultured with CpG for 24 hours and supernatants analyzed for IL-12p40 by ELISA (Figure 5.1.2A). Both DC subsets from B6 mice at day 17 p.i. exhibited a decrease in their ability to produce IL-12p40 and after CpG stimulation relative to naïve controls, although the $CD11c^+CD11b^+$ DCs, which make up on average 75 percent of the splenic DC population, was the most affected. Consistent with our *in vivo* observations, both $CD11c^+CD11b^-$ and $CD11c^+CD11b^+$ DCs upregulated IL-10 in response to stimulation with CpG (Figure 5.1.2B). With respect to DCs from infected IL-10 KO mice, $CD11c^+CD11b^+$ cells produced higher levels of IL-12p40 in response to CpG compared to their naïve counter

parts. There were no significant differences in IL-12p40 production between CD11c+CD11b- cells from naïve and day 15 p.i. mice (Figure 5.1.2A). This suggests, like what is observed in IL-10 KO mice *in vivo*, also occurs at the level of the most abundant population of DCs.

Using intracellular cytokine staining to measure inducible IL-12p40 production in naïve and infected IL-10 KO mice, we further show that DCs from IL-10 KO mice retain the ability to upregulate IL-12p40 in the face of infection. Here, while DCs from infected mice upregulate significantly less IL-12p40 following CpG stimulation than their naïve counterparts, DCs from infected IL-10 KO mice were capable of inducing more ($p = 0.09$ two sided student's t test) IL-12p40 relative to DCs from naïve IL-10 KO mice (Figure 5.1.3).

When splenic DCs from IL-10 KO were sorted based on $CD11c^{+}MHCII^{+}CD19^{-}CD3^{-}NK1.1^{-}CD11b^{+/-}CD8^{+/-}$ then analyzed for message, the $CD11b^{-}CD8^{+}$ subset of these cells downregulated IL-12 and TNF-α, a similar finding to WT DCs; whereas the $CD11b^{+}CD8^{-}$ subset showed increased IL-12p40 message relative to naïve controls in the face of TNF-α downregulation as seen in WT DCs (Figure 5.1.4), a finding similar to our *in vitro* culture data. This divergent role of IL-10 between these subsets of DCs remains unexplored.

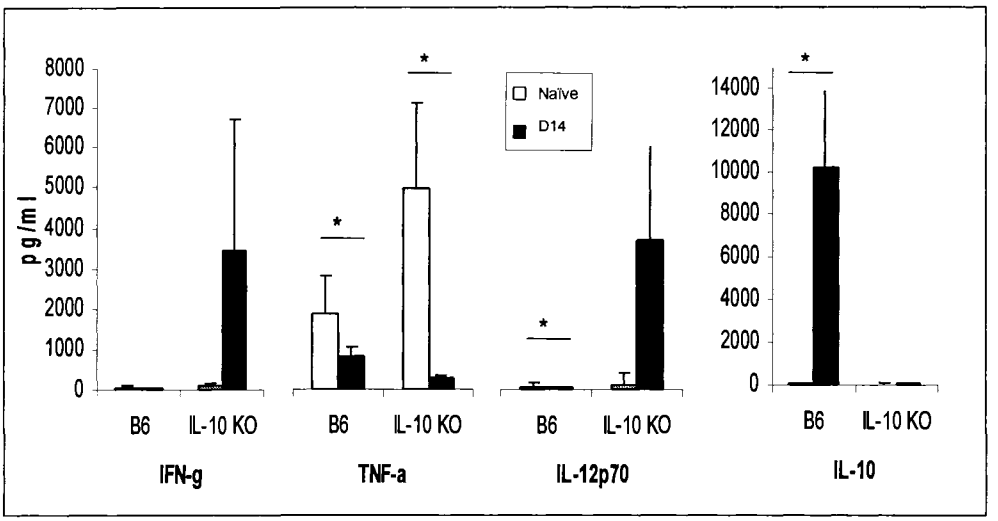


Figure 5.1.1 IL-10 mediates suppression of IL-12 and IFN-γ in response to LPS in vivo. Uninfected IL-10 KO (white bars n = 5) or day 15 p.i. mice (filled bars n = 5) were treated with 0.1 mg of LPS i.p. Serum was taken 3 hours post treatment and assayed for the indicated cytokines. All comparisons between day 0 and day 15 were statistically significant ($p < 0.01$). IL-10 was unmeasurable in both naïve and infected knockout mice

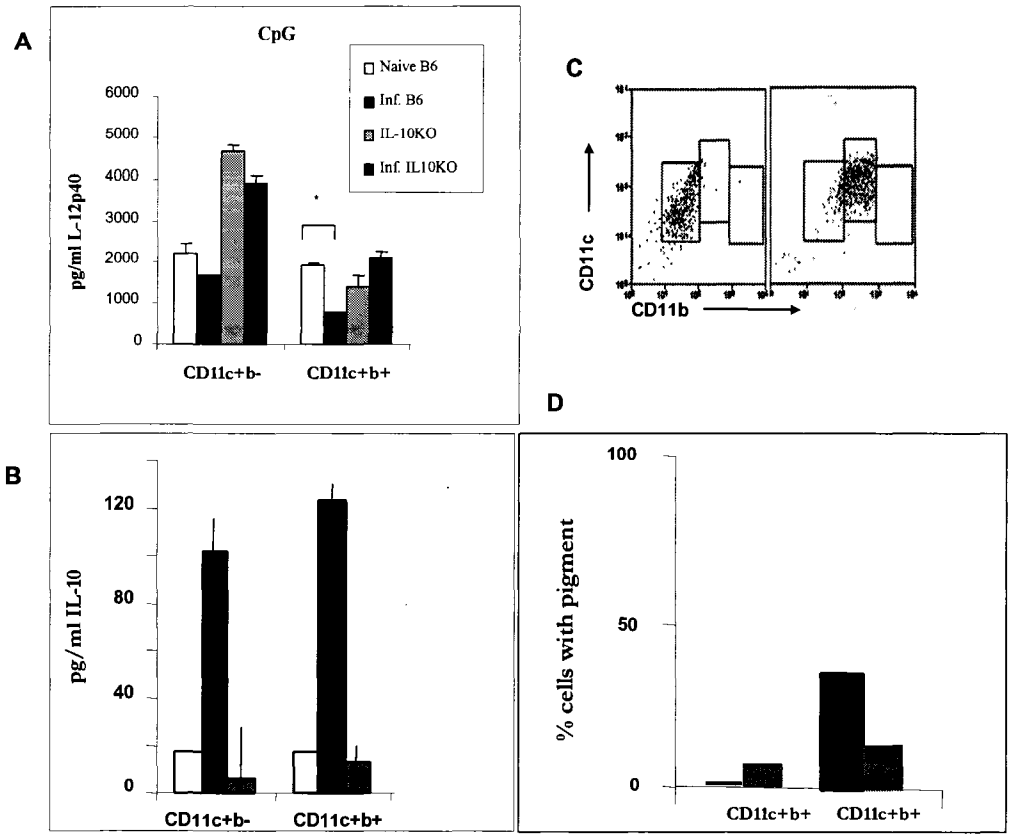


Figure 5.1.2. CD11c+ DC subsets from wild type and IL-10 KO mice reflect the in vivo response to TLR ligation. DCs from naïve B6, day 17 infected B6 and day 17 infected IL-10 KO mice were sorted based upon the level of CD11b expression, and then cultured with CpG for 24 hours. Sorted cells from infected B6 mice are shown; all sorted populations were greater than 85% pure. CD11c+ DC subsets from wild type and IL-10 KO mice reflect the in vivo response to TLR ligation. A and B) DCs from naïve B6 (white bars), day 17 infected B6 (shaded bars) and day 17 infected IL-10 KO mice (black bars) were sorted based upon the level of CD11b expression, and then cultured with CpG for 24 hours, and IL-12p40 (A) and IL-10 (B) production was measured. C) Sorted cells from infected B6 mice are shown; all sorted populations were greater than 85% pure. D) The percentage of cells containing pigment in each population is shown for infected B6 (shaded bars) and IL-10 (black bars).

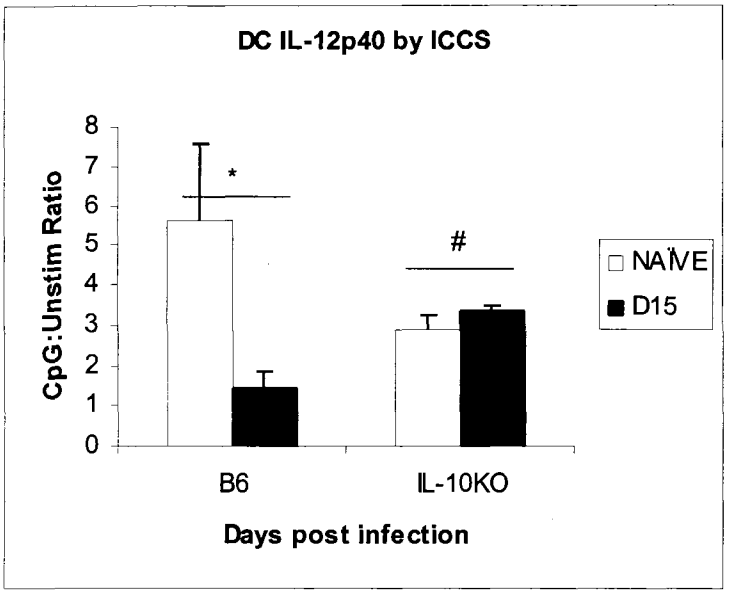


Figure 5.1.3: DCs from IL-10 knockout mice fail to down regulate IL-12p40 in response to CpG as infection progresses. CD11c+CD3-CD19-NK1.1- cells were analyzed for intracellular IL-12p40 after 8hrs of culture with 1ug/ml CpG and 10ug/ml BFA. * $p < 0.05$; # $p = 0.09$.

