The role of IL-4Rα signalling in gene deficient mice during asexual-stage *Plasmodium chabaudi* AS infection

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DECLARATION

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

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ABSTRACT

BALB/c mice infected with *P. chabaudi* AS develop immunity to erythrocytic-stage infection with early Th_1 responses followed by a switch towards Th_2 responses later to mediate protection during chronic disease. In order to determine the importance of the Th2 cytokines, IL-4/IL-13, in inducing protective immunity, the course of *P. chabaudi* infection was monitored in IL-4Rα-deficient mice. Interestingly, an early delay in the onset of peak parasitaemia in IL-4Rα$^{-/-}$ compared with WT control BALB/c mice was evident. Consequently, we demonstrated that IL-4Rα deficiency resulted in mice becoming more susceptible to chronic *P. chabaudi* infection with increased recrudescence, mortality and an impaired Th_2 immune response compared with WT control mice. Similar results in the overall disease and immunological profiles between IL-4R$\alpha^{-/-}$ and wild-type mice were obtained whether male or female mice or the AJ or AS strains of *P. chabaudi* were used to infect mice. Thus, the protective role of IL-4Rα signalling during chronic disease was not parasite strain-specific or host gender dependent. However, males were significantly more susceptible than female mice and consequently further studies involving cell-type IL-4R$\alpha^{-/-}$ mice, utilized female mice to identify functional targets of IL-4/IL-13 protection. Abrogated IL-4Rα expression on macrophages/neutrophils (LysM^{cre}IL-4Rα$^{-/lox}$) mice had minimal effect on the outcome of *P. chabaudi* AS chronic infection and was comparable to WT mice implicating no major role for alternatively activated macrophages during chronic infection. In contrast, CD4$^+$ T-cell-specific IL-4Rα$^{-/-}$ (Lck^{cre}IL-4Rα$^{-/lox}$) mice infected with *P. chabaudi* AS developed increased recrudescence, increased mortality and impairment of Th_2 immunity during the chronic infection similar to that of the global IL-4Rα$^{-/-}$ mice. This highlights the importance of signalling via CD4$^+$ T-cells signalling via IL-4Rα for protective immunity during chronic infection. Paradoxically, CD4$^+$CD8$^+$ T-cell-specific IL-4Rα$^{-/-}$ (iLck^{cre} IL-4Rα$^{-/lox}$) mice displayed a similar disease profile to WT control mice but manifested a delayed Th_2 phenotype during the latter stage of the disease with enhanced splenomegaly in comparison to the WT and IL-4Rα$^{-/-}$ mice. Thus while protection during chronic infection with *P. chabaudi* AS appears dependent on CD4$^+$ T-cells responsive to IL-4, CD8$^+$ T cells responsive to IL-4 have a more complex and more difficult role to interpret.

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ABBREVIATIONS

α	Alpha
αß	Alpha-Beta
AMA-1	Apical membrane antigen 1
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
bp	Base pairs
ß	Beta
CO_2	Carbon dioxide
ConA	Concanavalin A
CSP	Circumsporozoite protein
^oC	Degrees celcius
DC	Dendritic cell
DNA	Deoxyribonucleotide acid
ELISA	Enzyme-linked Immunosorbant Assay
EBA	Erythrocyte binding antigen
FCS	Foetal calf serum
γ	Gamma
γδ	Gamma-Delta
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol
g	Gram
HRP	Horse radish peroxidase
IL-4Rα	IL-4/IL-13 receptor
Ig	Immunoglobulin
IFN-γ	Interferon gamma
IL	Interleukin
I.P	Intra-peritoneal
I.V	Intravenous
JAK	Janus Kinase
KDa	Kilodalton
KO	Knock-out
LPS	Lipopolysaccharide

LSA1	Liver-stage antigen 1
MHC	Major histocompatibility complex
MSP	Merozoite surface protein
MW	Molecular weight
µg	Microgram
µl	Microlitre
µm	Micrometers
Mg	Milligram
Ml	Millitre
ng	Nanogram
nm	Nanometers
NK	Natural killer cells
PAMPs	Pathogen associated molecular patterns
pNPP	P nitrophenyl phosphate
PCR	Polymerase chain reaction
PBS	Phosphate Buffered Saline
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PRR	Pathogen recognition receptor
RBCs	Red blood cells
RNA	Ribonucleic acid
rpm	Rounds per minute
SERA	Serine-rich antigen
STARP	Sporozoite threonine and asparagine rich protein
STAT	Signal Transducers and Activators of Transcription
H_2SO_4	Sulphuric acid
TMB	Tetramethylbenzidine
TLR	Toll-like receptors
T_h	T-helper lymphocyte
Th_1	T-helper type 1 response
Th_2	T-helper type 2 response
Temp	Temperature
Treg	Regulatory T-cells
TRAP	Thrombosponsin-related adhesive protein
TGF	Transforming Growth Factor

TNF-α	Tumour necrosis factor alpha
VATS	Variable Antigen Types
WHO	World Health Organization

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1. INTRODUCTION

1.1 Malaria Epidemiology

Compared with 30 years ago, malaria remains one of the greatest burdens for the developing world with increased infections, morbidity and mortality rates. Infection is endemic in most parts of sub-Saharan Africa and in other tropical regions. Approximately half of the world's population is at risk of malaria. An estimated 250 million cases and 1 million deaths, mostly among African children under five years of age and pregnant women, occur annually amongst the 3.3 billion people at risk (WHO World Malaria Report, 2008)

There are over 200 species of *Plasmodium*, in the phylum Apicomplexa, and they collectively infect humans and other mammals, birds and lizards although with a significant amount of specificity for host species. They represent a successful group of parasites due to their invasion and host immunity avoidance capabilities. There are four types of human malaria. The majority of severe cases and deaths are caused by *Plasmodium falciparum*. Relatively infrequent causes of morbidity are caused by *P. malariae* and *P. ovale* while *P. vivax* is a common cause of severe acute febrile illness especially in Asia and South America but is rarely fatal (Price *et al.*, 2009, 2007, Stevenson and Riley, 2004). In recent years, some human cases of malaria have also occurred with *Plasmodium knowlesi*, a primate malaria that occurs in certain forested areas of South-East Asia (Sabbatani *et al.*, 2010).

Malaria transmission occurs via the bite of female *Anopheles* mosquitoes, which bite mainly between dusk and dawn. Intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment. There are about 100 species of *Anopheles* which can transmit malaria with 30-40 regularly transmitting the parasite. About 20 different *Anopheles* species occur around the world and breed in shallow collections of freshwater puddles, rice fields, and hoof prints. Transmission is more intense in areas where the mosquito is relatively long-lived which enables the malaria parasite to complete its development inside the mosquito, and where the mosquito prefers to bite humans rather than other animals. For example, the long lifespan and strong human-biting habit of the African vector species, *Anopheles gambiae,* is the underlying reason why more than 85% of the world's malaria deaths occur in Africa. In areas of intense transmission, human immunity is another important

factor. Immunity to malaria infection is developed over years of exposure to the parasite but still does not provide complete protection (Hafalla *et al.*, 2011; Crompton *et al.*, 2010; Marsh and Kinyanjui, 2006). It does, however, reduce the risk of severe disease. This explains why the majority of deaths occur in young children. In areas where there is lower transmission and low immunity, all age groups are at risk of infection and clinical disease. Climatic conditions such as rainfall patterns, temperature and humidity is another factor affecting the transmission rate of malaria by the mosquito. Malaria epidemics can occur when climate conditions suddenly favour transmission (Paaijmans *et al.*, 2010, 2009; Parham and Michael, 2010) in areas where people have little or no immunity to malaria. Alternatively this can occur when people with low immunity move into areas with intense malaria transmission, for instance to find work, or as refugees.

Malaria is an acute febrile illness. Only the malaria parasite multiplying in the red blood cell (RBC) will cause disease. Clinical symptoms associated with the blood-stage infection usually include bouts of fever, headaches, chills and vomiting which may be mild and difficult to sometimes recognize as malaria. If not treated within 24 hours of clinical symptoms first appearing, *P. falciparum* infection can progress to severe malaria often leading to death. Young children with severe disease where malaria is endemic frequently develop severe anaemia and respiratory distress in relation to metabolic acidosis, which appears between seven and fifteen days after an infective bite. Malaria during pregnancy may cause severe disease in the mother, and may lead to premature delivery or delivery of a low-birth-weight baby. In *P. vivax* and *P. ovale* infections, clinical relapses may occur weeks to months after the first infection. These relapses arise from dormant liver forms of the parasite termed hypnozoites that reactivate and which are absent in *P. falciparum* and *P. malariae* infections (WHO, 2010). Special treatment targeted at these liver stages is mandatory for a complete cure. In addition, host inflammatory mediators have also been implicated to play a role in immune-mediated pathology in humans and a number of well-characterized animal models (Couper *et al.*, 2008, Riley *et al.*, 2006, Engwerda *et al.*, 2005, Artavanis-Tsakonas *et al.*, 2003, Hunt and Grau, 2003, Omer *et al.*, 2000, Kwiatkowski *et al.*, 1997).

A number of factors have been proposed to be responsible for the failure to develop lasting immunity to natural malaria infections and for the difficulty of inducing strong protective immunity against malaria in trials of prototype vaccines. These factors include the following: the complex *Plasmodium* life cycle, antigenic diversity in and variation by the malaria parasites (Hafalla *et al.*, 2011, Marsh and Kinyanjui, 2006), genetic polymorphism and malnutrition of the human host, an immature immune system in young children (Marsh and Kinyanjui, 2006, Good, 2001, Shankar, 2000) and the consequence of socio-economic deficits and warfare in human populations (Trape *et al.*, 2002). Furthermore, concurrent helminth infections, highly prevalent in malaria endemic areas, have recently been recognized as a possible contributing factor modulating immune responses to pathogens including the malaria parasite (Melo *et al.*, 2010, Achidi *et al.*, 2008, Su *et al.*, 2005, Nacher, 2000).

1.2 Life-cycle (*Plasmodium falciparum*)

Plasmodium parasites' genome encode for at least 5600 genes and have a complex life cycle. The malaria parasite has two stages of development, first, in the human host (asexual stage) and secondly in the mosquito vector (sexual stage). The first stage is divided into two phases, namely, the liver phase (pre-erythrocytic phase) and the blood phase (erythrocytic phase). The second stage within the mosquito vector involves fusion of gametes and further parasite propagation via sporogony (Cowman and Crabb, 2006, Bannister and Mitchell, 2003, Phillips, 2001). An excellent animation of the life-cycle of *P. falciparum* can be viewed on the following website: http://www.wehi.edu.au/education/wehitv/malaria_lifecycle_part_1_human_host/ (Drew Berry, Biomedical Animator, The Walter and Elisa Hall Institute of Medical Research, WEHI-Tv, 2008).

ASEXUAL STAGE

Pre-erythrocytic Phase

Investigation of pre-erythrocytic stages remains restrictive because *P. falciparum* only infects humans and a few primate species (Herrera *et al.*, 2002, Mazier *et al.*, 1985). *P. berghei* and *P. yoelii*, which are rodent malaria parasites, provide good models for pre-erythrocytic stage biology studies and described below.

Plasmodium sporozoites are injected into the skin by the bite of an infected mosquito, which then move through dermal cells, enter a blood capillary and are rapidly distributed to the liver in the blood circulation. Some sporozoites are immobilized in the skin before reaching the blood circulation and eventually undergo incomplete transformation in the skin before being eliminated by phagocytes. However, in a *P. berghei* model, it was shown that after 24 hours about 10% of sporozoites develop in the epidermis and dermis where they can survive for weeks, differentiate into merozoites and possibly persist (Gueirard *et al.*, 2010). Some sporozoites are removed by the lymphatic system to the draining lymph node where they provide a source of antigens for activation of T-cell responses (Hafalla *et al.*, 2011).

Sporozoites cross the liver sinusoidal barrier possibly through Kupffer cells. Kupffer cells are specialized macrophages lining the liver sinusoids. In the liver parenchyma, sporozoites do not immediately switch to productive invasion but rather actively traverse/glide through numerous hepatocytes (Amino *et al.*, 2008) before invading a target cell by forming a parasitophorous vacuole, where the parasite differentiates into an exo-erythrocytic form (EEF). The parasites actinomyosin motor, a family of transmembrane proteins called thrombosponsin-related adhesive protein (TRAP), powers this gliding motility (Baum *et al.*, 2006, Menard, 2001). After multiple rounds of replication, mature EEFs release membrane-shielded merosomes that contain infectious merozoites. These merosomes are transported away and eventually rupture in the lung microvasculature, releasing thousands of modified merozoites directly into the bloodstream for a brief extracellular period of about 20-40 seconds (Hafalla *et al.*, 2011).

Erythrocytic Phase

The life-cycle for the human malarial parasites is relatively similar and is described here for *P. falciparum* (Cowman and Crabb, 2006, Bannister and Mitchell, 2003, Phillips, 2001).

Merozoites (2-3μm long) are well suited to invade and establish themselves within erythrocytes and initiate the pathogenic blood-stage of a malaria infection. Merozoites contain the organelles, micronemes, rhoptries and dense

granules, which contain many of the key proteins needed for its apical reorientation, then formation of an irreversible "tight" junction and finally entry into the red blood cell (RBC) in a parasitophorous vacuole. Invasion in the RBC appears to be via a receptor ligand-interaction mechanism. Within the erythrocyte, the parasite undergoes a number of maturation steps from mature trophozoites to mature schizonts, ultimately containing 16-24 merozoites each. Some of the changes to the erythrocytic surface in *P. falciparum* in the last third of each asexual cycle are linked with the infected erythrocytes sticking, via a cytoadherant molecule called *P. falciparium* erythrocyte membrane protein 1 (PfEMP1), to endothelial cells lining capillary venules and as a result stop circulating in the peripheral blood. This process is known as sequestration. 48 hours after merozoite invasion, the RBC ruptures releasing the merozoites into the blood-stream where they either invade further RBC's, repeating the cycle or differentiate into gametocytes. Rupturing of numerous infected RBC's occurs at the same time and results in the release of pyrogens and the characteristic periodic fever associated with malaria infection. Combined with the destruction of RBC's and haemoglobin, the malaria parasite produces haemozoin, a molecule formed via haeme-catalyzed lipoperoxidation as a detoxification product and is released together with other cell debris (Hänscheid *et al.*, 2007).

SEXUAL STAGE

When a new mosquito takes a blood meal from an infected human host, the male and female gametocytes would be taken up by this mosquito and develop into male and female gametes. The gametes fertilize, form a zygote which differentiates into a motile ookinete. The ookinete develops into an oocyst on the midgut wall of the mosquito within which the sporozoites develop. Rupturing of the oocyst releases numerous sporozoites into the haemocoel in the mosquito and these migrate to the salivary glands of the mosquito ready to begin the cycle again (Figure 1.1).

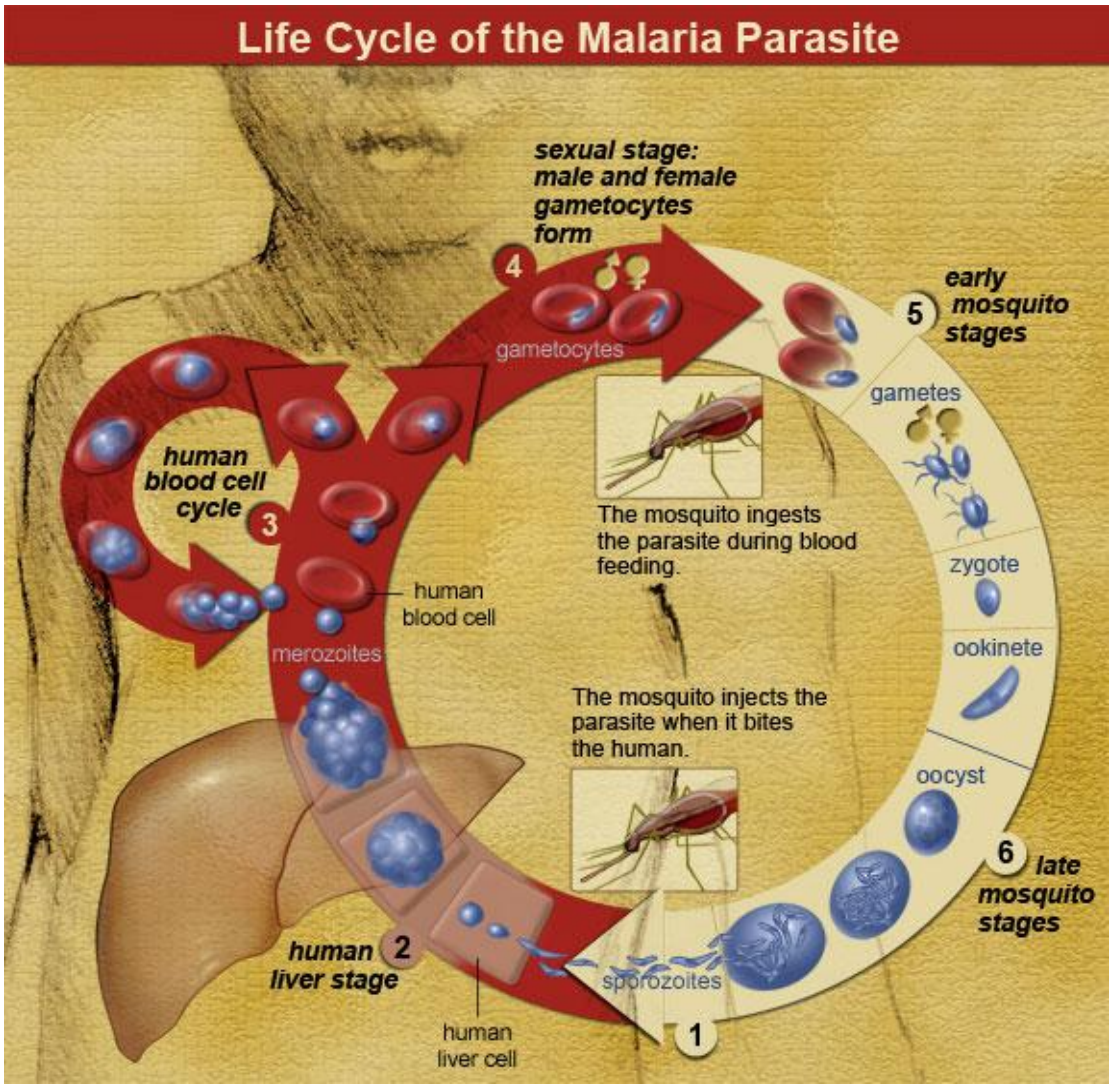


Figure 1.1: The lifecycle of *Plasmodium falciparum*. The figure depicts the asexual stage in the human host and the sexual stage in the mosquito vector. The asexual stage consists of two phases namely the pre-erythrocytic phase in the liver and the erythrocytic phase in blood-stream. Picture taken from the National Institute of Allergy and Infectious Diseases (NIAID) website at www.niaid.nih.gov/.../pages/lifecycle.aspx.

1.3 Parasite-specific proteins

Sporozoites must invade and develop within hepatocytes in order to produce merozoites. The hepatic infection is a clinically silent phase of the parasite's life-cycle. These unique characteristics make the pre-erythrocytic phase an ideal target for vaccine interventions and prophylactic drug discovery as this would prevent both disease and transmission. In the early 1980's it was discovered that the most abundant protein on the sporozoite surface is the circumsporozoite protein (CSP) (Nussenzweig and Nussenzweig, 1985). The CSP is a multifunctional protein required for sporozoite development and likely mediates several steps of this journey. It was the first pre-erythrocytic antigen to be cloned and sequenced, appears to be present in all malaria species and displays a similar

structure across the species with a central region of repeats and two conserved regions. The CSP is essential for parasite development and participates in binding the sporozoite to hepatocytes. CSP's are thus targets of anti-sporozoite neutralizing antibodies (Phillips, 2001, Plebanski and Hill, 2000). Most pre-erythrocytic vaccine candidates are based on the CSP, expressed in various cell lines, microorganisms, or as the corresponding DNA. Studies in which radiation attenuated sporozoites, of *P. falciparum* and *P. vivax* in humans, and *P. berghei* and *P. yoelii* in mice, were injected, the recipients showed resistance to infection for variable periods of time thereafter (Marsh and Kinyanjui, 2006, Nardin *et al.*, 1999,). Thus, development of vaccines against the pre-erythrocytic stages has been attempted although it is not clear why vaccination with attenuated sporozoites induces strong immunity compared to that induced by natural infections. Irradiated sporozoites undergo partial development which seems to be essential. However, since infected irradiated mosquitoes are unavailable for large scale vaccination, the alternative is to develop subunit vaccines. Naturally infected humans have a wide range of specificities for *P. falciparum* CSP (Calle *et al.*, 1992) and other pre-erythrocytic antigens including liver-stage antigen 1 (LSA1) and thrombosponsin-related adhesive protein (TRAP) (John *et al.*, 2003) as well as to antigens recently known to also occur in the sexual stage, namely, STARP (Pasquetto *et al.*, 1997) and apical membrane antigen 1 (AMA-1) (Silvie *et al.*, 2004). Antibodies, affinity purified on most of these proteins, were shown to block *P. falciparum* sporozoite invasion into hepatocytes in *in vitro* studies (Silvie *et al.*, 2004, Pasquetto *et al.*, 1997). Currently, however, there is no clear evidence that the presence or level of antibodies recognizing sporozoites is related to protection against infection or disease.

Initial erythrocyte contact by the merozoite is thought to be partly via the merozoite surface protein 1 (MSP-1), an immuno-dominant antigen and the most abundant protein on the surface of the merozoite. MSP-1 was suggested to be essential for parasite survival and is a major vaccine candidate (O'Donnell *et al.*, 2000). Although there is some evidence for the role of *P. falciparum* MSP-1 in initial contact of the host erythrocyte in *in vitro* studies (Goel *et al.*, 2003), definitive proof is lacking. It, however, remains possible that MSP-1 is involved in the early invasive recognition and attachment events but these events still

remain to be defined (Cowman and Crabb, 2006, Bannister and Mitchell, 2003). Furthermore, *P. falciparum* invasion is facilitated by the erythrocyte binding antigen (EBA 175), an erythrocyte glycophorin A binding protein (Cowman and Crabb, 2006, Sim *et al.*, 1994); AMA-1, a micronemal protein thought to engage in apical junction formation by secretion into the merozoite apex before invasion (Mitchell *et al.*, 2004, Narum *et al.*, 1994); the serine-rich antigen (SERA), secreted in the parasitophorous vacuole and the dense granules are also associated with the invasive mechanism of the merozoite into its host erythrocyte (Bannister and Mitchell, 2003). In *P. falciparum* the VAR genes are responsible for antigenic variation and code for PfEMP-1, expressed on the surface of infected erythrocytes. This cytoadherent protein is the major antigenically variant protein and due to its variability creates difficulties for recognition by the immune system and for vaccine development (Arnot and Jensen, 2011, Bannister and Mitchell, 2003, Phillips, 2001).

