The Role of $CD4^+$ $Foxp3^+$ Naturally-Occurring Regulatory T Cells in the Host Immune Response to *Plasmodium chabaudi* AS

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ABSTRACT

Naturally-occurring $CD4^+Foxp3^+$ regulatory T cells (nT_{reg}) play a central role in maintaining immune self-tolerance as well as modulating immunity towards pathogens. Pathogens may establish chronic infections in immunocompetent hosts by engaging nT_{reg} in order to promote immunosuppression. The goal of the research described here is to test the hypothesis that nT_{reg} modulate protective immunity to malaria, and consequentially affect susceptibility to the parasite. To investigate this question, the functional dynamics of $CD4^+Foxp3^+$ nTreg cells were evaluated in mice infected with blood-stage *Plasmodium chabaudi* AS. Adoptive transfer of nT_{reg} to infected wild-type C57BL/6 (B6) mice or infection of transgenic B6 mice over-expressing Foxp3 resulted in increased parasitemia and reduced survival compared to control mice. Moreover, while resistant B6 mice exhibited decreased splenic nT_{reg} frequencies at day 7 post infection, susceptible A/J mice maintained high numbers of nT_{reg} at this time. Investigation of the effects of nT_{reg} regulation on immune cell function in *P. chabaudi* AS-infected mice revealed that increased nT_{reg} frequencies led to decreased malaria-specific lymphoproliferation and increased systemic levels of IL-10. Unlike B6 mice, increased splenic nT_{reg} frequencies in infected A/J mice correlated with decreased effector T cell proliferation and IFN-γ secretion, decreased B cell and NK cell proliferation as well as deficient IFN-γ secretion by NK cells. Finally, nT_{reg} proliferated within infected sites in both B6 and A/J mice, albeit to a greater extent in susceptible A/J mice. Altogether, these results demonstrate that nT_{reg} suppressed anti-malarial immunity, and in turn promoted parasite growth and persistence.

ABRÉGÉ

Les cellules T régulatrices naturelles $CD4^+Foxp3^+$ (nT_{reg}) jouent un rôle essentiel dans la tolérance immunitaire et sont impliquées dans le contrôle de la réponse immunitaire contre les pathogènes. De nombreux pathogènes peuvent établir une infection chronique chez leur hôte immunocompétent en recrutant les nT_{reg} afin de promouvoir l'immunosuppression. Dans cette étude, nous avons postulé que les nT_{reg} suppriment la réponse immunitaire anti-paludique et ainsi promeuvent la survie du pathogène. La fonction des nT_{reg} a été évaluée chez des souris infectées par le pathogène paludique, *Plasmodium chabaudi* AS. Le transfert adoptif de nT_{reg} dans des souris C57BL/6 (B6) infectées, ou l'infection de souris B6 transgéniques sur-exprimant Foxp3, a eu comme conséquences d'accroître la parasitémie et de décroître la survie de ces souris par rapport à des souris contrôle. De plus, alors que les souris résistantes B6 ont montré une diminution de la fréquence des nT_{reg} dans la rate, les souris susceptibles A/J ont maintenu une fréquence élevée de nT_{reg} 7 jours après l'infection avec *P. chabaudi* AS. L'analyse des effets régulateurs des nT_{reg} sur la fonction des cellules immunitaires dans les souris infectées par *P. chabaudi* AS a révélé que des fréquences accrues en nT_{reg} décroissent la réponse proliférative anti-paludique des lymphocytes et accroît le niveau systémique d'IL-10. À la différence des souris B6, les fréquences accrues de nT_{reg} chez les souris infectées A/J corrèlent avec une diminution de la prolifération et de la sécrétion d'IFN-γ par les cellules T effectrices, avec une réduction de la prolifération des cellules B et NK ainsi qu'une production réduite d'IFN-γ par les cellules NK. Finalement, les nT_{reg} ont proliféré spécifiquement dans la rate chez les souris infectées B6 et A/J, quoiqu'à un plus haut degré chez les souris susceptibles A/J. Enfin, ces résultats démontrent que les nT_{reg} paralysent l'immunité anti-paludique, et ainsi, favorisent la croissance et la persistance du parasite.

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
B6	C57BL/6
CCR	Chemokine (C-C motif) receptor
ConA	Concanavalin A
CSA	Chondroitic sulphate A
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
CXCL10	Chemokine (C-X-C motif) ligand 10
DC	Dendritic cells
DTH	Delayed-type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
FACS	Fluorescence activated cell sorting
Foxp3	Forkhead box P3
GFP	Green fluorescent protein
GITR	Glucocorticoïd-induced tumor necrosis factor receptor
GPI	Glycolsylphosphatidylinositol
ICAM-1	Intercellular adhesion molecule 1
ICS	Intracellular cytokine staining
IFN	Interferon
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IL-2R	IL-2 receptor
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
i.p.	Intraperitoneally
iT_{reg}	Induced regulatory T cell
i.v.	Intravenous
LAG3	Lymphocyte activation gene-3
LFA-1	Lymphocyte function-associated antigen-1

LPS	Lipopolysaccharide
Ly6	Lymphocyte antigen 6
MACS	Magnetic cell sorting
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex (MHC)
MSP1	Merozoite surface protein 1 (MSP1)
NK	Natural killer cell
NKT	Natural killer T cell
NO	Nitric oxide
NOD	Non-obese diabetic, mouse
nT_{reg}	Naturally-occurring regulatory T cell
OVA	Ovalbumin
PHA	Phytohemagglutinin
p.i.	Post-infection
PBMC	Peripheral blood mononuclear cells
PD1	Programmed-death 1
pfEMP1	*P. falciparum*-encoded erythrocyte membrane protein 1
PMA	Phorbol 12-myristate 13-acetate
pRBC	Parasitized red blood cells
RAG	Recombination activating genes
RANTES	Regulated upon activation, normal T cell expressed, and secreted
RBC	Red blood cells
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
SE	Standard error
SP	Single-positive, single-positive CD4 thymocyte
TCR	T cell receptor
T_{eff}	Effector T cell
Tg	Transgenic, Foxp3Tg
TGF-β	Transforming growth factor-beta

Th1	T helper 1
Th2	T helper 2
Th3	T helper 3
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tr1	T regulatory cell 1
T_{reg}	Regulatory T cell
WT	Wild type

INTRODUCTION

1. Malaria: epidemiology, disease and life cycle

1.1 Epidemiology and clinical pathogenesis

Malaria is an endemic parasitic disease, caused by protozoa of the *Plasmodium* genus. It causes over 500 million clinical cases and more than 2 million deaths per year worldwide, mostly in tropical areas of the world, such as sub-Saharan Africa, South-East Asia, Latin America, India and the Pacific. The *P. falciparum* specie is the main causative agent of malaria-associated morbidity and mortality in humans. *P. vivax* is a frequent cause of febrile malarial illness but is rarely fatal, whereas *P. ovale* and *malariae* are infrequent causes of benign malaria. Malarial disease mainly affects children under the age of five and pregnant women in sub-Saharan Africa, although humans of all ages and conditions are afflicted in Asian countries. Malarial symptoms and pathology include severe anemia, respiratory distress, and cerebral malaria, and can be accompanied by hypoglycemia and lactic acidosis due to an increase in the parasite's metabolic rate[1,2]. Approximately 40% of the global population is at risk of malaria[3]. This disease is a huge financial burden on those afflicted and hinders the economic growth of countries where it is endemic[4].

1.2 *Plasmodium* life cycle

Malaria is transmitted by the female *Anopheles* mosquito. During a blood meal, approximately 1 to 100 sporozoites, which develop in the mosquito's mid-gut, are injected from the disease vector into the bloodstream of its human victim (Figure 1). Sporozoites then migrate to the host's liver where they infect hepatocytes and replicate as exo-erythrocytic schizonts. Of note, unlike *P. falciparum*, *P. vivax* and *P. ovale* can form hypnozoites, a dormant stage of the parasite, which remain latent in hepatocytes but might cause malarial relapses

months or even years after initial infection. The clinically silent hepatic stage of infection typically lasts a week, but sometimes up to 30 days. Its end is marked by the rupture of hepatic schizonts, releasing thousands of merozoites into the human bloodstream. Each merozoite can infect a red blood cell (RBC) and divide mitotically to give rise to approximately 20 daughter merozoites, which can themselves infect other erythrocytes. This asexual stage of *Plasmodium* reproduction is responsible for most of the malaria associated morbidity and mortality. As merozoites differentiate in infected RBC, they form trophozoites, or ring stages, which are easily detectable during microscopic analysis. In the bloodstream, some merozoites differentiate into female and male gametocytes, which can be ingested by other mosquitoes during a blood meal. In the new mosquito's mid-gut, gametocytes fuse to form an ookinete, which eventually differentiates into a sporozoite-containing oocyst[1,2,5].

The life cycles of all *Plasmodium* species are similar, including those that infect rodent laboratory models, such as *Plasmodium chabaudi* AS. *P. chabaudi* AS was first isolated from shiny thicket rats in Africa in 1965, and, since then, has been extensively used in laboratory mice for the study of resistance to anti-malarial drugs, anti-malarial immunity, and the genetic susceptibility to malaria[2,6]. *P. chabaudi* AS is similar to *P. falciparum* in many ways: no hypnozoites are generated during infection, its genome is of a comparable size and is also A/T rich. In addition, *P. chabaudi* AS infection can be transmitted to mice in the laboratory by the bite of *Anopheles* mosquitoes. However, since blood-stage malaria largely accounts for the pathogenesis of *Plasmodium* infections, most laboratories bypass the liver stage of *P. chabaudi* AS by infecting mice with malaria merozoite-parasitized red blood cells (pRBC) rather than with sporozoites. Injection of pRBC is preferably done intraperitoneally (i.p.) rather than intravenously (i.v.), due to the technical difficulty of i.v. injections in mice and thus the risk of inconsistency of infectious doses within experimental groups. I.p. injections require higher infectious doses of pRBC to achieve reasonable levels of parasitemia, since most pRBC will not gain access to the bloodstream once injected into the peritoneum.

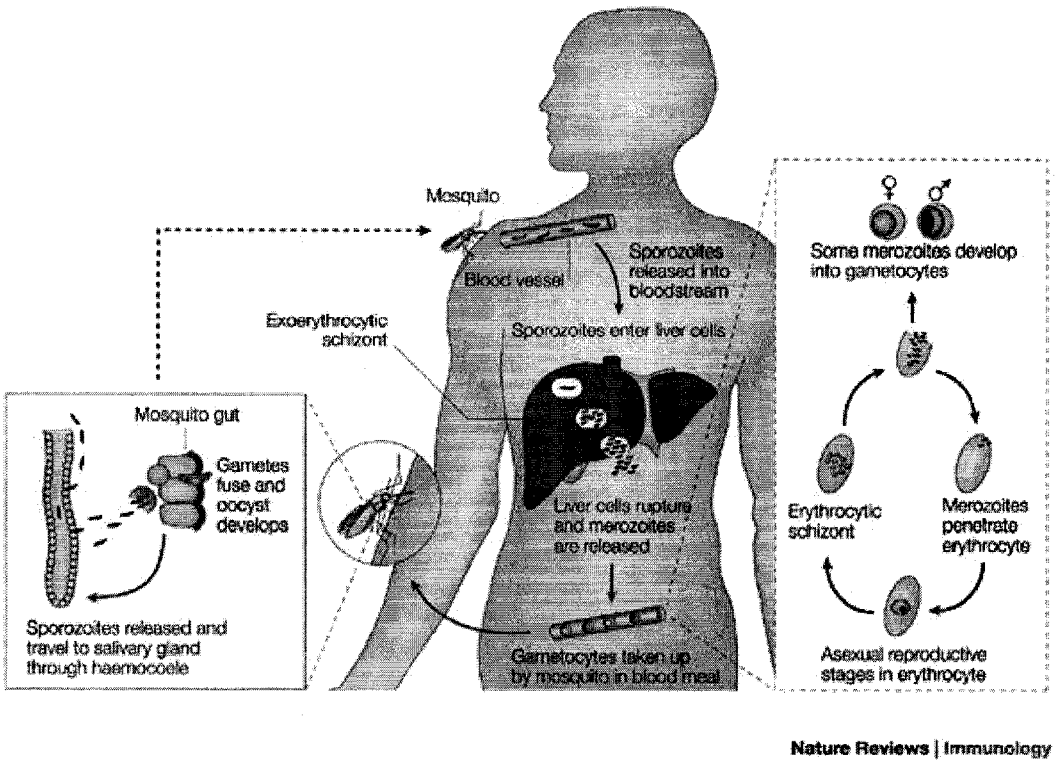

Figure 1. The life cycle of *Plasmodium* species.

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2. Anti-malarial immune responses

With the intricate life cycle of *Plasmodium* parasites, comes a complex anti-malarial immune response. Immunity to malaria has been extensively studied, mainly because it is thought that malaria is partially an immune-mediated disease[7] and because of the urgent need to develop an effective anti-malarial vaccine. Although a single laboratory mouse model of *Plasmodium* infection cannot replicate all of the features associated with human malaria[8], infection of laboratory mice with *Plasmodium chabaudi* AS, *Plasmodium yoelii*, *Plasmodium berghei* or *Plasmodium vinckei* has been an invaluable tool in studying the pathogenesis[7], immune mechanisms[9] and susceptibility genes[10] involved in the host immune response to malaria, but also for anti-malarial vaccine research[11] and

drug design[12]. Even though malaria parasites infect RBC, which do not express surface major histocompatibility complex (MHC) molecules, infected RBC do express malaria-specific antigens as approximately 8% of *P. falciparum*'s proteome, or about 400 proteins, are exported to the surface of the infected erythrocyte. Therefore, pRBC express malaria-specific surface antigens that can easily be recognized by the innate and adaptive immune systems (Figure 2).

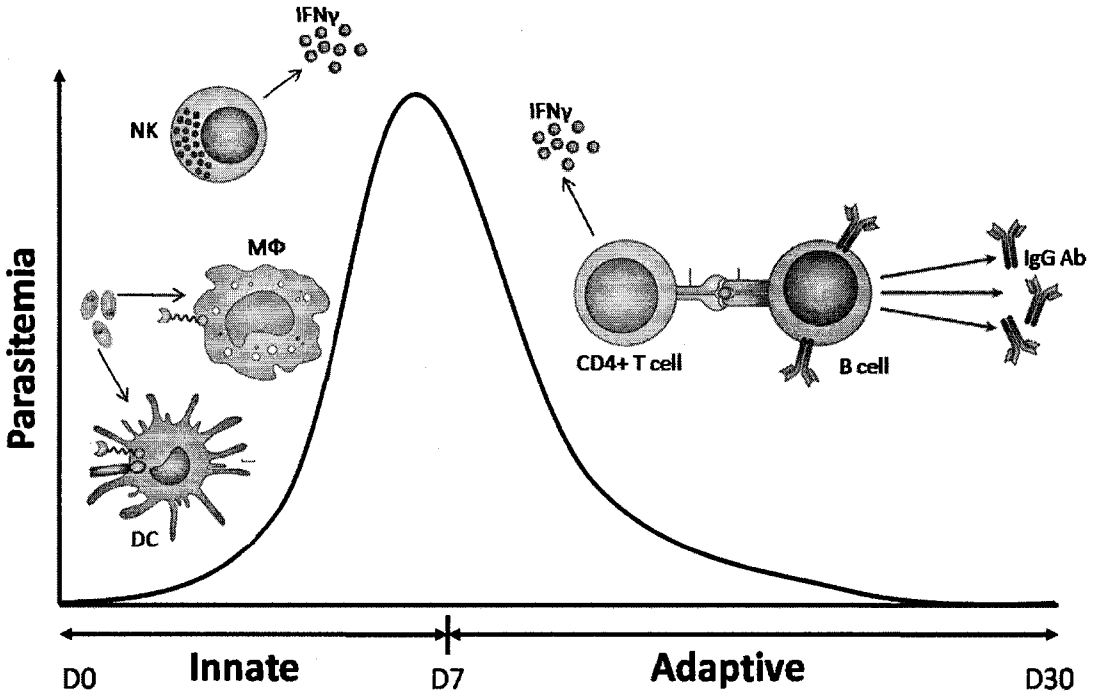


Figure 2. Immunity to blood-stage malaria in mice.

During the innate phase of anti-malarial immunity, which occurs within the first week of infection, NK cells produce large amounts of the pro-inflammatory cytokine IFN-γ. In addition, both DC and macrophages can phagocytose pRBC and present malaria antigen to $CD4^+$ T cells in the context of MHC. Around peak parasitemia, adaptive anti-malarial immunity is initiated, mainly with the activation of $CD4^+$ T cells that secrete IFN-γ and promote antibody secretion and isotype class switching by B cells. The production of large amounts of malaria-specific antibody leads to the resolution of parasitemia around day 30 post-infection DC, dendritic cell; NK, Natural Killer cell; MΦ, macrophage; IgG, immunoglobulin class G antibody. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Stevenson and Riley[2]) copyright (2004).

2.1 Innate immunity to malaria

2.1.1 Dendritic cells

Immunity to malaria consists of both the innate and adaptive branches of immune responses. Innate immunity to malaria is mediated mainly by dendritic cells (DC), macrophages and natural killer (NK) cells, and is initiated within 24 hours post-infection (p.i.) in murine models of malaria[13]. DC are professional antigen-presenting cells with the specific task of sampling the microenvironment and taking up antigens that are then presented by DC in the context of MHC. Upon maturation, DC migrate to nearby lymphoid tissues where they prime antigen-specific T cells by up-regulating co-stimulatory molecules, such as CD80 and CD86, and secreting immunostimulatory cytokines, such as IL-12p70. DC are therefore a central and critical link between innate and adaptive immunity[14].

Hence, it is not surprising that DC play an important role in mediating anti-malarial immune responses. In fact, DC effectively and preferentially uptake *P. chabaudi*-infected RBC resulting in their maturation and ability to stimulate T cell priming[15]. Although the molecular basis of DC-malaria parasite interactions remains unclear, it is thought that DC can interact with pRBC by means of the scavenger receptor CD36 and the integrin CD51[16]. In addition, although this is a very controversial area of research, it is thought that *P. falciparum* schizonts might activate human and murine DC through a MyD88-dependant signaling pathway[17], probably by direct interaction of the malaria pigment hemozoin with Toll-like receptor 9 (TLR-9)[18]. Splenic DC expand and migrate from the marginal zone of the spleen into the $CD4^+$ T cell areas of the white pulp by day 5 p.i. with *P. chabaudi* AS[19]. This pattern of migration indicates that DC are strategically located within the spleen, and are thus able to promote the maturation and differentiation of malaria-specific $CD4^+$ T cells. However, analysis of DC function throughout the course of infection with various *Plasmodium* species in mice has yielded contradictory results. pRBC-exposed bone marrow-derived DC upregulate class II MHC, secrete large amounts of bioactive IL-12p70, and induce

T cell proliferation and differentiation into IFN-γ-secreting cells[15]. On the other hand, other studies indicate that *P. chabaudi* AS pRBC impair LPS-mediated DC maturation *in vitro* and *in vivo*[20]. Some evidence indicates that this suppression might be mediated by hemozoin in both murine[20] and human[21] malaria. Despite these contradicting results, it is likely that DC play a central role in shaping the host immune response to malaria by modulating the initiation of malaria-specific adaptive immunity.

2.1.2 Macrophages

Other subsets of innate immune cells are also activated during malaria infection and contribute to the immune effector mechanisms involved in anti-malarial immunity. These include macrophages, which are responsible for the clearance of malaria-infected RBC in the spleen[3]. Macrophages uptake pRBC via interaction of surface CD36 with *P. falciparum*-encoded erythrocyte membrane protein 1 (PfEMP1) on infected cells[22]. Also, *P. falciparum* glycolsylphosphatidylinositol (GPI) can bind TLR-2 and promote TNF-α secretion by macrophages[23]. However, the function of these cells might also be impaired during malaria infection as hemozoin-loaded splenic macrophages are unable to repeat phagocytosis and to generate an oxidative burst[24]. The primary role of macrophages in the clearance of malaria parasites seems to occur in the adaptive stages of immunity when $CD4^+$ T cell-derived IFN-γ and TNF-α promote macrophage anti-parasitic activity by enhancing phagocytosis of pRBC and secretion of reactive oxygen species (ROS) and nitric oxide (NO)[25]. In fact, NO expression in the spleen correlates with resistance to *P. chabaudi* AS[26]. Macrophages might also contribute to parasite-induced pathology, such as cerebral malaria, due to their ability to secrete pro-inflammatory cytokines and chemokines, such as TNF-α, CXCL10, MCP1, and RANTES, both systemically and in the brain of affected mice[7]. Nevertheless, macrophages play an important role throughout malaria infection by secreting immune mediators, removing pRBC from the circulation, and thus potentially capturing and presenting malaria antigen, which promotes the generation of acquired anti-malarial immunity.

2.1.3 Natural killer cells

In addition to macrophages and DC, NK cells play an essential role in the innate immune response to malaria. In fact, there is a positive correlation between NK cell activity and the extent of blood parasitemia in malaria-infected children[27]. It is thus not surprising that the anti-malarial protective immunity induced by infection of BALB/c mice with *P. yoelii* sporozoites or by infection of resistant C57BL/6 mice with *P. chabaudi* AS, is abrogated by depleting NK cells prior to malaria challenge using anti-asialo GM1[28] or anti-NK1.1 antibodies[29], respectively. Although NK cells are directly cytotoxic to *P. falciparum* infected erythrocytes by means of Fas and Granzyme B[30], it appears that NK cell-derived IFN-γ, not their cytotoxic activity, may contribute to resistance to malaria infection[31]. NK cells are the major source of IFN-γ during the initial phase of infection, prior to the development of T cell-specific anti-malarial responses. This IFN-γ secretion is IL-12- and IL-18-dependant and occurs within 6 hours of *in vitro* exposure to infected erythrocytes[32]. Although the exact molecular basis of NK cell recognition of pRBC remains unclear, it has been suggested that NK cell secretion of the pro-inflammatory cytokine IFN-γ is due to direct interactions of PfEMP1 with its host receptor chondroitic sulphate A (CSA) as well as ligation of NK cell surface ICAM-1 with LFA-1 on macrophages[33]. Since high NK cell cytotoxicity and cytokine secretion correlate with malaria-resistance in mice[13,31], NK cells likely play an essential role in parasite killing and contribute to the shaping of the adaptive immune response by secreting large amounts of IFN-γ, which in turn promotes Th1 immunity.

2.2 Adaptive immunity to malaria

Though innate immune responses are efficient at controlling parasite growth during the initial stages of an infection with *Plasmodium* species, adaptive immunity is required for the ultimate clearance of parasites and the prevention of parasite-induced pathology. Since adaptive immune responses are responsible for the development of immunological memory, the induction of this branch of the

immune system is essential to prevent recurrent malaria infections. The generation of protective immunity to blood-stage malaria in mice is dependent on the effector functions of $CD4^+$ T cells and B cells[34].

2.2.1 B cells and malaria-specific antibodies

An important component of the anti-malarial immune response is the production of malaria-specific antibodies, which ultimately lead to the clearance of parasites from the blood of infected humans and mice. Antibodies were first demonstrated to be important for the resolution of malaria in both human and mice by passive transfer of immune sera[35,36]. The exact mechanism of action of antibodies during a malaria infection remains unclear. Antibody-mediated fixation of complement is not required for protective anti-malarial immunity as mice deficient in components of either the classical or alternative complement activation pathway are still able to clear an infection with *P. chabaudi* AS[37]. Antibodies specific for *P. falciparum* MSP-1 can inhibit proteolytic cleavage of MSP-1, which is required for the erythrocytic invasion by the parasite[38]. Therefore, antibodies might operate by preventing parasite invasion of erythrocytes rather than direct killing of free merozoites in the blood.

B cells are necessary for the development of protective immunity to malaria as demonstrated by the fact that SCID mice reconstituted with only $CD4^+$ T cell survive malaria infection but display patent parasitemia, which is only alleviated by co-transfer of B cells to these mice. This clearance of parasites in the blood correlates with the presence of malaria-specific immunoglobulin at later stages of infection[34]. In addition, μ-MT mice, which are deficient in mature B cells, or depletion of B cells using anti-μ antibodies develop chronic parasitemia[39,40]. Thus, B cell-deficient mice can control infection but develop persistent and relapsing blood parasitemia. $CD4^+$ T cells play an important role in promoting the production of antibody by secreting cytokines, such as IL-4, which help B cells undergo isotype switching, from production of low affinity IgM to secretion of large amounts of high affinity IgG isotypes. γδ T cells have also been shown to

influence the malaria-specific immunoglobulin response mainly by influencing the Th1/Th2 balance during a malaria infection[41]. Thus, differential cytokine patterns influence the outcome of the malaria-specific antibody responses. Indeed, IFN-γ production promotes the production of IgM, IgG2a and IgG3, whereas IL-4 and IL-5 restricts immunoglobulin secretion to the IgG1 isotype during infection with *P. chabaudi* AS[42,43].