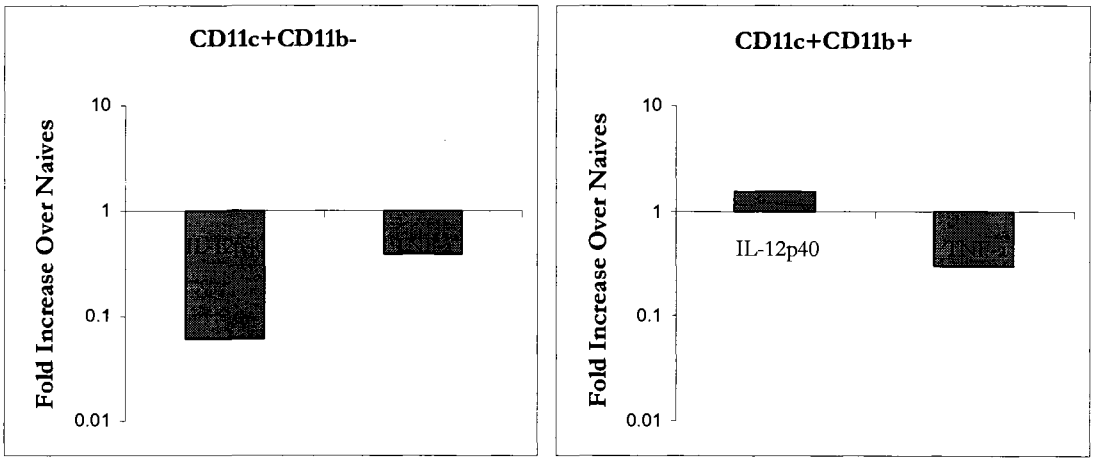


Figure 5.1.4: CD11c+ selected cells from 10 naïve or infected IL-10 knockout mice were further sorted based on CD19-CD3-NK1.1-CD11c+MHCII+CD11b+/-. RNA was harvested and analyzed for IL-12p40 and TNF-α message as shown.

5.2 IL-10 upregulation as infection progresses partially depends on MAP kinase p38 and ERK 1/2, but is NF-κB p50 independent

In an attempt to dissect the molecular mechanisms associated with IL-10 production during *P. yoelii* infection and its role in IL-12 and IFN-γ downregulation, we looked specifically at NF-κB p50. Several reports have implicated this transcription factor in both IL-10 production as well as IL-10's downstream effector mechanisms such as the down regulation of IL-12p70 and IFN-γ (Driessler, Venstrom et al. 2004; Cao, Zhang et al. 2006). We hypothesized that in the absence of p50 infected mice would fail to upregulate IL-10 and therefore retain the ability to produce IL-12p70 and IFN-γ.

Using p50 KO mice, we found that while these mice become more parasitemic, especially early in infection (Figure 5.2.1), they retain the ability to down-regulate both and IL-12p70 and IFN-γ, as well as TNF-α, as infection progresses in response to *in vivo* LPS stimulation (Figure 5.2.2). Message data with respect to IL-12p40 and IL-10 were not significantly different from WT mice treated in a similar fashion (Figure 5.2.3).

We then hypothesized that the switch from IL-12p40 production at day 3 p.i. to IL-10 production at day 15-17 p.i. in DCs is mediated by changes in p38 and ERK 1/2 activities as these molecules have been shown to differentially regulate IL-12 and IL-10 production in the context of other infections (Mathur, Awasthi et al. 2004). In order to test this hypothesis, we cultured magnetically selected CD11c+ DCs from infected day 15 p.i. mice and stimulated them with CpG in the presence of specific inhibitors of p38

(SB203580) and ERK 1/2 (PD098059), or not (Figure 5.2.4). Inhibition of p38 led to a significant abrogation of IL-12p40 at this time point where inhibition of ERK 1/2 had no significant effect on its production, albeit, there was a trend toward an increase in IL-12p40 production with the inhibition of ERK 1/2. IL-10 production was below the limits of detection in this study (data not shown). Further studies using FAC sorted DCs may provide a more sensitive method for achieving significance. These studies are currently underway in our laboratory.

Sorted DCs were also tested for the presence of activated (phosphorylated) p38, ERK 1/2 and p65 by western blot. By this method, we were unable to detect significant differences between infected and naïve mice (data not shown) nor changes following stimulation with CpG (Figure 5.2.5). One possible explanation for this is that the process of cell sorting induces the phosphorylation of these signaling molecules.

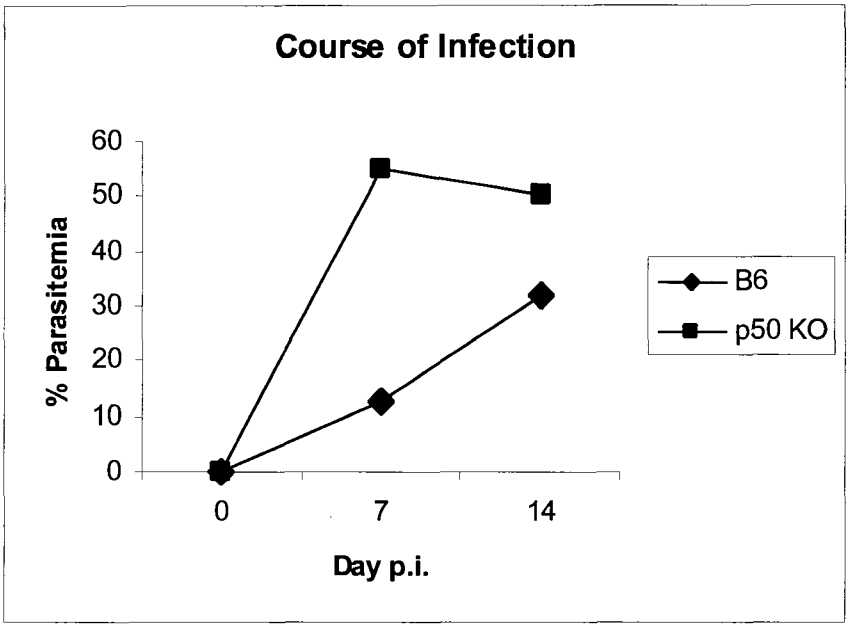


Figure 5.2.1: Average parasitemia obtained from 8 individual mice as a function of time post infection in WT B6 mice and p50 KO mice. Data points are representative of two individual experiments of three mice in each group.

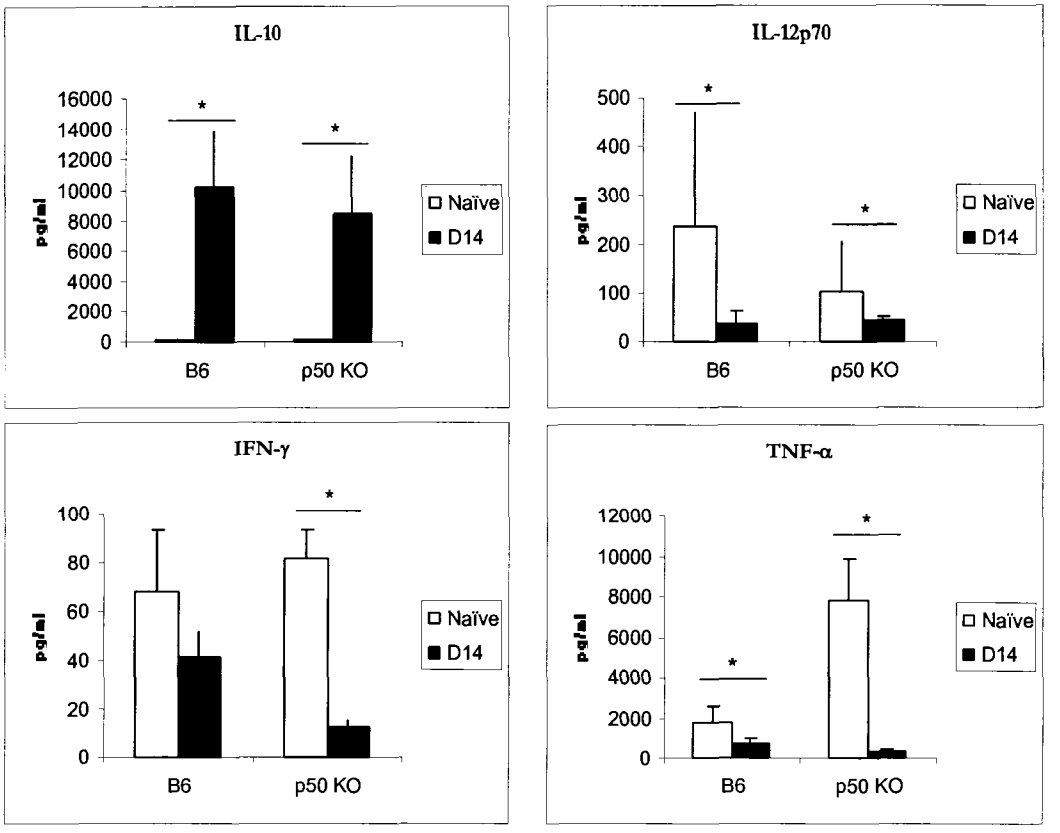


Figure 5.2.2: Serum cytokine levels following LPS treatment of p50 KO mice infected with *P. yoelii.* NF-κB does not appear to play a role in the production of IL-10 nor the inhibition of LPS induced IL-12, IFN-γ or TNF-α. B6 and p50 knockout mice were infected with P. yoelii then at day 13 p.i. challenged with 100ug LPS IP and sacrificed 3 hours later. Serum levels of IL-10, IL-12p70, IFN-γ and TNF-α were measured by CBA assay.

