REGULATION OF ADAPTIVE IMMUNE RESPONSES TO *PLASMODIUM CHABAUDI* MALARIA INFECTION

by

Victor H. Carpio Rojas

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Dedication

To Charlotte, for always believing in me and loving me.

To my parents, for giving me their unconditional love and support.

To my grandma, for showing me how to love.

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REGULATION OF ADAPTIVE IMMUNE RESPONSES TO *PLASMODIUM CHABAUDI* MALARIA INFECTION

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Victor H. Carpio Rojas, B.S.

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Malaria killed an estimated of 437,000 people last year, most of them children. While there is a vaccine nearly licensed, it only induces limited and short-lived protection. A major problem in the development of an effective vaccine is the poor understanding of the mechanisms that generate protective immunity. In malaria, IFN-γ^+ CD4 T cell responses, called T helper type 1 (Th1), are necessary to control acute parasite growth. On the other hand, IL-21^+ T follicular helper (Tfh) cells are essential for generating high-affinity antibodies for parasite clearance. Whereas the transcription factors Bcl6, Blimp-1, and STAT3 regulate the development of Tfh cells, T-bet and STAT4 direct the commitment of Th1 cells. Since our understanding of the regulation of adaptive protective responses against malaria is poor, the present dissertation aimed to study the development of CD4 T cell responses using the murine parasite *Plasmodium chabaudi*, which models chronic human malaria immunology and pathology. First, we show that the CD4 T cell response against malaria consists mostly of a newly described Th1-like Tfh hybrid population. We demonstrated that these cells express both T-bet and Bcl6 in the nucleus and survive into the memory pool. Second, we used Bcl6, Blimp-1 and STAT3 T cell-specific conditional knock out (TKO) mice to study their roles in the generation of the Th1-like Tfh subset. We found that the Bcl6/Blimp-1 axis regulates the expression of the Tfh chemokine receptor CXCR5, but only Blimp-1 deficiency increased the proportions of Th1-like Tfh cells. STAT3 TKO mice had more IFN-γ^+ Th1 memory cells, and were protected from reinfection. Since persistent immune stimulation can promote Tfh development, we treated mice at day 3 post infection with the antimalarial mefloquine (MQ) to reduce immune stimulation early in our system. This early MQ treatment resulted in a great expansion of IFN-γ^+ Th1 cells and a great reduction of Th1-like Tfh cells. In conclusion, our studies have provided vital information describing the adaptive immune responses during chronic malaria infection. Moreover, our data has identified several molecules for future studies to help the rational development of a much-needed vaccine.

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WHO	World Health Organization
iRBCs	Infected red blood cells
DCs	Dendritic cells
p.i.	Post infection
NK	Natural killer
Th17	T helper 17
CyTOF	Mass cytometry
AhR	Aryl hydrocarbon receptor
PCs	Short-lived plasma cells
LCMV Arm	LCMV Armstrong
LCMV Cl13	LCMV Clone 13
uMT	B cell deficient mice
BF	Bright Field
Eomes	Eomesodermin
CSP	Circumsporozoite protein
KI mice	*Ifng/Thy1.1* knock-in mice
KO	Knock Out
WT	Wild type
APC	Antigen Presenting cells
PBMC	Peripheral blood mononuclear cells
CHMI	Controlled Human Malaria infection
ROI	Reactive oxygen intermediates
GC	Germinal Center
IRF3	IFN regulatory factor 3
BTLA	B and T Lymphocyte Attenuator
Tmem	Memory T cells
Tem	T effector memory cells
NO	Nitric oxide
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
Th1	T helper 1
Th2	T helper 2
Tfh	T follicular helper
Treg	Regulatory T cells
RORγt	Orphan retinoid receptor
FoxP3	Forkhead transcriptional repressor
TCR	T cell receptor
NGS	Next generation sequencing
VL	Visceral leishmaniasis
Ig	Immunoglobulin
ADCI	Antibody-dependent cell inhibition
STAT	Signal transducer and activator of transcription
FMO	Fluorescence minus one
CTV	Cell Trace Violet
Teff	Effector T cells
MQ	Mefloquine
iNOS	Inducible nitric oxide synthase
TKO	T cell-specific deficient mice
PfSPZ	Plasmodium falciparum sporozoites
PMIF	Macrophage migration inhibitory factor
cGAS	Cyclic GMP-AMP synthase
DBL	Duffy biding-like domain
IFN	Interferon

CHAPTER 1: INTRODUCTION

MALARIA

Current status and control efforts

Malaria is one of the most prevalent diseases around the world with approximately 216 million people infected in 2016. Most cases are found in sub-Saharan Africa (1). Malaria generally results in an uncomplicated febrile disease, but it some instances it progresses into severe malaria resulting in death. Tragically, children aged 2 to 10 years old are the population with the highest infection rate. Five *plasmodium* species that can cause malaria have been identified in humans: *P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi*. In 2016, there were an estimated 445,000 deaths due to malaria, and the vast majority of these deaths were due to *P. falciparum* infections (99%). The Global Technical Strategy (GTS) for Malaria of the World Health Organization (WHO) aims to reduce the malaria incidence and mortality rates globally by 90% by 2030. Moreover, they hope to eliminate malaria from at least 35 countries in which malaria was evident in 2015. Even though this goal seems difficult to achieve, in 2015, 17 countries eliminated malaria (defined as zero indigenous cases for 3 years or more). In 2016, Kyrgyztan and Sri Lanka were certified and added to this list by the WHO. Therefore, there is great hope that this target will be met.

In the year 2000, the leading world organizations recognized malaria as a worldwide health disaster. As a result, the Roll Back Malaria initiative and the United Nations Millennium Development Goals (MDGs) set a goal to control malaria in sub-Saharan Africa. Currently, the

total funding for malaria control and elimination was estimate at US$2.7 billion, with the United States of America being the largest funder. As previously reported, the main interventions for malaria control are insecticide-treated bed nets (ITNs), indoor residual spraying (IRS), and treatment of clinical cases with artemisinin-based combination therapy (ACT). Unfortunately, the emergence of resistance to artemisinins and insecticides is putting at risk the current success over malaria; therefore there is an urgent need to develop an effective vaccine to employ in conjunction with these other interventions.

Malaria vaccine efforts

Many efforts have been made to control malaria. The current vaccine agenda primarily targets the pre-erythrocytic stages of the parasite. This approach is intuitive as it aims to stop sporozoites from reaching the liver. A key protein for invasion of hepatocytes is the circumsporozoite protein (CSP), which is highly expressed on the sporozoite surface. Currently there is a licensed malaria vaccine, RTS,S/AS01. Unfortunately, recent studies show the vaccine confers partial protection that wanes over time (2). RTS,S aims to elicit a strong humoral response against CSP; thereby, stopping sporozoite invasion of the liver. Other vaccine efforts include immunization with radiation-attenuated *Plasmodium falciparum* sporozoites (PfSPZ) delivered through mosquito bites (3); intravenous inoculation of purified, radiation-attenuated PfSPZ (4-6); or inoculation of infectious PfSPZ by mosquitoes to volunteers under chemoprophylactic treatment using the anti-malarials chloroquine or mefloquine (7, 8). All of these approaches have achieved significant protection in controlled human malaria infection (CHMI) studies; however, the immune correlates of protection have not been clearly defined yet.

To date, high titers of antibodies against CSP and polyfunctional (IFN-γ^{+}TNF^{+}IL-2^{+}) CD4 T cell responses are the strongest correlates of protection to the RTS,S vaccine, but there is no cut off that is predictive of protection (9-11). Therefore, understanding the molecular determinants for the generation of polyfunctional T cell populations could help the rational development of an effective vaccine.

THE LIFE CYCLE OF *PLASMODIUM* PARASITE

Despite the high prevalence of malaria, immunity to malaria clinical symptoms can be achieved in the field. However, naturally acquired immunity is reached only after repeated exposure, and sterile immunity is never achieved (12, 13). Repeated infection promotes continued immunity to clinical disease, and emigration out of endemic areas can reduce protection from disease acquired over years (14-16). This is similar to concomitant immunity, where chronic infection maintains protection, and it is also observed in many other parasitic infections (17-20). Effective malaria immunity relies on both humoral and cellular immune responses (21, 22). However, mechanisms of regulation of these responses are poorly understood. Differences exist between the adaptive response to liver and blood stages of *Plasmodium* infection. Therefore, we have to take in consideration the life cycle of the *Plasmodium spp*, and the possible immune mechanisms for control and elimination of the parasite *in vivo*.

The life cycle of *Plasmodium spp*. parasites involves two hosts: humans and female mosquitos of the genus *Anopheles* (Fig. 1.1). Overall, the parasite life cycle can be divided into

three stages: Pre-erythrocytic (liver stage), erythrocytic, and the mosquito life cycle. During a blood meal, the mosquito probes the skin injecting saliva that contains anti-coagulants and vasodilators to increase the chances of encountering a blood vessel. Through this process, 15 to 200 of the sporozoite form of the *Plasmodium* parasite, which resides in the mosquito salivary gland, are deposited under the skin (23, 24). Using gliding motility, the sporozoites reach a blood vessel and enter systemic circulation to travel to the liver [Fig. 1.1A, (25)]. In the malaria mouse model *Plasmodium berghei*, the estimated time for sporozoites to reach the liver after being deposited into the skin is 30 minutes to three hours (26). Inside the hepatocytes, the sporozoites develop and multiply into the schizont form, which is composed of thousands of merozoites [Fig. 1.1B, (27, 28)]. The liver stage is asymptomatic and it is thought of more as an amplification stage that takes between 2 to 9 days in the case of *P. falciparum*. Eventually, through signals that still remain unknown, merozoites are released into the bloodstream in a merosome (Fig. 1.1C). In the bloodstream, merozoites invade erythrocytes where they develop asexually through ring, trophozoite, and schizont stages (Fig. 1.1D). Each schizont contains between 8 and 32 merozoites, which are released from erythrocytes synchronously (every 48 hours in the case of *P. falciparum*) causing the classic symptoms of malaria disease. Some parasites will develop intraerythrocytically into male and female gametocytes, which are taken up by the mosquito during the next blood meal [Fig. 1.1 E, (29)]. One point in the life cycle that can be targeted for malaria control is the migration of sporozoites from the skin to the liver, or their differentiation and division in hepatocytes. Another vulnerability of the *Plasmodium* parasite is during the erythrocytic stage, which leads to clinical symptoms and also transmission to a mosquito. Finally, other efforts focus on blocking transmission by preventing uptake of gametocytes by mosquitos.

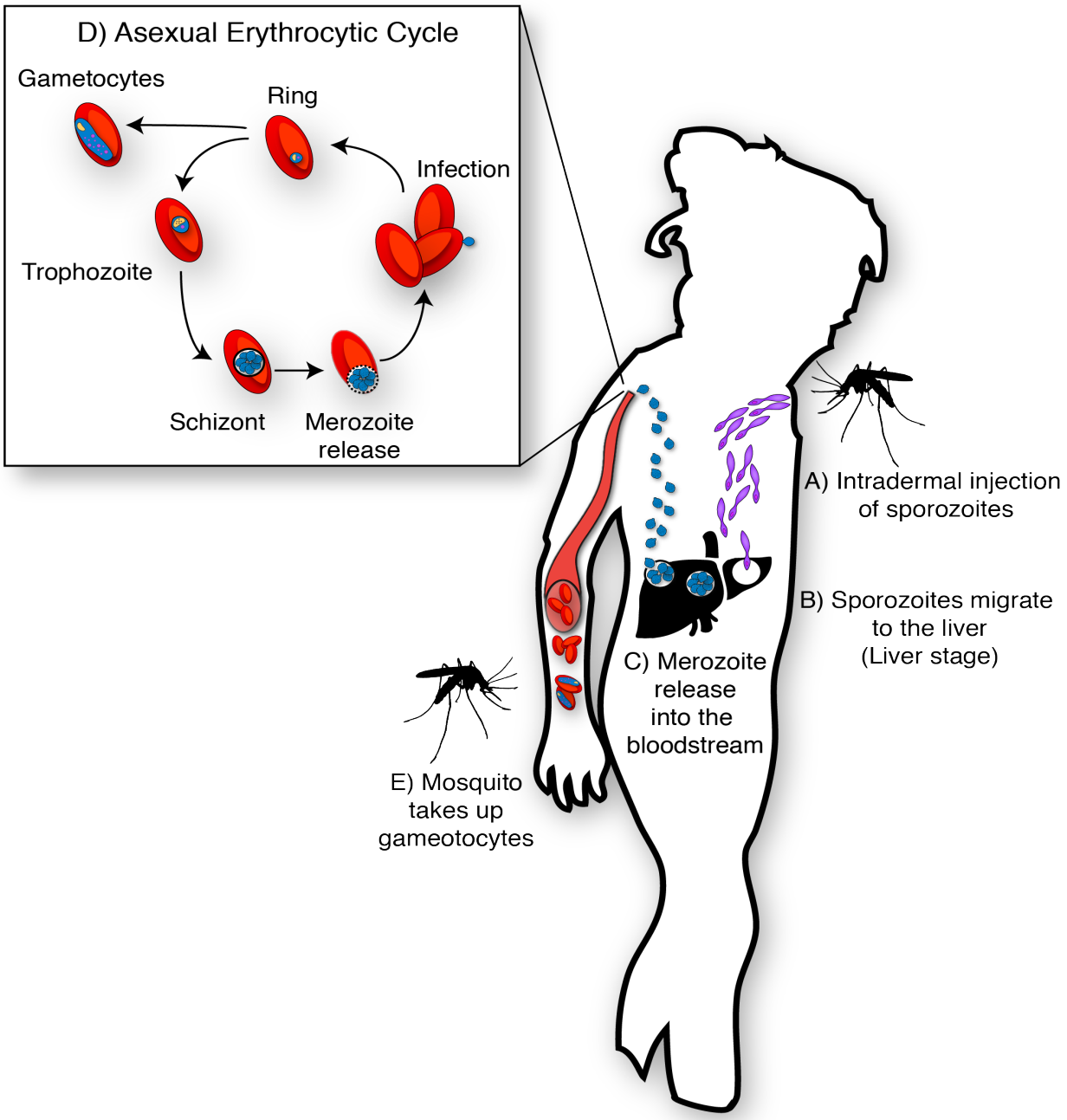

Figure 1.1 The life cycle of *Plasmodium* parasites. A) Malaria infection begins with the intradermal injection of *Plasmodium* sporozoites from the mosquito salivary gland during a blood meal. B) Sporozoites reach the systemic circulation enabling them to access the liver, where they undergo schizogony. C) After a period of incubation, tens of thousands of merozoites are released into the bloodstream. D) In the bloodstream, merozoites infect red blood cells. All clinical malaria symptoms are evident during this asexual erythrocytic stage. E) Some intraerythrocytic parasites will develop into male and female gametocytes, which are taken up by the mosquito during the next blood meal.

MALARIA DISEASE AND MOUSE MODELS

Clinical Symptoms of malaria

Clinical symptoms of malaria are driven by massive synchronous rupture of red blood cells and parasite escape into the bloodstream. In general, the presentation of malaria disease resembles many common viral infections with patients experiencing fever, headache, chills, myalgias, arthralgias, and fatigue. This leads to a significant number of false diagnoses (30). *Plasmodium* infection of healthy adults has been used as an experimental approach to study the immune response and test vaccine efficacy. Short-term infection with chloroquine sensitive *P. falciparum* and cure upon PCR positivity has been used since 1985 (31) and it has provided important information regarding malaria clinical manifestations (32). From a retrospective study of eighteen different challenge trials between 1985 and 1992, malaria symptoms are first shown from 6 to 23 days after the exposure to an infected-mosquito bite. The frequency of these symptoms increases after parasites are detectable in peripheral blood using a thick blood smear. Individuals that have not been previously exposed to *P. falciparum* will show parasite in their blood by microscopy 11 days on average after exposure, at which point they received antimalarial treatment (33-35). Malarial fever was also used as a cure for neurosyphilis before penicillin and data from these infections was also carefully curated showing chronicity of *P. falciparum* for up to a year.

The duration of *Plasmodium* infections

In endemic areas, children are constantly exposed to different strains of *Plasmodium* parasites. Younger children (below the age of 5 years old) are the ones at the highest risk of

developing the severe form of disease, which could result in death. However, with recurrent infections, their chance of falling in a life-threatening episode is significantly reduced. Eventually protection from clinical symptoms, such as fever, is greatly reduced. However, in endemic areas, parasite can be detected in asymptomatic children. Parasite numbers in these children are very low, which supports the idea that malaria is a persistent infection. This should be considered different than chronic infection, where typically the pathogen is found at higher number accompanied by clinical symptoms (36, 37). Epidemiological studies done in Asar village in eastern Sudan, where malaria transmission is restricted to the rainy season that lasts 2 to 3 months, showed that some people shift from symptomatic to asymptomatic infections after treatment, but remain infected (38). Using genetic analysis it was confirmed that the genotypes of diverse clones can coexist within single individuals, but most importantly, these infections can sustain the generation of gametocytes supporting transmission prevalence in the area (39). These observations support the current paradigm that clinical immunity precedes acquisition of immunity against the parasite.

Studying immunity to malaria in humans

The first studies that allowed for the description of immunity against *Plasmodium* infections come from the neurosyphilis patients who were treated with malaria back in the early 1900s. Dr. Wagner-Jauregg made the observation that there was a correlation between improvements for patients with psychosis after feverish episodes. This observation led him to speculate whether it would be favorable to induce fever using infectious agents as a therapy for patients with psychosis. Later on he was able to identify that patients with “General Paralysis of

the Insane" (GPI), a severe neuropsychiatric disorder developed late during syphilis infection due to chronic meningoencephalitis, were the population that responded the best to the treatment. After trying streptococci from erypsipelas, tuberculin, and dead staphylococci, he used tertian malaria as therapy. The treatment consisted of blood transfusion from malaria-infected patients followed up by the administration of the anti-malarial quinine bisulphate after seven to twelve "attacks of fever". In 1927 Dr. Wagner-Jauregg was bestowed Honorary Membership in the German Society for Psychiatry and the Nobel Prize (40). One important piece of data obtained from this era is in the case of patients that had recurrent neuropsychiatric episodes. When they were treated again with tertian malaria, they did not develop high fever episodes. This suggests that immunity to malaria clinical symptoms can be achieved after one exposure.

Several groups are able to study natural infection in malaria-endemic areas (35). These studies will vary depending on the differences between transmission patterns (sporadic, seasonal, or year-round), transmission intensity (low or high), and mosquito breeding, among others. These types of studies allow using the healthy preinfection subjects as their own control. This approach helps to elucidate immune parameters or profiles that will correlate with protection, or even to determine if acute or persistent malaria infections modulate immune responses differently. These research models, in combination with animal models, have shown immense potential to helps us understand malaria immunity. Sterilizing immunity to the malaria parasite can be induced in animal models [mice, nonhuman primates (41, 42)] and humans (3) using attenuated-irradiated sporozoites. Sterilizing immunity can also be achieved using genetically attenuated parasites (43) or by inoculation of infectious *P. falciparum* by mosquitoes to volunteers under chemoprophylactic treatment using the anti-malarial chloroquine (8). Chloroquine only targets blood-stage parasites; therefore, both of the previously mentioned

approaches allow for exposure to full parasites from the skin to the liver stage, but only transient and minimal exposure to blood-stage parasite. Thus far, studies using exposure to sporozoites have shown to be effective only to re-challenge with homologous parasites, an event that would rarely happen in endemic areas (44).

Plasmodium chabaudi as a malaria mouse model

Currently, there are many *plasmodium* parasites available that can infect rodents. As previously mentioned, all the malaria symptoms are shown during the blood stage. Different species of malaria parasite have different life cycles, which results in particular pathology developments (Table 1). Furthermore, the immune responses elicited by these infections have clear differences. For example, *Plasmodium chabaudi chabaudi* (AS) is a synchronous parasite like *P. falciparum*. Moreover, both of these parasites invade normocytes and reticulocytes (45). This is important, as the host cell preference of infection for the rodent malaria parasites influences heavily their virulence. In the case of non-lethal *P. yoelii* 17XNL, the infection is acute in wild type (WT) mice and it only infects reticulocytes. However, the lethal strain *P. yoelii* 17XL, which is closely related to *P. yoelii* 17XNL, has no restriction in the range of erythrocytes it infects, resulting in faster and higher parasitemia (46). *P. chabaudi*-infected red blood cells (iRBCs), unlike those infected with other rodent *Plasmodium* parasites, sequester in different organs by adhering to endothelial cells, and causes RBCs rosetting by adhering to uninfected RBCs (45, 47-50). In *P. falciparum*, parasite sequestration and RBCs rosetting have been related to the development of cerebral malaria (51, 52). However, *P. chabaudi* does not sequester in the brain (53), but it induces cerebral edema and hemorrhage in IL-10 deficient mice

(54). Recent studies have demonstrated that exacerbated neuroinflammation in these mice correlates with development of cerebral malaria behavioral symptoms (55).

Parasite	Liver Stage (h)	Synchrony in blood	Persistency
P. c. chabaudi	52-53	Synchronous	Persistent
***P. berghei* ANKA**	50	Asynchronous	Acute
***P. y. Yoelii* 17XNL**	43-50	Asynchronous	Acute

Table 1. 1 Life-cycle characteristic of the main rodent malaria parasites

The overall symptomatology observed during *P. chabaudi* infection in C57BL/6J mice is shown in Figure 1.2. As in humans, most of these symptoms can be linked to systemic inflammation driven by the parasite. One difference is that mice infected with *P. chabaudi* develop hypothermia instead of fever. Anemia is another key feature of malaria in individuals that is very similar in *P. chabaudi*. Anemia in humans is cause by several mechanisms, such as death of infected erythrocytes, suppression of erythropoiesis, elimination of uninfected RBCs by phagocytosis, and dyserythropoiesis (56). This process has been shown to be similar in *P. chabaudi* infection (57, 58). Importantly, both IFN-γ and TNF-α can inhibit renewal of hematopoietic stem cells during systemic inflammatory responses (59). However, in *Mycobacterium avium* infection, a chronic bacterial infection, IFN-γ can help to maintain hematopoietic stem cells as a reservoir of immune effector cells (60). One study showed that IFN-γ Knock Out (KO) mice infected with a low dose (10^4 iRBCs) of *P. yoelii* 17XNL had significantly lower parasitemia during the early stages of infection (61). They found that in the

absence of IFN-γ there was a great reduction in the percentage of reticulocytes (70% in WT vs 20% in IFN-γ KO), which is the preferred cell to infect by the parasite. These results suggest that IFN-γ has a role in malaria infection beyond an immune inflammatory cytokine. Moreover, these results indicate that *P. yoelii* 17XNL mouse malaria infection might be in a disadvantage as a model to study malaria.

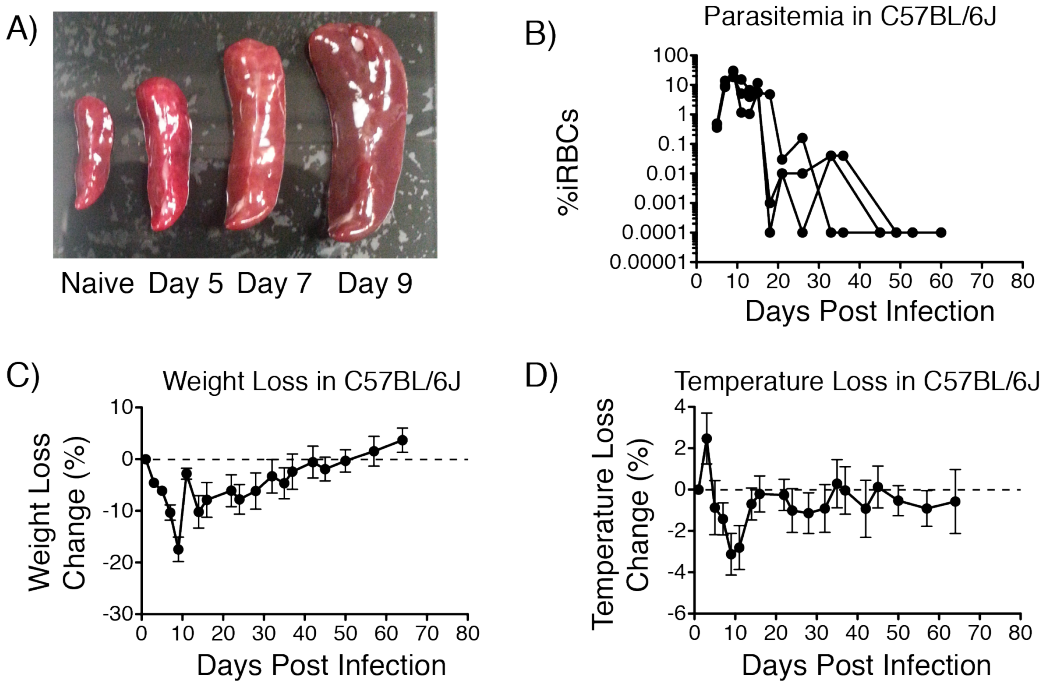

Figure 1.2 Overview of *P. chabaudi* pathology in C57BL/6J mice. Wild Type C57BL/6J mice survive a *P. chabaudi* infection using 10^5 iRBCs dose intraperitoneally. A) Spleens from naive (uninfected) and infected C57BL/6J mice at day days 5, 7, and 9 (p.i.) are shown. Splenomegaly is a characteristic of *P. chabaudi* infection. B) Parasitemia curve is shown in a log scale for 3 mice as percentage of infected red blood cells over total red blood cells (%iRBCs). Circulating parasite is measured using Giemsa-stained thin blood smears. Parasitemia peaks aroun days 8-10 p.i. After a recrudescence period around day 30 p.i, parasitemia falls to a sub-patent level (for up to 2 more months) detectable by blood transfer into a naive mouse. Infected mice gradually loose C) weight and D) temperature after the peak of infection (days 10-11 p.i.). These parameters return to normal as parasite clearance is accomplished.

P. chabaudi pathology severity, as *P. falciparum*, depends on both host (62-64) and parasite genetics (65). For example, in *P. chabaudi* the percentage of parasitemia depends on

several factors, such as sex, mouse strain, and nutritional status. Parasitemia in male A/J mice is higher (closer to 60%) when compared to the resistant strain C57BL/6 (30-40%). In human malaria, host genetic factors that correlate with protection are pyruvate kinase (PK) deficiency, Glucose-6-Phosphate deficiency, Sickell cell anemia, Thalasemias, Duffy blood group antigens, among others (66). In mice, the regions containing genetic markers related to susceptibility or resistance are named *chabaudi* resistance (*Char*) loci (67, 68). In the case of parasite genetics, different strains differ in characteristics such as erythrocytic manipulation and cell adhesion (69 , 70). In *P. falciparum*, variant surface antigens (largely divided into the multigene families *rif*, *stevor*, and *var*) have been extensively related to disease severity (71). In the case of rodent malaria, the multigene families are called *pir* genes (72), and depending on the species acquire a specific nomenclature (i.e. *cir* in *P. chabaudi*). In *P. chabaudi*, a study showed that passage of the parasite once through mosquitoes changed the expression of a single *cir* gene, which resulted in decreased virulence (73) suggesting that the multigene families have a similar role in virulence than in *P. falciparum*.

Blood-stage immune responses in humans are comparable to the one observed in *P. chabaudi* mouse malaria infection (12). T and B cell responses are pivotal to control parasite growth and pathology during this stage (74). The main role of B cells in human malaria is to reduce parasite numbers, demonstrated by transfer of purified IgG from malaria-immune adults to children with ongoing malaria infection (75, 76). In the case of *P. chabaudi*, B cell-deficient mice are able to control primary acute infection but unable to eliminate parasite during the chronic phase (77). CD4 T cells are important for survival in *P. chabaudi* infection. Immunodeficient mice can be rescue from lethal blood-stage *P. chabaudi* infection by CD4 T

cell transfer (78). In both *P. falciparum* and *P. chabaudi* infections, the blood-stage induces a response characterized by IFN-γ production by natural killer (NK) cells, γδ T cells, and αβ T cells (79-81). Importantly, in both infections Th1 cells, and not T regulatory (Treg) cells, are the main source of the immunoregulatory cytokine IL-10 (82, 83). In conclusion, *P. falciparum* and *P. chabaudi* share many aspects in pathology, parasite immune evasion genetics, and elicited immune mechanisms.

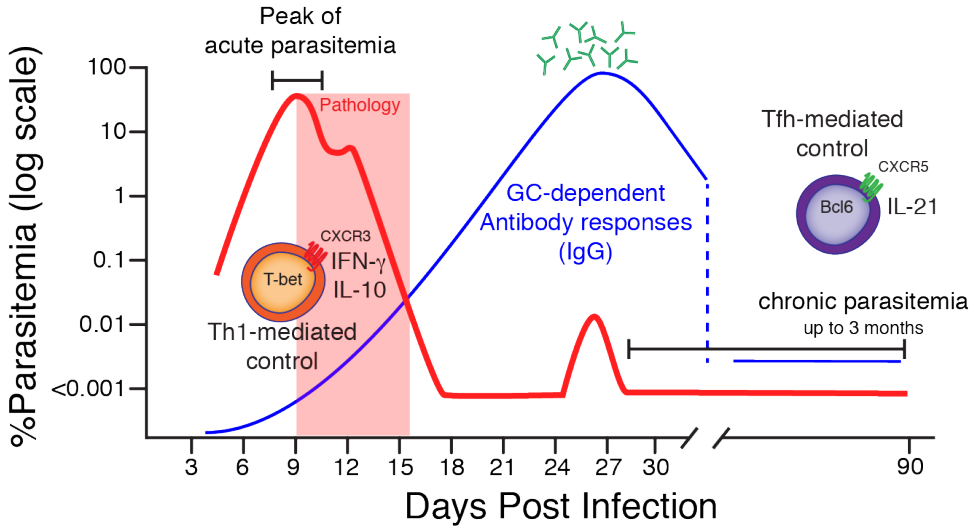

Figure 1.3 CD4 T cells play a major role in *Plasmodium chabaudi* infection. *P. chabaudi* infection shares many aspects with *P. falciparum* infection, such as the importance of CD4 T helper responses in immuno-pathology during the blood-stage. Here we show a cartoon depicting the parasitemia curve during blood-stage *P. chabaudi* infection (red line). During the first week after infection, *P. chabaudi* expands exponentially. Peak parasitemia is reached around day 8-10 p.i. At this time, IFN-γ^{+} Th1 cells are crucial to control parasite growth. Around day 10-12 p.i, after the peak parasitemia, we observe the IFN-γ-mediated pathology characteristic of malaria infection. At this time Th1 cells are extremely important to control the immune-pathology since they are the main producers of the immune-regulatory cytokine IL-10. After the peak, IL-21^{+} Tfh cells and malaria-specific antibodies are required to achieve parasite clearance. However, parasitemia is not cleared, but reduced to sub-patent levels and it can last for up to 3 months at this levels.

IMMUNITY TO MALARIA

The early stages

Albeit malaria immunity from natural infection can be generated, it decays rapidly with removal from endemic areas (84, 85). These observations suggest that continuous exposure to the parasite is required to confer immuno-protection, but the mechanisms driving this response are still unknown. In humans, infection at the skin and liver stages is considered immunologically silent, as no signs of inflammation are triggered during these stages (86). This idea is supported by the observation that naturally acquired immunity is inefficient at neutralizing the parasite at the skin or liver stages. Moreover, the skin (87) and liver (88) are inherently immunoregulatory, which could be exploited by the parasite. Guilbride and colleagues conducted an unbiased meta-analysis of nearly 2000 malaria vaccine studies suggesting that *Plasmodium*-specific regulatory T cells (Tregs) are induced during skin vaccination, which could result in increased immune tolerance to sporozoites and the following blood stage infection (89). In mice, Tregs and dendritic cells (DCs) migrate to the skin within 30 min post-inoculation of *Plasmodium* sporozoites through a mosquito bite (90). Importantly, migratory DCs show downregulation of MHC-II and the co-stimulatory molecule CD86, suggesting a tolerogenic response. Some studies have shown a modulatory effect on DCs by *P. falciparum in vivo* and *in vitro* (91, 92).

A large body of literature has addressed the role of DCs in malaria and relevant findings regarding their role in CD4 development have been made (93-95). RBCs infected with *P. chabaudi* can activate DCs *in vivo* and *in vitro*, although there is evidence that there is a "turn-around" from inflammatory DCs to immunoregulatory DCs as infection progresses (94, 96, 97).

Plasmodium parasites can active Antigen Presenting Cells (APCs) through many mechanisms, such as iRBCs (98), free parasites in the blood stream, and parasite products (99). The *Plasmodium* parasite contains activators of Toll-like receptor 2 (TLR2) and TLR9 (100, 101). The parasite can trigger these responses by glycosylphosphatidylinsitol (GPI) anchors and DNA trapped in the metabolic byproduct hemozoin (102, 103). However, infection of TLR2 KO, TLR4 KO, TLR6 KO, and TLR9 KO mice with *P. chabaudi* showed no differences when compared with WT mice (104). Nevertheless, MyD88 deficient mice had impaired production of TNF-α and IFN-γ and had less malaria symptoms (body weight and temperature loss).

The role of monocytes during bacterial and viral infections, autoimmunity, and cancer has been widely studied, while their role in protozoan parasitic infections have been less characterized (105). Innate cells from the monocyte lineage play a crucial role during malaria infection. Dendritic cells (DCs), monocytes, and macrophages sense and respond to several signals in infected red blood cells (iRBC) and other parasite products (106). The role of monocytes has been more elusive than DCs, mostly due to the heterogeneity of their responses, as well as their phenotypical similarities with macrophages (107). The contribution of monocytes to protection and modulation of adaptive immune responses in malaria is still under debate (108). Monocytes, and not resident macrophages, have been shown to be able to restrict parasite growth by antibody-dependent cell inhibition (ADCI) (109-111). Monocytes originate from bone marrow precursors and depending on their exposure to growth factors, cytokines, and microbial products, they can differentiate into macrophages or dendritic cell populations in the tissue (112). In *Plasmodium chabaudi* infection, $CD11b^{high}Ly6C^{+}$ monocytes recruited to the spleen produce reactive oxygen intermediates (ROI) and phagocytize parasites (113). *Ccr2* KO mice, where

monocyte migration from bone marrow is impaired, showed uncontrolled parasitemia after the peak of infection. Importantly, adoptive transfer of $CD11b^{high}Ly6C^{+}$ cells rescued the mice from this phenotype.

The erythrocytic stage

The most obvious mechanisms that elicit an immune response during the blood stage are free merozoites and iRBCs. Since red blood cells lack the antigen presenting apparatus (MHC-I or -II), it is expected that humoral responses are the most important immune defense mechanism. However, it is well established that CD4 T cells mediate proper humoral responses (74, 78).

In malaria endemic areas, children acquire resistance to life-threatening malaria at the age of five years old (16). After this, children remain susceptible to malaria clinical outcomes, such as fever and anemia, until they reach adulthood. Clinical immunity is achieved late in life and disease is basically controlled by inflammatory signals, immunoregulatory responses, and acquisition of an extensive anti-plasmodium antibody repertoire (22). In malaria, fever and other symptoms are a results of proinflammatory responses directed by cytokines such as IL-1β, IL-6, IL-8, IL-12(p70), IFN-γ, and TNF (114-116). However, this inflammatory response is also responsible for early parasite control. In some cases, excessive inflammatory signals can result in fatal or severe malaria, and this is related to individuals with very low or no exposure at all to the parasite (117). However, individuals that are constantly exposed in endemic areas will develop blood-stage infections that result in mild febrile episodes or no symptoms at all. This idea that constant exposure confers protection from clinical symptoms is supported by early studies in

humans, showing that risk of developing febrile episodes decreases with each parasite infection (118). In *P. chabaudi* infection, TNF and IFN-γ production are greatly increased at the peak of infection [from days 5 to 10 post infection (p.i.)] and IFN-γ deficient mice die at the peak of infection (119). The anti-inflammatory cytokines IL-10 and Transforming Growth Factor (TGF)-β regulate this proinflammatory condition, and both cytokines are required for mice survival (120, 121). Cross-sectional studies in malaria endemic areas have shown that higher ratios of pro- over anti-inflammatory responses correlate with severe disease (114, 122). Moreover, a study used a systems biology approach to determine that PBMCs from children in a malaria endemic area had a pro-inflammatory response when exposed to iRBCs. However, following natural malaria infection, their PBMCs response to iRBCs shifted to a more anti-inflammatory response, characterized by IL-10 and TGF-β production (123). These findings suggest that children re-exposed to *P. falciparum* acquire a more immunoregulatory response presenting an explanation to the acquisition of clinical immunity in the field. Nevertheless, it remains unknown if these mechanisms are just another way of immunoregulation by the parasite to establish persistent infection without killing the host.

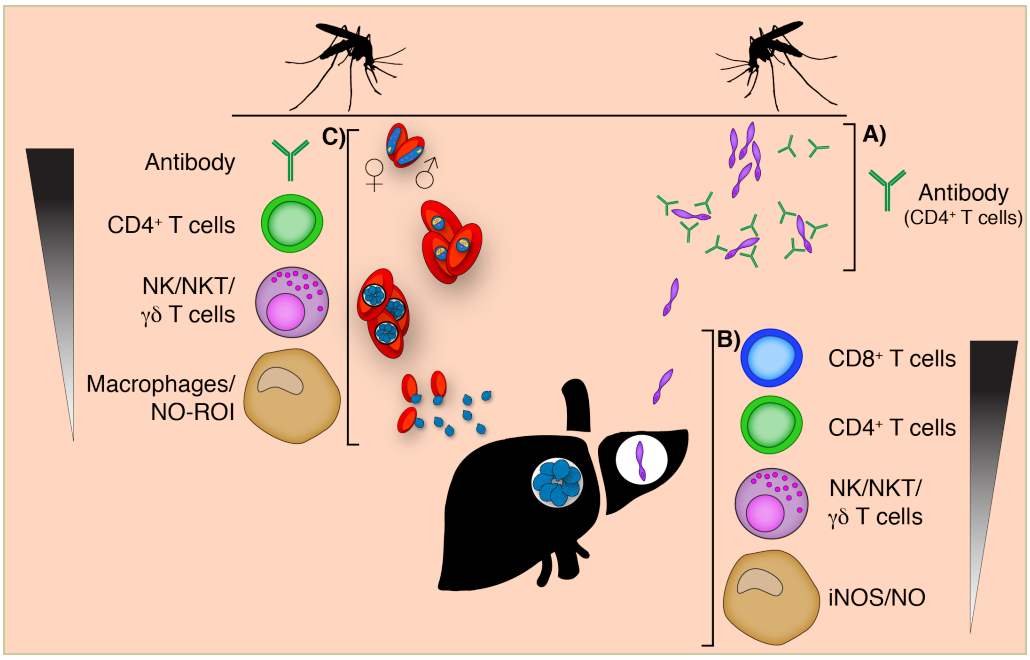

Figure 1.4 Immune players at the different stages of *Plasmodium spp.* life cycle. A) During the skin stage the infection is clinically silent with no clear evidence that immunity can be achieved during naturally infection in the field. However, other immunization procedures, such as live-attenuated sporozoites, have shown to reduce sporozoite migration from the skin to the liver. The sporozoite blockage is mediated by antibodies, which require CD4 T cell help. B) As in the skin, the liver stage in humans is clinically silent. No sterilizing immunity is achieved in the field. Nevertheless, live-attenuated sporozites can induce sterilizing immunity at this stage. This immunity relies on $CD8^+$ and $CD4^+$ T cell responses; effective production of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) by innate immune cells, and IFN-γ-producing natural killer (NK) cells, NKT cells, and γδ T cells. C) Malaria clinical symptoms are observed during the blood-stage. During this stage, both innate and adaptive immune responses are readily detected. The most important effector response during this stage is CD4-mediated antibody responses. NK, NKT, γδ T cells also play a role. Macrophages and monocytes help to clear infected red blood cells and free merozoite through the production of iNOS and NO. A few parasites will differentiate into sexual gametocytes, which are taken up by a mosquito in the next blood meal. (*adapted from Langhorne et. al. 2008*(12))

T CELL MEDIATED IMMUNITY

Defining CD4 T cell subsets

Adaptive immune responses are involved in protection and elimination of *Plasmodium* parasites at all the stages of the parasite life cycle. Generation of long-lived humoral responses

against sporozoites could, in theory, stop their migration and block liver infection. In mouse studies, elimination of pre-erythrocytic parasites requires mainly CD8 T cells that produce IFN-γ that mediate elimination of infected hepatocytes (124). During the erythrocytic stage, B cells and antibodies are extremely important in eliminating the parasite (125). It is well established that CD4 T cells can "help" at all these stages. Memory B cells can be generated to many pre-erythrocytic antigens such as, Circumsporozoite protein (CSP), liver-stage antigen 1 (LSA1), and sporozoite threonine-asparagine-rich protein (STARP) (126-128). Importantly, malaria-specific CD4 T cells against pre-erythrocytic antigens can be generated after immunization (129) and natural infection (130). CD4 T cells can contribute to CD8 responses against liver stages (131-134); however, CD8 T cells are thought to be mainly primed in the skin-draining lymph node after the sporozoite infection (135). It is possible that CD4 T cells specific to pre-erythrocytic antigens are generated in the same way, providing a scenario where they provide help to liver-stage specific CD8 T cells responses.