CSP and MSP parasite-specific proteins in recent years have been vaccine candidates which could induce immune responses to prevent invasion of the parasite within the host cell. However, several problems are associated with vaccine development, namely (1) parasitic antigens are specific for each stage of its life-cycle, (2) host genetic susceptibility and immune status and (3) antigenic diversity in immunologically dominant polymorphic antigens (Stevenson and Zavala, 2006, Phillips, 2001).

After many years of research and testing, a partially efficacious first generation vaccine, the RTS,S/AS01E vaccine against *P. falciparum*, is currently undergoing pivotal phase III trials in Africa (Cohen *et al.*, 2010). One of the obstacles facing the development of a licensed malaria vaccine is the extensive heterogeneity of many of the current malaria vaccine candidate antigens. To counteract this antigenic diversity, an effective malaria vaccine may need to elicit an immune response against multiple malaria antigens, thereby limiting the negative impact of variability in any one antigen. Since most of the malaria vaccine antigens that have been evaluated in people have not elicited a protective immune response, there is a need to identify additional protective antigens. In a study by Limbach and colleagues, the efficacy of three pre-erythrocytic stage malaria antigens (PY0311, PY03424 and PY03661) was evaluated in a *P. yoelii* model of

protection. Their results suggested that vaccine combinations with vectors that express PY03011 and PY03424, or PY03011, PY03424 and PY03661 protected mice at significantly higher levels than vaccines that express the individual antigens (Limbach *et al.*, 2011). Other studies have also shown that combination vaccines can enhance protection, as well as circumvent the HLA restricted protection observed with some single antigen vaccines in rodent (Doolan *et al.*, 1996) and primate models (Weiss *et al.*, 2007). Combination vaccines, however, can have several disadvantages. A multi-component vaccine may be more expensive to manufacture than a single component vaccine. In addition, there is a risk that one vaccine component can have an immunosuppressive effect on the other components. For example, a vaccine containing nine different DNA-*P.falciparum* vectors elicited significantly lower immune responses against each individual antigen than a vaccine containing the individual vectors (Sedegah et al., 2004). Therefore, combining vaccine antigens would need to be evaluated thoroughly to see if synergistic, additive or antagonistic responses are observed.

A detailed understanding of the host immune response to malaria parasites in terms of identifying mechanisms, which will either suppress or induce protection within the host, will in addition to vaccine studies aid the development of an effective and safe vaccine.

2. IMMUNITY TO MALARIA

In humans, natural acquired immunity, takes several years to develop and is followed by repeated exposure to the parasite in endemic regions (Stevenson and Riley, 2004, Baird, 1995). Naturally acquired immunity is targeted against the erythrocytic stage of the parasite and only protects against the clinical manifestations of the disease and does not provide complete/sterile protection. The malaria parasites have evolved intricate mechanisms to avoid the development of effective anti-parasitic immune responses that would protect the human host against future re-infections. Other factors influencing immunity to malaria infection include the parasite infective dose, age, genetic background, pregnancy, nutritional and immune status of the human host.

It has been widely established that immunity in humans and mice to blood-stage malaria infection is characterized as a parasite-specific innate and acquired immune response involving both cell-mediated and antibody-dependent mechanisms (Burns *et al.*, 2004), essential for the control of parasitaemia levels and hence parasite-induced pathology (Good, 2005, Good *et al.*, 2004, Langhorne *et al.*, 2004, Taylor-Robinson and Phillips, 1994, Taylor-Robinson *et al.*, 1993, Stevenson *et al.*, 1992). Although host immunity may be protective, it has also been implicated as a significant contributor to the pathology of malaria (Artavanis-Tsakonas *et al.*, 2003, Heddini, 2002). Upon malaria infection, activation of specific cells and their effector mechanisms during innate immunity is vital as it greatly influences the nature and magnitude of the adaptive response (Stevenson and Zavala, 2006). The nature of the immune response induced depends on the following factors: the parasite and the pathogen recognition receptor (PRR) and toll-like receptors (TLR) involved; the cytokines and chemokines; the strength of the activation signal between the antigen presenting cell (APC) and the pathogen receptors; the antigen dose and the influence of cell memory (Langhorne *et al.*, 2004). The innate and adaptive immune effector mechanisms in malaria infection can limit peak parasitaemia, prevent severe pathology and reduce the levels of circulating infected erythrocytes but ultimately, they usually fail to eliminate the infection completely leading to a long-lasting or even persistent low-grade parasitaemia which might persist for months to years (Stevenson and Riley, 2004, Franks *et al.*, 2001).

2.1 Rodent *Plasmodium* species causing malaria

P. chabaudi, P. berghei, P. yoelii and P. vinckei, isolated from small rodents in central and West Africa, have been used as mouse models to mimic human malaria infections (Table 1.1). At present, there is no single mouse model available to replicate all the features of human infection in terms of pathology and immune responses (Hunt and Grau, 2003, Langhorne *et al.*, 2002). However, mouse models have proved useful in the investigation of immune mechanisms and pathology, identification of genes regulating susceptibility to infection and for vaccine development studies (Dechamps et al., 2010, Langhorne *et al.*, 2002, de Souza *et al.*, 2002, Fortin *et al.*, 2002, Shear *et al.*, 1998). Genetic variations among inbred strains of mice have been shown to influence susceptibility to

infection with the mouse *Plasmodium* species (Fortin *et al.*, 2002). In a *P. chabaudi* AS model, non-lethal infections occur in BALB/c, C57BL/6 and C57BL/10 mice while lethal infections occur in A/J and DBA/2 mice (Langhorne *et al.*, 2004, Langhorne *et al.*, 2002, Cross and Langhorne, 1998, Stevenson *et al.*, 1992).

Table 1.1: *Plasmodium* parasites that cause malaria in mice (Stevenson and Riley, 2004)

Plasmodium chabaudi	• *P. chabaudi chaudi AS, P. chabaudi adami* • Study immune mechanisms, immunoregulation by cytokines, identify susceptibility loci & immune basis of pathology • *P. chabaudi chabaudi AS*: non-lethal in resistant mice & lethal in susceptible mice. • *P. chabaudi adami*: mild, non-lethal infection
Plasmodium berghei	• *P. berghei* ANKA, *P. berghei* K173 • Widely used to study pathogenesis. *P. berghei* ANKA: serves as an experimental model of cerebral malaria (ECM) • There is genetic variation in the development of ECM between inbred mouse strains which correlates with the production of pro-inflammatory cytokines
Plasmodium yoelii	• *P. yoelii* 17XL, *P. yoelii* 17XNL, *P. yoelii* YM • Study immune mechanisms and pathogenesis & ECM, as recombinant MSP-1 is available • *P. yoelii* 17 XL widely used to identify vaccine-induced immunity
Plasmodium vinckei	• Study pathogenesis and for chemotherapy studies • Causes lethal infection • *P. vinkei petteri* causes a non-lethal infection & used to study immune mechanisms

2.2 *Plasmodium chabaudi* AS model of blood-stage infection

In a review by Phillips and colleagues, the immune response to blood-stage malaria parasites was characterized using NIH mice infected with *P. chabaudi* AS (Figure 1.2) (Phillips *et al.*, 1997). NIH mice are immunologically impaired and can survive an acute infection that is followed by one or two patent recrudescences. The course of infection resembles that in the natural host and therefore used as an accessible model for further studies.

An unusual feature of immunity in the *P. chabaudi* AS model compared with other parasitic infections is that $CD4^+$ Th_1 and $CD4^+$ Th_2 cells play an important role in protection. It is worth remembering that the *Plasmodium* parasite has the ability to undergo antigenic variation, a factor that has affected vaccine studies. In *P. chabaudi* infection, antigenic variation occurs at high rates and because different variable antigen types (VATS) switched at different rates provides for their expression in a hierarchical sequence and consequent immune evasion by the parasite. Consequently, the increasing parasitaemia is largely composed of the parental VAT with minor VATS emerging at different rates as the infection progresses. During the rising primary parasitaemia, immune effector mechanisms that are effective against all VATS, are initially dominated by NK cells and $CD4^+$ Th_1 cells which release IFN-γ. IFN-γ in turn activates macrophages and Kupffer cells in the liver, which is a major site of parasite sequestration. Macrophage functions include anti-parasitic mechanisms (including TNF-α and IL-12 production) (Namazi and Phillips, 2010) and phagocytosis. IFN-γ production then tails off, one or two days before peak parasitaemia, implying that non-specific mechanisms are declining. Consequently, initial protection against rising parasitaemia is mediated through cytotoxic and cytostatic (NO) non-specific effector mechanisms largely driven by Th_1 cells. All VATS present might be expected to be vulnerable to the early immune clearance. Thus, the antigenic stimulus for this Th_1 acquired immune response would mainly occur from the parent VAT. At peak parasitaemia, there are more specific effector mechanisms that come into play and are mediated through IgG2a and IgM with possibly some early IgG1 antibody production which in turn remove the parental VAT and leave behind the minor VATS. Thereafter, there is a switch from being predominantly $CD4^+$ Th_1 to predominantly $CD4^+$ Th_2 cells with IL-4 driving the major anti-parasitic effector mechanisms mediated by IgG1 antibodies to the individual VATS and the recrudescence is controlled (Namazi and Phillips, 2010, Phillips *et al.*, 1997) as shown in Figure 1.2. Consequently, the erythrocytic stage of *P. chabaudi* AS infection depends on a complex and dynamic interaction of immunological events and parasite immune evasion strategies. Thus, both the parasite and host contribute to this balance (Phillips *et al.*, 1997).

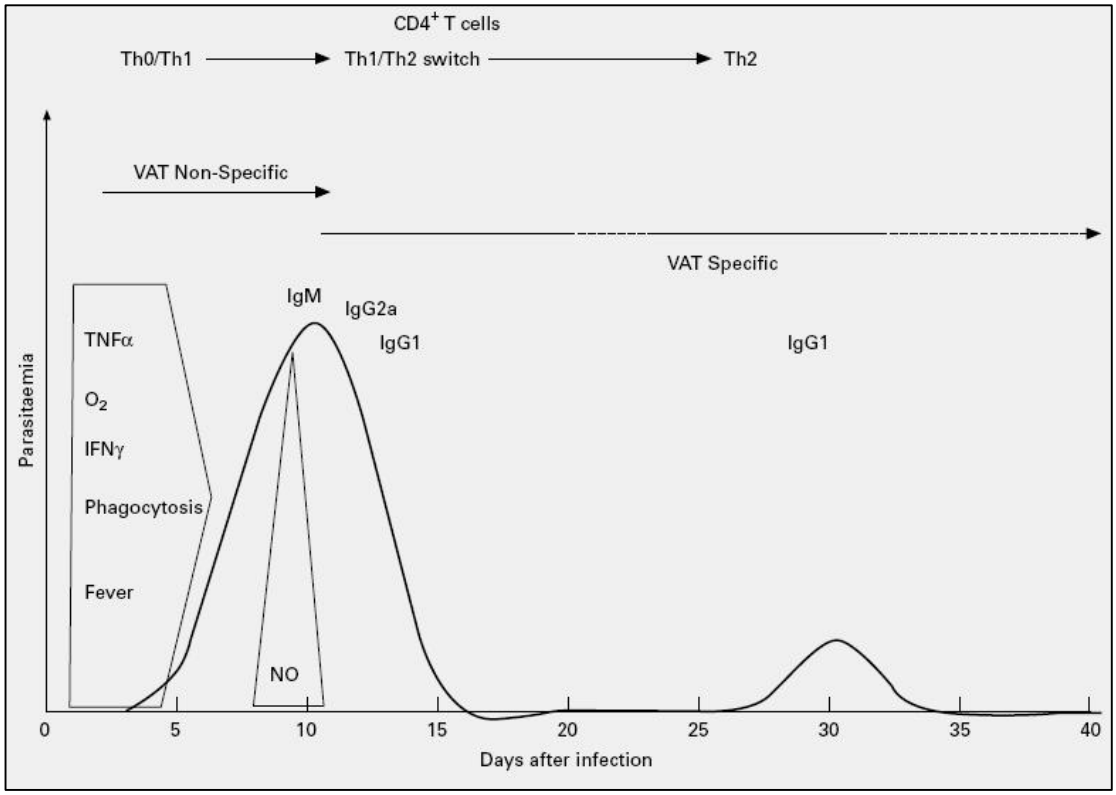


Figure 1.2: The immune response to the erythrocytic stage of *P. chabaudi* AS infection. The initial control is mediated by $CD4^+$ Th1 cells and after the primary parasitaemia there is a switch to $CD4^+$ Th2 regulated responses. This diagram suggests how the cytokine network drives the immune effector mechanisms. Immunopathology can also be a consequence (Phillips *et al.*, 1997).

Other studies using *P. berghei and P.yoelii* infection models, demonstrated that humoral immunity was responsible for parasite clearance and protection (Stevenson and Riley, 2004, Phillips, 2001, Roberts *et al.*, 1977). In contrast, *P. chabaudi adami* required antibody-independent immunity as B-cell deficient mice were able to control the infection (Stevenson and Riley, 2004, Phillips, 2001, Grun and Weidanz, 1981). On the other hand, again, clearance of *P. chabaudi* infection has been shown to be associated with a switch from a B-cell independent to B-cell dependent immune response (Stevenson and Riley, 2004, Taylor-Robinson *et al.*, 1993). Supporting studies have demonstrated that both T-cell (McDonald and Phillips, 1978) and B-cell responses are crucial for protective immunity and both antibody dependent and independent mechanisms are involved (Stevenson and Riley, 2004, Taylor-Robinson and Phillips, 1994). Depletion or deficiency in cell-mediated responses alters the course of infection during both the acute and chronic phases whereas depletion or deficiency of B-cells alters the course of infection during the chronic phase only (Figure 1.3).

Evidence from the *P. chabaudi* AS model of blood-stage malaria highlights the importance of adaptive, $CD4^+$ T-cell dependent mechanisms for control. However, although initial studies have indicated that control of parasitaemia levels, after the primary peak parasitaemia, is dependent on a Th_2 response, other investigations have indicated that Th_1 responses are required not only during acute infection to promote cell-mediated immunity but also during the chronic stage of infection to promote antibody responses (Stevenson and Riley, 2004, Su and Stevenson, 2002).

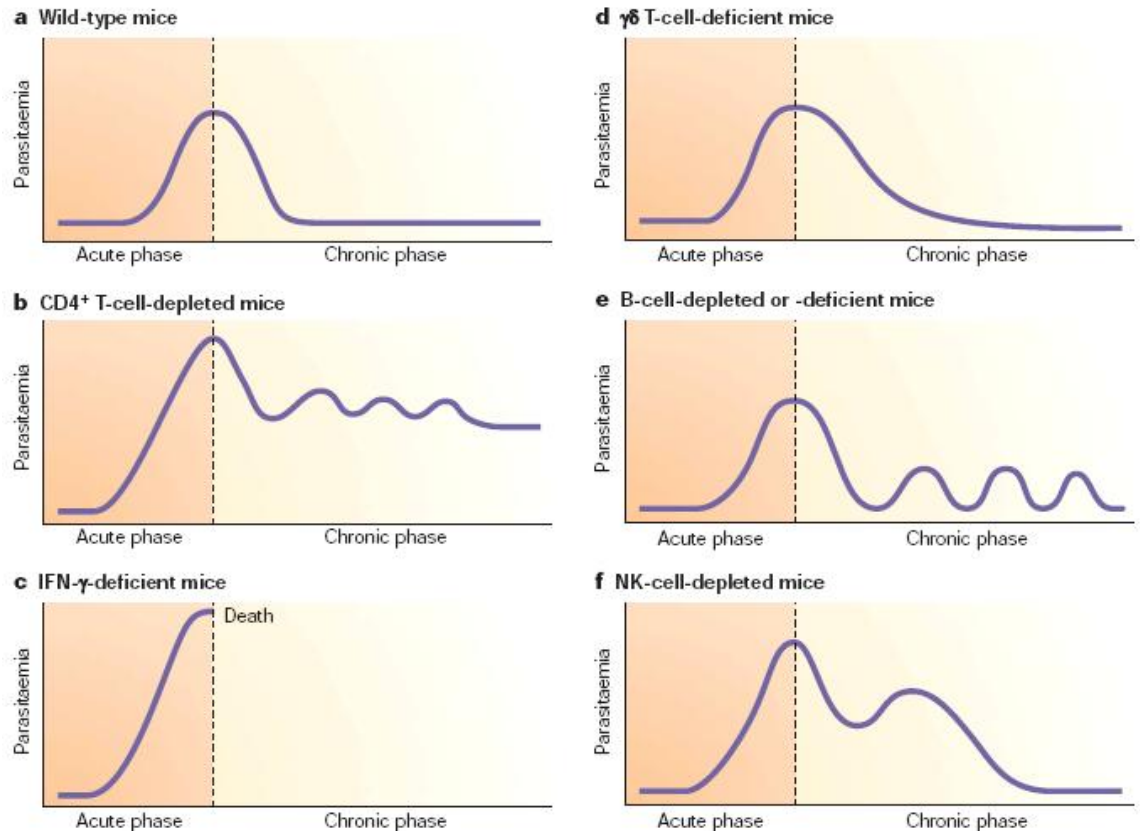


Figure 1.3: Course of infection with *P. chabaudi chabaudi* AS in C57BL/6 mice. Shown for **(a)** C57BL/6 wild-type, **(b)** $CD4^+$ T-cell depleted mice, **(c)** IFN-γ deficient mice, **(d)** γδ T-cell deficient mice, **(e)** B-cell depleted or deficient mice and **(f)** NK cell depleted mice. The infection consists of an acute and chronic phase. In intact C57BL/6 wild-type mice, peak parasitaemia was controlled during the acute phase by a $CD4^+$ T-cell (Th_1), IFN-γ dependent mechanism that is antibody independent. The parasite was eliminated during the chronic phase by a mechanism that requires both $CD4^+$ T-cells and malaria-specific antibody. Depletion or deficiency of $CD4^+$ T-cells or NK cells alters the course of infection during acute and chronic infection, whereas depletion or deficiency of B-cells alters the course of infection during the chronic phase only. γδ T-cells are not essential for resolution of infection. (Stevenson and Riley, 2004).

2.3 CELL-MEDIATED IMMUNITY

2.3.1 Antigen presenting cells (APC's)

Many parasitic infections are controlled by cell-mediated immunity. A key question that needs to be resolved is the identity of the antigen-presenting cells (APCs) that activate T-cells to produce IFN-γ and mediate class switching to the protective IgG2a and IgG2b (in mice) and IgG1 and IgG3 (in humans) antibodies during an acute *Plasmodium* infection (Su and Stevenson, 2002). Bone-marrow derived macrophages, DC's and B-cells isolated from immune mice have showed that they have the ability to present malarial antigen to T-cells (Quin *et al.*, 2001).

Macrophages

In the early stages of inflammation/infection, neutrophils and macrophages kill most microbes and macrophages remove the apoptotic bodies and present antigen to T-lymphocytes, thereby initiating the mechanisms of acquired immunity, which ends in the production of antibodies and cytokines and memory cells. Macrophage activity then switches from being pro-inflammatory to anti-inflammatory, whereby they remove all the tissue debris, thus achieving healing (Celada, 2009) (Figure 1.4).

The signals encountered by developing macrophages during migration determine their functional properties at sites of inflammation or infection (Claasen *et al.*, 2009, Celada, 2009). Among these signals, cytokines, which can act synergistically or have opposing effects, are responsible for the development of highly divergent macrophage phenotypes. The classification of macrophage activation is described for in figure 1.5.

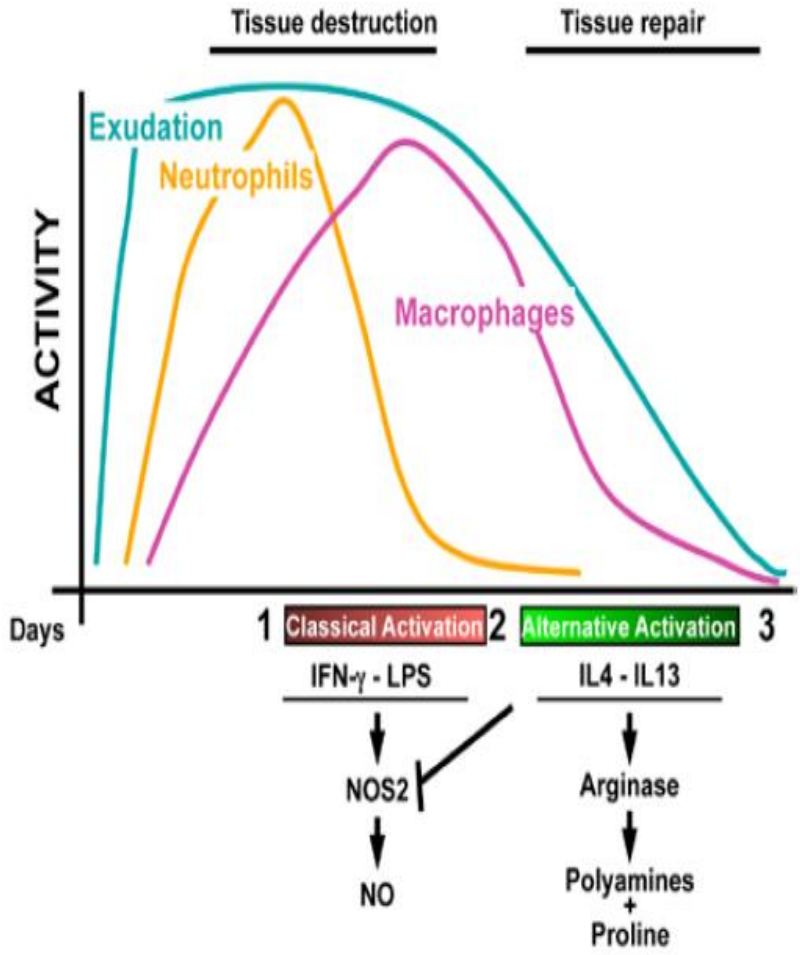


Figure 1.4: Dual activity of macrophages at the inflammatory loci. In the initial stages of inflammation, macrophages eliminate the remaining microbes that escape neutrophils, remove the apoptotic bodies of dead neutrophils and present antigen to T-lymphocytes, thereby initiating the mechanisms of acquired immunity, which ends in the production of antibodies and cytokines and memory cells. Macrophage activity then switches from being pro-inflammatory to anti-inflammatory, whereby they remove all the tissue debris, thus achieving healing (Taken and adapted from Celada, 2009).