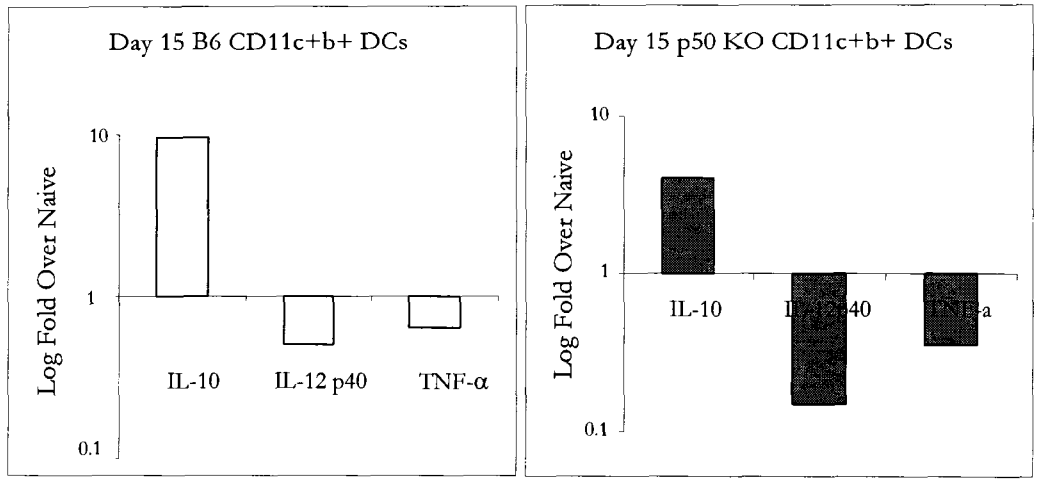


Figure 5.2.3: Cytokine mRNA levels in p50 knockout mice compared with WT B6 controls. Message levels from DC11c+b+ DCs are shown. Adequate RNA levels could not be obtained from sort CD11c+11b- DCs due to a limited number of p50 knockout mice used in these experiments. Data is representative of two sort experiments with sorting parameters similar to those previously described.

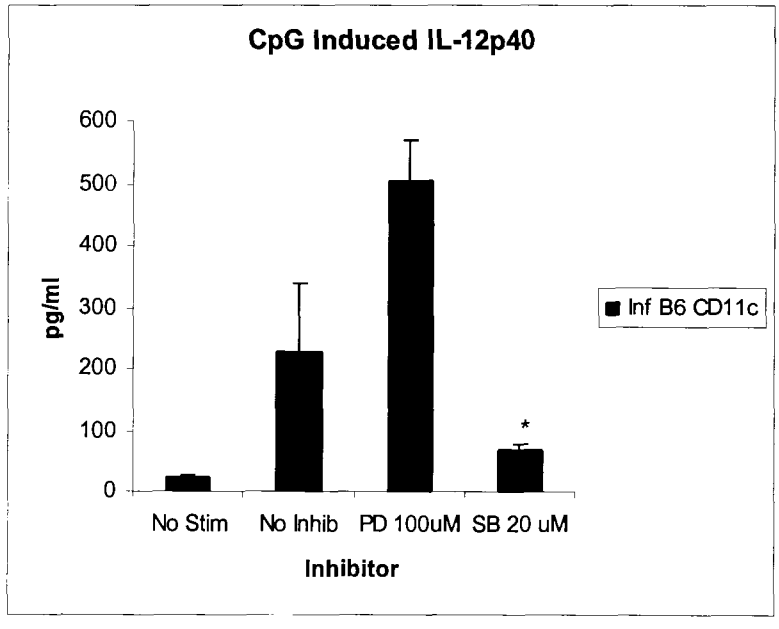


Figure 5.2.4: Only SB (selective p38 inhibitor) significantly affected IL-12p40 production at 24hrs and 72hrs of culture following CpG stimulation. While PD (selective ERK 1/2 inhibitor) show a trend at enhancing IL-12p40 production, these data did not achieve statistical significance. The data shown is representative of 3 individual mice with treatments in duplicate.

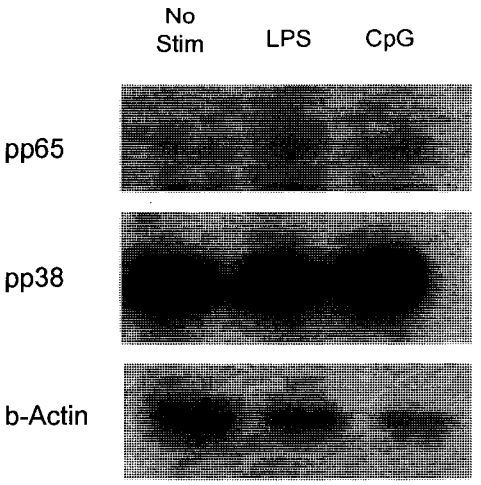


Figure 5.2.5: NF-κB p65 and MAP kinase p38 become activated following DC sorting and are subsequently refractory to TLR ligand stimulation. DCs sorted from naïve mice based on CD11c+MHCII+CD19-CD3-NK1.1- expression were stimulated for 30 minutes then whole cell lysates analysed by western blot for phosphorylation of p65 and p38.

5.3 In the absence of IL-10, significant anemia and liver pathology ensues.

In addition to the laboratory differences associated with the lack of IL-10, clinically, IL-10 knockout mice show more pathology relative to their degree of parasitemia compared to wild type mice. Specifically, despite lower parasitemia, IL-10 KO mice show more extensive hepatocellular damage and develop significantly higher anemia to parasitemia ratios and than wild type mice.

In the first of several experiments comparing the maximum change in total RBC count to maximum parasitemia, IL-10 knockout mice had a maximum change in RBC count (10^6/ul) to maximum parasitemia (%) of 4.3 compared to 0.2 in B6 controls ($p < 0.001$) (Figure 5.3.1 and 5.3.2). These findings were repeated a minimum of three times and

achieved significance ($p < 0.05$). In addition to the degree of anemia in these mice, when sacrificed at day 14 the mean serum alanine transferase (ALT), a measure of hepatocellular damage, was 732 g/dl as compared to 100 mg/dl in B6 mice at the same day p.i., which was statistically significant ($p < 0.05$)(Figure 5.3.3B). Additionally, based on histopathologic analysis of the livers of these mice following sacrifice, the IL-10 knockout mice had multifocal hepatocellular coagulative necrosis throughout the parenchyma (Figure 5.3.3A and 5.3.4). It is important to note that in addition to lower parasitemias, IL-10 knockout had a shorter duration of parasitemia with respect to infected B6 controls (Figure 5.3.5).

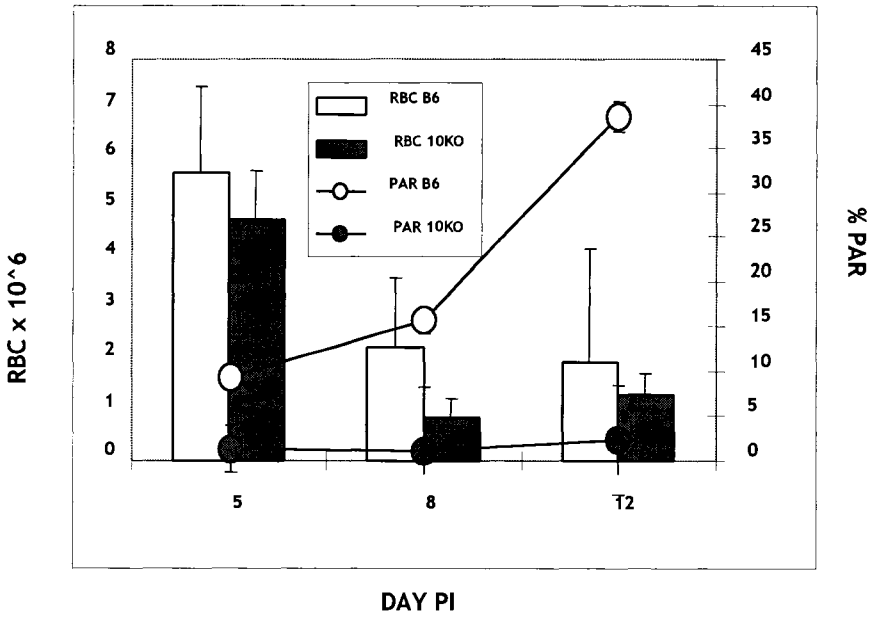


Figure 5.3.1: IL-10 knockout mice show significantly higher anemia relative to parasitemia. Erythrocyte counts (10^6 cells/ul) are displayed on the right axis and average percent parasitemia on the right axis. Data is representative of 3 independent experiments of 5 mice per experiment.