In the 1980s, Mossmann and Coffman proposed that CD4 T helper cells could acquire one of two possible final and stable fates: either an IFN-γ-producing T helper 1 (Th1) phenotype, which was necessary to control intracellular pathogens, or an IL-4-producing T helper 2 (Th2) phenotype, which mediated immunity against helminths and provided help to B cells for antibody production (136). For years this Th1/Th2 paradigm was the rule of CD4 differentiation in the mist of infection. To support this idea, each subset depended in the expression of lineage-defining transcription factors (T-bet for Th1, GATA3 for Th2) and the overexpression of these "master regulators" forced the differentiation of naive T cells into each one of the lineages (137-139). This model of CD4 differentiation was not strange to malaria researchers. In *P. chabaudi*,

P. berghei, and *P. yoelii* infections, it was widely recognize that the acute phase of infection was characterized by IFN-γ production (140-142). The same results were found in *P. falciparum* (143) in accordance with Th1 responses being triggered by intracellular parasites. Moreover, it was described in *P. chabaudi* a noticeable shift in the response from Th1 to Th2 promoting the antibody production required for complete parasite clearance (141). This whole concept of stable and terminally differentiated CD4 T cells made sense form the point of view that memory T cells had to "remember" to respond appropriately to specific insults.

Even though this dualistic view of CD4 T cell differentiation benefited the studies of immunological responses against pathogens, it suffered when new subsets were discovered. The discovery of T regulatory (Treg) cells and its master regulator the forkhead transcriptional repressor, Foxp3 (144, 145), demonstrated that naive cells could acquire an immunoregulatory phenotype. The next lineage to be discovered was characterized by its signature cytokine IL-17; therefore named T helper 17 (Th17), and its own master regulator, the orphan retinoid receptor, RORγt (146, 147). T follicular helper (Tfh) cells are yet another CD4 T subset with a defined function, to help B cell maturation and antibody isotype class-switch through its signature cytokine IL-21 (148, 149). Tfh cells have their own master regulator, Bcl6 (150, 151). It has been extensively reported that in the absence of Bcl6, B cell responses are greatly affected, with no development of Germinal Centers (specialized regions within secondary lymphoid organs that are required for proper B cell activation) (152, 153). An interesting fact is that Bcl6 is a transcriptional repressor and not a transcriptional activator, such as T-bet or GATA3. Bcl6 directs Tfh differentiation by direct interaction and inhibition of genes that direct the differentiation of the other subsets (154, 155). Therefore, it is still a debate if the Tfh phenotype

is a true lineage comparable to Th1 or Th2, or a default state of differentiation for naive CD4 T cells that are in close interaction with B cells (148, 156, 157).

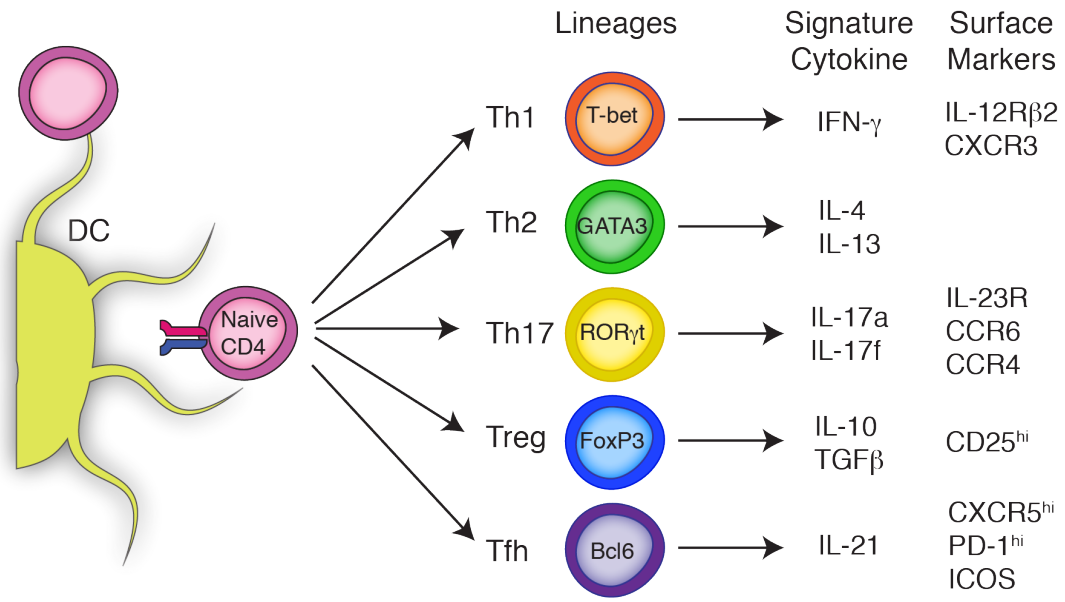

Figure 1.5 CD4 T helper differentiation lineages. *In vitro* studies helped defined the initial subsets Th1 and Th2. However, the plasticity of CD4 T cells in their cytokine production insinuated that CD4 lineages might not be as stable as previously thought. (*adapted from O'shea 2010* (158))

CD4 T cell plasticity

The concept of "T cell plasticity" is becoming more common as new technology is use to study immune responses. Multicolor flow cytometry, mass cytometry (CyTOF), highly multiplex single cell qPCR, and Next Generation Sequencing (NGS), among others, have increasingly been used in immunology shedding light into the nature of T cell differentiation (159). Once thought a Th2 cytokine, IL-10 now has been identified in Th1 cells (160). It is fairly common to find "alternative" Th17 cells that co-produce IL-17 and IFN-γ (161). *In vivo* Th1 polarization using intracellular bacterial or viral pathogens only resulted in 80%-90% of T cells becoming

"committed" to the Th1 lineage (demonstrated by only producing IFN-γ) (162). Importantly, CD4 memory T cells that maintained their plasticity conferred protection against *Mycobacterium tuberculosis* infection, suggesting a beneficial role for plasticity *in vivo*.

Tfh cells can produce IFN-γ or IL-4 pending on the nature of the infection (163). This finding raises questions: Are Tfh capable of acquiring Th1, Th2, and possibly Th17 cytokine profiles? Or is the Tfh phenotype a property that Th1, Th2, and possibly Th17 can acquire? Weinstein et al. recently addressed these questions by creating an IL-21-IL-4 dual-reporter mouse (164). In a series of elegant experiments, they demonstrated that Tfh cells progressively went from IL-21- to IL-4-producing T cells during a helminth infection. Interestingly, Tfh cells localization, cytokine production, and surface ligand expression differentiated their capability to modulate GC formation. IL-21^{+}IL-4^{-} Tfh cells facilitated selection of high-affinity B cell clones, while IL-4^{+}IL-21^{-} Tfh cells enabled B cell differentiation to antibody-secreting plasma cells. These results highlight that cytokine expression determines unique roles on Tfh cells. Moreover, it is possible that immune responses against infectious agents do not result in one stable cytokine expression, but that flexible cytokine production is required to mount a proper and efficient immune response. Nevertheless, if T cell plasticity is a phenomenon that occurs at the single cell level or at the population level remains to be determined.

The idea that T cell subsets can loose or gain different cytokines pending on the duration and quality of immune stimulus can be seen as logical. In the context of chronic infections, IFN-γ^{+}T-bet^{+}Foxp3^{-} Th1 cells are the main producers of IL-10 (82, 165, 166). Successful elimination of a persistent infection might require a strong and prolonged inflammatory response. Therefore,

having self-regulating IL-10^{+} Th1 cells could be beneficial to limit damage to the host. However, it is possible that this immunoregulatory mechanism is exploited by the pathogen to cause persistence in the host. The concept that might be harder to fit within the theory of fixed T helper lineages is the expression of more than one master regulator. This situation is true in the case of Foxp3^{+}T-bet^{+} Tregs during acute *L. monocytogenes* bacterial infection (167), T-bet^{+}GATA3^{+} Th1/Th2 hybrid T cells during persistent parasitic *H. polygyrus* and *S. mansoni* helminth infections (168), and T-bet^{+}Bcl6^{+} Th1/Tfh hybrid T cells during persistent parasitic *P. chabaudi* and *T. gondii* infection (169, 170). Therefore, flexibility in expression of cytokines and lineage-determining factors in helper T cells *in vivo* is more common than previously recognized. The mechanisms that control this plasticity are elusive; however, T helper hybrid phenotypes seemed to be characteristic of persistent infections (168, 169).

Th1 cells in malaria infection

IFN-γ^{+} Th1 cells, directed by the transcription factor T-bet, have been shown to be crucial in controlling the acute phase of the erythrocytic stage of *Plasmodium spp*. rodent infections (119, 171-174). Both, IFN-γ^{+} effector CD4^{+} T cells and IFN-γ^{+} effector memory CD4^{+} T cells confer partial protection from *P. chabaudi* infection (175). Importantly, in human malaria protection from reinfection has been correlated with IFN-γ-mediated responses (176). One possible mechanism for the IFN-γ induced protection in malaria infection is priming the innate immune responses for parasite clearance during re-challenge (177). This mechanism has been observed in a model of systemic *L. monocytogenes* as well (178). In this study, vaccination with attenuated bacteria generated memory T cells that quickly secreted IFN-γ upon challenge with

live bacteria. This study showed that this rapid IFN-γ-mediated response instructs innate cells to become potent effector cells to control pathogen growth. In that study, IFN-γ from both $CD4^+$ and $CD8^+$ memory T cells was important to activate innate cells. However, in the case of *P. chabaudi* it remains to be determined whether IFN-γ from CD4, CD8, or both is required for protection from reinfection. One study proposed that PD-1 mediates the loss of anti-parasitic function of $CD4^+$ and $CD8^+$ T cells in *P. chabaudi* (179). Less than 70% of PD-1 KO mice did not developed chronic phase parasitemia and these results correlated with increased IFN-γ^+ $CD4^+$ and $CD8^+$ T cells. Unexpectedly, 100% of PD-1 KO mice developed chronic malaria when CD8 T cells were depleted suggesting a role for this population during chronic infection.

The innate immune signals that control the differentiation of Th1 are still been elucidated. In *P. berghei* ANKA mouse malaria infection, type I interferon (IFN) signaling inhibited the development of IFN-γ^+T-bet$^+$ Th1 cells and IFN-αR1 deficient mice were able to control parasite growth (180). The effect of type I IFN signaling was specifically on $CD8^-$ DCs population (93). Importantly, the same results were found in healthy volunteers infected for the first time with *P. falciparum* (181). Moreover, type I IFNs promoted the development of IL-10^+ Th1 cells. Other groups have found that T-bet deficient mice, which is necessary for the differentiation of Th1 cells, had less IFN-γ^+ Th1 cells and less cerebral pathology during *P. berghei* ANKA infection (174). However, mice succumbed to infection due to uncontrollable parasite growth. In acute *P. yoelii* 17XNL mouse malaria infection, T-bet deficiency resulted in better parasite control (182). In this infection, IFN-γ CD4 responses did not change but antibody responses were drastically changed, with no detectable IgG2a and increased IgG1. These last results insinuate that *P. yoelii* acute malaria infection control depends more on antibody responses than IFN-γ-mediated

responses. The cytokine IL-27 has been shown to be detrimental for Th1-mediated pathology in *P. berghei* NK65 mouse malaria model (183, 184). T cells from mice deficient of IL-27Rα (WSX-1 KO mice) had more T-bet and IFN-γ expression, and were hyper-responsive to IL-12 activation (184). Overall, $CD4^+$ T cells are expose to a complex signaling environment that promotes and regulates Th1 differentiation.

IL-10 producing Th1 cells

As in many other persistent infections, *Plasmodium spp* infection promotes the differentiation IL-10^+ Th1 cell (165, 166, 169). These cells are referred to as IL-10^+ Th1s or Tr1s, and their phenotype is IL-10^+IFN-$\gamma^+$$CD25^-$T-$bet^+$$FoxP3^+$ (160). In *P. falciparum*, the CD4 response is characterized by the co-secretion of IFN-γ and IL-10 (185). The expression of IL-10 by Th1 cells decreases with age in children in endemic areas, allowing the expansion of IFN-γ^+IL-10^- CD4 T cells, which correlates with parasite control (186). Moreover, IFN-γ^+IL-10^+ Th1 cells are more prevalent in children with uncomplicated malaria than in children with severe malaria (83). The importance of this population has been demonstrated by infection of IL-10 deficient mice with *P. chabaudi* that succumbed to the infection during the peak due to uncontrolled inflammatory responses (120, 121). In *P. chabaudi*, the production of IL-10 by Th1 cells is directed by IL-27 and Blimp-1 (82, 187). In *P. falciparum*, IFN-γ^+IL-10^+ CD4 T cells express high levels of T-bet and Blimp-1, suggesting a similar transcriptional control than the ones observed during *P. chabaudi* infection.

In other persistent infections it has been demonstrated that Blimp-1 controls the development of IL-10^{+} Th1 cells (188, 189), suggesting a common transcriptional control during persistent infection. However, the control of IL-10 in T cells is fairly complex. In Th1 *in vitro* systems, IL-10 production requires STAT4 activation by IL-12 signaling and ERK2 MAP kinase activation through high sustained T Cell Receptor (TCR) activation (190). Other report showed that IL-21 induces IL-10 secretion by Th1 cells *in vitro* (191). Other group showed that IL-27 (through the expression of the transcription factor c-maf), the cytokine IL-21, and the costimulatory receptor ICOS can induce IL-10 production in Th1 cells (192). *In* vitro experiments have demonstrated that IL-27 can also induce the transcription factor aryl hydrocarbon receptor (AhR) that works in concert with relies to promote IL-10 production in Th1 cells (193). IL-27 and IL-21 seem to be involved in the control of IL-10 production in humans with visceral leishmaniasis (VL) (194). Antigen-specific IL-10^{+} Th1 cells from VL patients can augment their IL-10 production by the addition of recombinant human IL-21. In CD8^{+} T cells it has been shown that IL-27 can induce Blimp-1-dependent IL-10 expression (195). But Th1 cells are not the only CD4 subset that can acquire IL-10. Th17 cells can secrete IL-10 under the control of IL-27 and IL-6 that signal through the transcription factor STAT3 (196). Similarly, a recent study has shown that in Th1 cultures, IL-12- and IL-27-dependent STAT1 and STAT3 signaling can promote epigenetic modifications in the *il10* gene (197). Together, these studies demonstrate a very complex control of immunoregulatory Th1 cells. It is important to understand what are the signals that control their generation, as they are crucial to control malaria pathology.

Th2 cells in malaria infection

The role of IL-4$^+$ Th2 responses is less clear than the role of IFN-γ$^+$ Th1 cells. In *P. falciparum*, IL-4$^+$ CD4$^+$ T cells specific to a small semi conserved area of the Duffy biding-like domain (DBL)α-domain of the variant surface antigen *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) correlated with protection from future malaria episodes. In *P. yoelii* mouse malaria infection, IL-4$^+$ CD4$^+$ T cells have been shown to be necessary to secure the survival of effector and memory CD8$^+$ T cells induced by irradiated attenuated sporozoites in the liver stage (131, 132).

The beginning of the erythrocytic phase of *P. chabaudi* infection is clearly marked by a Th1 response. This response is followed by a B cell help response that mediates the generation of malaria-specific antibodies for parasite control during the chronic phase. This change in type of CD4 help was thought to be a shift from inflammatory Th1 to antibody promoting Th2 cells (141), and B cells regulated the shift (198, 199). Moreover, it was described a correlation between IL-4$^+$ CD4 T cells and the production of specific antibodies in individuals that achieved immunity by natural infection (200). Together, these results suggested that Th2 responses were responsible to promote humoral responses against *Plasmodium spp*. However, the frequencies of IL-4$^+$ CD4 T cells within the population that was able to provide B cell help were very low (141). Furthermore, infection of IL-4 deficient mice with *P. chabaudi* did not affected significantly malaria-specific IgG responses (201). Therefore, the proposed Th1/Th2 biphasic CD4$^+$ T cell response during malaria infection did not fully explained the shift from inflammatory to humoral oriented adaptive immune response.

T follicular helper cells in malaria

The generation of long lasting protective antibody responses relies on the formation of GCs within secondary lymphoid organs. When a naive B cells encounter antigen for the first time, they can differentiate into either short-lived plasma cells (PCs) or GC B cells. PCs secrete the first wave of low affinity antibodies that help control infection, while GC B cells will undergo somatic hypermutation generating high affinity Immunoglobulin (Ig). These GC B cells will eventually differentiate into PCs and migrate to the bone marrow, or memory B cells that circulate until reencounter with their cognate antigen. The development of GC requires the help of the specialized CD4 T cells named T follicular helper (Tfh), defined by CXCR5 expression (202) and IL-21 production (149).

In *P. falciparum*, antibodies play a critical role in protection. Several studies have demonstrated that passive immunization with purified IgG antibodies from malaria-immune individuals to children can reduce parasite load and pathology (75, 76). However, in malaria endemic areas it takes time to acquire a large repertoire of antibodies (125). There are mainly two reasons why this happens: 1) it takes time to be exposed to a large number of *P. falciparum* clones and/or 2) there is an inefficient generation of memory B cell responses against the parasite. Furthermore, chronic malaria exposure in endemic areas has been correlated with the generation of "atypical" memory B cells (203, 204), which express high amounts of inhibitory molecules and exhibit poor effector function (205, 206). It is possible that dysregulated Tfh responses could affect B cell responses. Indeed, therapeutic *in vivo* blockade with anti-PD-L1 and anti-LAG-3 resulted in increased Tfh cells and improved humoral responses that resulted in

better parasite control in *P. yoelii* infection (207). In the same model, stimulation of the costimulatory molecule OX40 using agonistic antibodies improved $CD4^+$ T and B cell responses improving antiparasitic immunity (208). Furthermore, treatment with anti-CTLA4, an immune inhibitory protein on T cells, blocked the interference of Tregs with the Tfh-B cell interaction resulting in improved parasite control and cross-species protection (209). These results suggest that interventions targeting host immune mechanisms might be feasible and beneficial to improve malaria control.

Tfh responses are extremely important during mouse malaria infection. Recent research from Dr. Jean Langhorne's laboratory, suggest that Tfh, and not Th2, cells drive the protective antibody responses in *P.chabaudi*. In a series of elegant experiments, Perez *et al*. demonstrated that IL-21, the Tfh signature cytokine, is required to control parasite growth during the chronic phase (211). The main producers of IL-21 are CD4 T cells, and the peak of IL-21 expression is right at the peak of parasite growth (around day 8 p.i.). They identified that IL-21^+ CD4 T cells expressed CXCR5, PD-1, and Bcl6; therefore, they defined them as Tfh cells. However, these cells also expressed IFN-γ and IL-10, and they expanded greater than 14-fold and 60-fold in their percentages and numbers upon *P. chabaudi* infection at the peak. This is surprising because *P. chabaudi* infection is characterized by a late development of malaria-specific IgG responses that are detectable 30 days p.i. This data suggests that either Tfh cells are unable to help B cells at the peak or that *P. chabaudi* infection hinders Tfh function at the peak. Mice deficient of Bcl6, the lineage determining factor of Tfh, only in T cells (Bcl6 TKO) are unable to control chronic parasitemia (212). Nevertheless, IL-21-receptor KO and Bcl6 TKO mice controlled peak parasitemia similar to WT, demonstrating that the control of acute parasite growth is Tfh

independent. However, IL-21-receptor KO mice failed to reduce parasitemia beyond day 15 p.i. reaching up to 50% iRBCs, while Bcl6 TKO mice were able to maintain parasitemia below 1%. Importantly, Bcl6 TKO mice had IL-21$^+$ CD4 T cells, suggesting that the effect of IL-21 is not restricted to Tfh-dependent B cell responses. We have demonstrated that IL-21$^+$IFN-γ$^+$CXCR5$^+$ Th1-like Tfh cells arise in the absence of Bcl6 during *P. chabaudi* infection, suggesting this population do not belong to the classical Tfh lineage (169). These findings are discussed in Chapter 2 of this thesis.

Regulatory controls of Tfh and Th1 flexibility

There are several reports showing that in the context of persistent infection CD4$^+$ T helper cells can acquire a mixed Th1/Tfh phenotype. A comparison between acute LCMV Armstrong (Arm) and chronic LCMV Clone 13 (Cl13) infection demonstrated that the later promoted CD4 differentiation away from Th1 (213). Importantly, CD4 memory T cells from chronic LCMV Cl13 infection expressed more Tfh-related genes, such as Bcl6 and CXCR5. It was previously described that viral persistency could redirect CD4$^+$ T cells from a Th1 to a Tfh phenotype and this shift was the result of prolonged TCR stimulation (214). This relationship between Th1 and Tfh phenotypes was also observed *in vitro*, where naive CD4$^+$ T cells early during Th1 differentiation under high IL-12 concentrations express many Tfh markers (170). Furthermore, a study proposed a mathematical model were constant TCR stimulation inhibited IL-12Rβ2 expression; and therefore, T-bet maintenance hindering Th1 development (215). The role of TCR signaling in CD4 differentiation was studied in the context of duration of TCR stimulation. Using a series of peptides with different kinetic behaviors in regards the TCR-

peptide(p):MHC dissociation time, Tubo *et al*. showed that longer TCR-p:MHC "dwell time" favored Tfh differentiation, while shorter dwell time resulted in Th1 development (216). Another aspect to take in consideration is the metabolism of T cells. One group reported that IL-2 mediated activation of mTORc1, a metabolic regulator, was both necessary and sufficient to shift the differentiation of $CD4^+$ T cells from Tfh to Th1 (217). Many signals that are common to inflammatory conditions seem to play a role in the development of Th1 and Tfh cells. The signal transducer and activator of transcription 3 (STAT3) has been shown to promote Tfh differentiation (218, 219), while type I interferon signaling can inhibit Tfh and promote Th1 differentiation (219). One study showed that *in vitro* co-culture of salmonella-infected human B cells with naive CD4 T cells induced the co-expression of Th1 (T-bet and IFN-γ) and Tfh (Bcl6, IL-21, and CXCR5) genes in the T cells (220). Importantly, it has been shown that T-bet and Bcl6 can bind to each other and behave like a protein complex (221, 222). In this complex, Bcl6 is unable to bind to its target genes, since it interacts with T-bet through its DNA-binding zinc finger. However, T-bet interacts with Bcl6 through its C-terminal domain, leaving its DNA-biding domain free. Nevertheless, the repressor domain of Bcl6 is still functional and it is "adopted" by T-bet (221). These sets of data show the complex regulation between Th1 and Tfh differentiation, and many of these aspects have been corroborated during mouse malaria infection.

The signals that control the differentiation of Th1 and Tfh in malaria are still been discovered. In *P. berghei* ANKA severe mouse malaria infection, infection-mediated inflammation inhibits GC formation and Tfh differentiation (223). Moreover, Tfh cells co-express Th1-related markers, such as T-bet and CXCR3. We found the same results in *P.*

chabaudi infection (169). Interestingly, deletion of T-bet or blockade of IFN-γ and TNF together restored the formation of GC and Tfh differentiation (223). In *P. falciparum*, a population of $CXCR3^{+}CXCR5^{+}PD\text{-}1^{+}$ Th1-like Tfh cells has been identified (224). Importantly, this population is preferentially activated during acute infection in children. These cells were shown to be Th1 polarized, since they express T-bet and produce IFN-γ upon restimulation. Moreover, $CXCR3^{+}$ Th1-like Tfh cells were less efficient in stimulating B cell activation *ex vivo* and promoted the differentiation of naive B cells to $T\text{-}bet^{hi}$ atypical B cells (225). This findings support vaccine strategies that promote $CXCR3^{-}$ Tfh cells to improve protection. Nevertheless, in attenuated whole-parasite vaccines, protection seems to be mediated by generation of protective $CD4^{+}$ T cells, IFN-γ, and nitric oxide (NO) rather than malaria-specific antibodies (226, 227). A recent study showed that the ATP sensor P2X7 promotes Th1, while inhibiting Tfh differentiation, suggesting a role of metabolic byproducts in $CD4^{+}$ T cell differentiation in malaria (228). Metabolism plays a role in $CD4^{+}$ T differentiation in *P. chabaudi* infection, as suggested by the fact that inhibition of fatty acid synthesis at day 3 p.i. increases the generation of effector T cells reducing parasitemia at the peak (229).

There is also a role of innate immune signals in the differentiation of Th1/Tfh cells in malaria. Recently it has been shown in *P. yoelii* 17XNL infection that mice deficient in the pattern recognition receptor cyclic GMP-AMP synthase (cGAS) have increased parasitemia, decrease type I IFN, and decrease Tfh and B cell responses (231). IFN regulatory factor 3 (IRF3) deficient mice infected with *P. chabaudi* had less Th1, more Tfh cells, and increased IgG antibodies (232). However, the effect of IRF3 seems to be intrinsic to B cells since uninfected mice had increased GC B cells without changes in the $CD4^{+}$ T cell populations. All together,

these results suggest that in order to develop an effective vaccine against malaria a balance between Th1 and Tfh memory responses should be considered as an objective.

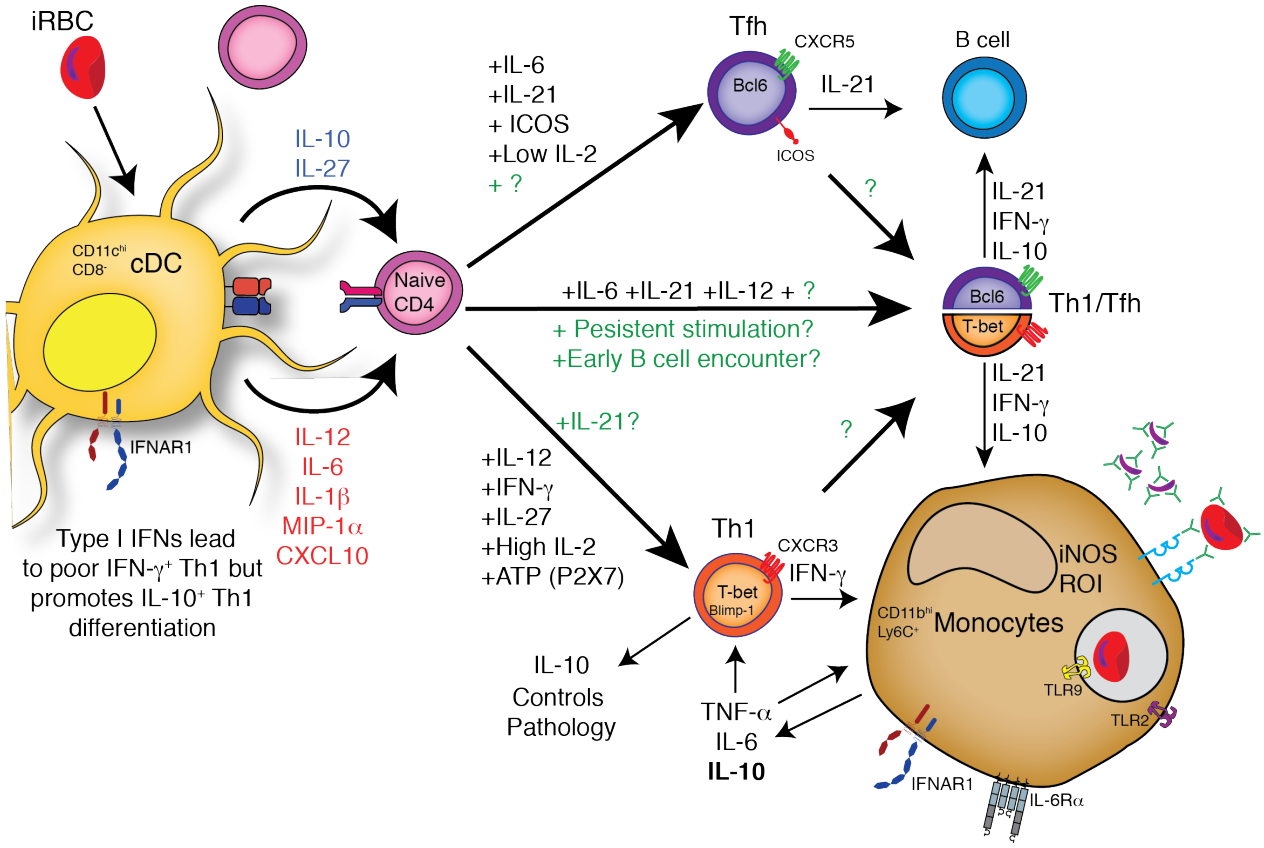

Figure 1.6 Th1 and Tfh polarization during blood-stage *Plasmodium spp.* infection. The signals that shape the differentiation of $CD4^+$ T cells during malaria infection are complex and not fully understood. In the case of *P. chabaudi* mouse malaria infection, $CD8^-$ DCs present malaria antigens to naive $CD4^+$ T cells. A previous report demonstrated that there is change in the DC population from pro-inflammatory to anti-inflammatory as the infection progress (230). Naive T cells are exposed to many different cytokines, inflammatory (red) and immuno-regulatory (blue), that can direct them to either IFN-γ^+IL-10^+ Th1 or IL-21^+ Tfh cells. However, recent reports have shown that a Th1-like Tfh population is predominantly generated during malaria infection (169, 211, 223, 224). The signals that direct the differentiation of Th1-like Tfh cells remain unknown. The possible signals that have not been tested yet are in green. Moreover, it remains to be tested if Th1 or Tfh cells can acquire this Th1-like phenotype.

OBJECTIVES OF THIS DISSERTATION

$CD4^+$ T cell subsets can acquire a stable lineage characterized by the expression of a determined transcription factor, the production of signature cytokines, and the expression of specific surface markers. However, plasticity of $CD4^+$ T cells is also a possibility. In the context of *P. chabaudi* persistent mouse malaria infection, to what extend is beneficial to achieve a stable lineage or to maintain T helper flexibility, and what are the $CD4^+$ T helper populations that correlate with protection is still unresolved. Thus, it is important to determine the molecular orchestrators of $CD4^+$ T helper cells *in vivo*. Knowing that IFN-γ^+ Th1 cells are crucial for the control of acute parasitemia in *P. chabaudi* infection, the development of Th1 cells *in vivo* will be examined in Chapter 2 using an IFN-γ reporter mouse. Previous reports have shown that there is a shift from Th1- to Th2- mediated immune responses in *P chabaudi*. Now we are aware that the previously described Th2 responses are most likely Tfh. However, no studies have address if the Th1 cells generated during the acute phase change their phenotype once they survive into the memory phase, or if new T helper cells arise during the chronic phase with a Tfh phenotype. This subject will be study by adoptive transfer experiments of $CD4^+$ T cells during *P. chabaudi* infection.

The molecular control of $CD4^+$ T cells is complex. During malaria infection, $CD4^+$ T cells express many transcription factors that are involved in T cell differentiation. Using T cell-specific Knock Out (TKO) mice, we will study in Chapter 3 the role of Bcl6 (Tfh master regulator), Blimp-1 (Tfh repressor), and STAT3 (Tfh promoter) in the differentiation of $CD4^+$ T helper cells *in vivo*. We analyze in detail the adaptive immune responses in these mice, and

determined if the deletion of this transcription factor affected parasite control and protection from reinfection. Collectively, this dissertation aims to increase our knowledge regarding protective adaptive immune responses to persistent malaria infection. Understanding the molecular control of protective immune responses may aid in the development of a much needed malaria vaccine.

CHAPTER 2: IFN-γ AND IL-21 DOUBLE PRODUCERS ARE NOT BCL6-DEPENDENT IN *PLASMODIUM CHABAUDI* AND SURVIVE INTO THE MEMORY PHASE[1]

Introduction

Immunity against intracellular pathogens, such as the blood-stages of the rodent malaria parasite *Plasmodium chabaudi,* requires both antibodies and Th1-type responses (74, 233). This rodent parasite shares many traits with human malaria parasites, such as *P. falciparum*, and generates immunity that parallels that observed in human malaria (50). The CD4 T cell response starts with strong IFN-γ production, which reduces the initial parasite growth, followed by a marked change in the response to promote antibody and B cell involvement. This is critical because antibody is required for complete parasite clearance (141). CD4 T cells isolated from *P. chabaudi* infected mice on day 40 and cultured for two weeks with parasite antigen lose their IFN-γ production capacity, but gain the ability to generate IL-4 and provide help to B cells, suggesting that they are not of pure Th1 lineage (141).

The transition from Th1 to antibody promoting T cells in response to *P. chabaudi* is likely regulated by B cells, as T cells from infected B cell deficient (μMT) mice produce more IFN-γ and less IL-4, and become inefficient to help antibody formation (198). Furthermore,

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during the early phases of this infection there is a switch in the type of antigen presenting cells, which reduces IFN-γ production (94). This change in T cell function includes acquiring the ability to secrete the regulatory cytokine IL-10, and the antibody-promoting cytokine IL-21 (82, 211). This response seems appropriate to achieve an adequate balance between parasite control and immunopathology. Despite this controlled regulation, serum IFN-γ and IFN-γ^+ T cells correlates with resistance to *P. falciparum* in African children (123, 176). Therefore, understanding the generation of IFN-γ-producing memory T cells is important for the rational creation of a malaria vaccine.

It was recently reported that IL-21 generated by IFN-γ^+IL-10^+ T cells is critical to generate antibodies that control chronic infection and re-infection (211). This new data suggests that the earlier reported switch from IFN-γ^+ Th1 immunity relates to an increase in CXCR5^+IL-21^+ T follicular helper cells (Tfh) (234). Indeed, a recent study in Malian children uncovered that CXCR5^+PD-1^+CXCR3^+ Th1-like Tfh cells are the predominant response against acute malaria. Importantly, these Th1-like Tfh cells were unable to mount an optimal antibody response, albeit produced the highest levels of IL-21 (224). Th1 cells are the major source of IL-10 during this infection, as in other chronic parasitic infections, and it is induced by IL-27 (82, 165, 166, 235). Importantly, IL-27 can also induce IL-21 (192), and promote Tfh development (236). The transcriptional regulation of IL-21 expression in T cells is not clearly defined and may involve Bcl6, as well as Maf and STAT3 (151, 237, 238).

IL-21 has a pivotal role in B cell differentiation and germinal center formation, but can also have effects on T cell biology, including inhibition of IFN-γ production (239). However,

this finding may be limited in scope as CD4 T cells cultured in vitro under Th1 polarizing conditions can produce significant levels of IL-21 (237). Conversely, although IL-21 is the signature cytokine of the Tfh subset (149), these cells can simultaneously express other cytokines, including IFN-γ, depending on the nature of the cytokine milieu (163). For example, experiments using an influenza infection model in IL-21 reporter mice showed that $CXCR5^{+}PD\text{-}1^{+}IL\text{-}21^{+}$ Tfh cells can express IFN-γ, IL-10, and T-bet (240). Therefore, it is not clear whether the unusually large amount of IL-21 observed in this chronic infection is made by Tfh- or Th1-lineage derived cells, and if they are able to survive into the memory phase.

Herein, we investigated IFN-γ-producing effector T cells elicited during *P. chabaudi* infection for molecular evidence of Th1 commitment, and their ability to generate $IFN\text{-}\gamma^{+}IL\text{-}21^{+}$ memory T cells. Using an *Ifng/Thy1.1* reporter mouse, we observed that a majority of $IFN\text{-}\gamma^{+}$ T cell responders expressed several Tfh markers. In line with previous findings (211, 224), the dominant $IFN\text{-}\gamma^{+}$ Teff population identified was $CXCR5^{+}$, and these cells produced high levels of IFN-γ, in addition to IL-10 and IL-21. An $IFN\text{-}\gamma^{+}CXCR5^{hi}PD\text{-}1^{hi}IL\text{-}21^{+}$ GC Tfh population was also observed. The $CD4^{+}IFN\text{-}\gamma^{+}$ effector T cells also expressed both T-bet and the Tfh lineage-promoting transcription factor Bcl6. As expected, deficiency of Bcl6 regulated the $CXCR5^{hi}PD\text{-}1^{hi}$ GC Tfh subset. On the other hand, Bcl6 did not regulate the $CXCR5^{+}IL\text{-}21^{+}IFN\text{-}\gamma^{+}$ population. We also studied IL-10 deficient mice, which have increased T-bet and IFN-γ in T cells to promote Th1 development. We found that in response to *P. chabaudi* infection, these mice generated increased levels of both $CXCR5^{+}IL\text{-}21^{+}IFN\text{-}\gamma^{+}$ T cells and $IFN\text{-}\gamma^{+}$ GC-Tfh. During the memory phase, we found that $IFN\text{-}\gamma^{+}$ T cells at day 60 post-infection were able to produce IL-21. Adoptive transfer of CFSE-labeled $IFN\text{-}\gamma^{+}$ T cells revealed that T-bet and IFN-γ

expression are only maintained by cell division in the memory phase. Together, these findings suggest that a heterologous T helper memory cell population is critical to the malaria immune response because it maintains both cellular and humoral immunity through IFN-γ, IL-21, and CXCR5, and regulates pathology via IL-10. Importantly, this subset is not dependent on Bcl6 suggesting is not of the Tfh lineage. These results have significant implications for our understanding of the protective responses against malaria, and intend support the development of effective vaccines to control and prevent malaria.

Materials and Methods

Animals and infections

C57BL/6J (B6), B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (CD45.1) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10 deficient) were purchased from The Jackson Laboratory (Bar Harbor, ME), and CD4-Cre^{+} mice from Taconic (Hudson, NY). *Ifng/Thy1.1* Knock-In and *Ifng/Thy1.1* BAC-In mice were a kind gift of Casey Weaver (University of Alabama, Birmingham, AL). Bcl6$^{fl/fl}$ x CD4-Cre mice (241) (Indiana University School of Medicine, Indianapolis, IN) were bred at UTMB. The floxed allele was genotype by PCR using the primers for the 3' loxP site: forward 5'-TCACCA ATCCCAGGTCTCAGTGTG-3'; reverse 5'-CTTTGTCATATTTCTCTGGTTGCT-3'. All mice were maintained in our specific pathogen free animal facility with ad libitum access to food and water. Mice 6–12 weeks old were infected with 10^{5} *Plasmodium chabaudi chabaudi* (AS) courtesy of Jean Langhorne (MRC NIMR, London, UK) infected erythrocytes i.p. Parasites were counted in thin blood smears stained with Giemsa (Sigma, St. Louis, MO) by light microscopy (121).