In addition to mediating humoral immunity, evidence has shown that B cells can influence T cell-mediated anti-malarial immune responses. In fact, mice depleted of B cells do not exhibit a typical Th1 to Th2 switch at peak of infection with *P. chabaudi* AS[39,44]. Instead, these mice display low frequencies of IL-4-secreting T cells and thus persistent Th1 immunity, with high levels of serum IFN-γ, IL-2 and NO up to 50 p.i. Transfer of immune B cells into anti-μ treated mice restores Th2 responses later on during infection[45]. Therefore, B cells not only produce large amounts of protective anti-parasite antibodies, but are also involved in shaping the anti-malarial immune response by promoting an appropriate Th1/Th2 balance throughout the infection, a process which is essential for the generation of protective immunity to the parasite and to host survival, as described below.

2.2.2 $CD4^+$ T helper cells

The role of T cells expressing an αβ T cell receptor (TCR) in immunity to malaria was first demonstrated with thymectomized or athymic nude mice, which display enhanced susceptibility to malaria[46]. Early studies on the role of $\alpha\beta^+$ T cells in anti-malarial immunity involved studying $CD8^+$T cell responses. It is now widely accepted that $CD8^+$ T cells specific for MHC class I-restricted *Plasmodium* antigens are essential for the development of immunity to infective sporozoites and throughout the liver-stages of malaria infection. However, evidence suggests that $CD8^+$ T cells are involved only in the immune response to pre-erythrocytic stages of malaria and do not play a major role in immunity to blood-stage malaria[47]. In addition, $CD8^+$ T cell anti-malarial memory responses to

sporozoites and liver-stage parasites might strongly depend on the secretion of IL-4 by malaria-specific $CD4^+$ T cells[48]. This finding indicates that $CD4^+$ T cells might be the central players in T cell-mediated anti-malarial immunity to pre-erythrocytic stages, thereby playing a pivotal role in controlling both parasite growth and the overall host anti-malarial immune response.

The importance of the $CD4^+$ subset of T cells in controlling blood-stage malaria was first revealed by depletion studies whereby *P. chabaudi* AS-resistant C57BL/6 mice treated with anti-CD4 monoclonal antibody exhibited high levels of blood parasitemia throughout infection as well as decreased titers of malaria-specific IgM and IgG antibodies and loss of immunity to re-infection[49]. Since then, it has become increasingly clear that $CD4^+$ T cells are the central players of the anti-malarial adaptive immune response to blood-stage parasites since they promote antibody secretion by B cells as well as the activation of macrophages and other cells that can produce anti-parasitic molecules, such as TNF-α, NO and ROS.

$CD4^+$ T cell anti-malarial effector functions

Although T cell-mediated induction of antibody production appears to be crucial for the ultimate resolution of malaria infection in mice[46], it is clear that there are antibody-independent $CD4^+$ T cell effector mechanisms, which result in the control of parasitemia during the acute stage of infection[50]. $CD4^+$ T cells can carry out either a Th1 or Th2-type of immune response, which influences the ultimate outcome of an infection by determining either resistance or susceptibility to malaria[51]. Production of the Th1 cytokine IFN-γ by splenic $CD4^+$ T cells within 1 week of infection with *P. chabaudi* AS occurs in resistant C57BL/6 (B6) mice but not in susceptible A/J mice, which produce high levels of IL-5, an important Th2 cytokine[52]. This early predominant Th1 immune response seen in resistant animals is dependent on the production of bioactive IL-12p70, most probably by DC and splenic macrophages during the early stages of infection[53]. Production of Th1-skewing IL-12p70 is observed as early as 2 days post-infection in B6 mice

and correlates with resistance to *P. chabaudi* AS as infected A/J animals produce significantly less of this cytokine[54]. Furthermore, treatment of susceptible A/J mice with recombinant IL-12 during the first 5 days of infection results in lower parasitemia and increased survival[55]. Thus, the main function of activated $CD4^+$ T cells during the early stages of infection is the production of pro-inflammatory cytokines, such as IFN-γ. Although NK cells produce large amounts of this cytokine in response to malaria, $CD4^+$T cells are the main source of IFN-γ just before peak parasitemia[56]. The importance of this cytokine is highlighted by depletion studies whereby anti-IFN-γ treatment in resistant mice during *P. chabaudi* AS infection leads to significantly higher peak parasitemia[42,57]. In addition, *P. chabaudi* AS infected, IFN-γ-deficient mice display increased parasite loads and mortality, lower serum levels of IL-12p70 and TNF-α, decreased malaria-induced macrophage activation, lower parasite-specific immunoglobulin as well as an overall decrease in splenic macrophage, NK cell and T cell numbers when compared to infected wild type (WT) mice[43]. Thus, it is likely that $CD4^+$ T cell-derived IFN-γ plays an essential role in shaping the immune response to blood-stage malaria.

Although the primary $CD4^+$ T cell-mediated, anti-malarial immune response is mainly Th1, there is a switch to a Th2 cytokine response post peak parasitemia. This suggests that Th1 immunity is required for the control of peak parasitemia, whereas Th2 immunity is responsible for the ultimate clearance of malaria parasites. IFN-γ and IL-2 producing cells dominate the $CD4^+$ T cell response during the first 2 weeks of infection with *P. chabaudi* AS, after which IL-4 and IL-5-secreting Th2 cells are generated[52,58,59]. These Th2, $CD4^+$ T cells provide proper co-stimulation and secrete the appropriate cytokines which give B cells the help needed to expand and produce large amounts of protective malaria-specific antibodies[60]. In addition to promoting humoral immunity, the generation of a Th2 anti-malarial response might also be essential for immunity to re-infection as IL-4 knock-out mice on the B6 background develop parasitemia from a secondary infection with *P. chabaudi* AS, whereas WT mice achieve full immunity to malaria after primary infection[9].

Initiation of $CD4^+$ T cell-dependant anti-malarial immunity

To initiate adaptive immunity against infection, including malaria, $CD4^+$ T cells must be activated by professional APCs. It is known that parasite material is proteolytically degraded and presented by surface MHC class I and II on APCs in order to activate T cells[61]. $CD4^+$ T cell-dependant immunity to blood-stage malaria is dependent on both MHC class II antigens and co-stimulation through the B7/CD28 pathway as MHC class II and CD28 knock-out mice fail to resolve parasitemia after infection with either *P. chabaudi* AS or *P. yoelii*[62,63]. In addition, the switch from Th1 to Th2 immunity observed during a *P. chabaudi* AS infection, as described above, might actually be under the control of DC, which present malaria antigen to T cells in the context of MHC class II. In fact, both $CD11c^+$ $CD8^+$ and $CD11c^+$ $CD8^-$ DC can effectively present malaria-antigen to $CD4^+$ T cells; however, $CD8^-$ DC from *P. chabaudi* AS-infected mice preferentially induce T cells to secrete IL-4 and IL-10[64]. Interestingly, this $CD8^-$ subset dominates the DC repertoire at peak malaria infection, which coincides with the observed switch to a Th2 immune response[64]. Although DC are likely the cells responsible for the initiation of $CD4^+$ T cell-mediated, anti-malarial immunity, it is unknown whether proper presentation of malarial antigens and up-regulation of co-stimulatory molecules is achieved in all malaria infections in order to properly instruct malaria-specific T cells to become activated and divide. Improper activation of T cells due to insufficient co-stimulatory signals might result in anergy, tolerance or even deletion of malaria-specific T cell clones. Specific deletion of $CD4^+$ T cells has been observed both in *P. berghei* infection, during which 99% of malaria-specific $CD4^+$ T cells are deleted upon infection[65], and in *P. yoelii* malaria, where Fas-mediated apoptosis of T cells specific for a 19-kDa fragment of merozoite surface protein 1 (MSP1), but not OVA-specific T cells, was observed as a result of malaria infection[66]. The exact outcome and significance of the deletion of malaria-specific T cells are unclear, however, this phenomenon might partially explain the poor immunogenicity of parasite antigens and may be the underlying cause of malaria-induced immunosuppression, which

is a common feature of *Plasmodium* infections, as discussed in a subsequent section.

Trafficking of $CD4^+$ T cells during anti-malarial immunity

It is well known that B cells locate within the splenic red pulp and bone-marrow upon differentiation into antibody-secreting plasma cells, allowing them to secrete large amounts of malaria-specific antibody in the bloodstream of malaria-infected mice[3]. Yet, little is known about the migratory pattern of activated T cells during blood-stage malaria. T cells are likely to also migrate to the splenic red pulp, where they can interact with B cells and secrete large amounts of pro-inflammatory IFN-γ in order to promote pRBC phagocytosis and destruction by macrophages[3]. Chemokines likely play a pivotal role in T cell migration during malaria infection since they are known to promote leukocyte recruitment to inflammatory sites and participate in cell-mediated immunity during various parasitic infections[67]. Whereas the significance of chemokines in the immune response to blood-stage malaria remains unclear, multiple studies suggest that these molecules play an important role in malaria pathogenesis, such as development of severe anemia and cerebral malaria. In fact, low serum levels of RANTES is associated with suppressed erythropoiesis[68] and cerebral malaria[69] in *P. falciparum*-infected children. On the other hand, significantly higher mRNA levels of RANTES and its receptors CCR1, CCR3 and CCR5 are observed in the brain of *P. yoelii* XL-infected mice compared to non-infected mice[70]. $CD4^+$ T cell recruitment to the brain of mice suffering from cerebral malaria seems to be dependent on CXCR3 expression. A recent study showed that NK cell-derived IFN-γ enhances CXCR3 expression on $CD4^+$ T cells from *P. berghei*-infected mice and increases $CD4^+$ T cell responsiveness to IFN-γ-inducible protein 10 (IP-10) during malaria infection[71]. Another study indicated that CCR5 expression might also contribute to cerebral malaria as $CCR5^{-/-}$ mice have decreased neutrophil and $CD4^+$ T cell infiltrations in the brain of *P. berghei*-infected mice. Therefore, both the CCR5 and CXCR3 chemokine pathways may play important roles in the development of cerebral malaria by promoting the recruitment of

pathogenic $CD4^+$ T cells to the brain of infected mice. However, whether these chemokine pathways also play a role in blood-stage malaria remains to be determined.

3. Malaria-induced immunosuppression

Studies in laboratory mouse models have shown that an infection with malaria can elicit the generation of protective immunity to *Plasmodium* parasites. However, these studies as well as human epidemiological studies have also shown that malaria infections induce a state of malaria-specific and generalized immunosuppression.

3.1 Malaria induces generalized immunosuppression

Studies have shown that malaria-induced suppression is commonly observed during blood-stage infection and affects both T and B cell responses. Nigerian children afflicted with acute blood-stage *P. falciparum* malaria have significantly reduced antibody responses to tetanus toxoid and *Salmonella typhi* antigen[72]. This impaired humoral response in malaria-infected children correlated with decreased efficacy of vaccinations for *S. typhi* and *N. meningitidis*[73]. Thus, concurrent malaria infection might contribute to increased susceptibility to various respiratory and gastrointestinal infections often observed in children from malaria-endemic areas. In addition, acute *Plasmodium* infection can impair both malaria-specific and non-specific, cell-mediated immune responses. A study in Thai adults with either acute blood-stage infection or cerebral malaria showed that they have decreased delayed-type hypersensitivity (DTH) skin reactions in responses to several antigens[74]. Peripheral blood mononuclear cells (PBMC) isolated from acutely infected Gambian children display significantly lower *P. falciparum*-specific proliferative responses as well as impaired responses to various mitogens and to *Candida*, an unrelated antigen[75]. Proliferative responses are, however, restored during the convalescent stage of the infection[75]. Interestingly, malaria-specific, IFN-γ production is also decreased during acute *P. falciparum* infection[75], and PBMCs from malaria-infected Thai adults display

reduced IL-2 production in response to both malaria and streptococcal antigen[76]. Therefore, malaria infection in humans results in severe defects in both malaria-specific and non-specific immune responses. This generalized immunosuppresion is illustrated by decreased antibody production as well as impaired cell-mediated immunity as demonstrated by decreased lymphocyte proliferation, reduced DTH reactions, and impaired pro-inflammatory cytokine production.

Similar observations have been made in mouse models of malaria. BALB/c mice infected with *P. berghei*[77] as well as *P. chabaudi* AS infection in both resistant B6 and susceptible A/J mice[78] results in decreased primary antibody responses to sheep erythrocytes. In addition to deficient humoral responses, spleen cells isolated from malaria-infected animals also display impaired proliferative responses to mitogens, such as PHA, LPS and ConA[79,80]. The deficiency in cell-mediated immunity is also apparent by reduced DTH responses against chicken gamma globulin, 2,4-dinitrofluorobenzene, and oxazolone in mice infected with *P. berghei*[80,81]. As discussed previously, it has also been shown in experimental mouse models that malaria-specific $CD4^+$ T cells are specifically deleted during various *Plasmodium* infections. Therefore, experimental models of malaria also exhibit a broad malaria-induced immunosuppression, which mimics that observed in acutely infected humans.

3.2 Malaria induces poor immunological memory

Another indication that malaria infection can severely impair the host immune response is the fact that malaria induces very poor immunological memory. This is demonstrated by the observation that naturally acquired immunity does not develop after a primary malaria infection, unlike infections with other pathogens. In addition, most field trials of candidate malaria vaccines have yielded very unsatisfactory results. Although there are many potential malaria vaccine candidates awaiting clinical trials, perhaps the biggest disappointment came when the highly anticipated three-dose recombinant protein vaccine, RTS,S/AS02, showed 71% efficacy in preventing *P. falciparum* infection

in Gambian adults within the first nine weeks post-vaccination but displayed 0% efficacy in the following 6 weeks of surveillance[5,82]. These results, in addition to multiple other studies, reinforce the concept that immunity to malaria is relatively short-lived; repeated infections are required for the partial development of protective immunity, persistent infection with low grade parasitemia is frequent, and protective immunity declines when a previously immune individual moves away from a malaria endemic region. However, the fact that the majority of clinical malaria cases among individuals residing in endemic areas are seen in children and not adults indicates that partial immunity to malaria may develop over time with frequent exposure to the parasite. Indeed, in sub-Saharan Africa where *P. falciparum* is prevalent, it is only young children between the ages of 1 and 5, after passive maternal immunity has subsided, who suffer mostly from malarial disease, with elevated parasitemia, severe morbidity, and high mortality rates associated with high prevalence of malarial anemia and cerebral malaria. Thus, protection against severe disease and death is acquired through early exposure to the parasite, but does not protect against infection *per se* and does not prevent the development of mild disease[83]. Sterile immunity to malaria is never achieved, though a marked reduction of 10 000 to a million fold in parasite burden is observed in immune individuals[84]. This demonstrates that there are efficient immune effector mechanisms generated in response to a malaria infection, although not sufficient to provide life-long sterile immunity. This provides indirect evidence that there are likely immunoregulatory mechanisms that contribute to the development of an inadequate malaria-specific immune response.

4. Naturally-occurring regulatory T cells

Immune responses must occur within certain limits, allowing for the development of optimal protection of the host but limiting immunopathology. Thus, all immune responses require a form of regulation to maintain an optimal range of efficiency, with a proper balance between antibody secretion, production of inflammatory mediators, and expansion of various immune cell subsets. The

concept of immunoregulation is typically illustrated by the control of immune reactivity to "self" antigens, or autoimmunity. It was especially after the discovery that the human immune system harbors self-reactive T cells, with specificities for peripheral tissue antigens, such as myelin basic protein[85], that the notion of immunoregulation was reinforced. Such autoreactive T cells are present in all individuals, however, only 5% of the population suffers from autoimmune disease[86]. In the periphery, self-reactivity and aggressive autoimmunity can be prevented by mechanisms such as immunological ignorance, anergy and peripheral deletion of T cell clones. However, in the 1970s, evidence emerged that a specialized subset of T cells, termed suppressor T cells, might have the specific task of hindering the activation of helper T cells. The observation that stimulation of the immune system using thymus-dependant antigens leads to the development of T cells capable of preventing the activation of T cells supported this notion[87]. Since then, suppressor T cells, renamed regulatory T cells (T_{reg}), have been extensively studied; however, multiple questions still remain unanswered, such as the ontogeny of T_{reg} as well as the molecular and cellular mechanisms that allow these cells to prevent overt immune responses.

4.1 Definition and characteristics of nT_{reg}

The first evidence indicating that T cells could down-regulate immune responses came with the early observation that thymectomy in 3 day old non-autoimmune prone BALB/c mice leads to ovaritis[88]. It was not until 1995 that studies performed by Sakaguchi *et al.* convincingly demonstrated the existence of T_{reg}. This study confirmed that thymectomy in BALB/c mice at 3 days after birth provokes multi-organ specific autoimmunity, including gastritis, thyroiditis, oophoritis, orchitis, prostatitis, and pancreatitis. Further characterization of T_{reg} was also achieved with the demonstration that adoptive transfer of a $CD4^+CD25^+$ T cell population to thymectomized mice prevents poly-autoimmune disease[89]. Thus, Sakaguchi *et al.*'s seminal study identified T_{reg} as a $CD4^+CD25^+$ T cell subset capable of preventing the activation of $CD4^+CD25^-$ T cells, defined as potential effector T cells (T_{eff}). Similar findings have been obtained in various

experimental settings, thus reinforcing the notion of T_{reg}. In fact, reconstitution of immunodeficient athymic nude mice with CD25-depleted $CD4^+$ T cells leads to multi-organ autoimmunity similar to that observed following thymectomy of 3 day old mice[89]. In addition, reconstitution of SCID mice with $CD45RB^{hi}$ T cells, in the absence of a $CD45RB^{lo}$ subset thought to include $CD4^+CD25^+$ T_{reg}, results in the development of inflammatory bowel disease[90]. Thus, it is now widely accepted that T_{reg} can regulate the immune response to self-antigens and hence prevent the development of autoimmune disease in mice.

The T_{reg} identified by Sakaguchi *et al.* are termed "naturally-occurring", because they acquire their regulatory phenotype in the thymus during development and are present in the periphery prior to antigenic challenge. These naturally-occurring T_{reg} (nT_{reg}) make up 5-10% of total peripheral $CD4^+$ T cells in mice and approximately 1 to 2% in humans. Other subsets of T_{reg} have been described as "induced", as they acquire suppressive activity upon antigenic-stimulation and in particular environmental conditions (Table 1). For example, naïve T cells can acquire a Tr1 regulatory phenotype upon exposure to the immunomodulatory cytokine IL-10[91]. Th3 cells differentiate upon exposure to large doses of oral, intradermal or intranasal antigens and have been shown to produce large amounts of TGF-β[92]. Other T_{reg} subsets include NKT cells[93], γδ T cells[94] and $CD8^+$ T cells[95], although the origins, phenotypes and mechanisms of suppression of these cells as well as their significance in immunoregulation remain elusive. Unlike induced T_{reg} (iT_{reg}), nT_{reg} constitutively express the surface marker CD25, which is the α-chain of the high affinity heterotrimeric IL-2 receptor (IL-2R). nT_{reg} have been shown to express other surface markers that distinguish them from conventional T_{eff}. These biomarkers include CD103, galectin-1, Ly6, OX-40 (CD134), 4–1BB (CD137), CTLA-4, glucocorticoïd-induced tumor necrosis factor (TNF) receptor (GITR), TNFR2, TGF-βR1, programmed cell death 1 (PD1), neuropilin-1 as well as the chemokine receptors CCR4, CCR8 and CCR5[96-98]. However, none of these markers are exclusive to nT_{reg}. Although CD25 has been extensively used to identify and characterize nT_{reg}, this marker is also expressed by non-regulatory T cells upon activation and

differentiation into T_{eff}. Indeed, prior to the discovery of nT_{reg}, CD25 was thought to be an exclusive activation marker for $CD4^+$ T cells. It is thus essential to recognize that CD25 is a very poor marker for nT_{reg}, unless referred to in steady state. In an inflammatory context, such as autoimmunity or any infectious disease, CD25 cannot confidently be used as an absolute marker for nT_{reg}.

	Subset	Phenotype	Origin	Mechanisms of action
Naturally-occurring	nT_{reg}	$CD4^+CD25^+$ $Foxp3^+$	Thymus	Cell-cell contact and/or cytokine secretion (IL-10, TGF-β)
Induced	Tr1	$CD4^+$	Periphery	Secretion of IL-10
	Th3	$CD4^+$	Periphery	Secretion of TGF-β
	$CD8^+$	$CD8^+Foxp3^{+/-}$	Periphery	Secretion of IL-10 and IFN-γ
	γδ T cell	γδ TCR	Periphery	Unclear, perhaps by IL-10 secretion and cytolysis
	NKT cell	$NK1.1^+CD1d^+$	Thymus	Secretion of large amounts of Th1 or Th2 cytokines

Table 1. Phenotype, origin and mechanisms of action of the various Treg subsets

4.1.1 Foxp3 is a novel biomarker for nT_{reg} and required for nT_{reg} development

It is only recently that a biomaker specifc for nT_{reg} was discovered. In 2003, several research groups simultaneously reported that Forkhead box protein 3 (Foxp3), a member of the forkhead/winged-helix family of transcriptional regulators, was essential for the thymic development and suppressive function of nT_{reg}[99-101].Genetic analysis revealed that mutations in the human *FOXP3* gene was

the cause of a rare autoimmune lymphoproliferative disorder, termed IPEX for immune dysfunction, polyendocrinopathy, enteropathy, X-linked[102,103]. Subsequent studies also revealed a spontaneous frameshift mutation in the murine *Foxp3* gene as a direct cause of *scurfy* syndrome, the murine equivalent of IPEX[104]. This phenotype could be rescued by the introduction of a *Foxp3*-transgene in *scurfy* mice[104], and *Foxp3* mRNA expression was shown to be confined to the $CD4^+CD25^+$ T cell subset[99]. Thus, these studies indicated that T cell hyperresponsiveness in *scurfy* mice[105] is due to a deficiency in peripheral nT_{reg} numbers and not an intrinsic defect in T_{eff}. Most importantly, however, is the fact that only nT_{reg}, and not T_{eff}, constitutively express *Foxp3* in mice. Therefore, these studies established a more rigorous definition of nT_{reg} as $CD4^+CD25^+Foxp3^+$ T cells.

The generation of a reporter $Foxp3^{gfp}$ transgenic mouse expressing a Foxp3-green fluorescent protein (GFP) fusion transgene confirmed on a protein level that the previous definition is in fact accurate since GFP^+ cells in this model are suppressive and exhibit a regulatory phenotype[106]. Also, it was shown that Foxp3 is not up-regulated in putative $CD4^+Foxp3^-$ T_{eff} during an acute immune response upon conventional TCR engagement but can be induced via certain stimulatory conditions. Indeed, TGF-β has been shown to induce *Foxp3* expression in peripheral $CD4^+Foxp3^-$ T cells both *in vivo* and *in vitro*, a mechanism which is referred to as peripheral conversion[107-109]. The $Foxp3^{gfp}$ model also indicated that there might exist a second population of nT_{reg}. As demonstrated by expression of GFP, although the majority of $Foxp3^+$ cells are also $CD25^+$, a proportion of cells, up to 2% of total $Foxp3^+$ cells, do not express this T_{reg} marker[106]. These $CD4^+CD25^-Foxp3^+$ T cells represent up to 50% of total $Foxp3^+$ T cells in the lung of uninfected and *Mycobacterium tuberculosis*-infected $Foxp3^{gfp}$ mice, and as a result cannot be regarded as a negligible regulatory population[106]. In addition, these cells differ slightly in their transcriptional profile when compared to $CD4^+CD25^+Foxp3^+$ T cells, with higher expression of ICOS, IL-10, CCR2, CXCR3, CCR5, and β1-integrin as well as higher proliferative capacity[106]. Most importantly, these cells have potent *in vitro* suppressive activity, comparable to

the classical $CD25^+Foxp3^+$ nTreg population[106]. However, the significance and function of this second regulatory population remains unclear. It has been proposed that the $CD25^-$ nT_{reg} subset might result from a transient down-regulation of the IL-2Rα chain on previously activated $CD25^+$ nT_{reg}, either due to activation-induced proliferation or to the restricted availability of IL-2 in peripheral tissues. It is also possible that $CD25^-Foxp3^+$ T cells are a completely distinct regulatory population or simply precursors to the $CD25^+Foxp3^+$ population[106]. Nonetheless, the discovery of this important cell subset not only highlights the limitations of the use of CD25 as a marker for nT_{reg}, but also indicates that there might be two functionally distinct regulatory populations with the capabilities of modulating immunity at different times and/or by different mechanisms.