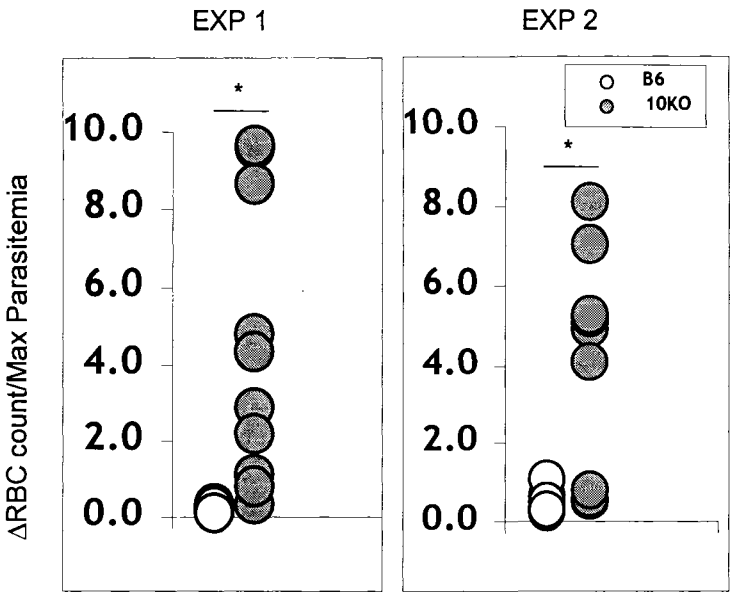


Figure 5.3.2: IL-10 knockout mice show significantly higher anemia relative to parasitemia. Each data point represents the maximum change in RBC count over the maximum parasitemia in a single mouse. * $p < 0.05$

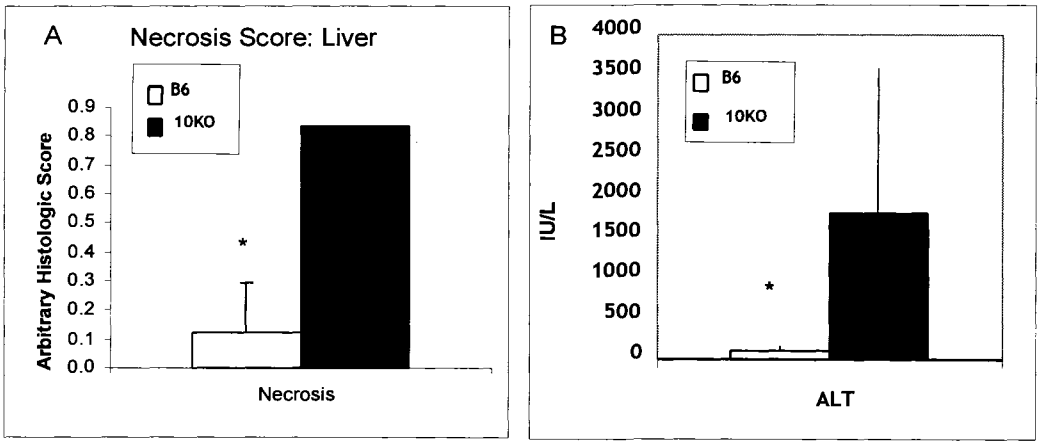


Figure 5.3.3 : A) Histologic scoring set a 0-5 scale. The average score was significantly higher in IL-10 knockout mice (* $p < 0.01$). B) Mean ALT values for D15 post infected B6 and IL-10 KO mice infected with *P. yoelii* 17 XNL. Data is representative of 3 independent experiments ($p<0.01$).

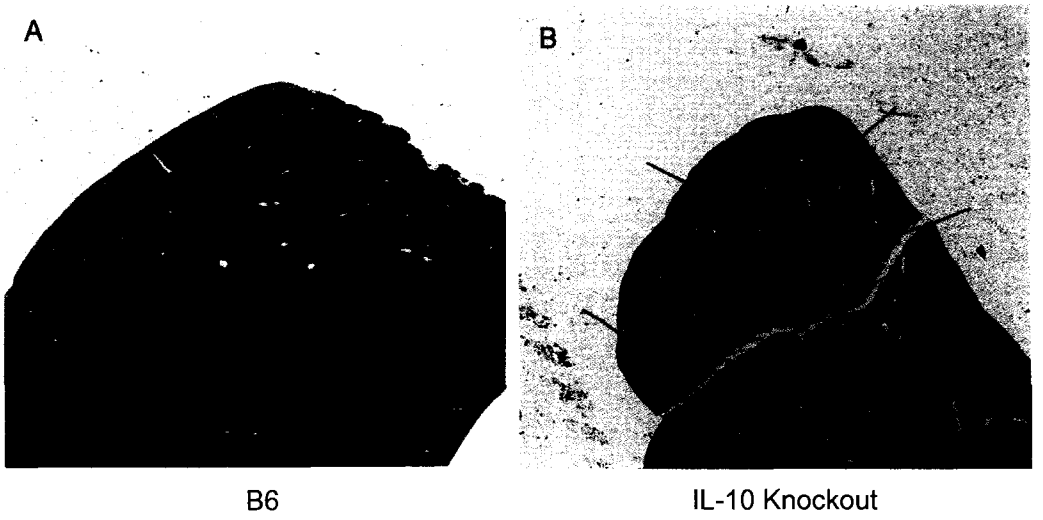


Figure 5.3.4: Interleukin-10 knockout mice show significantly more liver damage associated with infection. Livers from 15 IL-10 knockout and 15 infected wild type B6 controls (3 independent experiments of 5 mice each) were compared. (B) Multifocal hepatocellular coagulative necrosis (Arrows) relative to similarly infected wild type mouse liver (A).

Discussion:

Role of IL-10 in down regulating immune responses:

Studies done in our lab have shown that not only is IL-10 upregulated as infection progresses, it also plays a pivotal role in the suppression of some, but not all pro-inflammatory cytokines. First, in chapter 2, we show that blockade of IL-10 in cultures wild type DCs from infected mice and TCR-Tg T cells and antigen results in significantly improved IFN-γ production by the responding T cells but had little effect on IL-2 production (Figure 2.3.1). In this chapter we show IL-10 knockout mice retain the ability to produce IL-12 and IFN-γ throughout infection in response to LPS administered *in*

vivo. Additionally, in the absence of IL-10 mice experience significantly higher anemia to parasitemia ratios and more hepatocellular damage and necrosis during *P. yoelii* infection. Li et al. also showed that this lack of IL-10 leads to greater weight loss, hypoglycemia and hypothermia associated with *P. chabaudi* infection relative to infected wild type controls (Li, Corraliza et al. 1999). Experiments in which IL-12 or IFN-γ are blocked in IL-10 deficient mice are necessary to definitively link IL-12 and/or IFN-γ to the observed pathology. Interestingly, the absence of IL-10 did not affect the downregulation of TNF-α production, indicating that initiation of tolerance with respect to TNF-α production is mediated by other means, i.e. TGF-β. Other groups have partially addressed this in studies where anti-TGF-β was administered together with anti-IL-10 receptor antibody (but neither alone) leading to enhanced TNF-α and IFN-γ production during *P. yoelii 17XL* infection (Omer, de Souza et al. 2003). Another group found that *in vivo* administration of anti-TGF-β marginally enhanced TNF-α levels in the serum of infected IL-10 knockout mice (Li, Sanni et al. 2003), suggesting that this cytokine may exert some additional specific control over TNF-α production. Recently TGF-β was shown to play a central role in endotoxin tolerance (Sly LM 2004), further supporting this idea. Further studies are necessary for the resolution of this dichotomy between IL-12 and TNF-α regulation in wild type and IL-10 knockout mice during malaria infection.

The response to *P. falciparum* derived GPI characterized by Zhu *et al.* suggested one mechanism for this differential regulation, in that inhibition of the ERK 1/2 MAP kinase pathway resulted in upregulation of IL-12 but not TNF-α (Zhu, Krishnegowda et al.

2005). Other studies looking at differential production, rather than specific downregulation, of IL-12 and TNF-α following stimulation with *Brucella abortus* found that both IL-12 and TNF-α production is dependent on MyD88, however, IL-12 production was mediated through TLR9 and TNF-α via TLR2 (Huang, Aliberti et al. 2003; Huang, Ishii et al. 2005). In the context of our results, a reasonable hypothesis would be that IL-10 specifically inhibits signaling through TLR9 while another factor such as TGF-β may be involved in inhibiting signals through TLR2. It would be interesting to block TGF-β production *in vivo* during infection then see if there is a similar loss of TNF-α tolerance as seen with IL-12 in IL-10 knockout mice following LPS stimulation. Other studies looking specifically upstream of the TNF-α and IL-12p40 (and IL-12p35) genes looking for candidate regulators that are disparate between these two cytokines may prove informative.

Many other disease models have looked at the role of IL-10 in reducing inflammatory processes and limiting autoimmunity. For example, Appleton's group from Cornell University recently showed that IL-10 production protects against the occurrence of hepatitis in mice infected with *Trichonella spiralis* (Bliss, Bliss et al. 2007). Similarly, Erhardt *et al* showed that IL-10 production in the liver is essential for protection against ConA mediated hepatic inflammatory cell infiltrate and hepatocellular necrosis (Erhardt 2007).

The primary sources of this protective source of IL-10 during malaria infection and the other studies just mentioned remain elusive. Studies to date show that nearly all cells of

the immune system are capable of its production (Fickenscher, Hor et al. 2002). Mauri et al. showed B cell derived IL-10 is required for the protection against collagen induced arthritis (Mauri, Gray et al. 2003; Asako Chiba 2004). Helminth infection protects mice from anaphylaxis in a B cell derived IL-10 dependent manner. (Mangan 2004).
Mice devoid of B cells through anti-Ig treatment fail to down-regulate the IFN-γ response during *P. chabaudi chabaudi* infection (Taylor-Robinson and Phillips 1994). Unfortunately, IL-10 production was not measured in this studie. A more recent report showed that B cell knockout mice do have the capability to secrete high levels of IL-10 during the chronic phase of malaria infection (Weidanz, Batchelder et al. 2005). Based on these two studies, using two different systems, it is difficult to clearly define the role of B cells in the induction of an anti-inflammatory phenotype. Further studies are being done in our lab using B cell KO mice to address this discrepancy. Interestingly, preliminary data shows that IgH mice, even following adoptive transfer of B cells from naïve mice, are highly susceptible to acute infection with non-lethal *P. yoelii* and die of excessive parasitemia (mean = 88%) by day 7 p.i. (Perry unpublished data). This is in contrast to wild type controls at this day of infection who appear clinically healthy and harbor a mean parasitemia of 13% at this time-point ($p < 0.001$).