Animal Care Statement

All animal experiments were carried out in compliance with the protocol specifically approved for this study by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Flow Cytometry and Imaging Flow Cytometry

Single-cell suspensions from spleens were made in HEPES buffered Hank's Balanced Salt Solution (Gibco, Lifetechnologies, Grand Island, NY), incubated in red blood cell lysis buffer (eBioscience, San Diego, CA), and stained in PBS 2% FBS (Sigma, St. Louis, MO) and 0.01% sodium azide with anti-CD16/32 (2.4G2) supernatant (BioXcell, West Lebanon, NH) followed by combinations of FITC–, PE–, PerCP-Cy5.5, PE/ Cyanine 7 (Cy7), Allophycocyanin monoclonal antibodies (all from eBioscience, San Diego, CA), CD127-PE/Cy5, CD44-Brilliant Violet 785 (BV785), CXCR3 BV421 (Biolegend, San Diego, CA), CXCR5-Biotin (BDbioscience, San Jose, CA) followed by either Streptavidin-eFluor 450,–PE or–BV650. For experiments using KI and $Bcl6^{fl/fl}CD4^{Cre}$ mice, CXCR5 staining was performed using rat anti-mouse purified CXCR5 (BDbioscience, San Jose, CA) for 1 hour at 4°C followed by 30 min incubation with biotin conjugated AffiniPure Goat anti-rat (H+L) (Jackson Immunoresearch, West Grove, PA) followed by Streptavidin (242). For intracellular staining, total cells were stimulated for 2 h with PMA (50 ng/mL), ionomycin (500 ng/mL), and Brefeldin A (10 μg/mL) in complete Iscove's Media (cIMDM) (all from Sigma), 10% FBS, 2mM L-glutamine, 0.5 mM sodium pyruvate, 100 U penicillin, 100 μg streptomycin, and 50 μM2-ME (all from Gibco, Lifetechnologies). Cells were fixed in 2% paraformaldehyde (Sigma), permeabilized using Permeabilization buffer (Perm, eBioscience) and incubated for 40 minutes with anti-IFN-γ-FITC

(XMG1.2), IL-10-PE (JES5-16E3), T-bet-efluor 660 (eBio4B10, eBioscience), or Bcl6-Alexa Fluor 488 (K112-91).

For IL-21 staining, cells were incubated for 40 minutes with recombinant mouse IL-21R-Fc chimera (1 μg, R&D systems, Minneapolis, MN) in Perm and washed twice, followed by 30 min with Alexa Fluor 647 F(ab')2 goat anti-human IgG (0.3 μg, Fcγ Specific, Jackson ImmunoResearch, West Grove, PA) in Perm buffer. After three washes in FACS buffer, cells were collected on a LSRII Fortessa in the UTMB Flow Cytometry and Cell Sorting Core Facility using FACSDiva software (BDbiosciences, San Jose, CA) and analyzed in FlowJo version 9.7 (TreeStar, Ashland, OR). Compensation was performed in FlowJo using single stained splenocytes (using CD4 in all colors). In adoptive transfer figures, data from 3–4 mice is concatenated to achieve sufficient cell numbers for presentation and Boolean gating analysis, after each mouse was analyzed and averages and SEM calculated. Nuclear co-localization of T-bet and Bcl6 was collected using ImageStream MARKII and analyzed with IDEAS1 ImageStream (EMD Millipore, Seattle). For Boolean gating analysis, the distribution of used markers was analyzed with

SPICE 5.35 software (243).

Cell Sorting and Microbead Purification

Splenic $CD4^+$ T cells from uninfected mice were enriched using EasySep biotin Selection Kit (STEMCELL, Vancouver, Canada) and a cocktail of biotinylated anti-CD8α (55–6.7), B220 (RA3-6B2), CD11b (MI/70), CD11c (N418), F4/80 (BM8) and Ter119 (all from eBioscience). Enriched T cells were then Naïve ($CD44^{lo}CD25^-$) sorted with anti-CD4-FITC, CD44-

Allophycocyanin- Cy7, and CD25-PE (all eBioscience) on a FACSAriaI with FACDiva software (BDbiosciences). Thy1.1^{+}CD4^{+} T cells were isolated via direct magnetic bead separation (Miltenyi Biotec, San Diego, CA) after CD4^{+} T cell enrichment. Cells were washed and resuspended in calcium- and magnesium-free PBS at 10^{7} cells/mL before incubation with 5 μMCell Trace Violet (CTV, Invitrogen) for 10 minutes at 37°C with shaking, then quenched with FCS. After washing, $2x10^{6}$ cells were transferred into each mouse i.p.

Real Time PCR

RNA from Thy1.1^{+}CD4^{+} T cells was extracted (RNeasy, Qiagen, Valencia, CA) and treated with DNAse (DNAse I, Invitrogen), adjusted to 40–100 ng/μL and reverse transcribed in a final volume of 20 μL (High capacity reverse transcription kit (Applied Biosystems, Grand Island, NY). Between 2–40 ng of reverse transcribed RNA was amplified using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) with the following primer pairs: *bcl6*, forward, 5' CCGGCACGCTAGTGATGTT 3', reverse, 5' TGTCTTATGGGCTCTAAACTGCT 3', and qSTAR qPCR primer pairs (Origene, Rockville, MD) against *tbx21* (MP216689), *prdm1* (MP211365), *eomes* (MP204243). The level of expression was determined by the comparative threshold method ($2^{-}\Delta\Delta^{CT}$) compared to naïve (CD44^{lo}CD25^{-}) CD4 T cells from uninfected BAC-In mice as a calibrator sample and the 18s ribosomal RNA (*rp18s*) gene as a reference gene for normalization. Samples were collected using a ViiA7 Real-Time PCR system (Applied Biosystems).

Statistics

Statistical analysis was performed in Prism (GraphPad, La Jolla, CA) using Student's t-test one-tail or two-tail when indicated. $p<0.05$ was accepted as a statistically significant difference.

Results

Kinetics of Th1 differentiation in response to *P. chabaudi* infection

P. chabaudi malaria infection reaches maximal parasitemia by day 9 and persists at low levels for up to three months (244). To investigate Th1 differentiation during *P. chabaudi* infection, we used IFN-γ reporter (*Ifng/Thy1.1* knock-in, KI) mice, where the Thy1.1 reporter gene is expressed transiently during active transcription of *Ifng* (245). Direct *ex vivo* analysis of splenocytes by flow cytometry on day 9 post-infection (p.i.) revealed a small *Ifng/Thy1.1*$^+$ CD4$^+$ T cell population (average of 2.41%, **Fig 2.1A**). The majority of these Thy1.1$^+$CD4$^+$ T cells had an IL-7Rα negative (96.6% CD127$^-$), effector phenotype (**Fig 2.1B**). Similar expansion kinetics were observed for all Teff and *Ifng/Thy1.1*$^+$ effector T cells along with parasite levels, which all peaked at day 9 p.i. (**Fig 2.1C**).

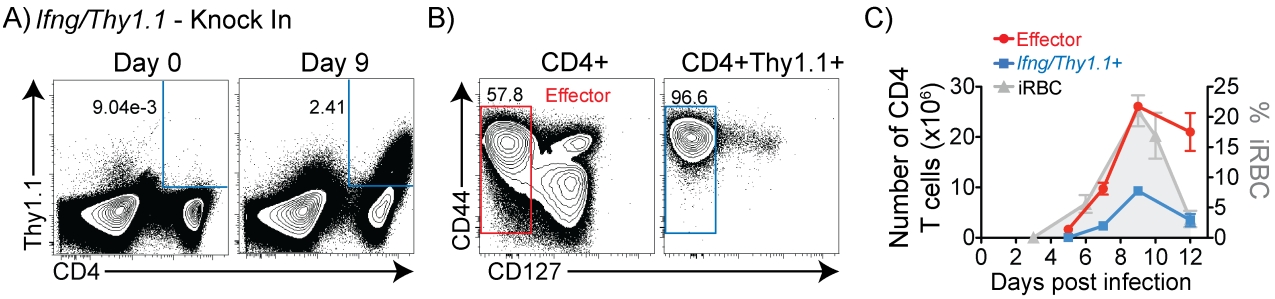

Fig 2.1. IFN-γ-producing effector cells expand and contract with kinetics similar to parasitemia. *Ifng/Thy1.1* Knock-In (KI) mice were infected with *P. chabaudi* infected RBC (iRBC). Splenocytes were harvested and analyzed by flow cytometry on various days post-infection (p.i.) and (A) were gated on CD4$^+$*Ifng/Thy1.1*$^+$, shown at day 0 and 9 p.i. Average percentages from three animals are shown on plots. (B) CD4$^+$*Ifng/Thy1.1*$^+$ (as gated in A)

showing $CD4^+CD127^-$ effector gate (red box) and $Thy1.1^+CD127^-$ effector gate (blue box) at day 9 p.i. (C) Total numbers of CD4+ effector T cells and $CD4^+$*Ifng/Thy1.1*$^+$ T cells (as gated in B) per spleen overlaid with parasitemia curve (%infected RBCs/total RBCs). Data are representative of eight independent experiments with three animals per time point. Error bars represent SEM.

In order to investigate the degree of Th1 differentiation of IFN-γ^+ cells during the effector phase of the response, we measured expression of Th1 markers on CD4 T cells at timepoints leading up to the peak of *P. chabaudi* infection. We used T-bet, the master regulator of Th1 differentiation, as a marker of Th1 commitment, since high levels of this transcription factor are expressed in Th1 cells fully committed to making IFN-γ. On day 5 p.i, minimal effector cells were detectable and most of the *Ifng/Thy1.1*$^+$ cells expressed T-bet (average, 79%) above that of the T-bet isotype control. Further, 55% of the cells were above fluorescence minus one (FMO) multi-color staining control for CXCR3, the IFN-γ-induced homing receptor for inflamed tissues (**Fig 2.2A**). An average of 47% of $Thy1.1^+CD4^+$T-bet$^+$ T cells co-expressed CXCR3 on day 5 p.i. On day 7 p.i, Teff appeared Th1-like, with 95% of $Thy1.1^+CD4^+$ T cells expressing T-bet and an average of 70.4% of $Thy1.1^+$ cells co-expressing CXCR3. However, at day 9 p.i, we observed a downregulation of T-bet in *Ifng/Thy1.1*$^+$ CD4 T cells, accompanied by a significant downregulation of CXCR3. However, the downregulation of CXCR3 has been reported to be required for proliferation in CD8 T cells in the red pulp, suggesting this may be independent of IFN-γ production (246). Runx3, a chromatin-remodeling factor critical for commitment to IFN-γ production by Th1 cells (247), also peaked on day 7, and was downregulated in *Ifng/Thy1.1*$^+$ CD4 T cells by day 9 (**Fig 2.2A and 2.2B**). This data is summarized in Fig 2.2C by Boolean gating analysis of the Th1 phenotype of the *Ifng/Thy1.1*$^+$ CD4 T cell population through the peak of *P. chabaudi* infection. We found that on day 5 p.i, 16% of the $Thy1.1^+CD4^+$ T cells coexpressed T-bet, CXCR3, and Runx3 with a similar percentage on day 7; on day 9, only 0.5%

of the Thy1.1^{+}CD4^{+} T cells maintained the expression of these three markers. These results indicate the generation of a strong Th1 response by day 7 p.i, characterized by the expression of Tbet, CXCR3, Runx3, and the production of IFN-γ. However, this is followed by T-bet downregulation, and decreased CXCR3 and Runx3 by day 9 p.i, despite a continued increase in the number of IFN-γ-producing T cells.

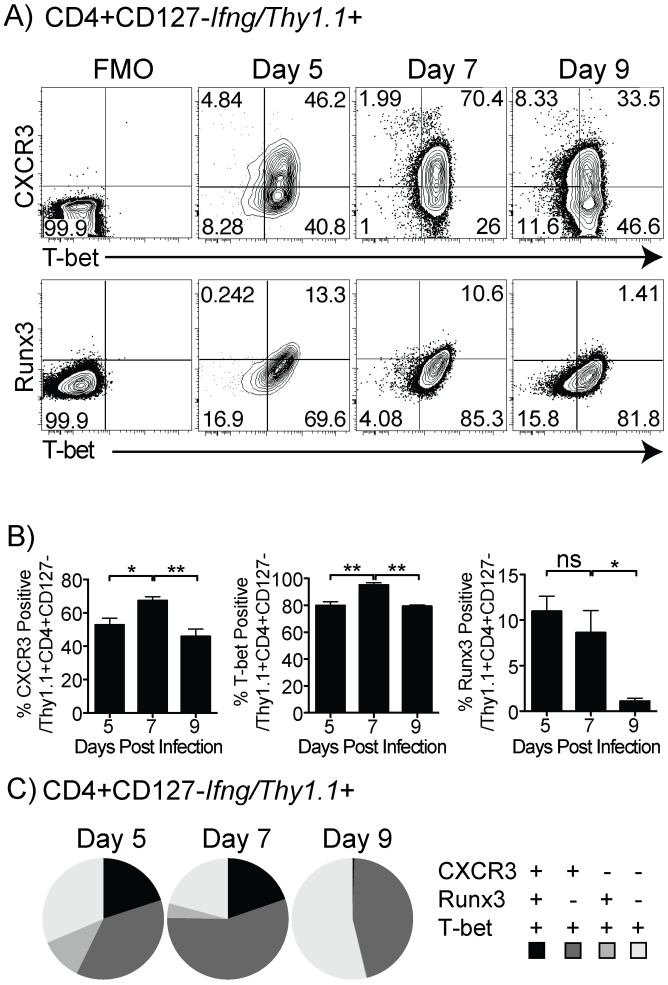

Fig 2.2. Expression of markers of Th1 differentiation is reduced at the peak of IFN-γ production. *Ifng/Thy1.1* KI mice were infected with *P. chabaudi* iRBC, and splenocytes were analyzed. (A) Contour plots show expression of CXCR3, T-bet, and RUNX3 gated on CD4^{+}*Ifng/Thy1.1*$^{+}$ on days 5, 7, and 9 post-infection. The numbers depicted on the plots are average percentages. Gates were drawn using fluorescence minus one (FMO, CXCR3) or isotype (Tbet, Runx3) controls for each day, as shown to the left. (B) Bar graphs showing percentages of CXCR3, T-bet, and Runx3 positive cells in Thy1.1^{+} Teff population on each day. Data is

summarized in (C) pie charts of Boolean gating analysis of all possible combinations of CXCR3$^+$, Runx3$^+$, and T-bet$^+$ within CD4$^+$*Ifng/Thy1.1*$^+$ effector T cells. *Ifng/Thy1.1*$^+$ T cells expressing all three Th1 markers are shown in black. Two markers are shown in dark grey, and one marker is indicated by light grey. Data are representative of three independent experiments with three animals per time point. Statistical significance was obtained using Students t test. Error bar represents SEM; *p < 0.05, **p < 0.01, ns = not significant.

IFN-γ^{hi}CXCR5$^+$ effector T cells are the main source of IL-21 and IL-10 in *P. chabaudi* infection

Upon investigation of responsive T cells in this infection, we observed a dramatic increase in CXCR5, ICOS, and SLAM. Given the presumed predominance of Th1 cells at this timepoint (141), we investigated expression of several Tfh markers on *Ifng/Thy1.1*$^+$ effector T cells. Surprisingly, at day 7 p.i, *Ifng/Thy1.1*$^+$ and *Ifng/Thy1.1*$^-$ effector T cells expressed comparable levels of CXCR5 and BTLA (B and T Lymphocyte Attenuator), while expression of Blimp-1, ICOS, and SLAM was higher on *Ifng/Thy1.1*$^+$ (**Fig 2.3A**). This unusual profile suggests that these IFN-γ producers also have some features of Tfh cells. To differentiate Tfh and GC Tfh cells, we used CXCR5, the chemokine receptor that determines localization to B cell areas in lymphoid tissue; and PD-1, which is highly expressed on GC-Tfh (248). Despite a majority of PD-1^{int} Teff, as observed in the CD4 response to chronic *M. tuberculosis* (249), we detected both CXCR5^{hi}PD-1^{hi} (GC Tfh) cells and CXCR5$^+$ (Tfh) cells within the *Ifng/Thy1.1*$^+$ effector T cell population on day 7 p.i. (**Fig 2.3B**). After gating on these populations, we found that IL-21 production by CXCR5$^+$ *Ifng/Thy1.1*$^+$ effector T cells was greater than in the *Ifng/Thy1.1*$^-$ Teff (**Fig 2.3C and 2.3D**).

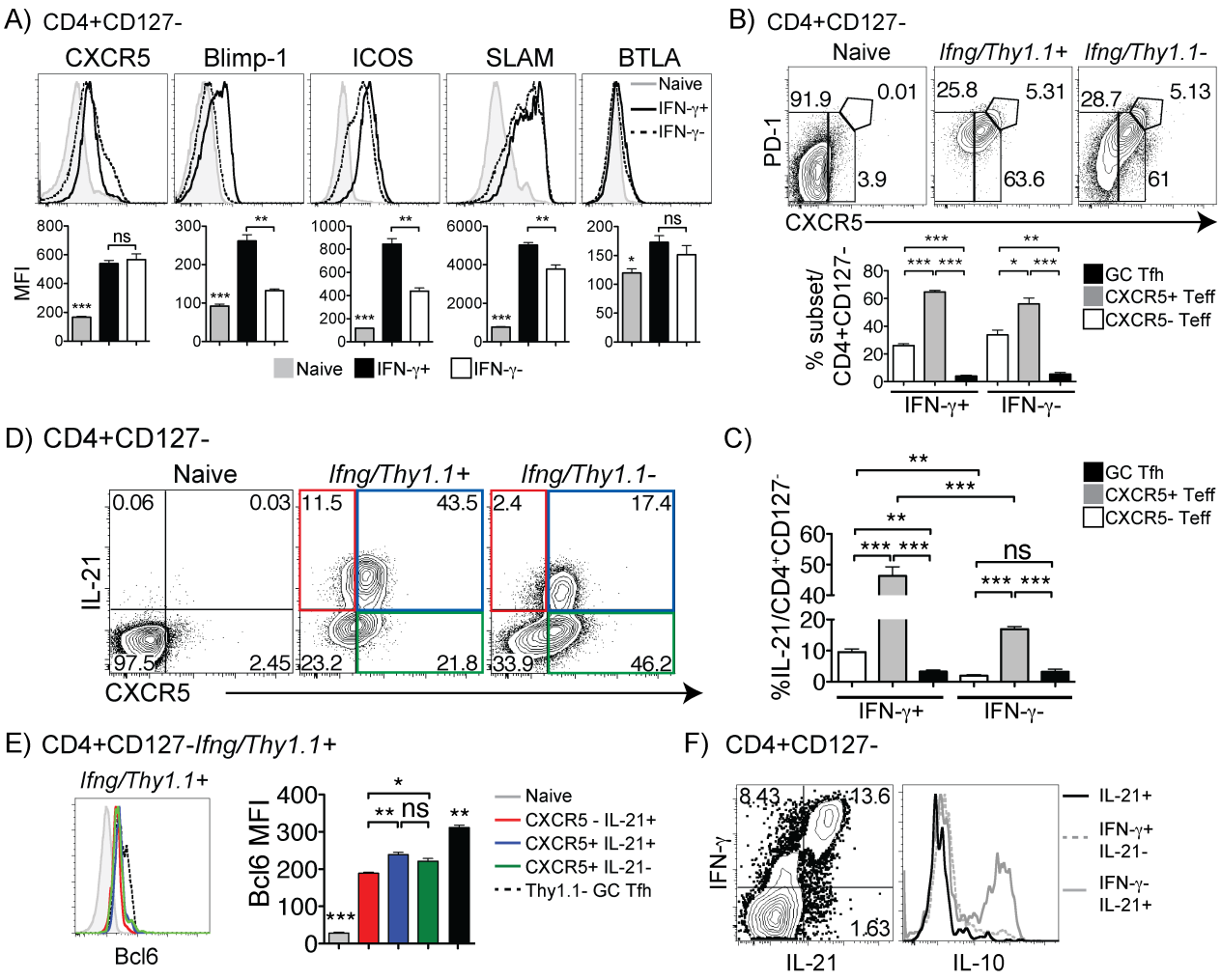

Fig 2.3. The *Ifng/Thy1.1*$^{+}$ T cell population expresses Tfh markers. Splenocytes from day 7 p.i (A) C57Bl/6J mice were analyzed for expression of CXCR5, Blimp-1, ICOS, SLAM, and BTLA in naïve (CD44^{lo}CD127^{+}, grey), IFN-γ^{+} (black line), and IFN-γ^{-} (dotted line) effector T cells (CD127^{-}) as determined by intracellular cytokine staining. Bar graphs show mean fluorescence intensity (MFI). (B) Contour plots of PD-1 and CXCR5 from *Ifng/Thy1.1* knock-in (KI) mice effector T cells (CD4^{+}CD127^{-}, as shown in Fig 2.2) are gated on naïve T cells from uninfected, *Ifng/Thy1.1*$^{+}$, and *Ifng/Thy1.1*$^{-}$ mice. Bar graph shows percentages of *Ifng/Thy1.1*$^{+/—}$ subsets out of total effector T cells. (C) Contour plot of IL-21 and CXCR5 expression. (D) Percentages of IL-21 within effector subsets. (E) Histogram showing Bcl6 expression in CXCR5^{-} IL-21^{+} (C, red box), CXCR5^{+}IL-21^{+} (C, blue box), and CXCR5^{+}IL-21^{-} (C, green box) subsets relative to naïve and *Ifng/Thy1.1*$^{-}$ GC Tfh T cells. Bar graph shows average MFI of Bcl6 staining shown for each subset. (F) Contour plots for IFN-γ, IL-21 and IL-10 by intracellular cytokine staining in C57Bl/6J splenocytes day 7 p.i. Data are representative of four independent experiments with 3 mice per group. Statistical significance was obtained using Students t test. Error bar represents SEM; *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant.

Quantifying the fraction of IL-21 in the three populations, shown in Fig 2.3B, revealed that CXCR5^{+}IFN-γ^{+} cells generated the most IL-21. These levels were even greater than that produced by CXCR5^{hi}PD-1^{hi} GC Tfh cells and CXCR5^{-} effector cells (**Fig 2.3D**). Moreover, the mean fluorescence intensity (MFI) of IL-21 in *Ifng/Thy1.1*$^{+}$ effector T cells was slightly higher (average, 611) than the *Ifng/Thy1.1*$^{-}$ population (536, $p<0.05$). Given the similarity of the Th1 cells to Tfh, we measured expression of Bcl6, the Tfh lineage-determining transcription factor, in the *Ifng/Thy1.1*$^{+}$ and IL-21 or CXCR5 expressing populations shown in Fig 2.3D at day 7 p.i. (**Fig 2.3E**). All *Ifng/Thy1.1*$^{+}$ effector subsets expressed a higher level of Bcl6 than naïve (CD44^{lo}CD127^{+}) CD4 T cells, but lower than *Ifng/Thy1.1*$^{-}$ CXCR5^{hi}PD-1^{hi} GC Tfh. Intriguingly, we also found that the majority of IL-21 producers also made IFN-γ and IL-10 (**Fig 2.3F**), and only the IFN-γ^{hi} Th1 cells produced both IL-21 and IL-10.

Bcl6 T cell deficiency abolishes CXCR5^{+} Germinal Center T follicular helper cells, but not CXCR5^{+}IL-21^{+}IFN-γ^{+} T cells

Bcl6 is the primary transcription factor that determines Tfh cell lineage (151). However, in Th1 cultures, Bcl6 has been shown to be driven by IL-12 and to oppose expression of IFN-γ by direct association with T-bet (222). Therefore, we examined the co-expression and localization of Bcl6 and T-bet, and tested the correlation of Bcl6 and T-bet expression with *Ifng*, as well as the requirement of Bcl6 to generate T cell populations making IL-21 or expressing CXCR5. To enrich the *Ifng*$^{+}$ cells for imaging flow cytometry analysis, *Ifng/Thy1.1*$^{+}$ cells were purified from infected *Ifng/Thy1.1* Knock-In reporter animals on day 7 p.i. *Ifng/Thy1.1*^{hi} and *Ifng/Thy1.1*^{lo} populations were gated (**Fig 2.4A**) and expression of Bcl6 and T-bet was quantified

(**Fig 2.4B**). We observed a correlation between high expression of Thy1.1 to high T-bet or low Bcl6. Using the bright detail similarity score, which compares the brightness of two probes in the image, and DAPI to stain the nucleus, we observed both T-bet and Bcl6 in the nucleus of Thy1.1^{+} effector (CD127^{-}) T cells (**Fig 2.4C**). However, in some cells both T-bet and Bcl6 could also be detected in the cytoplasm. This has been previously reported for T-bet (250).

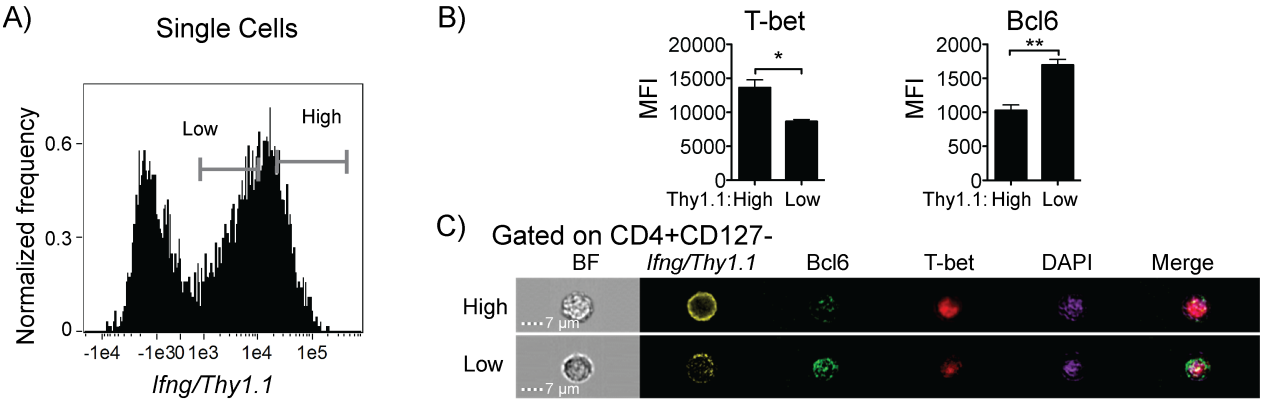

Fig 2.4. Level of T-bet in the nucleus correlates with *Ifng/Thy1.1* expression. *Ifng/Thy1.1* BAC-In reporter mice were infected with *P. chabaudi* and splenocytes were analyzed on day 7 post-infection. After positive selection of Thy1.1 cells, cells were stained for surface CD4, CD127, and Thy1.1 (yellow), followed by intracellular staining for Bcl6 (green) and T-bet (red). Just before analysis, nuclei are stained with DAPI (purple), and analyzed by imaging flow cytometry. (A) Histogram showing *Ifng/Thy1.1* expression is gated on single focused cells. Cells were gated on CD4^{+}CD127^{-} Teff and (B) expression of Bcl6 and T-bet was measured within *Ifng/Thy1.1* high and low gates as shown in (A). (C) Representative images of individual cells from imaging flow cytometry. Bright Field (BF) and DAPI/Bcl-6/T-bet merged images are shown. Statistical significance was obtained using Students t test. Error bar represents SEM; *p < 0.05, **p < 0.01.

As low levels of T-bet coincides with low Bcl6 expression and correlates with reduced *Ifng/Thy1.1* expression, we tested the role of Bcl6 in induction of the mixed Th1 and Tfh phenotype observed here. We infected Bcl6$^{fl/fl}$Cre^{CD4} T cell-specific KO (TKO), where the Zn finger-encoding exons of the Bcl6 gene are flanked with loxP sites and deleted specifically from all CD4^{+} T cells, as previously verified (241). This domain of Bcl6 is involved in both DNA binding, and the protein-protein interaction with T-bet (222, 251). Therefore, the potential Bcl6-

mediated regulation of Th1 lineage loci and binding to T-bet are both deficient. As Bcl6 TKO mice do not make Germinal Center B cells (241), which are likely to be essential for clearance of *P. chabaudi* (198, 211), we sorted naïve CD4 T cells ($CD44^{lo}CD25^{-}$) from Bcl6 TKO mice (CD45.2), labeled them with the CFSE analog cell trace violet (CTV), and adoptively transferred them into CD45.1 congenic mice, followed by *P. chabaudi* infection (**Fig 2.5A**). Both groups of recipients exhibited a similar course of parasitemia (**data not shown**), and were expected to make antibodies normally. On day 7 p.i, responding $CTV^{-}CD45.2^{+}$ effector T cells were identified and the Bcl6- versus wild type-derived cells were characterized and compared (**Fig 2.5B**). As expected, Bcl6 deficient T cells did not generate $CXCR5^{hi}PD\text{-}1^{hi}$ GC Tfh cells (**Fig 2.5C**). While the frequency of $CXCR5^{+}$ effector T cells did not change between groups, we did observe a decrease in the MFI of CXCR5 on the Bcl6 TKO T cells (**Fig 2.5D**). However, Bcl6 deficiency had no effect on IFN-γ or IL-21 production (**Fig 2.5E**). Although we observed a decrease in IFN-γ^{-}IL-21^{+} cells in this experiment, this effect was not repeatable. There was no change in IFN-γ^{+}IL-10^{+} effector T cells in the TKO cells either (**Fig 2.5F**). Because both groups of recipients are wild type, cell numbers followed the same trends shown here as percentages. Overall, these data demonstrate that Bcl6 deficiency in T cells reduces GC Tfh formation, as previously described (241). However, Bcl6 deficiency had no effect on the IFN-γ^{+}IL-10^{+}IL-21^{+} effector T cells, indicating that these cells are not derived from the Bcl6-dependent Tfh lineage. This was surprising, given that IFN-γ cells express so many markers indicative of Tfh.

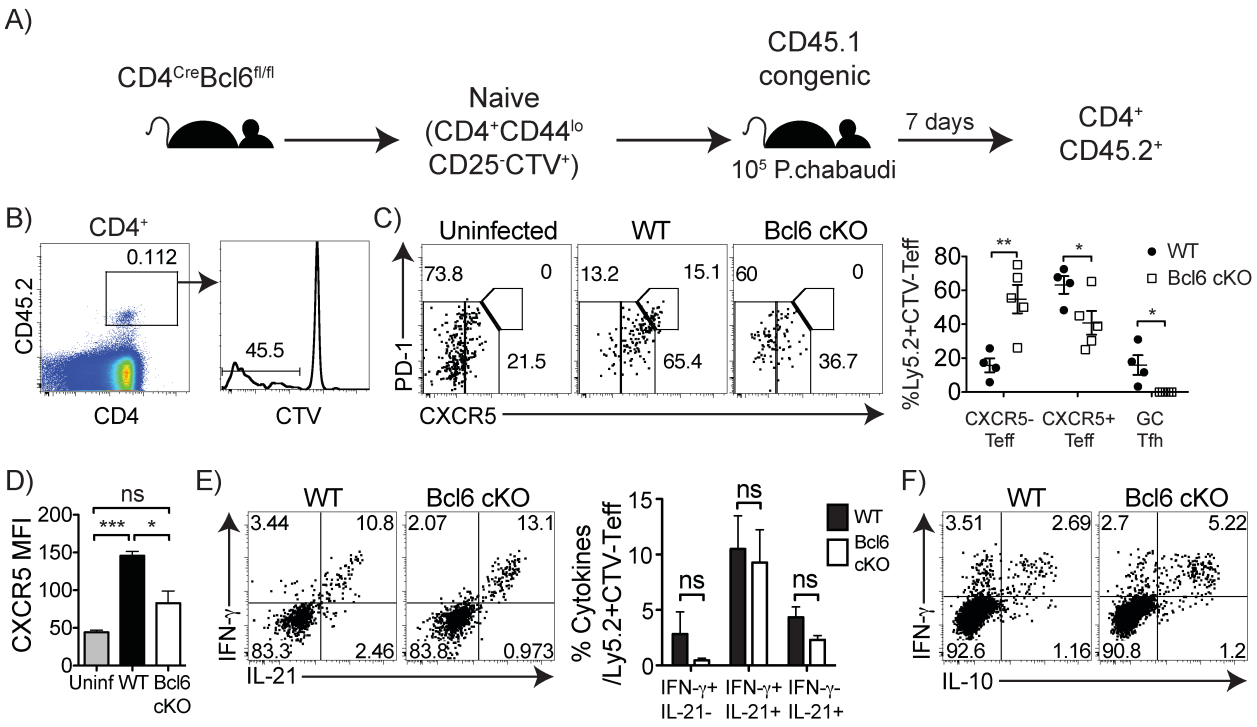

Fig 2.5. Bcl6 controls generation of GC Tfh, but not cytokine profile, in responding effector cells. (A) Naïve ($CD44^{lo}CD25^{-}$) CD4 T cells ($2x10^6$) from either $Bcl6^{fl/fl}$ $CD4^{Cre}$ (Bcl6 TKO) or $Bcl6^{fl/fl}$ (WT) were labeled with cell trace violet (CTV) and adoptively transferred into Ly5.1 (CD45.1) congenic mice, followed by *P. chabaudi* infection. On day 7 post-infection, splenocytes were harvested and stained with (B) CD4, CD45.2, CTV, (C, D) PD-1, CXCR5, (E) IFN-γ, IL-21, and (F) IFN-γ, IL-10. (B) Plots showing the gating on responding $CD4^{+}CD45.2^{+}CTV^{-}$ T cells. (C) Graph shows percentages for individual recipients of effector T cell subsets. No $CD45.2^{+}$ $CXCR5^{hi}PD\text{-}1^{hi}$ GC Tfh cells were detected in any recipient of Bcl6 cKO T cells. (D) Bar graph shows CXCR5 MFI of $CD4^{+}CD45.2^{+}CTV^{-}$ donor cells. (E) Plots and bar graph of average IFN-γ and IL-21 cytokine producers in the responding donor cells ($CD45.2^{+}CTV^{-}$) in recipients of WT and Bcl6 cKO T cells. (F) Dot plot showing intracellular cytokine staining. Data are representative of three independent experiments with 4–5 animals per group. Numbers within plots represent mean percentages. Statistical significance was obtained using Students t-test. Error bars represent the SEM; *$p < 0.05$, ***$p < 0.001$, ns = not significant.

In order to determine if these cells are regulated by IL-10, which downregulates IL-12 (120, 252), we infected IL-10 deficient mice. On day 7 of infection, as expected, effector T cells from IL-10 deficient animals showed an increase in the percentage of $CXCR3^{+}$T-bet^{+} Th1 cells within the IFN-γ^{+} effector T cell gate with no difference in cell numbers (**Fig 2.6A and data not shown**). As IL-12Rβ2 is required for reinforcement of Th1 differentiation (137), we determined

that the proportion and number of IL-12Rβ2^{+}IFN-γ^{+} effector T cells was significantly increased in the IL-10 deficient mice (**Fig 2.6B**). Along with this augmented CXCR3^{+}T-bet^{+}IL-12Rβ2^{+} Th1 phenotype, we observed an increase in the production of IFN-γ single-producers and IFN-γ-IL-21 double-producers, with a significant overall increase in IL-21 production in the IL-10 KO Teff (**Fig 2.6C**). Interestingly, this phenotype was also accompanied by an increase in cells with the CXCR5^{hi}PD-1^{hi} GC Tfh phenotype within the IFN-γ-producing effector T cells, but not within the IFN-γ- subset (**Fig 2.6D**). As infection and splenomegaly is similar in IL- 10 deficient and wild type (WT) animals, cell numbers follow the same trends shown. These results suggest that IL-10 regulates generation of both IL-21^{+}IFN-γ^{+} and IFN-γ^{+} GC Tfh cells. Collectively, these data suggest that IL-21, known to be essential for generation of a protective B cell response in *P. chabaudi*, is highly produced by Th1 cells.

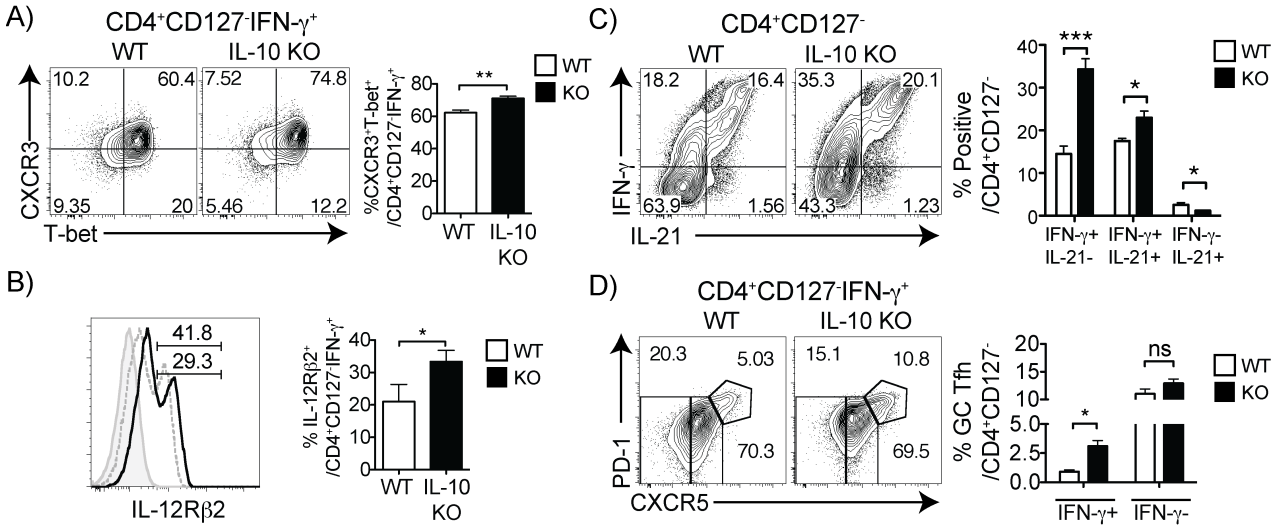

Fig 2.6. IL-10 regulates generation of both IL-21^{+}IFN-γ^{+} and IFN-γ^{+} GC Tfh cells. Wildtype (WT) and IL-10 deficient mice (IL-10 KO) were infected and 7 days later splenocytes were analyzed by intracellular staining. Contour plots and bar graphs from IL-10 KO and WT controls showing expression of (A) CXCR3 and T-bet, and (B) IL-12Rβ2 in the IFN-γ^{+} Teff (CD127^{-}) population; (C) IFN-γ and IL-21 in all Teff, and (D) PD-1 and CXCR5 in the IFN-γ^{+} Teff population. Data are representative of two independent experiments with 3–4 animals per group. Statistical significance from Students t-test. Error bars represent the SEM; *p < 0.05, **p < 0.01, ***p < 0.001), ns = not significant.

T-bet$^+$ IFN-γ$^+$ T cells decay in the effector to memory transition, but are promoted by chronic infection

We have previously shown that the chronic phase of infection with *P. chabaudi* generates memory-phenotype specific CD4 T cells, and maintains excellent protection from re-infection. We have proposed that this improved immunity during chronic infection is mediated by TNF$^+$IFN-γ$^+$IL-2$^-$ Th1 cells, as production of these cytokines depends on the chronic infection (175, 244). Furthermore, both protection and IFN-γ production decay in *P. chabaudi* (82, 141, 177). Although Th1 memory in this infection is not fully understood, it appears unlikely to be fully committed to make IFN-γ upon restimulation, especially without continuous stimulation. Therefore, we investigated markers of Th1 commitment on *Ifng*$^+$ cells later in infection using a different reporter system. In *Ifng/Thy1.1* BAC-In mice, cells with an accessible *Ifng* locus can be identified by prolonged expression of Thy1.1 because of an SV40 intron/polyA tail downstream of the Thy1.1 insert that stabilizes mRNA expression (245). We infected *Ifng/Thy1.1* BAC-In mice with *P. chabaudi*, harvested splenocytes on day 60 p.i, and analyzed expression of Th1 markers in *Ifng/Thy1.1*$^+$ CD4 T cells. We identified *Ifng*$^+$ memory T cells as CD4$^+$ *Ifng/Thy1.1*$^+$ CD44^{hi}CD127$^+$ on day 60 p.i, and observed that they all expressed CXCR3, but only 14% expressed T-bet (**Fig 2.7A**) above the isotype control baseline. The transcription factors Runx3 and Eomesodermin (Eomes) were also undetectable by flow cytometry in the memory Th1 cells (**data not shown**). Interestingly, the Tfh chemokine receptor CXCR5 was expressed on a significant subset of the *Ifng/Thy1.1*$^+$ memory T cells (average of 12%), though Bcl6 was not detectable (**Fig 2.7A**). However, *Ifng*$^+$ CXCR5$^+$ and T-bet$^+$ populations did not overlap significantly. As imaging flow cytometry is more sensitive than conventional flow cytometry, we

analyzed Thy1.1^{+} T cells harvested from BAC-In mice 60 days p.i. by imaging flow cytometry to detect Bcl6 and T-bet (**Fig 2.7B**). Similar to effector T cells, *Ifng/Thy1.1*^{hi} memory cells displayed higher levels of T-bet than *Ifng/Thy1.1*^{lo} cells. On the contrary, while expression of Bcl6 was detectable, it did not change in *Ifng/Thy1.1*^{hi} cells. Representative images of Thy1.1^{hi} and Thy1.1^{lo} cells showing Bcl6 and T-bet expression are shown in Fig 2.7C. Real-time PCR confirmed expression of both Bcl6 and T-bet (*tbx21*) in the memory population (**Fig 2.7D**). Notably, we also detected *prdm1* (Blimp-1) mRNA, suggesting that Bcl6 fails to completely repress its target genes at the memory stage. Interestingly, CD8 effector memory cells also express Blimp-1 [44]. *Eomes* mRNA was slightly decreased in the day 60 cells compared to naïve controls.