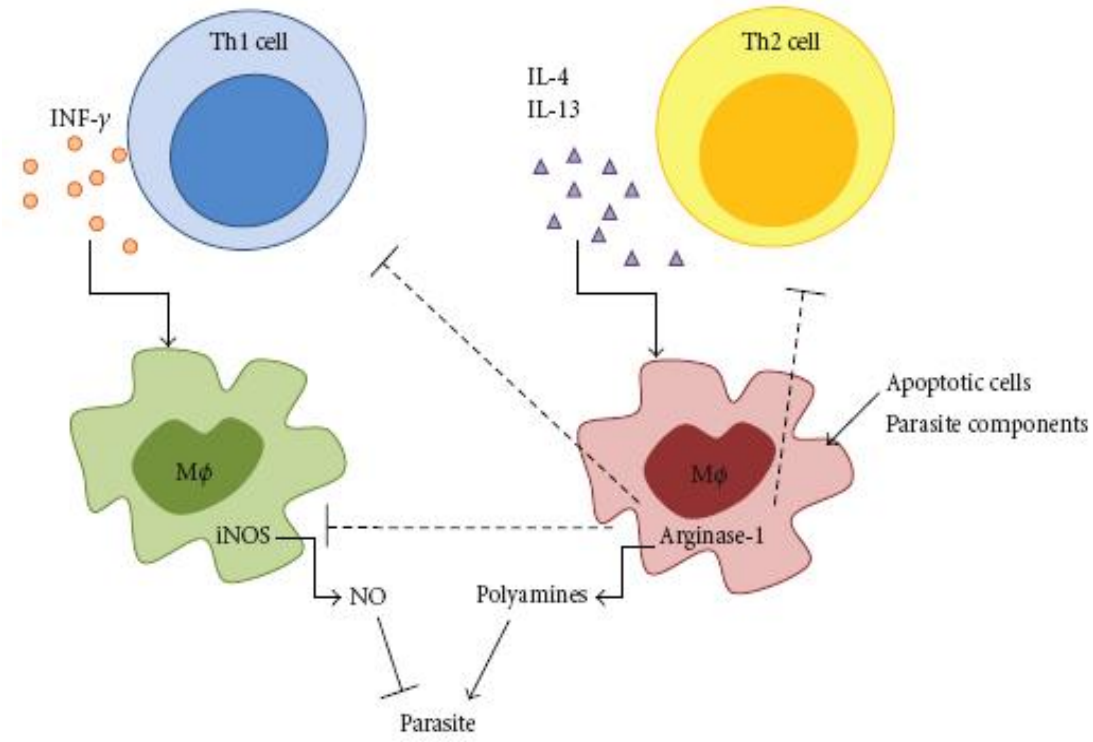


Figure 1.5: Classification of macrophage activation. Type-1 cytokine-dependent proinflammatory responses induce classically activated macrophages (caMac) which leads to nitric oxide (NO) production and the synthesis of several products of NO reaction. CaMac are crucial for parasite control during protozoan infections but can also contribute to the development of immunopathological disease symptoms. Type-2 cytokines such as IL-4 and IL-13 antagonize caMac inducing alternatively activated macrophages (aaMac) that upregulate arginase-1 expression. Arginase-1 can also be induced during the infection by apoptotic cells or even directly by parasites or parasite components. Arginase-1 limits caMac-dependent parasite clearance promoting parasite proliferation. Additionally, arginase-1 suppresses T cell response. Therefore, generation of alternative activation states of macrophages could limit collateral tissue damage because of excessive type-1 inflammation. However, they affect disease outcome by promoting parasite survival and proliferation (Taken and adapted from Stempin *et al.*, 2010).

During an early, acute-phase microbial infection, for example with bacteria (McCaffrey *et al.*, 2004, Nau *et al.*, 2002) or protozoa (Walther *et al.*, 2006, Plebanski and Hill, 2000, Kropf *et al.*, 2004, Peluffo *et al.*, 2004), macrophages are activated by pathogen-associated molecular patterns (PAMPs) which allow them to recognize, engulf and kill invading pathogens (Zhang *et al*, 2010). Interaction with different PAMPs and cytokines leads to different states of macrophage activation (Zhang *et al*, 2010). Microbial products such as lipopolysaccharide (LPS) through engagement of pattern recognition receptors (PRRs) and/or the presence of Th1-type proinflammatory cytokines particularly IFN-γ but also TNF-α and IL-12 (Stempin *et al.*, 2010, Mosser, 2003), induces classically activated macrophages (caMac), also known as M1 cells (Zhang *et al*, 2010). The prime function of caMac is initial microbial destruction, which is

controlled by the production of reactive oxygen, and nitrogen intermediates such as nitric oxide (NO) from inducible NO synthase (iNOS/NOSII) (Claasen *et al.*, 2009, Gordon, 2003, Yu *et al.*, 1999). During infections with protozoan parasites such as *Plasmodium* (Walther *et al.*, 2006, Plebanski and Hill, 2000), *Leishmania* (Kropf et al., 2004) or *Trypanosoma* (Peluffo *et al.*, 2004), an IFNγ-dependent proinflammatory response triggering the development of caMac is required for controlling parasitaemia particularly during the acute stage of infection (Stempin *et al* 2010). Consequently, caMac are essential components of host defense but their activation must be securely regulated since the cytokines and mediators that they produce can lead to host-tissue damage and pathology (Stempin *et al.*, 2010, Celada, 2009).

Alternatively, depending on the parasite species or parasite virulence, host genotype and stage of infection, type-2 cytokines such as IL-4 and IL-13, are produced by the host and antagonize caMac and induce arginase-1 expression in macrophages. These macrophages, termed alternatively activated macrophages (aaMac), are commonly found during parasitic infections (Noël *et al.*, 2004) and have been associated with the downregulation of type-1 immunity and the survival of both protozoa (Raes *et al.*, 2007) and helminths (Reyes and Terrazas, 2007). IL-4 and IL-13 upregulate the expression of the mannose receptor and major histocompatibility complex (MHC) II molecules on macrophages and as a result stimulate endocytosis and antigen presentation and induce the expression of selective chemokines. The concept of alternative activation has been less well studied than macrophage activation by IFN-γ or innate activation by lipopolysaccharide (LPS) or microbes. Studies with the latter led to the discovery of toll-like receptors (TLR's) and signalling pathways that are distinct from those used by the IL-4/IL-13 receptor (IL-4Rα) (Menzies *et al.*, 2010). Apart from IL-4 and IL-13 activation (Martinez *et al.*, 2009) factors such as IL-10, TGF-ß (Gordon, 2003) and phagocytosis of apoptotic cells (Freire-De-Lima *et al.*, 2000) also antagonize caMac and induce alternative non-M1 activation states in macrophages.

During *P. falciparum* infection, as red blood cells and haemoglobin are destroyed, the malaria parasite produces hemozoin, a molecule formed via heme-catalyzed lipoperoxidation as a detoxification product and released together with other cell debris (Hänscheid *et al.*, 2007). Hemozoin induces the release of endogenous ligands lipoxin A4 (LXA4), 5,15-diHETE and 15-HETE, that can activate a peroxisome proliferator-activated receptor (PPAR) which suppresses production of type-1 proinflammatory cytokines and favour the development of aaMac which control inflammation and pathology (Chan *et al.*, 2010). Significantly, IL-4 and IL-13 also activate PPAR (Chan *et al.*, 2010). Overall studies would indicate that aaMac not only inhibit T-cell proliferation via an arginase induced hypo-responsiveness (Kropf *et al.*, 2005), but also promote resolution of inflammation and fibrogenesis. They actively express a set of genes enabling them to regulate anti-inflammatory processes, induce tolerance and wound healing (Chan *et al.*, 2010, Celada, 2009). These anti-inflammatory regulatory mechanisms can act as a counterbalance to limit disease severity and protect the host from detrimental effects of an excessive type-1 response.

In particular and with reference to the present study, IL-4Rα deficient mice and more recently macrophage/neutrophil specific IL-4Ra deficient mice have been exploited in various parasitic and infectious disease models to examine the importance of alternative activation of macrophages (Bryson *et al.*, 2011, Hölscher *et al.*, 2006, Herbert *et al.*, 2004, Gordon, 2003).

Dendritic cells

Dendritic cells (DCs) are the most important heterogeneous potent antigen-presenting-cells (APC) with a key role in the initiation and regulation of innate and adaptive immune responses (Gordon, 2003, Banchereau *et al.*, 2000). DCs have the remarkable ability to sample sites of pathogen entry, integrate signals coming from the environment, take up and process antigen and deliver this antigen to naïve T-cells. In turn, DCs activate these and memory T-cells through antigen presentation via receptor interaction bound to the MHC inducing the appropriate response for the initial stimuli (Terrazas *et al.*, 2010, Langhorne *et al.*, 2004, Sher *et al.*, 2003, Guermonprez *et al.*, 2002). DCs can be broadly divided into plasmacytoid DCs (pDC) and conventional DCs (cDC) based on the

expression of a variety of cell surface markers and their response to the pathogen molecules (Colonna *et al*., 2006, Banchereau *et al*., 2000).

Toll-like receptors (TLRs), expressed by cell-types including DCs and macrophages, have a vital role in triggering innate immunity and orchestrating the acquired immune response during infection. In order to initiate an immune response, TLRs recognize certain pathogen associated molecular patterns (PAMPs) (Gazzinelli and Denkers, 2006). The role of TLRs in immunity to malaria has not been firmly established, although this area is under investigation. In a model of cerebral malaria, Adachi and colleagues has analyzed the involvement of TLRs in cerebral malaria by using a mouse infection *P. berghei* model whereby myeloid differentiation primary response gene 88 (MyD88), an essential adaptor molecule for most TLRs, was critical for IL-12 induction by *P. berghei* NK65 parasites and caused liver injury (Adachi *et al*., 2001). Furthermore, glycosyl-phosphatidylinositol (GPI) and hemozoin (a parasite heme metabolite) derived from *P. falciparum* has been identified as the ligands for TLR2, TLR4 and TLR9 and induce TNF-α synthesis (Coban *et al*., 2006, Coban *et al*., 2005, Krishnegowda *et al.,* 2005). Other heat-labile molecules derived from the malaria parasite are still to be clarified for TLR9-mediated recognition (Coban *et al*., 2006, Pichyangkul *et al*., 2004).

DCs are widely accepted as having a crucial role in Th_1-cell versus Th_2-cell differentiation but DC plasticity *in vivo* and *in vitro* makes it inappropriate to equate different types of DCs (pre-DC, DC1, DC2 and plasmacytoid DC) with Th-cell subsets or with corresponding macrophage phenotypes. Depending on their maturity and modulation, DCs can activate or suppress Th-cell responses (Gordon, 2003, Pulendran *et al*., 1999) or induce Th-cell anergy (Gordon, 2003).

In addition, modulation of TLRs by parasites to evade effects of the host immune system has also been shown in *P. falciparum* and *P. yoelii* infection studies (Gazzinelli and Denkers, 2006). DCs and phagocytes infected with the *Plasmodium spp*. became unresponsive to LPS-induced activation resulting in defects in T-cell activation. Furthermore, in *P. chabaudi* infection, phagocytosis of malaria haemozoin, a TLR9 agonist, resulted in non-responsive of DCs to LPS and thus their inability to activate T-cells (Millington *et al*., 2006). A more

recent review by Terrazas and colleagues discusses in detail how *Plasmodium* species through varied mechanisms (human and murine) modulate DC function in order to survive (Terrazas *et al.*, 2010). For example, modification of DC function results in a decrease in the total number of DCs and an altered ratio of myeloid versus plasmacytoid cell subsets, the latter involved in the induction of Tregs and IL-10. Furthermore, the reduced capacity of *Plasmodium*-exposed DCs to prime T-cells results in the inability to establish prolonged interactions with naïve $CD4^+$ T-cells. Together, this suggests that *Plasmodium* species have the ability to switch an aggressive immune response into a more permissive one in order to survive within its host.

2.3.2 Natural Killer (NK) cells and Gamma-Delta T-cells (γδ T-cells)

The early source of IFN-γ remains controversial with both NK cells and γδ T-cells being indicated as a potential source of this critical cytokine that is necessary not only during the innate immune response to pathogens but also in activation of the adaptive immune response and development of protective immunity (Urban *et al.*, 2005, Stevenson and Riley, 2004, Choudhury *et al.*, 2000, Troye-Blomberg *et al.*, 1999). The mechanism by which they accomplish this appeared to be mediated via their secretion of IFN-γ induced by cytokines such as IL-12, TNF-α, and IL-6 produced by other components of the innate immune system, including macrophages and DCs (Ing and Stevenson, 2009, Robinson *et al.*, 2009, van der Heyde *et al.*, 2006, Stevenson *et al.*, 2001, Mohan *et al.*, 1997). NK cells are rapid IFN-γ responders to *Plasmodium falciparum*-infected erythrocytes (*Pf*RBC) *in vitro* and are involved in controlling early parasitaemia in murine models, yet little is known about their contribution to immune responses following malaria infection in humans. Therefore, McCall and colleagues have studied the dynamics of and requirements for *in vitro* NK cell responses to *P. falciparum* infected RBC (PfRBC) in malaria-naïve volunteers undergoing a single experimental malaria infection under highly controlled circumstances, and in naturally exposed individuals. They concluded that NK cells make a significant contribution to total IFN-γ production in response to PfRBC but their innate role was questioned because of their dependency on (memory) T-cell help (McCall *et al.*, 2010).

P. chabaudi malaria is more severe in wild-type (WT) mice treated with IFN-γ neutralizing antibody and in IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice, as indicated by the increased magnitude and duration of parasitaemia and mortality in the IFN-$\gamma^{-/-}$ mice compared to the intact controls (Su and Stevenson, 2000, van der Heyde *et al.*, 1997, Meding *et al.*, 1990). Earlier genetic studies failed to correlate susceptibility to *P. chabaudi* infection with NK activity (Urban *et al.*, 2005, Skamene *et al.*, 1983). Subsequently, Mohan and colleagues reported that depletion of NK cells during acute *P. chabaudi* AS infected C57BL/6 mice led to a rapid increase in blood-stage parasitaemia levels and less efficient resolution of the infection (Mohan *et al.*, 1997). The mode of action by which NK cells function appeared to be via the secretion of cytokines rather than direct cytotoxicity against blood-stage malaria parasites (Mohan *et al.*, 1997).

Contrary to the findings of Mohan and colleagues, other studies indicate similar *P. chabaudi* parasitaemia in depleted mice and intact controls after NK1.1 monoclonal antibody (MAb) depletion of NK cells (Taniguchi *et al.*, 2007, Yoneto *et al.*, 1999, Kitaguchi *et al.*, 1996). Kim and colleagues showed that by using microarray analysis of blood cells from *P. chabaudi*-infected mice, a rapid production of IFN- γ and activation of IFN-γ-mediated signalling pathways as early as 8 hours existed after infection. However, NK cells did not express IFN-γ or exhibit IFN-γ-mediated pathways in their analysis (Kim *et al.*, 2008). At this time, NK cells are replicating and migrating from the spleen to the blood. In humans with *P. falciparum* infection, increased IFN-γ production by peripheral blood mononuclear cells (PBMC) in response to parasitized RBCs correlated with protection from acute parasitemia and clinical malaria (D'Ombrain *et al.*, 2008, 2009). Early IFN-γ production by PBMC obtained from malaria naive donors was primarily produced by γδ T cells and not by NK cells (Robinson *et al.*, 2009). Animal models do not exactly mimic the human condition and experimental malaria in mice is species specific from those that infect humans. Nevertheless, analysis of protective immunity by murine models provides important information on how a protective immune response to *Plasmodium* may be elicited. Whether both NK cells and γδ T-cells have essential roles during the early stages of immunity to blood-stage malaria remains to be determined and whether these cells function early in cell-mediated immunity (CMI) to malaria parasites is unknown. To address these issues, Weindanz and colleagues infected

NK cell-or-γδ-T-cell-depleted $J_H^{-/-}$ mice with blood-stage *P. chabaudi*. Their data indicated that the suppression of acute *P. chabaudi* infection by CMI was γδ T-cell dependent, was independent of NK cells, and was highlighted by the deficient IFN-γ response seen early in γδ T-cell-depleted mice (Weindanz *et al.*, 2010).

2.3.3 $CD8^+$ T-cells

Research studies have shown that immune protection against pre-erythrocytic stages of *Plasmodium* infection depends on $CD8^+$ T-cell responses (Good and Doolan, 1999). However, immunity against the erythrocytic stage is largely antibody-mediated although $CD4^+$ T-cells may also be protective (Good and Doolan, 1999). Mounting evidence in murine models has also shown that $CD8^+$ T-cells contribute to the pathology of experimental cerebral malaria (ECM) although the precise mechanisms remain unclear (Renia *et al.*, 2006, Belnoue *et al.*, 2002, Hermsen *et al.*, 1997, Yanez *et al.*, 1996).

Studies implicating $CD8^+$ T-cells in ECM required reinterpretation in light of recent evidence, which demonstrated that DCs expressing CD8α mediate priming of T-cell responses to pathogens such as viruses (Belz *et al.*, 2005, Smith *et al.*, 2003) and bacteria (Belz *et al.*, 2005). For example, studies using depletion with anti-CD8α antibody to implicate $CD8^+$ T-cells in pathology may be reinterpreted as implicating CD8α DCs in $CD4^+$ T cell priming. Similarly, studies implicating $CD8^+$ T-cells in ECM that used perforin-deficient mice might reflect a role for NK cells rather than $CD8^+$ T cells in disease. Lundie and colleagues have recently resolved the role of $CD8^+$ T cell responses to *Plasmodium* blood-stage infection. In order to examine *Plasmodium*-induced $CD8^+$ T cell responses to blood-stage malaria in the absence of known MHC class I (MHC I) restricted epitopes, they generated transgenic *P. berghei* parasites expressing a variety of model T-cell epitopes for which T-cell receptor (TCR) transgenic mice were available. Using those parasites, they demonstrated that antigens expressed by blood-stage *P. berghei* were captured and cross-presented by CD8α DC to stimulate naive $CD8^+$ T cell proliferation and lytic function (Lundie *et al.*, 2008).

2.3.4 $CD4^+$ T-cells

$CD4^+$ T-cells play a central role in the immune response to blood-stages of *P. chabaudi* AS infection (Langhorne *et al.*, 2002). They produce cytokines that amplify the phagocytic and parasitocidal response of the innate immune system, as well as dampening this response later on to limit immunopathology (Stephens and Langhorne, 2006, Stephens *et al.*, 2005, Langhorne *et al.*, 2002). $CD4^+$ T-cells are also required to help B-cells produce the antibody that is essential for parasite clearance (Stephens *et al.*, 2005, Langhorne *et al.*, 2002). Therefore, understanding the mechanisms by which T-helper cells undergo priming, expansion and development of specific and effective T-cell memory and immunity would aid the formulation of suitable treatment therapies (Stephens and Langhorne, 2010).

Initial priming and activation of T-cells by APCs is vital for effective cell-mediated and antibody-mediated responses. Thus, athymic mice, deficient in T-cells, have been shown to be unable to control a primary infection, and die with fulminating malaria following infection with *P. berghei*, *P.yoelii* (Weinbaum *et al.*, 1976) and *P. chabaudi adami* (Cavacini *et al.*, 1990) indicating the importance of T-cells during early infection. In *P. chabaudi* AS infection, there is debate over the extent to which T-cells protect via Th1 cytokine mediated mechanisms or by the Ab's that they help to produce (Stephens *et al.*, 2005). Both mechanisms dominate the T-cell response to *P. chabaudi* in turn with an early Th_1 type cytokine response that has been shown to provide protection during the acute patent parasitaemia which switches later to a Th_2 response which provides effective help for malaria-specific Ab production as the infection becomes chronic (Stephens *et al.*, 2005, Smith and Taylor-Robinson, 2003, Langhorne, 1989, Langhorne *et al.*, 1989). In support of these observations, $CD4^+$ T-cells isolated during acute *P. chabaudi* AS infection were shown to display a typical Th_1 phenotype while $CD4^+$ T-cells during the chronic phase displayed a Th_2 phenotype (Stevenson and Tam, 1993, Taylor-Robinson and Phillips, 1992). During chronic infection, $CD4^+$ T-cells are activated to produce additional cytokines such as IL-4 and IL-10 and it has been suggested that this change in Ag-presenting splenic DC population is responsible for the switch away from the predominantly IFN-γ-producing T-cells present in the acute infection (Sponaas *et*

al., 2006). A similar T-cell profile seems to occur during human *P. falciparum* infection (Riley *et al*., 1993, Brown *et al*., 1990).

Enhanced IFN-γ, TNF-α and IL-12 production are all associated with an acute infection with *P. chabaudi* AS (Taylor-Robinson and Phillips, 1994). IL-12 is the critically important cytokine in the establishment and differentiation of Th_1 cells (Manetti *et al*., 1993) and is also required for NK activity (Kobayashi *et al*., 1989). T-cells and NK cells are important for IFN-γ production, which is an essential mediator in immunity to *P. chabaudi* AS infection (Su and Stevenson, 2000, van der Heyde *et al*., 1997, Favre *et al*., 1997). IL-12 also acts synergistically, while participating in cellular immunity, it also contributes to IgG2a production required for clearance of the parasite following peak parasitaemia (Su and Stevenson, 2002).

In-vivo studies have shown that neutralization of IFN-γ significantly elevates peak parasitaemia during *P. chabaudi* AS infection (Meding *et al*., 1990, Stevenson *et al*., 1990). In addition, IFN-γ has been shown to induce IgG2a antibody production during *P. chabaudi* AS infection (Stevenson and Tam, 1993). *P. chabaudi* AS infection studies provided further evidence that in the absence of IFN-γ the Th_1 associated IgG2a antibody response was significantly reduced and thought to account for the failure to clear parasitaemia levels (Su and Stevenson, 2000). Furthermore, IFN-γ deficient C57BL/6 mice infected with *P. chabaudi* AS demonstrated higher morbidity and mortality compared to the controls (Yoneto *et al*., 2001, Balmer *et al*., 2000, Su and Stevenson, 2000).

TNF-α on the other hand exerts a dichotomy of functions during *Plasmodium* infection, providing protection but also contributing much to the pathology associated with infection (Beutlar and Cerami, 1988). TNF-α is a potent activator of macrophages and nitric oxide production (Liew *et al*., 1990) but not a necessity for the development of immunity whereby other cytokines are sufficient to replace its actions.

While $CD4^+$ Th_1 cells and their role in acute malaria infection have been widely investigated, the mechanisms governing the Th_1 to Th_2 switch and the resulting protective mechanisms that occur during chronic malaria infection await elucidation. IL-4 has been shown to be the key cytokine driving a Th_2 type response (Gordon, 2003, Brombacher, 2000). Studies have shown that in 129SVxC57BL/6 mice deficient for IL-4 have elevated Th_1 responses during *P. chabaudi* AS infection as measured by increased IFN-γ production (Balmer *et al.*, 2000, Von der Weid *et al.*, 1994). These mice were able to control and clear the infection similar to the wild-type mice although Balmer and colleagues noted that the IL-4 deficient mice demonstrated a significantly elevated peak parasitaemia (Balmer *et al.*, 2000) whereas von der Weid and colleagues showed increased recrudescent infection in these mice (von der Weid *et al.*, 1994). The Th_2-type response initiated in the deficient mice was delayed but not absent. Evidence for the protective Th_2 response in the IL-4 deficient mice was attributed to IL-13 to substitute for the action of IL-4 (Balmer *et al.*, 2000). Taken together, the ability of the IL-4 deficient mice to control infection in the absence of early Th_2 responses indicated the importance of the Th_1 response in inducing IFN-γ and IgG2a antibody production thereby promoting parasite clearance. It is notable that in the same series of experiments, IFN-γ deficient mice rapidly died at the primary patent parasitaemia (Balmer *et al.*, 2000).