4.1.2 Function of Foxp3 in nT_{reg} suppression

The exact means by which Foxp3 renders T cells suppressive is not entirely understood, as the unravelling of the complex molecular mechanism of Foxp3-mediated transcriptional regulation has only begun. It is known that promoters of pro-inflammatory cytokine genes are a major target for the Foxp3 transcription factor. In fact, strong evidence shows that Foxp3 can prevent NFAT/AP-1 binding to promoters of several cytokine genes, thus decreasing expression of these target genes[110]. In addition, ectopic expression of *Foxp3* in T cells results in decreased secretion of IL-2, IL-4 and IFN-γ upon activation *in vitro* and *in vivo*[111]. Although these studies reinforce the notion that Foxp3 acts as a transcriptional repressor, recent evidence has shown that Foxp3 can also promote transcriptional activation[112,113]. Microarray analysis confirms that Foxp3 modulates expression of genes involved mainly in TCR triggering, pro-inflammatory cytokine secretion as well as signal transduction from TCR and co-stimulatory molecules. These target genes are differentially regulated in thymic and peripheral nT_{reg}. Thus, although the molecular basis of a regulatory phenotype in T cells, as dictated by the effects of Foxp3 transcriptional regulation, is extremely complex and only starting to be

deciphered, understanding it will be crucial in order to fully comprehend nT_{reg} development and function.

Of note, the Foxp3 transcription factor has also been identified as an important marker for nT_{reg} in humans. Transduction of human non-regulatory $CD4^+CD25^-$ cells with a *FOXP3*-expressing retrovirus renders these cells suppressive[114]. However, unlike its murine counterpart, human Foxp3 has two isoforms and other unidentified factors, besides Foxp3 expression, might be required for nT_{reg} suppressive activity in humans[115]. In addition, the use of Foxp3 is more limited in the study of human peripheral blood nT_{reg}, as human T_{eff} have been shown to express Foxp3 upon activation and achieve a regulatory phenotype[116]. Therefore, unlike its murine counterpart, human Foxp3 seems to be an activation-induced marker similar to CD25. Despite this ambiguity, Foxp3 remains the most reliable marker for the study of nT_{reg} in both human and mouse.

4.1.3 Proliferative capacity of nT_{reg}

Until recently, it was thought that an important characteristic of nT_{reg} is that they are anergic in nature. Early studies demonstrated that $CD4^+CD25^+$ T_{reg} cells failed to proliferate *in vitro* in response to IL-2, anti-CD3, ConA or anti-CD28 stimulation, unlike $CD4^+CD25^-$ T_{eff} that proliferate vigorously in response to these stimulations[117,118]. However, nT_{reg} unresponsiveness could be reversed by stimulation with both IL-2 and anti-CD3[117]. In addition, it was thought that nT_{reg} were unresponsive to antigen-driven proliferation *in vivo*, although these cells underwent homeostatic proliferation in lymphopenic mice[119]. Therefore, it was previously accepted that nT_{reg} were hypo-responsive to TCR stimulation, both *in vitro* and *in vivo*.

Newly emerging evidence contradicts the notion that nT_{reg} are naturally anergic and suggests that nT_{reg} might respond to antigen stimulation *in vivo*. In fact, nT_{reg} harbouring a transgenic TCR specific for ovalbumin (OVA) proliferate when adoptively transferred into OVA-immunized mice, despite being previously anergic to antigenic stimulation *in vitro*[120]. These transgenic nT_{reg} also proliferate

specifically in pancreatic lymph nodes when transferred into RIP-mOVA transgenic mice, which express OVA antigen exclusively in pancreatic islets[120]. TGF-β might play a role in nT_{reg} expansion *in vivo* as intra-islet treatment of non-obese diabetic (NOD) mice with TGF-β can promote nT_{reg} proliferation and inhibit the onset of autoimmune diabetes[121]. Interestingly, some more recent reports also suggest that nT_{reg} might not be anergic *in vitro*. In fact, *in vitro* proliferation of nT_{reg} can be achieved with anti-CD3 in the presence of DC and high doses of LPS through a TLR-4-dependant mechanism[122]. $CD4^+CD25^+$ T_{reg} can also be rendered responsive both *in vitro* and *in vivo* by antigen-loaded mature and immature DC, indicating that nT_{reg} might be able to proliferate in both steady-state and inflammatory conditions[123]. DC-driven nT_{reg} proliferation appears to be dependent on DC-T cell interactions, B7 co-stimulation and low levels of IL-2 produced by nT_{reg} themselves[123]. Although controversial, these data suggest that nT_{reg} are likely responsive to TCR stimulation both *in vitro* and *in vivo* and might proliferate *in vivo,* both in the steady-state to maintain peripheral tolerance and during inflammatory conditions to regulate immune responses.

4.2 Thymic Development

It was previously thought that nT_{reg} from mice developed in peripheral tissues, and that nT_{reg} isolated from the thymus were merely re-circulating T cells which had acquired a regulatory phenotype by antigenic stimulation in the periphery[124]. It is now clear that nT_{reg}, unlike iT_{reg}, develop in the thymus like conventional $CD4^+$ and $CD8^+$ T cells. A recent study has shown that most thymic $Foxp3^+$ T cells express high levels of recombination activating gene (RAG) proteins[125,126], indicating that these cells are in fact undergoing TCR rearrangement and confirming that T cells commit to the nT_{reg} lineage within the thymus. This commitment occurs during the late stages of T cell development, since at least 85% of thymic $Foxp3^+$ cells are found within the CD4 single-positive (SP) thymocyte pool[125]. Unlike conventional T cells, the kinetics of nT_{reg} development is relatively slow: within the first day of birth, only 0.1% of CD4 SP thymocytes express Foxp3. The biggest increase in Foxp3 expression within this

subset of developing thymocytes occurs between days 3 and 4 after birth, explaining the aggressive autoimmunity observed in thymectomized 3 day old mice. The number of Foxp3-expressing cells within the CD4 SP thymocyte population steadily but slowly increases, reaching a maximum of 10% of total $CD4^+$ T cells at approximately 3 weeks of age[125]. This delay in nT_{reg} development relative to conventional T cells might be explained by the delayed maturation of their physiological niche within the thymus. $Foxp3^+$ cells primarily reside within the thymic medulla[106], and the steady increase in $Foxp3^+$ CD4 SP thymocytes after birth correlates with the development of thymic medulla[125]. In addition, mutations in signalling proteins involved in proper medullary architecture hinders nT_{reg} generation[124]. Therefore, it is possible that T cells require specific signals from the thymic medullary microenvironment in order to commit to the nT_{reg} lineage.

nT_{reg} require specific signals for their development in the thymus. These include the interaction of TCR with self-peptides in the context of MHC II. Although nT_{reg} are thought to have a diverse polyclonal TCR repertoire and hence are able to recognize a breadth of antigens, there is evidence that nT_{reg} specificity might be skewed towards self-antigens[124]. Recent studies have also indicated that the TCR repertoire of T_{eff} and nT_{reg} only partially overlap[127]. It is recognized that nT_{reg} are likely selected for their high affinity interactions with self-peptide-MHC II complexes during T cell development in the thymus. In fact, evidence suggests that nT_{reg} undergo an altered negative selection process within the medulla, whereby the affinity threshold of their TCR is below the strength that would normally lead to the clonal deletion of self-reactive thymocytes. It has been proposed that the presence of Foxp3 in nT_{reg} allows them to survive the apoptotic program that normally ensues after the triggering of high affinity TCR interactions during negative selection within the thymus[124].

In addition to peptide-MHC interactions, other co-factors have been shown to be essential for T cell commitment to a regulatory lineage. These include the co-stimulatory molecule CD28, which is known to enhance TCR signalling and

IL-2 production by binding to CD80 and CD86 on activated APC. Thymic medullary DC express high levels of the latter two surface molecules. There are indications for a role of these accessory molecules in nT_{reg} development, as mice deficient in either CD28 or CD80 and CD86 exhibit reduced numbers of peripheral nT_{reg} and often succumb to autoimmune disease[128]. This is also in agreement with the necessity of an intact thymic medulla for T cell commitment to a regulatory phenotype although these signals may also be needed in the periphery. Most importantly, studies indicate that CD28 ligation in the thymus promotes Foxp3 expression as well as up-regulation of nT_{reg}-associated markers, such as GITR and CTLA-4[129]. Thus, CD28 appears to be important for initiation of the nT_{reg} developmental program. Perhaps nT_{reg} require two signals for Foxp3 expression and proper lineage commitment: TCR-peptide/MHC interactions and a second signal, such as CD28 ligation.

4.2.1 Role of cytokines in nT_{reg} development

In addition to appropriate co-stimulation, there is evidence indicating that nT_{reg} development might also require the action of cytokines, such as IL-2, as second co-factors. IL-2 is an essential cytokine required for homeostatic proliferation of T cells in peripheral tissues, especially activated T_{eff}. Co-stimulation by CD28 ligation is known to promote the production of IL-2 by activated T cells. Thus, it is not surprising that CD28 ligation in the thymus leads to IL-2 production within the thymic medulla, and that thymic IL-2 production is sufficient to sustain nT_{reg} development[129]. Early studies suggested that IL-2 was essential for nT_{reg} development, as mice deficient in *IL-2* or its receptors *CD25* or *IL-2Rβ* suffer from a severe lymphoproliferative disorder and immunopathology[130]. However, the current view is that IL-2 may be important, but not essential for nT_{reg} development in the thymus as CD28-mediated Foxp3 expression does not require IL-2[129]. In addition, studies using mice carrying the *Foxp3*^{gfp} reporter gene revealed the presence of thymic GFP^+ cells, albeit reduced by 50% to 80%, in the absence of the *IL-2* or *CD25* gene[131].

Besides IL-2, other cytokines likely play a vital role for the commitment to a regulatory lineage. In fact, mice lacking the common γ-chain receptor CD132 are completely deficient in Foxp3-expressing cells in the thymus[131]. As the common γ-chain receptor is necessary for the binding and proper signalling of various cytokines, such as IL-4, IL-5, IL-7, IL-15, and IL-21, it is probable that at least one, or a combination of, these proteins are essential for nT_{reg} development. These cytokines might have a redundant function in thymic nT_{reg} development and thus could compensate for a lack of IL-2. Interestingly, the immunomodulatory cytokine TGF-β seems not to be required for nT_{reg} development. Since the severe inflammatory disease observed in TGF-β knockout mice is not due to the absence or non-functionality of nT_{reg}[132]. Cytokines might also be required for peripheral homeostasis of nT_{reg}. For example, IL-2 is required for the regulation of nT_{reg} metabolism and cell growth[131].

Finally, it is likely that nT_{reg} require two signals to develop within the thymus: first, they must encounter self antigen with high affinity TCR binding, and second, they require additional cofactors, such as CD28 ligation and signals from one or several of the members of the vast family of common γ-chain cytokines.

4.3 Mechanisms of suppression

In addition to sustaining tolerance to self, recent evidence suggests that nT_{reg} also control a wide array of immune responses, such as those initiated against allergens[133], allogeneic organ transplants[134], tumour cells[135] as well as pathogenic and commensal microorganisms[136]. The cellular and molecular mechanisms by which nT_{reg} modulate these various immune responses remain elusive, although there are indications that multiple mechanisms might be used either alone or simultaneously, depending on the inflammatory context and tissue. It is apparent that nT_{reg} can suppress the activation and expansion of various immune cell subsets. Although nT_{reg} primarily down-regulate the activation, proliferation and cytokine secretion (mainly IL-2) of both $CD4^+$ and $CD8^+$ T cells[117,137], recent

evidence indicates that they might also prevent the proliferation, antibody production and isotype switching of B cells[138,139]. nT_{reg} can also inhibit both cytotoxicity and cytokine secretion by NK[140] and NKT[141] cells, and the maturation and function of DC[142]. Finally, nT_{reg} have been shown to impair memory responses mediated by both $CD4^+$ [143] and $CD8^+$ [144] T cells. This broad spectrum of action indicates that nT_{reg} likely employ various means in order to suppress immune responses.

4.3.1 Role of surface molecules in nT_{reg} suppression

As described in section 4.2.1 above, nT_{reg} require TCR triggering[145] in order to carry out their suppressive function but CD28 ligation does not appear to be essential for this activity *in vitro*[146]. Since a semi-permeable membrane between nT_{reg} and their target cells abrogates suppressive activity, nT_{reg} suppression is thought to be contact-dependant *in vitro*[117]. CTLA-4 might also play an important role in nT_{reg}-mediated suppression. In fact, CTLA-4 blockade abrogates nT_{reg} suppression *in vitro*, and mice deficient in *CTLA-4* suffer from multi-organ specific autoimmunity[147]. CTLA-4 might mediate nT_{reg} suppression either directly by promoting the activation of nT_{reg} or indirectly by inducing indoleamine 2,3-dioxygenase (IDO) in DC by interactions with CD80 and CD86[148]. IDO catalyzes the conversion of tryptophan into metabolites that possibly have potent immunosuppressive effects[149]. Although controversial, lymphocyte activation gene 3 (LAG3), which binds to MHC II and is expressed by nT_{reg} upon activation, might also mediate nT_{reg} suppression. In fact, $CD4^+CD25^+$ cells isolated from LAG3 knock-out mice display no suppressive activity, and blockade of LAG3 *in vitro* abrogates nT_{reg} suppression[150]. Whether or not LAG3 contributes to nT_{reg} suppression remains contentious, however, LAG3-mediated nT_{reg} suppression might explain how nT_{reg} can suppress a broad range of targets, such as DC, B cells, and macrophages, cells which all express MHC II[148]. All of these studies suggest that nT_{reg} utilize cell surface molecules, such as CTLA-4 and LAG3 to mediate suppression of T_{eff} functions.

4.3.2 Role of cytokines in nT_{reg} suppression

Although contact-dependant suppression has been demonstrated *in vitro*, there is no direct evidence that nT_{reg} function by such a manner *in vivo*. Cytokines have been implicated in nT_{reg}-mediated suppression *in vivo*. Some cytokines, such as IL-10 and TGF-β, are widely recognized for their immunosuppressive capacities. It is not surprising that there is mounting evidence that nT_{reg} can secrete these proteins in order to modulate immune responses to various antigens. $Foxp3^+$ T cells control inflammatory bowel disease and experimental allergic encephalomyelitis (EAE) by secreting IL-10 in affected tissue, such as the colon and central nervous system, respectively[151,152]. nT_{reg} have been demonstrated to promote tolerance to allogeneic skin grafts by means of IL-10 secretion[153]. TGF-β, on the other hand, is mostly important for the function of induced Th3 cells, but this cytokine has been suggested to induce nT_{reg} in the periphery by up-regulating Foxp3 expression in naïve T cells and supporting peripheral maintenance of nT_{reg}[154]. Although a controversial area of study, it has also been proposed that nT_{reg} might express high levels of membrane bound TGF-β, which could be involved in contact-dependant suppression of activated T cells and NK cells[148]. Thus, nT_{reg} secretion of cytokines, especially IL-10, is likely to allow them to mediate suppression *in vivo*.

The signals that trigger nT_{reg} to mediate this suppression are currently unknown. Clearly, activation of nT_{reg} by TCR ligation is essential, and thus TCR specificity of nT_{reg} might be important. It is known that nT_{reg} can suppress in an antigen non-specific manner[145] and are capable of mediating bystander suppression. However, nT_{reg} can probably also mediate suppression in an antigen-specific manner. This is supported by the fact that the nT_{reg} repertoire contains cells with TCR specific to foreign antigen, such as microbes, and that these cells can inhibit the immune response to infections in an antigen-specific manner[155]. Therefore, recognition of specific antigens by nT_{reg}, amongst other signals, might be essential for the initiation of their regulatory functions during an inflammatory response.

5. Role of regulatory T cells in parasitic infections

Since nT_{reg} can recognize foreign antigen, it is not surprising that recent evidence has demonstrated that nT_{reg} modulate the host immune response to various microbial infections, such as viruses, fungi, bacteria and parasites[156]. Interestingly, nT_{reg} seem to mainly influence immunity to pathogens that tend to establish chronic infections, such as parasites. In fact, nT_{reg} have been implicated in all parasitic diseases investigated so far, including leishmaniasis, schistosomiasis, filariasis, intestinal nematodes and importantly, malaria[157,158]. Parasites have intricate life cycles that must be completed in their vertebrate hosts, and hence they must establish long-term interactions with their host. Parasites can achieve this by manipulating the host immune response and establishing an environment that is most favorable for their growth and survival. As nT_{reg} can down-regulate host immune responses, these cells are excellent targets for parasites: nT_{reg} could preferentially be recruited to allow parasites to survive for a long period within their host. Many chronic parasitic infections also induce prolonged immune responses, which can lead to immunopathology, an important contributor to the morbidity and mortality associated with such infections. Therefore, as depicted in Figure 3, nT_{reg} might play a dual role in parasite infections. On one hand, nT_{reg} may be an intricate part of the microbe's life cycle and be actively recruited to suppress host anti-parasite immunity. On the other hand, nT_{reg} may be triggered as a consequence of parasite-induced immunopathology in order to limit tissue damage in the host.

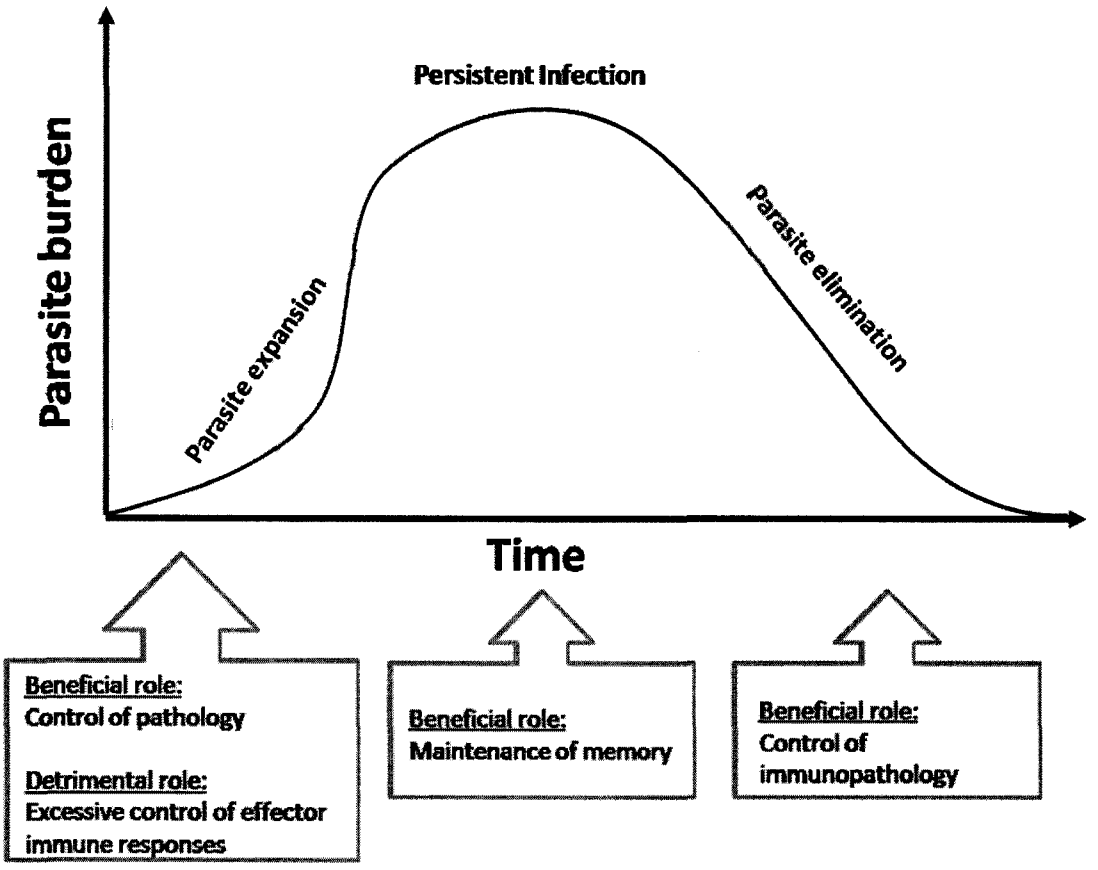


Figure 3. Dual role of nTreg during parasitic infection

Throughout parasite infection, nT_{reg} immunoregulation can be both beneficial and detrimental to the infected host. Adapted from Current Opinion in Immunology, Vol 18, Yasmine Belkaid, Cheng Ming Sun and Nicolas Bouladoux, Parasites and immunoregulatory T cells, Page 407, Copyright (2006), with permission from Elsevier.

Although most studies so far have investigated the role of nT_{reg} in immunity to parasites, it is important to recognize that other T_{reg} might regulate anti-parasitic immunity. In fact, various studies have identified that iT_{reg}, such as Th3 and Tr1 cells, might be induced by parasites during infection. *In vitro* treatment of DC with *S. mansoni*'s phosphatidylserine rendered these cells capable of inducing

IL-10 producing iT_{reg}[159]. Similarly, Tr1 cells can be generated *in vitro* by stimulation of T cells with *Onchocerca volvulus* antigen, and Tr1 cells can be isolated from the dermis of patients suffering from onchocerciasis[160]. TGF-β secreting Th3 cells might also be involved in *O. volvulus*-induced immunosuppression in humans[161]. Interestingly, both *Schistosoma japonicum*[162] and *Heligmosomoides polygyrus*[163] infection in mice induce T_{reg} which have the ability to suppress the development of asthma, possibly in an IL-10-dependant manner. Thus, regulation of anti-parasitic immunity is likely mediated by various regulatory immune cells, including both nT_{reg} and induced T_{reg}.

5.1 Beneficial roles of nT_{reg} during parasitic infections

Evidence that nT_{reg} play an important role in preventing overt immune responses and the development of immunopathology comes from studies demonstrating their role in maintaining gastrointestinal homeostasis and tolerance. The gastrointestinal tract is populated by thousands of microbial species and various studies suggest that nT_{reg} are essential for maintaining intestinal tolerance and preventing intestinal inflammatory diseases[164]. Gastrointestinal parasitic infections, such as *Cryptosporidium muris*, can provoke immunopathology unless kept in check by nT_{reg}[164]. Chronic non-intestinal infections, such as *Schistosoma mansoni*, can also lead to significant tissue pathology due to the continuous activation of the host immune system. *S. mansoni* is a trematode parasite that can survive within the human liver for up to 30 years[165]. Murine models of schistosomiasis have indicated that host survival is dependent on the control of immunopathology initiated by the strong immunogenicity of the parasite's eggs. It has recently been shown that Foxp3-expressing T_{reg} control overt host immunity to egg antigens by inhibiting the production of IL-12 by DC, potentially by an IL-10-dependant mechanism[166,167]. Interestingly, by preventing excessive pro-inflammatory immune responses, nT_{reg} can also limit parasite growth and survival. Non-healing *Leishmania amazonensis* infection has been attributed to the activation of pathogenic Th1 cells. A recent study revealed that accumulation of IL-10-producing nT_{reg} within skin lesions and draining lymph nodes of infected

mice decreases the production of IL-2 and IFN-γ by pathogenic T_{eff}, significantly limiting the development of pathogenesis. In addition, removal of nT_{reg} leads to an increase in parasite load[168]. Paradoxically, nT_{reg} inhibit *L. amazonensis*-specific immunity, however, this results in a more efficient control of parasites in the skin. Therefore, it seems that the establishment of an appropriate balance between nT_{reg} and T_{eff} allows for both the control of infection and prevention of immunopathology.

In addition to the beneficial roles of nT_{reg} to the host pertaining to their control of parasite-induced immunopathology, parasites themselves might also profit from the recruitment of nT_{reg} to the site of infection. There is the possibility that nT_{reg} might mediate a mutually beneficial relationship between parasites and their host. For example, a paper published by Belkaid and Piccirillo *et al.*[169] demonstrated that IL-10-producing nT_{reg} accumulate within dermal lesions during *L. major* infection in B6 mice and contribute to the long-term persistence of *Leishmania* parasites within these lesions. Depletion of nT_{reg} using anti-CD25 monoclonal antibody leads to the sterile cure of infected animals, but surprisingly, also impairs host immunity to re-infection. It seems that immunological memory to *Leishmania* requires persistent low-grade tissue infection that is maintained by the presence of nT_{reg} at the site of infection. Therefore, immunosuppression mediated by nT_{reg} can provide benefits to both the parasite, by providing a transmission reservoir within the host, and to the host, by maintaining life-long immunity to re-infection.