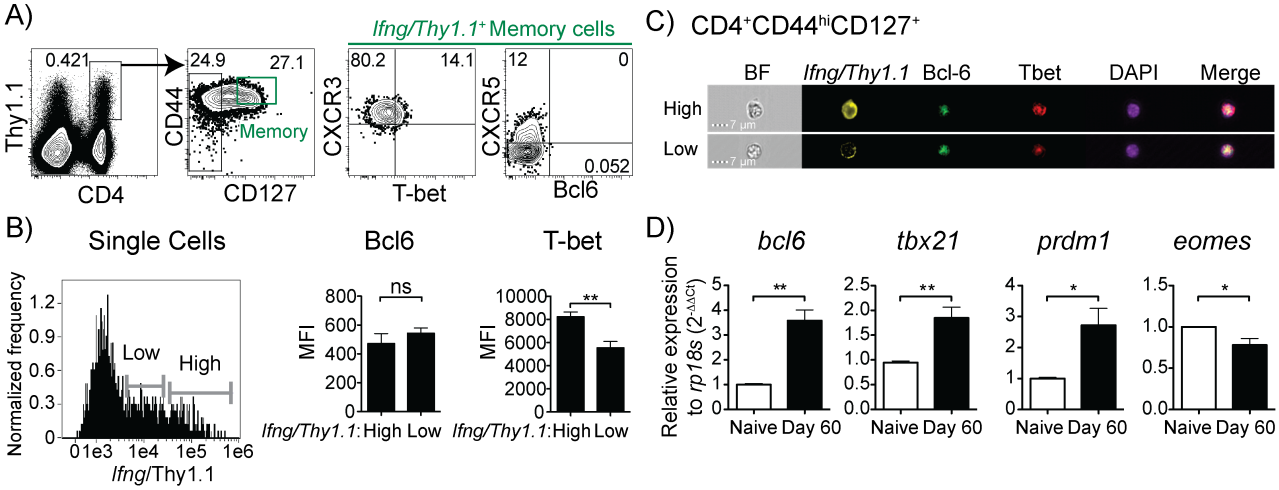

Fig 2.7. IFN-γ^{+} CD4 memory T cells maintain CXCR5 expression, but little T-bet. BAC-In mice were infected and splenocytes were analyzed by flow cytometry on day 60 post-infection. (A) CD4^{+}*Ifng/Thy1.1*$^{+}$ memory (CD44^{hi}CD127^{+}, green box, gate set on CD4^{+}) T cells were gated and expression of CXCR3, T-bet, CXCR5, and Bcl6 was measured. Numbers represent mean percentages. (B, C) Surface staining of CD4^{+} T cells with CD4, CD44, CD127, and Thy1.1 (yellow) was followed by intracellular staining with Bcl-6 (green), T-bet (red), and nuclei with DAPI (purple). Cells were analyzed by imaging flow cytometry. (B) Histogram showing expression of *Ifng/Thy1.1* on single focused cells. Bar graphs show MFI of Bcl6 and T-bet within Thy1.1 gates (C) Representative images of individual *Ifng/Thy1.1* high and low cells showing Bcl6 and T-bet expression and localization in relation to the DAPI stained nucleus. Bright field (BF; left) and DAPI (nucleus)/Bcl6/T-bet merged images also shown (right). (D) Real time PCR analysis of *bcl6*, *tbx21*, *prdm1*, and *eomes* of CD4^{+}Thy1.1^{+} sorted T cells. Results were

normalized to control gene *rp18s*. RNA from FACS Sorted naïve ($CD44^{lo}CD25^{-}$) cells from uninfected, aged-matched BAC-In mice was used as control. Data are representative of three (A) and one (B, C, D) independent experiments with 3–4 animals per group. Statistical significance shown using Students t-test. Error bars represent the SEM;*$p < 0.05$, **$p < 0.01$, ns = not significant.

In order to investigate whether the cumulative T-bet^{lo} memory cells observed in the previous experiment are derived from IFN-γ^{+} effector T cells, we adoptively transferred *Ifng/Thy1.1*$^{+}$ effector T cells (99.7% purity) from *Ifng/Thy1.1* BAC-In animals on day 7 p.i. into infection-matched CD45.1 recipients, as illustrated in Fig 2.8A. At day 60 p.i., we collected recipient splenocytes and analyzed the $CD45.2^{+}$ (formerly $Thy1.1^{+}$) memory cell phenotype by flow cytometry. Surprisingly, we observed that the majority of the cells that were transferred from day 7 infected donors, had downregulated Thy1.1 by day 60 p.i (>94%, **Fig 2.8B**). The loss of *Ifng/Thy1.1* expression suggests a less accessible *Ifng* locus, and was accompanied by a significant decrease in CXCR3 and T-bet expression in Ifng/Thy1.1- cells (**Fig 2.8C**). However, the *Ifng/Thy1.1*$^{+}$ T cells maintained both CXCR3 and T-bet expression, suggesting a stronger Th1 phenotype in this small fraction of the recovered cells. When cells transferred at day 7 were labeled with CTV, we observed that by day 60 p.i. an average of 78% of the transferred cells had divided more than six times. Essentially only these cells included *Ifng/Thy1.1*$^{+}$ cells and contained the highest levels of T-bet (**Fig 2.8D**). Taken together, these findings suggest that Th1 commitment, as defined by T-bet expression, is maintained by division in this infection. Interestingly, after *ex vivo* restimulation we found that a significant fraction of effector cells that survived into the memory phase still co-produced IFN-γ and IL-21 at day 60 p.i. (**Fig 2.8E**). These data suggest that the mixed Th1/Tfh population entered the memory pool, and that maintenance of IFN-γ production and T-bet expression by Th1 cells is linked to further parasite-driven proliferation.

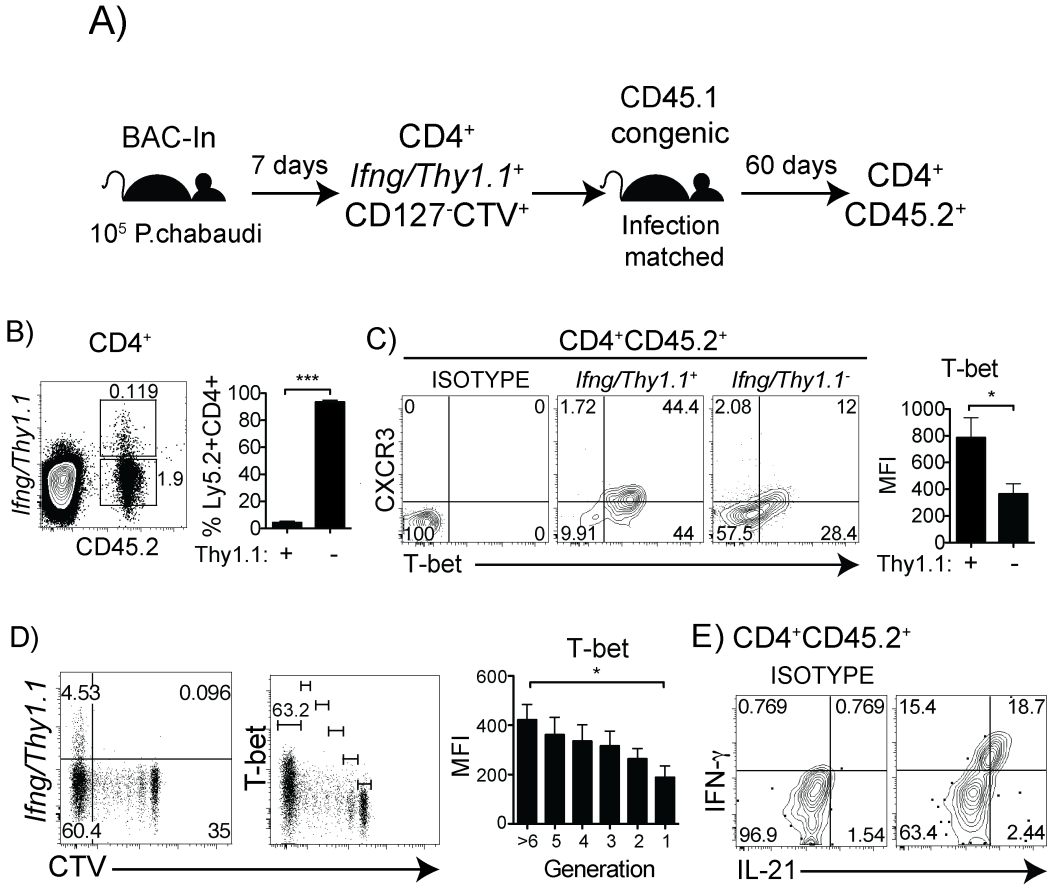

Fig 2.8. IFN-γ$^+$ memory cells co-produce IL-21, and *Ifng*-accessibility and T-bet are maintained by division during chronic malaria infection. (A) BAC-In mice (CD45.2) were infected and at day 7 post-infection, CD4$^+$*Ifng/Thy1.1*$^+$ T cells were purified, CTV labeled, and adoptively transferred into infection-matched CD45.1 recipients. At day 60 post-infection, splenocytes were analyzed by flow cytometry. (B) Dot plot and bar graph shows expression of *Ifng/Thy1.1* in recovered donor cells. (C) Contour plots gated on CD4$^+$CD45.2$^+$ show expression of CXCR3 and T-bet in recovered *Ifng/Thy1.1*$^+$ and *Ifng/Thy1.1*$^-$ populations. Bar graph shows T-bet MFI. (D) *Ifng/Thy1.1* and T-bet within the CTV$^-$ maximally divided cells. Bar graph shows T-bet MFI in each division (E) IFN-γ and IL-21 expression ex vivo in CD4$^+$CD45.2$^+$ donor cells. Data are representative of two independent experiments with 3–4 mice per experiment. Statistical significance shown using Students t-test. Error bars represent SEM; *p < 0.05, ***p < 0.001.

Discussion

The mechanisms of concomitant immunity, which is defined as protection from reinfection during persistent infection, are poorly understood. In acute parasitic infection, fully committed Th1 cells can be generated (162, 253). However, in chronic parasitic infection, T cells in the memory phase require IL-12 for continuous IFN-γ production, which suggests that a lack of intrinsic commitment to IFN-γ production is promoted by chronic antigen stimulation (254-256). Here, in a model of malaria infection that lasts for up to 90 days (244), we studied commitment to IFN-γ production by effector CD4 T cells and their phenotype in the memory phase. The present data demonstrate that during the effector response to *P. chabaudi* infection, responding IFN-γ-producing T cells do not maintain a robust Th1 (T-bet$^+$CXCR3$^+$Runx3$^+$) phenotype. Instead, Teff in this infection consist largely of IFN-γ$^+$ Tfh-like cells that only maintain expression of IFN-γ and T-bet into the memory phase with proliferation. Weak maintenance of the Th1 phenotype into the memory phase was evident as only 6% of adoptively transferred IFN-γ$^+$ Teff maintained *Ifng*-accessibility, T-bet, and CXCR3 expression. Importantly, Bcl6, IL-21, and CXCR5 expression persisted in the IFN-γ$^+$ cells as well. Our data showing a loss of T-bet expression in the absence of proliferation supports evidence that IFN-γ$^+$ T cells decay over time after exposure to malaria, correlating with the documented loss of immunity with time observed in animals (82). This is also reported in humans with reduced parasite-exposure upon emigration from endemic areas (14). Therefore, maintenance of IFN-γ production by Th1 memory cells in malaria depends on antigen or cytokines generated by chronic infection, as in other parasitic infections (177, 254-256). This data highlights the challenge of classifying these protective Th1 cells as effector, long-lived effector, or long-lived

effector memory T cells, despite some evidence supporting each of these conclusions (175, 257-259). Studies of T cell immunity in chronic viral infection suggest that the landscape of the T cell response to chronic infection includes IFN-γ^+IL-21^+ multifunctional cytokine producers and T helper cell phenotype plasticity (211, 213, 260-263). Fahey *et al*. have shown that prolonged TCR stimulation during a persistent viral infection can re-direct Th1 cells towards the Tfh lineage in a TCR-dependent manner (214), however, they did not investigate expression of IL-21 in this context.

Here, we confirm that IL-10 and IL-21 are both produced by IFN-γ^+CXCR5^+ T cells (211). This suggests that the IFN-γ^+IL-10^+ double-producing Teff population previously shown to protect animals from pathology in *P. chabaudi* and *P. yoelli*, are the same population that also enhance the B cell response via IL-21 (82, 211). IL-21 has been shown to be critical for isotype-switched antibody production and parasite clearance in this infection (211). We also show that in addition to CXCR5, IFN-γ^+IL-10^+IL-21^+ T cells express the Tfh markers Bcl6, ICOS, BTLA, and SLAM. However, we have now shown that generation of the majority of IL-21 producers is Bcl6-independent, suggesting that they are not of the Tfh lineage. The IFN-γ^+IL-21^+ cells are also increased in an IL-10 deficient environment, which promotes Th1 development. Interestingly, IFN-γ^+ GC Tfh cells were increased in this context as well. This data suggests that the majority of the Teff in *P. chabaudi* infection are Th1-type cells that express many of the markers of Tfh, and may be similar to those defined in chronic LCMV as "exhausted" due to reduced homeostatic proliferation (213), which is also a feature of effector memory T cells (Tem) (264). Maintenance of Tem has also been shown to depend on ICOS (265). Previous studies demonstrated a phenotypic overlap between Th1 and Tfh cells during *Toxoplasma gondii*

infection, where Tbet expression was required for downregulation of IL-21 and CXCR5 to achieve Th1 commitment (170).

We also observed a separate, very small population of IFN-γ^+ cells that expressed high levels of CXCR5 and PD-1, generated IL-21, and were regulated by Bcl6, thus, confirming their GC Tfh lineage. The B cell response to *P. chabaudi* begins with a strong extrafollicular antibody producing cell response and IgM, followed by a delayed specific IgG response. While IL-21 and CXCR5, widely considered Tfh-related molecules, are both predominantly expressed by IFN-γ^+ cells in this infection, this population is not regulated by Bcl6. Importantly, both types of multifunctional T cell populations (IFN-γ^+CXCR5$^+$ and IFN-γ^+IL-21$^+$) entered the memory pool. For this reason, the memory cell population likely maintains T helper function through the expression of IL-21 and CXCR5. Some surviving cells showed improved maintenance of *Ifng*-locus accessibility, and expressed T-bet, but this correlated with extensive division after the effector phase. Importantly, the majority of memory T cells that maintained IFN-γ expression produced higher levels of IL-21 *ex vivo*, as well.

While there is a predominantly Th1 cytokine profile in CD4 T cells in healthy rural African individuals who are exposed frequently to malaria (266), these T cells also express high levels of IL-10 and IL-21. Interestingly, both cytokines are co-expressed with IFN-γ, though the three together were not tested in the human studies. In children with acute *P. falciparum* infection, increased plasma IL-21 levels correlated with IgG1 and IgG3 antibodies and the development of clinical immunity (267). In *Plasmodium spp*, IL-10 is induced in Th1 cells by IL-27 (82, 183). Moreover, a previous study has shown that IL-21 can also be induced through

this pathway (192), suggesting a mechanism for the generation of these multi-cytokine producers. In other types of chronic diseases, IFN-γ-producing cells are also the major source of IL-21 (240, 268, 269), although, in those studies the cells were also $CXCR5^{+}PD\text{-}1^{hi}$. In a study of chronic LCMV, CD4 T cells had a weak Th1 transcriptional profile compared to those from acute infection. Moreover, none of the other T helper lineages were favored in these T cells, suggesting increased plasticity in the face of chronic infection (213). This chronically stimulated population also made IFN-γ and TNF-α, as well as IL-10 and IL-21. Importantly, in chronic LCMV infection, IL-21 is required to prevent CD8 exhaustion sustaining effector activity (260, 261, 263), however, its effect on chronic CD4 responses is less clear (82, 270, 271).

Bcl6 is considered the lineage-defining transcription factor of the Tfh subset [22]. Bcl6 regulates Th subset differentiation by inhibiting Th1, Th2, Th17, and Treg transcription factors and cytokine genes (272). However, Tfh cells can acquire cytokine profiles and maintain the master regulatory transcription factors of other Th subsets, such as IFN-γ, IL-4, or IL-17, suggesting flexibility in this less committed subset (163). This data suggests a new paradigm where Tfh cells co-express Bcl6 and also another lineage-defining transcription factor so that the function of CD4 T cells avoid terminal differentiation and remain responsive to chronic infection producing cytokines. While $CXCR5^{hi}PD1^{hi}$ GC Tfh cells, which can make IL-21, differentiate in a Bcl6-dependent manner [25], Tfh markers are not solely controlled by Bcl6. For example, IL-21 production by Tfh cells is controlled by STAT3 and c-Maf (273), and c-Maf can also induce CXCR5 expression (238). Furthermore, IFN-γ production by CD4 T cells from Bcl6 TKO mice in response to sheep red blood cells was not affected (241). It is interesting to note that in vitro Th1 differentiation induces IL-21 production without expression of CXCR5 (237). In agreement

with this data, we found that only the IFN-γ^{+}CXCR5^{hi}PD-1^{hi} GC Tfh population was regulated by Bcl6. Therefore, the CXCR5^{+} Tfh-like phenotype found in IFN-γ^{+} cells is likely to be a consequence of Bcl6-inducing cytokines (such as IL-6, IL-21 or IL-27) or B:T cell interactions involving ICOS, which is also highly upregulated on all effector cells in this infection, and is reported to promote Tem survival (265).

The majority of IL-21 was produced by the IFN-γ^{+}CXCR5^{+} T cell subset in accordance with Peréz-Mazliah (211). Infection of IL-10 KO animals results in increased Th1 polarization (120), however, the proportion of the CXCR5^{hi}PD-1^{hi} GC Tfh subset within the IFN-γ^{+} population also increased significantly in these animals. This suggests that IL-10 also regulates the CXCR5^{hi}PD-1^{hi}IFN-γ^{+} GC Tfh phenotype. Additionally, there is precedence for IL-10 regulating CXCR5^{hi}BTLA^{hi} Tfh cells (274).

In summary, memory T cells in this infection show poor maintenance of *Ifng/Thy1.1* expression in the BAC-In reporter animals. This was accompanied by low T-bet expression, and continuous expression of IL-21 and Bcl6. This conclusion was presaged by previous work in this infection that indicated poor Th1 commitment, as measured by IL-4 production and help for malaria-specific antibody by T cells late in infection (198). Interestingly, T-bet is not essential for IFN-γ production in *Plasmodium yoelii* infection, and actually inhibits parasite killing (174, 182). Therefore, we propose that the mixed Th1/Tfh phenotype reported here may actually be a beneficial response. Indeed, plasticity in T cells has been shown to benefit the host in tuberculosis infection (182, 275), and this mechanism is still being investigated. Our data is

relevant for the understanding of protective responses required for development of effective protective vaccines to control malaria pathology.

CHAPTER 3: TRANSCRIPTION FACTOR STAT3 REGULATES DIFFERENTIATION OF T HELPER 1 MEMORY CELLS IN PERSISTENT MOUSE MALARIA INFECTION

Introduction

Of the *Plasmodium* species that are currently used to study malaria infection in laboratory mice, *P. chabaudi* is unique in that it is synchronous, does not have a red blood cell maturation stage preferences, and causes persistent infection, as does *P. falciparum*, which can persist for a year or more (38, 50). An increased ratio of IFN-γ to IL-10 correlates with resistance to reinfection in malaria endemic areas (176). While the initial response to *P. chabaudi* is IFN-γ production, this is quickly downregulated, corresponding with production of antibodies (141). CD4 T helper (Th) cells promote host survival and reduction of the peak of *P. chabaudi* (78, 276). It is thought that IFN-γ^+ Th1 cells promote parasite phagocytosis controlling peak parasitemia (119), followed by IL-21^+ T follicular helper (Tfh) cells required to help B cell responses for complete parasite clearance (234). In acute infections and *in vitro* systems, Th1 and Tfh subsets have been defined independent of each other, however, there is flexibility between both subsets (262). In *P. chabaudi* infection, one hybrid T helper subset produces both, IFN-γ and IL-21 (169, 211). Moreover, these hybrid Th1-like Tfh cells are the main source of the immuno-regulatory cytokine IL-10. A recent study in a malaria endemic area showed that Th1-like Tfh cells preferentially expand during acute malaria infection (224). This hybrid phenotype has been described in other chronic infections as well, and it has been identified as a "dysfunctional" T helper cell (165, 170, 213).

There is a great interest in understanding the differentiation of T helper cells in malaria infection and their role in disease outcome. In acute *P. yoelii* infection, OX-40 agonistic antibody treatment resulted in increased proportions of IFN-γ^{+}T-bet^{+} Th1 cells, which improved early parasite control (208). However, they found no differences in the proportions of Tfh cells or Germinal Center generation. They showed that treatment with recombinant IFN-γ constrained T-cell mediated humoral responses, which resulted in poor parasite control after the peak. Using *P. berghei* ANKA, a lethal malaria mouse model, another group showed that severe inflammatory signals and T-cell-intrinsic T-bet expression inhibit Tfh differentiation and humoral responses (223). They restored Tfh responses by using lethally irradiated parasites (iPbA) or treating mice with anti-IFN-γ together with anti-TNF antibody starting at day 1 post-infection (p.i.). Importantly, drug-cured mice on day 5 p.i, did not have the same phenotype as iPbA-infected mice suggesting that CD4 T cell dysregulation is established earlier during disease. Importantly, T-bet Knock Out (KO) mice infected with *P. berghei* ANKA have dramatically increased parasite burden than WT mice reinforcing the importance of Th1 responses in malaria (174).

The molecular control of Th1 and Tfh cell differentiation involves many transcription factors (262). T-bet is the Th1 master regulatory transcription factor (137), and Th1 cells express CXCR3 and IL-12Rβ2 (277). The Tfh cell lineage-determinant transcription factor is Bcl6 (150, 151). Tfh cells are identified as CXCR5^{high}PD-1^{high} T cells. However, Bcl6 is also expressed in IL-12-driven Th1 cells (170). Bcl6 also has been shown to oppose expression of IFN-γ (170, 222). Another molecule necessary for Tfh development is STAT3 (278). The cytokines IL-12, IL-27, IL-6, and IL-21, signal via STAT3 and can promote Tfh, while inhibiting Th1 differentiation (170, 184, 219, 279). Blimp-1 has been shown to inhibit both Tfh and Th1

differentiation, via Bcl6 and T-bet, respectively (150, 280) and in the context of chronic infection, Blimp-1 also controls IL-10 production by Th1 cells (188, 189). Therefore, we investigated the roles f Bcl6, Blimp-1, and STAT3 in the development of Th1-like Tfh cells during persistent malaria infection.

Persistent infection has been shown to be capable of directing T cells away from the Th1 to the Tfh phenotype (214, 215, 281). It has been shown that increased antigen dose and duration of stimulation directs the divergence of naive cells into Tfh rather than Th1 phenotypes in acute *L. monocytogenes* infection (216). We show that duration of *P. chabaudi* infection beyond 3 days leads to CD4 T cell dysfunction during malaria infection. To investigate the transcriptional regulators of IFN-γ^{+}IL-21^{+} Th1-like Tfh cells during persistent malaria infection, we infected Bcl6 ($Bcl6^{fl/fl}CD4^{cre}$), Blimp-1 ($prdm1^{fl/fl}CD4^{cre}$), and STAT3 ($STAT3^{fl/fl}CD4^{cre}$) T-cell specific Knock Out (TKO) mice and determined the effects on IFN-γ^{+}IL-21^{+} T cells. Bcl6 TKO animals showed a dramatic decrease in generation of $CXCR5^{int}$ T cells, while Blimp-1 TKO had slightly more, with only small effects on the cytokines. Infection of STAT3 TKO mice generated a significantly lower proportion of IFN-γ^{+}IL-21^{+} Teff, and $CXCR5^{int}$ T cells. Differences were seen in primary parasitemia of STAT3 TKO, and they were completely protected from reinfection. Importantly, memory T cells generated in the STAT3 TKO showed a more committed IFN-γ^{+}IL-21^{-}T-bet^{hi} Th1 phenotype that correlated with protection from reinfection. We found that Bcl6, Blimp-1 and STAT3 work in concert to guide the differentiation of these cells with STAT3 as a key player regulating the cytokine plasticity of memory T cells in malaria. Understanding the molecular control of protective T cells against such disease might aid in the rational development of effective vaccines.

Materials and methods

Animals and infections

C57BL/6J (B6), B6.129S1-*Stat3*^{tm1Xyfu}/J (STAT3$^{fl/fl}$), and B6.129-*Prdm1*^{tm1Clme}*/J* (Blimp-1$^{fl/fl}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred to B6.Cg-Tg (CD4-Cre)1Cwi N9 mice from (Taconic Hudson, NY). Bcl6$^{fl/fl}$ x CD4-Cre mice (Indiana University School of Medicine, Indianapolis, IN) were bred at UTMB. Six to twelve weeks old animals of both sexes were used for all the experiments. The floxed allele for STAT3$^{fl/fl}$ mice was genotype by PCR using the primers: forward 5'-TTGACCTGTGCTCCTACAAAAA-3'; reverse 5'-CCCTAGATTAGGCCAGCACA-3'. The floxed allele for Blimp-1$^{fl/fl}$ mice was genotype by PCR using the primers: forward 5'-CAATGCTTGTCTAGTGTC-3'; reverse 5'-AGTAGTTGAATGGGAGC-3'. The floxed allele for Bcl6$^{fl/fl}$ mice was genotype by PCR using the primers for 3' loxP site: forward 5'-TCACCAATCCCAGGTCTCAGTGTG-3'; reverse 5'-CTTTGTCATATTTCTCTGGTTGCT-3'. The *cre* gene was genotype by PCR using the primers: forward 5'-CGATGCAACGAGTGATGAGG-3'; reverse 5'-GCATTGCTGTCACTTGGTCGT-3'. All mice were maintained in our specific pathogen free animal facility with *ad libitum* access to food and water. Mice were infected i.p. with 10^5 (or 10^7 for reinfection) *Plasmodium chabaudi chabaudi* (AS) infected red blood cells (iRBCs), courtesy of Jean Langhorne (Francis Crick Institute, London, UK). Parasites were counted by light microscopy of thin blood smears stained with Giemsa (Sigma, St. Louis, MO). In some experiments, mice were treated with mefloquine hydrochloride (MQ, 4mg/kg body weight, Sigma, St. Louis, MO) by oral gavage daily for 4 days, staring on day 3 post-infection (p.i.). In

some experiments mice were treated three times every other day starting 10 weeks p.i. with 50 mg/kg body weight per animal of Chloroquine (Sigma, St. Louis, MO) in Saline (Sigma).

Animal Care Statement

All animal experiments were carried out in compliance with the protocol specifically approved for this study by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Flow Cytometry

Single-cell suspensions from spleens were made in HEPES buffered Hank's Balanced Salt Solution (Gibco, Lifetechnologies, Grand Island, NY), incubated in red blood cell lysis buffer (eBioscience, San Diego, CA), and stained in PBS + 0.5% BSA + 0.1% sodium azide + 2% Normal Mouse Serum (NMS) and 2% FBS (Sigma, St. Louis, MO) followed by rat anti-mouse purified CXCR5 (BDbioscience, San Jose, CA) for 1 hour at 4°C, followed by 30 min incubation with biotin conjugated AffiniPure Goat anti-rat (H+L) (Jackson Immunoresearch, West Grove, PA) for 30 min at 4°C followed by Streptavidin-eFluor 450, –PE or –Brilliant Violet 650 (BV650), and combinations of FITC–, PE–, PerCP-Cy5.5, PE/ Cyanine 7 (Cy7), Allophycocyanin monoclonal antibodies (all from eBioscience, San Diego, CA), CD127-PE/Cy5, CD44-Brilliant Violet 785 (BV785). For B cell staining we used B220-PE/Cy5, MHC-II(I-A/I-E)-APC, CD38-PE, GL-7-FITC (all from eBioscience, San Diego, CA). For intracellular staining, total cells were stimulated for 2 h with PMA (50 ng/mL), Ionomycin (500 ng/mL), and Brefeldin A (10 μg/mL) in complete Iscove's Media (cIMDM) (all from Sigma), 10% FBS, 2mM L-glutamine, 0.5 mM sodium pyruvate,, 100 U/ml penicillin, 100ug/ml streptomycin, and

50 μM 2-β-Mercaptoethanol (ME) (all from Gibco, Lifetechnologies). Cells were fixed in 2% paraformaldehyde (Sigma), permeabilized using Permeabilization buffer (10X Permeabilization buffer, eBioscience) and incubated for 40 minutes with anti-IFN-γ- Brilliant Violet 605 (BV605, XMG1.2), IL-10-PE (JES5-16E3), T-bet-efluor 660 or -PerCP-Cy5.5 (eBio4B10, eBioscience), Bcl6-Alexa Fluor 488 or –PE (K112-91), and/or Blimp-1-Alexa Fluor 647 (6D3, BDbioscience). For IL-21 staining, cells were incubated for 40 minutes with recombinant mouse IL-21R-Fc chimera (1 μg, R&D systems, Minneapolis, MN) and washed twice in Perm buffer, followed by 30 min with Alexa Fluor 647 goat anti-human IgG $F(ab')_2$ (0.3 μg, Fcγ-specific, Jackson ImmunoResearch, West Grove, PA) in Perm buffer. After three washes in FACS buffer, cells were collected on a LSRII Fortessa in the UTMB Flow Cytometry and Cell Sorting Core Facility using FACSDiva software (BDbiosciences, San Jose, CA) and analyzed in FlowJo version 9.4.11 (TreeStar, Ashland, OR). Compensation was performed in FlowJo using single stained splenocytes (using CD4 in all colors).

ELISAs

Serum samples were obtained on days 7, 14, and 35 p.i. by bleeding mice from the tail vein under a heath lamp. Nunc-Immuno Plates (MaxiSorp™) were coated with whole freeze-thaw parasite lysate (transfer from N_2(l) to 37°C, 4-5 times). Plates were blocked with 2.5% BSA + 5%FCS in PBS. Bound antibody was detected using Alkaline Phosphatase (AP)-conjugated goat anti-mouse IgM (Sigma) and IgG (Oncogene Research Product, La Jolla, CA), which was revealed with a 1 mg/ml 4-Nitrophenyl phosphate disodium salt hexahydrate (PNPP, Sigma) solution. Plates were analyzed with a FLUOstar Omega plate reader (BMG Labtech, Cary, NC).

Statistics

Statistical analysis was performed in Prism (GraphPad, La Jolla, CA) using Student's *t*-test. $p < 0.05$ was accepted as a statistically significant difference, * $p \leq 0.05$, ** $p \leq 0.01$, ** $p \leq 0.001$. For pie comparison, statistical analysis was performed in SPICE software (http://exon.niaid.nih.gov/spice/) version 5.35.

Results

P. chabaudi-specific CD4 effector T cells upregulate Th1- and Tfh-related genes

To determine the functional phenotype of CD4 effector ($CD44^{hi}CD127^{-}$) T cells (Teff) at the peak of infection, we infected C57Bl6/J mice with *P. chabaudi* and determined the expression of Th1 and Tfh markers by flow cytometry at day 7 and 9 p.i. in splenocytes (Fig. 1A,B). CD4 Teff at day 7 and 9 p.i. expressed CXCR5, IL-21 and IFN-γ (Fig. 1A). In accordance with the previously described features of the Teff population in *P. chabaudi* (169, 211) and based in the co-expression of T-bet, IFN-γ, IL-21, CXCR5, and Bcl6, we defined Th1-like, Tfh-like, and Th1-like Tfh cells (Fig. 1B). Boolean gating analysis showed a significant difference between the phenotypical distribution of CD4 Teff at day 7 and 9 p.i. At day 7 p.i, the majority of Teff showed a Th1-like Tfh phenotype (light gray slices in the pie). The proportion of Th1-like cells (black slices in the pie) was more on day 7 than day 9 p.i. The proportion of Tfh-like cells (dark gray slice of the pie) had a discrete increase on day 9 p.i. Moreover, T-bet

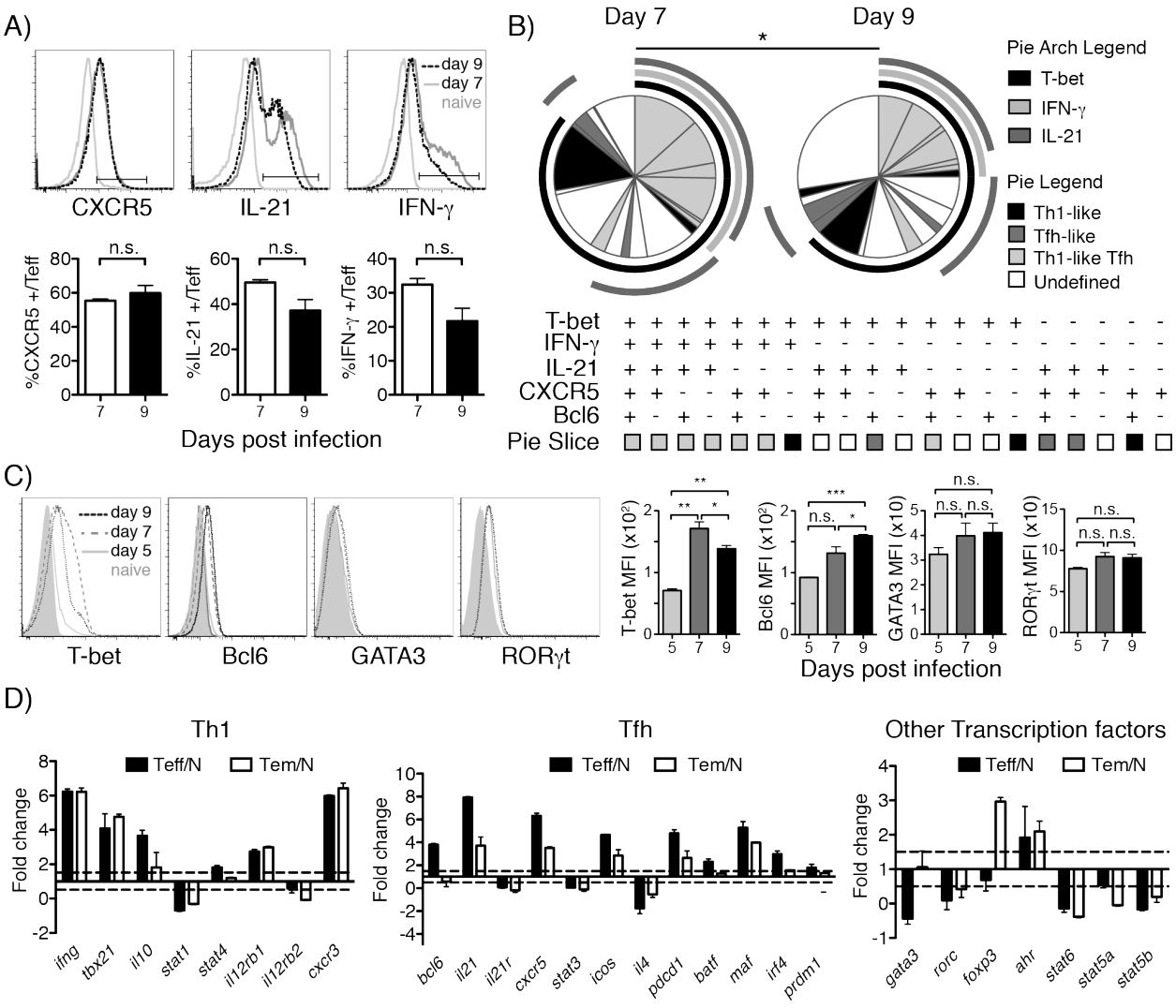

Figure 3.1. T helper gene expression profile during *P. chabaudi* infection is Th1 and Tfh related. A-C) C57BL/6J mice were infected with 10^5 iRBC. Splenocytes were harvested and analyzed by flowcytometry. A) Histograms show CXCR5, IL-21 and IFN-γ expression in effector $CD4^+$ T cells ($CD44^{hi}CD127^-$, Teff) cells at days 7 (gray line) and 9 (black dotted line) p.i, and naive (gray filled line). Bar graphs shows average percentage positive of CXCR5, IL-21 and IFN-γ at days 7 (white bars) and 9 (black bars) p.i. B) Pie graphs showing subsets from Boolean gating analysis of all possible combinations of T-bet, IFN-γ, IL-21, CXCR5, and Bcl6 at days 7 and 9 above (cut off 0.5%). C) Histograms show T-bet, Bcl6, GATA3, and RORγt expression in Teff cells at days 5 (gray line), 7 (gray dotted line), and 9 (black dotted line) p.i, and naive (gray filled line). Bar graphs shows average MFI of T-bet, Bcl6, Gata3 and RORγt at days 5 (light gray), 7 (dark gray), and 9 (black) p.i. D) B5 TCR-Tg mice were infected with 10^5 iRBC and splenocytes were purified into Teff ($CD44^{hi}CD127^-CD62L^-CD27^+$) from day 8 p.i. and effector memory ($CD44^{hi}CD127^-CD62L^-CD27^+$) $CD4^+$ T cells (Tem) from day 60 p.i. and naive T cells ($CD44^{lo}CD25^-$) from uninfected mice and subject to transcriptomic analysis of T helper profiling genes. Relative expression is shown as relative expression of Teff over naive (black bars) or Tem over naive (white bars) of Th1-, Tfh- related genes, and other transcription

factors directly from microarray. Microarray was repeated twice on biological replicates. Statistical significance was obtained using Students *t* test and Pie comparison in SPICE software. Error bar represents SEM; *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant.

expression increased from day 5 to day 7 p.i; however, it was downregulated at day 9 p.i, never reaching T-bet hi levels described in full Th1 differentiation. Bcl6 expression increased from day 5 to day 9 p.i. We found no differences in the expression of the Th2 determining transcription factor, GATA3, or Th17 determining factor, RORγt, at any of the studied timepoints compared to naive ($CD44^{lo}CD25^{-}$) T cells. The Teff and T effector memory (Tem) gene profile (GEO accession number GSE89555) suggests features of genes expressed by both Th1 and Tfh subsets, with only AhR from other subsets, despite a documented lack of IL-17 or IL-22 (Fig. 1D). As previously reported (187), Teff cells also expressed *il10* and *prdm1*, which encodes for Blimp-1 (Fig. 1D). These results indicate that persistent malaria infection promotes Th1 and Tfh differentiation and that these phenotypes are maintained into the memory phase.