$CD4^+$ T-cell priming, expansion and memory in response to model antigens is also an important factor to consider since it has been suggested that continuous exposure to the parasite may be required for the maintenance of immunological protection from malaria as has also been suggested for *Leishmania major* (Uzonna *et al.*, 2001) and other (Hansen *et al.*, 2009) chronic infections (Stephens and Langhorne, 2010). Recent work with *P. chabaudi* demonstrated that the diminishing protection is replicated in mouse models and that this may be determinined by a decay in the memory T-cell function (Freitas do Rosário *et al.*, 2008).

Although the role of $CD4^+$T cells in immunity to blood-stage malaria is well established (Yoneto *et al.*, 2001) the complexity of the antigen repertoire of the *Plasmodium* parasite, makes it difficult to identify which antigens are effective in inducing protective $CD4^+$ T-cell–dependent immunity. Thus, the development

and characterization of antimalarial immune responses in a $CD4^+$ T-cell antigen receptor (TCR) transgenic mouse with a TCR specific for *Plasmodium chabaudi*-derived MSP-1, a major vaccine candidate antigen has been investigated (Stephens and Langhorne, 2010, Stephens *et al.*, 2005) and could provide another tool to ascertain the role of $CD4^+$ T-cells in antimalarial immunity.

2.4 ANTIBODY-MEDIATED IMMUNITY

There is longstanding evidence that naturally acquired immunity to the erythrocytic stages of malaria is strongly dependent on antibodies (Abs) (Osier *et al.*, 2008, Cavanagh *et al.*, 2004, Metzger *et al.*, 2003, Conway *et al.*, 2000). Malaria infection induces both polyclonal and specific immunoglobulin (Ig) production and although the different Ig isotypes may have protective functions, IgG is seen as the most important (Table 1.3). Passive antibody protection has been described in *P. falciparum* infection (Diggs *et al.*, 1995) as well as in animal studies. During *P. falciparum* infection, specific antibodies for a number of the parasite antigens such as MSP-1 and MSP-2 (O'Donnell *et al.*, 2001, Egan *et al.*, 1996, Al-Yaman *et al.*, 1994) and PfEMP1 (Marsh *et al.*, 1989, Celada *et al.*, 1982) have been correlated with resistance. Similarly, passive transfer experiments in *P. chabaudi* infection have also shown the protective role of antibodies during *P. chabaudi* infection where treatment with anti-serum at the time of infection in mice subsequently induced a delay in patent parasitaemia levels (Falanga *et al.*, 1984, McLean *et al.*, 1982). Furthermore, *P. chabaudi* AS infection involving the switch from a Th_1 to Th_2 response that induced the characteristic switch from IgG2a to IgG1 antibody production (Phillips *et al.*, 1997, Stavnezer, 1996, Taylor-Robinson and Phillips, 1994). Thus, during the acute infection, IgG2a antibodies were shown to predominate while during the chronic stage, IgG1 antibodies were associated with protection. Earlier mouse studies with *P. yoelii* (White *et al.*, 1991) and *P. berghe*i (Waki *et al.*, 1995) infections also showed that antibody-dependent protection and parasite clearance was related to IgG2a production. In addition, a more recent study by Couper and colleagues has demonstrated that IgM also has a significant role to play in controlling both primary and recrudescent *P. chabaudi* AS parasitaemias (Couper *et al.*, 2005). The effector mechanisms of IgE antibody (generated during a Th2 response) during *Plasmodium* infection in humans and mouse models are not as clear. However, in *P. falciparum* infection in humans, total

and specific parasite IgE levels were shown to be increased (Perlmann *et al.*, 1994). Furthermore, parasite-specific IgE antibodies were shown to be associated with pathogenesis of disease when total IgE and specific IgE Ab's were higher in Thai patients with severe *P. falciparum* infection. Specific IgE Ab's detected in apprpoximately 65% of the patients were also positively correlated to parasitaemia (Perlmann *et al.*, 2000). In contrast, others have demonstrated that high parasite-specific IgE antibody levels in asymptomatic individuals were associated with the reduced risk of clinical episodes (Bereczky *et al.*, 2004). Thus, the significance of IgE, which is present in increased levels in severe malaria, remains unknown.

Both experimental malaria models and human malaria, have shown evidence for loss of memory or activated $CD4^+$ T-cells, B-cells and plasma cells and short-lived malaria specific Abs after a primary acute infection (Langhorne *et al.*, 2008, Wykes *et al.*, 2005, Struik and Riley, 2004, Xu *et al.*, 2002). This suggested that some of the components contributing to the humoral response might be short-lived. Moreover, other studies have suggested that maintenance of malaria-specific Abs was dependent on the presence of chronic parasitaemia (Akpogheneta *et al.*, 2008). However, there are conflicting reports on the life-span of Ab reponses to *Plasmodium*. In some longitudinal studies short-lived Ab responses with reduced half lives have been reported (Akpogheneta *et al.*, 2008, Kinyanjui *et al.*, 2007, Kinyanjui *et al.*, 2003, Cavanagh *et al.*, 1998), whereas other studies report that Ab responses persist and are protective (Osier *et al*, 2008, Drakeley *et al.*, 2005, Taylor *et al.*, 1996). Nonetheless, it still has to be determined whether there are any deficiencies in the generation and maintenance of *Plasmodium*-specific memory B cells and Abs. Long term production of Abs was maintained by a combination of short-lived and long-lived Ab secreting cells (ASC). Although short-lived ASC die within 3–5 days, Ab levels can be maintained by continuous proliferation and differentiation of memory B cells into short-lived ASC upon continuous re-activation either by persistent antigen during chronic infection (Gatto *et al.*, 2007, Zinkernagel and Hengartner, 2006) or by polyclonal stimulation (Crompton *et al.*, 2009, Bernasconi *et al.*, 2003, Bernasconi *et al.*, 2002). Alternatively, long-term production of Ab is maintained by long-lived ASC, which migrate to survival niches within the bone marrow (Manz *et al.*, 1997, Slifka *et al.*, 1995) and spleen

(Slifka *et al.*, 1998) and can exist for the life-time of the mouse (Manz and Radbruch, 2002, Manz *et al.*, 1997), and this is probably also the case in humans (Cambridge *et al.*, 2003). In a more recent study by Ndungu and colleagues, they showed that *P. chabaudi*-MSP-1-specific B-cells and plasma cells were still detectable after eight months following infection and that both long-lived memory B cells and plasma cells secreting anti-$MSP1_{19}$ Abs can be generated after a single infection (Ndungu *et al.*, 2009).

Table 1.2: Associated antibodies and their functional properties during *Plasmodium* infection (Taken from Couper, 2003)

IgM	Complement activation against merozoites Opsonization Neutralization of merozoites Agglutination of merozoites and pRBC's Immune complex formation and clearance in the spleen
IgG2a	Complement activation against merozoites Opsonization Neutralization of merozoites Agglutination of merozoites and pRBC's Immune complex formation and clearance in the spleen Cytophillic and may induce ADCC by macrophages and PMN's
IgG1	Neutralization of merozoites Agglutination of merozoites Immune complex formation and clearance in the spleen
IgE	Binds CD23 and may induce pathology by upregulating type 1 cytokine production
IgD	Function largely unknown

2.5 PROTECTION VERSUS PATHOLOGY

Immunity to malaria infection clearly involves a complex network of cells and cytokines, as well as both pro-inflammatory and regulatory mechanisms. Innate immunity involving rapid pro-inflammatory responses is of great benefit in controlling primary infection but rapid and potent cytokine development can lead to severe malaria either directly or by amplifying the effects of the adaptive response (Riley, 1999). The ability of the immune response to control

circulating levels of pro-inflammatory cytokines so that they facilitate parasite clearance but do not trigger pathology is one of the hallmarks of acquired immunity to malaria. However, the mechanisms by which this may be achieved are still unknown. In mice (Omer and Riley, 1998, 2003, Li *et al.*, 2003) and humans (Schmieg *et al.*, 2003, Kurtzhals *et al.*, 1998) the key regulatory cytokines are IL-10 and TGF-ß. The primary function of IL-10 and TGF-ß is to prevent the pathological consequences of both Th_1 and Th_2 responses. Both cytokines can be produced by cells of the innate (macrophages) or adaptive immune systems (T-cells) and can regulate either system (O'Garra and Vieira, 2007). The source of IL-10 was thought to be via T-cell subsets (Th1, Th2, Tr1 cells, Treg cells) (Niikura *et al.*, 2011) but today, it is known that the sources of IL-10 is not only $CD4^+$ T-cells but also almost all leukocytes (Niikura *et al.*, 2011). IL-10 has been shown to inhibit IL-12 production by DCs and macrophages thereby downregulating IFN-γ production by NK cells and T-cells (Niikura *et al.*, 2011, D'Andrea *et al.*, 1993). The depletion of IL-10, using neutralizing IL-10-specific or IL-10 receptor (IL-10R)-specific monoclonal antibodies or $IL\text{-}10^{-/-}$ mice, has been shown to increase the resistance of mice to a number of intracellular pathogens namely, *Listeria monocytogenes, Toxoplasma gondii, Mycobacterium avium, Mycobacterium bovis* bacillus Calmette–Guérin (BCG), *P. chabaudi chabaudi, Candida albicans, Trypanasoma cruzi* (O'Garra and Vieira, 2007). However, in a model of *P. chabaudi chabaudi*, inhibition of IL-10 also resulted in immunopathology (Moore *et al.*, 2001). TGF-ß has also been shown to inhibit pro-inflammatory cytokine responses resulting in unconstrained parasite growth in *P. yoelii* (Omer *et al.*, 2003) and *P. chabaudi* (Tsutsui and Kamiyama, 1999) mouse infection studies by antibody depletion. Other studies have also shown that TGF-ß can inhibit human IFN-γ production from NK cells directly (Ortaldo *et al.*, 1991).

Recently there has been considerable interest as to the mechanisms by which parasites can modulate the immune response. For example, it has been shown that malaria parasites could directly activate endogenous latent TGF-ß to its bioactive form indicating that the parasite itself can manipulate the innate response of the host (Omer *et al.*, 2003). In addition, the malaria parasite has been implicated in modulating DC function as mentioned in chapter 2.3.1 (Millington *et al.*, 2006).

2.6 ROLE OF IL-4-RECEPTOR-ALPHA (IL-4Rα) IN IMMUNITY/PATHOLOGY

The role of the Th_2 response in immunity to malaria is particularly intriguing. Classically Th_2 responses and the associated cytokines IL-4 and IL-13 are thought to counter-regulate Th_1 responses (Brombacher, 2000). Consequently, during early *P. chabaudi* infection when it has been recognised that protective immunity is Th_1 dependent it might be expected that a Th_2 response would serve primarily to limit pathology associated with a pro-inflammatory type-1 response. Similarly, a Th_2 response early in infection may limit the effectiveness of a Th_1 response and promote parasite growth. Thus, in the absence of IL-4/IL-13 or IL-4/IL-13 signalling reduced parasitaemias and increased pathology might be expected. Conversely, during chronic infection with *P. chabaudi*, immunity is primarily dependent on a Th_2 response and IgG1 production. Consequently, in the absence of IL-4/IL-13 signalling it might be expected that enhanced parasite growth would result. However, recent evidence would suggest that the roles of Th_2 cytokines might not be as clear-cut as early publications would suggest. In certain disease and immune-modulatory models, Th_2 responses can be induced in the absence of IL-4 or IL-4/IL-13 signalling (Brewer *et al*, 1999). Furthermore, infectious and inflammatory disease models have provided a substantial body of evidence indicating that the typical Th_2 cytokines, IL-4/IL-13, can not only counter-regulate Th_1 responses but also in particular examples actually drive, facilitate or promote Th_1 responses (Alexander and McFarlane, 2008). In various disease models such as *Toxoplasma gondii* (Suzuki *et al.*, 1996), *Listeria monocytogenes* (Flesch *et al.*, 1997), *Cryptosporidium parvum* (Menacacci *et al.*, 1998), *Leishmania major* (McDonald *et al.*, 2004) and *Leishmania donovani* (Alexander *et al.*, 2000, Biedermann *et al.*, 2001, Stager *et al.*, 2003, Murray *et al.*, 2006), IL-4/IL-13 has been shown to drive protective Th_1 responses as well as in an experimental autoimmune uveoretinitis and murine model of colitis (Fort *et al.*, 2001, Ramanathan *et al.*, 1996), IL-4/IL-13 has been shown to aggravate the Th1 responses (Alexander and McFarlane, 2008). Taken together (Alexander and McFarlane, 2008), it has been suggested that IL-4/IL-13 mediate their functions by inducing IL-12 production from macrophages and/or dendritic cells (McDonald *et al.*, 2004, Hochrein *et al.*, 2000), enhance IFN-γ production (Noble and Kemeny, 1995) or synergise with IFN-γ for enhanced antimicrobial activity (Lean *et al.*, 2003, Bogdan *et al.*, 1993).

Previous work utilising IL-$4^{-/-}$ mice on the B6 and/or 129 backgrounds has indicated no significant role for IL-4 in malaria pathology (Balmer *et al.*, 2000, van der Heyde, 1997, von der Weid *et al.*, 1994) but potential roles for IL-4 in controlling parasite growth in either the early acute phase (Balmer *et al*, 2000) of infection or during chronic infection (von der Weid *et al.*,1994). The former observation with regard to parasite numbers during primary infection in IL-$4^{-/-}$ mice would be contra-intuitive if IL-4 was indeed inhibiting a Th_1 response, while the latter observation might be expected if control of chronic infections was indeed Th_2 dependent. The moderate alteration in susceptibility observed in IL-$4^{-/-}$ mice as opposed to a more significant effect was attributed to the related cytokine IL-13 compensating for a deficiency in IL-4 and promoting protective immunity.

It has been documented that IL-4 and IL-13 share 30% homology (Brombacher, 2000, McKenzie *et al.*, 1993) with functional overlaps. They have broadly similar effects on macrophages and other cell types because they share a common IL-4Rα chain (Figure 1.6) but there are also differences with regards to the range of cell types that respond to each cytokine which correspond with the presence of different receptor subunits. The signalling pathway involves members of the Janus-activated kinase (JAK) and signal transducer and activator of the transcription (STAT) families (Brombacher, 2000, Zurawski and de Vries., 1994, Minty *et al.*, 1993).

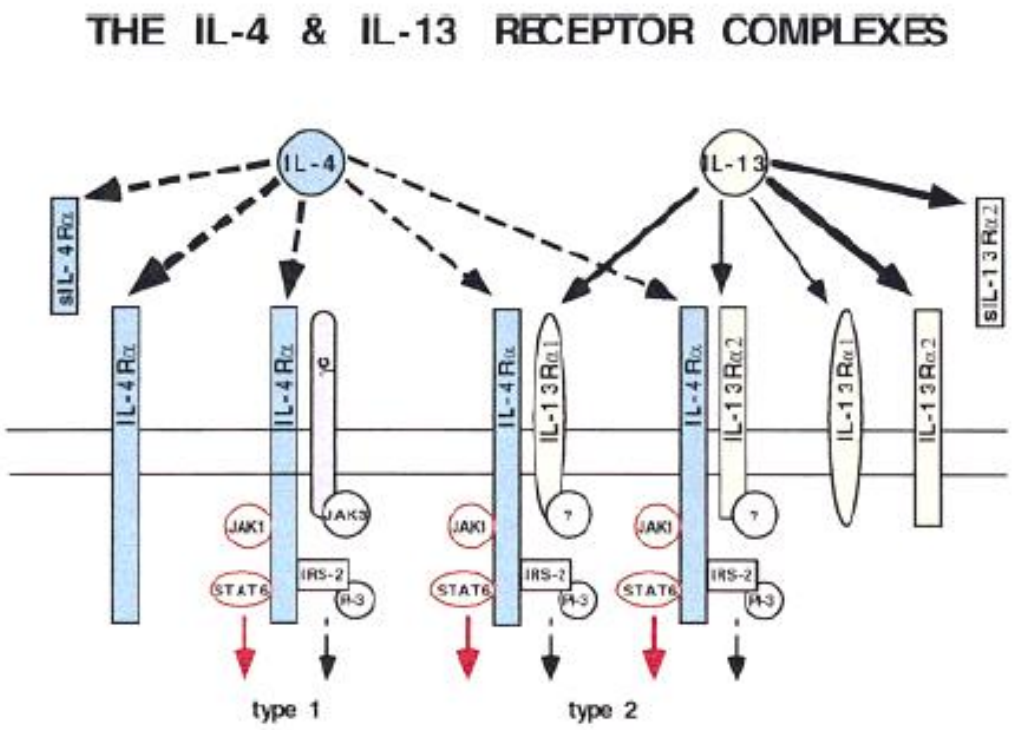


Figure 1.6: IL-4 and IL-13 receptor complexes. IL-4 interacts with the IL-4Ra binding protein in combination with either gc (IL-4 type 1 receptor), IL-13Ra1 or IL-13Ra2 (IL-4 type 2 receptor). IL-13 interacts with IL-13Ra1 or IL-13Ra2 binding proteins in combination with the IL-4Ra chain. The IL-4Ra chain is the signal transducing receptor for IL-4 and IL-13 and signal are transmitted via STAT6 or IRS-2 pathways. Increasing thickness of the arrows indicate increasing binding affinities (Kd) (Taken from Brombacher, 2000).

2.7 AIMS OF STUDY

In order to determine whether, in the absence of IL-4, IL-13 might drive a Th2 response and promote parasite clearance during *P. chabaudi* infections preliminary studies were carried out in our laboratory to compare the course of *P. chabaudi* infection in IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ BALB/c mice that are deficient in both IL-4 and IL-13 signalling (Couper *et al*, 2003). In agreement with previous studies IL-$4^{-/-}$ mice displayed delayed parasite clearance and enhanced recrudescent infection compared with wild-type mice but were able to clear infections to sub–patent levels. IL-13 contributed to parasite control as shown by elevated recrudescent infections in IL-4R$\alpha^{-/-}$ mice compared with IL-$4^{-/-}$ animals. However, in the absence of IL-4, but not IL-4 and IL-13 signalling, mortality increased significantly suggesting an additional exacerbatory role for IL-13 during *P. chabaudi* infection. Thus, our preliminary work indicated that both IL-4 and IL-13 played immunomodulatory roles during *P. chabaudi* infection.

However, IL-4 and IL-13 are pleiotropic cytokines and numerous cell types of both the innate and adaptive immune responses produce these cytokines as well as express their receptors. In order to differentiate better the specific role of IL-4/IL-13 responding cells from global effects *in vivo*, tissue specific IL-4R$\alpha^{-/-}$ mice have been produced in the laboratory of our co-principal investigator Professor Frank Brombacher in the University of Cape Town. So far macrophage/neutrophil specific (Lys*M*^{cre}IL-4R$\alpha^{-/lox}$) (Herbert *et al.*, 2004), CD4^{+} T cell specific (Lck^{cre}IL-4R$\alpha^{-/lox}$) (Radwanska *et al.*, 2007) and pan T-cell specific (iLck^{cre}IL-4R$\alpha^{-/lox}$) (Dewals *et al.*, 2009) IL-4R$\alpha^{-/-}$ mice have been generated.

Consequently, the aims of the present project were:

1. To re-establish an appropriate murine model of *P. chabaudi* infection in the laboratory that was compatible with current ethical guidelines and was reproducible.

2. To determine whether the results previously obtained in the laboratory utilizing IL-4R$\alpha^{-/-}$ mice were reproducible and whether results were mouse gender and/or parasite strain dependent.

3. To identify a potential role for alternative macrophage activation by comparing disease in macrophage/neutrophil specific (Lys*M*^{cre}IL-4R$\alpha^{-/lox}$) mice and wild-type equivalent mice.

4. To identify potential roles for IL-4 signaling via CD4^{+} T-cells in pathology/immunity by utilizing CD4^{+} T-cell (Lck^{cre}IL-4R$\alpha^{-/lox}$) IL-4R$\alpha^{-/-}$ mice.

5. To identify a potential role for IL-4 signalling via T-cells in pathology/immunity by utilizing pan T-cell (iLck^{cre}IL-4R$\alpha^{-/lox}$) IL-4R$\alpha^{-/-}$ mice.