5.2 Detrimental roles of nT_{reg} during parasitic infections

5.2.1 nT_{reg} impair anti-parasitic immunity

Recruitment of nT_{reg} during parasitic infection can also have obvious detrimental effects since nT_{reg} might hinder the initiation of protective anti-parasitic immunity. There are multiple examples where excessive nT_{reg} function results in disruption of a proper T_{reg}/T_{eff} balance, resulting in inefficient anti-parasitic immune responses and thus contributing to the morbidity and mortality

of parasitic diseases. This includes the concept of disease reactivation, which might occur due to the presence of nT_{reg} during the chronic stages of parasitic disease. Disease reactivation often occurs as a result of immunosenescence due to aging, immunosuppression, or environmental stress, although the exact causes are unclear. Recent evidence has indicated that nT_{reg} play a pivotal role in this process. In fact, an increase in nT_{reg} numbers in B6 mice chronically infected with *L. major* is sufficient to trigger disease reactivation[170]. In this model, nT_{reg} were shown to adversely affect the generation of memory T_{eff} responses. Therefore, although nT_{reg} are beneficial to the host by providing life-long immunity to re-infection, alteration of the proper T_{reg}/ T_{eff} balance can be detrimental to the host by triggering disease reactivation.

The presence of nT_{reg} during the early stages of a parasitic infection might also result in an impairment of the initiation of anti-parasitic immunity and contribute to pathogenesis of the disease. Human filariasis is characterized by a profound state of chronic immunosuppression, which also characterizes murine models of this disease. Mice infected with *Brugia pahangi* exhibit decreased protective Th1 immunity, which correlates with the accumulation of Foxp3-expressing T cells throughout infection[171]. In addition, infection of mice with *Litomosoides sigmodontis* is characterized by the accumulation of nT_{reg} in the thoracic cavity[172]. Treatment of mice with anti-CD25 and anti-GITR antibodies simultaneously during *L. sigmodontis* infection results in a decrease in parasite burden and an increase in parasite-specific immunity, characterized by significantly higher Th2 cytokine secretion. Thus, immune hyporesponsiveness and high parasite burden during infection with parasites causing filariasis might be attributable to the accumulation of suppressive nT_{reg} at the site of infection

5.2.2 nT_{reg} modulate anti-malarial immunity

nT_{reg} possibly also play a role in promoting malaria-induced pathogenesis. A study conducted in *P. falciparum*-infected humans showed a strong correlation between high levels of parasitemia and both an increase in bio-active TGF-β

throughout infection and an up-regulation of *FOXP3* mRNA between days 7 and 10 p.i.[173]. Monocytes and not nT_{reg} were identified as the major source of TGF-β in this study. However, high concentrations of TGF-β correlated with up-regulation of *FOXP3* expression in peripheral blood $CD3^+CD4^+CD25^{hi}CD69^-$ lymphocytes in infected individuals, indicating that the regulatory cells might be induced and not naturally-occurring T_{reg}. *In vitro* re-stimulation of PBMC from infected patients after depletion of $CD25^{hi}$ cells was found to result in a two-fold increase in malaria-specific proliferation, but had no effect on malaria-specific T cell IFN-γ production. Thus, it appears that a population of T cells with a regulatory phenotype can suppress anti-malaria immunity in humans, resulting in high parasite burdens. Murine studies have also investigated the role of nT_{reg} in malaria pathogenesis and immunosuppression. A study by Hisaeda *et al.* demonstrated that depletion of nT_{reg} using anti-CD25 during infection with *P. yoelii* strain XL increases survival to a normally lethal infection[174]. *In vivo* depletion of nT_{reg} results in increased malaria-specific splenocyte proliferation, indicating that nT_{reg} are likely responsible for the immune hyporesponsiveness associated with lethal *P. yoelii* infection in mice. A subsequent study by this group demonstrated that the lethal stain of *P. yoelii*, in contrast to the non-lethal strain, preferentially recruits and induces the expansion of nT_{reg}, and that these cells can suppress in a malaria-specific manner[175]. These observations indicate that nT_{reg} might be directly induced by malaria parasites, and that this activation might be strain-specific. Thus, studies in both murine models and in humans indicate that nT_{reg} likely play an important role in anti-malarial immunity by inhibiting T_{eff} expansion responses and promoting parasite growth and survival. Unfortunately, these studies do not provide any insight as to which mechanisms are involved in nT_{reg}-mediated suppression of anti-malarial immune responses. In addition, the major caveat of these studies is the use of CD25, and not Foxp3, as the main biomarker for nT_{reg}. Therefore, the ontogeny of the cells mediating anti-malarial suppression, either nT_{reg} or iT_{reg}, remains elusive.

5.2.3 nT_{reg} decrease vaccine efficacy

Strong evidence also indicates that nT_{reg} might decrease the efficacy of vaccination. As described in the mouse model of *L. major* infection, nT_{reg} can actively prevent memory T_{eff} responses[176], the development of which is essential for the efficacy of vaccination. In addition, trials of the anti-leishmania LACK and TRYP vaccines in a murine model of *L. major* indicated that IL-10-producing T_{reg} prevent the success of these vaccines, most probably by counteracting protective pro-inflammatory IFN-γ responses[176]. Although the regulatory cells identified in this study were most probably iT_{reg}, these results suggest that T_{reg}, including nT_{reg}, might contribute to the failure of all anti-parasitic vaccines to date.

5.3 Manipulation of nT_{reg} by parasites

Lessons learned from various parasitic infections in mice strongly suggest that parasites can actively recruit nT_{reg} for their own benefit and the establishment of a chronic infection in their host. The methods by which parasites do this remain elusive. Two mechanisms have been shown to potentially contribute to nT_{reg} recruitment and/or retention at the site of parasitic infection: parasites can promote the production of cytokines that favor nT_{reg} peripheral maintenance and homeostasis, and/or they can increase the chemokine responsiveness of these regulatory cells[177]. A prime example of the former view is that of *Plasmodium* parasites, which are known to promote the production of TGF-β by various immune cell subsets in both mice and humans[178]. Production of TGF-β during a malaria infection might promote the survival and function of nT_{reg}. This is demonstrated by a recent study by Walther *et al.*, which showed a positive correlation between high levels of monocyte-derived TGF-β and an increase in *FOXP3* mRNA in PBMCs of *P. falciparum* infected humans, which correlated with higher blood parasitemia[173].

In addition to cytokine production, parasites might promote the retention of nT_{reg} within infected tissue by inducing the up-regulation of adhesion molecules

on the surface of nT_{reg}. For example, *L. major* infection in mice results in the retention of CD103-expressing nT_{reg} within skin lesions[179]. Exposure to *Leishmania* antigen leads to CD103 up-regulation on the surface of nT_{reg} and correlates with susceptibility to the parasite and establishment of a chronic infection. Up-regulation of CD103 on nT_{reg} also occurs during infection with *S. mansoni* in mice[180]. A recent study showed that nT_{reg} from *L. major*-infected mice preferentially express the CCR5 chemokine receptor[97]. High levels of CCR5 ligands, such as MIP-1α, MIP-1β, or RANTES, were shown to be produced during leishmania infection. Thus, this study indicates that nT_{reg} likely express certain receptors, which allow them to localize to the site of parasitic infection and mediate immunsuppression. However, it is currently unknown whether parasites themselves are directly responsible for the increased expression of these receptors on nT_{reg}, or whether they mediate the production of specific chemokines in order to actively recruit nT_{reg}. Since nT_{reg} express many chemokine receptors, including CCR4 and CCR8[98], it is quite possible that parasites have evolved one or both such mechanisms.

In addition to expressing chemokine receptors, nT_{reg} express a wide array of TLRs, such as TLR-4, TLR-5, TLR-7, and TLR-8. As these receptors are known to bind various conserved microbial products, nT_{reg} might be able to directly respond to microbial infections. Interestingly, some parasites also express TLR ligands, and these include malarial hemozoin, which binds TLR-9[18], as well as *Trypanosoma cruzi* GPI[181] and *Toxoplasma gondii*'s profilin-like protein[182], which bind TLR-2 and TLR-11, respectively. Since TLR ligation can directly influence the function and expansion of nT_{reg} *in vitro* and *in vivo*[183], it has been postulated that parasites might enhance immunosuppression during an infection by directly interacting with nT_{reg} through binding of their microbial products to TLRs on the surface of nT_{reg}. However, to date, there is no direct evidence that parasites manipulate the host immune response in this manner.

6. Rationale and objectives

Malaria is one of the most prevalent infectious diseases in humans. Infected individuals exhibit generalized immunosuppression characterized by inefficient cellular and humoral responses against the parasite as well as bystander antigens in addition to inadequate parasite-specific immunological memory demonstrated by poor immunity to re-infection and the failure of anti-malarial vaccines. Since nT_{reg} can suppress the development of various immune responses, these cells likely play an important role in diseases, which result in immunosuppression. In fact, it is now widely accepted that nT_{reg} modulate the immune response to parasitic infections, mainly by suppressing the development of appropriate T_{eff} responses but also by providing protection against parasite-induced immunopathology. In the specific case of malaria, previous studies suggest that T_{reg} might render mice susceptible to malaria infection and impair malaria-specific lymphoproliferative responses in an antigen-specific manner[174,175]. Observational data from humans indicate that high expression of *FOXP3* during *P. falciparum* infection correlates with higher parasitemia and production of immunomodulatory TGF-β[173]. However, a number of questions regarding the role of T_{reg} in anti-malarial immunity remain unanswered. What is the ontogeny of T_{reg} during malaria infection, induced or naturally-occurring? What are the specific immune cell targets of T_{reg} during suppression of malaria immunity? What are the cellular and molecular mechanisms that allow T_{reg} to suppress anti-malarial immunity? For example, do T_{reg} require direct-contact with their immune cell targets and/or mediate suppression by secreting immunomodulatory cytokines? Do T_{reg} play a role in the pathogenesis of malaria infections?

In light of these issues, the objective of the present study is to determine whether and how $CD4^+Foxp3^+$ nT_{reg} contribute to the inefficacy of anti-malarial immunity by inhibiting the development of innate and adaptive cell-mediated immune responses, therefore promoting parasite growth and survival. To address this question, we investigated the role and functions of nT_{reg} in anti-malarial immunity using the model of *P. chabaudi* AS blood-stage infection in mice.

Firstly, we confirmed that nT_{reg} modulate the anti-*P. chabaudi* AS immune response by promoting parasite growth and thus increasing susceptibility to malaria. Secondly, the effects of nT_{reg} on anti-malarial immunity were assessed, particularly regarding the impact of nT_{reg} on response of NK cells, $CD4^+$ T_{eff} cells and B cells. Finally, the cellular mechanisms by, which nT_{reg} regulate the malaria-specific immune responses were determined. The present study also investigated the requirement of immunomodulatory cytokines, such as IL-10, for nT_{reg} modulation of anti-malarial immunity, as well as the proliferative capacity of nT_{reg} during *P. chabaudi* AS.

MATERIALS & METHODS

1. Mice, parasites and infections

C57BL/6, A/J (The Jackson Laboratory, Bar Harbor, ME), pUbi-GFP transgenic mice (kind gift from Brian C. Schaefer, Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA) and Foxp3 transgenic line 2826 (Foxp3Tg; kind gift from Fred Ramsdell, ZymoGenetics Inc., Seattle, WA, USA) mice were bred and housed in the Lyman Duff animal facility, McGill University (Montreal, Quebec, Canada), under specific pathogen-free conditions. All animal studies were conducted according to the guidelines of the Canadian Council for Animal Care and from the Guide for the Care and Use of Laboratory Animals, Animal Resources, McGill University. Blood-stage *P. chabaudi* AS malaria parasites were maintained in A/J mice by weekly passage as described by Podoba and Stevenson[184]. For experimental infection, mice were injected i.p. with 10^6 *P. chabaudi* AS-parasitized red blood cells (pRBC). Malaria parasitemia was monitored on blood smears collected at the indicated time points for up to 30 days p.i. and stained with Diff-Quik (Fisher Scientific, ON, Canada). In some experiments, mice were sacrificed at the indicated time points and blood was obtained by cardiac puncture. Sera were collected and stored at -20°C for determination of cytokine levels. Unless specified, mice used in all experiments were males, 6–8 weeks of age. Mice were age and sex-matched for all experiments, and at least 3 mice were included in each experimental group.

2. Cell cultures and *in vitro* proliferation assays

Spleens from infected mice were removed aseptically. In some experiments, splenomegaly in infected Tg and B6 mice was determined based on the spleen index (splenic weight (mg)/body weight (g)). Body weight was measured prior to the sacrifice of each animal and splenic weight was measured right after resection.

Perfusion and mechanical shearing using a sterile metal mesh allowed for the preparation of single-cell suspensions from spleens. Red blood cells were lysed with 0.15 M NH_4Cl (Sigma-Aldrich, St. Louis, MO, USA) and cell suspensions were filtered through sterile gauze to remove debris. The viability of cells was always >95% as determined by trypan blue exclusion (Invitrogen Life Technologies, Carlsbad, CA, USA). Preparation of single-cell suspensions and splenocyte cultures were conducted in complete medium: RPMI 1640 was supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (20μg/ml), 2mM L-glutamine, 10 mM Hepes, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Invitrogen Life Technologies), and 50μM 2-mercaptoethanol (Sigma-Aldrich).

For proliferation assays, $5x10^5$ splenocytes were cultured alone (to determine background level proliferation) or stimulated with $5x10^6$ pRBC, $5x10^6$ uninfected red blood cells (RBC) as a negative control, or 2.5 μg/ml of concanavalin A (ConA) as a positive control, in triplicate wells in a 96-well, flat-bottom microtiter plates at a final volume of 200 μl/well for 72 hours at 37°C in 5% CO_2. Cells were pulsed with 0.5 μCi ^{3}H-thymidine for the last 12 hours to assess the extent of proliferation. RBC and pRBC were purified by a method described by Ing *et al.*[185]. Briefly, blood was collected by cardiac puncture from naive mice (RBC) and *P. chabaudi* AS-infected mice (pRBC) using a heparinised syringe and washed twice with 1x PBS. Blood was then diluted with 1 ml of 1x PBS and loaded onto a 74% Percoll (Sigma-Aldrich) density gradient for pRBC and a 90% Percoll density gradient for RBC. Blood-loaded density gradients were then centrifuged at 7800 rpm at room temperature for 20 minutes, using a table microcentrifuge. The top bands were collected, washed twice with 1x PBS and purified RBC and pRBC were resuspended in complete medium.

For intracellular IFN-γ staining, single cell suspensions were prepared from infected and non-infected Foxp3Tg and B6 mice, and $4x10^6$ cells were stimulated in the presence of 20 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin (Sigma-Aldrich, St. Louis, MO, USA) for 5 hours, with the addition of

2 µl/well of monensin (GolgiStop, BD Biosciences) during the last hour of culture in a final volume of 2 ml in a 24-well flat-bottom culture plate. Cells were then harvested and stained for FACS analysis as described below.

3. Flow cytometry

In order to determine the phenotype of lymphocyte populations, $2x10^6$ spleen cells from experimental and control mice were washed in 1x PBS with 1% FCS and stained with anti-CD16/32 (2.4G2;BD PharMingen) to block F_c receptors for 15 minutes at 4°C. Without washing, cells were then stained for an additional 15 minutes at 4°C with a combination of the following conjugated antibodies: allophycocyanin (APC) anti-CD25 (PC61, eBioscience, San Diego, CA, USA), phycoerythrin cytocrome 7 (PECy7) or fluorescein isothiocyanate (FITC) anti-CD4 (L3T4, eBioscience), APC anti-CD49b (DX5, eBioscience), phycoerythrin (PE) anti-B220 (RA3-6B2, eBiosciene), PeCy7 anti-CD8α (53-6.7, eBioscience). For intra-nuclear Foxp3 staining, cells were stained with antibodies against surface antigens and then were fixed, permeabilized, and stained with PE anti-Foxp3 (FJK-16s, eBioscience) in the presence of anti-CD16/32 (2.4G2;BD PharMingen) to block F_c receptors, using the eBioscience Foxp3 staining kit according to the manufacturer's protocol (eBioscience). For intracellular cytokine staining or analysis of lymphoproliferation, cells were stained following the same protocol as for Foxp3 staining, with the addition of either APC anti-IFN-γ (XMG1.2, eBioscience) or FITC anti-Ki67 (B56, BD Bioscience) during staining with PE anti-Foxp3.

FACS acquisition was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). At least 25 000 gated events were acquired per sample, and data were analysed using FlowJo software (TreeStar, Ashland, OR, USA).

4. Purification of T cell subsets and adoptive transfers

Splenocytes from naïve C57BL/6 or pUbi-GFP mice were surface stained with 5 μg/100x10^6 cells of FITC anti-CD4 (eBioscience) and PE anti-CD25 (eBioscience). $CD4^+CD25^+$ cells were isolated using anti-PE magnetic beads (Miltenyi Biotec, Auburn, CA) with a double sensitive positive selection strategy using an autoMACS, according to the manufacturer's protocol (Miltenyi Biotec). $CD4^+CD25^-$ cells were isolated using anti-FITC magnetic beads (Miltenyi Biotec) on CD25-depleted splenocytes with a positive selection strategy using an autoMACS, according to the manufacturer's protocol (Miltenyi Biotec). Total $CD4^+$ splenocytes from Foxp3Tg mice were purified using positive selection after staining with anti-CD4 magnetic beads, according to the manufacturer's protocol (Miltenyi Biotec). The purity of isolated total $CD4^+$ or $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were typically >95% and >85%, respectively. For adoptive transfer experiments, an aliquot of 200 μl of sterile 1x PBS (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 5x10^5 or 10x10^6 MACS-purified $CD4^+CD25^+$ cells, $CD4^+CD25^-$ cells or total $CD4^+$ were injected i.v. into naïve C57BL/6 or Foxp3Tg mice. Control mice were injected with 200 μl of sterile 1x PBS. One day post-adoptive transfer, mice were challenged i.p. with 10^6 pRBC and parasitemia was monitored.

5. Cytokine ELISAs

Cytokines were detected in serum blood samples of infected animals, using cytokine-specific sandwich ELISA. IFN-γ and IL-10 ELISA were performed as previously described[43]. Briefly, round bottom microtiter plates (Immulon II; Dynatech Laboratories, Inc., Chantilly, Va.) were coated with 50 μl/well (3 ug/ml) of DB-1 anti-IFN-γ, produced in house, or with JES5.2A5 MAb (American Type Culture Collection, Manassas, Va.) capture antibodies. The plates were incubated overnight at 4°C and then washed three times with washing buffer which consisted of 1x PBS with 0.1% Tween 20 (Sigma-Aldrich). Blocking was performed for 2 hours at room temperature by adding 200 μl/well of blocking

buffer which consisted of 1x PBS + 0.1% Tween 20 + 1.0% BSA (Invitrogen). Plates were then washed three times with washing buffer and 50µl of each sample was added to the plate in triplicate and incubated overnight at 4°C for 1 hour. After three washes with washing buffer, 50 µl/well of a 1/500 dilution in blocking buffer of biotinylated rabbit polyclonal anti-mouse IFN-γ (PharMingen) or IL-10 (PharMingen) detection antibodies was added to each well and incubated at room temperature for 1 hour. After washing three times with washing buffer, ELISA reactivities were revealed by adding 50 µl/well of a 1/1000 dilution of streptavidin-HRP conjugate (R&D) for 1 hour at room temperature, followed by three washes and the addition of 200 µl/well of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) substrate (Boehringer Mannheim, Laval, Quebec, Canada). Optical density (OD) values were read in a microplate reader at 405 nm with a reference wavelength of 492 nm. The concentrations of cytokine in samples were calculated against the standard curve generated using recombinant cytokines for IFN-γ and IL-10 (R&D System, Minneapolis, MN).

6. Statistical analysis

Data are presented as means ± standard errors (SE) of the means. The statistical significance of the differences in means between experimental and control groups was analysed by Student's *t* test (two-tailed *P*-value) using the GraphPad Prism software. $P<0.05$ was considered significant.

RESULTS

1. $CD4^+Foxp3^+$ nT_{reg} enhance susceptibility to malaria

nT_{reg} have been shown to play a pivotal role in various parasitic diseases by being either beneficial or detrimental to the development of host anti-parasitic immunity[158]. Recent evidence indicates that $CD4^+CD25^+$ T cells might also influence anti-malarial immune responses[173-175]. However, since CD25 is a poor marker for nT_{reg} in an inflammatory setting, these studies do not convincingly demonstrate a functional role for nT_{reg} in the immune response to malaria, nor do they identify the phenotype of these regulatory cells as being either naturally-occurring or induced in nature. In light of this, an experimental setup was devised which allowed us to formally determine whether nT_{reg} modulate immunity to malaria (Fig. 4A). We established a mouse model whereby malaria infection induced by *P. chabaudi* AS would be characterized immunologically in mice over-expressing the *Foxp3* transcription factor (Foxp3Tg) and harbouring a supraphysiological peripheral pool of nT_{reg}. Foxp3Tg (Tg) mice are on the resistant B6 background but express 16 copies of a *Foxp3* transgene under the control of its natural promoter, resulting in no ectopic expression of this nT_{reg}-specific gene[186]. As shown in Figure 4B, up to 80% of splenic $CD4^+$ T cells from Foxp3Tg mice are $Foxp3^+$ in comparison to 15% in wild type (WT) B6 mice. Thus, Tg mice have a five-fold increase in the cellular frequency of peripheral nT_{reg}, which renders these mice profoundly immunosuppressed.

In order to determine whether increased nT_{reg} frequencies enhance susceptibility to malaria, Tg mice and their WT littermate controls were infected i.p. with 10^6 *P. chabaudi* AS pRBC; parasitemia levels and survival were monitored for 30 days post-infection (p.i.). Tg mice displayed a significantly higher level of peak parasitemia, with a peak parasitemia of 48% in comparison to 35% in B6 mice (Fig. 5A; $48 \pm 2\%$ vs. $35 \pm 1\%$, $p<0.05$). Unlike WT mice that all survived and cleared the parasites within 30 days p.i., Tg mice exhibited 100%

mortality to *P. chabaudi* AS infection (Fig. 5B). These data suggest that the increase in nT_{reg} pool impaired the host's control of parasite replication as well as clearance of parasites, therefore decreasing the survival to a malaria infection.

To investigate whether susceptibility of Tg mice to malaria can be transferred to normally resistant WT B6 mice, $10x10^6$ $CD4^+$ T cells MACS-purified from the spleens of naïve Tg mice were injected i.v. into WT recipient mice prior to infection with *P. chabaudi* AS. Over 80% of MACS-purified Tg $CD4^+$ T cells were $Foxp3^+$ (data not shown). Our results show that B6 mice adoptively transferred with nT_{reg} from Tg mice exhibited enhanced parasite burdens around peak of infection (days 7 and 8 p.i.) when compared to non-transferred, infected B6 mice (Fig. 5C). In addition, some adoptively transferred B6 mice (33%, 4 out of 12 total mice) succumbed to the infection. Infected B6 recipients displayed a 15% increase in peak parasitemia when compared to non-transferred control mice (55 ± 3 vs. 41 ± 1 % parasitemia, $p<0.05$). The increase in parasite burden in B6 recipients was similar to that observed in Tg mice compared to non-transgenic controls (Fig. 5A; 48 ± 2 vs. 35 ± 1 % parasitemia, $p<0.05$). These data provide further evidence that increased susceptibility to *P. chabaudi* AS in Tg mice was likely due to $CD4^+Foxp3^+$ T cells. Furthermore, these results support previously published data indicating that *in vivo* depletion of CD25-expressing T cells, including $CD4^+CD25^+$ nT_{reg}, in BALB/c mice decreases susceptibility to a normally lethal strain of *P. yoelii*[174]. Although Tg mice provide a good model for studying the effects of an increased nT_{reg} pool on immune responses, these mice have been reported to have a two-fold decrease in total circulating $CD4^+$ T cells[186]. This caveat might indicate that our findings in Tg mice were due to an overall decrease in T_{eff} rather than the effects of nT_{reg}. However, adoptive transfer of $10x10^6$ splenic $CD4^+CD25^-$ T_{eff} from WT mice into Tg mice prior to *P. chabaudi* AS infection did not rescue the susceptible phenotype of Tg mice (data not shown). This suggests that nT_{reg}-mediated regulation, and not impaired T_{eff} immunity, was directly responsible for the increased susceptibility of Tg mice to malaria infection. Taken together, these

findings indicate that resistant hosts, such as B6 mice, can be rendered susceptible to malaria by the presence of high numbers of circulating nT_{reg}.