Another recent study points at T cells as perhaps the most important producers of IL-10. Using *T. gondii* infection, Roers *et al.* showed that targeted deletion of the IL-10 locus in T cells results in a phenotype similar to mice completely devoid of IL-10 (Roers, Siewe et al. 2004). This finding is supported by studies previously mentioned looking at ConA induced liver damage, where CD4+ T cells play a sufficient role in the pathogenesis (Erhardt 2007). Kupfer cells (resident macrophages within the liver) and NK T cells

were also implicated in ConA mediated liver pathology (Erhardt 2007). More specifically, T regulatory type 1 (T_r1) cells, characterized as being derived from IL-10 producing DCs and phenotypically $CD4^+Foxp3^-$, have been implicated as a major T cell source of IL-10 in multiple systems (Awasthi, Carrier et al. 2007).

Unpublished data from our lab also implicates the importance of T cells in this process. We see that T cells isolated from late stages of infection as well as from mice having cleared the infection produce significantly higher levels of IL-10 than those T cells isolated from naïve mice. This was initially shown by ELISA using T cells isolated from infected mice. Subsequent experiments looking at T cells from infected mice *ex vivo* revealed that these same IL-10 producing T cells also co-express IFN-γ and the T_H1 marker, T-bet (Kiser/Perry unpublished data). A similar report of IFN-γ^+IL-10^+ co-expressing T cells was recently published by Jankovic et al. in response to *T. gondii* infection where these cells were further characterized as $CD4^+$T-bet^+FoxP3^- T_H1 effector T cells (Jankovic, Kullberg et al. 2007).

Naturally occurring regulatory T (Treg) cells also likely play a role as well in limiting inflammation as infection progress. Again, few reports exist specifically looking at the role of Treg cells during malaria infection. One study reported that depletion of $CD4^+CD25^+$ T cells improved survival in a lethal model of *P. yoelii* infection. However, this group did not explore the consequence of $CD25^+$ cell depletion had on IL-10 production within these mice (Hisaeda, Maekawa et al. 2004). Subsequent studies from this group showed that DCs, specifically those being activated through TLR 9 ligation, favor the production of $CD4^+CD25^+$ T cells with a regulatory phenotype (Hisaeda,

Tetsutani et al. 2008). Further studies are required to investigate the role of T cells in the initiation and maintenance of IL-10 production late during infection, and how their production of IL-10 effects other cell populations, including DCs. Some of these studies looking at T cells and IL-10 are currently underway in our laboratory. Interestingly, preliminary studies in our lab using nude mice in a T cell adoptive transfer model suggest that T cells play a minor role in the upregulation of IL-10 as malaria infection progresses. This is evidenced by the observation that nude mice receiving IL-10 knockout T cells produce significantly ($p < 0.05$) more of IL-10 upon LPS challenge *in vivo* relative to nude mice receiving WT T cells (data not shown). This work was done in conjunction with Patti Kiser's dissertation in the lab.

Interleukin-10 production by dendritic cells likely represent an underlying mechanism by which the host can down-regulate innate pro-inflammatory cytokine responses and instruct the adaptive immune system to do the same. In order for such "regulatory", IL-10 producing DCs' to transmit the down-regulatory signal to the adaptive immune system, they must maintain their ability to activate naïve T cells as we demonstrate here in Chapter 3. Maintenance of high levels of co-stimulatory proteins and MHC class II may be the result of TLR signaling through a pathway not tolerized by interaction with *Plasmodium* derived molecules, such as the TLR4-dependent, MyD88 independent pathway (Hemmi, Takeuchi et al. 2000) or by CD40-CD40L interaction. Because IL-10 producing DCs can activate naïve T cells to do the same, it is reasonable to assume that DCs play a key role in generating an adaptive immune response that is subsequently required for perpetuating an anti-inflammatory state. This could be one mechanism for

generating anti-disease immunity in *P. falciparum* infected people (Gupta, Snow et al. 1999).

Molecular mechanisms leading to IL-10 production:

The mechanism by which IL-10 is upregulated during malaria infection has not been established. Here we tested two hypotheses; first, that IL-10 production was controlled by the NF-κB subunit p50. Second, that IL-10 production was driven by the MAP kinase ERK 1/2. For the most part, these questions remain unresolved, especially in the context of malaria infection. NF-κB p50 has been implicated in both IL-10 production and its downstream effector functions, specifically, macrophages from $p50^{-/-}$ mice exhibit aberrant cytokine responses to LPS, characterized by a decrease in IL-10 production and significant increases in IL-12 and TNF-α. Additionally, these mice are more susceptible to lethal endotoxemia (Cao, Zhang et al. 2006). Studies done in our laboratory show little difference in IL-12 and TNF-α production in response to LPS in infected p50 KO mice, suggesting that p50 does not play a major role in IL-10 upregulation or its downstream affects on IL-12 and TNF-α downregulation in the context of malaria infection. Furthermore, the course of anemia relative to parasitemia in p50 KO mice was the same as in WT mice, suggesting that p50 does not play a role in the mechanisms by which IL-10 protects from severe anemia potentially independent of IL-12 and TNF-α.

In addition to NF-κB p50, we asked whether the MAP kinases p38 and ERK 1/2 play a role in pro- and anti-inflammatory cytokine production in DCs during malaria infection,

as several reports suggest their involvement in similar inflammatory processes (Lu HT 1999; Yi, Yoon et al. 2002). We first attempted to measure levels of these signaling molecules in sorted naïve DCs with or without TLR ligation and found that the process of sorting led to significant activation as measured by phosphorylation, and subsequent TLR ligation did not lead to further activation. This problem might be circumvented if sorted DCs are allowed to incubate in culture for a period of time prior to stimulation with TLR ligands. However, due to the number of mice necessary for each sort, we chose to abandon this method and attempt to inhibit p38 and ERK 1/2 *in vitro* to gain insight into their function regarding IL-10 and IL-12 production.

In *in vitro* experiments where p38 and ERK 1/2 were inhibited with SB203580 PD098059, respectively, we found a significant role for p38 in the production of IL-12 and a minor, but not statistically significant role of ERK 1/2 in IL-12 inhibition. The studies described herein were done using magnetically selected CD11c+ cells. Further, large scale, sorting experiments are necessary to resolve the role of p38 and ERK 1/2, and are currently underway in our laboratory.

Several other mechanisms of IL-10 mediated inhibition of pro-inflammatory cytokine production have recently been discussed. A role for suppressor of cytokine signaling 3 (SOCS-3) has been suggested by several groups in IL-10 mediated suppression of pro-inflammatory cytokine production by DCs and macrophages (Cassatella, Gasperini et al. 1999; Li, Chu et al. 2006). Another candidate molecule associated with the down regulation of IL-12 and other pro-inflammatory cytokines is ST2. Addition of soluble ST2 to mice prior to LPS treatment resulted in significantly reduced IL-12, TNF-α and

IL-6 production, whereas blockade of ST2 signaling significantly upregulated the production of these cytokines in response to LPS (Sweet, Leung et al. 2001; Leung, Xu et al. 2004; Trajkovic, Sweet et al. 2004). ST2 was also shown to shift the T helper response from T_H1 to T_H2 in Balb/c mice infected with *L. major* and ST2 blockade led to mouse survival in an otherwise fatal model of *L. major* infection (Xu, Chan et al. 1998). A preliminary study done in our lab showed only slightly elevated expression of ST2 on DCs at day 21 p.i. relative to naïve controls ($p = 0.05$). Further studies are necessary to confirm this observation and to determine its biologic significance.

Another important question is whether IL-10 production by DCs is required to induce its production in naïve T cells, in the context of malaria infection; to date, no studies have addressed this conundrum. However, when infected IL-$10^{-/-}$RAG$^{-/-}$ mice were reconstituted with naive WT $CD4^+$ T cells, IL-10 mRNA was induced, albeit to a lesser extent than in all other IL-10-competent groups (Hesse, Piccirillo et al. 2004). These findings indicate that IL-10-producing T cells can develop in the absence of an innate source of IL-10 but perhaps less effectively in the absence of DC derived IL-10. The results also confirm that IL-10 can be induced independently in both T cell and non-T cell populations. Consistent with the more modest responses reported in IL-10 KO mice versus WT controls, mice receiving WT $CD4^+$ cells consistently displayed smaller increases in serum ALT/AST than did animals receiving IL-10 KO $CD4^-$ T cells (Hesse, Piccirillo et al. 2004). Similar results were obtained in our laboratory when WT CD45.1 T cells were transferred to IL-10 KO or WT mice. While these T cells did support IL-10 production in the KO environment as a result of infection, the amounts of IL-10 produced by these cells was significantly lower than that observed in CD45.1 T cells

transferred to infected WT mice suggesting that paracrine derived IL-10 is required for maximal IL-10 production by T cells (data not shown).