Duration of stimulus regulates Th1-like Tfh differentiation

Generation of IFN-γ^{+}IL-21^{+} CD4 T cells has been documented in several persistent infections (170, 213) suggesting that their derivation is due to prolonged stimulation. Therefore, we tested if limiting the duration of stimulation by shortening the period of infection would regulate the generation of Th1-like Tfh cells. *P. chabaudi*-infected animals were treated with the antimalarial drug Mefloquine (MQ) starting at day 3 or 5 p.i. (Fig. 2). Previous studies have determined that MQ itself has no effect on immune responses in mice at this dose (282). We found that MQ treatment day 3, but not day 5, p.i. significantly reduced the proportions of $CXCR5^{+}$ Teff (NTx, 40.2% ± 2.173; +MQ, 22.3% ± 2; 45% reduction, Fig. 2A). Day 3 MQ

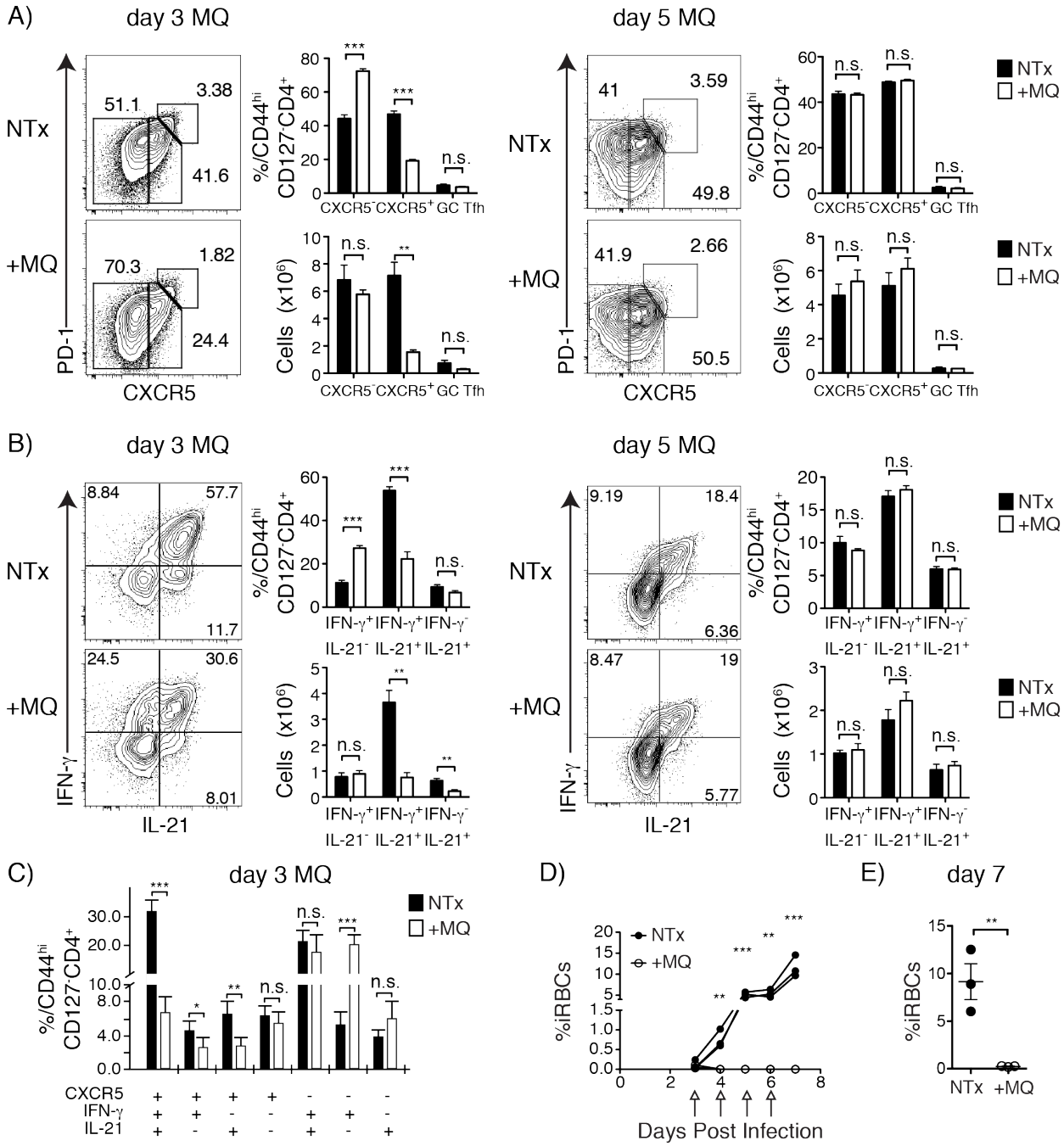

Figure 3.2. Mefloquine treatment starting at day 3 p.i, but not at day 5, results in Th1 Teff. C57BL/6 mice were infected with 10^5 iRBC and one group was given mefloquine (+MQ) by oral gavage on days 3-6 p.i, other group was given MQ on days 5-6 p.i, and other group was left untreated (NTx). Splenocytes were harvested and analyzed by flowcytometry at day 7 p.i. Contour plots show expression of A) PD-1 and CXCR5, and B) IFN-γ and IL-21 in effector ($CD44^{hi}CD127^{-}$) $CD4^{+}$ T cells (Teff). Bar graphs shows percentages out of total Teff and numbers of A) $CXCR5^{-}$, $CXCR5^{+}$, $CXCR5^{hi}PD^{hi}$ (GC Tfh), and B) IFN-γ^{+}IL-21^{-}, IFN-γ^{+}IL-21^{+}, and IFN-γ^{-}IL-21^{+} subsets. C) Bar graph showing percentages of subsets from

Boolean gating analysis of all possible combinations of CXCR5, IFN-γ and IL-21 subsets of NTx (black bars) and +MQ (white bars) groups. D) Parasitemia (%infected RBCs/total RBCs) curve from NTx (black filled circles) and day 3 MQ treated (open circles) groups. Arrows depict treatement days. E) Day 7 p.i. parasitemia of NTx and day 5 MQ treated groups. Data representative of 3 (day 3 treatment) or 1 (day 5 treatment) experiments with two to four animals per group. Statistical significance was obtained using Students *t* test. Error bars represent SEM; *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant.

treatment decreased the overall cytokine production of Teff cells. Moreover, only treatment starting at day 3, but not day 5, p.i. led to a dramatic increase in the percentages of IFN-γ^{+}IL-21^{-} Th1-like and a reduction of IFN-γ^{+}IL-21^{+} Th1-like Tfh cells (Fig. 2B). We used the markers CXCR5, IFN-γ and IL-21 to conduct a more comprehensive phenotypical analysis of Teff at day 7 p.i. (Fig. 2C). The boolean analysis indicated that day 3 MQ treatment reduced the proportions of CXCR5^{+}IL-21^{+}IFN-γ^{+} Th1-like Tfh by 80% (NTx, 32.4% ± 1.760; +MQ, 6.763 ± 0.903) and increased by 270% the proportions of IFN-γ^{+}CXCR5^{-}IL-21^{-} Th1-like cells (NTx, 5.46% ± 0.699; +MQ, 20.38 ± 1.726) compared to continuously infected animals. MQ treatment d3-d7 p.i. resulted in undetectable parasite by day 7 p.i. (Fig. 2D), while MQ treatment d5-d7 p.i. reduced parasitemia to 0.24 ± 0.03% iRBCs at day 7 p.i. (Fig. 2E). These results show that the stimuli driving the hybrid Th1-like Tfh cell phenotype is determined before day 5 of infection. These results support the hypothesis that extended stimulation by infection reduces Th1 cytokine commitment, a phenomenon first identified in chronic LCMV as dysfunction (213).

Bcl6 controls Germinal Center T follicular helper cell formation

To test the role of Bcl6 in the differentiation of Th1-like Tfh cells, we infected Bcl6$^{fl/fl}$CD4^{Cre} (Bcl6 TKO) and Bcl6$^{fl/fl}$ (WT) control animals with *P. chabaudi*. As previously

shown, Bcl6-deficient T cells were unable to developed into PD-1^{hi}CXCR5^{hi} fully differentiated Germinal Center T follicular helper cells (GC Tfh), and the proportion and numbers of CXCR5^{+} Pre-Tfh-like Teff significantly decreased at day 7 p.i. (Fig. 3A) (169, 212). The percentages of IFN-γ^{+}IL-21^{+} Teff did not change significantly; however, the numbers of IFN-γ^{+}IL-21^{+} Th1-like Tfh cells decreased in Bcl6 TKO (29% reduction; WT, 2.21 x10^{6} ± 0.076x10^{6}; Bcl6 TKO, 1.57x10^{6} ± 0.153x10^{6}; Fig. 3B). CXCR$5^{+/int}$ T cells have been shown to be pre-Tfh-like in *P. berghei* ANKA infection, as they can make GC Tfh in IRF4 deficient recipients (223). The proportion of IFN-γ^{+}IL-21^{-} Th1-like cells increased moderately, though the total number remained the same. Bcl6 deficiency resulted in the reduction of IL-21^{+}IFN-γ^{-} Tfh-like cells (45% reduction; WT, 25.2% ± 2.234; Bcl6 TKO, 13.87 ± 1.184; Fig. 3B) and CXCR5^{+}IL-21^{+}IFN-γ^{-} Teff (67% reduction; WT, 19.3% ± 1.57; Bcl6 TKO, 6.373 ± 0.86) without affecting the Th1-like Tfh cell populations (Fig. 3C). No significant differences in parasitemia were observed between Bcl6 TKO and WT animals during the early stage; however, Bcl6 TKO mice were unable to completely eliminate parasite in later stages, as previously reported (Fig. 3D)(212). Previously, we have shown that T-bet is downregulated from day 7 to day 9 p.i, despite the fact day 9 is the peak of IFN-γ^{+} Teff expansion (169). T-bet expression was maintained in Teff from day 7 to day 9 p.i. from Bcl6 TKO animals (Fig. 3E) suggesting that co-expression of Bcl6 and T-bet in Th1like Tfh cells drives the decrease of T-bet. Bcl6 deficient T cells had lower CXCR5 and IL-21 expression, but similar levels of IFN-γ than WT (Fig. 3E). Overall, these results confirm that Bcl6 absolutely controls the development of fully differentiated GC Tfh cells during *P. chabaudi* infection, but only partially regulates development of CXCR$5^{+/int}$ pre-Tfh cells. We showed this previously on adoptive transfer of CD4 T cells from Bcl6 TKO (169).

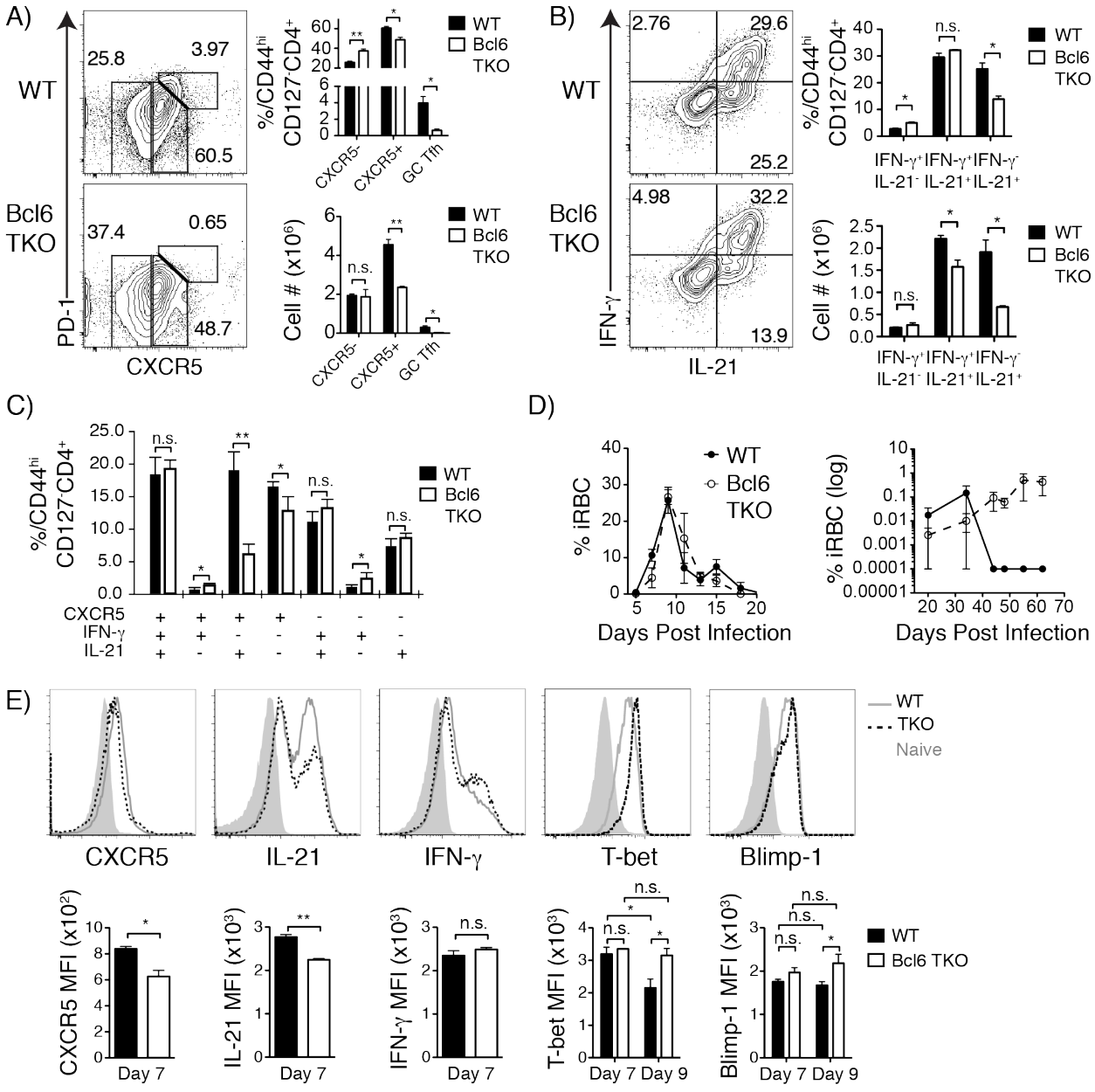

Figure 3.3. Bcl6 deficiency reduces GC Tfh differentiation in persistent malaria infection. $Bcl6^{fl/fl}CD4^{Cre}$ (TKO) and $Bcl6^{fl/fl}$ (WT) animals were infected with 10^5 iRBC. Splenocytes were harvested and analyzed by flowcytometry at day 7 post-infection (p.i.). Contour plots show expression of A) PD-1 and CXCR5, and B) IFN-γ and IL-21 in Teff. Bar graphs shows percentages out of total Teff and cell numbers of A) $CXCR5^-$, $CXCR5^+$, $CXCR5^{hi}PD^{hi}$ (GC Tfh), and B) $IFN\text{-}\gamma^+IL\text{-}21^-$, $IFN\text{-}\gamma^+IL\text{-}21^+$, and $IFN\text{-}\gamma^-IL\text{-}21^+$ subsets. C) Bar graph showing percentages of subsets from Boolean analysis of all possible combinations of CXCR5, IFN-γ and IL-21 within WT (black bars) and Bcl6 TKO (white bars) Teff. D) Parasitemia curve of WT (black filled circles) and Bcl6 TKO (open circles) animals. E) Histograms show expression of CXCR5, IL-21, and IFN-γ from day 7 p.i, and T-bet, and Blimp-1 from day 9 p.i. in Teff cells from WT (gray line), Bcl6 TKO (black dotted line) and naive (gray filled line) T cells. Bar graphs shows

average MFI of show CXCR5, IL-21, IFN-γ, T-bet, and Blimp-1 of WT (black bars) and Bcl6 TKO (white bars). Data representative of 3 experiments with three animals per group. Statistical significance was obtained using Students *t* test. Error bar represents SEM; *$p < 0.05$, ** $p < 0.01$, n.s. = not significant.

However, here we extend our findings showing that infected Bcl6 TKO mice have slightly more IFN-γ^{+}IL-21^{-} Th1-like and less IL-21^{+}IFN-γ^{-} Tfh-like Teff cells. These results show that Bcl6 regulates cytokine production in persistent malaria infection.

Blimp-1 negatively regulates the differentiation of CXCR5 on Th1-like Tfh cells

Blimp-1 is a direct transcriptional repressor of Bcl6, and consequently antagonistic to the GC Tfh program (150). Therefore, we studied the role of Blimp-1 in Th1-like Tfh cell differentiation using *prdm1*$^{fl/fl}$CD4^{Cre} (Blimp-1 TKO) and *prdm1*$^{fl/fl}$ (WT) animals during *P. chabaudi* infection. Blimp-1 deficiency resulted in a modest but significant increase in the percentage of CXCR5^{+} Teff (Fig. 4A,D). The numbers of CXCR5^{-} Teff were decreased in Blimp-1 TKO mice, while the CXCR5^{+} Teff and GC Tfh population remained unchanged. We observed a decrease in the proportions of IFN-γ^{+}IL-21^{-} Teff in Blimp-1 TKO Teff and an increase in the fraction of IFN-γ^{+}IL-21^{+} Teff cells (Fig. 4B). The numbers of these populations followed the same trend; however, they did not reach statistical significance. We conducted the same analysis done in Figure 3C, revealing that the proportion of Th1-like Tfh Teff was significantly increased in Blimp-1 TKO animals (Fig. 4C). As expected, T-bet and Bcl6 expression was increased in Blimp-1 deficient T cells at day 7 p.i. (Fig. 4D). Moreover, Blimp-1 deficient T cells had increased expression levels of CXCR5 and IFN-γ (Fig. 4D). Blimp-1 has been shown to regulate IL-10 production in Th1 cells during chronic LCMV and *T. gondii*

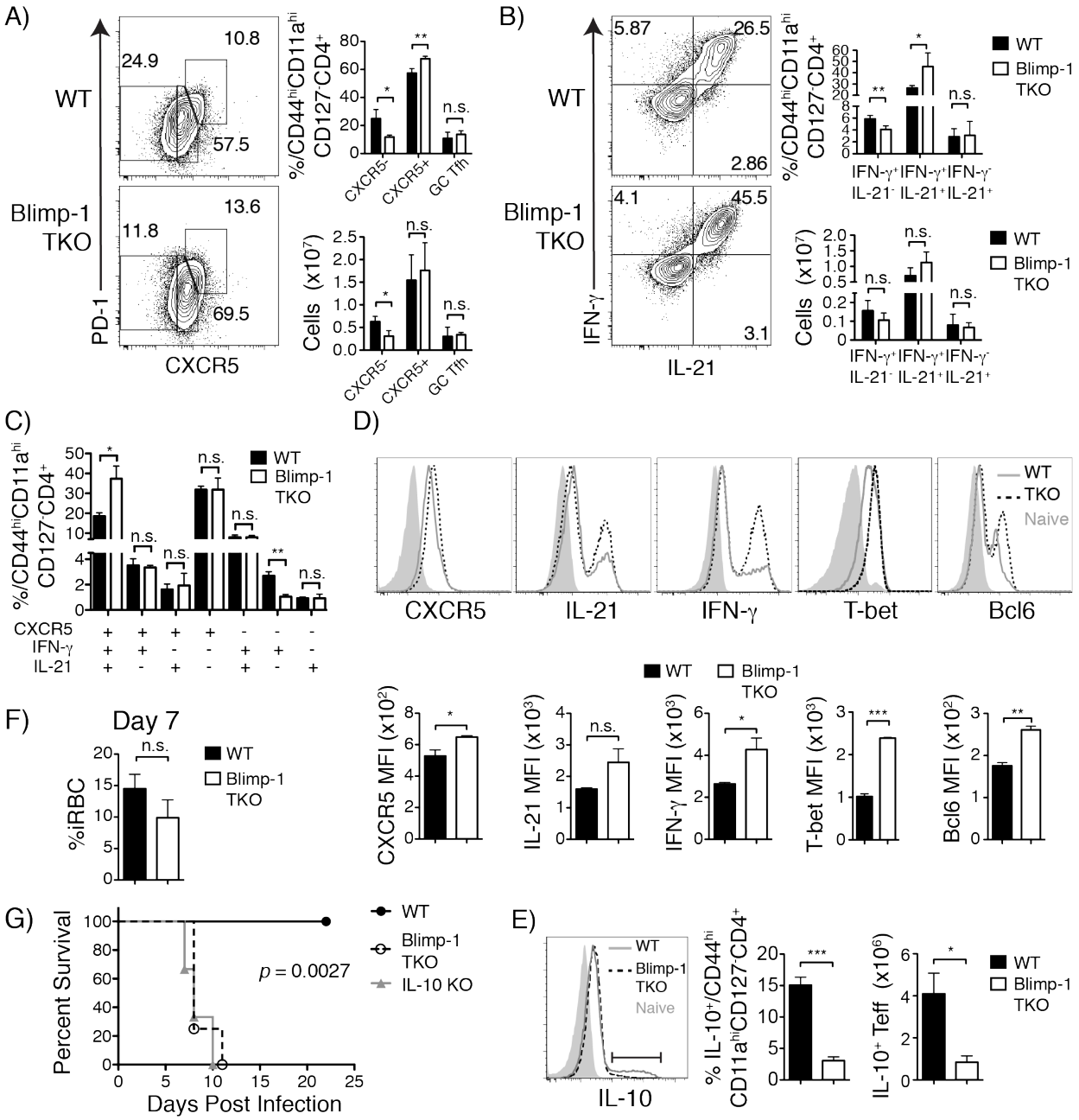

Figure 3.4. Blimp-1 deficient effector T cells preferentially differentiate into Th1-like Tfh cells. Blimp-$1^{fl/fl}CD4^{Cre}$ (TKO) and Blimp-$1^{fl/fl}$ (WT) animals were infected with 10^5 iRBC. Splenocytes were harvested and analyzed by flowcytometry at day 7 post-infection (p.i.). Contour plots show expression of A) PD-1 and CXCR5, and B) IFN-γ and IL-21 in Teff. Bar graphs shows percentages out of total Teff and numbers of A) $CXCR5^-$, $CXCR5^+$, $CXCR5^{hi}PD^{hi}$ (GC Tfh), and B) IFN-γ^+IL-21^-, IFN-γ^+IL-21^+, and IFN-γ^-IL-21^+ subsets. C) Bar graph showing percentages of subsets from Boolean analysis of all possible combinations of CXCR5, IFN-γ and IL-21 within WT (black bars) and Blimp-1 TKO (white bars) Teff. D) Histograms show expression of CXCR5, IL-21, and IFN-γ, T-bet, and Bcl6 from day 7 p.i. in Teff cells from WT (gray line), Blimp-1 TKO (black dotted line) and naive (gray

filled line) T cells. Bar graphs shows average MFI of CXCR5, IL-21, IFN-γ, T-bet, and Blimp-1 of WT (black bars) and Blimp-1 TKO (white bars). E) Histogram showing IL-10 expression by Blimp-1 TKO (dotted line), WT (gray line) Teff, and naive (gray filled line) cells. Bar graphs shows percentages out of total Teff and numbers of IL-10^+ Teff. F) Day 7 p.i. parasitemia of WT (black bars) and Blimp-1 TKO (white bars) animals. G) Survival curve of WT (black circles), Blimp-1 TKO (open circles) and IL-10 KO (filled gray triangles) mice. Data representative of 3 experiments with three to four animals per group. Statistical significance was obtained using Students *t* test. Error bar represents SEM; *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant.

infections (188, 189). IL-10 production by Teff was reduced by 80% in Blimp-1 TKO animals suggesting that IL-10 in the IFN-γ^+IL-21^+ Teff is regulated by Blimp-1 as reported for Th1 cells (Fig. 4E; (189)). We found no differences in parasitemia between Blimp-1 TKO and control animals at day 7 p.i (Fig. 4F). However, Blimp-1 TKO animals succumbed to infection starting at day 8 p.i. similar to IL-10 KO mice (Fig. 4G ;(120)). In summary, Blimp-1 negatively regulates the development of IFN-γ^+IL-21^+CXCR5^+ Teff during persistent malaria infection.

STAT3 T cell-specific deficient mice have reduced generation of Th1-like Tfh cells

STAT3 promotes the GC Tfh phenotype (218, 236, 283) and it is recognized as a critical regulator of differentiation and function of T cells (278). IL-6 signaling through STAT3 has been shown to be important for Bcl6 induction and IL-2Rα downregulation in CD4 T cells during LCMV infection (283). IL-2 signaling through STAT5 induces the expression of Blimp-1 inhibiting Tfh differentiation (284, 285). Therefore, we hypothesized that STAT3 deficiency would result in reduced generation of Th1-like Tfh cells as well. To test this hypothesis, we infected STAT$3^{fl/fl}$CD4^{Cre} (STAT3 TKO) and STAT$3^{fl/fl}$ (WT) animals with *P. chabaudi* and analyzed splenocytes at day 7 p.i. by flowcytometry.

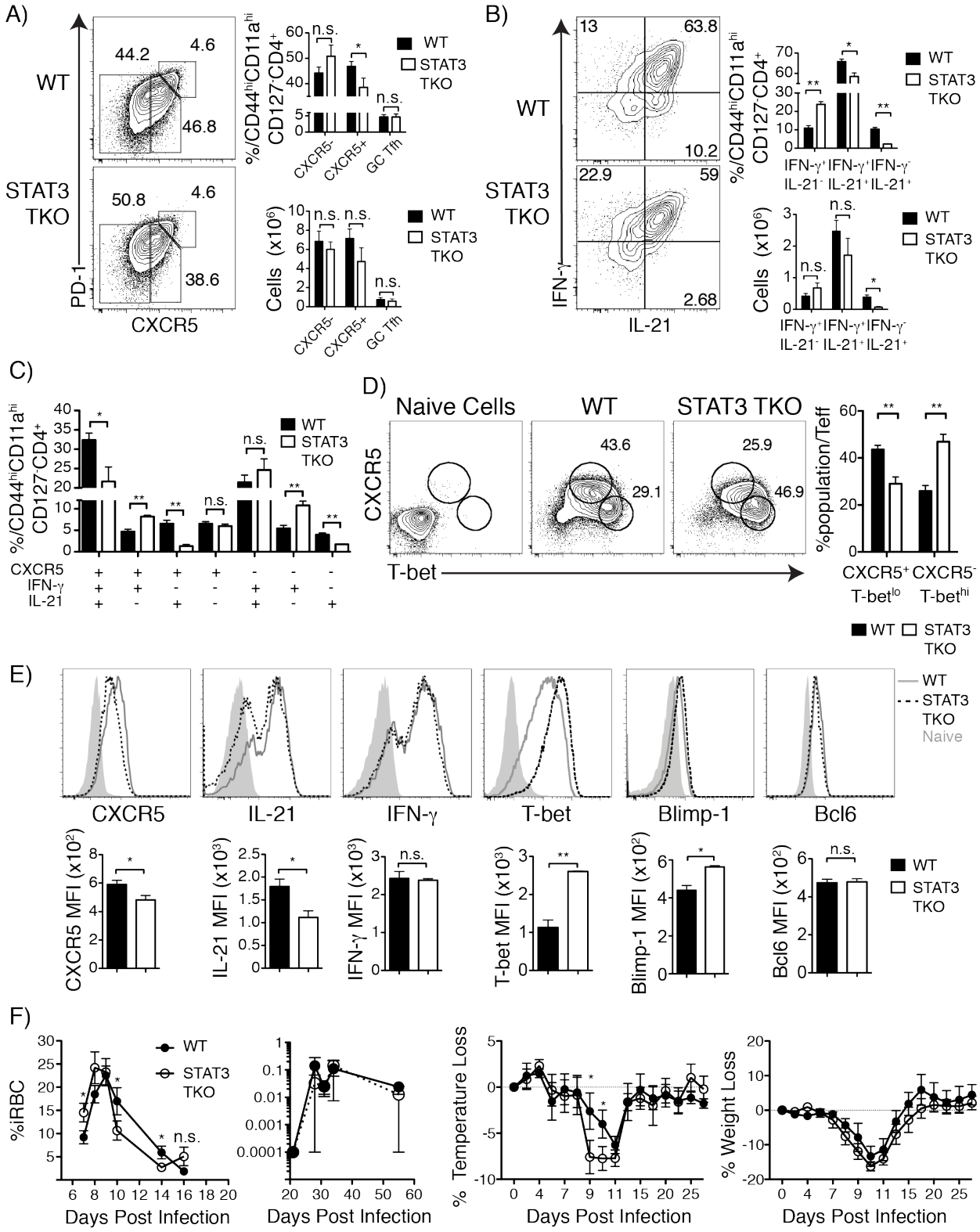

Figure 3.5. STAT3 regulates Th1-like Tfh differentiation. $STAT3^{fl/fl}CD4^{Cre}$ (TKO) and $STAT3^{fl/fl}$ (WT) animals were infected with 10^5 iRBC. Splenocytes were harvested and analyzed by flowcytometry at day 7 p.i. Contour plots show expression of A) PD-1 and CXCR5, and B)

IFN-γ and IL-21 in Teff. Bar graphs shows percentage out of total Teff and numbers of A) $CXCR5^-$, $CXCR5^+$, $CXCR5^{hi}PD^{hi}$ (GC Tfh), and B) IFN-γ^+IL-21^-, IFN-γ^+IL-21^+, and IFN-γ^-IL-21^+ subsets. C) Bar graph showing percentages of subsets from Boolean gating analysis of all possible combinations of CXCR5, IFN-γ, and IL-21 within WT (black bars) and STAT3 TKO (white bars) Teff. D) Contour plots show expression of CXCR5 and T-bet in Teff. Bar graph shows percentages of $CXCR5^+$T-bet^{lo} and $CXCR5^-$T-bet^{hi} subsets in Teff. E) Histograms show expression of CXCR5, IL-21, and IFN-γ, T-bet, Blimp-1 and Bcl6 from day 7 p.i. in Teff cells from WT (gray line), STAT3 TKO (black dotted line) and naive (gray filled line) T cells. Bar graphs shows average MFI of CXCR5, IL-21, and IFN-γ, T-bet, Blimp-1 and Bcl6 of WT (black bars) and STAT3 TKO (white bars). F) Parasitemia curve and Percentage Temperature and Weight loss of WT (black filled dots) and STAT3 TKO (open circles) animals. Data representative of 3 experiments with three to four animals per group. Statistical significance was obtained using Students *t* test. Error bar represents SEM; *$p < 0.05$, ** $p < 0.01$, n.s. = not significant.

STAT3 deficienct T cells showed reduced CXCR5 expression, and the fraction of $CXCR5^+$ T cells compared to WT at day 7 p.i. (Fig. 5A,E). We found no significant differences in proportions of GC Tfh or numbers at this timepoint. STAT3 deficiency also resulted in an increased proportion of IFN-γ^+IL-21^- Th1-like cells, while reducing the percentages of IFN-γ^+IL-21^+ and IFN-γ^-IL-21^+ Teff (Fig. 5B). STAT3 deficiency in T cells significantly decreased the fraction of IFN-γ^-IL-21^+CXCR5^+ Tfh cells (Fig. 5C). Furthermore, the fraction of IFN-γ^+IL-21^+CXCR5^+ Th1-like Tfh cells was decreased, as well. We observed an inverse correlation between T-bet and CXCR5 expression in response to *P. chabaudi*, which was skewed towards T-bet expression in STAT3 TKO mice, as demonstrated by an average increase of 80% (WT, 25.95 ± 2.343; STAT3 TKO, 46.95 ± 3.15) in the $CXCR5^-$T-bet^{hi} population (Fig. 5D). STAT3 deficient Teff cells had more T-bet and Blimp-1 protein at day 7 p.i, but Bcl6 expression was not affected (Fig. 5E). Moreover, STAT3 deficient T cells had less CXCR5 and IL-21, but the levels of IFN-γ expression were the same as WT (Fig. 5E). Parasitemia was better controlled in STAT3 T cell deficient mice than WT animals (Fig. 5F). STAT3 TKO mice had an earlier drop in body temperature, but similar weight loss than WT. We found no differences in parasitemia at the later

stage of infection. The shift towards Th1 in STAT3 deficient animals indicates that STAT3 regulates the differentiation of Th1-like Tfh cells during *P. chabaudi* infection.

Control of Th1-like Tfh cell differentiation by Bcl6, Blimp-1, and STAT3 is stronger in acute infectious stimulation

The functions of Th1 and Tfh determining transcription factors are largely understood from experiments *in vitro* and in acute infection. As the Th1-like Tfh phenotype is promoted by infection longer than 3 days, we tested the effect of deficiency in the transcriptions factors Bcl6, Blimp-1 and STAT3 in T cells during *P. chabaudi* infection, comparing Teff cytokines induced by persistent infection with those from infection shortened by MQ treatment. TKO animals were infected and one group was treated with MQ starting at day 3 p.i. and T cell intracellular cytokines were measured at day 7 p.i. (Fig. 6). In the case of Bcl6 TKO mice, the generation of both IFN-γ^{+}IL-21^{-} Th1-like and hybrid IFN-γ^{+}IL-21^{+} Th1-like Tfh Teff were only significantly increased in the short infection (Fig. 6A). Rather than Bcl6 driving hybrid T cell cytokine production, this suggests that Bcl6 actually acts to limit this phenotype. In the case of Blimp-1 TKO, IFN-γ^{+}IL-21^{-} Th1-like decreased and IFN-γ^{+}IL-21^{+} Th1-like Tfh increased only in persistent *P. chabaudi* infection (Fig. 6B). In STAT3 TKO animals, the biggest change was that IFN-γ^{+}IL-21^{-} Th1-like cells were increased compared to WT (Fig. 6C). This effect was less in Teff from infected and treated compared to untreated mice. There was a significant decrease in IFN-γ^{+}IL-21^{+} Th1-like Tfh in persistent and acute infection in STAT3 TKO compared to WT animals, suggesting that STAT3 regulates Th1-like Tfh in *P. chabaudi* infection.

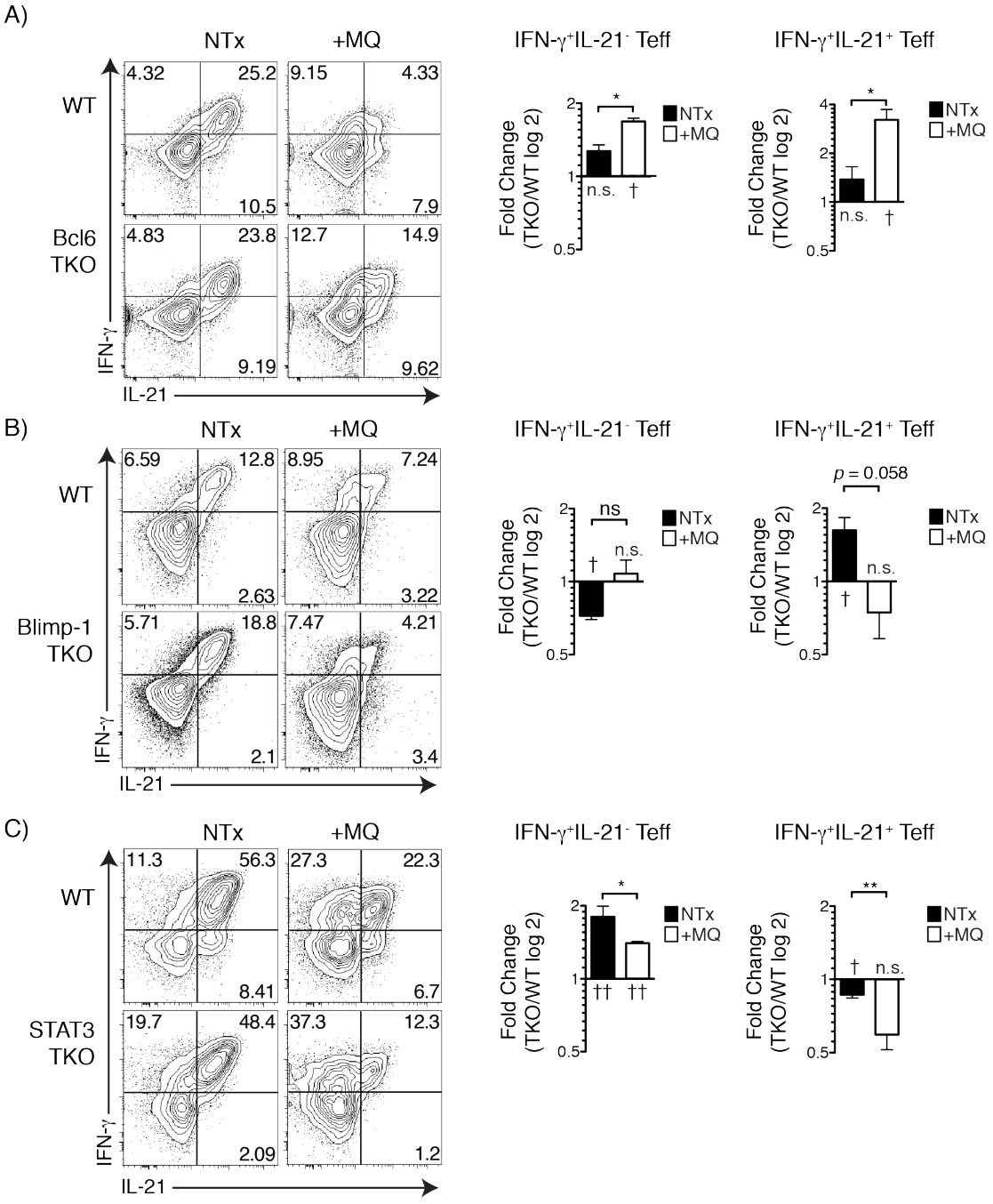

Figure 3.6. Bcl6, Blimp-1, and STAT3 have different regulatory effects on Teff during Short stimulus duration. TKO and WT animals were infected with 10^5 iRBC. One group was given mefloquine (+MQ) by oral gavage on days 3-6 p.i. and other group was left untreated (NTx). Splenocytes were harvested and analyzed by flowcytometry at day 7 p.i. Contour plots show expression of IFN-γ and IL-21 in Teff. Bar graphs shows average of fold change (%TKO/%WT) in a log2 scale of percentages of IFN-γ^+IL-21$^-$ and IFN-γ^+IL-21$^+$ Teff from NTx (black bars) and +MQ (white bars) from A) Bcl6 TKO, B) Blimp-1 TKO, and C) STAT3 TKO mice. Data representative of 1 (C) and 2 (A, B) experiments with two to three animals per group. Statistical significance was obtained using Students *t* test. Error bar represents SEM; *$p < 0.05$,

** $p < 0.01$, n.s. = not significant. † $p < 0.05$, †† $p < 0.01$ are statistical comparison of WT and TKO animals within NTx and +MQ groups.

The decrease in percentage of IFN-γ^+IL-21^+ Th1-like Tfh cells during MQ treatment in STAT3 deficient T cells was significantly more than in persistent infection. This could be due to the role of TGF-β (286). Together, these results suggest that the role of Bcl6, Blimp-1, and STAT3 on regulation of IFN-γ and IL-21 expression is different depending on the duration of stimuli.

STAT3 TKO mice have more Th1 memory cells and are protected from reinfection

Giving the strong increase of Th1-like cells in STAT3 TKO mice, we re-infected STAT3 TKO animals to test for protection. As mice in these experiments did not clear infection, they were treated with the anti-malarial Chloroquine (CQ) at 10 weeks p.i, as shown in experimental schematic Fig. 7A. Previous reports have demonstrated that CQ treatment is effective in eliminating low levels of *P. chabaudi* as in chronic infection (287). STAT3 TKO mice controlled secondary infection dramatically with low to not detectable iRBCs as early as day 3 post re-infection (p.r, Fig. 7B). WT mice showed significantly higher parasitemia that peaked around day 4 p.r. but was controlled by day 7 p.r. The relative concentration of *P. chabaudi*-specific IgM was not affected in STAT3 TKO; however, IgG was significantly less at day 35 p.i. in STAT3 TKO compared to WT (Fig. 7C). In addition, GC B cells ($B220^+$MHC-$II^{hi}CD38^{int}$GL-7^{hi}) at days 20 and 55 p.i. were quantitated by flow cytometry. The proportions of GC B cells were significantly reduced in STAT3 TKO mice at both timepoints (Fig. 7D, data not shown).