Nonetheless, Tg mice are generally low responders to immune challenges[186], and little is known about their response to infection *in vivo*. Thus, in order to study the influence of nT_{reg} on anti-malarial immunity in a more physiological setting, we performed experiments in infected WT mice, which were adoptively transferred with nT_{reg} enriched from uninfected B6 mice. $CD4^+CD25^+$ nT_{reg} and $CD4^+CD25^-$ T_{eff} were MACS-purified from naïve B6 mice and injected i.v. into B6 recipients, one day prior to infection with 10^6 pRBC. Parasitemia levels were monitored up to 30 days p.i. in the three experimental groups, which were defined as follows: group A mice received $5x10^5$ T_{reg}, group B recipients were injected with $5x10^5$ T_{eff}, and group C controls received no cells. As shown in Figure 5D, B6 mice receiving T_{eff} (group B) had a significantly lower parasitemia at days 8 and 9 p.i. when compared to non-transferred control animals (group C; $p<0.05$). Interestingly, B6 mice receiving as few as $5x10^5$ nT_{reg} (group A) had significantly higher parasitemia around peak *P. chabaudi* AS infection (days 5, 8, 9, 10 and 11 p.i.; $p<0.05$) when compared to control mice (group C). In addition, recipients from group A displayed a marked recrudescence in parasite burden post-peak parasitemia (days 14, 16 and 18 p.i.) when compared to mice receiving T_{eff} (group B) or no cells (group C). In fact, adoptive transfer of nT_{reg} to some B6 mice delayed clearance of parasites up to a week later than T_{eff} recipients and non-transferred controls. Thus, these findings indicate that nT_{reg} were sufficient to transfer susceptibility to malaria to a normally resistant host and promote parasite growth and persistence.

Splenomegaly is a common clinical feature of malaria infection and is a marker for malaria-specific proliferation of immune cells and increased splenic erythropoiesis[187]. Therefore, we investigated whether nT_{reg} can influence the development of splenomegaly during malaria by determining splenic indices in infected Tg and WT B6 mice. Whereas B6 mice exhibited a significant increase in splenic indices at days 6 and 8 p.i. with *P. chabaudi* AS compared to naïve B6

mice, the splenic indices of Tg mice remained at levels comparable to non-infected Tg mice at these time points (Fig. 5E). Interestingly, splenic indices were not increased at later time points (days 6 and 8 p.i.) in Tg compared to B6 mice, and were significantly less than those of B6 mice (5.1 ± 0.8 vs. 9.6 ± 0.7 and 4.3 ± 0.9 vs. 12.5 ± 2.3 at days 6 and 8 p.i., respectively, $p<0.05$). Thus, these data suggest that nT_{reg} may impair malaria-specific lymphoproliferation and that nT_{reg} may contribute to the pathological sequelae of malaria by promoting malaria-induced anemia. Indeed, preliminary observations in our laboratory suggest that reticulocyte levels were lower in infected Tg compared to infected B6 mice (data not shown), although the direct involvement of nT_{reg} in erythropoiesis remains to be investigated.

In conclusion, our findings from *P. chabaudi* AS-infected Tg mice as well as those from adoptive transfer experiments of nT_{reg} into B6 mice prior to infection with malaria demonstrated that nT_{reg} impaired anti-malarial immunity and increased susceptibility to malaria.

2. $CD4^+Foxp3^+$ nT_{reg} modulate malaria-specific immune effector responses

Our data suggest that nT_{reg} represented an important checkpoint in the modulation of anti-malarial immunity. Previous studies have demonstrated the suppressive activity of nT_{reg} during malaria infection, but the exact anti-malarial immune effector functions impaired by nT_{reg} suppression have not been identified[175]. Therefore, we sought to determine which elements of the anti-malarial immune response are affected by nT_{reg} suppression. To this end, the effects of nT_{reg} on the malaria-specific lymphoproliferation and cytokine production by splenocytes were determined *in vitro*. Splenocytes from Tg and B6 were isolated at various time points p.i. with *P. chabaudi* AS and their ability to proliferate in a malaria-specific fashion was assessed following *in vitro* re-stimulation with pRBC by means of a ^{3}H-thymidine incorporation assay. Whereas splenocytes from infected B6 mice proliferated vigorously in response to *in vitro* stimulation with malaria antigen, cells from Tg mice proliferated up to 78%, 84%

and 42% less than B6 mice at days 4, 6 and 8 p.i., respectively (Fig. 6A, $p<0.05$). Consistent with reduced splenic indices in Tg mice (Fig. 5E), these data provide evidence that nT_{reg} impaired malaria-specific lymphoproliferative responses.

It is widely accepted that both pro-inflammatory and immunomodulatory cytokines play a pivotal role in resistance to malaria[188,189], and an appropriate balance of cytokine secretion must be achieved to ensure proper parasite clearance. Early IFN-γ secretion is a measure of resistance to *P. chabaudi* AS infection[43], while immunomodulatory cytokines, such as IL-10 are paradoxically also required for proper parasite clearance[189]. Additionally, IL-10 secretion has been suggested as a possible mechanism by which nT_{reg} might modulate the host immune response to various inflammatory conditions as well as parasitic diseases[190]. Therefore, we determined whether nT_{reg} suppress anti-malarial immune responses by inhibiting the production of IFN-γ and whether this nT_{reg} regulation might be IL-10 dependent. To address these questions, serum levels of IFN-γ and IL-10 in *P. chabaudi* AS-infected Tg and B6 mice were determined by ELISA. Our results showed that serum levels of IFN-γ were significantly increased in infected B6 mice at days 6 and 8 compared to naïve B6 controls (Fig. 6B; 935.3 ± 79.3 and 433.3 ± 183.7 at days 6 and 8, respectively, vs. 18.0 ± 9.0 at day 0; $p<0.05$). Unlike B6 mice, Tg mice did not exhibit increased levels of IFN-γ at days 6 and 8 p.i. with *P. chabaudi* AS compared to non-infected Tg controls. Indeed, IFN-γ production at days 6 and 8 p.i. was decreased five-fold in Tg compared to B6 mice (Fig. 6B; 178.7 ± 30.6 vs. 935.3 ± 79.4 pg/ml and 75.0 ± 7.0 vs. 433.3 ± 183.7 pg/ml, $p<0.05$). Intriguingly, the levels of IL-10 were also significantly reduced in Tg mice compared to B6 at both day 6 (Fig. 6C; 446.5 ± 266.2 vs. 4865.3 ± 1058.4 pg/ml, $p<0.05$) and day 8 p.i. (Fig. 6C; 208.7 ± 55.5 vs. 1995.3 ± 338.4 pg/ml, $p<0.05$). Unlike infected Tg mice, which displayed no significant increase in serum IL-10 levels compared to non-infected Tg controls, increased IL-10 levels were observed in infected B6 mice compared to non-infected B6 controls (4865.3 ± 1058.5 and 1995.3 ± 338.4 pg/ml at days 6 and 8, respectively, vs. not detected at day 0, $p<0.05$). Reduced systemic IFN-γ observed

in *P. chabaudi* AS-infected Tg mice suggest that nT_{reg} likely impaired pro-inflammatory production during malaria infection.

However, the observation that Tg mice exhibited low serum levels of IL-10 during *P. chabaudi* AS infection might be attributable to the fact that nT_{reg} dominate the T cell repertoire in these mice, and as such circumvents the requirement for IL-10 in the regulation of malaria infection. In addition, low IFN-γ production in these mice might be due to the overall decrease in T_{eff} frequencies in the spleens of naïve Tg compared to naïve WT B6 mice[186], which might result in a generalized decreased pro-inflammatory cytokine production during malaria infection. To investigate whether IL-10 contributes to nT_{reg}-mediated suppression of anti-malarial immunity, a more physiological model of nT_{reg} enrichment in B6 mice prior to infection with *P. chabaudi* AS was used. To this end, we adoptively transferred B6 mice with $5x10^5$ $CD4^+CD25^+$ nT_{reg} (group A) or $CD4^+CD25^-$ T_{eff} (group B) purified from spleens of naïve B6 mice, one day prior to infection with *P. chabaudi* AS. B6 mice receiving no cells (group C) were included as negative controls. Total systemic IL-10 levels were then quantified by ELISA in serum samples of infected, transferred mice at various time points p.i. Mice from all three experimental groups exhibited increased serum levels of IL-10 at days 6, 8 and 21 p.i. with *P. chabaudi* AS compared to naïve mice. In contrast to what was observed in Tg mice (Fig. 6C), mice receiving $5x10^6$ nT_{reg} (group A) had significantly higher levels of IL-10 at day 6 p.i. compared to mice receiving $5x10^5$ T_{eff} (group B) or no cells (group C) (Fig. 6D; 3086.0 ± 280.1 pg/ml vs. 1559.3 ± 466.8 and 2192.7 ± 65.7 pg/ml, $p<0.05$). No significant differences in IL-10 levels between experimental groups were observed on days 8 and 21 p.i. Thus, nT_{reg} induced the production of IL-10 during the early phase of *P. chabaudi* AS infection, at a time point which corresponds to the initiation of the adaptive immune response to malaria. In addition, no differences in IFN-γ production were observed between experimental groups at any time points analysed (Fig. 6E). These findings suggest that nT_{reg} promote high levels of IL-10 in malaria-infected mice prior to peak parasitemia and thus likely influence the balance between pro-inflammatory and immunomodulatory cytokines during the early stage of *P.*

chabaudi AS infection. As in other models of pathogen infections[156,191], increased levels of IL-10 were associated with increased disease severity, as demonstrated by higher parasite burdens in *P. chabaudi* AS-infected nT_{reg} recipient mice.

In conclusion, supraphysiological frequencies of circulating nT_{reg} in Tg mice lead to deficient levels of both IFN-γ and IL-10 during *P. chabaudi* AS infection in these mice. However, a more physiological model of nT_{reg} enrichment in B6 mice prior to infection with *P. chabaudi* AS indicated that nT_{reg} likely did not influence systemic levels of IFN-γ but promoted increased levels of IL-10 during early infection. These findings suggest that nT_{reg} might regulate the balance between Th1 and Th2 anti-malarial immune responses. Although our data are contradictory, we believe that Tg mice might not be an ideal model for studying the mechanisms of nT_{reg} suppression due to the relative dominance of these cells in the T cell repertoire of these mice, and that our adoptive transfer experiments are likely a better indication of the effects of nT_{reg} suppression on the immune response to *P. chabaudi* AS.

3. $CD4^+Foxp3^+$ nT_{reg} accumulate in the spleen of infected mice and modulate malaria-specific T_{eff} IFN-γ production

Since the spleen is the major site of immunological priming and parasite clearance during a malaria infection[3], we examined the kinetics of nT_{reg} and T_{eff} bio-distribution and accumulation in spleen during *P. chabaudi* AS infection in susceptible Tg and resistant B6 mice. Peripheral lymph nodes cells (inguinal, brachial, axial and mesenteric) were also analysed, but no significant differences were observed between genotypes or in infected mice compared to their uninfected controls (data not shown). As Tg mice succumbed to infection within 10 days p.i. (Fig. 5B), only early time points of *P. chabaudi* AS infection were analysed. To monitor frequencies of splenic $CD4^+$ $Foxp3^+$ nT_{reg} and $CD4^+$ $Foxp3^-$ T_{eff} throughout the evolution of the disease, splenocytes were isolated from malaria-infected mice at days 0, 5, and 7 p.i. and $CD4^+$ $Foxp3^+$ nT_{reg} frequencies were analysed by FACS. Interestingly, nT_{reg} numbers were significantly decreased

in the spleens of *P. chabaudi* AS-infected B6 mice at days 5 and 7 p.i., compared to day 0 controls (Fig. 7A; $24.0x10^6 \pm 1.8x10^6$ and $18.0x10^6 \pm 2.2x10^6$ cells at days 5 and 7, respectively, vs. $98.0x10^6 \pm 2.1x10^6$ cells at day 0; $p<0.05$ for both). On the other hand, a sharp increase in splenic T_{eff} was observed at day 5 and 7 in infected B6 mice compared to uninfected mice ($13.0x10^7 \pm 2.0x10^7$ and $19.0x10^7 \pm 2.2x10^7$ cells at days 5 and 7, respectively, vs. $19.0x10^6 \pm 2.7x10^6$ cells at day 0; $p<0.05$ for both). This increase in T_{eff} corresponded to the initiation of the anti-malarial adaptive immune response and peak parasite burden (Fig. 5A). Therefore, nT_{reg} numbers were decreased in resistant B6 mice whereas T_{eff} numbers increased, likely indicating the generation of a pro-inflammatory response leading to protective immunity against *P. chabaudi* AS.

Interestingly, nT_{reg} numbers were significantly increased at days 5 and 7 p.i. in Tg mice compared to day 0 ($99.0x10^6 \pm 9.9x10^6$ and $94.2x10^6 \pm 3.8x10^6$ at days 5 and 7, respectively, vs. $15.4x10^7 \pm 1.9x10^6$ cells, $p<0.05$ for both). This day 5 and 7 increase in the frequency of nT_{reg} correlated with the time of T_{eff} increase in B6 mice. However, levels of $CD4^+Foxp3^-$ T_{eff} remained low throughout infection with *P. chabaudi* AS in Tg mice, and an increase in T_{eff} numbers in these mice was not observed at day 5 and 7 p.i. as occurred in B6 mice. Therefore, susceptible Tg mice displayed an increase in nT_{reg} frequencies during the early stage of infection, which likely impaired the expansion of T_{eff} in the spleen of infected mice. On the other hand, malaria infection promoted the accumulation of T_{eff} cells and not nT_{reg} within the spleen, site of immunological priming in resistant B6 mice. Thus, these observations suggest that nT_{reg} likely responded to a malaria infection in a time-dependant manner and potentially optimally suppressed the development of a T cell-mediated anti-malarial response.

An increase in T_{eff} numbers in the spleen of resistant B6 mice within the first week of infection (Fig. 7A) was likely responsible for the decreased parasite burdens, which occurred at day 8 p.i. in B6 mice (Fig. 5A). In fact, the expansion of T_{eff} cells upon antigenic stimulation is directly linked to the acquisition of their effector function and often leads to the production of inflammatory mediators,

such as cytokines[192]. Therefore, we investigated whether decreased T_{eff} accumulation in the spleen of *P. chabaudi* AS-infected Tg mice was associated with impaired T_{eff} functions. In order to address this question, we examined the production of IFN-γ by intracellular cytokine staining (ICS) on splenocytes isolated from infected B6 and Tg mice. Splenocytes were re-stimulated *in vitro* for 5 hours in the presence of PMA/ionomycin and FACS analysis was performed on gated $CD4^+Foxp3^-$ cells in order to assess the production of intracellular IFN-γ in this population. The frequency of IFN-γ-producing $CD4^+Foxp3^-$ T_{eff} increased significantly in both B6 and Tg mice at day 7 p.i. with *P. chabaudi* AS relative to non-infected control mice (Fig. 7B; 12.1 ± 0.9 vs. 0.1 ± 0.02 pg/ml for B6 mice, 4.9 ± 0.7 vs. 0.08 ± 0.01 pg/ml for Tg mice, $p<0.05$). However, the relative increase in IFN-γ^+ T_{eff} at day 7 compared to day 0 p.i. was noticeably lower in Tg compared to B6 mice. Indeed, the frequency of IFN-γ^+ T_{eff} was significantly lower in Tg compared to B6 mice at day 7 p.i. (4.9 ± 0.7 vs. 12.1 ± 0.9 %, $p<0.05$) In accordance with our finding of reduced levels of IFN-γ in the serum of infected Tg mice (Fig. 6B), these results indicate that the presence of high numbers of nT_{reg} was associated with a decreased $CD4^+$ T cell-mediated *P. chabaudi* AS-specific pro-inflammatory response. These findings suggest that nT_{reg} not only prevented the accumulation of T_{eff} in the spleen of malaria-infected mice, but also suppressed the T_{eff} anti-malarial response by inhibiting the production of IFN-γ.

4. $CD4^+Foxp3^+$ nT_{reg} preferentially accumulate within the spleen of malaria infected mice

Previous findings indicate that nT_{reg} require specific localization to the draining lymph node or affected target tissue in order to carry out their suppressive function and mediate their regulation of inflammatory responses[193]. An example of this is the requirement for CCR5 expression on nT_{reg} in order for these cells to localize within dermal lesions of *L. major*–infected mice and promote chronic infection[97]. However, it is unknown whether localization of nT_{reg} is important for modulation of anti-malarial immunity. In order to specifically address whether nT_{reg} preferentially accumulate in the spleen of *P. chabaudi* AS-

infected mice, we performed an adoptive transfer experiment whereby naïve B6 mice were injected i.v., one day prior to infection with *P. chabaudi* AS, with $5x10^5$ $CD4^+CD25^+$ nT_{reg} (group A) or $CD4^+CD25^-$ T_{eff} (group B) MACS-sorted splenocytes from naïve pUbI-GFP transgenic reporter mice. These mice are on the B6 background and ubiquitously express green fluorescent protein (GFP)[194], which enabled us to track the *in vivo* bio-distribution of transferred cells by FACS. Mice receiving no cells were used as a control (group C). Of note, GFP^+ cells, both nT_{reg} and T_{eff}, were only detected in adoptively transferred B6 mice at day 7 p.i. with *P. chabaudi* AS and not at day 21 p.i. (data not shown), possibly due to the limitation of detecting injected GFP^+ cells within an enlarged malaria-infected spleen. Interestingly, GFP^+ cells were not detected in peripheral lymph nodes or in blood of any group of adoptively transferred mice. However, GFP^+ cells were detected in the spleens of *P. chabaudi* AS-infected nT_{reg} and T_{eff} recipients at day 7 p.i. (Fig. 8). Importantly, significantly higher numbers of GFP^+ cells were recovered from nT_{reg} recipients (group A) compared to T_{eff} recipients (group B; $p<0.05$). These data suggest that both T_{eff} and nT_{reg} accumulate within the site of immunological priming during a malaria infection. However, our findings indicate that there was preferential accumulation of nT_{reg}, and not T_{eff} within the spleen of *P. chabaudi* AS-infected B6 mice.

5. Susceptible A/J mice have high numbers of nT_{reg} throughout malaria infection

A/J mice are the most common model for studying the susceptibility to *P. chabaudi* AS infection[10], as they exhibit significantly higher parasitemia when compared to resistant B6 mice and succumb to infection within 10 days of infection (Fig. 9A). In light of the observation that both A/J and Tg mice strains were susceptible to *P. chabaudi* AS infection (Fig. 5A and Fig. 9A), and that our results indicate that nT_{reg} suppressed anti-malarial immunity, we sought to investigate whether nT_{reg} might contribute to the susceptibility of A/J mice to *P. chabaudi* AS infection. To address this question, the relative frequencies of $CD4^+Foxp3^+$ cells in the spleens of *P. chabaudi* AS-infected A/J and B6 mice during the first 7 days of infection were examined. Tg mice were also included in

our study as positive controls for nT_{reg}-mediated susceptibility to malaria. As expected, Tg mice had high frequencies of nT_{reg} throughout *P. chabaudi* AS infection (Fig. 9B). As described above, resistant B6 mice displayed a significant decrease in the mean number of splenic Foxp3-expressing $CD4^+$ T cells at day 7 p.i., relative to naïve B6 mice ($p<0.05$). However, in contrast to infected B6 mice, *P. chabaudi* AS-infected A/J mice displayed no such decrease in splenic nT_{reg} numbers. In fact, nT_{reg} numbers in the spleen of A/J were maintained throughout *P. chabaudi* AS infection, and were significantly higher than the number of Foxp3-expressing cells in B6 mice at day 7 p.i. ($28.0x10^5 \pm 4.5x10^5$ vs. $8.2x10^5 \pm 2.3x10^5$ cells, $p<0.05$). Thus, the presence of higher frequencies of nT_{reg} in the spleen of infected A/J mice was associated with their impaired control of parasite growth and subsequent susceptibility to the disease.

6. High numbers of splenic nT_{reg} correlates with impaired anti-malarial immune effector functions in susceptible A/J mice

Multiple lines of evidence have indicated that nT_{reg} not only impair T_{eff} functions but can also modulate the effector functions of other immune cells, such as NK cells and B cells[195]. Our findings suggested that nT_{reg} suppressed $CD4^+$ T cell-mediated anti-*P. chabaudi* AS immunity (Fig. 7A and B). However, it remains unclear how nT_{reg} influence T cell-mediated anti-malarial immunity and whether nT_{reg} also modulate the function of other immune cells during a malaria infection. Since our results demonstrated that susceptible A/J mice maintained high frequencies of splenic nT_{reg} at days 5 and 7 p.i. with *P. chabaudi* AS (Fig. 9B), we investigated the impact of nT_{reg} on the expansion and functions of T_{eff}, NK cells and B cells in these mice. To determine the effects of nT_{reg} on malaria-induced T_{eff} proliferation, FACS analysis of Ki67 expression, which is a specific marker for proliferating cells, was performed on splenocytes from *P. chabaudi* AS-infected A/J, B6 and Tg mice. Our results showed that the frequency of Ki67-expressing T_{eff} increased only modestly at day 5 p.i. but considerably at day 7 p.i. in all three genotypes compared to their respective non-infected controls (Fig. 10A). However, *P. chabaudi* AS-infected Tg mice exhibited only a modest

increase in the frequency of Ki67-expressing T_{eff} at days 5 and 7 p.i. compared to naïve Tg mice (14.2 ± 1.9 and 23.5 ± 1.5 pg/ml at days 5 and 7, respectively, vs. 9.1 ± 0.4 pg/ml at day 0). B6 mice displayed the highest increase in the frequency of proliferating T_{eff} as over 50% of $CD4^+Foxp3^-$ cells expressed the Ki67 marker at day 7 p.i. compared to less than 10% at day 0. Although the frequency of proliferating T_{eff} was also increased in the spleen of A/J mice at day 7 p.i. compared to non-infected controls, the frequency of $Ki67^+CD4^+Foxp3^-$ T_{eff} cells was significantly lower in A/J mice compared to B6 mice at this time point (39.4 ± 1.2 vs. 54.5 ± 0.1 %, $p < 0.05$). These data suggest that the extent of T_{eff} proliferation in response to *P. chabaudi* AS infection was inversely associated with the number of nT_{reg} present in the spleen of malaria infected mice.

NK cells play an essential role in the innate immune response to *P. chabaudi* AS, as the extent of their production of pro-inflammatory IFN-γ during the early phase of infection influences the course and outcome of an infection[196]. Therefore, we investigated whether nT_{reg} modulate NK cells during malaria infection by determining the extent of Ki67 expression in $CD3^-DX5^+$ splenocytes during *P. chabaudi* AS infection in A/J, B6 and Tg mice. The frequency of Ki67-expressing NK cells was increased in B6, A/J and Tg mice at both days 5 and 7 p.i. with *P. chabaudi* AS compared to non-infected controls. However, $CD3^-DX5^+$ splenocytes from A/J mice had significantly lower expression of Ki67 at day 5 p.i. compared to B6 mice, to a level comparable to that of Tg mice (Fig. 10B). These findings indicated that at day 5 p.i., when innate anti-malarial immunity should be fully developed, NK cells from A/J mice displayed impaired proliferation. However, this does not indicate whether or not NK cell function is impaired in A/J mice. In order to address this question, FACS analysis of intracellular IFN-γ production in $CD3^-DX5^+$ splenocytes was performed. The frequencies of IFN-γ^+ NK cells were increased on days 5 and 7 in infected A/J, Tg and B6 mice compared to their respective day 0 controls. However, similar to Tg mice, NK cells from A/J mice expressed significantly lower levels of intracellular IFN-γ at day 5 p.i. compared to B6 mice (Fig. 10C; 6.07 ± 0.3 vs. 10.85 ± 0.6 %, $p<0.05$). No significant differences in the frequency of IFN-γ^+ NK cells were observed at

day 7 p.i. in A/J and Tg mice compared to B6, indicating that higher frequencies of nT_{reg} in these mice did not impair NK cell IFN-γ production during the late phase of innate immunity to *P. chabaudi* AS. These data indicate that NK cells from A/J mice displayed both deficient expansion and IFN-γ production during the early innate phase of the malaria immune response. However, whether nT_{reg} are directly responsible for deficient NK cell responses to malaria remains to be determined.

Because of the critical role of antibody production by B cells in the protective immune response to malaria[197], we investigated whether nT_{reg} modulated anti-malarial B cell immunity. To this end, $CD3^-B220^+$ splenocytes from *P. chabaudi* AS-infected A/J, B6 and Tg mice were stained for intracellular Ki67 and the *in vivo* proliferation of B cells was assessed. The extent of B cell proliferation was significantly reduced in A/J mice compared to B6 mice at days 5 and 7 of *P. chabaudi* AS infection (Fig. 10D). In fact, similar to Tg mice, the frequency of $KI67^+$ B cells in infected A/J mice remained similar to those of uninfected A/J mice at all time points studied, whereas a sharp increase in proliferating B cells was observed in B6 mice compared to non-infected B6 controls at day 7 p.i. B cells from A/J mice expressed up to five-fold less Ki67 at day 7 p.i. compared to B6 mice (5.2 ± 0.7 vs. 23.4 ± 1.2 %, $p<0.05$). Therefore, similar to the proliferative responses of T_{eff} and NK cells, a decrease in B cell expansion in A/J mice during *P. chabaudi* AS infection was associated with increased nT_{reg} numbers in the spleen of infected mice.