Based on the findings in this chapter, we suggest that increased production of IL-10 by DCs as infection progresses, and the T cells they subsequently activate, can be correlated with the observed reduction in IL-12 and IFN-γ seen globally. Decreased pro-inflammatory cytokine production by DCs is likely the result of tolerance to microbial stimulation through toll like receptors similar to the state of endotoxin tolerance described after the administration of sub-lethal doses of LPS. While the underlying mechanisms remain obscure, it is clear that the tolerance phenomena has evolved as a complex orchestrated counter regulatory response to inflammation resulting in the preferential production of anti-inflammatory cytokines such as IL-10 and TGFβ and reduced production of proinflammatory cytokines such as IL-12, TNF-α and IFN-γ. It is this switch to an anti-inflammatory phenotype that likely protects hosts from severe inflammatory and autoimmune disease associated with infection and associated tissue pathology as has been observed in other system (Tanaka Y 1996).

References:

Asako Chiba, S. O. K. M. H. H. T. Y. S. M. (2004). "Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of ?-galactosylceramide." Arthritis & Rheumatism **50**(1): 305-313.

Awasthi, A., Y. Carrier, et al. (2007). "A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells." Nat Immunol **advanced online publication**.

Bliss, S. K., S. P. Bliss, et al. (2007). "IL-10 Regulates Movement of Intestinally Derived CD4+ T Cells to the Liver." J Immunol **178**(12): 7974-7983.

Boutlis, C. S., T. W. Yeo, et al. (2006). "Malaria tolerance - for whom the cell tolls?" Trends in Parasitology **22**(8): 371-377.

Cao, S., X. Zhang, et al. (2006). "NF-{kappa}B1 (p50) Homodimers Differentially Regulate Pro- and Anti-inflammatory Cytokines in Macrophages." J. Biol. Chem. **281**(36): 26041-26050.

Carapau, D., M. Kruhofer, et al. (2007). "Transcriptome profile of dendritic cells during malaria: cAMP regulation of IL-6." Cellular Microbiology **9**(7): 1738-1752.

Cassatella, M. A., S. Gasperini, et al. (1999). "Interleukin-10 (IL-10) Selectively Enhances CIS3/SOCS3 mRNA Expression in Human Neutrophils: Evidence for an IL-10-Induced Pathway That Is Independent of STAT Protein Activation." Blood **94**(8): 2880-2889.

Dong, C., R. J. Davis, et al. (2002). "MAP KINASES IN THE IMMUNE RESPONSE." Annual Review of Immunology **20**(1): 55-72.

Dragoi AM, F. X., Ivanov S, Zhang P, Sheng L, Wu D, Li GC, Chu WM. (2005). "DNA-PKcs, but not TLR9, is required for activation of Akt by CpG-DNA." EMBO J. **24**(4): 779-89.

Driessler, F., K. Venstrom, et al. (2004). "Molecular mechanisms of interleukin-10-mediated inhibition of NF-kB activity: a role for p50." Clin Exp Immunol **135**(1): 64-73.

Erhardt, A., Markus Biburger Thomas Papadopoulos Gisa Tiegs (2007). "IL-10, regulatory T cells, and Kupffer cells mediate tolerance in concanavalin A-induced liver injury in mice." Hepatology **45**(2): 475-485.

Fan H, C. J. (2004). "Molecular mechanisms of endotoxin tolerance." J Endotoxin Res. **10**(2): 71-84.

Fickenscher, H., S. Hor, et al. (2002). "The interleukin-10 family of cytokines." Trends in Immunology **23**(2): 89-96.

Ghosh, S. and M. Karin (2002). "Missing Pieces in the NF-[kappa]B Puzzle." Cell **109**(2, Supplement 1): S81-S96.

Ghosh, S., M. J. May, et al. (1998). "NF-κB AND REL PROTEINS: Evolutionarily Conserved Mediators of Immune Responses." Annual Review of Immunology **16**(1): 225-260.

Gupta, S., R. W. Snow, et al. (1999). "Immunity to non-cerebral severe malaria is acquired after one or two infections." Nature Medicine **5**(3): 340-343.

Hemmi, H., O. Takeuchi, et al. (2000). "A Toll-like receptor recognizes bacterial DNA." Nature **408**(6813): 740-745.

Hesse, M., C. A. Piccirillo, et al. (2004). "The Pathogenesis of Schistosomiasis Is Controlled by Cooperating IL-10-Producing Innate Effector and Regulatory T Cells." J Immunol **172**(5): 3157-3166.

Hisaeda, H., Y. Maekawa, et al. (2004). "Escape of malaria parasites from host immunity requires CD4+CD25+ regulatory T cells." Nat Med **10**(1): 29-30.

Hisaeda, H., K. Tetsutani, et al. (2008). "Malaria Parasites Require TLR9 Signaling for Immune Evasion by Activating Regulatory T Cells." J Immunol **180**(4): 2496-2503.

Huang, L.-Y., J. Aliberti, et al. (2003). "Heat-Killed Brucella abortus Induces TNF and IL-12p40 by Distinct MyD88-Dependent Pathways: TNF, Unlike IL-12p40 Secretion, Is Toll-Like Receptor 2 Dependent." J Immunol **171**(3): 1441-1446.

Huang, L.-Y., K. J. Ishii, et al. (2005). "Th1-Like Cytokine Induction by Heat-Killed Brucella abortus Is Dependent on Triggering of TLR9." J Immunol **175**(6): 3964-3970.

Jankovic, D., M. C. Kullberg, et al. (2007). "Conventional T-bet+Foxp3- Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection." J. Exp. Med. **204**(2): 273-283.

Karin, M. and M. Delhase (2000). "The I[kappa]B kinase (IKK) and NF-[kappa]B: key elements of proinflammatory signalling." Seminars in Immunology **12**(1): 85-98.

Leung, B. P., D. Xu, et al. (2004). "A Novel Therapy of Murine Collagen-Induced Arthritis with Soluble T1/ST2." J Immunol **173**(1): 145-150.

Li, C., I. Corraliza, et al. (1999). "A Defect in Interleukin-10 Leads to Enhanced Malarial Disease in Plasmodium chabaudi chabaudi Infection in Mice." Infect. Immun. **67**(9): 4435-4442.

Li, C., L. A. Sanni, et al. (2003). "Pathology of *Plasmodium chabaudi chabaudi* infection and mortality in interleukin-10 deficient mice are meliorated by anti-tumor necrosis factor alpha and exacerbated by anti-transforming growth factor β antibodies." Infection and Immunity **71**(9): 4850-4856.

Li, Y., N. Chu, et al. (2006). "Dendritic Cells Transduced with SOCS-3 Exhibit a Tolerogenic/DC2 Phenotype That Directs Type 2 Th Cell Differentiation In Vitro and In Vivo." J Immunol **177**(3): 1679-1688.

Lu HT, Y. D., Wysk M, Gatti E, Mellman I, Davis RJ, Flavell RA (1999). "Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice." EMBO J. 1999 Apr 1;18(7):1845-57 **18**(7): 1845-57.

Lucas, M., X. Zhang, et al. (2005). "ERK Activation Following Macrophage Fc{gamma}R Ligation Leads to Chromatin Modifications at the IL-10 Locus." J Immunol **175**(1): 469-477.

Mathur, R. K., A. Awasthi, et al. (2004). "Reciprocal CD40 signals through p38MAPK and ERK-1/2 induce counteracting immune responses." Nat Med **10**(5): 540-544.

Mauri, C., D. Gray, et al. (2003). "Prevention of Arthritis by Interleukin 10-producing B Cells." J. Exp. Med. **197**(4): 489-501.

Omer, F. M., J. B. de Souza, et al. (2003). "Differential Induction of TGF-{beta} Regulates Proinflammatory Cytokine Production and Determines the Outcome of Lethal and Nonlethal Plasmodium yoelii Infections." J Immunol **171**(10): 5430-5436.

Ouaaz, F., J. Arron, et al. (2002). "Dendritic Cell Development and Survival Require Distinct NF-[kappa]B Subunits." Immunity **16**(2): 257-270.

Perry, J. A., A. Rush, et al. (2004). "Dendritic Cells from Malaria-Infected Mice Are Fully Functional APC." J Immunol **172**(1): 475-482.

Pulendran, B., J. Lingappa, et al. (1997). "Development pathways of dendritic cells in vivo." Journal of Immunology **159**(5): 2222-2231.

Roers, A., L. Siewe, et al. (2004). "T Cell-specific Inactivation of the Interleukin 10 Gene in Mice Results in Enhanced T Cell Responses but Normal Innate Responses to Lipopolysaccharide or Skin Irritation." J. Exp. Med. **200**(10): 1289-1297.

Sen, R. and D. Baltimore (1986). "Inducibility of [kappa] immunoglobulin enhancer-binding protein NF-[kappa]B by a posttranslational mechanism." Cell **47**(6): 921-928.

Sly LM, R. M., Kalesnikoff J, Song CH, Krystal G. (2004). "LPS-induced upregulation of SHIP is essential for endotoxin tolerance." Immunity **21**: 227-239.

Stevenson, M. M. and E. M. Riley (2004). "Innate immunity to malaria." Nat Rev Immunol **4**(3): 169-180.

Sweet, M. J., B. P. Leung, et al. (2001). "A Novel Pathway Regulating Lipopolysaccharide-Induced Shock by ST2/T1 Via Inhibition of Toll-Like Receptor 4 Expression." J Immunol **166**(11): 6633-6639.

Tanaka Y, T. A., Watanabe K, Takayama K, Yahata T, Habu S, Nishimura T. (1996). "A pivotal role of IL-12 in Th1-dependent mouse liver injury." Int Immunol. 1996 Apr;8(4):569-76 **8**(4): 569-76.

Taylor-Robinson, A. W. and R. S. Phillips (1994). "B cells are required for the switch from Th1- to Th2-regulated immune response to *Plasmodium chabaudi chabaudi* infection." Infection and Immunity **62**: 2490-2498.