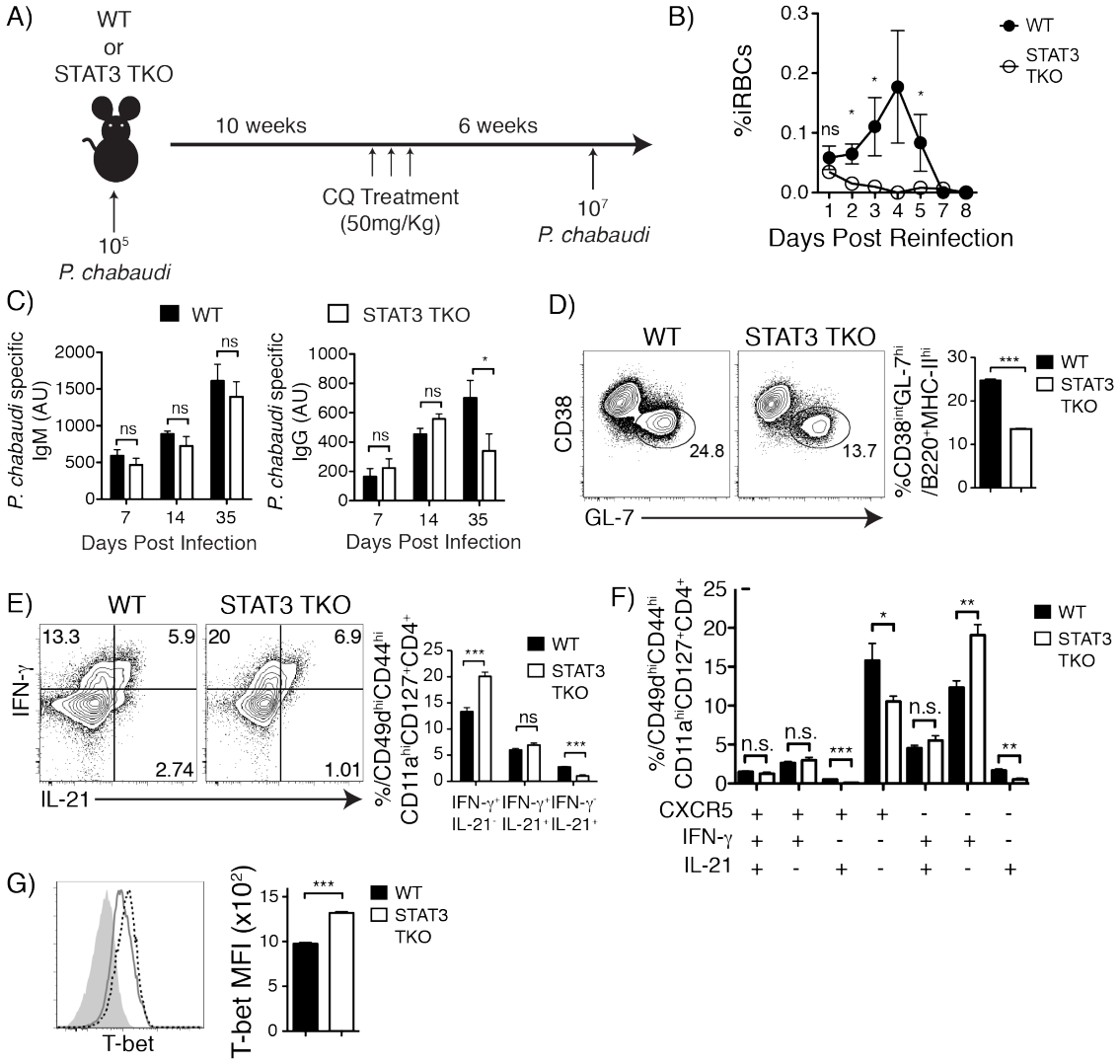

Figure 3.7. STAT3 deficient T cells preferentially differentiate into Th1 memory cells in persistent malaria infection and STAT3 TKO mice are protected from reinfection. STAT3 TKO and WT animals were infected with 10^5 iRBC. Splenocytes were harvested and analyzed by flowcytometry at day 55 p.i. A) Scheme describing the experimental approach for reinfection experiment. B) Parasitemia curve from WT (black filled circles) and STAT3 TKO (open circles) animals after reinfection. C) Bar graphs showing IgM (left graph) and IgG (right graph) antibodies specific for a lysate of *P. chabaudi*-infected red blood cells determined by ELISA. Antibody units (AU) were calculated based on the *P. chabaudi*-specific antibody levels of a hyper-immune standard plasma defined as 1000U. D) Contour plots show expression of CD38 and GL-7 in activated B cells ($B220^+MHC\text{-}II^{hi}$) at day 20 p.i. in WT and STAT3 TKO mice. Bar graph shows percentages of Germinal Center B cells ($CD38^{int}GL\text{-}7^{hi}$) from WT (black bars) and STAT3 TKO (white bars) at days 20 p.i. E) Contour plots show expression of IFN-γ and IL-21 in memory ($CD49d^{hi}CD44^{hi}CD11a^{hi}CD127^+$) $CD4^+$ T cells (Tmem). Bar graphs shows percentages out of total Tmem of IFN-γ^+IL-21^-, IFN-γ^+IL-21^+, and IFN-γ^-IL-21^+ subsets from WT (black bars) and STAT3 TKO (white bars) mice. F) Bar graph showing percentages of subsets from Boolean gating analysis of all possible combinations of CXCR5, IFN-γ, and IL-21

in WT (black bars) and STAT3 TKO (white bars) Tmem. G) Histogram showing T-bet expression in Tmem from WT (dotted black line) and STAT3 TKO (gray line) animals, and naive ($CD49d^{lo}CD44^{lo}CD11a^{lo}CD127^{+}$, gray filled line) cells at day 55 p.i. Bar graphs shows average MFI of T-bet in Tmem from WT (black bars) and STAT3 TKO (white bars) mice at day 55 p.i. Data representative of 1 experiment with four to five animals per group. Data in B is 2 combined experiments with 7-8 mice per group. Statistical significance was obtained using Students *t* test. Error bar represents SEM; *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant.

To determine if the changes caused by STAT3 deficiency in T cells observed at the peak of infection were maintained into the memory phase, we recovered antigen-experienced memory T cells (Tmem, $CD49d^{hi}CD11a^{hi}CD44^{hi}CD127^{hi}$) from infected STAT3 TKO and WT animals at day 55 p.i. CD11a and CD49d have been reported to be co-expressed on T cells in a TCR-stimulation dependent manner, and retained by memory T ells (288). Indeed, STAT3 deficient Tmem had higher fraction of IFN-γ^{+}IL-21^{-} Th1-like cells (Fig. 7E). Boolean analysis revealed that STAT3 TKO mice generated significantly more $CXCR5^{-}$IL-21^{-}IFN-γ^{+} Th1 memory cells (Fig. 7F). While there were some $CXCR5^{+}$IFN-γ^{+} T cells, the proportion of $CXCR5^{+}$IFN-γ^{+}IL-21^{+} Th1-like Tfh cells was low at day 55 p.i. and we did not find significant differences between the TKO vs WT animals. Supporting increased Th1 cell survival, T-bet expression was higher in STAT3 deficient Tmem (Fig. 7G). These data suggest that STAT3 regulates the differentiation of Th1 memory cells in persistent malaria infection and that STAT3 deficiency in T cells confers protection from reinfection independently of malaria-specific antibody.

Discussion

The classical view of T helper differentiation relies on the idea of “master regulators” controlling stable T helper programs. However, there is plasticity in CD4 T cell differentiation

(158, 277, 289) particularly during persistent infection (213). Prolonged antigenic stimulation directly affects T cell differentiation (281). Acute LCMV and Listeria infections induce a stable Th1 memory phenotype (ref-Lm, SS Way, ref for LCMV). However, chronic LCMV, Leishmania, and *T. gondii* infections skewed the T cell responses away from T-bet expression and Th1 cytokines, and favor the expression of GATA3 and Bcl6 along with markers of other T helper subsets (165, 170, 213). Uninterrupted TCR stimulation during Th1 differentiation *in vitro* leads to inhibition of IL-12Rβ2 and T-bet expression, compared to termination of antigen signaling, resulting in inhibition of Th1 commitment (215). Long antigen stimulation also promotes IL-10 production from Th1 cells (290). CD4 T cell responses in adults from malaria endemic areas have a phenotype skewed to Th1, with high proportions of IFN-γ, IL-10, and IL-21 CD4 T cell producers (266). In chronic LCMV infection, IL-21 production by CD4 T cells is essential to ensure a CD8 T cell effector activity and survival (260). Unfortunately, neither of these studies addressed the generation of IFN-γ^{+}IL-10^{+}IL-21^{+} triple producing CD4 T cells. *P. chabaudi* infection lasting more than three days drives differentiation of Th1-like Tfh cells over committed Th1 (Fig. 2), and this is controlled by continuous stimulation, as treatment of infection promoted more IFN-γ^{+}IL-21^{-} Th1-like cells.

Importantly, many of the effects on Tfh differentiation previously reported in Bcl6, Blimp-1, and STAT3 TKO mice, were not observed during persistent infection. Our results suggest that Bcl6 drives hybrid T cell cytokine production in acute conditions, which supports reports of Bcl6 inhibition of T-bet, inhibiting IFN-γ expression *in vitro* (221, 222), and in fact, T-bet protein was increased in Bcl6 TKO Teff (Fig. 3E). This data supports the report that Bcl6 and IL-21 expression in *in vitro* Th1 differentiation is transient, due to increasing T-bet; however, it

suggests that the IFN-γ^+IL-21^+ Th1-like Tfh in this persistent infection may be regulated in another way. However, T-bet expression is increased in all of theses transcription factor KO T cells, suggesting that T-bet expression does not strongly regulate production of IFN-γ in this infection. This is supported by studies in *Tbx21* deficient animals where levels of IFN-γ are not dramatically changed in *P. yoelii* infected T-bet KO (182). Blimp-1 deficiency increased the proportions of IFN-γ^+IL-21^+ Th1-like Tfh only in persistent stimulation (Fig. 6B), suggesting an inhibitory effect of Blimp-1 on Th1-like Tfh phenotype in persistent stimulation. This effect may be independent of its inhibition of Bcl6, as it is not opposite to the effects seen in T cells from Bcl6 deficient animals. Blimp-1 and STAT3 deficiency did not regulate the fraction of GC Tfh dramatically, as previously shown (150, 219), however this may be due to the delayed appearance of GCs in this infection. STAT3 deficiency also did not decrease Bcl6 expression as seen in other systems (219), though Blimp-1 deficiency did. STAT3 can promote Bcl6 expression in cooperation with Ikaros family transcription factors (291), or inhibit Bcl6 (292); however STAT3 TKO did not have different levels of Bcl6 in this infection (Fig. 5E).

There is a close relationship between the Tfh and Th1 programs (262), partly because Bcl6 can modulate Th1 differentiation by direct interaction with T-bet, leading to Bcl6-mediated repressor activity on T-bet-driven genes, such as IFN-γ (221, 222). Bcl6 deficiency reduces the unusual $CXCR5^+$ Pre-Tfh-like subset by 50%, as observed on transfer of these cells (169), suggesting that the requirement is intrinsic to the T cells. We have shown that Bcl6 and Blimp-1 coordinately regulate the expression of CXCR5, while only Bcl6 regulates GC Tfh production in persistent malaria infection. The effects observed inthe absence of Blimp-1 most likely are not dependent on IL-10 signaling, as IL-10 KO mice greatly favored IFN-γ^+IL-21^- Th1-like cells

(169), while Blimp-1 TKO mice had the opposite effect promoting IFN-γ^{+}IL-21^{+} Th1-like Tfh cells (Fig. 4C).

IL-10^{+} Th1 cells are a hallmark of persistent infections (82, 166) and Blimp-1 has been identified as a major controller of this phenotype (165, 188). CD4 T cells from children living in areas with high malaria transmission co-produce IFN-γ and IL-10, and these cells are positive for T-bet and Blimp-1 while IFN-γ^{+}IL-10^{-} T cells are not (186). In *P. chabaudi* infection, IL-27 promotes this IFN-γ^{+}IL-10^{+} phenotype (82). Our results confirm that Blimp-1 controls IL-10 production in Teff, as in *P. yoelii* (187), suggesting a model where IL-27 and Blimp-1 work in concert to control the generation of IL-10^{+} Th1 cells. As IL-21 is produced by the same IFN-γ^{hi} T cells that make IL-10, it is intriguing that the mechanisms that regulate IL-21 expression are so different. This is particular so given that the main functions of IL-21 are in regulation of B cell function, which IL-10 also does (ref). However, the role of IL-10 in malaria pathology regulation is more critical (120). It is not known if there is a role of IL-21 in promoting CD4 T cell responsiveness in chronic infection, as in CD8 (260, 261, 263).

The balance of Th1 and Tfh programs is controlled by multiple overlapping cytokine programs, such as the lvels of IL-2 and IL-12 or IL-27 present during priming (262). Many cytokines that regulate T cell differentiation during malaria infection signal through STAT3, including IL-6, IL-10, and IL-27 (183, 196, 278). STAT3 has been shown to be required for the differentiation of Tfh cells during acute viral infection in mice (219). Moreover, humans with STAT3-deficient mutations have reduced Tfh cell generation (293). We have not determined the cytokine(s) responsible for the STAT3 phenotype observed here, as inhibition of any of these

three cytokines did not change T cell cytokine production detectably (data not shown). STAT3 deficiency reduced the proportion of $CXCR5^{+}IFN\text{-}\gamma^{+}IL\text{-}21^{+}$ Th1-like Tfh and increased $IFN\text{-}\gamma^{+}IL\text{-}21^{-}$ Th1-like cells suggesting it plays a regulatory role inhibiting Th1 differentiation. This effect was maintained into the memory phase, resulting in STAT3 deficient T cells developing preferentially into $IFN\text{-}\gamma^{+}IL\text{-}21^{-}$ Th1 memory cells (Fig. 7 F, G). STAT3 TKO animals showed earlier development of pathology than WT mice (Fig. 5F), the major cytokines regulating T cell induced pathology in *P. chabaudi* are IL-10 and TGF-β (121). We did not see any change in IL-10 production in STAT3 TKO mice (data not shown), suggesting that T cell intrinsic STAT3 is not what regulates IL-10 production, at least inchronic infection. Similarly to Bcl6 and Blimp-1, STAT3 deficiency resulted in increased T-bet expression in T cells, which suggests overlapping mechanisms for tight control of T-bet during chronic infection. The most compelling result of our studies is that re-directing T cell differentiation from a Th1-like Tfh to a more conventional Th1 phenotype was sufficient to improve protection from reinfection (Fig. 7B). This data suggests that the hybrid Th1-like Tfh phenotype, of the majority of the Teff generated in response to *P. chabaudi*, but not *P. yoelii,* is maladaptive, though further studies will be required to establish their functionality in controlling parasitemia, pathology and B cell help. More studies need to be performed to fully understand the full molecular network governing the differentiation of CD4 T cells during persistent infections.

In conclusion, we have shown that the persistence of *P. chabaudi* induces Th1-like Tfh cells, and that multiple transcription factors working in concert regulate various aspects of thisphenotype. The most striking is that STAT3, as T cell-specific STAT3 deletion, promotes the differentiation of Th1 memory cells. T cell-specific STAT3 deficiency clearly resulted in less

malaria-specific antibodies, and better protection from reinfection, suggesting that stable production of IFN-γ from T cells reduces parasitemia quickly upon reinfection. The crucial role of IFN-γ in controlling parasitemia is consistent with studies of human malaria, as well as earlier studies of animal models (119, 176, 177). In addition, vaccine strategies that induce both antibodies and IFN-γ from T cells promote protection from re-infection (10, 294). It is important to note that while antibodies are required for parasite clearance, protection from reinfection to malaria in mice and human can be antibody-independent (294, 295). These results critically identify a unique role for transcription factors acting in concert to regulate Th subset differentiation in response to persistent infection, which is different from that in acute stimulation. A better understanding of the molecular differentiation of the immune response to persistent stimuli is critical to development of improved vaccination strategies for prolonged infections.

CHAPTER 4: SUMMARY AND DISCUSSION

The previous chapters (2 and 3) have been presented as independent manuscripts. The results and conclusion for each chapter are presented within each chapter. In this section, I will present a broader view of the results and how they complement and integrate with each other. Moreover, I will present some unpublished data that hopefully will help to expand the impact of the previous results and increase the relevance of these findings in the context of malaria infection. Finally, I will present future research directions that can rest on the results presented in the current thesis.

THE CD4 TEFF RESPONSE TO *P. CHABAUDI* INFECTION

Previously in *P. chabaudi* infection it has been described a noticeable shift in the CD4 T cell response from a Th1 to a Th2 response, which coincides with the concept of antibody production requirement for complete parasite clearance (141). In *P. yoelii* and *P. berghei* ANKA mouse malaria models, CD4 T cells co-express the Th1 and Tfh chemokine receptors CXCR3 and CXCR5, respectively (296). In *P. chabaudi* and *P. berghei* ANKA models, CD4 T cells co-express the Th1 and Tfh lineage-determining transcription factors T-bet and Bcl6 (137, 150, 151, 169, 223). Therefore, this hybrid phenotype could be defined by IFN-γ and IL-21 co-production, CXCR3 and CXCR5 co-expression, and/or T-bet and Bcl6 co-expression. Early during the acute phase, we find CD4 T cells that express many different levels and combinations of Th1 and Tfh markers (**Fig 3.1**). The production of IFN-γ is not restricted to Th1 cells (210). The epigenetic control of the *Ifng* locus involves many *cis* elements that allow for other Th subsets, besides Th1, to acquire IFN-γ production when needed (297). We used multicolor flow cytometry and

Boolean gating analysis to compare the overall phenotype of CD4 T cells responses during the acute phase (day 7 p.i.) of *P. chabaudi* versus acute LCMV Arm infection (**Fig. 4.1**). We observed a very different response to *P. chabaudi* characterized by CXCR5^{+}IFN-γ^{+}IL-21^{+} Teff. This population was completely absent in acute LCMV infection. We showed in chapter 2 that the population of T-bet positive cells is decreased from day 7 to day 9 (**Fig 2.2**), suggesting a control over Th1 differentiation as parasite growth reaches its maximum. This idea of T-bet control, as a control of Th1 responses, could be an immuno-regulatory mechanism to limit Th1-mediated pathology during malaria infection. Another possibility is that the parasite dampens the Th1 response as a mechanism to ensure persistence. Indeed, studies have demonstrated that *Plasmodium spp.* express an ortholog of macrophage migration inhibitory factor (PMIF) that can interfere with the development of CD4 T cell responses (298, 299). More studies need to be done to determine if the parasite produces effector molecules that directly shape CD4 T cell development.

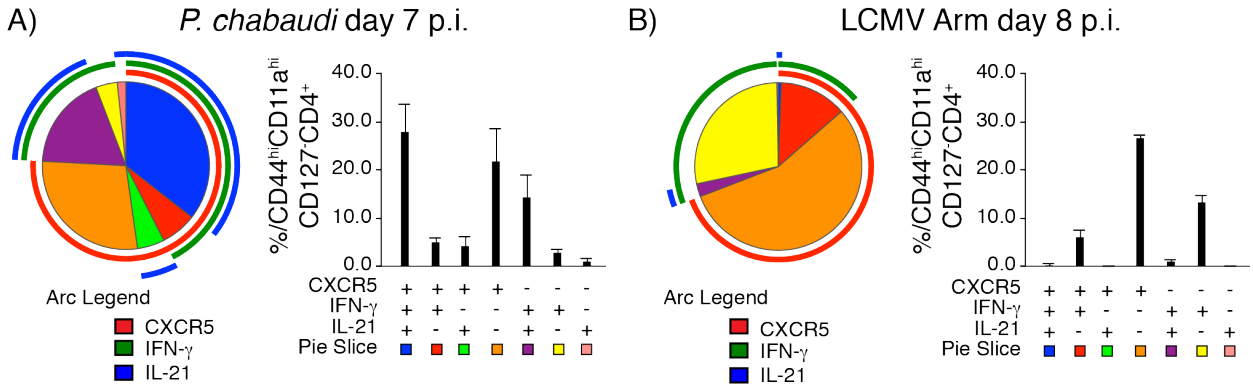

Fig 4.1. *P chabaudi* induces a characterist CXCR5^{+}IFN-γ^{+}IL-21^{+} T CD4 Teff response. C57Bl/6J mice were infected with 10^5 iRBCs or $2x10^5$ LCMV Arm PFU i.p. A) At day 7 and B) 8 p.i. we recovered splenocytes and performed a multicolor flow cytometric analysis to determine the phenotype of CD4 Teff responses. CD4 Teff from *P. chabaudi* infection preferentially differentiated into CXCR5^{+}IFN-γ^{+}IL-21^{+} Th1-like Tfh cells, while this population was not present in LCMV infection. Data is representative of one experiment with 3 animals per group.

The impact of Th1 responses in *P. chabaudi* infection might be stronger than previously thought. STAT3 deficient T cells preferentially differentiated into Th1 cells (**Fig. 3.5**). Moreover, STAT3 TKO mice showed peak temperature losses as early as day 9 p.i. (**Fig. 3.5**). This pathology phenotype makes sense in a disease where pathology is greatly driven by Th1 responses (119, 121). STAT3 TKO mice had lower parasitemia at day 10 and 14 p.i. suggesting that increasing IFN-γ^+ Th1 responses can improve parasite control after the peak. Th1-like responses have been shown to be extremely beneficial in murine models of malaria. A recent study has demonstrated that Th1-like Ly6C$^+$ Tmem cells generated in *P. yoelii* exhibit functional features of Tfh cells and provide robust B cell help upon reinfection (296). Moreover, *in vitro* generated Th1 cells using dead *P. yoelii* parasite, conferred sterile immunity to immunodeficient mice independent of antibody responses (300). In *P. chabaudi* infection, T effector memory (Tem) cells with Th1 characteristics correlate with protection (175). However, uncontrolled Th1 responses can lead to disease (120). Infection of IL-10 deficient mice with *P. chabaudi* can lead to symptoms and behavior of cerebral malaria (55), suggesting that Th1 responses might be involved in the development of this syndrome. Therefore, understanding the development of CD4 responses to malaria might be beneficial to create treatments for patients in the field.

HUMORAL CONTROL BY CD4 T CELLS DURING *P. CHABAUDI* INFECTION

In *P. berghei* ANKA, T-bet and IFN-γ inhibit Tfh responses (223). These results have lead to the idea that Th1-polarizing cytokine responses to malaria are detrimental for Tfh development; and therefore, inadequate to achieve proper humoral responses (301). However, we

found that mice treated with the anti-malarial MQ had more IFN-γ Th1-like responses and more GC B cells as early as day 7 p.i. (**Fig. 4.2**). Furthermore, our data demonstrates that tilting the balance from Th1-like Tfh to a more Th1-like correlates with protection from reinfection (**Fig. 3.7**). STAT3 deficiency in T cells affected GC B cell differentiation (**Fig. 3.7**). Nevertheless, a significant reduction in malaria-specific antibodies did not impaired protection from reinfection. On the contrary, STAT3 deficient mice were protected from reinfection, while WT mice had detectable parasite after rechallenge (**Fig. 3.7**). Previous studies suggest that sterile immunity to malaria can be achieved even in the absence of detectable malaria-specific antibodies (294). Furthermore, previous studies have shown that in *P. chabaudi* infection protection from reinfection can be antibody-independent (295). Previous studies have suggested that IFN-**γ** and Nitric oxide responses contribute to human and rodent malaria immunity (176, 302-304). IFN-γ seems to be required during protection from reinfection to maintain a priming of innate immune responses (177). This mechanism might be extrapolated to other infection, such *L. monocytogenes*, where IFN-γ production was required for the rapid activation of monocytes, macrophages, and dendritic cells for pathogen clearance (178). In malaria, Tem Th1 cells are maintained by chronic infection (175), which would explained why in the field constant malaria exposure is protective.

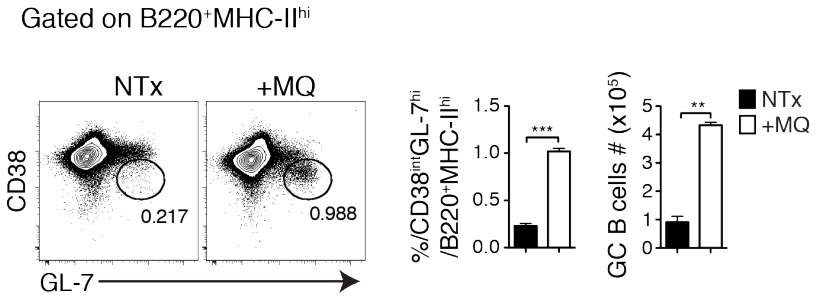

Fig. 4.2. Day 3 Mefloquine (MQ) treatment restores Germinal Center B cell responses. C57Bl/6J mice were infected with 10^5 iRBCs and one group was treated with the anti-malarial

mefloquine (MQ) from day 3 p.i. Splenocytes were recovered on day 7 p.i. MQ treatment resulted in increased proportions of IFN-γ^+ Th1-like cells (Fig. 3.2) and more $CD38^{int}GL\text{-}7^{hi}$ GC B cells. Data is representative of three experiments with 2-3 animals per group. Statistical significance from Students t-test. Error bars represent the SEM; **$p < 0.01$, ***$p < 0.001$.

In human and mouse malaria infection, CD4 T cells mainly develop into Teff that co-produce IFN-γ, IL-10, and IL-21 (169, 211, 224). This subset is inefficient to provide proper humoral responses, which might lead to parasite persistence. It is understood from previous experiments that antibodies are extremely important for parasite control. Transfer of purified IgG from immune adults to infected children resulted in a dramatic reduction in parasite load in the blood (75, 76). Unfortunately, the large genome and genetic mutation rate of the *Plasmodium spp*. parasites make really challenging to predict which proteins are the ones eliciting this protection. IgG1 and IgG3 are thought to be the main antibodies driving parasite control, since these two subclasses have shown to be good opsonins and mediators of Antibody-dependent cell-mediated cytotoxicity (ADCC). However, antibodies also help with neutralization, inhibition of parasite sequestration in peripheral organs, and re-entry into red blood cells. Bcl6 promotes the differentiation of Tfh cells by direct inhibition of genes that promote other T helper subsets, such as T-bet (Th1), Gata3 (Th2), and RORγt (Th17) (155). The character of Tfh cells seems to be more plastic than the other T helper subsets, and it is possible for Tfh cells to co-produce signature cytokines of other T helper subsets (163). Hence, classifying T cells as of the Tfh lineage *in vivo* has shown to be challenging (305). One of the novel finding in chapter 2 (confirmed in chapter 3) was that Bcl6 was not required for the generation of $CXCR5^+IFN\text{-}\gamma^+IL\text{-}21^+$ Teff cells. As expected, Bcl6 deficiency resulted in complete elimination of Germinal Center (GC) Tfh cells (212). The chemokine receptor CXCR5 guides T cells into the follicles by interaction with CXCL13. In the follicles, T cells encounter follicle B cells, which reinforces the

upregulation of Bcl6 and; consequently, the Tfh phenotype. It is then possible that the role of Bcl6 in T cell differentiation during malaria infection to be closely related to direction of T cells into the follicles (306). As expected, Blimp-1 deficiency had opposite results than Bcl6 deficiency in CXCR5 expression, but we did not find differences in GC Tfh formation. This result could be explained by the fact that in *P. chabaudi* infection there are no detectable GCs at day 7 p.i. (307), and B:T interactions are necessary for the progression of T cells into GC Tfh cells (164). However, Bcl6 TKO mice were able to control parasite growth to some extend during the chronic phase, regardless of their inability to generate GC B cells (Fig. 4.3). Conversely, IL-21 receptor deficient mice have uncontrolled parasite growth after the peak (212). These results emphasize that IL-21 signaling is more critical than GC formation for parasite control during the chronic phase. However, it is important to emphasize that GC formation is essential for parasite control during the chronic phase. This is demonstrated in Fig. 3.3D and Fig. 4.3D, where we can see that Bcl6 TKO mice are unable to maintain low parasite levels and generate GC structures. Other groups have shown the same results (212) and have shown that Bcl6 TKO mice do not have detectable levels of IgG in plasma reinforcing the idea that GC organization is required for isotype class-switch.

Tmem from chronically infected Bcl6 TKO mice had higher proportions of IFN-γ^+IL-21^+ Th1-like Tfh cells and T-bet expression (**Fig. 4.3**). It remains to be determined if the amount of persistent parasitemia plays a role in the maintenance of the Th1-like Tfh phenotype. In chronic LCMV infection, IL-21 production by CD4 T cells is required for survival and maintenance of CD8 T cells and control of persistent infection (260, 261, 263). Although IL-21 by itself has minimal effect on CD8 T cell proliferation, it has been shown to synergize with either IL-7 or IL-

15 to promote proliferation and IFN-γ production. Surprisingly, CD8 T cells can also produce IL-21 in an IL-27-dedendent fashion resulting in autocrine induction of granzyme B production. It might be possible that IL-21 in malaria infection has a role beyond support for B cell maturation and isotype class-switching. One study has shown that CD8 T cell responses are important to control parasite growth during the chronic phase (179). Therefore, more studies need to be done to address the role of IL-21 on CD8s during *P. chabaudi* infection, and its role in persistent parasite growth.

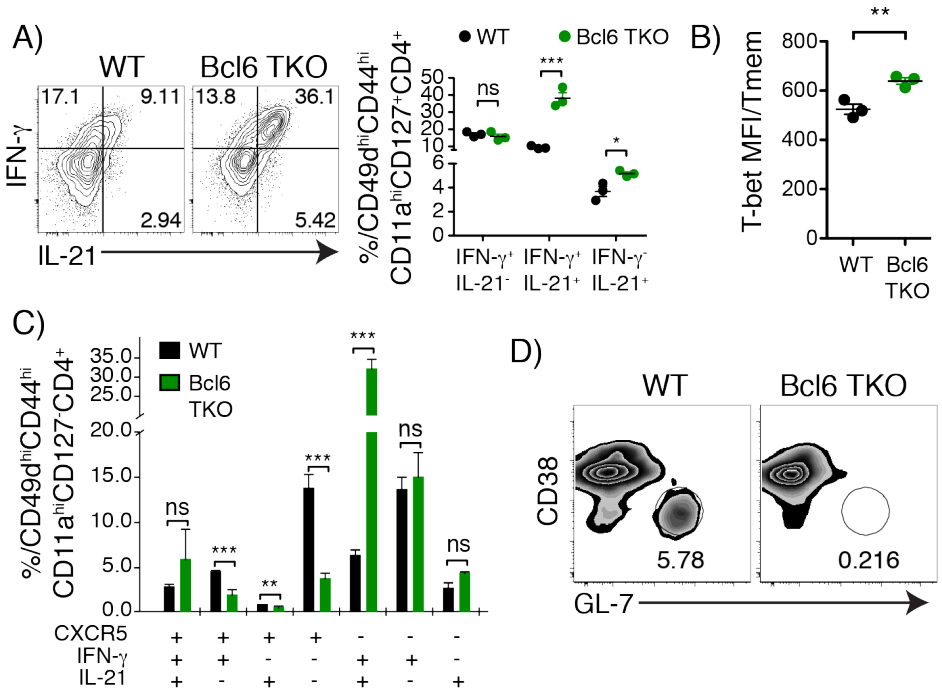

Fig. 4.3. Bcl6 TKO mice have more IFN-γ$^+$IL-21$^+$ Th1-like Tfh proportions during the chronic phase. Bcl6$^{fl/fl}$CD4^{Cre} (Bcl6 TKO) or Bcl6$^{fl/fl}$ (WT control) mice were infected with 10^5 iRBCs and splenocytes were recovered on day 55 p.i. Bcl6 TKO mice had more parasite load during the chronic phase (**Fig. 3.3**) and A,C) higher proprotions of IFN-γ$^+$IL-21$^+$ Th1-like Tfh cells and B) more T-bet expression. D) As expected, Bcl6 TKO mice were not able to mount GC B cell responses. Data is representative of one experiment with 3-4 animals per group. Statistical significance from Students t-test. Error bars represent the SEM; **p < 0.01, ***p < 0.001, ns = not significant.

In Figure 4.4, we summarize the big picture regarding regulation of adaptive immune responses based on the results presented in the current thesis. We propose that *P. chabaudi* infection induces the differentiation of Th1-like, Tfh-like, and Th1-like Tfh cells, been the later favored over the other two. This response will result in a delayed formation of GCs and the production of malaria-specific IgG antibodies. However, this infection confers poor protection from reinfection. In the absence of STAT3 signaling, CD4 T cells preferentially differentiate into Th1-like instead of Th1-like Tfh cells. This immune response will result in the formation of GCs, but they are smaller than in WT mice. As a result, STAT3 deficient mice have diminished malaria-specific IgG antibodies. As expected, extrafollicular antibody responses (IgM) are not affected by this. Nevertheless, upon re-challenge the STAT3 TKO mice had significantly lower levels of parasitemia, corroborating that IFN-γ memory responses correlate with protection from reinfection (176, 294). However, it still remains to be clarified that IFN-γ is the solely responsible for this protection. Moreover, we have not elucidated which cell population is the main source of IFN-γ.

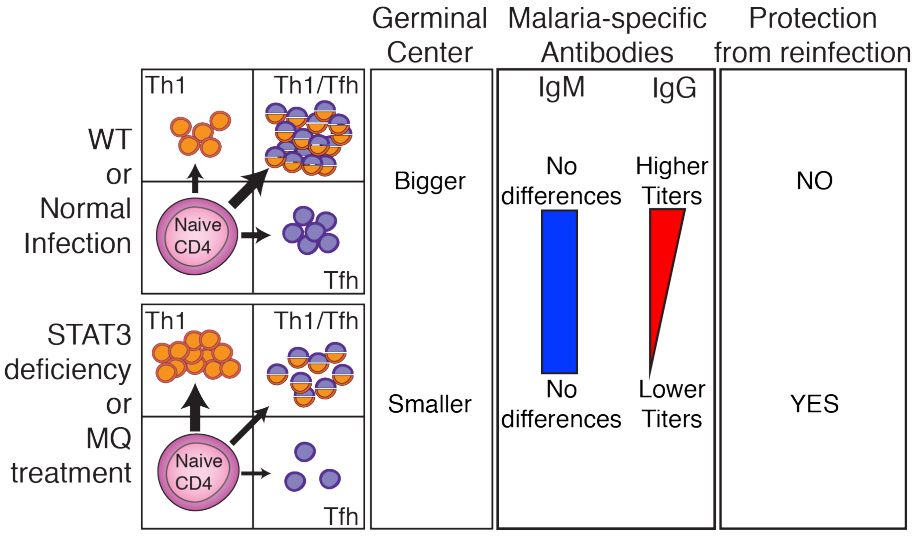

Fig 4.4. Proposed model for regulation of T cell responses in the absence of STAT3 and its relationship with protection.

THE CONTROL OF CD4 DIFFERENTIATION DURING *P. CHABAUDI* INFECTION

In chapter 3, we discuss in detail the effects of Bcl6, Blimp-1, and STAT3 deficiency on CD4 T cell differentiation during *P. chabaudi* infection. In general, we concluded that Bcl6 and Blimp-1 oppose to each other in regards CXCR5 regulation. However, both of them control T-bet expression in the same way. Blimp-1 controls IL-10 production; and therefore, it is crucial for host survival. STAT3 controls T-bet expression the same as Bcl6 and Blimp-1. In the absence of STAT3, CD4 Teff expressed lower levels of CXCR5 and IL-21 at day 7 p.i. However, at day 55 p.i the expression of IL-21 was invariant in STAT3 deficient T cells, while the production of IFN-γ was increased. Importantly, T-bet expression was higher in STAT3 deficient memory T cells. These results let us to propose a model of the molecular control of CD4 T cell differentiation during *P. chabaudi* infection.

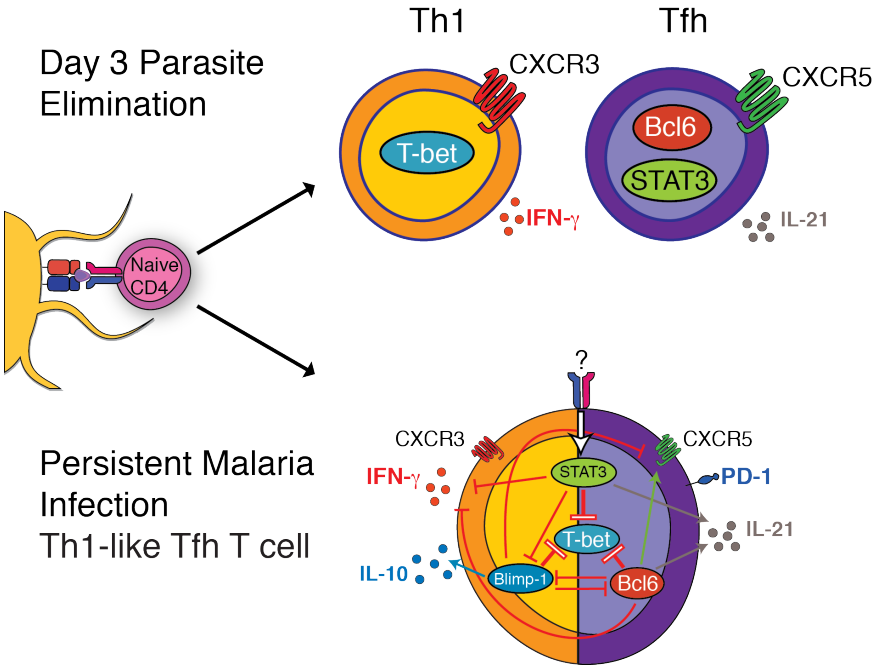

Fig 4.5 Proposed model of the molecular control of CD4 differentiation during persistent malaria infection. Persistent malaria infection preferentially expands $CXCR5^{+}IFN\text{-}\gamma^{+}IL\text{-}21^{+}$ Th1-like Tfh cells. Under MQ treatment at day 3 p.i, the CD4 T cells differentiate mainly into $CXCR5^{-}IFN\text{-}\gamma^{+}IL\text{-}21^{-}$ Th1-like and $CXCR5^{+}IFN\text{-}\gamma^{-}IL\text{-}21^{+}$ Tfh-like cells. During persistent malaria infection, Bcl6 and Blimp-1 control the expression of CXCR5. Bcl6 and STAT3 regulate IL-21 and IFN-γ production. Blimp-1 controls IL-10 production. Bcl6, Blimp-1, and STAT3 regulate T-bet expression. The cytokines that signal through STAT3 to control the differentiation Th1-like Tfh cells remain unknown.

We propose that persistent malaria infection preferentially expands Th1-like Tfh cells. In our studies, changes in CD4 differentiation by MQ-mediated parasite elimination was only effective in treatment starting at day 3 p.i. Treatment starting at day 5 p.i. had no effect on CD4 T cell differentiation (**Fig. 3.2**). In *P. berghei* ANKA model, day 5 antimalarial treatment did not rescue Tfh and humoral responses (223). Therefore, there seems to be an early check point were the immune system is shifted from regular Th1 and Tfh responses to a Th1-like Tfh response. If this mechanism is solely control by the parasite or a host survival mechanism remains to be determined. However, the dysregulation of CD4 responses seems to be a feature of persistent infections and not exclusive to *Plasmodium spp.* infections (165, 166, 170, 213, 308).