7. Expansion of $Foxp3^+CD25^-$ nT_{reg} during *P. chabaudi* AS infection

Studies indicate that nT_{reg} may proliferate in an antigen-dependant manner in response to various inflammatory conditions and that expansion of nT_{reg} may be required for their suppressive activity *in vivo*[198,199]. However, whether nT_{reg} expand in response to a malaria infection remains unknown. In order to address this question, FACS analysis of Ki67 expression in splenic nT_{reg} from *P. chabaudi* AS-infected A/J, B6 and Tg mice was performed. As shown in Figure 11A,

$CD4^+Foxp3^+$ splenocytes from *P. chabaudi* AS-infected A/J, B6 and Tg mice all expressed higher levels of Ki67 on day 7 p.i. compared to their respective non-infected controls. However, nT_{reg} from Tg mice had decreased Ki67 expression compared to nT_{reg} from both A/J and B6 mice at days 5 and 7 p.i. This discrepancy may be accounted for by the fact that nT_{reg} dominate the T cell repertoire in Tg mice and their expansion might not be required in order to suppress an immune challenge. Interestingly, although the frequency of $Ki67^+Foxp3^+$ nT_{reg} were similar in A/J mice compared to B6 mice at day 5 p.i., the frequencies of proliferating $CD4^+Foxp3^+$ cells was significantly higher in A/J mice compared to B6 mice at day 7 p.i. (Fig. 11A; 40.2 ±2.5 vs. 20.7 ± 0.2 %, $p< 0.05$). This high frequency of proliferating nT_{reg} in A/J mice was associated with a lower frequency of $Ki67^+CD4^+Foxp3^-$ T_{eff} cells (Fig. 10A). In addition, the frequency of Ki67 expression in nT_{reg} from peripheral lymph nodes (inguinal, brachial, axial and mesenteric) of B6 and A/J mice did not increase upon infection with *P. chabaudi* AS (data not shown), which indicates that malaria-induced nT_{reg} proliferation was specific to the spleen. These data suggest that the susceptibility of A/J mice to *P. chabaudi* AS was associated with high frequencies of proliferating splenic nT_{reg}, which likely results in decreased T_{eff} expansion. Since the spleen is the main site of parasite clearance during a malaria infection, the finding that only nT_{reg} from the spleen, and not lymph nodes, of infected mice expressed high levels of Ki67 suggests that *P. chabaudi* AS infection may promote antigen-specific triggering of nT_{reg} expansion. However, this issue requires further investigation.

Recently, the generation of a reporter $Foxp3^{gfp}$ mouse model allowed for the speculation that two populations of nT_{reg} might exist in mice[106]. In fact, although the majority of nT_{reg} are $CD4^+Foxp3^+CD25^+$, a small proportion of nT_{reg} cells do not express CD25. Although controversial, it is thought that $CD25^+$ and $CD25^-$ nT_{reg} may represent distinct subsets of nT_{reg}, but the transitional nature of these subsets cannot be excluded. Interestingly, a recent study has shown that the frequency of $CD4^+Foxp3^+CD25^-$ T cells increases upon *M. tuberculosis* infection in mice[106], suggesting that these cells might be important in regulating immunity

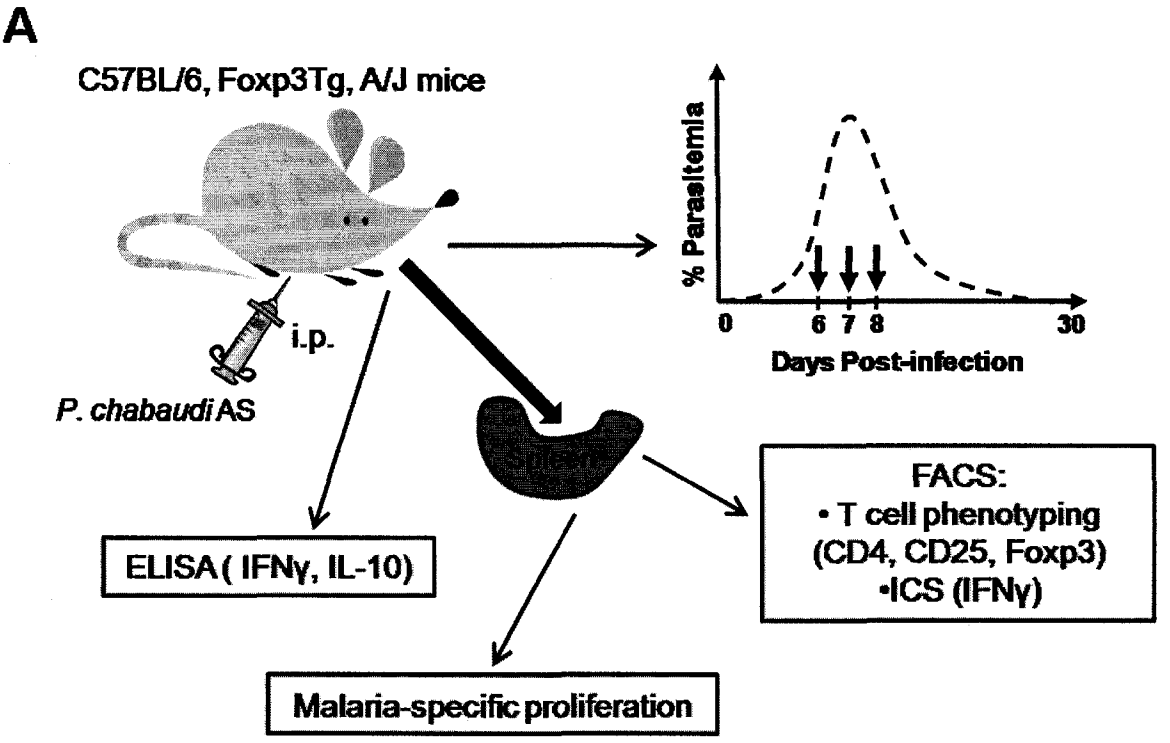
to infectious diseases. Therefore, we investigated whether differential expression of CD25 on nT_{reg} might result in different proliferative responses during *P. chabaudi* AS infection. To this end, FACS analysis for expression of Ki67, Foxp3 and CD25 was performed on splenocytes from infected A/J, B6 and Tg mice. The "classical" subset of $CD4^+Foxp3^+CD25^+$ nT_{reg} from A/J and B6 mice expressed higher Ki67 levels at days 5 and 7 p.i. with *P. chabaudi* AS compared to their respective non-infected controls (Fig. 11B). In fact, Ki67 expression in these cells increased three-fold within five days of *P. chabaudi* AS infection in A/J and B6 mice, although no significant differences were observed between these two strains at this time point. A small, albeit significant, difference in proliferating $Foxp3^+CD25^+$ cells was observed at day 7 p.i., whereby cells from A/J mice had 4% higher Ki67 expression compared to B6 mice. A/J mice also exhibited significantly higher frequencies of $KI67^+$ $Foxp3^+CD25^+$ cells at day 0 p.i. compared to B6 mice ($p<0.05$) On the other hand, striking differences were observed when the proliferative capacities of $Foxp3^+CD25^-$ cells from A/J mice were compared to those of B6 and Tg mice (Fig. 11C). Ki67 expression in $CD25^-$ nT_{reg} from A/J mice was significantly higher than in B6 mice at days 5 and 7 of *P. chabaudi* AS infection. In fact, the frequency of $Ki67^+Foxp3^+CD25^-$ cells increased 1.5-fold in A/J mice by day 7 p.i, at levels which were 10% higher than in B6 mice (46.3 ± 4.3 vs. 32.5 ± 1 %, $p<0.05$). Overall, the proliferative capacity of $CD25^-$ nT_{reg} in response to *P. chabaudi* AS was higher than for $CD25^+$ nT_{reg} in both B6 and A/J mice. In fact, 46.3 ± 3.0 % of $CD25^-$ nT_{reg} expressed Ki67 compared to 26.5 ± 0.1 % of $CD25^+$ nT_{reg} in day 7 *P. chabaudi* AS-infected A/J mice. Similarly, in B6 mice, only 22.9 ± 0.5 % of $CD25^+$ nT_{reg} were $Ki67^+$ at day 7 p.i., whereas 32.5 ± 0.7 % of $CD25^-$ nT_{reg} were proliferating. These findings suggest that $CD25^-$ nT_{reg} preferentially expanded in response to malaria. In addition, our data indicate that malaria-induced expansion of both $CD25^+$ and $CD25^-$ nT_{reg} was higher in A/J compared to resistant B6 mice, which may contribute to the susceptibility of A/J mice to malaria.

Nonetheless, the ability of both $CD25^+$ and $CD25^-$ nT_{reg} to proliferate in response to *P. chabaudi* AS infection did not differ significantly between A/J and

B6 mice. In fact, both $CD25^+$ and $CD25^-$ nT_{reg} from A/J and B6 mice exhibited two-fold increases in Ki67 expression from day 0 to 7 p.i. (Fig. 11B and 11C insets). However, Ki67 expression in both nT_{reg} populations was higher in A/J compared to B6 mice at day 0 p.i. These results suggest that nT_{reg}, whether $CD25^+$ or $CD25^-$, have inherently higher proliferative capacities in A/J compared to B6 mice. In addition, although these findings suggest that $CD25^+$ and $CD25^-$ nT_{reg} behave as distinct cell subsets during malaria infection, we cannot exclude the possibility that what appeared to be two distinct cell populations was solely a result of differential surface CD25 expression during a malaria infection. In fact, it is possible that "classical" $CD25^+$ nT_{reg} might downregulate their surface CD25 expression following expansion in an inflammatory site, such as the spleen during a malaria infection.

FIGURES

Figure 4. Experimental Set-up



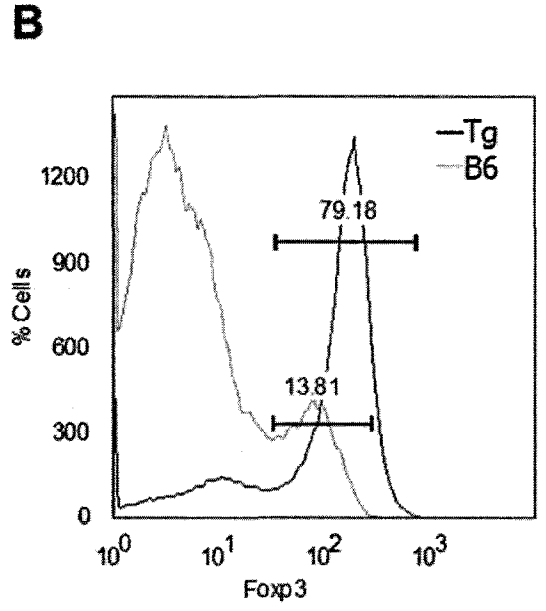


Figure 4: Experimental setup. (**A**) A/J, C57BL/6 or Foxp3Tg mice were infected with 10^6 *P. chabaudi* AS-infected RBC by i.p. injection. For all experiments, parasite load, as expressed by % parasitemia, was monitored throughout infection, up to 30 days p.i. Immunological studies (FACS, malaria-specific proliferation, and cytokine production) were performed on purified splenocytes at various time points p.i., notably prior to (day 6), on (day 7), or post peak parasitemia (day 8). In some experiments, systemic cytokine levels were quantified by ELISA on serum samples from infected mice. (**B**) FACS analysis of the frequencies of total $Foxp3^+$ splenocytes, gated on $CD4^+$ cells, from naïve Foxp3Tg (Tg) and C57BL/6 (B6). ICS, intracellular cytokine staining.

Figure 5. $CD4^+Foxp3^+$ nT_{reg} enhance susceptibility to malaria

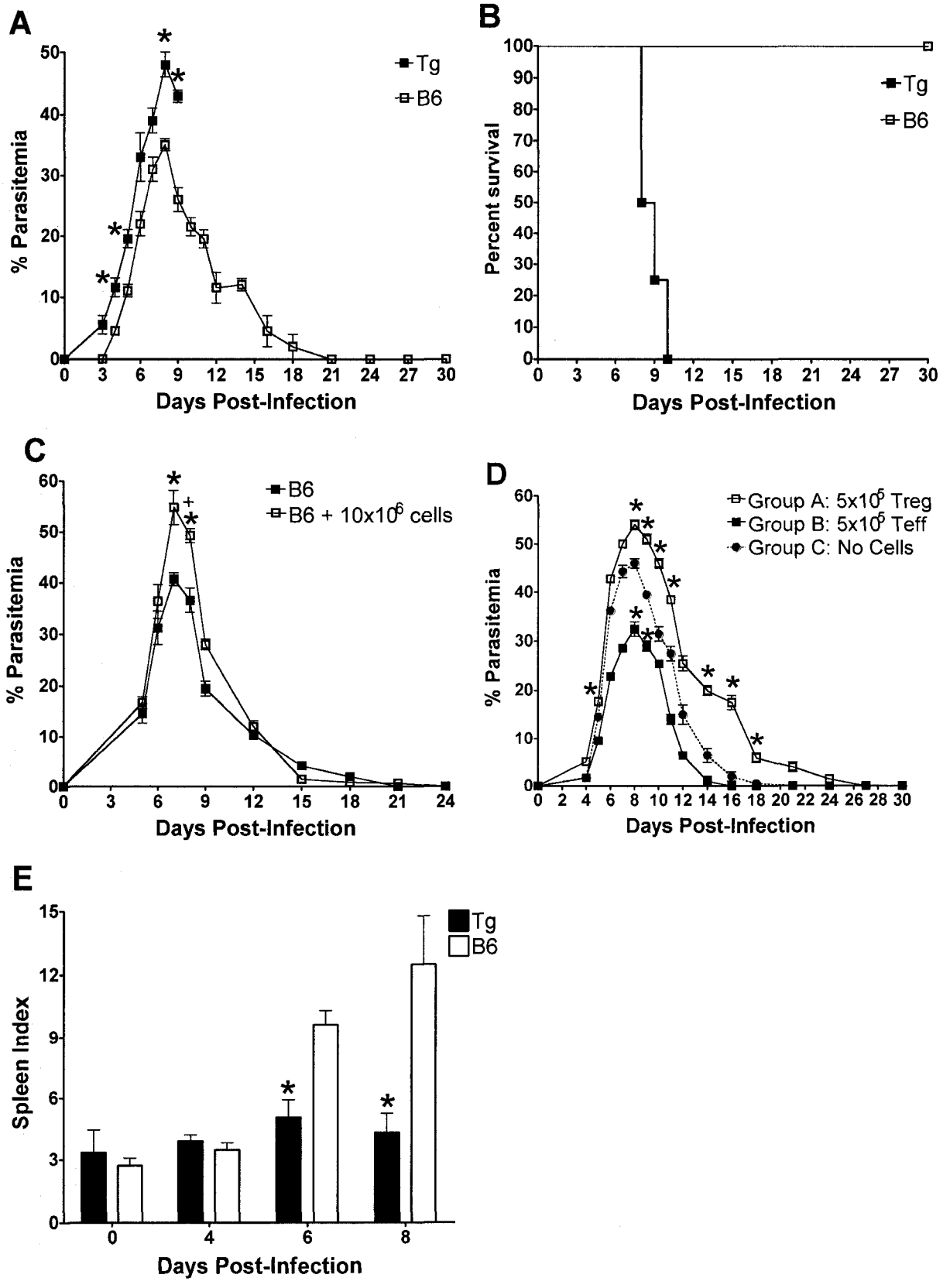


Figure 5: $CD4^+Foxp3^+$ nT_{reg} enhance susceptibility to malaria. Levels of (**A**) parasitemia and (**B**) survival of Foxp3Tg (Tg) and wild-type C57BL/6 (B6) mice after infection with 10^6 pRBC. (**C**) Levels of parasitemia in B6 mice adoptively transferred with $10x10^6$ total $CD4^+$ splenocytes purified from naïve Tg mice. All mice were infected i.p. with 10^6 pRBC on day 0. B6 mice receiving no cells were used as controls. (**D**) B6 mice were adoptively transferred with $5x10^5$ MACS-purified nT_{reg} (group A) or T_{eff} (group B), or with no cells (group C) as a negative control. Levels of parasitemia in each group of mice after infection with 10^6 pRBC. (**E**) Spleen indices of Tg and B6 mice at various time points p.i. with 10^6 pRBC. For all experiments, data are expressed as the mean ± SE of n = 4 mice per group from one of three replicate experiments. * $p<0.05$ compared to B6 animals, ** $p<0.05$ compared to Tg animals. +, time of deaths of adoptively transferred B6 mice.

Figure 6. $CD4^+Foxp3^+$ nT_{reg} modulate malaria-specific immune effector responses

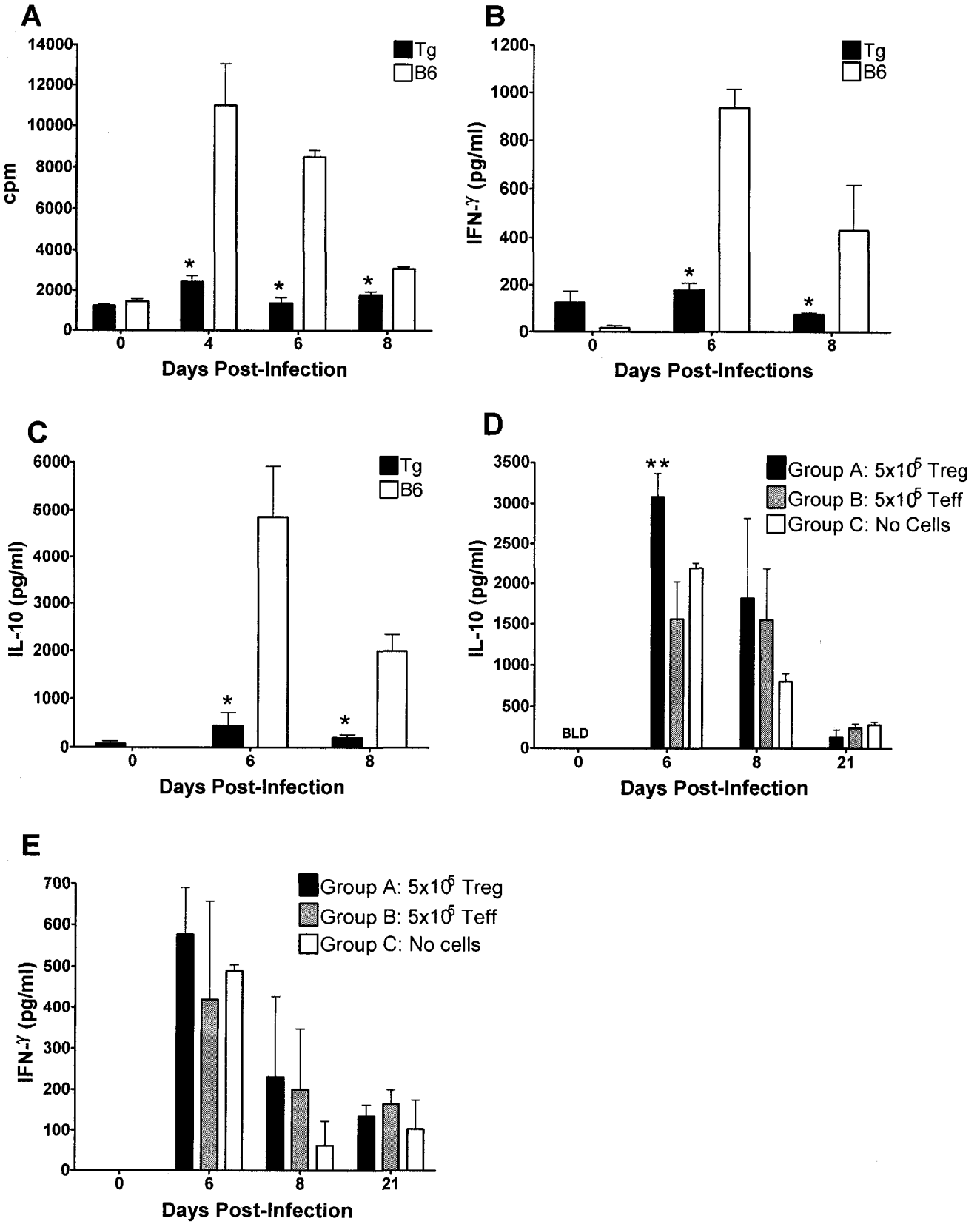
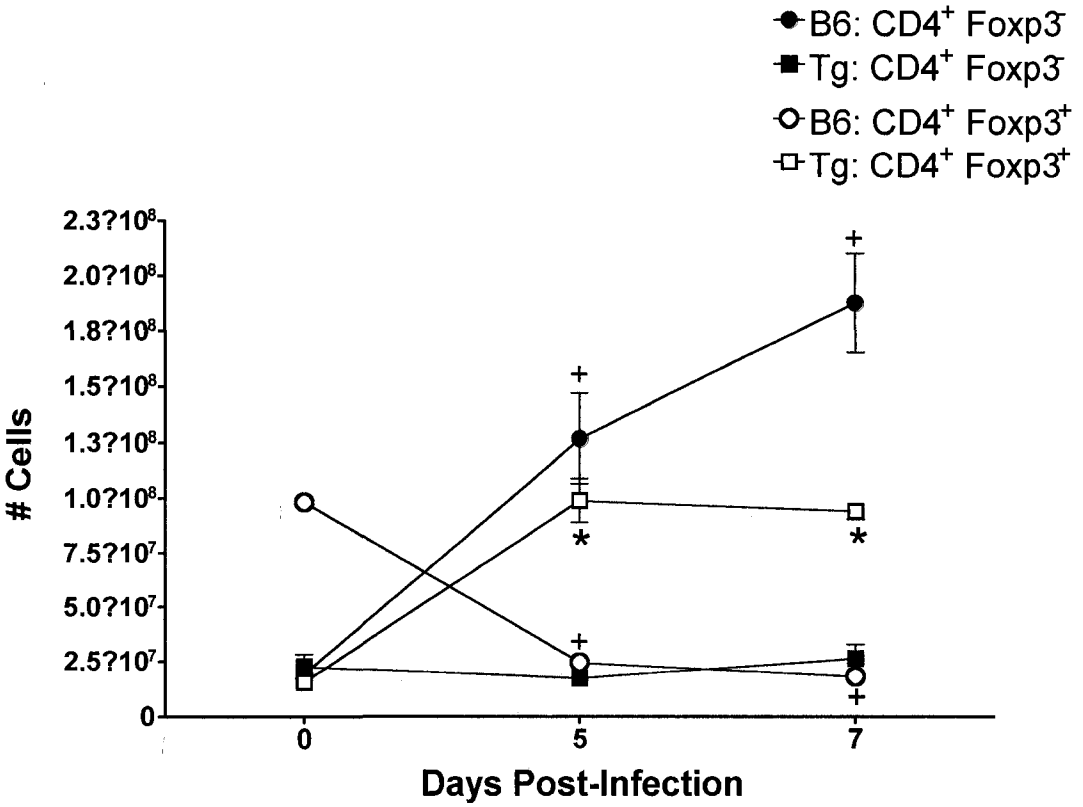


Figure 6: $CD4^+Foxp3^+$ nT_{reg} modulate malaria-specific immune effector responses. (**A**) Malaria-specific lymphoproliferation was measured by *in vitro* re-stimulation of $5x10^5$ splenocytes from *P. chabaudi* AS-infected Foxp3Tg (Tg) and C57BL/6 (B6) mice with purified pRBC, as described in *Materials & Methods*. In (**B**) and (**C**), serum samples were collected from infected Tg and B6 at the indicated time points and IFN-γ and IL-10 levels were quantified by ELISA. B6 mice were adoptively transferred with $5x10^6$ MACS-purified nT_{reg} or T_{eff} or with no cells as a negative control. ELISA quantifications of (**D**) IL-10 and (**E**) IFN-γ in serum samples collected from each group of adoptively transferred mice were performed at the indicated time points. For all experiments, data are expressed as the mean ± SE of n = 4 mice per group from one of three replicate experiments. * $p<0.05$ compared to B6 animals, ** $p<0.05$ compared to T_{eff} transferred mice or non-transferred controls. BLD, below level of detection.