6. To characterize the immune mechanisms underlying any disease phenotypes characterized.

Chapter Two

Methodology

2. GENERATION AND BREEDING STRATEGY OF TISSUE-SPECIFIC IL-4-Rα-DEFICIENT (IL-4Rα$^{-/-}$) BALB/c MICE

Tissue-specific IL-4Rα$^{-/-}$ mice were created using homologous recombination in embryonic stem (ES) cells in combination with the *Cre*/*loxP* recombinase system. Gene targeting in BALB/c ES cells and *Cre*/*loxP*-specific site-specific recombination was performed to generate floxed IL-4Rα$^{lox/lox}$ BALB/c mice which resulted in a silent mutation of the IL-4Rα gene (Mohrs *et al*., 1999). Mice that specifically express the Cre recombinase in macrophages/neurtophils under the control of the lysozyme M promoter (LysM^{cre} mice) were generated by a *knock-in* approach (Clausen *et al*., 1999). This approach was similar for mice expressing Cre recombinase in CD4^{+} T-cells under the control of the Lck promoter (Lck^{cre} mice) (Gu *et al*., 1994) and for mice expressing Cre recombinase in all T-cell populations under the control of the iLck promoter (iLck^{cre} mice) (Garvin *et al*., 1990). These mice (kindly provided by Prof. F. Brombacher, University of Cape Town) were first backcrossed to BALB/c for nine generations and then inter-crossed with global IL-4Rα$^{-/-}$ BALB/c mice (Mohrs *et al*., 1999) to establish double transgenic LysM^{cre}IL-4Rα$^{-/-}$ BALB/c, Lck^{cre}IL-4Rα$^{-/-}$ BALB/c and iLckM^{cre}IL-4Rα$^{-/-}$ BALB/c mice respectively in specific pathogen-free conditions in individual ventilated cages. These mice were further inter-crossed with IL-4Rα$^{lox/lox}$ BALB/c mice to generate cell-type-specific LysM^{Cre}IL-4Rα$^{-/lox}$ (McFarlane *et al*., 2011, Brombacher *et al*., 2009, Herbert *et al*., 2004), Lck^{Cre}IL-4Rα$^{-/lox}$ (Bryson *et al*., 2011, Radwanska *et al*., 2007) and iLck^{Cre}IL-4Rα$^{-/lox}$ (Dewals *et al*., 2009) BALB/c mice respectively. The Cre-negative IL-4Rα$^{lox/-}$ littermates served as "wild-type" controls for cell-type specific LysM^{cre}IL-4Rα$^{-/lox}$, Lck^{cre}IL-4Rα$^{-/-}$, and iLckM^{cre}IL-4Rα$^{-/-}$ BALB/c mice. An example of how the tissue-specific IL-4Rα gene-deficient mice were generated, namely for LysM^{cre}IL-4Rα$^{-/lox}$ mice, is shown in Figure 2.1

LysM^{cre}IL-4Rα$^{-/lox}$ Generated BALB/c mice

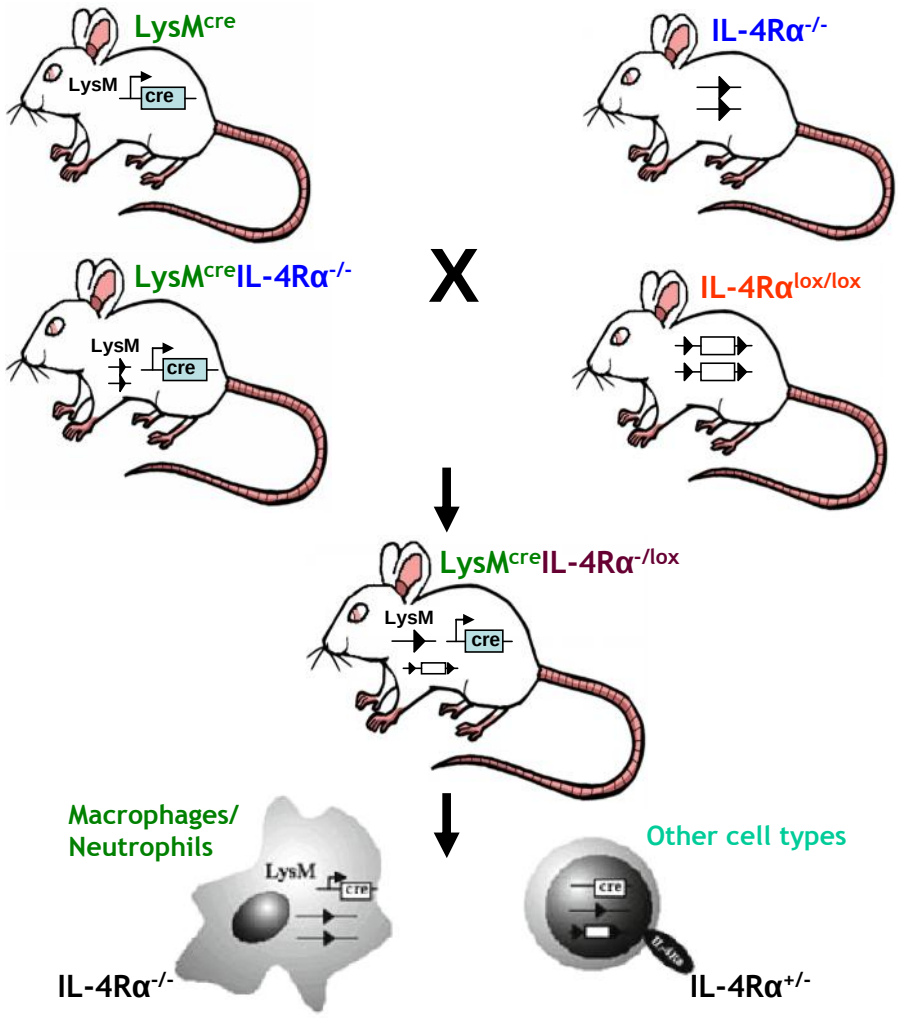


Figure 2.1: Principle of macrophage/neutrophil specific deletion of IL-4Rα. Conditional IL-4Rα$^{lox/-}$ mice were generated by homologous recombination. In these mice exons of the IL-4Rα are flanked by two loxP sites (*triangles*) that are recognized by the Cre recombinase. Mice that specifically express the Cre recombinase in macrophages/neutrophils under control of the lysozyme M promoter (LysM^{cre} mice) were first intercrossed with global IL-4Rα$^{-/-}$ mice. To facilitate efficient Cre-mediated recombination, LysM^{cre}IL-4Rα$^{-/-}$ mice were crossed with conditional IL-4Rα$^{lox/lox}$ mice. Because the Cre recombinase is only expressed in macrophages and neutrophils of hemizygote LysM^{cre}IL-4Rα$^{-/lox}$ offspring, loxP-flanked exons of the IL-4Rα locus on one allele was deleted only in these cell types. Hemizygote mice were used to reduce the substrate for the enzyme and to increase efficiency of Cre-mediated deletion in macrophages/neutrophils. In other cell types, the enzyme was not active and a functional IL-4Rα was still expressed. In experiments, Cre-negative IL-4Rα$^{lox/-}$ littermates served as controls for macrophage/neutrophil-specific LysM^{cre}IL-4Rα$^{-/lox}$ mice. Adapted from Brombacher and colleagues (Brombacher *et al.*, 2009).

2.1 Genotyping of the tissue-specfic IL-4Rα$^{-/lox}$ mice

One capillary (Hawksley and Sons Ltd, West Sussex, UK) of blood was removed from the tail of the mouse being genotyped and was placed into a RNA/DNA free eppendorf (Axygen Scientific, California, USA). To lyse erythrocytes, 1ml of Boyle's solution (1:9 v/v 0.17M Tris: 0.16M ammonium chloride) was added to the sample and was incubated at room temperature for 5 minutes. The blood was centrifuged at 13, 000 rpm for 3 minutes and the supernatant removed. The resulting pellet was resuspended in 100µl Tris-HCL and freeze-thawed at -80^{o}C. The sample was then ready for use in a PCR reaction.

2.1.1 Cre determination by PCR

Two PCR reactions were carried out on the samples: a Cre PCR to determine which mice were positive for Cre-recombinase and a second PCR reaction to determine if the mice were IL-4Rα-deficient or flox (presence of IL-4Rα gene on Macrophages/Neutrophils, CD4^{+} T cells and pan-T-cells flanked by lox-p sequences). All reagents were kept on ice at all times and all plasticware was RNA/DNA free. The primers and primer sequences used are summarised in Table 1.1. All PCR reactions used 13µl total volume: 6µl 2x Readymix (ABgene, Epsom, UK), 0.5µl of the relevant primers (obtained from Invitrogen, Paisley, UK) and an appropriate volume of molecular grade water (Sigma Aldrich, Poole, UK) to give 12µl. Lastly, 1µl of blood sample, prepared in 2.1, was added to give a final volume of 13µl.

A relevant positive control was included in the PCR studies and molecular grade water was used as a negative control for PCR reactions. The product sizes for the relevant genes are shown in Table 1.2 and the PCR conditions are shown in Table 1.3. Samples were maintained at 4^{o}C until an electrophoresis of samples could be completed. Electrophoresis of the PCR products for each tissue-specific group is shown in Figure 2.2.

Table 2.1: The primer sequences for each of the primers used in the genotyping of the LysM^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and Lck^{cre}IL-4Rα$^{-/flox}$ BALB/c mice. All primers were obtained from Invitrogen (Paisley, UK).

PRIMER	**PRIMER SEQUENCE**
LysM Cre F	5'- CTT GGG CTG CCA GAA TTT CTC –3'
LysM Cre R	5'– CCC AGA AAT GCC AGA TTA CG -3'
iLck Cre F	5'- GAG GGT GGA ATG AAA CTC TCG GT –3'
iLck Cre R	5'– CAG GTA TGC TCA GAA AAC GCC TGG -3'
Lck Cre F	3'– TGC ATG ATC TCC GGT ATT GAA AC
Lck Cre R	5'– GGT GAA CGT GCA AAA CAG GCT CTA
IL-4R P2	5'- CCT TTG AGA ACT GCG GGC T -3'
Flox IL-4R Intron 6.2 F	5'- CCC TTC CTG GCC CTG AAT TT -3'
Flox IL-4R Intron 6 R	5'- GTT TCC TCC TAC CGC TGA TT -3'
Glyceraldehyde-3-phosphate dehydrogenase (GADPH) F	AGA TTG TTG CCA TCA AAC GAC
GADPH R	ATG ACA AGC TTC CAT TTC TTC

Table 2.2: The band sizes (base pairs) for the LysM Cre, iLck Cre, Lck Cre, IL-4Rα deleted and flox PCR gene products.

GENE	**BAND SIZE (bp)**	**REFERENCE**
LysM Cre	450	Herbert *et al.*, 2004
LysM IL-4Rα deleted	1300	Herbert *et al.*, 2004
Lck Cre	450	Dewals *et al.*, 2009 Radwanska *et al.*, 2007
Lck IL-4Rα deleted	471	Dewals *et al.*, 2009 Radwanska *et al.*, 2007
Lox-p	188 (floxed) 94 (wild-type)	Dewals *et al.*, 2009 Radwanska *et al.*, 2007 Herbert *et al.*, 2004

Table 2.3: The thermal profile for the PCR reactions carried out to determine the genotype of the LysM^{cre}IL-4Rα$^{-/flox}$, iLck^{cre}IL-4Rα$^{-/flox}$ and Lck^{cre}IL-4Rα$^{-/flox}$ BALB/c mice.

TEMPERATURE	TIME	STEP
94 ^{o}C	1 minute	Enzyme activation
94 ^{o}C	30 seconds	Denaturation
60 ^{o}C	30 seconds	Annealing
72 ^{o}C	1 minute	Extension (40 cycles)
72 ^{o}C	5 minutes	Final extension

2.1.2 Electrophoresis

An electrophoresis gel was prepared using 1.6% w/v agarose (Bioline, London, UK) in TBE buffer (0.5 strength: 10.8g Tris base, 5.5g boric acid and 7.54g EDTA). The agarose was dissolved by microwaving the suspension for 1-2 minutes then cooled to prevent the release of a vapour when adding ethidium bromide (Sigma Aldrich, Poole, UK). 2µl ethidium bromide/100 ml of agarose/TBE was added and the solution was poured into a gel tank containing a sealed gel bed. Running buffer (0.5 strength TBE) was added to the gel tank and the samples were added to the gel along with a DNA ladder Forever Marker (Seegene, supplied from Insight Biotechnology, Wembley, UK). Samples were exposed to an electrophoresis voltage of 150 V. Once the samples had run sufficiently far along the gel, the gel bed was removed and the gel was viewed using an ultraviolet transilluminator (Viber Lourmat) and photographed for a permanent record.

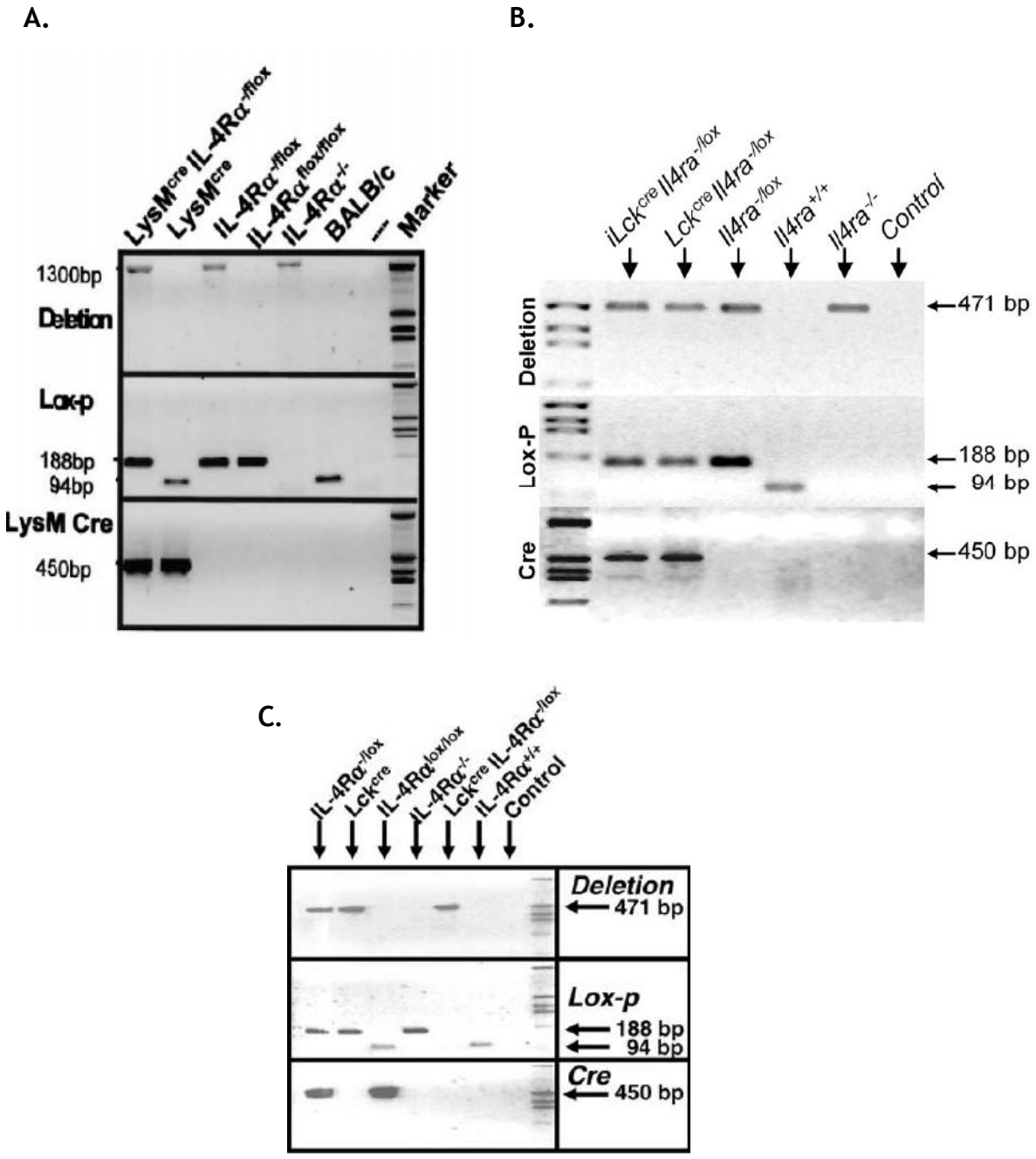


Figure 2.2: Genotyping. Genotyping of the transgene bearing **(A)** LysM^{cre}, **(B)** iLck^{cre} and **(C)** Lck^{cre} IL-4R$\alpha^{-/lox}$ deletion PCR fragment of **(A)** 1300bp and **(B+C)** 471bp, the loxP-specific PCR product of 188bp and the 94bp product is present in the wildtype configuration. The Cre-specific PCR generates a 450bp PCR product (Dewals *et al.*, 2009, Radwanska *et al.*, 2007, Herbert *et al.*, 2004).

2.2 MICE

2.2.1 Infection and Monitoring of the course of *P. chabaudi* AS infection

8-9 week old BALB/c wild-type mice, IL-4Rα$^{-/-}$ and tissue specific gene deficient BALB/c mice were housed in the Strathclyde University animal facility and were kept in a reverse light/dark cycle from 19h00 to 07h00 and fed with the standard diet. The capsule of cryopreserved infected blood was taken from liquid nitrogen (N_2) storage and thawed in a 37^{o}C waterbath. To the thawed blood, an equal volume of 0.5ml 17.5% sorbitol was mixed and then inoculated intraperitoneally (ip) into a passage BALB/c mouse. Parasites were then maintained if required by subpassage through susceptible mice. Briefly, infected mice were sacrificed in a carbon dioxide (CO_2) chamber and bled by cardiac puncture into a syringe containing sodium heparin at 10 i.u. heparin per ml of blood. The parasitemia of the donor mouse was determined by examination of a Giemsa (10% R66 improved Giemsa-staining Gurr (BDH Limited) v/v PBS pH 7.4 for 20 minutes) stained thin blood smear. The infected blood was subsequently diluted to give the required infective doses of the parasitized red blood cells (pRBCs). Here, infective doses were 1 × 10^5 pRBCs for *P. chabaudi* AJ and *Plasmodium* AS. Subsequently, the experimental mice were infected intraperitoneally with 1X10^5 *Plasmodium chabaudi* AJ or AS parasitized red blood cells (pRBCs) obtained from 4-5 day infected passage BALB/c mice. The stabilates were stored at -80^{o}C or in liquid N_2 until required. *P. chabaudi* AJ stabilate was obtained from Dr. Owain Millington from the University of Strathclyde and *P. chabaudi* AS stabilates were obtained from Dr. Paul Hunt from the University of Edinburgh. Thin blood smears from 3-6 mice per group, depending on the group size, were performed daily initially and then every second to third day during the course of infection (0-47 days) to monitor parasitaemia. Smears were evaluated via microscopy following fixation for 1 minute in 100% methanol and Giemsa's staining. Daily and then on selected days weight loss was determined as a sign of morbidity and the mean red blood cell count was followed during infection to monitor malaria induced anaemia. On selected days, tail bleeds were performed to follow cytokine and antibody profiles during the infection.

2.2.2 Measurement of *P. chabaudi* AS induced anaemia

3 µl of non-infected and *P. chabaudi* infected mice venous blood obtained by tail tip bleeding were mixed with 597µl formaldehyde/saline buffer (10ml 40% formalin adjusted to 1 litre with 32g/l trisodium citrate). Following vortex mixing, the red blood cell concentration was then determined by microscopy using a haemocytometer

2.3 Antigen preparation

P. chabaudi antigen and control red blood cell antigen were prepared for use in *in vitro* stimulation of splenocytes and ELISA. *P. chabaudi* antigen was prepared from mature trophozoite or schizont-infected red blood cells as only these life cycle stages induce expression of parasite specific antigens on the red blood cell surface.

2.3.1 *P. chabaudi* antigen preparation

Late stage trophozoites or schizont-infected red blood cells were obtained at high pRBC to RBC ratio from BALB/c mice. The parasites were washed in 5% RPMI at 200G for 10 minutes, and then washed twice in sterile PBS at 200G for 10 minutes. Following washing the pellet was resuspended to the original volume and freeze-thawed 5 times in liquid nitrogen. The pRBC antigen containing supernatant was then collected by centrifugation at 1500G for 10 minutes and the protein concentration was determined using the Bradford assay.

2.3.2 RBC (-ve) control antigen preparation

Red blood cell control antigen was prepared by bleeding uninfected BALB/c mice. Following collection, the blood was subjected to snap-freeze thawing 5 times in liquid nitrogen. The supernatant was then collected following centrifugation at 1500G for 10 minutes. The protein concentration was determined using the Bradford assay.

2.3.3 The Bradford protein concentration estimation assay

300 µl Bradford reagent (100mg Coomaise brilliant blue dissolved in 50ml methanol and 100ml 85% orthophosphate acid and adjusted to 1 litre with deionised water) were mixed 1:4 with de-ionised water and added to 10 µl of BSA standards ranging from 1mg/ml to 0.0625µg/ml or 10µl of sample in a 96

well microtitre plate. The absorbance was read at 570nm using a Molecular Devices Spectramax Pro spectrophotometer.

2.4 Splenocyte Stimulation Assay

Mice spleens were removed aseptically and placed in petri dishes containing 5ml complete media (RPMI 1640 (Lonza, Belgium), 10% v/v heat inactivated FCS (Sigma Aldich, Poole, UK), 1% v/v of 2mM L-glutamine solution and 1% v/v of 100 IU/ml Penicillin-100 µg/ml Streptomycin (PAA Laboratories, GmbH, Austria). Half a spleen per mouse was used. Cell suspensions were prepared by teasing the spleens apart using nitex (monofilament filter nylon cloth 100mm, Cadisch Precision Meshes, London, UK) and a 2ml syringe (Becton Dickinson, Madrid, Spain) and the resultant suspension was centrifuged at 1200rpm for 5 minutes. The supernatant was poured off and 2ml Boyle's solution (1:9 v/v 0.17M Tris: 0.16M ammonium chloride) was added to lyse erythrocytes. The reagents were mixed before use and sterile filtered through a 0.22µm syringe driven filter unit (Millipore, Cork, Ireland). The resultant cell suspension was centrifuged for 5 minutes at 1200rpm. The pellet was washed twice in 5ml complete media by centrifuging at 1200rpm for 5 minutes. The pellet was then resuspended in 1ml complete media and the number of viable cells was then estimated by trypan blue exclusion using a haemocytometer (Assistant, Germany). The cell suspension was adjusted to $5x10^6$ cells/ml (equivalent to $5x10^5$ cells/well) and 100µl was added to the wells of a 96-well sterile flat-bottomed tissue culture plate (Iwaki, Japan) to which *P. chabaudi* antigen (prepared as previously described, 10; 50; 100µg/ml) and Concanavalin A (Sigma-Aldrich, Poole, UK, 10µg/ml) had previously been added. The plates were incubated at 37^oC and 5% CO_2/95% air for 60 hours before being stored at -20^oC for cytokine quantification.

2.5 Enzyme Linked Immunosorbent Assay (ELISA)

Serum *P. chabaudi* specific IgG2a and IgG1 isotype titres were quantified by indirect ELISA. Spleen cytokine (IFN-γ, IL-12 p40/70, IL-10, IL-4) levels were measured using sandwich ELISA protocols.

2.5.1 Anti *P. chabaudi* AS and RBC specific antibody ELISA

Blood was harvested at sacrifice, serum prepared and stored for up to 24 hours at 4°C. Clotted blood was centrifuged at 13 000 rpm for 10 minutes and the resulting serum collected and stored at -20°C until assayed. 96-well microtitre plates (Greiner Bio-One, Germany) were coated overnight with *P. chabaudi* antigen or RBC antigen at 2µg/ml. The plates were then washed 3 times in phosphate buffered saline (PBS) pH 7.4, 0.05% Tween 20 (Sigma-Aldrich, Poole, UK) and blocked using 200µl/well 10% Foetal calf serum (FCS) (Harlan Sera-Lab Ltd) in PBS pH 7.4, at 37°C for 1 hour. Thereafter, serum samples were serially diluted. Dilutions were performed in PBS pH 7.4, 10% FCS buffer. Plates were then incubated at 37°C for 2-3 hours. Thereafter, plates were washed 4 times and 100µl of horseradish peroxidise conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology, supplied by Cambridge BioScience Ltd, Cambridge, UK) diluted 1/20 000 or 1/10 000 v/v respectively in PBS pH 7.4, 10% FCS buffer were added to the plate. The plate was then incubated at 37°C for 1 hour then washed 4 times and 100µl of substrate tetramethylbenzidine (6mg/ml in DMSO) diluted 1/100 in 0.1M sodium acetate pH 5.5 containing hydrogen peroxide (which was added just before the substrate was applied to the plate) were added. The plate was covered in foil until an appropriate colour change was observed. The reaction was stopped with 50µl of 10% sulphuric acid added to each well. The absorbance was read at 450nm on a SOFTmax Pro (Molecular Devices, California, USA). The end point titres of parasite specific IgG1 and IgG2a for each sample were regarded as the last dilution to give an absorbance above background levels.