Trajkovic, V., M. J. Sweet, et al. (2004). "T1/ST2--an IL-1 receptor-like modulator of immune responses." Cytokine & Growth Factor Reviews **15**(2-3): 87-95.

Weidanz, W. P., J. M. Batchelder, et al. (2005). "Plasmodium chabaudi adami: Use of the B-cell-deficient mouse to define possible mechanisms modulating parasitemia of chronic malaria." Experimental Parasitology **111**(2): 97-104.

Xu, D., W. L. Chan, et al. (1998). "Selective Expression of a Stable Cell Surface Molecule on Type 2 but Not Type 1 Helper T Cells." J. Exp. Med. **187**(5): 787-794.

Yang, Z., D. M. Mosser, et al. (2007). "Activation of the MAPK, ERK, following Leishmania amazonensis Infection of Macrophages." J Immunol **178**(2): 1077-1085.

Yi, A.-K., J.-G. Yoon, et al. (2002). "Role of Mitogen-Activated Protein Kinases in CpG DNA-Mediated IL-10 and IL-12 Production: Central Role of Extracellular Signal-Regulated Kinase in the Negative Feedback Loop of the CpG DNA-Mediated Th1 Response." J Immunol **168**(9): 4711-4720.

Zanetti, M., P. Castiglioni, et al. (2003). "The Role of relB in Regulating the Adaptive Immune Response." Ann NY Acad Sci **987**(1): 249-257.

Zhong, H., M. J. May, et al. (2002). "The Phosphorylation Status of Nuclear NF-[kappa]B Determines Its Association with CBP/p300 or HDAC-1." Molecular Cell **9**(3): 625-636.

Zhu, J., G. Krishnegowda, et al. (2005). "Induction of Proinflammatory Responses in Macrophages by the Glycosylphosphatidylinositols of Plasmodium falciparum: The Requirement of Extracellular Signal-Regulated Kinase, p38, c-Jun N-Terminal Kinase and NF-kB Pathways for the Expression of Proinflammatory Cytokines and Nitric Oxide." J. Biol. Chem. **280**(9): 8617-8627.

Chapter 6: **Significance and Relevance of this work to the development of effective treatment strategies for malaria disease and infection.**

The work in this dissertation has provided several novel and confirmatory findings that have implications for our understanding of the host-parasite interactions that occur during malaria infection. Our findings also exemplify the difficulties associated with creating effective vaccine strategies against malaria infection and disease.

In the first experimental chapter we showed that heterologous T cell responses are specifically inhibited *in vitro* and *in vivo* as a result of malaria infection. *In vitro* findings show that macrophages contain the majority of the inhibitory capacity conferred by whole spleen cells when cultured with TCR-Tg T cells and antigen. We then go on to show in the subsequent chapters that in well adapted host-parasite interactions, such as that which occurs with infection of B6 mice with the non-lethal form of *P. yoelii* 17X, there is a well orchestrated shift from a pro-inflammatory state early to anti-inflammatory as infection progresses. This process occurs at least in part by modulating fully functional dendritic cells to either produce IL-12 or IL-10 as a function of the stage of infection. In less well adapted models of murine malaria infection, the importance of this shift become exquisitely apparent. For example, IL-10 knockout mice produce large amounts of IL-12 and IFN-γ early, but then fail to down regulate these cytokines as infection progresses, resulting in exacerbated pathology. Other studies have shown an equally detrimental outcome when pro-inflammatory cytokine production is either absent or down-regulated too early. Omer *et al.* found that one difference between lethal and non-lethal *P. yoelii* infection is that in lethal infection, large amounts of TGF-β are

produced early in infection – neutralization of the anti-inflammatory cytokines IL-10 and TGF-β resulted in increased pro-inflammatory cytokine production and resolution of infection rather than death. Similarly, mice devoid of IFN-γ also succumb to infection due to uncontrolled parasite proliferation (Su and Stevenson 2000; Omer, de Souza et al. 2003). Knowing the mechanism associated with these events is essential to our understanding of how to engineer effective vaccine modalities and immune system manipulation for the control against infection as well as the associated immune mediated disease.

The concept of TLR tolerance underlies the majority of the studies described in chapter 4 where IL-12 and TNF-α production by DCs is diminished late during infection in response to TLR ligation. Repeated signaling of cells with LPS *in vitro*, or repeated administration of sub-lethal levels of LPS to humans and animals alike *in vivo* results in a phenomenon that has been termed endotoxin tolerance (reviewed in(Favorite 1942; Cavaillon, Adrie et al. 2003; Fan H 2004)). As defined, cells (or animals) are considered tolerant because they make greatly reduced amounts of IL-12 or TNF-α in response to subsequent stimulation with LPS, and an otherwise lethal dose of LPS will not kill LPS tolerant mice. With the discovery of TLRs, the idea of endotoxin tolerance has been expanded to include the observation that cells which are unresponsive to LPS (a TLR4 ligand) are also unresponsive to signaling with multiple other TLR ligands, and that signaling through multiple TLR ligands can induce unresponsiveness to LPS (Hemmi, Takeuchi et al. 2000; Sato, Takeuchi et al. 2002; Yeo, Yoon et al. 2003), a phenomenon termed hetero- or cross-tolerance.

The term "tolerance" is a misnomer, because it implies a global unresponsiveness. This is not the case, as LPS tolerant dendritic cells and mice as a whole can continue to produce IL-10 following LPS treatment (Varma, Toliver-Kinsky et al. 2001; Wysocka, Robertson et al. 2001). Regardless of the terminology, understanding the signaling mechanisms controlling inflammatory cytokine production is pivotal to the research of both acute and chronic infectious diseases and immune disorders.

Based on our findings we hypothesize that malaria infection, likely in part through TLRs 2 (GPI) and 9 (hemazoin/CpG), induce a state of TLR tolerance in DCs and the animal as a whole. We also propose that this is a beneficial mechanism aimed at reducing the occurrence of immune mediated pathology rather than a dysfunctional or anergic state as advocated by other groups (Urban, Ferguson et al. 1999; Ocana-Morgner, Mota et al. 2003). These data suggest that DCs, in their 'tolerant' state, activate T cells in an efficient manner, albeit activating and expanding a population of T cells with anti-inflammatory properties. It is this that may allow individuals experiencing repeated malaria infections are capable of acquiring anti-disease immunity.

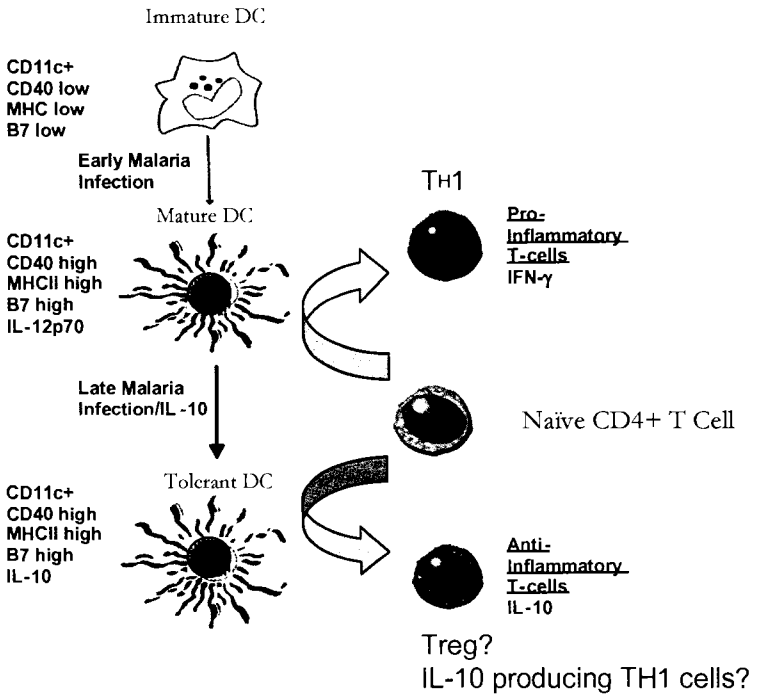


Figure 1: Schematic of DC activation and expression. DCs respond to microbial stimuli (or CD40 ligation—not shown) and mature into IL-12 producing professional APCs. As infection progresses, these DCs specifically lose the ability to secrete IL-12 and begin secreting IL10. Naïve DCs can also become tolerant following activation in the presence of IL-10 or antigen specific T-regs.

6.1 Implications of malaria immunology on immunotherapy and vaccine development.

Malaria parasites, unlike many of the organisms for which there are effective vaccines, present a significant conundrum to the host immune system and vaccinologists alike. The more simple approach of vaccine development against infectious agents is to present the immune system with pathogen derived antigens together with innate stimuli (adjuvants, general PAMPs or modified live vaccines). These approaches have proven inadequate in the prevention of malaria infection, most likely attributable to the

complexity of the *Plasmodium* life cycle, its antigenic heterogeneity with drift potential and the presence of pyogenic toxins such as GPIs . Ideally, one would produce a vaccine that rapidly eliminates parasites and infected cells without the induction of exuberant immune responses. The potential for inducing immunopathology in vaccinated individuals poses an alarming concern and has been one of many factors limiting the vaccine development (Reed, Verhuel et al. 1997; Jones, Stroncek et al. 2002).