Figure 7. $CD4^+Foxp3^+$ nT_{reg} accumulate in the spleen of infected mice and modulate malaria-specific T_{eff} IFN-γ production



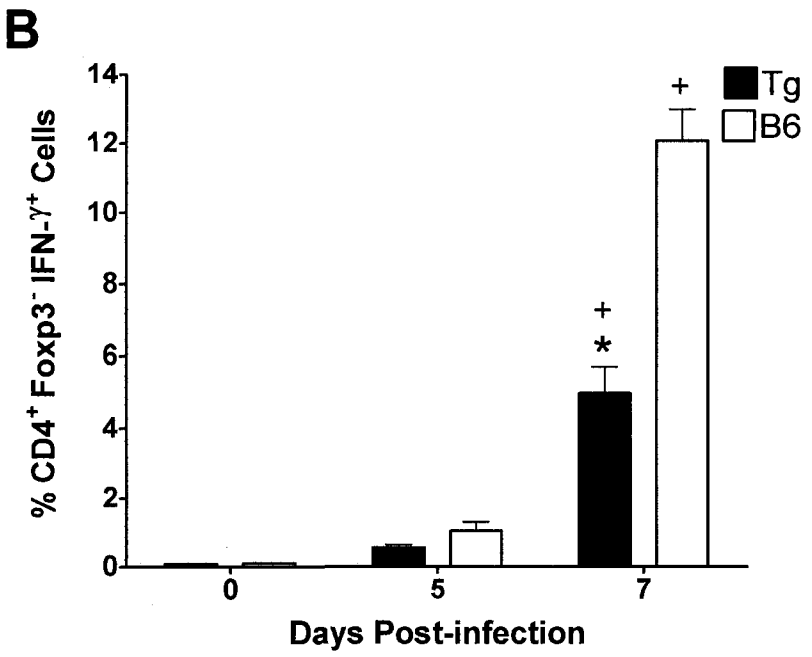


Figure 7: $CD4^+Foxp3^+$ nT_{reg} accumulate in the spleen of infected mice and modulate malaria-specific T_{eff} IFN-γ production. (**A**) FACS analysis of splenic nT_{reg} and T_{eff} numbers, gated on $CD4^+$ cells, in spleens of from Foxp3Tg (Tg) and C57BL/6 (B6) animals at various time points p.i. with 10^6 pRBC. (**B**) Intracellular IFN-γ production by $CD4^+Foxp3^-$ splenocytes at various days p.i. with *P. chabaudi* AS in Tg and B6 mice, as determined by FACS analysis. For all experiments, data are expressed as the mean ± SE of n = 4 mice per group from one of three replicate experiments. + $p<0.05$ compared to non-infected controls; * $p<0.05$ compared to infected B6 animals.

Figure 8. $CD4^+Foxp3^+$ nT_{reg} preferentially accumulate within the spleen of malaria infected mice

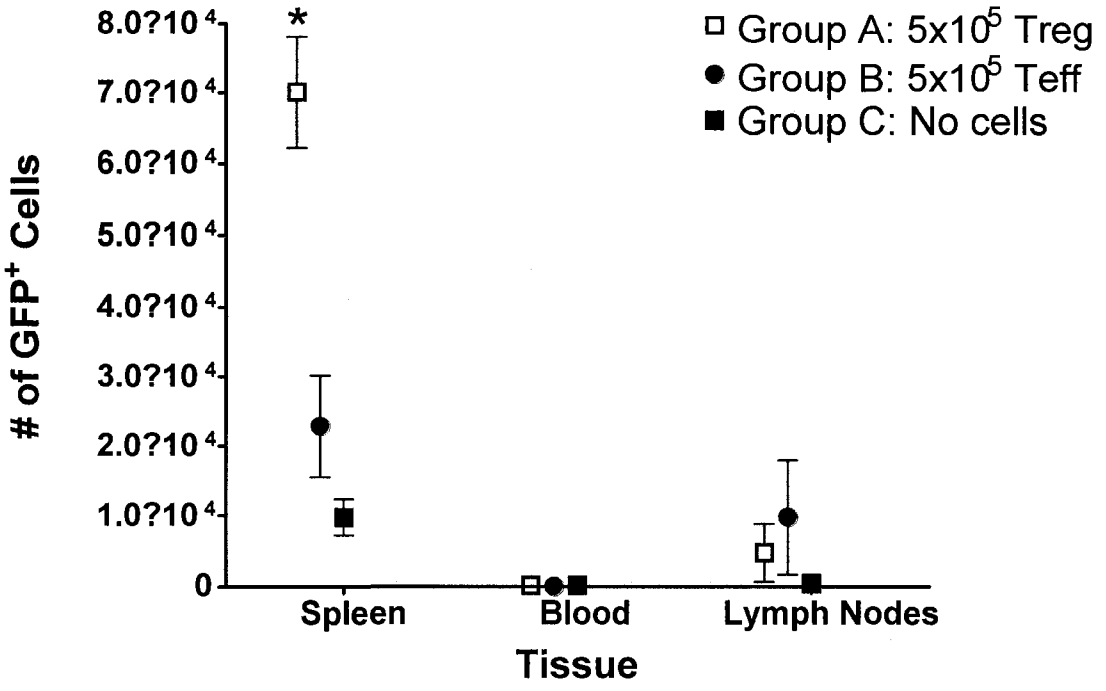


Figure 8: $CD4^+Foxp3^+$ nT_{reg} preferentially accumulate within the spleen of malaria infected mice. C57BL/6 (B6) mice were adoptively transferred with $5x10^5$ MACS-purified nT_{reg} (group A) or T_{eff} (group B) from pUbI-GFP transgenic mice or with no cells (group C) as a negative control. At day 7 p.i., numbers of GFP^+ cells were determined by FACS analysis on lymphocytes from lymph nodes, blood and spleen isolated from adoptively transferred mice. Data are expressed as the mean ± SE of n = 3 mice per group from one of three replicate experiments. * $p<0.05$ compared to T_{eff} transferred mice or non-transferred controls.

Figure 9. Susceptible A/J mice have high numbers of nT_{reg} throughout malaria infection

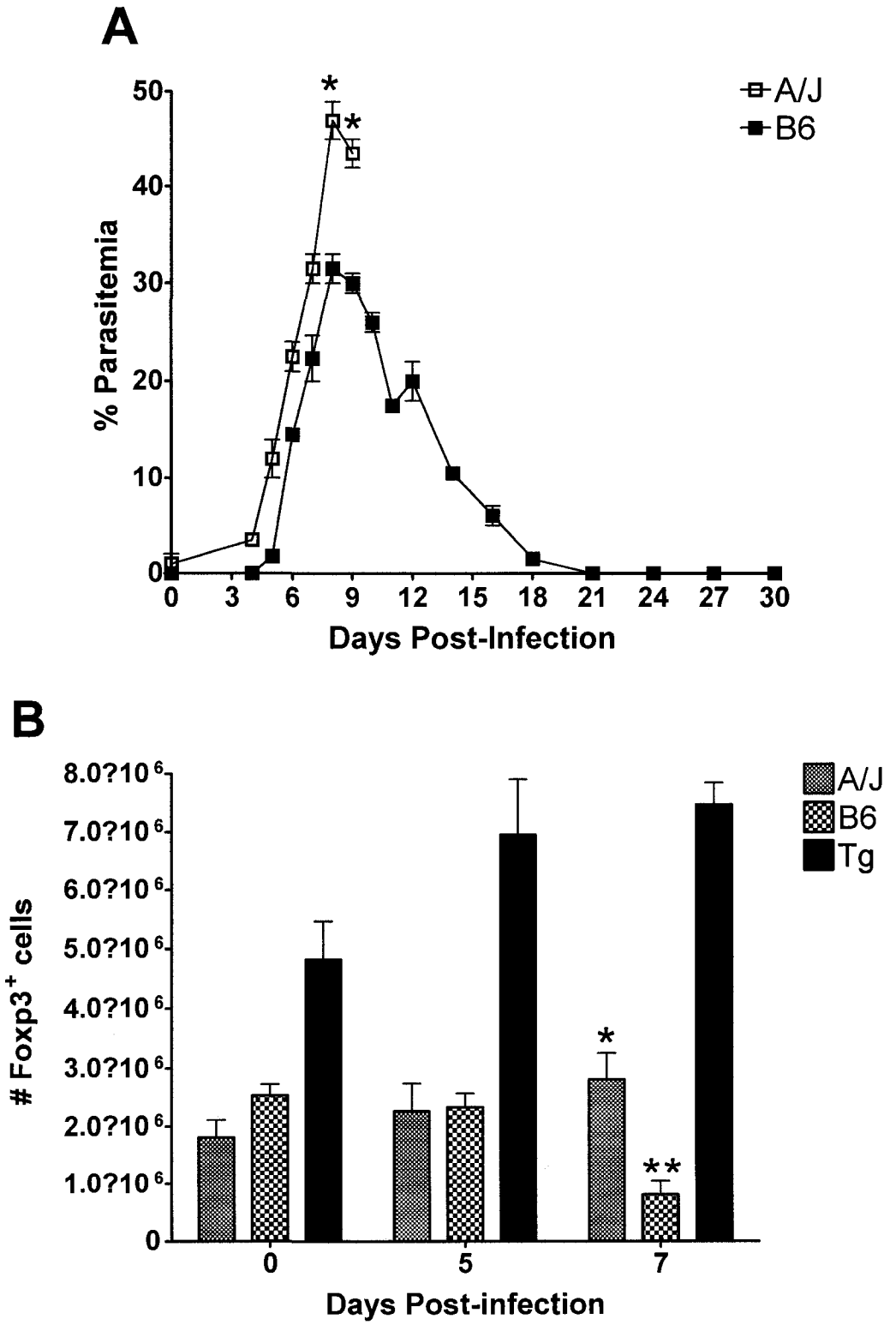


Figure 9: Susceptible A/J mice have high numbers of nT_{reg} throughout malaria infection. (**A**) Levels of parasitemia in A/J and C57BL/6 (B6) mice after infection with 10^6 pRBC. (**B**) FACS analysis of the total numbers of $Foxp3^+$ cells, gated on $CD4^+$ cells, in the spleens of *P. chabaudi* AS-infected A/J, B6 and Foxp3Tg (Tg) mice. For all experiments, data are expressed as the mean ± SE of n = 3 mice per group from one of three replicate experiments. * $p<0.05$ compared to infected B6 mice, ** $p<0.05$ compared to naïve B6 mice.

Figure 10. High numbers of splenic nT_{reg} correlates with impaired anti-malarial immune effector functions in susceptible A/J mice

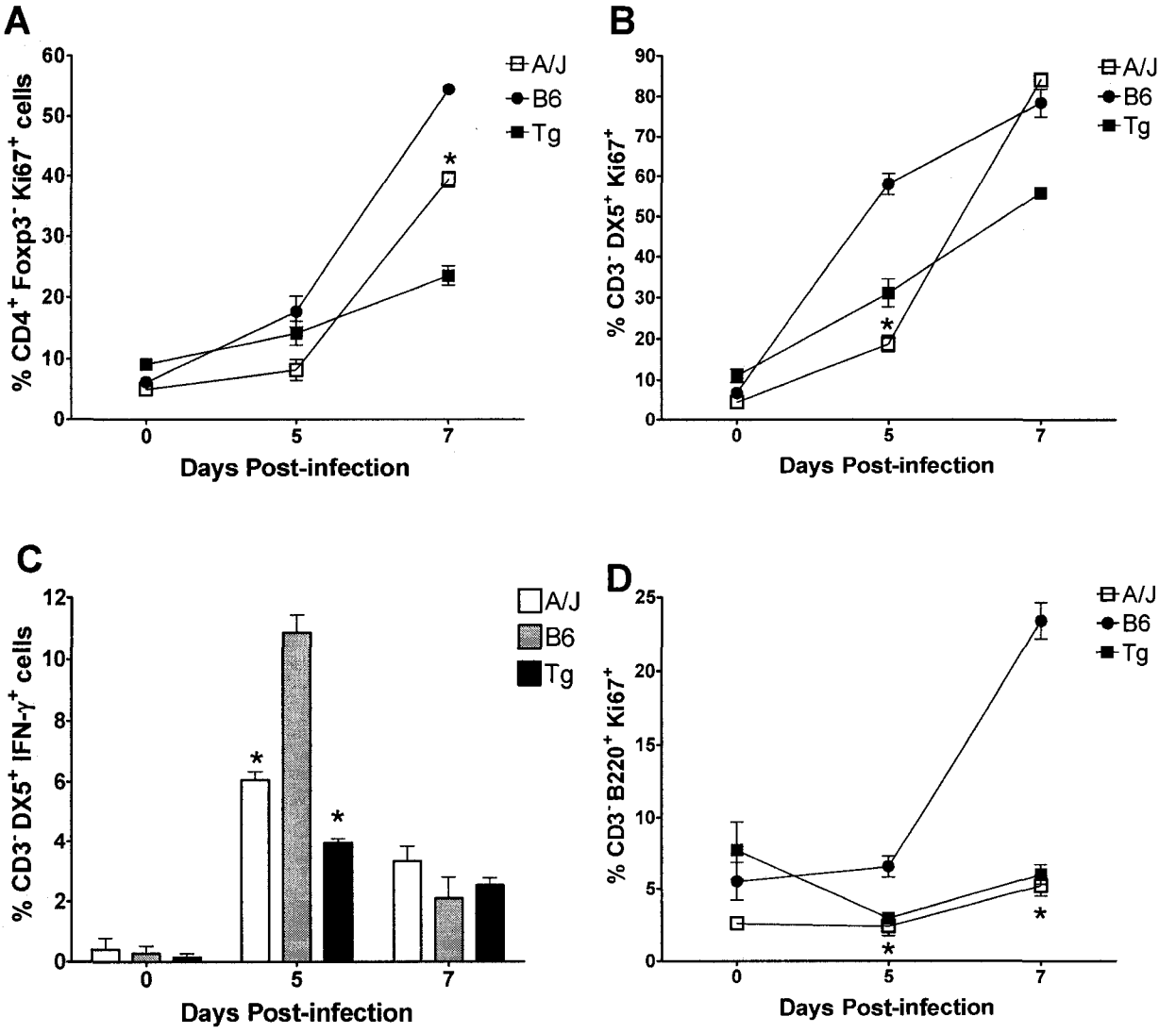


Figure 10: High numbers of splenic nT_{reg} correlates with impaired anti-malarial immune effector functions in susceptible A/J mice. FACS analysis of the frequency of $Ki67^+$ splenic (**A**) $CD4^+Foxp3^-$ T_{eff} and (**B**) $CD3^-DX5^+$ NK cells in *P. chabaudi* AS-infected A/J, C57BL/6 (B6) and Foxp3Tg (Tg) mice. (**C**) FACS analysis of intracellular IFN-γ expression in splenic $CD3^-DX5^+$ NK cells from *P. chabaudi* AS-infected A/J, B6 and Tg mice. (**D**) FACS analysis of the frequency of $Ki67^+$ splenic $CD3^-B220^+$ B cells in *P. chabaudi* AS-infected A/J, B6 and Tg mice. For all experiments, data are expressed as the mean ± SE of n = 3 mice per group from one of two replicate experiments. * $p< 0.05$ compared to B6 mice.

Figure 11. Expansion of Foxp3$^+$CD25$^-$ nT$_{reg}$ during *P. chabaudi* AS infection

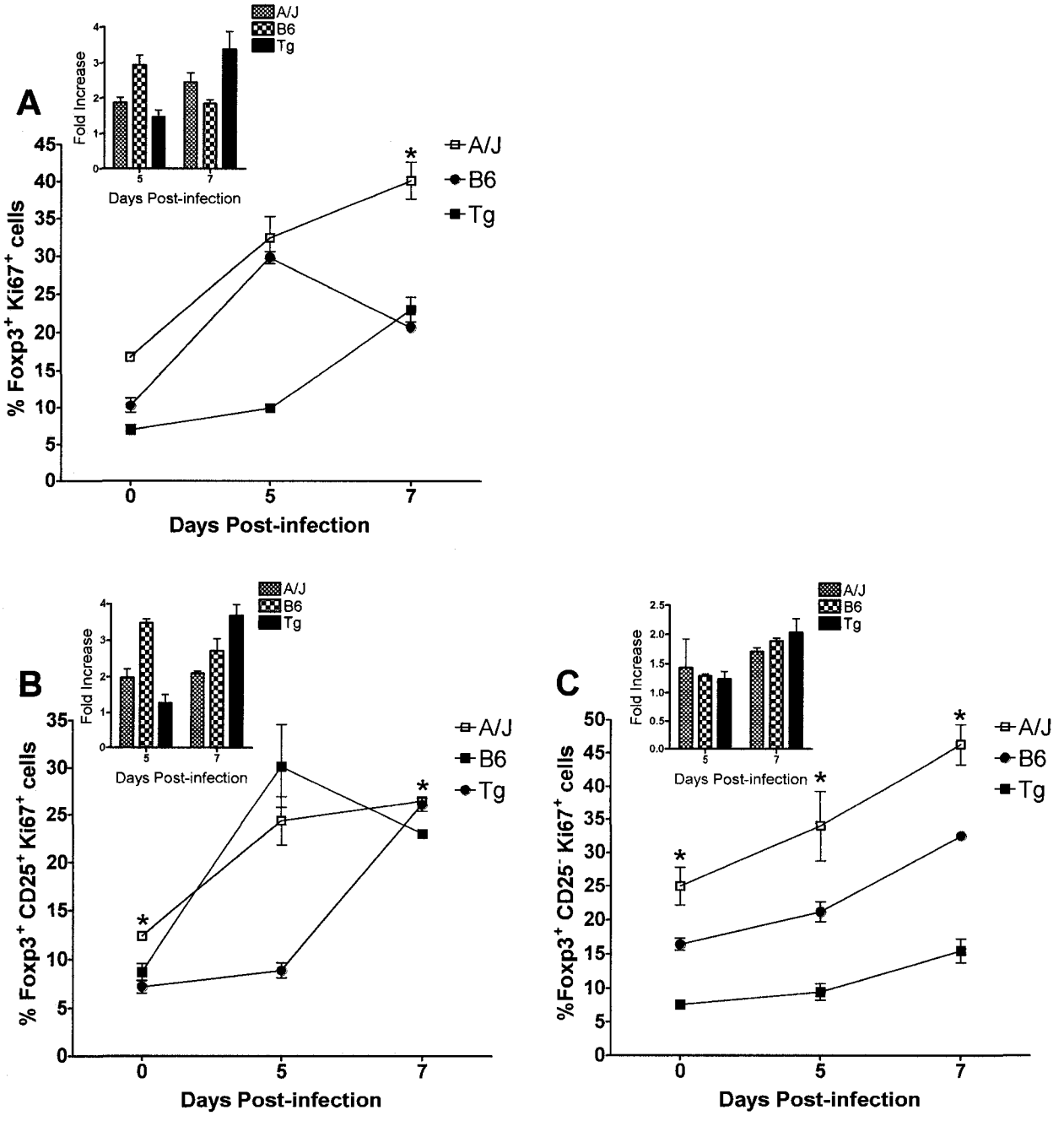


Figure 11: Expansion of Foxp3$^+$CD25$^-$ nT$_{reg}$ during *P. chabaudi* AS infection. FACS analysis of Ki67$^+$ cells within the (**A**) total Foxp3$^+$, (**B**) Foxp3$^+$CD25$^+$ and (**C**) Foxp3$^+$CD25$^-$ splenic CD4$^+$ cells in A/J, C57BL/6 (B6) and Foxp3T(Tg) mice at days 0, 5 and 7 p.i. with *P. chabaudi* AS. Insets represent the fold increase in proliferating cells from day 5 and 7 infected mice relative to naïve mice. Data are expressed as the mean ± SE of n = 3 mice per group from one of two replicate experiments. * $p < 0.05$ compared to B6 mice.

DISCUSSION

Multiple studies demonstrate that suppression of T and B cell immune responses occurs in both humans[5] and mice[77] infected with malaria. This immunosuppression is generalized and characterized by decreased immunoproliferation, cytokine production and antibody secretion in response to malaria or unrelated antigens[77,78,79,80,80,72,73,74,75,76]. A primary malaria infection in humans also induces poor immunological memory to subsequent re-challenges[5]. Furthermore, all field trials conducted so far for candidate anti-malarial vaccines have yielded unsatisfactory results by generating only limited protection against malaria re-infections. Persistent infection with low grade parasitemia is also frequently observed in human infected with malaria[5]. Recently, evidence indicates that $CD4^+Foxp3^+$ nT_{reg} modulate the immune response to various infectious diseases, including parasites[156,158]. nT_{reg} likely modulate anti-parasitic immunity by suppressing anti-parasitic immune effector cells, such as $CD4^+$ T helper cells, and also by impairing the generation of immunological memory, thus contributing to the failure of anti-parasitic vaccination. However, the role of nT_{reg} in the immune response to malaria is ill defined. The goal of the present study was to determine whether nT_{reg} regulate the host's immune response to malaria and contribute to disease susceptibility. By using the model of *P. chabaudi* AS infection in resistant B6, susceptible A/J and Tg mice, we provide compelling evidence that nT_{reg} suppressed innate and adaptive anti-malarial immune responses. Our findings also revealed potential mechanisms and immune cell targets of nT_{reg}-mediated suppression during malaria infection.

nT_{reg} modulate the host immune response to various pathogens, such as bacteria, viruses, fungi and parasites[156]. nT_{reg} likely play a dual role in the immune response to the pathogens by either suppressing the development of protective immunity and thus promoting pathogen persistence or by preventing the development of immunopathology as a consequence of overt anti-pathogen immune responses[177]. Recent reports indicate that nT_{reg} might regulate the host

immune response to various *Plasmodium* species[174,175,200,201] and promote the growth and survival of malaria parasites. Most of these studies use CD25 as a marker for $nT_{reg,}$ which does not allow for the discrimination of nT_{reg} from activated T_{eff} in an inflammatory setting. In addition, these reports fail to elucidate the ontogeny of regulatory T cells during malaria infection; that is, whether they are thymus-derived and naturally-occurring (nT_{reg}) or generated in the periphery as a consequence of malaria infection and are induced (iT_{reg}). Although the suppressive activity of nT_{reg} during malaria infection was confirmed in a recent study of *P. yoelii* infected mice[175], the cellular mechanisms involved in nT_{reg} regulation as well as the nature of the immune cells and effector functions targeted by nT_{reg} suppression were not identified. Our study addressed these questions by first confirming the role for nT_{reg} regulation during a malaria infection and then by shedding light on the mechanisms involved in the nT_{reg}-mediated suppression of anti-malarial immunity. As others have previously reported[52], we demonstrated that B6 mice displayed a non-lethal course of *P. chabaudi* AS infection, with a peak parasitemia at day 7 p.i. and parasite clearance by day 30 p.i.. Conversely, A/J mice exhibited significantly higher parasitemia compared to resistant B6 mice and succumbed 10 to 12 days p.i. Studies reveal that the susceptibility of A/J mice to *P. chabaudi* AS is likely due to the generation of a deficient anti-malarial immune response[52]. Indeed, A/J animals develop a predominantly Th2 response, whereas resistant B6 produce high levels of pro-inflammatory cytokines, especially IFN-γ, during the early stages of malaria infection and develop a predominantly Th1 response[51]. The imbalance in Th1/Th2 responses in infected A/J mice suggests that these mice might suffer from inappropriate immunoregulation. We hypothesized that inappropriate nT_{reg}-mediated regulation of anti-malarial immunity in A/J mice might contribute to their susceptibility to *P. chabaudi* AS infection. Unlike resistant B6 mice, which exhibited decreased splenic nT_{reg} numbers and lower parasitemia at day 7 p.i. with *P. chabaudi* AS, susceptible A/J mice maintained high nT_{reg} frequencies and higher parasite burdens at this time point. Consistent with the findings reported here, high *FOXP3* mRNA levels correlate with

increased blood parasitemia in humans infected with *P. falciparum*[173]. Altogether, this indicates that nT_{reg} might contribute to the susceptibility of both humans and mice to *Plasmodium* infections. However, nT_{reg} are likely to only be partially responsible for this susceptibility since multiple genetic loci, including some that regulate normal RBC metabolism, have been identified as contributing to the susceptibility of both humans[202] and mice[203] to malaria infection.