2.5.2 Cytokine ELISA

A 96 well microtitre plate (Greiner Bio-One, Germany) was coated with 50µl/well of 1µg/ml w/v of the appropriate purified anti-mouse capture antibody (IFN-γ R4-6A2, IL-12 p40/70, IL-10 JES5-2AS and IL-4 11B11 (PharMingen, supplied by Insight Biotechnology, Wembley, UK) diluted in PBS pH 9 respectively and incubated overnight at 4°C. The plates were then washed 3 times and the wells were blocked using 10% FCS in PBS pH 7.4, at 37°C for 1 hour. After washing 3 times, samples were added neat. Standards were prepared for each cytokine. Murine recombinant IFN-γ, IL-12 p40/70, IL-10 and IL-4 standards (R&D Systems Europe Ltd, Abingdon, UK) were diluted in 10% v/v

FCS/PBS. Standards started at 20ng/ml for IFN-γ, 10ng/ml for IL-12 p40/70, 10ng/ml for IL-10 and 1ng/ml for IL-4. The samples were incubated at 37°C for 3 hours before being washed 4 times and 100µl of the appropriate biotinylated rat anti-mouse monoclonal antibody at 2µg/ml (IFN-γ, IL-12 p40/70, IL-10 and IL-4) (PharMingen, supplied by Insight Biotechnology, Wembley, UK) diluted in 10% v/v FCS/PBS were added to the plate. The plate was then incubated for 1 hour at 37°C before being washed 4 times. 100µl Streptavidin-alkaline phosphatase (PharMingen, supplied by Insight Biotechnology, Wembley, UK) diluted 1/2000 in 10% v/v FCS/PBS were used for the IFN-γ, IL-12 p40/70 and IL-10 assays whilst 100µl of a streptavidin horseradish peroxidise conjugate diluted 1/4000 in 10% v/v FCS/PBS (PharMingen, supplied by Insight Biotechnology, Wembley, UK) were used for the IL-4 ELISA. The plate was incubated for 45 minutes at 37°C before being washed 4 times. 100µl/well of substrate p-nitrophenylphosphate (pNPP) (1mg/ml, Sigma-Aldrich, Poole, UK) dissolved in 0.1M glycine buffer pH 10.4 (7.51g glycine, 0.203g MgCl and 0.136g ZnCl) were used for the IFN-γ, IL-12 p40/70 and IL-10 ELISA. The substrate for the IL-4 ELISA was tetramethylbenzidine (6mg/ml in DMSO) diluted 1/100 in 0.1M sodium acetate pH 5.5 containing hydrogen peroxide (which was added just before the substrate was applied to the plate). The plates were covered in foil and incubated at 37°C until a colour change developed. For IL-4, the reaction was stopped by adding 50µl of 10% sulphuric acid to each well. The absorbance was read at 405nm for IFN-γ, IL-12 p40/70 and IL-10 ELISA and at 450nm for the IL-4 ELISA using a SOFTmax Pro (Molecular Devices, California, USA). The cytokine concentration (ng/ml) was determined by linear regression using the standard value.

2.6 Statistics

Values are given as mean ±SEM and significant differences were determined by the unpaired 2-tailed Student *t*-test using the computer software GraphpadPrism 4 (GraphPad Software Inc, San Diego, California). Values $P<0.05$ were considered statistically significant.

Chapter Three

The course of *Plasmodium chabaudi* AJ erythrocyte infection in wild-type (WT) and global IL-4R-alpha-deficient (IL-4Rα$^{-/-}$) female mice.

3.1 Abstract

Preliminary studies from our laboratory using male BALB/c mice infected with *P. chabaudi* AS indicated that IL-4Rα$^{-/-}$ mice were more susceptible than their wild-type counterparts as determined by greater recrudescent parasitaemia and severity of chronic infection. The present study was undertaken in the first instance to determine whether this result was parasite strain dependent and secondly whether it was host gender specific. Consequently, wild-type and IL-4Rα$^{-/-}$ mice were infected with *P. chabaudi* AJ. As with the previous study using AS strain parasites, IL-4Rα$^{-/-}$ mice were also more susceptible to *P. chabaudi* AJ infection than wild-type mice as measured by severity of recrudescent infection. Interestingly, there was an early delay in the onset of peak parasitaemia in IL-4Rα$^{-/-}$ mice compared to the wild-type counterparts. There was evidence of an enhanced Th_1 response early in infected IL-4Rα$^{-/-}$ mice as measured by increased ConA stimulated splenic IFN-γ production and elevated serum IgG2a antibody levels followed by a diminished Th_2 response during the chronic infection as measured by reduced splenic IL-10 and IL-4 cytokine responses and serum IgG1 antibody titres. These results are extremely similar to those previously obtained using the *P. chabaudi* AS strain indicating that the observation (chronic infection) is not parasite strain dependent. In addition, the present study used female mice suggesting that the observation (enhanced recrudescence in IL-4Rα$^{-/-}$ mice) is independent of host gender.

3.2 Introduction

The most characterized rodent model of malaria is *P. chabaudi* AS which can result in lethal and non-lethal infections in various mouse strains. Comparative studies in resistant and susceptible mice have shown that pro-inflammatory responses seem to be necessary for the subsequent development of protective immunity (Su and Stevenson, 2002, Sam and Stevenson, 1999). The current understanding of protective immunity towards the blood-stage of *P. chabaudi* AS infection is that initial protection is mediated through Th_1 responses that control the acute patent parasitaemia followed by a switch toward a Th_2 response that controls and eventually eliminates the chronic-stage of disease (Namazi and Phillips, 2010, Phillips *et al.*, 1997, Langhorne *et al.*, 1989).

Preliminary studies from our laboratory have shown that in comparison to BALB/c wild-type (WT) mice, IL-4-deficient (IL-$4^{-/-}$) mice survive *P. chabaudi* AS infection with only a slight but consistent elevation in peak parasitaemia and significantly higher recrudescent parasitaemias. Given that the role of antibody as an effector mechanism was well established, it was surprising that the IL-$4^{-/-}$ mice survived the infection and produced a Th_2 response (Couper, 2003, Balmer *et al*, 2000, von der Weid *et al.*, 1994). An explanation for this finding was that IL-13 can exert a functionally similar effect to IL-4 on macrophages and in the absence of IL-4 takes over its activities. In addition, it is well established that Th_2 responses can be induced independently of IL-4 (Brewer *et al.*, 1999). Subsequent findings by our group also demonstrated a role for IL-4Rα signalling in chronic *P. chabaudi* AS infection in male BALB/c mice (Couper, 2003). That is, that mice deficient in IL-4/IL-13 (IL-4R$\alpha^{-/-}$) were more susceptible to the chronic-stage of *P. chabaudi* AS infection contrary to their WT counterparts. In the present study, we aimed to further investigate whether the previous findings were reproducible and whether observations were parasite-strain specific and/or host gender dependent.

The absence of a suitable *P. chabaudi* AS strain early in the course of the project allowed us to study the outcome of *P. chabaudi* AJ strain infection in IL-4Rα$^{-/-}$ and wild-type BALB/c mice. Female BALB/c WT and IL-4Rα$^{-/-}$ mice were infected with *P. chabaudi* AJ and the infection was monitored over a 47-day period and was subsequently terminated. Overall, results of the present study were in agreement with previous findings and demonstrate a role for IL-4Rα signalling during the chronic stage of *P. chabaudi* infection with AJ as well as AS strain parasites. Recrudescent infections were significantly greater in IL-4Rα$^{-/-}$ mice than their wild-type counterparts.

3.3 Results

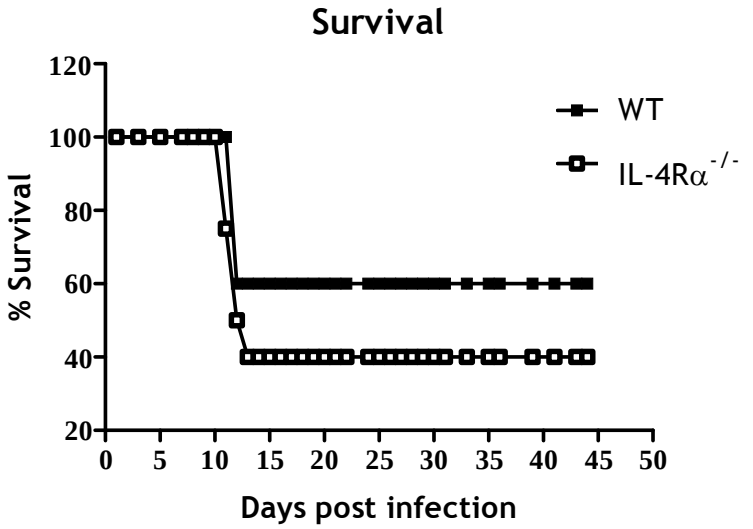
3.3.1 Comparison of the survival rates and disease phenotypes of wild-type (WT) and global IL-4R-alpha-deficient (IL-4Rα$^{-/-}$) female mice following *Plasmodium chabaudi* AJ infection.

The IL-4Rα$^{-/-}$ mice displayed an enhanced mortality rate of 60% by day 13 post-infection when compared to their WT counterpart, 40% mortality (Figure 3.1, A). Parasite burdens at day 5 were significantly lower in the IL-4Rα$^{-/-}$ mice compared to the WT control while peak parasitaemia was comparable between the groups (days 10-12). However, an elevated recrudescent episode was observed in the IL-4Rα$^{-/-}$ mice who were unable to effectively clear and control the infection compared to the WT mice on days 32 and 36 (Figure 3.1, B) thus highlighting an exacerbated infection in the absence of IL-4 and IL-13 function. Following day 36, IL-4Rα$^{-/-}$ mice suppressed the infection and was subsequently able to mediate the chronic infection until termination of the experiment on day 47 in a comparable manner to the WT controls. In addition, these findings were in accordance with a previous study conducted in our research group (Couper, 2003). In the latter study, male BALB/c WT and IL-4Rα$^{-/-}$ mice were infected with *P. chabaudi* AS parasites. Peak parasitaemia was comparable in both the WT and IL-4Rα$^{-/-}$ mice. Furthermore, the IL-4Rα$^{-/-}$ mice displayed elevated recrudescence on days 14-17 compared to the WT mice. In the IL-4Rα$^{-/-}$ mice, the infection was then controlled following day 21 in a comparable manner to the WT controls (Addendum One, Figure A).

WT and IL-4Rα$^{-/-}$ mice displayed similar weight loss during infection with no significant differences between the groups at peak and post infection (Figure 3.2, A). Maximal weight loss occurred between days 10-12 post infection and following parasite control a recovery in weight loss was observed in the WT and IL-4Rα$^{-/-}$ mice (Figure 3.2, A). Furthermore, Couper showed maximal weight loss in both WT and IL-4Rα$^{-/-}$ mice at day 9 and elevated weight loss in the IL-4Rα$^{-/-}$ mice on days 8-20 when compared to their WT counterparts (Couper, 2003) (Addendum One, Figure B).

Severe malarial induced anaemia was observed in both groups between days 10-12 post infection (Figure 3.2, B) corresponding to peak parasitaemia (Figure 3.1, B). Following parasite control, reduced RBC counts were only observed in the IL-4Rα$^{-/-}$ mice compared to the WT control at day 32 of the chronic infection (Figure 3.2, B). Results were similar to Couper who showed that peak anaemia was observed around day 10 in the WT and IL-4Rα$^{-/-}$ mice but the severity of anaemia was greater in the IL-4Rα$^{-/-}$ mice compared with the WT controls on day 14 post-infection (Couper, 2003) (Addendum One, Figure C).

A.



B.

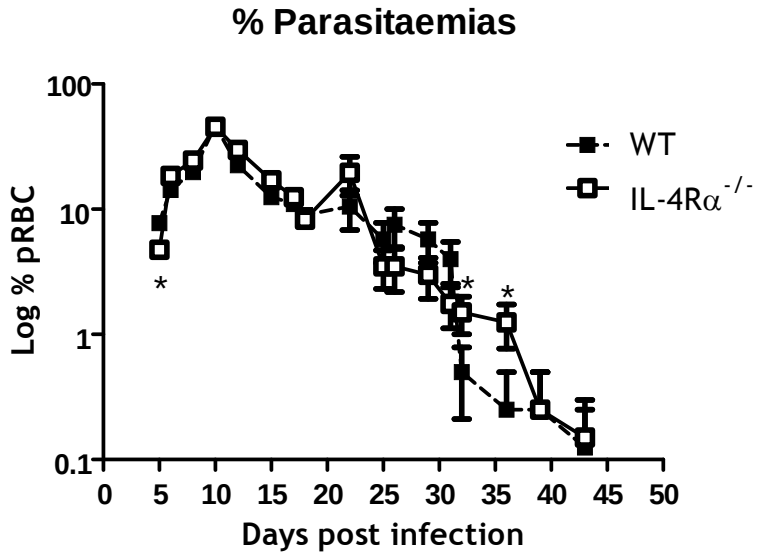
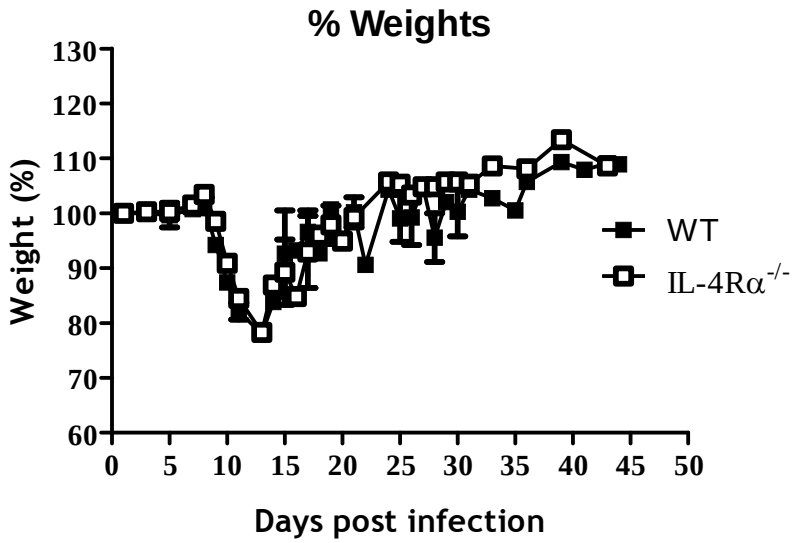


Figure 3.1: (A) Comparison of the survival rates of *P. chabaudi* AJ infection in WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. **(B)** Comparison of the disease parasitaemias of *P. chabaudi* AJ infection in WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Results are displayed as a log % of parasitaemia levels. Data are representative of two similar studies, n=3-4 and * denotes $p<0.05$.

A.



B.

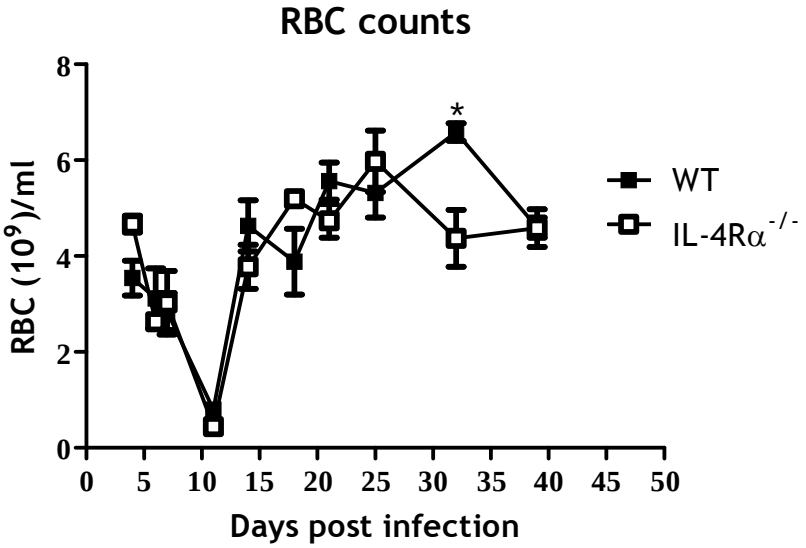


Figure 3.2 Comparison of **(A)** weight loss and **(B)** malaria induced anaemia during the course of *P. chabaudi* AJ infection in WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Data are representative of two similar studies, n=3-4 and * denotes $p<0.05$.

3.3.2 Comparison of the splenic cytokine production in wildtype (WT) and global IL-4Rα-deficient (IL-4Rα$^{-/-}$) female mice following *Plasmodium chabaudi* AJ infection.

IFN-γ production by IL-4Rα$^{-/-}$ derived splenocytes were significantly greater than WT derived splenocytes following ConA stimulation from day 12 infected mice while IFN-γ production was comparable following stimulation with antigen (Ag) (Figure 3.3, A). IL-12 production by IL-4Rα$^{-/-}$ derived splenocytes was significantly increased following *P. chabaudi* AJ Ag stimulation (Figure 3.3, B) compared to their WT counterparts (Figure 3.3, B). Furthermore, a significantly elevated IL-10 response was observed following ConA and *P. chabaudi* AJ antigen stimulation of IL-4Rα$^{-/-}$ derived splenocytes compared with WT derived splenocytes (Figure 3.3, C). IL-4 production was not significantly different between the groups in WT and IL-4Rα$^{-/-}$ mice (Figure 3.3, D).

At day 47, IFN-γ production by IL-4Rα$^{-/-}$ derived splenocytes were significantly lower than the WT derived splenocytes following any of the stimulation methods (Figure 3.4, A). IL-12 production by WT and IL-4Rα$^{-/-}$ derived splenocytes displayed no significant comparable differences upon any of the stimulation methods (Figure 3.4, B). IL-10 production from the IL-4Rα$^{-/-}$ derived stimulated splenocytes were below the sensitivity of the ELISA compared to the increased IL-10 production observed from WT derived splenocytes stimulated with ConA (Figure 3.4, C). In addition, IL-4 production was significantly reduced in IL-4Rα$^{-/-}$ derived splenocytes following ConA stimulation when compared to the WT derived splenocytes (Figure 3.4, D).

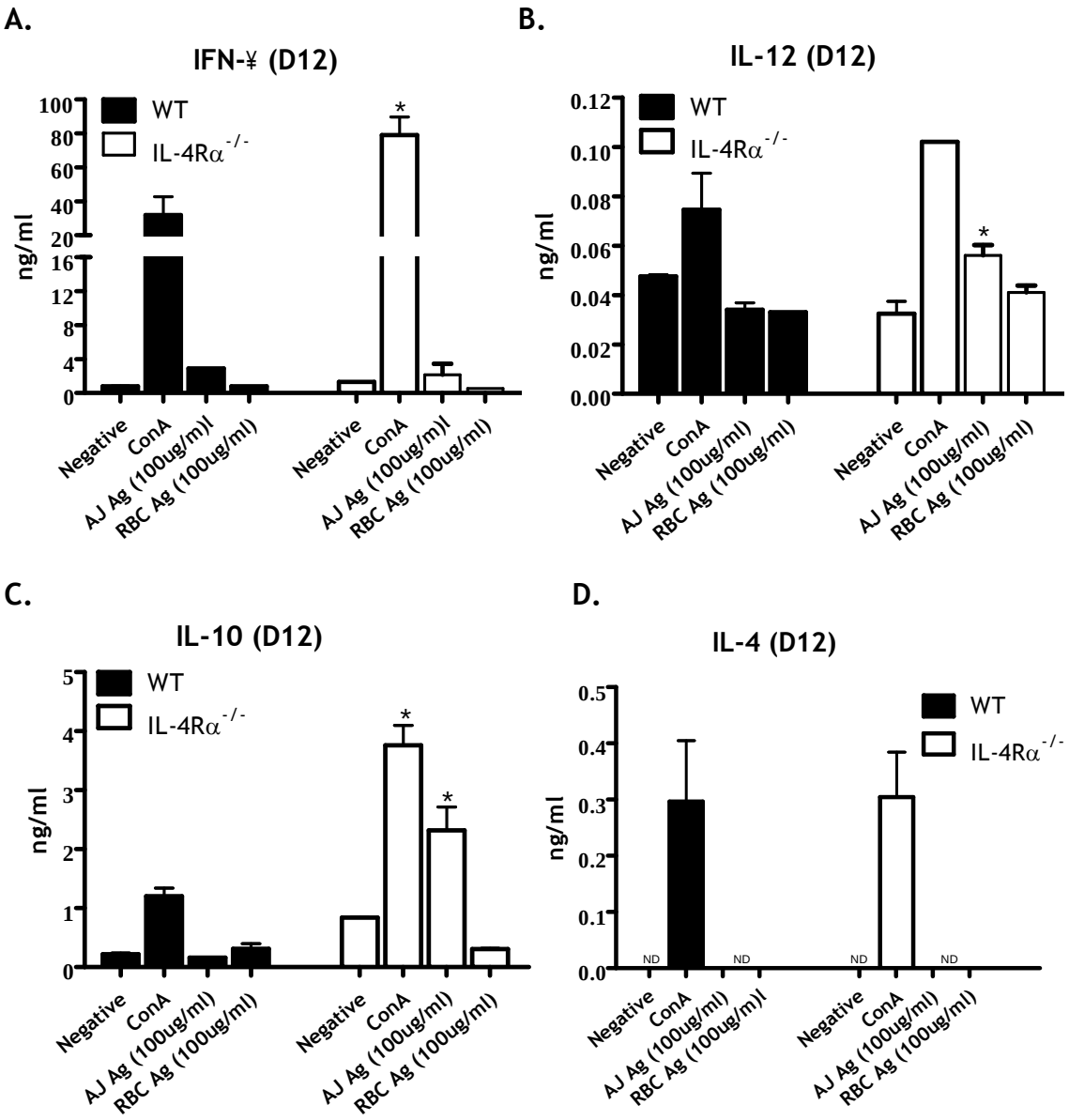


Figure 3.3 Comparison of day 12 splenic **(A)** IFN-γ, **(B)** IL-12,**(C)** IL-10 and **(D)** IL-4 production in *P. chabaudi* AJ infected WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Data are representative of two similar studies, n=3-4. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