With the potential for developing a vaccine that produces sterile, antiparasitic immunity being remote, it is reasonable then to attempt to develop a vaccine that provides immunity to disease rather than the infection itself. With sufficient exposure, immunity to disease does develop in individuals living in high transmission areas making this vaccination strategy plausible. Methods for developing such anti-disease vaccines would likely involve one or both of the following: 1) Activation of malaria specific regulatory T cells, or 2) development of vaccines against malaria-derived molecules that stimulate pro-inflammatory responses, such as the TLR ligands GPI and hemazoin. The efficacy of such strategies are extrapolated from studies where the presence, in one study using *P berghei*, or absence, in one study using the lethal form of *P. yoelii*, of T regs or treatment with anti-GPI antibodies alleviates disease severity (Schofield, Hewitt et al. 2002; Hisaeda, Maekawa et al. 2004). Examples of such anti-disease (anti-toxin) vaccines already on the market against other pathogens include those against tetanus and diphtheria. To date, there are no specific vaccines on the market which target the expansion of regulatory T cells.

Regulatory T cells can be generally defined as T cells that inhibit immune responses by other T cells or antigen presenting cells. There are two general classes of regulatory T cells: naturally occurring CD25+FoxP3+ T regs and induced Tr1, T_H3 or CD8+ T regs. Naturally occurring T regs develop in the thymus and appear to play a dominant role in control of autoimmunity under homeostatic conditions. Depletion of these cells from normal, healthy mice leads to the induction of immune mediated diseases such as thyroiditis, inflammatory bowel disease, pancreatic β cell destruction/type I diabetes, and many other autoimmune diseases (Sakaguchi 2004). The function of naturally occurring T regs in the context of malaria infection remains unresolved but appears to be host and parasite species specific, at least in mice. Infection of naïve Balb/c mice depleted of CD4+CD25+ T cells with *P. berghei* led to a slight but significant delay in parasite growth suggestive of enhanced parasite control in the absence of these naturally occurring T regs (Long, Nakazawa et al. 2003). A similar depletion of these cells in B6 mice prior to infection with the lethal strain of *P. yoelii* led to potentiation of the immune response resulting in protection against disease and increased survival in these mice (Hisaeda, Maekawa et al. 2004). It is important to note, that these studies show a benefit to eliminating T-regs in these otherwise lethal models of malaria infection. To date, no studies have looked at the role of T-regs in well adapted host-parasite interactions, but it can be hypothesized that elimination of these regulatory cells may have an opposite, detrimental, effect on outcome.

Inducible regulator T cells are just beginning to receive significant attention and based on studies in other infectious disease models, these cells likely play a significant role in malaria infection. Evidence suggests that this heterogeneous population of regulatory

cells is produced during infectious processes in order to control excessive inflammation. The two best defined populations of inducible regulatory T cells include Tr1 cells (Groux, O'Garra et al. 1997) and T_H3 cells (Fukaura 1996) which produce IL-10 and TGF-β in response to activation, respectively. Tr1 and T_H3 cells have been generated *in vitro* in an antigen specific manor in the presence of IL-10. A newly described T_H1-like regulatory T cells was shown to produce IFN-γ and IL-10 (Jankovic, Kullberg et al. 2007). A better understanding of the mechanism(s) by which these inducible regulatory T cells are generated *in vivo* may provide another point for therapeutic intervention. To this end, recent studies looking at the novel DC derived cytokine IL-27 have suggested a role for this cytokine in the induction of IL-10 expression in T cells (Hunter, Villarino et al. 2004; Kastelein, Hunter et al. 2007). Mice deficient in this cytokine ($WSX^{-/-}$ mice) develop a lethal pro-inflammatory response to *T. gondii* (Hunter, Villarino et al. 2004) or lethal immune mediated liver necrosis to *T. cruzi* (Hamano, Himeno et al. 2003). It is reasonable to hypothesize that exogenous administration of such an IL-10 polarizing cytokine may be beneficially implemented in anti-disease vaccines or for the treatment of ongoing immunopathology.

In reality, the ideal vaccine for malaria will incorporate all of these strategies—a traditional vaccine approach with immunogenic malarial antigens (MSP-1,), an anti-toxin (anti-GPI) component as well as specific immunomodulatory elements (IL-10, TGF-β, IL-27, etc.) to guide the production of regulatory T cells. Additionally, transmission-blocking vaccines, targeting gametocyte, gamete or ookinete antigens, while not directly benefiting the host, may reduce the spread of the parasites by inhibiting the sexual phase of their lifecycle. The practicality of such a vaccine, both due to cost and the likelihood

that such a vaccine would need to be administered temporally (i.e. first immunization would include the anti-toxin vaccine where strong adjuvants are necessary for neutralizing antibody production is necessary followed by subsequent immunizations against the parasite itself with or without immunomodulatory elements) is also likely to pose significant logistical issues.

In summary, the studies described here only touch the surface with regards to immunity to malaria, and for each hypothesis addressed, new questions remain unanswered. With continued research, especially those focused on the basic immunology of host-parasite interactions, a better understanding of the intricacies of the innate, adaptive and regulatory immune systems during malaria infection will likely lead to more effective vaccination strategies and therefore better protection against this devastating disease.

References:

Cavaillon, J. M., C. Adrie, et al. (2003). "Endotoxin tolerance: is there a clinical relevance?" Journal of Endotoxin Research **9**(2): 101-107.

Fan H, C. J. (2004). "Molecular mechanisms of endotoxin tolerance." J Endotoxin Res. **10**(2): 71-84.

Favorite, G. a. M., HR (1942). "Effects produced by the intravenous injectino in man of a toxic antigenic material derived from Eberthella typhosa: Clinical, hematological, chemical and serological studies." J Clin Invest. **21**(5): 589-599.

Fukaura, S. C. K., M J Pietrusewicz, S J Khoury, H L Weiner, and D A Hafler (1996). "Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients." J. Clin. Invest **98**(1): 70-77.

Groux, H., A. O'Garra, et al. (1997). "A CD4+T-cell subset inhibits antigen-specific T-cell responses and prevents colitis." Nature **389**(6652): 737-742.

Hamano, S., K. Himeno, et al. (2003). "WSX-1 Is Required for Resistance to Trypanosoma cruzi Infection by Regulation of Proinflammatory Cytokine Production." Immunity **19**(5): 657-667.

Hemmi, H., O. Takeuchi, et al. (2000). "A Toll-like receptor recognizes bacterial DNA." Nature **408**(6813): 740-745.

Hisaeda, H., Y. Maekawa, et al. (2004). "Escape of malaria parasites from host immunity requires CD4+CD25+ regulatory T cells." Nat Med **10**(1): 29-30.

Hunter, C. A., A. Villarino, et al. (2004). "The role of IL-27 in the development of T-cell responses during parasitic infections." Immunological Reviews **202**(1): 106-114.

Jankovic, D., M. C. Kullberg, et al. (2007). "Conventional T-bet+Foxp3- Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection." J. Exp. Med. **204**(2): 273-283.

Jones, T. R., D. F. Stroncek, et al. (2002). "Anemia in parasite- and recombinant protein-immunized aotus monkeys infected with Plasmodium falciparum." Am J Trop Med Hyg **66**(6): 672-679.

Kastelein, R. A., C. A. Hunter, et al. (2007). "Discovery and Biology of IL-23 and IL-27: Related but Functionally Distinct Regulators of Inflammation." Annual Review of Immunology **25**(1): 221-242.

Long, T. T. A., S. Nakazawa, et al. (2003). "Influence of CD4+CD25+ T cells on Plasmodium berghei NK65 infection in BALB/c mice." International Journal for Parasitology **33**(2): 175-183.

Ocana-Morgner, C., M. M. Mota, et al. (2003). "Malaria Blood Stage Suppression of Liver Stage Immunity by Dendritic Cells." J. Exp. Med. **197**(2): 143-151.

Omer, F. M., J. B. de Souza, et al. (2003). "Differential Induction of TGF-{beta} Regulates Proinflammatory Cytokine Production and Determines the Outcome of Lethal and Nonlethal Plasmodium yoelii Infections." J Immunol **171**(10): 5430-5436.

Reed, R. C., A. F. Verhuel, et al. (1997). "Rapid onset of malaria-induced mortality by immunizations with lipo-peptides: an experimental model to study deleterious immune responses and immunopathology in malaria." Vaccine **15**(1): 65-70.

Sakaguchi, S. (2004). "Naturally Arising CD4+ Regulatory T Cells for Immunologic Self-Tolerance and Negative Control of Immune Responses." Annual Review of Immunology **22**(1): 531-562.

Sato, S., O. Takeuchi, et al. (2002). "A variety of microbial components induce tolerance to lipopolysaccharide by differentially affecting MyD88-dependent and -independent pathways." International Immunology **14**(7): 783-791.

Schofield, L., M. C. Hewitt, et al. (2002). "Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria." Nature **418**(6899): 785-789.

Su, Z. and M. M. Stevenson (2000). "Central Role of Endogenous Gamma Interferon in Protective Immunity against Blood-Stage Plasmodium chabaudi AS Infection." Infect. Immun. **68**(8): 4399-4406.

Urban, B. C., D. J. P. Ferguson, et al. (1999). "Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells." Nature **400**(6739): 73-77.

Varma, T. K., T. E. Toliver-Kinsky, et al. (2001). "Cellular mechanisms that cause suppressed gamma interferon secretion in endotoxin-tolerant mice." Infection & Immunity **69**(9): 5249-5263.

Wysocka, M., S. Robertson, et al. (2001). "IL-12 Suppression During Experimental Endotoxin Tolerance: Dendritic Cell Loss and Macrophage Hyporesponsiveness." J Immunol **166**(12): 7504-7513.

Yeo, S. J., J. G. Yoon, et al. (2003). "CpG DNA induces self and cross-hyporesponsiveness of RAW264.7 cells in response to CpG DNA and lipopolysaccharide: alterations in IL-1 receptor-associated kinase expression." Journal of Immunology **170**(2): 1052-1061.