We used two approaches further investigate the contribution of nT_{reg} to malaria susceptibility. First, we infected mice over-expressing Foxp3 (Foxp3Tg) with *P. chabaudi* AS in order to determine whether a supraphysiological peripheral pool of nT_{reg} would compromise the host's ability to mount a protective anti-malarial immune response. Although these mice are on the resistant B6 background, a five-fold increase in peripheral nT_{reg} numbers in these mice rendered them highly susceptible to *P. chabaudi* AS infection, as demonstrated by increased blood parasitemia and mortality. Second, WT B6 mice adoptively transferred with nT_{reg} isolated from naïve mice also exhibited increased susceptibility to infection compared to mice receiving $CD4^+$ cells devoid of nT_{reg} or non-transferred controls. Together, these findings provide strong evidence that nT_{reg} modulated both control of parasite replication and clearance as well as survival during *P. chabaudi* AS infection and that nT_{reg} enrichment was sufficient to render normally resistant B6 mice susceptible to malaria infection. These observations are consistent with previous reports which indicate that depletion of $CD25^+$ cells, including $CD25^+Foxp3^+$ nT_{reg}, using anti-CD25 antibody exacerbates the clinical outcome of both blood-stage[174] and cerebral malaria[200,201]. Therefore, nT_{reg} likely play a role in the host immune response to malaria. However, even though T_{reg} expressed Foxp3 during malaria infection, the ontogeny of T_{reg} during a malarial infection still remains unclear. There is the possibility that nT_{reg} were not thymus-derived but arose from peripheral induction of Foxp3 expression in $CD4^+Foxp3^-$ cells during *P. chabaudi* AS infection. In fact, a study in humans infected with *P. falciparum* suggests that nT_{reg} might be induced from peripheral $CD4^+$ T cells during *P. falciparum* infection by a TGF-β-dependant mechanism[204]. TGF-β which is produced at high levels during malaria

infection in humans as well as in mice[205], induces the peripheral conversion of $CD4^+Foxp3^-$ cells into nT_{reg}[107-109]. Therefore, it is plausible that *P. chabaudi* AS infection engaged nT_{reg} that were a combination of both thymus-derived and peripherally converted $CD4^+Foxp3^-$ T cells, although this matter requires further investigation.

$CD4^+$ T_{eff} are essential for the generation of protective adaptive immunity to *P. chabaudi* AS[49]. Since nT_{reg} have been shown to suppress the cytokine production and proliferation of T_{eff}[117], we investigated whether nT_{reg} can regulate malaria-specific T_{eff} functions *in vivo*. Analysis of Ki67 expression in splenic T_{eff} during *P. chabaudi* AS infection in A/J and Tg mice revealed an association between high frequencies of splenic nT_{reg} and decreased T_{eff} proliferation at day 7 p.i. Consistent with our findings, a previous study demonstrated that depletion of $CD25^+$ splenocytes using anti-CD25 antibody increases *in vitro* lymphoproliferation of splenocytes isolated from *P. berghei*-infected mice[206]. However, our study showed additionally that decreased T_{eff} expansion was accompanied by decreased intracellular IFN-γ expression by T_{eff} in *P. chabaudi* AS-infected A/J and Tg mice compared to T_{eff} from B6 mice at day 7 p.i. Previous reports have also shown lower IFN-γ production by splenic $CD4^+$ T cells in *P. chabaudi* AS-infected A/J mice compared to infected resistant B6 mice within the first week of infection[52]. Since IFN-γ production is essential for resistance to *P. chabaudi* AS infection[57,207], nT_{reg} suppression of IFN-γ production by T_{eff} during the adaptive immune response to *P. chabaudi* AS might contribute to deficient parasite clearance and susceptibility in A/J and Tg mice. What remains to be determined, however, is whether nT_{reg} suppression of T_{eff} function occurs in an antigen-specific manner or as bystander activation by recognition of self-antigen during malaria infection. In fact, although nT_{reg} are thought to have a diverse polyclonal TCR repertoire, there is evidence that their TCR specificity might be skewed towards self-antigens[124]. Recent studies have indicated that nT_{reg} may be able to recognize certain antigens derived from infectious agents, such as *L. major*[155] and *P. yoelii*[175], and thus suppress the immune response generated against these parasites in an antigen-specific manner. However, whether this was

the case in our model of *P. chabaudi* AS infection in susceptible A/J and Tg mice was not determined.

NK cells play a pivotal role in the anti-malarial immune response, since they are a primary source of IFN-γ during the early stages of malaria infection and are essential for the development of adaptive immunity to *P. chabaudi* AS infection[31]. Indeed, NK cell-derived IFN-γ production during the early stages of *P. yoelii* infection correlates with resistance to infection[13]. Thus, we postulated that nT_{reg} suppressed NK cell responses during *P. chabaudi* AS infection in our model. Multiple studies have shown that nT_{reg} can inhibit NK cell functions in the context of anti-tumour immunity[208], and NK cell anti-tumour cytotoxicity is inversely correlated with nT_{reg} numbers in melanoma patients[208]. In addition, NK cell proliferation, as assessed by BrdU incorporation, is significantly enhanced in *scurfy* mice which lack nT_{reg} compared to WT mice[140]. Likewise, our results show that an association between high nT_{reg} frequencies and decreased NK cell proliferation and intracellular IFN-γ expression in the spleens of *P. chabaudi* AS-infected mice. It has been suggested that early production of IFN-γ by NK cells shapes the $CD4^+$ T cell-mediated anti-malarial immune response by promoting the development of a protective Th1 response against *P. chabaudi* AS[31]. NK cells have also been shown to be essential for the development of $CD8^+$ T cell-mediated immunity to liver-stage *P. yoelii* infection[28]. Altogether, this suggests that nT_{reg} suppression of NK cell IFN-γ production might severely impair the innate as well as the adaptive immune response to *P. chabaudi* AS, thus enhancing susceptibility to malaria. However, the mechanism involved in nT_{reg} suppression of NK cell function was not elucidated in our study. A possible mechanism by which nT_{reg} may modulate NK cell activity during malaria infection is by secreting or promoting the production of immunosuppressive TGF-β. In fact, TGF-β can directly inhibit NK cell-derived IFN-γ production and thus prevent the generation of a Th1 immune response[209]. Interestingly, high levels of TGF-β have been associated with increased *FOXP3* expression during *P. falciparum* malaria in humans[204], and TGF-β production correlates with murine susceptibility to malaria[205,210]. Although controversial, a recent study also

suggests that human nT_{reg} can directly interact with NK cells and suppress their cytokine production by means of TGF-β, most probably in its membrane-bound form[140]. However, it remains unclear whether nT_{reg} produce TGF- β during the immune response to malaria and whether nT_{reg} are directly responsible for deficient NK cell functions during *P. chabaudi* AS infection in mice. Nonetheless, to date, our findings are the only indication that nT_{reg} may regulate NK cell responses during the immune response to an infectious disease.

Malaria-specific antibody production by B cells is essential for the clearance of parasites from the blood of *P. chabaudi* AS-infected mice[34]. nTreg can directly suppress antibody production and isotype switching by B cells[138]. In our study, Ki67 expression analysis demonstrated that B cell proliferation during *P. chabaudi* AS infection was hindered in susceptible A/J and Tg mice compared to B6 mice. However, we did not assess whether this decreased B cell expansion correlated with deficient malaria-specific antibody production. Nonetheless, clonal expansion of B cells normally precedes their differentiation into antibody-secreting plasma cells[211]. It is possible that direct suppression of B cell proliferation by higher frequencies of splenic nT_{reg} also affected antibody secretion during *P. chabaudi* AS infection in susceptible A/J and Tg mice. In addition, suppression of T_{eff} activation by nT_{reg} in our model, as show by decreased proliferation and IFN-γ production, could have indirectly impaired B cell functions by decreasing T cell help to B cells during *P. chabaudi* AS infection in A/J and Tg mice. However, since antibody production is essential for anti-malarial immunity mainly in the chronic stage of infection[34] and our data show that susceptible A/J and Tg mice succumbed to *P. chabaudi* AS infection within 10 days of infection (acute infection), it is unlikely that nT_{reg} suppression of B cell antibody production could have been a main contributor to malaria susceptibility in these mice.

Multiple studies suggest that an imbalance between Th1 and Th2 immune responses contributes to the susceptibility to malaria. Indeed, production of the Th1 cytokine IFN-γ occurs during the first week of *P. chabaudi* AS infection in

resistant B6 mice, whereas susceptible A/J mice produce high levels of Th2 cytokines[52,51]. Interestingly, we observed that *P. chabaudi* AS-infected B6 mice adoptively transferred with nT_{reg} displayed significantly higher systemic IL-10 levels at day 6 p.i. compared to non-transferred controls or mice transferred with $CD4^+$ T cells devoid of nT_{reg}. However, no significant differences in serum IFN-γ levels were observed between nT_{reg} recipients and control mice at any time point studied. Since nT_{reg} recipients had similar IFN-γ responses but increased IL-10 levels compared to T_{eff} recipients or non-transferred controls, these findings suggest that nT_{reg} recipients had a lower IFN-γ/IL-10 ratio during the early stages of *P. chabaudi* AS infection, and this Th1/Th2 imbalance in nT_{reg} recipients might have contributed to their increased susceptibility to infection. Indeed, the balance between anti- and pro-inflammatory immunity is tightly controlled during *P. chabaudi* AS infection, and a Th1 to Th2 switch during the chronic stage of infection is required for resistance to malaria in B6 mice[52,58,59]. In addition, susceptibility to *P. chabaudi* AS is associated with high serum levels of the Th2-type cytokine IL-4 during the early phase of *P. chabaudi* AS infection[52,51]. It is also possible that nT_{reg} suppression of B cell responses, as observed in malaria-infected A/J and Tg mice, could contribute to an inappropriate Th1/Th2 balance in mice with increased splenic nT_{reg} frequencies. In fact, B cells promote a Th1 to Th2 switch during the acute phase of *P. chabaudi* AS infection that is essential for the generation of protective immunity to the parasite[212]. nT_{reg} suppression of B cell proliferation might impair the development of an efficient T cell-mediated anti-malarial immune response during the early stages of *P. chabaudi* AS infection, although extensive experimentation is required to further address this question. Nonetheless, our results suggest that nT_{reg} may play an important role in regulating the type of T cell-mediated, adaptive immunity generated in response to malaria. In accordance with our data, a previous report shows that nT_{reg} are capable of regulating the balance between Th1 and Th2 immunity, since T_{reg}-depleted BALB/c mice infected with *L. major* display enhanced IL-4 and decreased IFN-γ levels compared to non-depleted infected mice[213].

The cellular mechanisms involved in nT_{reg} suppression *in vivo* remain elusive, although multiple studies have indicated that nT_{reg} may resort to immunomodulatory cytokines, such as IL-10 and TGF-β, in order to regulate immune responses. Conflicting evidence suggests that nT_{reg} may or may not require IL-10 in order to control T_{eff}-mediated anti-parasitic immune responses. Indeed, although IL-10 is required for nT_{reg} suppression of immunity to *S. mansoni*[166], *L. amazonensis*[168], and the chronic phase of *L. major*[169] infection, nT_{reg} control of immunity to *L. sigmodontis*[172] occurs through an IL-10-independant mechanism. Our observation that B6 mice adoptively transferred with nT_{reg} exhibited significantly higher systemic levels of IL-10 during the early phase of *P. chabaudi* AS infection compared to mice receiving T_{eff} or no cells suggests that nT_{reg} suppression of anti-malarial immunity might be mediated by the production of IL-10. However, *P. chabaudi* AS-infected Tg mice produced decreased levels of IL-10 at days 6 and 8 p.i. Although our data seem contradictory, we believe that Tg mice might not be an ideal model for studying the mechanisms of nT_{reg} suppression due to the relative dominance of these cells in the T cell repertoire of these mice. Therefore, we cannot confirm nor omit the possibility that nT_{reg} suppress anti-malarial immunity in an IL-10-dependant manner. IL-$10^{-/-}$ mice on a resistant B6 background exhibit increased production of pro-inflammatory cytokines compared to WT mice and succumb to *P. chabaudi* AS infection during the chronic stage of infection, which indicates that high mortality in these mice is likely a consequence of overt immune effector responses and subsequent immunopathology rather than increased parasite burdens [214]. Therefore, it is possible that increased IL-10 levels observed in *P. chabaudi* AS-infected B6 mice transferred with nT_{reg} impaired immune effector functions by decreasing the production of pro-inflammatory cytokines, resulting in inefficient anti-malarial immunity and susceptibility of these mice. However, our study did not identify the cellular source of IL-10 in these nT_{reg} recipients. nT_{reg} were either the direct source of IL-10 in this model or induced other immune cells to produce IL-10. Both $CD4^+$ T cells and macrophages can be sources of IL-10 during malaria, as indicated by studies using *P. yoelii* infection in mice[215].

Determining whether the adoptive transfer of nT_{reg} isolated from IL-$10^{-/-}$ mice on the B6 background into naïve B6 mice also results in increased systemic levels of IL-10 upon *P. chabaudi* AS infection would allow us to determine whether or not nT_{reg} are in fact a source of IL-10 during malaria infection. In addition, monitoring parasite burdens in infected B6 mice transferred with IL-$10^{-/-}$ nT_{reg} would determine whether nT_{reg} suppression of anti-malarial immunity depends on their production of this immunomodulatory cytokine.

The requirement for nT_{reg} to localize within the sites of inflammation in order to regulate immune responses remains a topic of debate. Appropriate tissue localization is essential for nT_{reg} regulation of autoimmunity, tumour immunity, organ transplant rejection and infectious diseases[193]. Interestingly, selective nT_{reg} trafficking has been demonstrated in infections with *S. mansoni*[166], *L. sigmodontis*[172] and *L. major*[179], indicating that nT_{reg} localization to tissues where regulation is required might be of particular importance during parasitic infection. We examined the possibility that nT_{reg} require specific tissue localization in order to regulate immunity to *P. chabaudi* AS. FACS analysis of the bio-distribution of GFP^+ nT_{reg} or GFP^+ T_{eff} adoptively transferred into *P. chabaudi* AS-infected B6 mice revealed that nT_{reg} accumulated specifically within the spleen, and not in blood or lymph nodes, of infected mice within 7 days p.i. Lower numbers of transferred GFP^+ cells were found to have localized in the spleen of T_{eff} recipients. The increased nT_{reg} numbers observed in the spleen of *P. chabaudi* AS-infected mice may be a result of multiple non-mutually exclusive possibilities. nT_{reg} might preferentially home or expand within the spleen during malaria infection. In addition, there is the possibility that nT_{reg} and T_{eff} receive altered TCR signals, which lead to different migratory patterns during infection or exhibit different life spans within the spleen of *P. chabaudi* AS-infected mice. The fact that the spleen is the main site of parasite clearance during malaria infection also suggests that nT_{reg} might require antigen-specific signals in order to carry out suppression of anti-malarial immunity. The fact that nT_{reg} isolated from the dermis of mice suffering from cutaneous leishmaniasis are specific for *L. major* antigen[155] supports this possibility. The selective accumulation of nT_{reg} within

sites of inflammation, such as the spleen in malaria-infected mice, may also highlight the importance of nT_{reg} localization within target organs in order to modulate immune responses to infections. Although nT_{reg} retention in target tissue is necessary for suppression of immunity to *L. major*[97,216,179], whether nT_{reg} require localization to the infected spleen in order to suppress anti-*P. chabaudi* AS immunity was not determined in this study. There are possibly certain molecules and/or signals involved in the trafficking of nT_{reg} to the spleen during a malaria infection. For example, CCR5, which is essential for the retention of nT_{reg} within dermal lesions of *L. major*-infected mice[97], may be important for nT_{reg} localization during *P. chabaudi* AS infection. However, preliminary data in our laboratory suggest that nT_{reg} do not express CCR5 during *P. chabaudi* AS infection. Other surface molecules may be involved in nT_{reg} trafficking during malaria infection, including CD103, which is implicated in tissue homing of nT_{reg} during parasitic infection[179], or CCR4, CCR8[98], and CCR7[217] which have been shown to play a role in nT_{reg} function *in vivo*. Depending on the source of antigenic challenge, nT_{reg} may require different molecular signals in order to traffic to target tissues during parasitic infection.

Although nT_{reg} are anergic to *in vitro* stimulation[117,118], some studies indicate that nT_{reg} expand when triggered by inflammatory stimuli *in vivo*[198,199]. The use of Ki67 as a marker for proliferation revealed the ability of nT_{reg} to proliferate in response to *P. chabaudi* AS infection in both resistant B6 mice and susceptible A/J and Tg mice. Interestingly, nT_{reg} proliferation was increased in A/J mice compared to B6 mice, suggesting that enhanced nT_{reg} expansion might contribute to the susceptibility of A/J mice. nT_{reg} proliferation also occurs in response to *L. major*-infected murine DC[155] and during HIV[218] and acute dengue[219] infections in humans. However, several important questions remain to be addressed. Is nT_{reg} proliferation required for their *in vivo* suppression of anti-pathogen immunity, such as anti-malarial immunity? Is nT_{reg} proliferation antigen-specific and driven by the pathogen itself, or is it dictated by inflammation provoked by infection? Our data strongly suggests nT_{reg} proliferation during *P. chabaudi* AS infection is dictated by exposure to malaria

antigens since Ki67 expression in nT_{reg} was enhanced in the spleen, site of parasite clearance, and not in peripheral lymph nodes. Since 90% of nT_{reg} from the spleen of naïve non-infected mice express surface CD25, we investigated whether the surface expression of CD25 was reflective of the proliferative potential of nT_{reg} during malaria infection. Interestingly, we found that $CD25^-$ nT_{reg} had higher proliferative capacities than $CD25^+$ nT_{reg} during the early stages of *P. chabaudi* AS infection in both resistant B6 and susceptible A/J mice. Our findings, in addition to the results of a previous report indicating that 50% of $Foxp3^+$ cells isolate from the lungs of *M. tuberculosis*-infected mice are CD25-negative[106], raise the possibility that $CD25^-$ nT_{reg} may play an important role in regulating the immune response to pathogens. Our data also suggest that $CD25^-$ and $CD25^+$ nT_{reg} are distinct regulatory cell subsets that respond differently to antigenic challenge. Nonetheless, the ontogeny of $CD25^-$ nT_{reg} is a subject of debate. Multiple hypotheses have been proposed to clarify this discrepancy in CD25 expression on nT_{reg}[106]. Besides the possibility that $CD25^-$ and $CD25^+$ nT_{reg} are distinct cell populations, $CD25^-$ cells might be undifferentiated precursors to functional $CD25^+$ nT_{reg} or represent a different cyclical state of the same regulatory T cell. nT_{reg} may also up- or down-regulate CD25 expression in peripheral tissues, depending on the bioavailability of the growth factor IL-2. Although our data suggest that $CD25^-$ nT_{reg} are in fact a distinct population from $CD25^+$ nT_{reg}, we cannot omit the possibility that either one of these hypotheses might explain the nature and significance of the $CD25^-$ nT_{reg} analyzed in the present study. The exact signals that trigger nT_{reg} expansion during *P. chabaudi* AS infection also remain unclear. nT_{reg} might require antigen-specific triggering, and/or require other signals, such as IL-2, for their expansion during malaria infection. Our observation that $CD25^-$ nT_{reg}, which do not express a functional IL-2R, proliferate during *P. chabaudi* AS infection suggests that IL-2 might not be essential for nT_{reg} expansion during infection. Other common γ-chain cytokines, such as IL-15, might contribute to this nT_{reg} cycling. Indeed, although associated with resistance to *P. chabaudi* AS infection[220], IL-15 increases the survival, expansion and suppressive activity of antigen-specific human nT_{reg}[221].

In conclusion, the present study provides convincing evidence that nT_{reg} play an important role in regulating the host immune response to *P. chabaudi* AS infection and suggests that these cells contribute to the susceptibility to malaria. Whereas the role of nT_{reg} in the host immune response to other infectious diseases is limited to chronic stages of infection[156], our results indicate that nT_{reg} can also regulate immunity to acute infection. Altogether, our study and previous reports implicating nT_{reg} in anti-malarial immunity[173-175,200,201] provide compelling evidence that these cells might contribute to malaria-induced immunosuppression which is commonly observed in *P. falciparum*-infected humans[5,74,75,83]. nT_{reg} suppression might also explain poor results generated from field trials of candidate malaria vaccines, such as the RTS,S/AS02 and Spf66 vaccines[5]. We have demonstrated that nT_{reg} suppression targets both the innate and adaptive immune responses to malaria, and, to our knowledge, this is the first report indicating that nT_{reg} can modulate NK cell and B cell responses during the immune response to infectious diseases. A tentative model of how nT_{reg} modulate the immune response to *P. chabaudi* AS can be drawn from our findings (Figure 12). nT_{reg} inhibit the production of pro-inflammatory IFN-γ by both NK cells and $CD4^+Foxp3^-$ T_{eff}, and also impair the malaria-specific proliferation of T_{eff} and B cells. Although the mechanisms by which nT_{reg} mediate suppression of anti-malaria immunity remain elusive, our results suggest that nT_{reg} proliferate in response to infection and might require specific localization within the *P. chabaudi* AS-infected spleen in order to mediate suppression. We also provide some indication that nT_{reg} might regulate anti-malarial immunity by an IL-10-dependant mechanism. Our characterization of nT_{reg} modulation of anti-malarial immunity provides some insight on how to decrease susceptibility and enhance protective immunity and vaccination to malaria parasites. A key strategy for the success of an anti-malarial vaccine would be to minimize the induction of nT_{reg} function during immunization, since IL-10-producing T_{reg} can hinder the success of vaccination, as demonstrated by a study of anti-*L. major* vaccines in mice[176]. Modulating the activation state of DC would be a potential way of indirectly preventing nT_{reg} induction during anti-malarial vaccination. In fact, immunization

with certain antigens, such as those derived from *Bortella pertussis*, results in only partial maturation of DC, resulting in a Tr1-type of immune response[176,222]. Therefore, the careful choice of a malaria antigen that can induce a fully mature phenotype in DC might prevent the induction of nT_{reg} during vaccination and enhance protective immunity to malaria re-challenge. Finally, our characterization of nT_{reg} regulation of anti-malarial immunity expands our understanding of the pathogenesis of malaria and could potentially be applied to other infectious diseases, such as HIV, leishmania and tuberculosis, since it contributes to the comprehension of how pathogens evolve mechanisms to evade host immune responses.

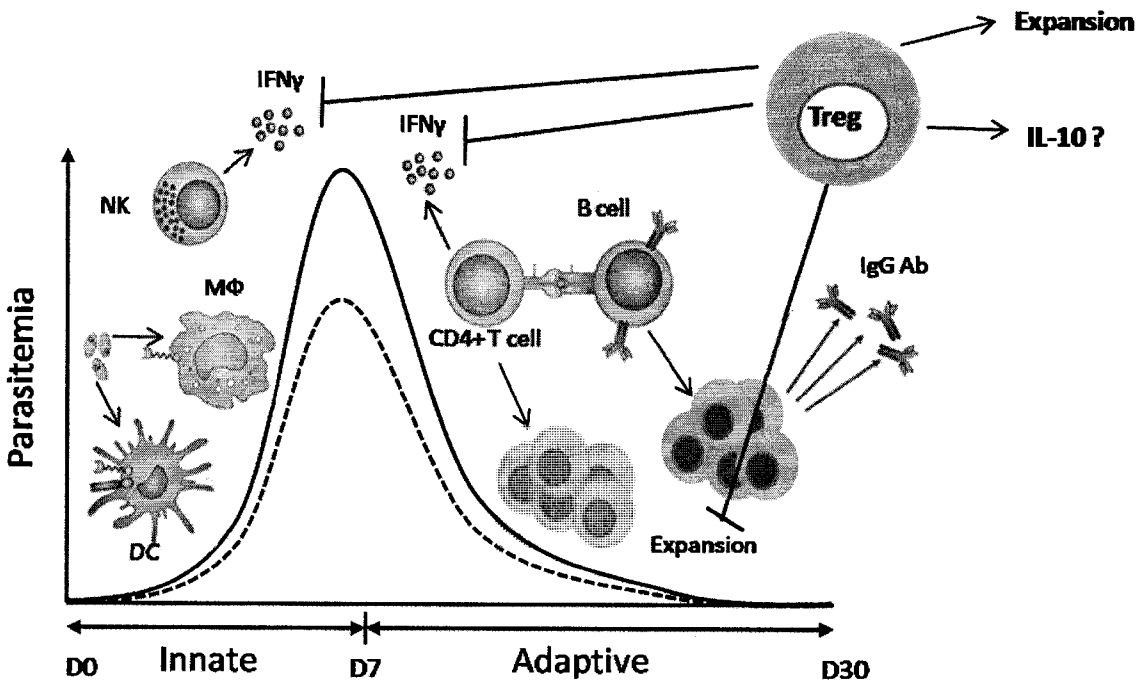


Figure 12. Simplified model of nTreg regulation of anti-malarial immunity in mice.

nT_{reg} regulate both the innate and adaptive immune response by expanding in response to a malaria infection and potentially by an IL-10-dependant mechanism. This results in an increase in parasitemia, illustrated by the full line compared to the dotted line. DC, dendritic cell; NK, Natural Killer cell; MΦ, macrophage; IgG, immunoglobulin class G antibody. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Stevenson and Riley[2]) copyright (2004).

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