All the transcription factors that we studied downregulated T-bet expression. We found that Bcl6 expression was required to downregulate T-bet expression at day 9 p.i. (**Fig. 3.3**). Moreover, Bcl6 deficiency resulted in a discrete, but significant, increase in $IFN\text{-}\gamma^{+}IL\text{-}21^{-}$ Teff, suggesting Bcl6 is involved in controlling Th1-related genes in Teff cells during persistent malaria infection. The control of the Tfh program by Bcl6 is extremely complex, and it involves many other transcriptions factors, such as AP-1 (154). Previous studies have demonstrated that Bcl6 and T-bet can bind to each other forming a complex that regulates Th1-related genes expression (221, 222). We used Imaging flow cytometry and *Ifng/Thy1.1* reporter mice to

demonstrate that at day 7p.i. CD4 T cells express both T-bet and Bcl6 in the nucleus (**Fig. 2.4**). Furthermore, Bcl6 expression correlated with *Ifng/Thy1.1*^{lo} T cells. We observed Bcl6 protein in the cytoplasm of T cells as well, but if Bcl6 outside of the nucleus has a function has not been defined. Interestingly, we did not see a significant difference in the proportions of IFN-γ^{+}IL-21^{-} Teff when Bcl6 TKO or WT T cells were transferred into WT recipients (**Fig 2.5**). This could represent a need for B:T interaction that is likely to occur less in the Bcl6 TKO, or may be due to the smaller number of cells recovered. There is a role for B:T interaction in CD4 T cell differentiation. Previous studies have shown that the "shift from Th1 to Th2" in *P. chabaudi* requires B cells (198). STAT3 TKO mice had reduced numbers of GC B cells but the effect on CD4 T cell differentiation was different than Bcl6 TKO mice. Therefore, there is also an intrinsic role for these transcriptions in T cell development. However, we cannot rule out an extrinsic effect mediated by other cell populations at the moment. Tregs (FoxP3^{+}CD4^{+} T cells) are recognized as an important component of the response against malaria. However, there are contradictory results regarding their role during infection. Treg depletion has a different effect pending on the type of mouse malaria parasite used, the timing of depletion, and the mouse background (83). A recent study showed that depletion of FoxP3^{+} T cells (using *foxp3*-DTR mice) during non-lethal *P. yoelii* was beneficial only at day 9 p.i. Depletion at days 0 and 2 p.i. resulted in the death of the infected mice (209). On the other hand, IL-10^{+}FoxP3^{+} Tregs were shown not to be required for protection from severe disease in *P. chabaudi* infection (82). It is clear that IL-10 is required for survival during *Plasmodium spp.i* infection since IL-10 deficient mice succumb to infection (54, 120,121). It seems to be that IL-10 helps to survival by controlling TNF-mediated pathology, since TNF blockade rescued IL-10 deficient mice from dying (120). A newly defined subset of Tregs, T follicular regulatory T cells (Tfr), which reside

in the GC has been shown to be impactful during *P. yoelii* infection (209). Tfr cells can be identify by the expression of neuropilin-1 (NRP-1) are have high CTLA-4 expression. Anti-CTLA-4 treatment during the T helper cell "hiatus" in *P. yoelii* infection resulted in improved humoral responses, accelerated parasite control and partially rescued the susceptible BALB/c mouse strain. Importantly, IL-10 blockade during the same window did not affected the T helper cell response. Moreover, the majority of human studies do suggest that the Treg population expands with plasmodium infections and lower frequencies of Tregs are associated with improved disease outcome.

While Blimp-1 inhibits Bcl6-promoted GC Tfh differentiation (150), it actually inhibits *tbx21* expression in CD4 and CD8 T cells (280, 309). Conversely, Blimp-1 TKO animals had less IFN-γ^{+}IL-21^{-} Teff and the fraction of IFN-γ^{+}IL-21^{+} Th1-like Tfh cells was significantly increased. These results are surprising, since previous reports have shown that Blimp-1 binds directly to a distal regulatory region of the *ifng* gene and at various sites of the *tbx21* gene, repressing the Th1 program (280). We did find more T-bet protein in Blimp-1 deficient T cells, supporting a repressive role of Blimp-1 on Th1 commitment in malaria persistent infection. Bcl6 expression was also increased in Blimp-1 TKO mice; therefore determining if the effect of Blimp-1 is direct or indirect on T-bet is not clear. However, T-bet expression was increased in Blimp-1 deficient T cells earlier than Bcl6 deficient T cells (day 7 vs day 9 p.i.) suggesting that Blimp-1 effect on T-bet is independent of Bcl6. An increase in Bcl6 could also result in less IFN-γ production due to the inhibitor Bcl6/T-bet complex.

It is well accepted that T helper differentiation depends on the stable expression of transcription factors that direct the acquisition of chemokines and signature cytokines. Nevertheless, more studies are showing that CD4 lineages can be flexible either at the CD4 population level or single cell level (158, 277, 289). This seems to be particularly true during persistent infections (213). Cytokines and costimulation play an important role directing the expression of the "master regulators" (289). Many cytokines that regulate T cell differentiation during malaria infection, such as IL-6, IL-10, IL-27, and IL-12, signal through STAT3 (170, 183, 196, 278). In general, STAT3 signaling affects CD4 T cell differentiation. IL-6 and IL-23 signaling through STAT3 play a critical role in Th17 differentiation (278). STAT3 induces the expression of the lineage-defining transcription factor RORγt, and regulates the production of IL-17A, IL-17F and IL-21, which promotes RORγt expression in an autocrine fashion. Importantly, STAT3 has been shown to promote Th17 differentiation by inhibition of *tbx21* (Th1), *gata3* (Th2), and *foxp3* (Treg) expression. Under Th2 conditions, murine STAT3 deficient T cells have diminished IL-4, IL-5, and IL-13 production, and less *gata3* expression. These effects are likely due to direct control by STAT3, which has been shown to directly bind to the Th2-related genes *gata3*, *batf*, and *cmaf*. STAT3 deficiency has a great effect on Tfh differentiation. Autosomal dominant hyper-IgE syndrome (AD-HIES) patients, who carry heterozygous mutations in *stat3*, have great reduction in circulating Tfh cells. CD4 T cell from AD-HIES patients are unable to differentiate *in vitro* into IL-21^{+} Tfh-like cells. Murine STAT3 deficient T cells are ineffective to acquire the Tfh phenotype *in vivo* due to impaired IL-6, IL-21 and/or IL-27 signaling. The reduction of Th1-like Tfh cells in STAT3 TKO mice suggests that one or more of these cytokines might play a role in their differentiation. It is well accepted that STAT3 regulates IL-21 production in T cells, especially in Th17 cells (310). The role of STAT3 on Tfh

differentiation is more complex. Early reports showed that STAT3 was required for Tfh differentiation through IL-21 signaling (218). Recent studies in LCMV corroborated a role in STAT3 signaling for proper Tfh and humoral responses (219). Moreover, people with functional mutations in STAT3 results in humoral defects (293). However, other groups have shown that STAT3 deficiency might be only required to secure the Tfh program once it is achieved (283). IL-6 can signal through STAT1 to induce a quick upregulation of Bcl6 and CXCR5 expression. In our model, STAT3 regulated T-bet expression and cytokines (**Fig 3.5**). STAT3 deficient T cells at the peak produced less IL-21, but similar amounts of IFN-γ. Conversely, STAT3 deficient Tmem cells produced more IFN-γ, but similar amounts of IL-21, when restimualted *ex vivo*. The role of STAT3 on Tmem development is not limited to cytokines production. Previous studies have demonstrate a role for STAT3 in functional maturation of CD8 T cells (311), as well as a requirement for survival of CD8 T cell during persistent infection (260, 261, 263). A recent study using IL-12 and IL-27 (a Th1 inflammatory condition *in vitro* system) showed that STAT3 deficient IFN-γ^+ T cells have less IL-10 expression (197). Nevertheless, in our studies STAT3 deficiency did not eliminate completely the development of Th1-like Tfh cells, revealing that other molecular controllers might be at play. For example, the transcription factor c-Maf can activate IL-21 production in CD4 T cells (312) and its activation is IL-27 dependent (192). IL-12 can induce IL-21 production in a STAT4-dependent manner (170), and Tfh differentiation of naive human CD4 T cells *in vitro* is induced by IL-12 (313). Mores studies need to be done to fully understand what are the key role players in CD4 T cell differentiation during malaria infection. Importantly, the results obtained might be extrapolated to other persistent infections. Understanding the molecular control of protective adaptive immune responses is important and

necessary, as it can aid in the development of novel treatment and the rational development of vaccine strategies.

IMPLICATIONS OF THE CURRENT THESIS FOR VACCINE DEVELOPMENT

Malaria elimination requires the development of an effective vaccine. Many groups around the globe are making great progress on different new concepts for malaria vaccination (Table 4.1). One of the main problems with malaria vaccine development is the difficulty in maintaining long-lived immune protection after immunization. The immune response to malaria is complex. Despite repeated infections in endemic areas, sterilizing immunity is hardly achieved. However, clinical immunity can be achieved, but only after repeated infections and later in life. Therefore, it is vital for malaria vaccine development to understand the immune response induced by vaccination and to refine the correlates of protection from infection or reinfection. Antibodies are the golden standard to predict protection (117). Nevertheless, malaria exposure is associated with the generation of atypical memory B cells that are ineffective in effector functions and Th1-polarized Tfh cells that are poor B cell helpers (224). Recent studies have shown that CTLA-4 blockade during the T cell "hiatus" results in increased CD4 and B cell memory responses that confer protection in malaria mouse models (209). These results suggest that modulating the immune responses is a feasible approach to induce protection in malaria.

Vaccine candidate	*Parasite stage*	*Vaccine classification*	*Status*
PfSPZ vaccine	Pre-erythrocytic	Whole organism (radiation attenuation)	Phase II
GAP vaccine	Pre-erythrocytic	Whole organism (genetic attenuation)	Phase I
RTS,S	Pre-erythrocytic	Subunit	Phase IV
CVac	Pre-erythrocytic	Whole organism (chemical attenuation)	Phase I
PfRH5	Blood stage	Subunit	Phase I
Pfs25	Mosquito stage	Subunit	Phase I
Pfs230	Mosquito stage	Subunit	Phase I

4. 1 Current malaria vaccine development efforts under clinical trials

The results presented in Chapter 3 suggest that STAT3 signaling within T cells during *P. chabaudi* infection regulates the differentiation of Th1 memory cells. Improving Th1 memory formation resulted in protection from reinfection. However, antibody responses declined in STAT3 T cell deficient mice when compared to WT. The importance of these findings resides in the fact that improving Th1 memory formation, despite a decrease in antibody responses, correlated with protection from reinfection. The current most advanced malaria vaccine, RTS,S, uses an adjuvant that promotes Th1 differentiation. It might be an option to add STAT3 inhibitors to the current vaccine regimen and determine if this would improve long-lasting protection. However, there is a chance that promoting Th1 memory formation could be troublesome. There is great risk on inducing strong proinflammatory responses through vaccination. Beyond the fact that strong IFN-γ-mediated responses could lead to a "cytokine storm", studies have indicated that IFN-γ and T-bet can inhibit Tfh responses, which are required for antibody isotype class switch (223). Conversely, CD4 T cells from T-bet deficient mice infected with *P. chabaudi* have almost no IFN-γ production and are unable to control parasite

growth during the chronic phase. These results suggest that Th1 responses are important for humoral responses. It would beneficial to start a more rigorous immunophenotype profiling of protective T cell responses against malaria. These approaches could bring a new perspective to vaccine development for other diseases as well.

CONCLUDING REMARKS

The current dissertation was aimed to understanding the development of protective CD4 T cell responses against *P. chabaudi* persistent malaria infection. Our results have revealed that a classic Th1, followed by a conventional Tfh, response, does not characterize the differentiation of CD4 T cells in malaria. The majority of the CD4 T cells responding to malaria infection are characterized by an IL-21^{+}IFN-γ^{+}CXCR5^{+}CXCR3^{+} Th1-like Tfh phenotype. Our studies have revealed that the acquisition of this phenotype depends on infections longer than 3 days, as MQ treatment on day 5 p.i. did not affected the phenotype of CD4 T cells. Nevertheless, persistent infection maintains T-bet and IFN-γ expression, suggesting a mechanism for improved protection during chronic infection. Finally, we have identified three major transcription factors that work in concert to regulate the generation of these cells. Our data points to STAT3 as a key role player in the regulation of Th1 memory formation in persistent malaria infection. STAT3 deficient Tmem cells had higher T-bet and IFN-γ expression. Importantly, these Th1 Tmem cells correlated with protection from reinfection. Our data suggest that immune modulation might be a feasible route to improve malaria control, and it points to Th1 memory cells as a candidate goal for vaccine strategies.

FUTURE DIRECTIONS

Many questions remain regarding the nature and function of hybrid Th1/Tfh cells. Is this a new CD4 T cell subset or a phase of differentiation of the CD4 population during persistent malaria infection? Are IFN-γ^{+}IL-21^{+} CD4 T cells beneficial for parasite or pathology control? Or both? What are other molecules controlling the differentiation of these cells? We can use IL-21/IFN-γ double reporters to address many of these questions. Cell sorting of IL-21/IFN-γ populations could help us address the main differences between these populations by Next Generation Sequencing studies or adoptive transfer of these cells into infection-matched and uninfected recipients. It would extremely beneficial to develop an *in vitro* system that promotes the differentiation of these. This approach would provide a great tool to conduct more mechanistic studies.

BIBLIOGRAPHY

1. Organization WH. World Malaria Report. (Geneva: World Health Organization). 2017.

2. Rts SCTP. Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. PLoS Med. 2014;11(7):e1001685.

3. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, et al. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. J Infect Dis. 2002;185(8):1155-64.

4. Ishizuka AS, Lyke KE, DeZure A, Berry AA, Richie TL, Mendoza FH, et al. Corrigendum: Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. Nat Med. 2016;22(6):692.

5. Ishizuka AS, Lyke KE, DeZure A, Berry AA, Richie TL, Mendoza FH, et al. Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. Nat Med. 2016;22(6):614-23.

6. Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, et al. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science. 2013;341(6152):1359-65.

7. Bijker EM, Bastiaens GJ, Teirlinck AC, van Gemert GJ, Graumans W, van de Vegte-Bolmer M, et al. Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity. Proc Natl Acad Sci U S A. 2013;110(19):7862-7.

8. Roestenberg M, Teirlinck AC, McCall MB, Teelen K, Makamdop KN, Wiersma J, et al. Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. Lancet. 2011;377(9779):1770-6.

9. Kazmin D, Nakaya HI, Lee EK, Johnson MJ, van der Most R, van den Berg RA, et al. Systems analysis of protective immune responses to RTS,S malaria vaccination in humans. Proc Natl Acad Sci U S A. 2017;114(9):2425-30.

10. Lyke KE, Ishizuka AS, Berry AA, Chakravarty S, DeZure A, Enama ME, et al. Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection. Proc Natl Acad Sci U S A. 2017;114(10):2711-6.

11. Mordmuller B, Surat G, Lagler H, Chakravarty S, Ishizuka AS, Lalremruata A, et al. Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. Nature. 2017;542(7642):445-9.

12. Langhorne J, Ndungu FM, Sponaas AM, Marsh K. Immunity to malaria: more questions than answers. Nat Immunol. 2008;9(7):725-32.

13. Richards JS, Beeson JG. The future for blood-stage vaccines against malaria. Immunol Cell Biol. 2009;87(5):377-90.

14. Deloron P, Chougnet C. Is immunity to malaria really short-lived? Parasitol Today. 1992;8(11):375-8.

15. Jelinek T, Schulte C, Behrens R, Grobusch MP, Coulaud JP, Bisoffi Z, et al. Imported Falciparum malaria in Europe: sentinel surveillance data from the European network on surveillance of imported infectious diseases. Clin Infect Dis. 2002;34(5):572-6.

16. Marsh K, Kinyanjui S. Immune effector mechanisms in malaria. Parasite Immunol. 2006;28(1-2):51-60.

17. Brown SP, Grenfell BT. An unlikely partnership: parasites, concomitant immunity and host defence. Proc Biol Sci. 2001;268(1485):2543-9.

18. Dubey JP, Jones JL. *Toxoplasma gondii* infection in humans and animals in the United States. Int J Parasitol. 2008;38(11):1257-78.

19. Mandell MA, Beverley SM. Continual renewal and replication of persistent *Leishmania major* parasites in concomitantly immune hosts. Proc Natl Acad Sci U S A. 2017;114(5):E801-E10.

20. Smith T, Felger I, Tanner M, Beck HP. Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections. Trans R Soc Trop Med Hyg. 1999;93 Suppl 1:59-64.

21. Beeson JG, Osier FH, Engwerda CR. Recent insights into humoral and cellular immune responses against malaria. Trends Parasitol. 2008;24(12):578-84.

22. Doolan DL, Dobano C, Baird JK. Acquired immunity to malaria. Clin Microbiol Rev. 2009;22(1):13-36, Table of Contents.

23. Ponnudurai T, Lensen AH, van Gemert GJ, Bolmer MG, Meuwissen JH. Feeding behaviour and sporozoite ejection by infected Anopheles stephensi. Trans R Soc Trop Med Hyg. 1991;85(2):175-80.

24. Vanderberg JP. *Plasmodium berghei*: quantitation of sporozoites injected by mosquitoes feeding on a rodent host. Exp Parasitol. 1977;42(1):169-81.

25. Vanderberg JP, Frevert U. Intravital microscopy demonstrating antibody-mediated immobilisation of *Plasmodium berghei* sporozoites injected into skin by mosquitoes. Int J Parasitol. 2004;34(9):991-6.

26. Yamauchi LM, Coppi A, Snounou G, Sinnis P. *Plasmodium* sporozoites trickle out of the injection site. Cell Microbiol. 2007;9(5):1215-22.

27. Mota MM, Rodriguez A. Migration through host cells: the first steps of *Plasmodium* sporozoites in the mammalian host. Cell Microbiol. 2004;6(12):1113-8.

28. Prudencio M, Rodriguez A, Mota MM. The silent path to thousands of merozoites: the *Plasmodium* liver stage. Nat Rev Microbiol. 2006;4(11):849-56.

29. Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. Cell. 2006;124(4):755-66.

30. Trampuz A, Jereb M, Muzlovic I, Prabhu RM. Clinical review: Severe malaria. Crit Care. 2003;7(4):315-23.

31. Chulay JD, Schneider I, Cosgriff TM, Hoffman SL, Ballou WR, Quakyi IA, et al. Malaria transmitted to humans by mosquitoes infected from cultured *Plasmodium falciparum.* Am J Trop Med Hyg. 1986;35(1):66-8.

32. Church LW, Le TP, Bryan JP, Gordon DM, Edelman R, Fries L, et al. Clinical manifestations of *Plasmodium falciparum* malaria experimentally induced by mosquito challenge. J Infect Dis. 1997;175(4):915-20.

33. Engwerda CR, Minigo G, Amante FH, McCarthy JS. Experimentally induced blood stage malaria infection as a tool for clinical research. Trends Parasitol. 2012;28(11):515-21.

34. Sauerwein RW, Roestenberg M, Moorthy VS. Experimental human challenge infections can accelerate clinical malaria vaccine development. Nat Rev Immunol. 2011;11(1):57-64.

35. Tran TM, Samal B, Kirkness E, Crompton PD. Systems immunology of human malaria. Trends Parasitol. 2012;28(6):248-57.

36. Monack DM, Mueller A, Falkow S. Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat Rev Microbiol. 2004;2(9):747-65.

37. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. Cell. 2009;138(1):30-50.

38. Babiker HA, Abdel-Muhsin AM, Ranford-Cartwright LC, Satti G, Walliker D. Characteristics of *Plasmodium falciparum* parasites that survive the lengthy dry season in eastern Sudan where malaria transmission is markedly seasonal. Am J Trop Med Hyg. 1998;59(4):582-90.

39. Nassir E, Abdel-Muhsin AM, Suliaman S, Kenyon F, Kheir A, Geha H, et al. Impact of genetic complexity on longevity and gametocytogenesis of *Plasmodium falciparum* during the dry and transmission-free season of eastern Sudan. Int J Parasitol. 2005;35(1):49-55.

40. Whitrow M. Wagner-Jauregg and fever therapy. Med Hist. 1990;34(3):294-310.

41. Gwadz RW, Cochrane AH, Nussenzweig V, Nussenzweig RS. Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of *Plasmodium knowlesi* and characterization of surface antigens of these parasites. Bull World Health Organ. 1979;57 Suppl 1:165-73.

42. Nussenzweig RS, Vanderberg J, Most H, Orton C. Protective immunity produced by the injection of x-irradiated sporozoites of *plasmodium berghei*. Nature. 1967;216(5111):160-2.

43. Vaughan AM, Wang R, Kappe SH. Genetically engineered, attenuated whole-cell vaccine approaches for malaria. Hum Vaccin. 2010;6(1):107-13.

44. Barry AE, Schultz L, Buckee CO, Reeder JC. Contrasting population structures of the genes encoding ten leading vaccine-candidate antigens of the human malaria parasite, *Plasmodium falciparum*. PLoS One. 2009;4(12):e8497.

45. Carter R, Walliker D. New observations on the malaria parasites of rodents of the Central African Republic - *Plasmodium vinckei petteri* subsp. nov. and *Plasmodium chabaudi Landau*, 1965. Ann Trop Med Parasitol. 1975;69(2):187-96.

46. Jayawardena AN, Mogil R, Murphy DB, Burger D, Gershon RK. Enhanced expression of H-2K and H-2D antigens on reticulocytes infected with *Plasmodium yoelii*. Nature. 1983;302(5909):623-6.

47. Cox J, Semoff S, Hommel M. *Plasmodium chabaudi*: a rodent malaria model for in-vivo and in-vitro cytoadherence of malaria parasites in the absence of knobs. Parasite Immunol. 1987;9(5):543-61.

48. Gilks CF, Walliker D, Newbold CI. Relationships between sequestration, antigenic variation and chronic parasitism in *Plasmodium chabaudi chabaudi*--a rodent malaria model. Parasite Immunol. 1990;12(1):45-64.

49. Mackinnon MJ, Walker PR, Rowe JA. *Plasmodium chabaudi*: rosetting in a rodent malaria model. Exp Parasitol. 2002;101(2-3):121-8.

50. Stephens R, Culleton RL, Lamb TJ. The contribution of *Plasmodium chabaudi* to our understanding of malaria. Trends Parasitol. 2012;28(2):73-82.

51. Seydel KB, Kampondeni SD, Valim C, Potchen MJ, Milner DA, Muwalo FW, et al. Brain swelling and death in children with cerebral malaria. N Engl J Med. 2015;372(12):1126-37.

52. Taylor TE, Fu WJ, Carr RA, Whitten RO, Mueller JS, Fosiko NG, et al. Differentiating the pathologies of cerebral malaria by postmortem parasite counts. Nat Med. 2004;10(2):143-5.

53. Brugat T, Cunningham D, Sodenkamp J, Coomes S, Wilson M, Spence PJ, et al. Sequestration and histopathology in *Plasmodium chabaudi* malaria are influenced by the immune response in an organ-specific manner. Cell Microbiol. 2014;16(5):687-700.

54. Sanni LA, Jarra W, Li C, Langhorne J. Cerebral edema and cerebral hemorrhages in interleukin-10-deficient mice infected with *Plasmodium chabaudi*. Infect Immun. 2004;72(5):3054-8.

55. Wilson KD, Stutz SJ, Ochoa LF, Valbuena GA, Cravens PD, Dineley KT, et al. Behavioural and neurological symptoms accompanied by cellular neuroinflammation in IL-10-deficient mice infected with *Plasmodium chabaudi*. Malar J. 2016;15(1):428.

56. Lamikanra AA, Brown D, Potocnik A, Casals-Pascual C, Langhorne J, Roberts DJ. Malarial anemia: of mice and men. Blood. 2007;110(1):18-28.

57. Belyaev NN, Brown DE, Diaz AI, Rae A, Jarra W, Thompson J, et al. Induction of an IL7-R(+)c-Kit(hi) myelolymphoid progenitor critically dependent on IFN-gamma signaling during acute malaria. Nat Immunol. 2010;11(6):477-85.

58. Mohan K, Stevenson MM. Dyserythropoiesis and severe anaemia associated with malaria correlate with deficient interleukin-12 production. Br J Haematol. 1998;103(4):942-9.

59. Sato T, Selleri C, Young NS, Maciejewski JP. Hematopoietic inhibition by interferon-gamma is partially mediated through interferon regulatory factor-1. Blood. 1995;86(9):3373-80.

60. Baldridge MT, King KY, Boles NC, Weksberg DC, Goodell MA. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. Nature. 2010;465(7299):793-7.

61. Okada H, Suzue K, Imai T, Taniguchi T, Shimokawa C, Onishi R, et al. A transient resistance to blood-stage malaria in interferon-gamma-deficient mice through impaired production of the host cells preferred by malaria parasites. Front Microbiol. 2015;6:600.

62. Mackinnon MJ, Mwangi TW, Snow RW, Marsh K, Williams TN. Heritability of malaria in Africa. PLoS Med. 2005;2(12):e340.

63. Driss A, Hibbert JM, Wilson NO, Iqbal SA, Adamkiewicz TV, Stiles JK. Genetic polymorphisms linked to susceptibility to malaria. Malar J. 2011;10:271.

64. Verra F, Mangano VD, Modiano D. Genetics of susceptibility to *Plasmodium falciparum*: from classical malaria resistance genes towards genome-wide association studies. Parasite Immunol. 2009;31(5):234-53.

65. Bautista JM, Marin-Garcia P, Diez A, Azcarate IG, Puyet A. Malaria proteomics: insights into the parasite-host interactions in the pathogenic space. J Proteomics. 2014;97:107-25.

66. Hedrick PW. Population genetics of malaria resistance in humans. Heredity (Edinb). 2011;107(4):283-304.

67. Fortin A, Stevenson MM, Gros P. Complex genetic control of susceptibility to malaria in mice. Genes Immun. 2002;3(4):177-86.

68. Min-Oo G, Willemetz A, Tam M, Canonne-Hergaux F, Stevenson MM, Gros P. Mapping of *Char10*, a novel malaria susceptibility locus on mouse chromosome 9. Genes Immun. 2010;11(2):113-23.

69. Heddini A, Chen Q, Obiero J, Kai O, Fernandez V, Marsh K, et al. Binding of *Plasmodium falciparum*-infected erythrocytes to soluble platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): frequent recognition by clinical isolates. Am J Trop Med Hyg. 2001;65(1):47-51.

70. Malpede BM, Tolia NH. Malaria adhesins: structure and function. Cell Microbiol. 2014;16(5):621-31.

71. Wahlgren M, Goel S, Akhouri RR. Variant surface antigens of *Plasmodium falciparum* and their roles in severe malaria. Nat Rev Microbiol. 2017;15(8):479-91.

72. Cunningham D, Lawton J, Jarra W, Preiser P, Langhorne J. The pir multigene family of *Plasmodium*: antigenic variation and beyond. Mol Biochem Parasitol. 2010;170(2):65-73.

73. Spence PJ, Jarra W, Levy P, Reid AJ, Chappell L, Brugat T, et al. Vector transmission regulates immune control of *Plasmodium* virulence. Nature. 2013;498(7453):228-31.

74. Meding SJ, Langhorne J. CD4+ T cells and B cells are necessary for the transfer of protective immunity to *Plasmodium chabaudi chabaudi*. Eur J Immunol. 1991;21(6):1433-8.

75. Cohen S, Mc GI, Carrington S. Gamma-globulin and acquired immunity to human malaria. Nature. 1961;192:733-7.

76. Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, Chantavanich P, et al. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am J Trop Med Hyg. 1991;45(3):297-308.

77. von der Weid T, Honarvar N, Langhorne J. Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection. J Immunol. 1996;156(7):2510-6.

78. Stephens R, Albano FR, Quin S, Pascal BJ, Harrison V, Stockinger B, et al. Malaria-specific transgenic CD4(+) T cells protect immunodeficient mice from lethal infection and demonstrate requirement for a protective threshold of antibody production for parasite clearance. Blood. 2005;106(5):1676-84.

79. Artavanis-Tsakonas K, Riley EM. Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live *Plasmodium falciparum*-infected erythrocytes. J Immunol. 2002;169(6):2956-63.

80. Ing R, Stevenson MM. Dendritic cell and NK cell reciprocal cross talk promotes gamma interferon-dependent immunity to blood-stage *Plasmodium chabaudi* AS infection in mice. Infect Immun. 2009;77(2):770-82.

81. Horowitz A, Newman KC, Evans JH, Korbel DS, Davis DM, Riley EM. Cross-talk between T cells and NK cells generates rapid effector responses to *Plasmodium falciparum*-infected erythrocytes. J Immunol. 2010;184(11):6043-52.

82. Freitas do Rosario AP, Lamb T, Spence P, Stephens R, Lang A, Roers A, et al. IL-27 promotes IL-10 production by effector Th1 CD4+ T cells: a critical mechanism for protection from severe immunopathology during malaria infection. J Immunol. 2012;188(3):1178-90.

83. Walther M, Jeffries D, Finney OC, Njie M, Ebonyi A, Deininger S, et al. Distinct roles for FOXP3 and FOXP3 CD4 T cells in regulating cellular immunity to uncomplicated and severe *Plasmodium falciparum* malaria. PLoS Pathog. 2009;5(4):e1000364.

84. Dicko A, Sagara I, Diemert D, Sogoba M, Niambele MB, Dao A, et al. Year-to-year variation in the age-specific incidence of clinical malaria in two potential vaccine testing sites in Mali with different levels of malaria transmission intensity. Am J Trop Med Hyg. 2007;77(6):1028-33.

85. Snow RW, Omumbo JA, Lowe B, Molyneux CS, Obiero JO, Palmer A, et al. Relation between severe malaria morbidity in children and level of *Plasmodium falciparum* transmission in Africa. Lancet. 1997;349(9066):1650-4.

86. Offeddu V, Thathy V, Marsh K, Matuschewski K. Naturally acquired immune responses against *Plasmodium falciparum* sporozoites and liver infection. Int J Parasitol. 2012;42(6):535-48.

87. Honda T, Miyachi Y, Kabashima K. Regulatory T cells in cutaneous immune responses. J Dermatol Sci. 2011;63(2):75-82.

88. Crispe IN. The liver as a lymphoid organ. Annu Rev Immunol. 2009;27:147-63.

89. Guilbride DL, Gawlinski P, Guilbride PD. Why functional pre-erythrocytic and bloodstage malaria vaccines fail: a meta-analysis of fully protective immunizations and novel immunological model. PLoS One. 2010;5(5):e10685.

90. da Silva HB, Caetano SS, Monteiro I, Gomez-Conde I, Hanson K, Penha-Goncalves C, et al. Early skin immunological disturbance after *Plasmodium*-infected mosquito bites. Cell Immunol. 2012;277(1-2):22-32.

91. Good MF, Doolan DL. Malaria vaccine design: immunological considerations. Immunity. 2010;33(4):555-66.

92. Urban BC, Mwangi T, Ross A, Kinyanjui S, Mosobo M, Kai O, et al. Peripheral blood dendritic cells in children with acute *Plasmodium falciparum* malaria. Blood. 2001;98(9):2859-61.

93. Haque A, Best SE, Montes de Oca M, James KR, Ammerdorffer A, Edwards CL, et al. Type I IFN signaling in CD8- DCs impairs Th1-dependent malaria immunity. J Clin Invest. 2014;124(6):2483-96.

94. Sponaas AM, Cadman ET, Voisine C, Harrison V, Boonstra A, O'Garra A, et al. Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells. J Exp Med. 2006;203(6):1427-33.

95. Wykes MN, Good MF. What really happens to dendritic cells during malaria? Nat Rev Microbiol. 2008;6(11):864-70.

96. Seixas E, Moura Nunes JF, Matos I, Coutinho A. The interaction between DC and *Plasmodium berghei/chabaudi*-infected erythrocytes in mice involves direct cell-to-cell contact, internalization and TLR. Eur J Immunol. 2009;39(7):1850-63.

97. Voisine C, Mastelic B, Sponaas AM, Langhorne J. Classical CD11c+ dendritic cells, not plasmacytoid dendritic cells, induce T cell responses to *Plasmodium chabaudi* malaria. Int J Parasitol. 2010;40(6):711-9.

98. Langhorne J, Meding SJ, Eichmann K, Gillard SS. The response of CD4+ T cells to *Plasmodium chabaudi chabaudi*. Immunol Rev. 1989;112:71-94.

99. Korbel DS, Norman PJ, Newman KC, Horowitz A, Gendzekhadze K, Parham P, et al. Killer Ig-like receptor (KIR) genotype predicts the capacity of human KIR-positive CD56dim NK cells to respond to pathogen-associated signals. J Immunol. 2009;182(10):6426-34.

100. Franklin BS, Parroche P, Ataide MA, Lauw F, Ropert C, de Oliveira RB, et al. Malaria primes the innate immune response due to interferon-gamma induced enhancement of toll-like receptor expression and function. Proc Natl Acad Sci U S A. 2009;106(14):5789-94.

101. Langhorne J, Albano FR, Hensmann M, Sanni L, Cadman E, Voisine C, et al. Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection. Immunol Rev. 2004;201:35-47.

102. Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, Uematsu S, et al. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. J Exp Med. 2005;201(1):19-25.

103. Pichyangkul S, Yongvanitchit K, Kum-arb U, Hemmi H, Akira S, Krieg AM, et al. Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. J Immunol. 2004;172(8):4926-33.

104. Franklin BS, Rodrigues SO, Antonelli LR, Oliveira RV, Goncalves AM, Sales-Junior PA, et al. MyD88-dependent activation of dendritic cells and CD4(+) T lymphocytes mediates symptoms, but is not required for the immunological control of parasites during rodent malaria. Microbes Infect. 2007;9(7):881-90.

105. Sheel M, Engwerda CR. The diverse roles of monocytes in inflammation caused by protozoan parasitic diseases. Trends Parasitol. 2012;28(10):408-16.

106. Liehl P, Mota MM. Innate recognition of malarial parasites by mammalian hosts. Int J Parasitol. 2012;42(6):557-66.

107. Jakubzick C, Gautier EL, Gibbings SL, Sojka DK, Schlitzer A, Johnson TE, et al. Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. Immunity. 2013;39(3):599-610.

108. Chua CL, Brown G, Hamilton JA, Rogerson S, Boeuf P. Monocytes and macrophages in malaria: protection or pathology? Trends Parasitol. 2013;29(1):26-34.

109. Bouharoun-Tayoun H, Oeuvray C, Lunel F, Druilhe P. Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. J Exp Med. 1995;182(2):409-18.

110. Galamo CD, Jafarshad A, Blanc C, Druilhe P. Anti-MSP1 block 2 antibodies are effective at parasite killing in an allele-specific manner by monocyte-mediated antibody-dependent cellular inhibition. J Infect Dis. 2009;199(8):1151-4.

111. Lunel F, Druilhe P. Effector cells involved in nonspecific and antibody-dependent mechanisms directed against *Plasmodium falciparum* blood stages in vitro. Infect Immun. 1989;57(7):2043-9.

112. Shi CL, Yu CH, Zhang Y, Zhao D, Chang XH, Wang WH. Monocyte chemoattractant protein-1 modulates invasion and apoptosis of PC-3M prostate cancer cells via regulating expression of VEGF, MMP9 and caspase-3. Asian Pac J Cancer Prev. 2011;12(2):555-9.

113. Sponaas AM, Freitas do Rosario AP, Voisine C, Mastelic B, Thompson J, Koernig S, et al. Migrating monocytes recruited to the spleen play an important role in control of blood stage malaria. Blood. 2009;114(27):5522-31.

114. Day NP, Hien TT, Schollaardt T, Loc PP, Chuong LV, Chau TT, et al. The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. J Infect Dis. 1999;180(4):1288-97.

115. Lyke KE, Burges R, Cissoko Y, Sangare L, Dao M, Diarra I, et al. Serum levels of the proinflammatory cytokines interleukin-1 beta (IL-1beta), 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. 2004;72(10):5630-7.

116. Walther M, Woodruff J, Edele F, Jeffries D, Tongren JE, King E, et al. Innate immune responses to human malaria: heterogeneous cytokine responses to blood-stage *Plasmodium falciparum* correlate with parasitological and clinical outcomes. J Immunol. 2006;177(8):5736-45.

117. Crompton PD, Moebius J, Portugal S, Waisberg M, Hart G, Garver LS, et al. Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. Annu Rev Immunol. 2014;32:157-87.

118. Collins WE, Jeffery GM. A retrospective examination of secondary sporozoite- and trophozoite-induced infections with *Plasmodium falciparum*: development of parasitologic and clinical immunity following secondary infection. Am J Trop Med Hyg. 1999;61(1 Suppl):20-35.

119. Su Z, Stevenson MM. Central role of endogenous gamma interferon in protective immunity against blood-stage *Plasmodium chabaudi* AS infection. Infect Immun. 2000;68(8):4399-406.

120. Li C, Corraliza I, Langhorne J. A defect in interleukin-10 leads to enhanced malarial disease in *Plasmodium chabaudi chabaudi* infection in mice. Infect Immun. 1999;67(9):4435-42.

121. Li C, Sanni LA, Omer F, Riley E, Langhorne J. 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. 2003;71(9):4850-6.

122. Dodoo D, Omer FM, Todd J, Akanmori BD, Koram KA, Riley EM. Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to *Plasmodium falciparum* malaria. J Infect Dis. 2002;185(7):971-9.

123. Portugal S, Moebius J, Skinner J, Doumbo S, Doumtabe D, Kone Y, et al. Exposure-dependent control of malaria-induced inflammation in children. PLoS Pathog. 2014;10(4):e1004079.

124. Schofield L, Villaquiran J, Ferreira A, Schellekens H, Nussenzweig R, Nussenzweig V. Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. Nature. 1987;330(6149):664-6.

125. Portugal S, Pierce SK, Crompton PD. Young lives lost as B cells falter: what we are learning about antibody responses in malaria. J Immunol. 2013;190(7):3039-46.

126. Crompton PD, Kayala MA, Traore B, Kayentao K, Ongoiba A, Weiss GE, et al. A prospective analysis of the Ab response to *Plasmodium falciparum* before and after a malaria season by protein microarray. Proc Natl Acad Sci U S A. 2010;107(15):6958-63.

127. Felgner PL, Roestenberg M, Liang L, Hung C, Jain A, Pablo J, et al. Pre-erythrocytic antibody profiles induced by controlled human malaria infections in healthy volunteers under chloroquine prophylaxis. Sci Rep. 2013;3:3549.

128. Nahrendorf W, Scholzen A, Bijker EM, Teirlinck AC, Bastiaens GJ, Schats R, et al. Memory B-cell and antibody responses induced by *Plasmodium falciparum* sporozoite immunization. J Infect Dis. 2014;210(12):1981-90.

129. Bijker EM, Teirlinck AC, Schats R, van Gemert GJ, van de Vegte-Bolmer M, van Lieshout L, et al. Cytotoxic markers associate with protection against malaria in human volunteers immunized with *Plasmodium falciparum* sporozoites. J Infect Dis. 2014;210(10):1605-15.

130. Reece WH, Pinder M, Gothard PK, Milligan P, Bojang K, Doherty T, et al. A CD4(+) T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural *Plasmodium falciparum* infection and disease. Nat Med. 2004;10(4):406-10.

131. Carvalho LH, Sano G, Hafalla JC, Morrot A, Curotto de Lafaille MA, Zavala F. IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. Nat Med. 2002;8(2):166-70.

132. Overstreet MG, Chen YC, Cockburn IA, Tse SW, Zavala F. CD4+ T cells modulate expansion and survival but not functional properties of effector and memory CD8+ T cells induced by malaria sporozoites. PLoS One. 2011;6(1):e15948.

133. Renia L, Grillot D, Marussig M, Corradin G, Miltgen F, Lambert PH, et al. Effector functions of circumsporozoite peptide-primed CD4+ T cell clones against *Plasmodium yoelii* liver stages. J Immunol. 1993;150(4):1471-8.

134. Takita-Sonoda Y, Tsuji M, Kamboj K, Nussenzweig RS, Clavijo P, Zavala F. *Plasmodium yoelii*: peptide immunization induces protective CD4+ T cells against a previously unrecognized cryptic epitope of the circumsporozoite protein. Exp Parasitol. 1996;84(2):223-30.

135. Chakravarty S, Cockburn IA, Kuk S, Overstreet MG, Sacci JB, Zavala F. CD8+ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes. Nat Med. 2007;13(9):1035-41.

136. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 1986;136(7):2348-57.

137. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100(6):655-69.

138. Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, et al. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity. 1998;9(5):745-55.

139. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature. 1996;383(6603):787-93.

140. Huang KY, Schultz WW, Gordon FB. Interferon induced by *Plasmodium berghei*. Science. 1968;162(3849):123-4.

141. Langhorne J, Gillard S, Simon B, Slade S, Eichmann K. Frequencies of CD4+ T cells reactive with *Plasmodium chabaudi chabaudi*: distinct response kinetics for cells with Th1 and Th2 characteristics during infection. Int Immunol. 1989;1(4):416-24.

142. Shear HL, Srinivasan R, Nolan T, Ng C. Role of IFN-gamma in lethal and nonlethal malaria in susceptible and resistant murine hosts. J Immunol. 1989;143(6):2038-44.