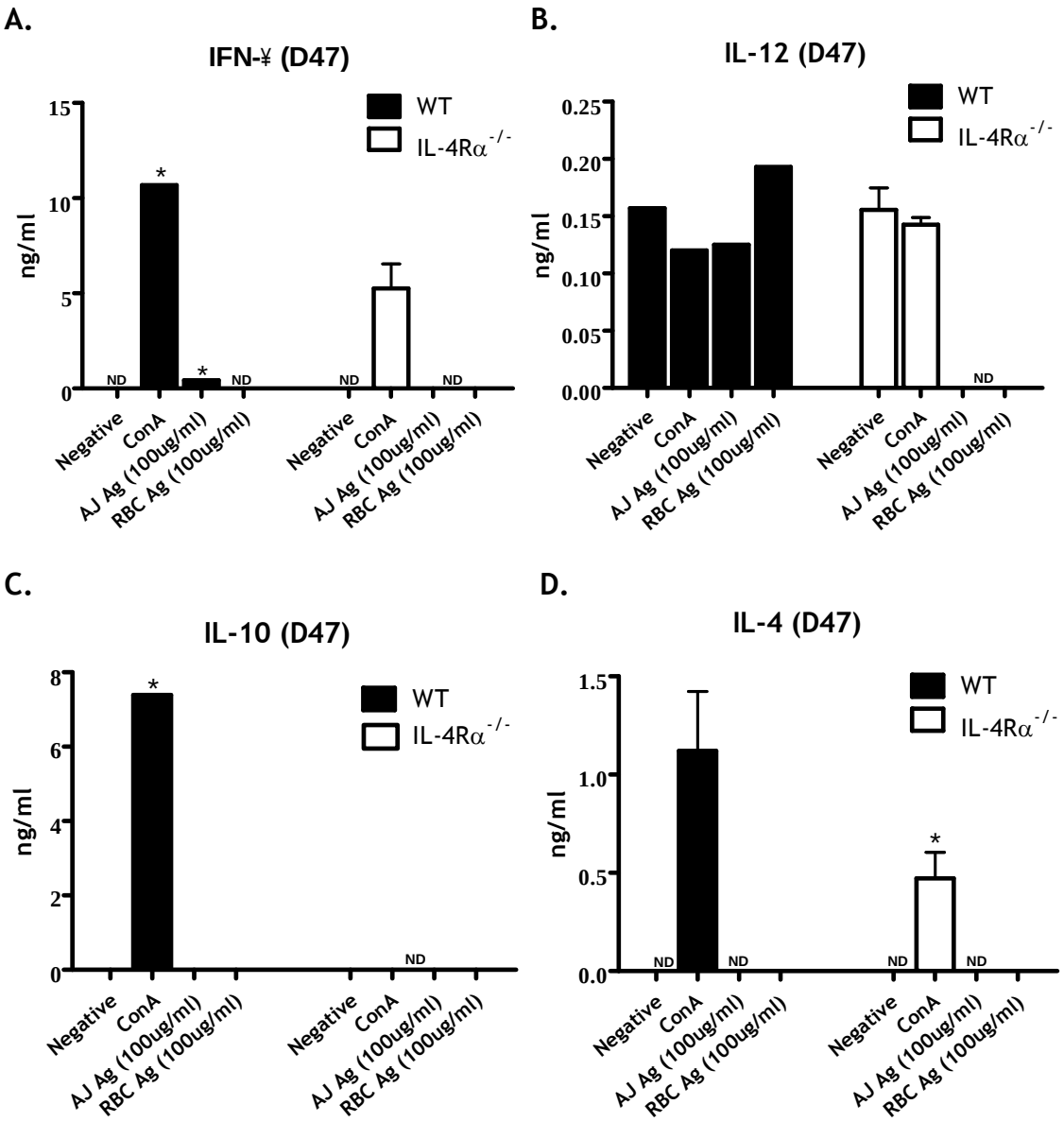


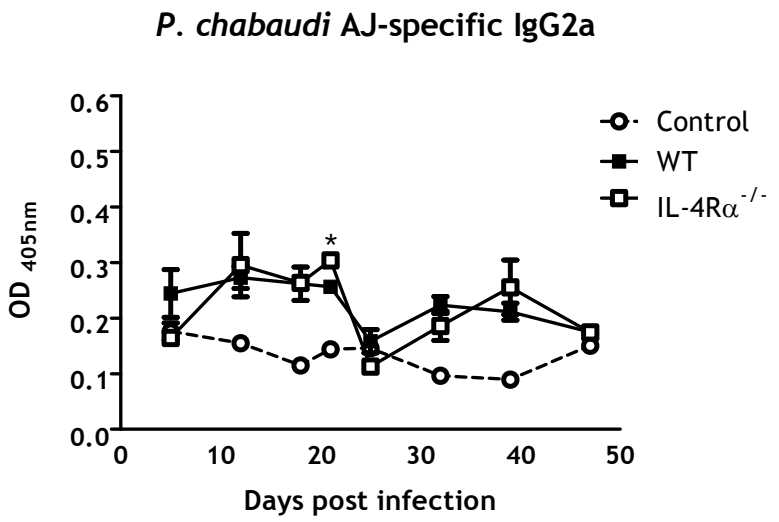
Figure 3.4 Comparison of day 47 splenic **(A)** IFN-γ, **(B)** IL-12, **(C)** IL-10 and **(D)** IL-4 production in *P. chabaudi* AJ infected WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Data are representative of two similar studies, n=3-4. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

3.3.3 Comparison of the IgG2a and IgG1 antibody responses in wildtype (WT) and global IL-4Rα-deficient (IL-4Rα$^{-/-}$) female mice following *Plasmodium chabaudi* AJ infection.

Significantly increased IgG2a antibody responses were observed in both infected groups between days 10-21 and again between days 30-45 when compared to the non-infected control mice (Figure 3.5, A). Comparable IgG2a antibody responses were observed between the infected WT and IL-4Rα$^{-/-}$ mice during the course of the infection except for day 21 when significantly elevated IgG2a Ab titres were observed in the IL-4Rα$^{-/-}$ mice compared to their WT counterparts (Figure 3.5, A).

IgG1 antibody responses were comparable in both the infected and the non-infected control groups during the course of the infection except for the infected WT mice which displayed elevated IgG1 Ab titres between days 28-45 (Figure 3.5, B). Furthermore, significantly decreased IgG1 Ab titres were observed in the infected IL-4Rα$^{-/-}$ mice on days 25, 32 and 39 post-infection when compared to their WT counterparts (Figure 3.5, B).

A.



B.

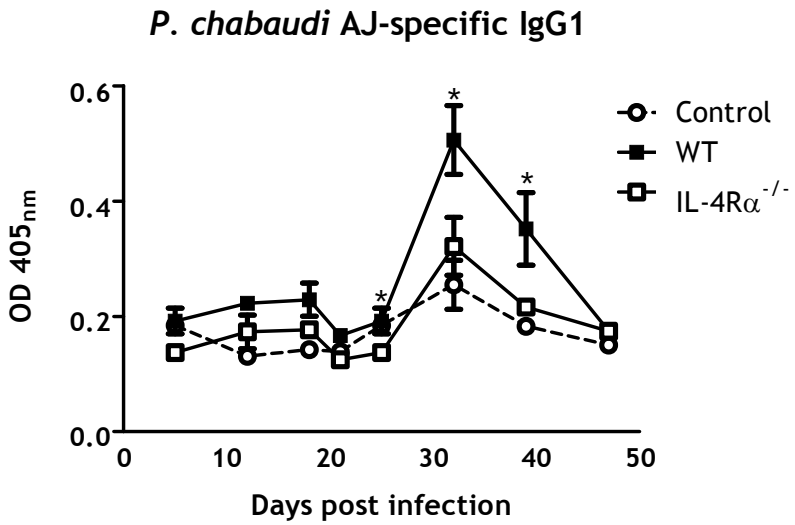


Figure 3.5 Comparison of the **(A)** IgG2a and **(B)** IgG1antibody responses of *P. chabaudi* AJ infected WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Data are representative of two similar studies, n=3-4. * denotes $p<0.05$.

3.4 Discussion

The data presented in this study demonstrate that IL-4Rα$^{-/-}$ female mice are more susceptible to *P. chabaudi* AJ during the chronic stage of infection than WT mice. These observations confirm previous results from our group which illustrated a role for IL-4 and IL-13 in the protective immune response to *P. chabaudi* AS infected male mice (Couper, 2003). Although a different parasite strain was used to the latter investigation due to strain availability at the time, similar observations were obtained. Consequently, our findings were also focussed on whether the two parasite strains reproduced similar disease profiles in the gene deficient mice.

It is established that Th_2 responses, driven by IL-4, are thought to control elimination of malarial parasites at the chronic stage of infection. However, studies have shown that male IL-4$^{-/-}$ (129SV X C57BL/6)F_2 mice were able to control *P.chabaudi* AS primary erythrocytic infection in a comparable manner to WT mice (Balmer *et al*., 2000, Von Der Weid *et al*., 1994) so IL-4 alone was not responsible for protection. It is known that IL-4 and IL-13 have similar fundamental roles in protection as they both signal via their shared receptor component, IL-4Rα.

In this study, WT and IL-4Rα$^{-/-}$ mice displayed comparable elevated peak parasitaemia but IL-4Rα$^{-/-}$ mice were unable to clear and control the infection as effectively as WT mice with recrudescence on days 32 and 36. This is comparable with previous findings in our group in which the lack of IL-4 and IL-13 does affect parasite clearance (Couper, 2003). It has also been shown in previous findings that IL-4Rα$^{-/-}$ mice displayed greater recrudescence than IL-4$^{-/-}$ mice, which indicated that in the absence of IL-4, IL-13 played a part in parasite control. IL-13 thus may replace the function of IL-4. Although IL-13 may be protective with regard to controlling parasite growth, greater mortality was found in IL-4$^{-/-}$ mice in comparison to IL-4Rα$^{-/-}$ mice infected with *P. chabaudi* AS during primary infection which suggested that IL-13 not only contributes to protection but also to the pathology of malaria disease. IL-13 may promote pathology by synergistic activity with IL-2 to promote IFN-γ production by NK cells or by directly affecting cellular function (Defrance *et al*., 1994, Punnonen

et al., 1993) thereby over stimulating the pro-inflammatory response. Despite the potential pathological role of IL-13 in contributing to mortality in the absence of IL-4 during *P. chabaudi* AS infection, it was also required to limit parasite burden since IL-4Rα$^{-/-}$ mice displayed elevated recrudescence compared with IL-4$^{-/-}$ mice (Couper,2003).

In the present study at day 5 of the early stage infection, IL-4Rα$^{-/-}$ mice showed a significantly reduced parastaemia count than WT mice. It may be of significance that IL-4Rα$^{-/-}$ mice have an enhanced Th_1 response compared to wild-type mice as measured by increased IFN-γ production from ConA stimulated splenocytes on day 12. Studies using IFN-γ or its receptor gene deficient mice provide conclusive evidence for the pivotal role for this cytokine in the control of acute parasitemia and survival of the host during primary infection (Sue and Stevenson, 2000, van der Heyde *et al.*, 1997, Favre *et al.*, 1997, Meding *et al.*, 1990). Studies in human malaria have also shown that IFN-γ production is associated with protection (Plebanski and Hill, 2000 Luty *et al.*, 1999). Furthermore, nitric oxide (NO) a hallmark of macrophage activation, causes direct parasite killing. In line with these findings, the present study showed that IFN-γ production is more pronounced in mice deficient in IL-4 and IL-13 during the very early stage of *P. chabaudi* AS infection. What we also found was that at day 47, IFN-γ production from stimulated splenocytes was significantly reduced in the IL-4Rα$^{-/-}$ mice compared to the WT control. Our findings were similar to that of Thawani and colleagues (Thawani *et al.*, 2009). Their data from malaria-infected STAT6-deficient mice had significantly lower IFN-γ levels than infected WT mice. Consequently, they suggest that there may be a link between IFN-γ levels and IL-4 dependent STAT6 signalling in malarial anaemia during acute blood-stage infection (Thawani *et al.*, 2009). Previous studies have also shown that IL-4 can promote Th_1 responses (Alexander and McFarlane, 2008, McDonald *et al.*, 2004, Lean *et al.*, 2003, Hochrein *et al.*, 2000, Noble and Kemeny, 1995, Bogdan *et al.*, 1993). It is clear that the cellular sources of IFN-γ induced by human and murine malarial infections have not been definitively identified but NK cells, $CD4^+$ T-cells, NKT cells, γδ-T cells and dendritic cells have all been proposed as potential sources (Couper *et al.*, 2007, D'Ombrain *et al.*, 2007, Soulard *et al.*, 2007, Newman *et al.*, 2006, Leisewitz *et al.*, 2004, Perry et al., 2004, Mohan *et al.*, 1997, Pichyangkul *et al.*, 1997). In addition, Charles and

colleagues have shown that an early host response to *P. chabaudi* infection is mediated by IFN-γ by an expansion of NK cells (Charles *et al.*, 2008). The relevance however of these responses to the generation of protective immunity is still to be addressed which provides another reason for utilizing the availability of cell-specific gene deficient mice to address these questions.

Interestingly, at day 12, antigen-specific splenocyte stimulation resulted in elevated IL-10 and IL-4 production in IL-4Rα$^{-/-}$ mice compared to WT mice. These findings indicate the production of non-specific IL-4 production in the IL-4Rα$^{-/-}$ mice. Studies have proved the existence of non-specific IL-4 because of the presence of soluble IL-4Rα and soluble IL-13Rα2 chains by alternate splicing or shredding and their ability to modulate IL-4 and IL-13 responses (Brombacher, 2000; Jung *et al.*, 1999; Zhang *et al.*, 1997).

The extent of the disease phenotype, characterized by reduced RBC counts, and reduced weight loss was evident in the WT and IL-4Rα$^{-/-}$ mice at peak infection with no differences observed. This is in line with previous studies conducted in our group (Couper, 2003). These phenotypic characteristics do not identify with the recrudescence and mortality observed in the IL-4Rα$^{-/-}$ mice during chronic *P. chabaudi* AS infection.

At peak infection, parasite burdens reach similar levels in both groups but recrudescence is only evident in the IL-4Rα$^{-/-}$ mice. Indeed, IL-4 inhibits IFN-γ production from T-cells and IL-12 production from macrophages thereby down-regulating a Th_1 response (Skeen *et al.*, 1996, Powrie *et al.*, 1993) and inducing the Th_2 switch (Phillips *et al*, 1994, 1997). Wild-type mice displayed the typical Th_1/Th_2 switch with elevated splenic IL-4, IL-10 and induction of IgG1 antibodies and promotion of Ab-mediated immunity against chronic *P. chabaudi* AS infection. It has been shown previously that IL-10 by itself and in co-operation with Th_1 cytokines such as IL-12 also regulates Th_2 responses (Wilson *et al.*, 2007, Hoffmann *et al.*, 2000, Joss *et al.*, 2000, Grunig *et al.*, 1997, Schandene *et al.*, 1994). Couper and colleagues reviewed the importance of IL-10 as a master regulator of immunity to infections. They discuss IL-10 as a key immunoregulator in various types of infections and ablation of IL-10 signalling results in the onset of severe, often fatal, immunopathology in different types of infections namely

Plasmodium spp, *Toxoplasma gondii*, *Trypanosoma* spp, *Mycobacterium* spp and HSV and can be seen in the table summarized in Addendum two (Couper *et al.*, 2008). The IL-4Rα$^{-/-}$ mice on the other-hand displayed reduced splenic IL-4 and IL-10 cytokine production and IgG1 antibody production at day 47 compared to WT mice. Since both IL-4 and IL-13 enhance B-cell function, IL-4Rα$^{-/-}$ mice showed an impaired B-cell antibody response with lower IgG1 antibody levels during the chronic stage of infection compared to the WT control. IL-10 production was only impaired in the IL-4Rα$^{-/-}$ mice and may therefore be controlled by IL-4 and IL-13 stimulation.

Ab mediated immunity, involving both B cell and CD4^{+} T cells is considered to play a major role in the resolution of the chronic stage of infection. The acute primary parasitaemia is accompanied by IgG2a antibody production which is preferentially induced by an IL-12 driven Th_1 response and IFN-γ production. Studies with *P. yoelii* (White *et al.*, 1991) and *P. berghei* (Waki *et al.*, 1995) models have also demonstrated that Ab-dependent protective immunity against these species is mediated by the Th_1-dependent IgG2a Ab. It is possible that IL-12 and associated Th_1 type cytokines, produced early during blood-stage *P. chabaudi* AS infection, modulate the Ab subclass response and consequently exert influence on Ab-mediated protective immunity for control of the chronic infections. Su and Stevenson further support the role for IL-12 and the type-1 response during both acute and chronic phases of blood-stage malaria (Su and Stevenson, 2002). They have demonstrated that IL-12 is required not only for activation of innate and cell mediated immune mechanisms to control acute primary infection, but also for the development of efficient Ab-dependent immunity to resolve the chronic phase of primary infection and to control challenge infection (Su and Stevenson, 2002). The present study shows that IgG2a is greater in IL-4Rα$^{-/-}$ mice at day 21 post infection compared with WT mice. In contrast to the WT mice, IL-4Rα$^{-/-}$ mice displayed reduced IgG1 Ab production at day 47. These findings may indicate that in the absence of IL-4 and IL-13 regulation, Ab-dependent protective immunity against *P. chabaudi* AS could still be mediated by Th_1-dependent IgG2a Ab.

In summary, IL-4Rα$^{-/-}$ mice have increased early resistance and diminished resistance during chronic infection compared with wild-type mice when infected with *P. chabaudi* AJ strain parasites. This is roughly concomitant with an enhanced Th_1 response as measured by IFN-γ early and diminished Th_2 response as measured by IgG1/IgG2a levels late in infection.

In particular, we have shown that the IL-4Rα$^{-/-}$ mice showed greater susceptibility to *P. chabaudi* AJ infection in comparison to their WT counterparts as measured by elevated recrudescence suggesting that IL-4 and IL-13 cytokines provide a protective function during the chronic stage of *P. chabaudi* AJ infection of female mice. These findings are in agreement with previous observations made in our group using *P. chabaudi* AS strain. Consequently, our results provide evidence that the immunological effects of infection are not parasite strain-specific as infection with both strains of the parasite produced a similar impaired Th_2 immune response during chronic protection in the IL-4Rα$^{-/-}$ mice. Thus, the effect of the absence of IL-4/IL-13 is the same with the different parasite strains. Secondly, our current findings in which female mice were utilized produced the same disease phenotype as was previously shown using male mice indicating that the observation obtained was not dependent on the host gender, the male and female mice demonstrated a similar disease profile.

Chapter Four

A comparison of the course of *Plasmodium chabaudi* AS erythrocyte infection in wild-type (WT) and global IL-4R-alpha-deficient (IL-4Rα$^{-/-}$) male and female mice.

4.1 Abstract

Findings in Chapter 3 using female BALB/c mice infected with *P. chabaudi* AJ indicated that the IL-4Rα$^{-/-}$ mice were more susceptible than WT controls during chronic infection and that these findings were similar to what has been previously obtained using the *P. chabaudi* AS strain. In this Chapter, with the availability of *P. chabaudi* AS strain, we firstly wanted to utilize the original *P. chabaudi* AS model system in male mice to further investigate the role of IL-4Rα signalling during murine malaria infection. However, we found that male mice, compared with female mice were extremely susceptible to infection with greater mortality observed. As a consequence, experiments using male IL-4Rα$^{-/-}$ and equivalent number of WT control mice were terminated at day 14. Nevertheless, lower survival rates were obtained in IL-4Rα$^{-/-}$ mice compared with their WT counterparts suggesting yet again the protective potential of IL-4/IL-13. As a result of the extreme susceptibility of male mice, we opted for the use of female mice infected with *P. chabaudi* AS as a more suitable model for subsequent investigations. Female IL-4Rα$^{-/-}$ mice in comparison to WT controls demonstrated an early delay in the onset of acute parasitaemia but were more susceptible to *P. chabaudi* AS infection as measured by the severity of the recrudescent infection. There was evidence of an enhanced Th_1 response early in the infected IL-4Rα$^{-/-}$ mice as measured by increased ConA stimulated splenic IFN-γ. This followed by a diminished Th_2 response in IL-4Rα$^{-/-}$ mice during the chronic-stage infection as measured by reduced splenic IL-10 and IL-4 cytokine responses following ConA stimulation and reduced serum IgG1 antibody titres at day 48. In conclusion, these results are similar to that obtained using the AJ strain of *P. chabaudi*.

4.2 Introduction

The optimal immune response to malaria infection comprises the rapid induction of inflammatory anti-parasitic responses that is followed by equally rapid resolution of inflammation that is mediated by anti-inflammatory cytokines to prevent immunopathology (Artavaris-Tsakonas *et al.*, 2003).

Protective immunity during the initial ascending and peak parasitaemias is controlled through innate NK and macrophage responses and $CD4^{+}$Th1 T cells, with IL-12, IFN-γ, TNF-α, ROI and NO candidates in parasite control (Langhorne *et al.*, 2004). IL-12 (Su and Stevenson, 2002) and IFN-γ-deficient (Balmer *et al.*, 2000) or depleted mice display increased peak parasitaemia and an impaired capacity to clear the infection. Th_2 protection is correlated to antibody production. Clearance of the acute primary parasitaemia occurs at the time corresponding to the switch from Th_1 to Th_2 biased immunity: B cells are essential for the switch to occur (Taylor-Robinson and Phillips, 1994). The exact mechanism inducing the biphasic T cell switch is, however, not as clear but may involve a gradual alteration in APC identity from DC's and macrophages to B-cells, and also IL-4/IL-13 may play a role. An IL-4 driven Th_2 response and associated cytokines such as IL-13 counter-regulate Th_1 responses and consequently Th_2 responses control the chronic infection. The decline of IL-12 production as infection develops along with IL-10 production has been suggested as contributing to the T-cell switch (Taylor-Robinson and Phillips, 1994). IgG2a associated with a Th_1 response has been demonstrated to be important in the control of infection as depletion of IgG2a and using IL-$12^{-/-}$ mice, which display reduced IgG2a responses, reduces the protective capacity of anti-sera (Cavinato *et al.*, 2001). A number of recrudescent episodes occur during *P. chabaudi* AS chronic infection, which is the result of antigenic switching of variant antigen types (VAT) and modulation of the immune response by the parasite (Phillips *et al.*, 1997).

It has been shown in previous studies that hormonal and immunological differences mediate sex differences in parasitic infections (Klein, 2004). Females typically have greater immune responses than males (Klein, 2000, Zuk and McKean, 1996) and in rodent malaria studies, mortality rates have shown to be greater in males than females and may involve immunological differences between the sexes (Klein, 2004). In *P. chabaudi* (Wunderlich et al., 1991) and *P. berghei* (Kamis and Ibrahim, 1989) infection studies, administration of testosterone was shown to increase mortality rates. Consequently, the immunomodulatory effects of testosterone may underlie the increased susceptibility to *Plasmodium* infections in males compared to females (Klein, 2004).

With the availability later in the project of *P. chabaudi* AS, the present study focused on using the original model as described by Couper (Couper, 2003) in male BALB/c mice to determine the immune response to infection in IL-4Rα$^{-/-}$ mice compared to WT controls. However, we found that male mice were highly susceptible to infection and suffered severe pathology and high levels of mortality. Consequently, they were not appropriate for further experimentation. Female mice were less susceptible and were used in all subsequent experiments. Moreover, the disease and immunological phenotypes produced in IL-4Rα$^{-/-}$ and wild-type BALB/c mice infected with *P. chabaudi* AS strain were similar to that using the AJ strain (previous chapter).

4.3 Results

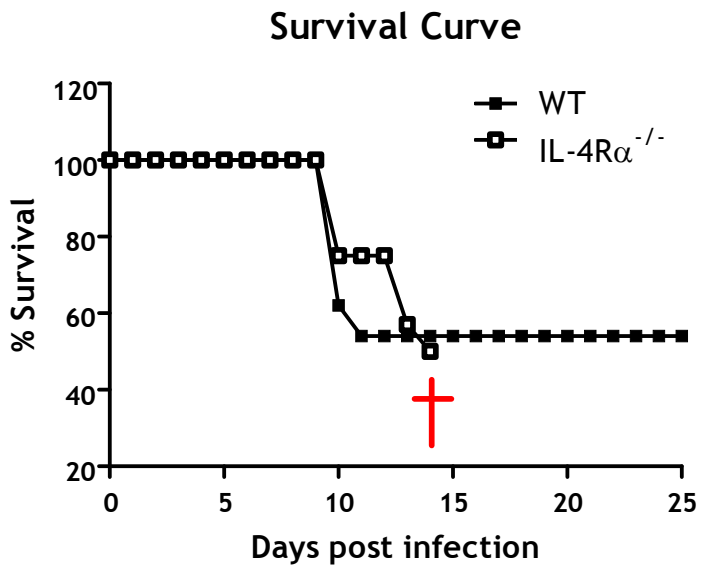
4.3.1 Disease phenotype and immunological responses in wild-type (WT) and global IL-4R-alpha-deficient (IL-4Rα$^{-/-}$) male mice following *Plasmodium chabaudi* AS infection.