143. Troye-Blomberg M, Andersson G, Stoczkowska M, Shabo R, Romero P, Patarroyo ME, et al. Production of IL 2 and IFN-gamma by T cells from malaria patients in response to *Plasmodium falciparum* or erythrocyte antigens in vitro. J Immunol. 1985;135(5):3498-504.

144. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. Immunity. 2009;30(5):616-25.

145. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell. 2008;133(5):775-87.

146. Stockinger B, Veldhoen M. Differentiation and function of Th17 T cells. Curr Opin Immunol. 2007;19(3):281-6.

147. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med. 2009;361(9):888-98.

148. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. Immunity. 2009;30(3):324-35.

149. Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol. 2011;29:621-63.

150. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science. 2009;325(5943):1006-10.

151. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. Science. 2009;325(5943):1001-5.

152. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. Science. 1997;276(5312):589-92.

153. Ye BH, Cattoretti G, Shen Q, Zhang J, Hawe N, de Waard R, et al. The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. Nat Genet. 1997;16(2):161-70.

154. Hatzi K, Nance JP, Kroenke MA, Bothwell M, Haddad EK, Melnick A, et al. BCL6 orchestrates Tfh cell differentiation via multiple distinct mechanisms. J Exp Med. 2015;212(4):539-53.

155. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity. 2014;41(4):529-42.

156. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. Annu Rev Immunol. 2008;26:741-66.

157. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. Immunity. 2009;30(5):646-55.

158. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. Science. 2010;327(5969):1098-102.

159. Chattopadhyay PK, Roederer M. A mine is a terrible thing to waste: high content, single cell technologies for comprehensive immune analysis. Am J Transplant. 2015;15(5):1155-61.

160. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. Nat Rev Immunol. 2010;10(3):170-81.

161. Peters A, Lee Y, Kuchroo VK. The many faces of Th17 cells. Curr Opin Immunol. 2011;23(6):702-6.

162. Curtis MM, Rowell E, Shafiani S, Negash A, Urdahl KB, Wilson CB, et al. Fidelity of pathogen-specific CD4+ T cells to the Th1 lineage is controlled by exogenous cytokines, interferon-gamma expression, and pathogen lifestyle. Cell Host Microbe. 2010;8(2):163-73.

163. Cannons JL, Lu KT, Schwartzberg PL. T follicular helper cell diversity and plasticity. Trends Immunol. 2013;34(5):200-7.

164. Weinstein JS, Herman EI, Lainez B, Licona-Limon P, Esplugues E, Flavell R, et al. TFH cells progressively differentiate to regulate the germinal center response. Nat Immunol. 2016;17(10):1197-205.

165. Anderson CF, Oukka M, Kuchroo VJ, Sacks D. CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis. J Exp Med. 2007;204(2):285-97.

166. Jankovic D, Kullberg MC, Feng CG, Goldszmid RS, Collazo CM, Wilson M, et al. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. J Exp Med. 2007;204(2):273-83.

167. Koch MA, Thomas KR, Perdue NR, Smigiel KS, Srivastava S, Campbell DJ. T-bet(+) Treg cells undergo abortive Th1 cell differentiation due to impaired expression of IL-12 receptor beta2. Immunity. 2012;37(3):501-10.

168. Peine M, Rausch S, Helmstetter C, Frohlich A, Hegazy AN, Kuhl AA, et al. Stable T-bet(+)GATA-3(+) Th1/Th2 hybrid cells arise in vivo, can develop directly from naive precursors, and limit immunopathologic inflammation. PLoS Biol. 2013;11(8):e1001633.

169. Carpio VH, Opata MM, Montanez ME, Banerjee PP, Dent AL, Stephens R. IFN-gamma and IL-21 Double Producing T Cells Are Bcl6-Independent and Survive into the Memory Phase in *Plasmodium chabaudi* Infection. PLoS One. 2015;10(12):e0144654.

170. Nakayamada S, Kanno Y, Takahashi H, Jankovic D, Lu KT, Johnson TA, et al. Early Th1 cell differentiation is marked by a Tfh cell-like transition. Immunity. 2011;35(6):919-31.

171. Meding SJ, Cheng SC, Simon-Haarhaus B, Langhorne J. Role of gamma interferon during infection with *Plasmodium chabaudi chabaudi*. Infect Immun. 1990;58(11):3671-8.

172. Sauvaget F, Fauconnier B. The protective effect of endogenous interferon in mouse malaria, as demonstrated by the use of anti-interferon globulins. Biomedicine. 1978;29(6):184-7.

173. Simon-Haarhaus B, Langhorne J, Meding S. CD4+T cell-dependent effector mechanisms important in the immune response to the erythrocytic stages of *Plasmodium chabaudi chabaudi* (AS). Behring Inst Mitt. 1991(88):94-8.

174. Oakley MS, Sahu BR, Lotspeich-Cole L, Solanki NR, Majam V, Pham PT, et al. The transcription factor T-bet regulates parasitemia and promotes pathogenesis during *Plasmodium berghei* ANKA murine malaria. J Immunol. 2013;191(9):4699-708.

175. Stephens R, Langhorne J. Effector memory Th1 CD4 T cells are maintained in a mouse model of chronic malaria. PLoS Pathog. 2010;6(11):e1001208.

176. Luty AJ, Lell B, Schmidt-Ott R, Lehman LG, Luckner D, Greve B, et al. Interferon-gamma responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. J Infect Dis. 1999;179(4):980-8.

177. da Silva HB, de Salles EM, Panatieri RH, Boscardin SB, Rodriguez-Malaga SM, Alvarez JM, et al. IFN-gamma-induced priming maintains long-term strain-transcending immunity against blood-stage *Plasmodium chabaudi* malaria. J Immunol. 2013;191(10):5160-9.

178. Soudja SM, Chandrabos C, Yakob E, Veenstra M, Palliser D, Lauvau G. Memory-T-cell-derived interferon-gamma instructs potent innate cell activation for protective immunity. Immunity. 2014;40(6):974-88.

179. Horne-Debets JM, Faleiro R, Karunarathne DS, Liu XQ, Lineburg KE, Poh CM, et al. PD-1 dependent exhaustion of CD8+ T cells drives chronic malaria. Cell Rep. 2013;5(5):1204-13.

180. Haque A, Best SE, Ammerdorffer A, Desbarrieres L, de Oca MM, Amante FH, et al. Type I interferons suppress CD4(+) T-cell-dependent parasite control during blood-stage *Plasmodium* infection. Eur J Immunol. 2011;41(9):2688-98.

181. Montes de Oca M, Kumar R, Rivera FL, Amante FH, Sheel M, Faleiro RJ, et al. Type I Interferons Regulate Immune Responses in Humans with Blood-Stage *Plasmodium falciparum* Infection. Cell Rep. 2016;17(2):399-412.

182. Oakley MS, Sahu BR, Lotspeich-Cole L, Majam V, Thao Pham P, Sengupta Banerjee A, et al. T-bet modulates the antibody response and immune protection during murine malaria. Eur J Immunol. 2014;44(9):2680-91.

183. Findlay EG, Greig R, Stumhofer JS, Hafalla JC, de Souza JB, Saris CJ, et al. Essential role for IL-27 receptor signaling in prevention of Th1-mediated immunopathology during malaria infection. J Immunol. 2010;185(4):2482-92.

184. Villegas-Mendez A, de Souza JB, Lavelle SW, Gwyer Findlay E, Shaw TN, van Rooijen N, et al. IL-27 receptor signalling restricts the formation of pathogenic, terminally differentiated Th1 cells during malaria infection by repressing IL-12 dependent signals. PLoS Pathog. 2013;9(4):e1003293.

185. Jagannathan P, Eccles-James I, Bowen K, Nankya F, Auma A, Wamala S, et al. IFNgamma/IL-10 co-producing cells dominate the CD4 response to malaria in highly exposed children. PLoS Pathog. 2014;10(1):e1003864.

186. Boyle MJ, Jagannathan P, Bowen K, McIntyre TI, Vance HM, Farrington LA, et al. The Development of *Plasmodium falciparum*-Specific IL10 CD4 T Cells and Protection from Malaria in Children in an Area of High Malaria Transmission. Front Immunol. 2017;8:1329.

187. Montes de Oca M, Kumar R, de Labastida Rivera F, Amante FH, Sheel M, Faleiro RJ, et al. Blimp-1-Dependent IL-10 Production by Tr1 Cells Regulates TNF-Mediated Tissue Pathology. PLoS Pathog. 2016;12(1):e1005398.

188. Neumann C, Heinrich F, Neumann K, Junghans V, Mashreghi MF, Ahlers J, et al. Role of Blimp-1 in programing Th effector cells into IL-10 producers. J Exp Med. 2014;211(9):1807-19.

189. Parish IA, Marshall HD, Staron MM, Lang PA, Brustle A, Chen JH, et al. Chronic viral infection promotes sustained Th1-derived immunoregulatory IL-10 via BLIMP-1. J Clin Invest. 2014;124(8):3455-68.

190. Saraiva M, Christensen JR, Veldhoen M, Murphy TL, Murphy KM, O'Garra A. Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. Immunity. 2009;31(2):209-19.

191. Spolski R, Kashyap M, Robinson C, Yu Z, Leonard WJ. IL-21 signaling is critical for the development of type I diabetes in the NOD mouse. Proc Natl Acad Sci U S A. 2008;105(37):14028-33.

192. Pot C, Jin H, Awasthi A, Liu SM, Lai CY, Madan R, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that

coordinately act together to promote differentiation of IL-10-producing Tr1 cells. J Immunol. 2009;183(2):797-801.

193. Apetoh L, Quintana FJ, Pot C, Joller N, Xiao S, Kumar D, et al. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. Nat Immunol. 2010;11(9):854-61.

194. Ansari NA, Kumar R, Gautam S, Nylen S, Singh OP, Sundar S, et al. IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral leishmaniasis. J Immunol. 2011;186(7):3977-85.

195. Sun J, Dodd H, Moser EK, Sharma R, Braciale TJ. CD4+ T cell help and innate-derived IL-27 induce Blimp-1-dependent IL-10 production by antiviral CTLs. Nat Immunol. 2011;12(4):327-34.

196. Stumhofer JS, Silver JS, Laurence A, Porrett PM, Harris TH, Turka LA, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. Nat Immunol. 2007;8(12):1363-71.

197. Hwang W, Lee CG, Lee C, Verma R, Rudra D, Park ZY, et al. Locus-Specific Reversible DNA Methylation Regulates Transient IL-10 Expression in Th1 Cells. J Immunol. 2018;200(5):1865-75.

198. Langhorne J, Cross C, Seixas E, Li C, von der Weid T. A role for B cells in the development of T cell helper function in a malaria infection in mice. Proc Natl Acad Sci U S A. 1998;95(4):1730-4.

199. Taylor-Robinson AW, Phillips RS. B cells are required for the switch from Th1- to Th2-regulated immune responses to *Plasmodium chabaudi chabaudi* infection. Infect Immun. 1994;62(6):2490-8.

200. Troye-Blomberg M, Riley EM, Kabilan L, Holmberg M, Perlmann H, Andersson U, et al. Production by activated human T cells of interleukin 4 but not interferon-gamma is associated with elevated levels of serum antibodies to activating malaria antigens. Proc Natl Acad Sci U S A. 1990;87(14):5484-8.

201. von der Weid T, Kopf M, Kohler G, Langhorne J. The immune response to *Plasmodium chabaudi* malaria in interleukin-4-deficient mice. Eur J Immunol. 1994;24(10):2285-93.

202. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J Exp Med. 2000;192(11):1553-62.

203. Portugal S, Doumtabe D, Traore B, Miller LH, Troye-Blomberg M, Doumbo OK, et al. B cell analysis of ethnic groups in Mali with differential susceptibility to malaria. Malar J. 2012;11:162.

204. Weiss GE, Crompton PD, Li S, Walsh LA, Moir S, Traore B, et al. Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. J Immunol. 2009;183(3):2176-82.

205. Portugal S, Tipton CM, Sohn H, Kone Y, Wang J, Li S, et al. Malaria-associated atypical memory B cells exhibit markedly reduced B cell receptor signaling and effector function. Elife. 2015;4.

206. Sullivan RT, Kim CC, Fontana MF, Feeney ME, Jagannathan P, Boyle MJ, et al. FCRL5 Delineates Functionally Impaired Memory B Cells Associated with *Plasmodium falciparum* Exposure. PLoS Pathog. 2015;11(5):e1004894.

207. Butler NS, Moebius J, Pewe LL, Traore B, Doumbo OK, Tygrett LT, et al. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage *Plasmodium* infection. Nat Immunol. 2011;13(2):188-95.

208. Zander RA, Obeng-Adjei N, Guthmiller JJ, Kulu DI, Li J, Ongoiba A, et al. PD-1 Co-inhibitory and OX40 Co-stimulatory Crosstalk Regulates Helper T Cell Differentiation and Anti-*Plasmodium* Humoral Immunity. Cell Host Microbe. 2015;17(5):628-41.

209. Kurup SP, Obeng-Adjei N, Anthony SM, Traore B, Doumbo OK, Butler NS, et al. Regulatory T cells impede acute and long-term immunity to blood-stage malaria through CTLA-4. Nat Med. 2017;23(10):1220-5.

210. Balasubramani A, Mukasa R, Hatton RD, Weaver CT. Regulation of the Ifng locus in the context of T-lineage specification and plasticity. Immunol Rev. 2010;238(1):216-32.

211. Perez-Mazliah D, Ng DH, Freitas do Rosario AP, McLaughlin S, Mastelic-Gavillet B, Sodenkamp J, et al. Disruption of IL-21 signaling affects T cell-B cell interactions and abrogates protective humoral immunity to malaria. PLoS Pathog. 2015;11(3):e1004715.

212. Perez-Mazliah D, Nguyen MP, Hosking C, McLaughlin S, Lewis MD, Tumwine I, et al. Follicular Helper T Cells are Essential for the Elimination of *Plasmodium* Infection. EBioMedicine. 2017;24:216-30.

213. Crawford A, Angelosanto JM, Kao C, Doering TA, Odorizzi PM, Barnett BE, et al. Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. Immunity. 2014;40(2):289-302.

214. Fahey LM, Wilson EB, Elsaesser H, Fistonich CD, McGavern DB, Brooks DG. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. J Exp Med. 2011;208(5):987-99.

215. Schulz EG, Mariani L, Radbruch A, Hofer T. Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. Immunity. 2009;30(5):673-83.

216. Tubo NJ, Pagan AJ, Taylor JJ, Nelson RW, Linehan JL, Ertelt JM, et al. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. Cell. 2013;153(4):785-96.

217. Ray JP, Staron MM, Shyer JA, Ho PC, Marshall HD, Gray SM, et al. The Interleukin-2-mTORc1 Kinase Axis Defines the Signaling, Differentiation, and Metabolism of T Helper 1 and Follicular B Helper T Cells. Immunity. 2015;43(4):690-702.

218. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity. 2008;29(1):138-49.

219. Ray JP, Marshall HD, Laidlaw BJ, Staron MM, Kaech SM, Craft J. Transcription factor STAT3 and type I interferons are corepressive insulators for differentiation of follicular helper and T helper 1 cells. Immunity. 2014;40(3):367-77.

220. de Wit J, Jorritsma T, Makuch M, Remmerswaal EB, Klaasse Bos H, Souwer Y, et al. Human B cells promote T-cell plasticity to optimize antibody response by inducing coexpression of T(H)1/T(FH) signatures. J Allergy Clin Immunol. 2015;135(4):1053-60.

221. Oestreich KJ, Huang AC, Weinmann AS. The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns. J Exp Med. 2011;208(5):1001-13.

222. Oestreich KJ, Mohn SE, Weinmann AS. Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. Nat Immunol. 2012;13(4):405-11.

223. Ryg-Cornejo V, Ioannidis LJ, Ly A, Chiu CY, Tellier J, Hill DL, et al. Severe Malaria Infections Impair Germinal Center Responses by Inhibiting T Follicular Helper Cell Differentiation. Cell Rep. 2016;14(1):68-81.

224. Obeng-Adjei N, Portugal S, Tran TM, Yazew TB, Skinner J, Li S, et al. Circulating Th1-Cell-type Tfh Cells that Exhibit Impaired B Cell Help Are Preferentially Activated during Acute Malaria in Children. Cell Rep. 2015;13(2):425-39.

225. Obeng-Adjei N, Portugal S, Holla P, Li S, Sohn H, Ambegaonkar A, et al. Malaria-induced interferon-gamma drives the expansion of Tbethi atypical memory B cells. PLoS Pathog. 2017;13(9):e1006576.

226. McCarthy JS, Good MF. Whole parasite blood stage malaria vaccines: a convergence of evidence. Hum Vaccin. 2010;6(1):114-23.

227. Pinzon-Charry A, McPhun V, Kienzle V, Hirunpetcharat C, Engwerda C, McCarthy J, et al. Low doses of killed parasite in CpG elicit vigorous CD4+ T cell responses against blood-stage malaria in mice. J Clin Invest. 2010;120(8):2967-78.

228. Salles EM, Menezes MN, Siqueira R, Borges da Silva H, Amaral EP, Castillo-Mendez SI, et al. P2X7 receptor drives Th1 cell differentiation and controls the follicular helper T cell population to protect against *Plasmodium chabaudi* malaria. PLoS Pathog. 2017;13(8):e1006595.

229. Ibitokou SA, Dillon BE, Sinha M, Szczesny B, Delgadillo A, Reda Abdelrahman D, et al. Early Inhibition of Fatty Acid Synthesis Reduces Generation of Memory Precursor Effector T Cells in Chronic Infection. J Immunol. 2018;200(2):643-56.

230. Sponaas AM, Belyaev N, Falck-Hansen M, Potocnik A, Langhorne J. Transient deficiency of dendritic cells results in lack of a merozoite surface protein 1-specific CD4 T cell

response during peak *Plasmodium chabaudi* blood-stage infection. Infect Immun. 2012;80(12):4248-56.

231. Hahn WO, Butler NS, Lindner SE, Akilesh HM, Sather DN, Kappe SH, et al. cGAS-mediated control of blood-stage malaria promotes *Plasmodium*-specific germinal center responses. JCI Insight. 2018;3(2).

232. James KR, Soon MSF, Sebina I, Fernandez-Ruiz D, Davey G, Liligeto UN, et al. IFN Regulatory Factor 3 Balances Th1 and T Follicular Helper Immunity during Nonlethal Blood-Stage *Plasmodium* Infection. J Immunol. 2018;200(4):1443-56.

233. Su Z, Stevenson MM. IL-12 is required for antibody-mediated protective immunity against blood-stage *Plasmodium chabaudi* AS malaria infection in mice. J Immunol. 2002;168(3):1348-55.

234. Perez-Mazliah D, Langhorne J. CD4 T-cell subsets in malaria: TH1/TH2 revisited. Front Immunol. 2014;5:671.

235. Stumhofer JS, Hunter CA. Advances in understanding the anti-inflammatory properties of IL-27. Immunol Lett. 2008;117(2):123-30.

236. Batten M, Ramamoorthi N, Kljavin NM, Ma CS, Cox JH, Dengler HS, et al. IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells. J Exp Med. 2010;207(13):2895-906.

237. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. PLoS One. 2011;6(3):e17739.

238. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. J Immunol. 2012;188(8):3734-44.

239. Wurster AL, Rodgers VL, Satoskar AR, Whitters MJ, Young DA, Collins M, et al. Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon gamma-producing Th1 cells. J Exp Med. 2002;196(7):969-77.

240. Luthje K, Kallies A, Shimohakamada Y, Belz GT, Light A, Tarlinton DM, et al. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. Nat Immunol. 2012;13(5):491-8.

241. Hollister K, Kusam S, Wu H, Clegg N, Mondal A, Sawant DV, et al. Insights into the role of Bcl6 in follicular Th cells using a new conditional mutant mouse model. J Immunol. 2013;191(7):3705-11.

242. Choi YS, Yang JA, Yusuf I, Johnston RJ, Greenbaum J, Peters B, et al. Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory. J Immunol. 2013;190(8):4014-26.

243. Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. Cytometry A. 2011;79(2):167-74.

244. Achtman AH, Stephens R, Cadman ET, Harrison V, Langhorne J. Malaria-specific antibody responses and parasite persistence after infection of mice with *Plasmodium chabaudi chabaudi*. Parasite Immunol. 2007;29(9):435-44.

245. Harrington LE, Janowski KM, Oliver JR, Zajac AJ, Weaver CT. Memory CD4 T cells emerge from effector T-cell progenitors. Nature. 2008;452(7185):356-60.

246. Shah S, Grotenbreg GM, Rivera A, Yap GS. An extrafollicular pathway for the generation of effector CD8(+) T cells driven by the proinflammatory cytokine, IL-12. Elife. 2015;4.

247. Djuretic IM, Levanon D, Negreanu V, Groner Y, Rao A, Ansel KM. Transcription factors T-bet and Runx3 cooperate to activate *Ifng* and silence *Il4* in T helper type 1 cells. Nat Immunol. 2007;8(2):145-53.

248. Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. J Immunol. 2007;179(8):5099-108.

249. Reiley WW, Shafiani S, Wittmer ST, Tucker-Heard G, Moon JJ, Jenkins MK, et al. Distinct functions of antigen-specific CD4 T cells during murine *Mycobacterium tuberculosis* infection. Proc Natl Acad Sci U S A. 2010;107(45):19408-13.

250. McLane LM, Banerjee PP, Cosma GL, Makedonas G, Wherry EJ, Orange JS, et al. Differential localization of T-bet and Eomes in CD8 T cell memory populations. J Immunol. 2013;190(7):3207-15.

251. Mascle X, Albagli O, Lemercier C. Point mutations in BCL6 DNA-binding domain reveal distinct roles for the six zinc fingers. Biochem Biophys Res Commun. 2003;300(2):391-6.

252. Gazzinelli RT, Wysocka M, Hieny S, Scharton-Kersten T, Cheever A, Kuhn R, et al. 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. 1996;157(2):798-805.

253. Hale JS, Youngblood B, Latner DR, Mohammed AU, Ye L, Akondy RS, et al. Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. Immunity. 2013;38(4):805-17.

254. Park AY, Hondowicz BD, Scott P. IL-12 is required to maintain a Th1 response during *Leishmania major* infection. J Immunol. 2000;165(2):896-902.

255. Stobie L, Gurunathan S, Prussin C, Sacks DL, Glaichenhaus N, Wu CY, et al. The role of antigen and IL-12 in sustaining Th1 memory cells in vivo: IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge. Proc Natl Acad Sci U S A. 2000;97(15):8427-32.

256. Yap G, Pesin M, Sher A. Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, *Toxoplasma gondii*. J Immunol. 2000;165(2):628-31.

257. Olson JA, McDonald-Hyman C, Jameson SC, Hamilton SE. Effector-like CD8(+) T cells in the memory population mediate potent protective immunity. Immunity. 2013;38(6):1250-60.

258. Scott P, Artis D, Uzonna J, Zaph C. The development of effector and memory T cells in cutaneous leishmaniasis: the implications for vaccine development. Immunol Rev. 2004;201:318-38.

259. Zaph C, Rook KA, Goldschmidt M, Mohrs M, Scott P, Artis D. Persistence and function of central and effector memory CD4+ T cells following infection with a gastrointestinal helminth. J Immunol. 2006;177(1):511-8.

260. Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. Science. 2009;324(5934):1569-72.

261. Frohlich A, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, et al. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. Science. 2009;324(5934):1576-80.

262. Weinmann AS. Regulatory mechanisms that control T-follicular helper and T-helper 1 cell flexibility. Immunol Cell Biol. 2014;92(1):34-9.

263. Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. Science. 2009;324(5934):1572-6.

264. Bouneaud C, Garcia Z, Kourilsky P, Pannetier C. Lineage relationships, homeostasis, and recall capacities of central- and effector-memory CD8 T cells in vivo. J Exp Med. 2005;201(4):579-90.

265. Hogan RJ, Cauley LS, Ely KH, Cookenham T, Roberts AD, Brennan JW, et al. Long-term maintenance of virus-specific effector memory CD8+ T cells in the lung airways depends on proliferation. J Immunol. 2002;169(9):4976-81.

266. Roetynck S, Olotu A, Simam J, Marsh K, Stockinger B, Urban B, et al. Phenotypic and functional profiling of CD4 T cell compartment in distinct populations of healthy adults with different antigenic exposure. PLoS One. 2013;8(1):e55195.

267. Mewono L, Matondo Maya DW, Matsiegui PB, Agnandji ST, Kendjo E, Barondi F, et al. Interleukin-21 is associated with IgG1 and IgG3 antibodies to erythrocyte-binding antigen-175 peptide 4 of *Plasmodium falciparum* in Gabonese children with acute falciparum malaria. Eur Cytokine Netw. 2008;19(1):30-6.

268. Monteleone G, Monteleone I, Fina D, Vavassori P, Del Vecchio Blanco G, Caruso R, et al. Interleukin-21 enhances T-helper cell type I signaling and interferon-gamma production in Crohn's disease. Gastroenterology. 2005;128(3):687-94.

269. Sarra M, Monteleone I, Stolfi C, Fantini MC, Sileri P, Sica G, et al. Interferon-gamma-expressing cells are a major source of interleukin-21 in inflammatory bowel diseases. Inflamm Bowel Dis. 2010;16(8):1332-9.

270. Strengell M, Sareneva T, Foster D, Julkunen I, Matikainen S. IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. J Immunol. 2002;169(7):3600-5.

271. Suto A, Wurster AL, Reiner SL, Grusby MJ. IL-21 inhibits IFN-gamma production in developing Th1 cells through the repression of Eomesodermin expression. J Immunol. 2006;177(6):3721-7.

272. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. Nat Immunol. 2010;11(2):114-20.

273. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature. 2007;448(7152):480-3.

274. Chacon-Salinas R, Limon-Flores AY, Chavez-Blanco AD, Gonzalez-Estrada A, Ullrich SE. Mast cell-derived IL-10 suppresses germinal center formation by affecting T follicular helper cell function. J Immunol. 2011;186(1):25-31.

275. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. Nat Immunol. 2007;8(4):369-77.

276. Xu H, Hodder AN, Yan H, Crewther PE, Anders RF, Good MF. CD4+ T cells acting independently of antibody contribute to protective immunity to *Plasmodium chabaudi* infection after apical membrane antigen 1 immunization. J Immunol. 2000;165(1):389-96.

277. Jiang S, Dong C. A complex issue on CD4(+) T-cell subsets. Immunol Rev. 2013;252(1):5-11.

278. Kane A, Deenick EK, Ma CS, Cook MC, Uzel G, Tangye SG. STAT3 is a central regulator of lymphocyte differentiation and function. Curr Opin Immunol. 2014;28:49-57.

279. Diehl S, Anguita J, Hoffmeyer A, Zapton T, Ihle JN, Fikrig E, et al. Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. Immunity. 2000;13(6):805-15.

280. Cimmino L, Martins GA, Liao J, Magnusdottir E, Grunig G, Perez RK, et al. Blimp-1 attenuates Th1 differentiation by repression of *ifng*, *tbx21*, and *bcl6* gene expression. J Immunol. 2008;181(4):2338-47.

281. Tubo NJ, Jenkins MK. TCR signal quantity and quality in CD4+ T cell differentiation. Trends Immunol. 2014;35(12):591-6.

282. Thong YH, Ferrante A, Rowan-Kelly B, O'Keefe DE. Effect of mefloquine on the immune response in mice. Trans R Soc Trop Med Hyg. 1979;73(4):388-90.

283. Choi YS, Eto D, Yang JA, Lao C, Crotty S. Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. J Immunol. 2013;190(7):3049-53.

284. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. J Exp Med. 2012;209(2):243-50.

285. Nurieva RI, Podd A, Chen Y, Alekseev AM, Yu M, Qi X, et al. STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. J Biol Chem. 2012;287(14):11234-9.

286. Schmitt N, Ueno H. Regulation of human helper T cell subset differentiation by cytokines. Curr Opin Immunol. 2015;34:130-6.

287. Hunt P, Cravo PV, Donleavy P, Carlton JM, Walliker D. Chloroquine resistance in *Plasmodium chabaudi*: are chloroquine-resistance transporter (crt) and multi-drug resistance (mdr1) orthologues involved? Mol Biochem Parasitol. 2004;133(1):27-35.

288. McDermott DS, Varga SM. Quantifying antigen-specific CD4 T cells during a viral infection: CD4 T cell responses are larger than we think. J Immunol. 2011;187(11):5568-76.

289. Murphy KM, Stockinger B. Effector T cell plasticity: flexibility in the face of changing circumstances. Nat Immunol. 2010;11(8):674-80.

290. Gabrysova L, Wraith DC. Antigenic strength controls the generation of antigen-specific IL-10-secreting T regulatory cells. Eur J Immunol. 2010;40(5):1386-95.

291. Read KA, Powell MD, Baker CE, Sreekumar BK, Ringel-Scaia VM, Bachus H, et al. Integrated STAT3 and Ikaros Zinc Finger Transcription Factor Activities Regulate Bcl-6 Expression in CD4(+) Th Cells. J Immunol. 2017;199(7):2377-87.

292. Wu H, Xu LL, Teuscher P, Liu H, Kaplan MH, Dent AL. An Inhibitory Role for the Transcription Factor Stat3 in Controlling IL-4 and Bcl6 Expression in Follicular Helper T Cells. J Immunol. 2015;195(5):2080-9.

293. Ma CS, Avery DT, Chan A, Batten M, Bustamante J, Boisson-Dupuis S, et al. Functional STAT3 deficiency compromises the generation of human T follicular helper cells. Blood. 2012;119(17):3997-4008.

294. Pombo DJ, Lawrence G, Hirunpetcharat C, Rzepczyk C, Bryden M, Cloonan N, et al. Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. Lancet. 2002;360(9333):610-7.

295. Grun JL, Weidanz WP. Antibody-independent immunity to reinfection malaria in B-cell-deficient mice. Infect Immun. 1983;41(3):1197-204.

296. Zander RA, Vijay R, Pack AD, Guthmiller JJ, Graham AC, Lindner SE, et al. Th1-like *Plasmodium*-Specific Memory CD4(+) T Cells Support Humoral Immunity. Cell Rep. 2017;21(7):1839-52.

297. Balasubramani A, Shibata Y, Crawford GE, Baldwin AS, Hatton RD, Weaver CT. Modular utilization of distal cis-regulatory elements controls *Ifng* gene expression in T cells activated by distinct stimuli. Immunity. 2010;33(1):35-47.

298. Augustijn KD, Kleemann R, Thompson J, Kooistra T, Crawford CE, Reece SE, et al. Functional characterization of the *Plasmodium falciparum* and *P. berghei* homologues of macrophage migration inhibitory factor. Infect Immun. 2007;75(3):1116-28.

299. Sun T, Holowka T, Song Y, Zierow S, Leng L, Chen Y, et al. A *Plasmodium*-encoded cytokine suppresses T-cell immunity during malaria. Proc Natl Acad Sci U S A. 2012;109(31):E2117-26.

300. Amante FH, Good MF. Prolonged Th1-like response generated by a *Plasmodium yoelii*-specific T cell clone allows complete clearance of infection in reconstituted mice. Parasite Immunol. 1997;19(3):111-26.

301. Hansen DS, Obeng-Adjei N, Ly A, Ioannidis LJ, Crompton PD. Emerging concepts in T follicular helper cell responses to malaria. Int J Parasitol. 2017;47(2-3):105-10.

302. Anstey NM, Weinberg JB, Hassanali MY, Mwaikambo ED, Manyenga D, Misukonis MA, et al. Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. J Exp Med. 1996;184(2):557-67.

303. Gyan B, Troye-Blomberg M, Perlmann P, Bjorkman A. Human monocytes cultured with and without interferon-gamma inhibit *Plasmodium falciparum* parasite growth in vitro via secretion of reactive nitrogen intermediates. Parasite Immunol. 1994;16(7):371-5.

304. Weinberg JB, Misukonis MA, Shami PJ, Mason SN, Sauls DL, Dittman WA, et al. Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. Blood. 1995;86(3):1184-95.

305. Yu D, Vinuesa CG. The elusive identity of T follicular helper cells. Trends Immunol. 2010;31(10):377-83.

306. Vinuesa CG, Cyster JG. How T cells earn the follicular rite of passage. Immunity. 2011;35(5):671-80.

307. Achtman AH, Khan M, MacLennan IC, Langhorne J. *Plasmodium chabaudi chabaudi* infection in mice induces strong B cell responses and striking but temporary changes in splenic cell distribution. J Immunol. 2003;171(1):317-24.

308. Li L, Jiang Y, Lao S, Yang B, Yu S, Zhang Y, et al. *Mycobacterium tuberculosis*-Specific IL-21+IFN-gamma+CD4+ T Cells Are Regulated by IL-12. PLoS One. 2016;11(1):e0147356.

309. Rutishauser RL, Martins GA, Kalachikov S, Chandele A, Parish IA, Meffre E, et al. Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. Immunity. 2009;31(2):296-308.

310. Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, Wei L, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. Immunity. 2010;32(5):605-15.

311. Cui W, Liu Y, Weinstein JS, Craft J, Kaech SM. An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. Immunity. 2011;35(5):792-805.

312. Hiramatsu Y, Suto A, Kashiwakuma D, Kanari H, Kagami S, Ikeda K, et al. c-Maf activates the promoter and enhancer of the IL-21 gene, and TGF-beta inhibits c-Maf-induced IL-21 production in CD4+ T cells. J Leukoc Biol. 2010;87(4):703-12.

313. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of naive human CD4(+) T cells to the T follicular helper (T(FH)) cell lineage is induced by IL-12. Immunol Cell Biol. 2009;87(8):590-600.

VITA

Victor Hugo Carpio Rojas was born in Lima, Perú on March 31, 1983 to Victor M. Carpio and Luz E. Rojas. He completed his undergraduate degree in Science with mention in Biology at Universidad Peruana Cayetano Heredia (UPCH), in Lima, Peru in 2004. In 2005, he worked at the Tropical Medicine Institute "Alexander von Humboldt". In 2010, he joined Dr. Stephens laboratory at UTMB as a Research Associate. In 2013, he joined the Ph.D. program in the Department of Microbiology and Immunology at UTMB. During his time at UTMB, Victor was recognized by different awards such as Robert Shope Endowed Scholarship (2015), Arun Kumar Chopra, Nirmal Rani Chopra and Dharan Dev Chopra Memorial Scholarship (2106), and Harold T. Sander Endowed Fellowship in Vaccine Development (2017). He was supported by the diversity supplement to Dr. Stephens' R01 (3 R01 AI089953 04S1) from 2014 to 2016, the James W. McLaughlin Pre-doctoral Fellowship from 2016 to 2017, and by the Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship (F31 – 1 F31 AI126809-01A1) from 2017 to 2018.

Victor H. Carpio Rojas can be contacted through his wife Charlotte H. Rivas at 8300 Red Bluff Rd. #1527 Pasadena, Texas, 77507.

This dissertation was typed by Victor H. Carpio Rojas.

EDUCATION

B.S. (Science with mention in Biology), October 2004. UPCH, Lima – Perú.

PUBLICATIONS

Victor H. Carpio, Michael M. Opata, Marelle E. Montañez, Pinaki P. Banerjee, Alexander L. Dent, and Robin Stephens. IFN-g and IL-21 Double Producing T cells are Bcl6-Independent and Survive into the Memory Phase in *Plasmodium chabaudi* Infection. *PLOS ONE 10(12): 0144654. https://doi.org/10.1371/journal.pone.0144654*

Victor H. Carpio, Kyle D. Wilson, Alexander L. Dent and Robin Stephens. Transcription Factor STAT3 Regulates Differentiation of T Helper 1 Memory Cells in Persistent Mouse Malaria Infection. *Journal of Immunology* 2018 (*manuscript submitted*)

Michael M. Opata, **Victor H. Carpio**, Samad A. Ibitokou, Brian E. Dillon, Joshua M. Obiero, and Robin Stephens. Early Effector Cells Survive the Contraction Phase in Malaria Infection and Generate Both Central and Effector Memory T cells. *J Immunol* 2015; 194:5346-5354.

Michael M. Opata, Samad A. Ibitokou, **Victor H. Carpio**, Karis M. Marshall, Brian E. Dillon, Jordan C. Carl, Kyle D. Wilson, Christine M. Arcari and Robin Stephens. Protection by and maintenance of CD4 effector memory and effector T cell subsets in persistent malaria infection. *PLOS Pathogens 14(4): e1006960. https://doi.org/10.1371/journal.ppat.1006960*

Kyle D. Wilson, Lorenzo F. Ochoa, Olivia D. Solomon, Rahul Pal, Sandra M. Cardona, **Victor H. Carpio**, Philip H. Keiser, Astrid E. Cardona, Gracie Vargas, Robin Stephens. Elimination of Intravascular Thrombi Prevents Early Mortality and Reduces Gliosis in Hyper-inflammatory Experimental Cerebral Malaria. *Journal of Neuroinflammation* 2018; 15:173 https://doi.org/10.1186/s12974-018-1207-4

Mingming Sun, Wei Wu, Liang Chen, Xiangsheng Huang, Caiyun Ma, Feidi Chen, Yi Xiao, Ye Zhao, Chunyan Ma, Suxia Yao, **Victor H. Carpio**, Robin Stephens, Sara M. Dann, Qihong Zhao, Zhanju Liu, and Yingzi Cong. Microbiota-derived short-chain fatty acids promote Th1 cell IL-10 production to maintain intestinal homeostasis. *Nature Communications* 2018 (*manuscript submitted*)

Inaia Phoenix, Shoko Nishiyama, Nandadeva Lokugamage, Terence E. Hill, Matthew B. Huante, Olga A.L. Slack, **Victor H. Carpio**, Allexander N. Freiberg, and Tetsuro Ikegami. N-Glycans on the Rift Valley Virus Envelope Glycoproteins Gn and Gc Redundantly Support Viral Infection via DC-SIGN. *Viruses* 2016; 8(5), 149. doi:10.3390/v8050149.

ABSTRACTS

Victor H. Carpio, Kyle D. Wilson, Alexander L. Dent, and Robin Stephens. Transcription factor STAT3 and parasite exposure regulate differentiation of protective T helper 1 cells in malaria infection. World Vaccine & Immunotherapy Congress in San Diego, California. Nov 30th – Dec 1st, 2017

Victor H. Carpio, Kyle D. Wilson, Gabriela M. Kaus, Alexander L. Dent, and Robin Stephens. Bcl6 and STAT3 orchestrate generation of IFN-g^{+}IL-21^{+} CD4 memory T cells during chronic mouse malaria infection. Gordon Research Conference on Tropical Infectious Diseases in Galveston, Texas. March 13^{th} – 17^{th}, 2017.

Victor H. Carpio, Michael M. Opata, Marelle E. Montañez , Alexander L. Dent , Pinaki P. Banerjee , and Robin Stephens. Characterization of mixed Th1/Tfh T cells in chronic malaria infection using multicolor Flowcytometry and SPICE analysis. FlowTex 2017 in Houston, Texas. February 7^{th}-8^{th}, 2017. (***Oral Presentation winner***)

Victor H. Carpio, Michael M. Opata , Marelle E. Montañez , Alexander L. Dent , Pinaki P. Banerjee , and Robin Stephens. IFN-g and IL-21 Double Producing T cells are Bcl6-Independent and Survive into the Memory Phase in *Plasmodium chabaudi* Infection. Keystone Symposia, Cell Biology and Immunology of Persistent Infection in Banff, Alberta, Canada. Jan 31^{st} – Feb 5^{th} 2016. (***Oral Presentation winner***)

Victor H. Carpio, Michael M. Opata , Marelle E. Montañez , Alexander L. Dent , Pinaki P. Banerjee , and Robin Stephens. Protective Il-10-producing Th1 cells also make Il-21, which is regulated by Il-10 in a model of chronic malaria (MPF7P.709). IMMUNOLOGY 2015 American Association of Immunologists in New Orleans, Louisiana. May 8^{th}-12^{th}, 2015.

Victor H. Carpio, Marelle Montañez, Michael M. Opata, and Robin Stephens. Memory CD4 T cells in *Plasmodium chabaudi* infection are not fully differentiated Th1 cells. Keystone Symposia "Emerging topics in Immune System Plasticity" in Santa Fe, New Mexico. Jan 15^{th}-20^{th}, 2013.