Mortality in the WT and IL-4Rα$^{-/-}$ mice (Figure 4.1, A) occurred between days 10-14 and coincided with the peak infection (Figure 4.1, B), maximum weight loss (Figure 4.2, A) and *P. chabaudi* AS induced anaemia (Figure 4.2, B) observed. However, parasite burdens around peak infection and upon termination of the experiment at day 14 were not significantly different between the IL-4Rα$^{-/-}$ and WT mice (Figure 4.1, B). Maximal weight loss occurred between days 10-12 (Figure 4.2, A). WT and IL-4Rα$^{-/-}$ mice displayed no significant difference in weight loss at the peak infection (Figure 4.2, A). Severe *P. chabaudi* AS induced anaemia was observed in both groups between days 10-12 of the acute infection (Figure 4.2, B). However, no significant differences in RBC count was observed between the IL-4Rα$^{-/-}$ and WT mice at peak infection (Figure 4.2, B).

While 54% of WT mice survived infection all remaining IL-4Rα$^{-/-}$ mice had to be sacrificed at day 14 because of severe pathology (Figure 4.1, A).

Immunological responses demonstrated that an enhanced Th_1 response occurred at day 10 of the peak infection in IL-4Rα$^{-/-}$ mice as measured by increased ConA stimulated splenic IFN-γ production compared to their WT counterparts (Figure 4.3, A). Furthermore, day 10 splenic IL-10 (Figure 4.3, B) levels were comparable and IL-4 (Figure 4.3, C) levels showed no significant differences between the IL-4Rα$^{-/-}$ and WT control groups. Similarly, at day 10 IgG2a (Figure 4.4, A) and IgG1 (Figure 4.4, B) antibody titres were comparable between the groups.

A.



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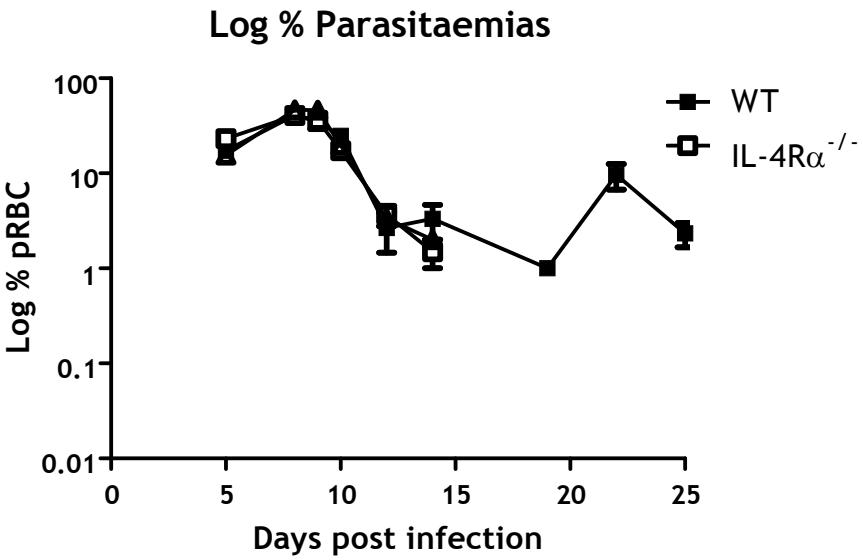
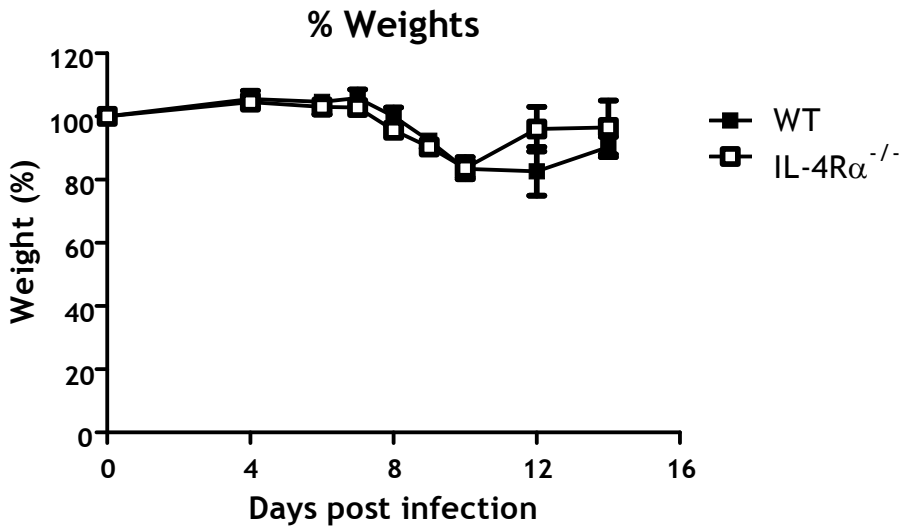


Figure 4.1: (A) Comparison of the survival rates of *P. chabaudi* AS infection in WT and IL-4Rα$^{-/-}$ male mice on a BALB/c background. **(B)** Comparison of the disease parasitaemias of *P. chabaudi* AS infection in WT and IL-4Rα$^{-/-}$ male mice on a BALB/c background. Results are displayed as a log % of parasitaemia levels. Data are representative of two independent studies, n=4-8. †, experiment was terminated because of severe pathology displayed by male IL-4Rα$^{-/-}$ mice.

A.



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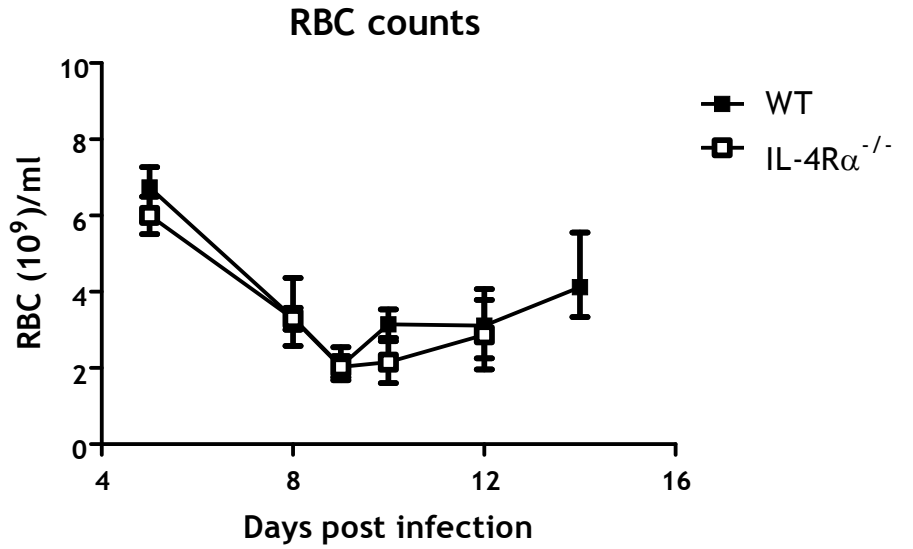


Figure 4.2: Comparison of **(A)** weight loss and **(B)** malaria induced anaemia during the course of *P. chabaudi* AS infection in WT and IL-4Rα$^{-/-}$ male mice on a BALB/c background. Data are representative of two independent studies, n=3-4.

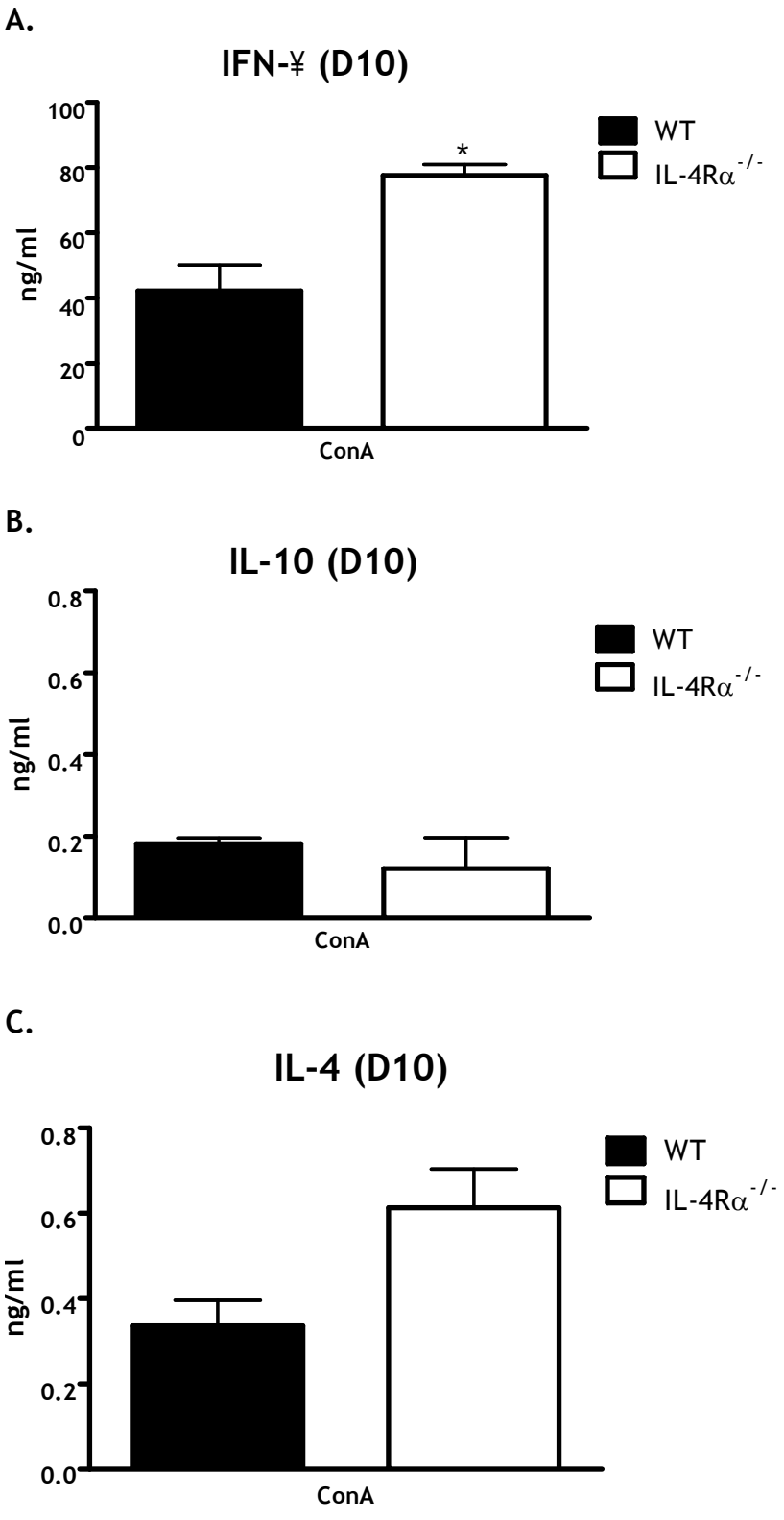


Figure 4.3: Comparison of day 10 splenic **(A)** IFN-γ, **(B)** IL-10 and **(C)** IL-4 production in *P. chabaudi* AS infected WT and IL-4Rα$^{-/-}$ male mice on a BALB/c background. Data are representative of two independent studies, n=3-4. * denotes $p<0.05$.

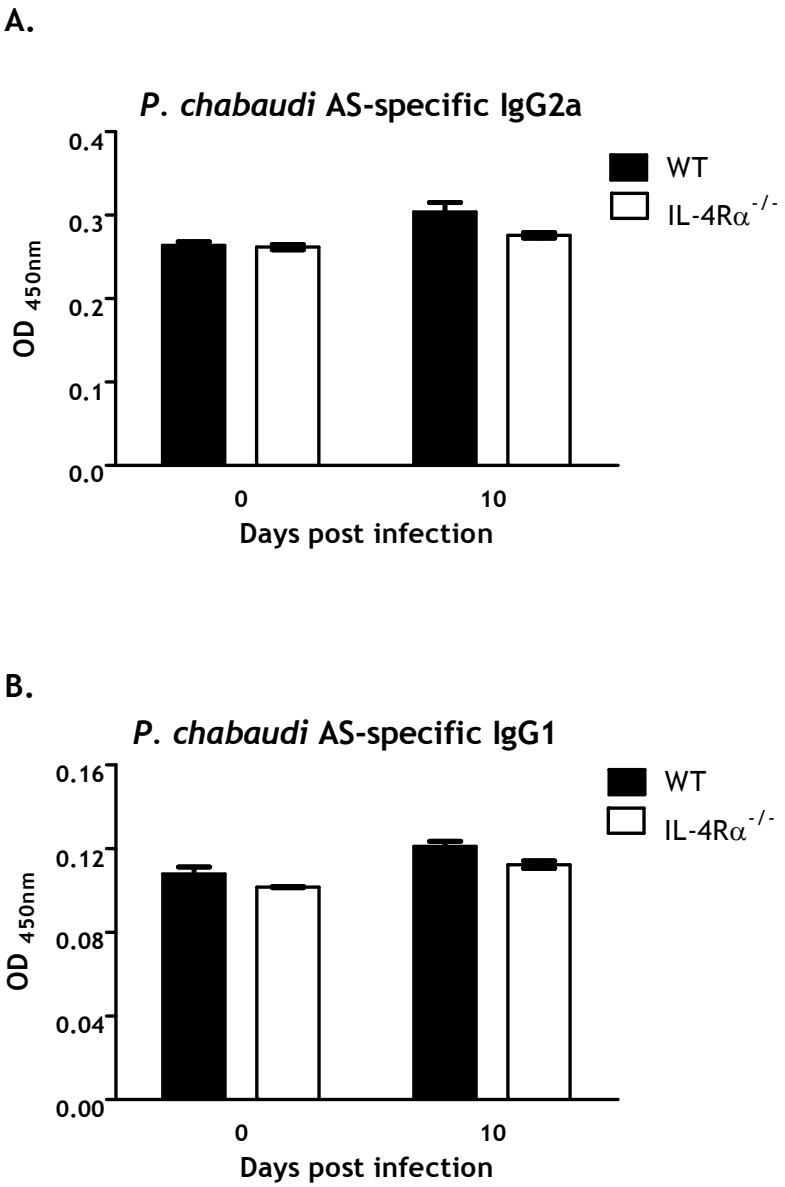


Figure 4.4: Comparison of the **(A)** IgG2a and **(B)** IgG1 antibody responses of *P. chabaudi* AS infected WT and IL-4Rα$^{-/-}$ male mice on a BALB/c background. Data are representative of two independent studies, n=3-4. * denotes $p<0.05$.

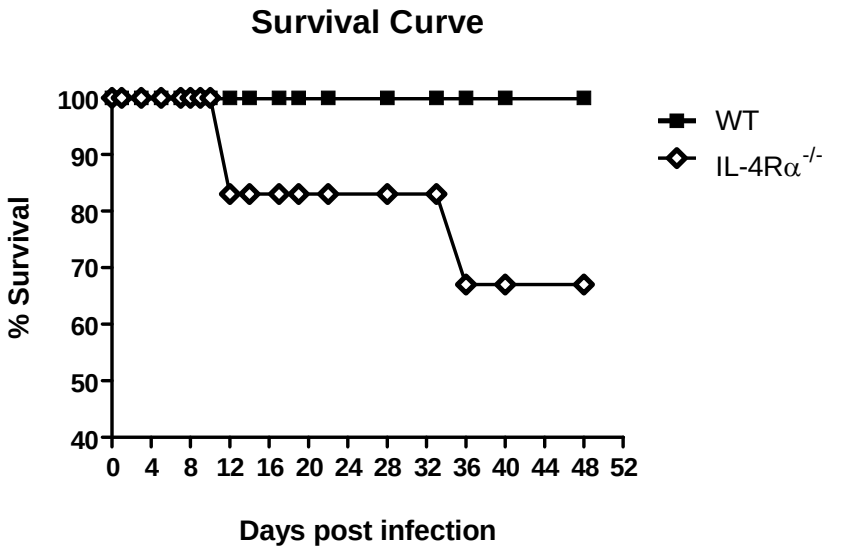
4.3.2 Comparison of the survival rates and disease phenotypes of wild-type (WT) and global IL-4R-alpha-deficient (IL-4Rα$^{-/-}$) female mice following *Plasmodium chabaudi* AS infection.

The IL-4Rα$^{-/-}$ mice displayed an enhanced mortality rate of 17% at day 12 and a further 33% at day 36 post-infection when compared to the WT mice where no deaths were recorded (Figure 4.5, A). Parasite burdens at day 3 were significantly greater in the WT mice compared to the IL-4Rα$^{-/-}$ mice while peak parasitaemia was comparable between the groups (days 7-12). However, an elevated recrudescent episode was observed in the IL-4Rα$^{-/-}$ mice that were unable to effectively clear and control the infection compared to the WT mice on days 17, 22 and 28 (Figure 4.5, B) thus highlighting an exacerbated infection in the absence of IL-4 and IL-13 signalling. Following day 28, IL-4Rα$^{-/-}$ mice suppressed the infection and were subsequently able to control the chronic infection until termination of the experiment on day 48 in a comparable manner to the WT counterparts.

WT and IL-4Rα$^{-/-}$ mice displayed similar weight loss during infection with no significant differences between the groups at peak and post infection (Figure 4.6, A). Maximal weight loss occurred on day 10 post infection and following parasite control a recovery in weight was observed in the WT and IL-4Rα$^{-/-}$ mice (Figure 4.6, A).

During the early stage of infection at day 5, RBC counts were significantly higher in the IL-4Rα$^{-/-}$ mice compared to the WT mice (Figure 4.6, B) corresponding to the lower parasitaemia levels observed in the IL-4Rα$^{-/-}$ mice around this time (Figure 3.1, B). Severe anaemia was observed in both groups at day 9-10 post infection (Figure 4.6, B) corresponding to peak parasitaemia (Figure 4.6, B). Following parasite control, no consistent difference in malarial induced anaemia was observed during chronic infection between the WT and IL-4Rα$^{-/-}$ mice (Figure 4.6, B).

A.



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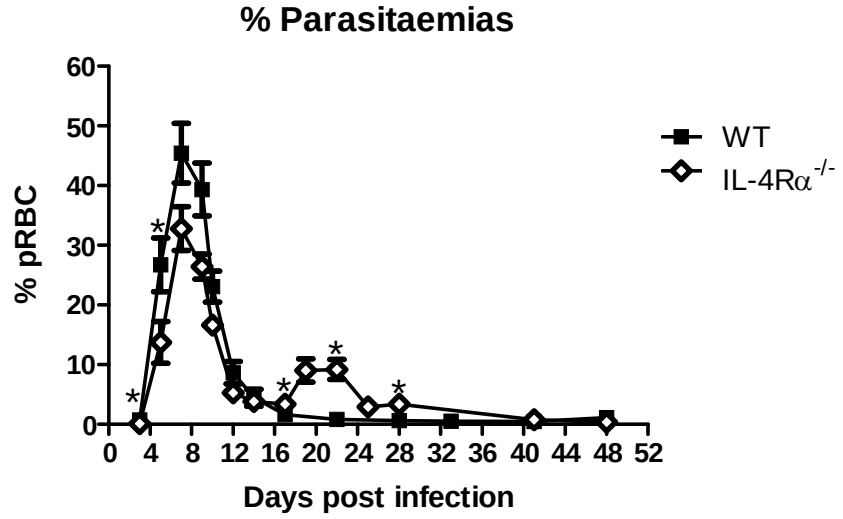


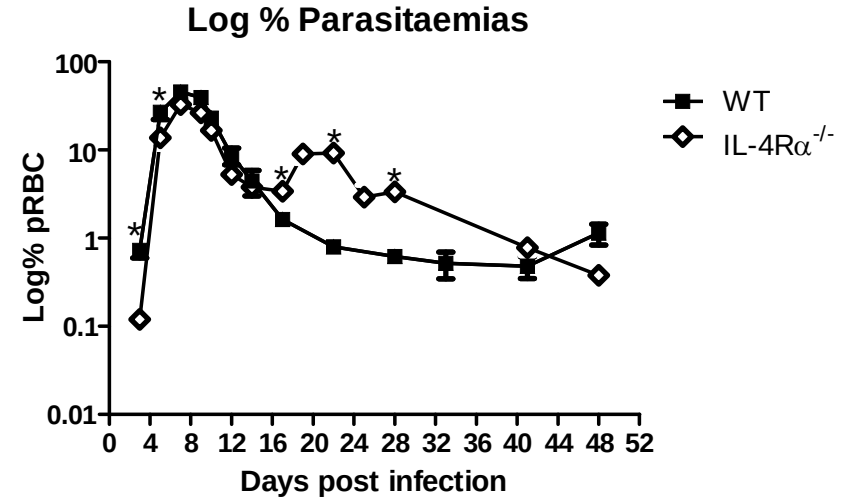
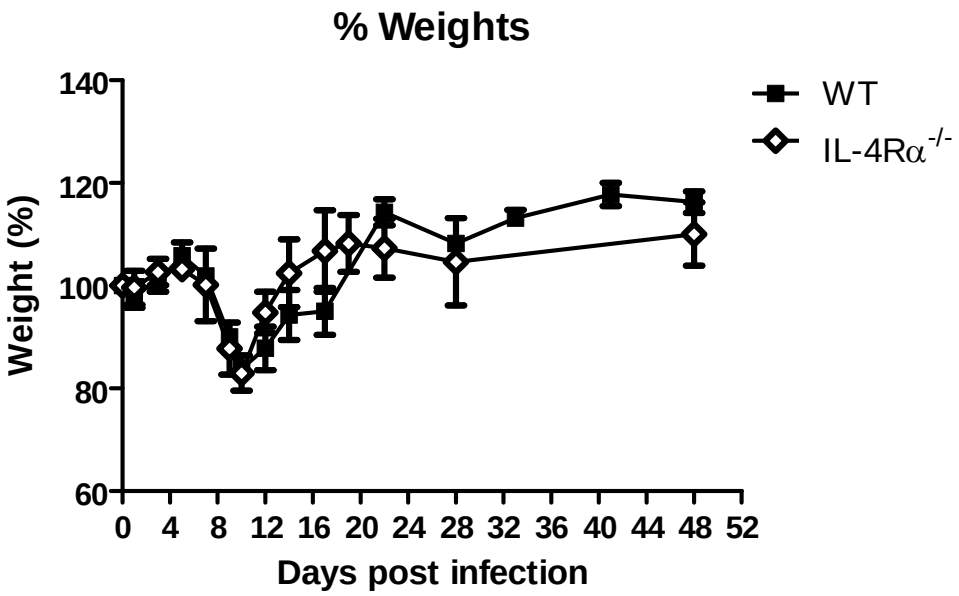


Figure 4.5: (A) Comparison of the survival rates of *P. chabaudi* AS infection in WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. **(B)** Comparison of the disease phenotype of *P. chabaudi* AS infection in WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Results are displayed as a % and as a log % of parasitaemia levels. Data are representative of two independent studies. WT n=14 and IL-4Rα$^{-/-}$ n=18. * denotes $p<0.05$.

A.



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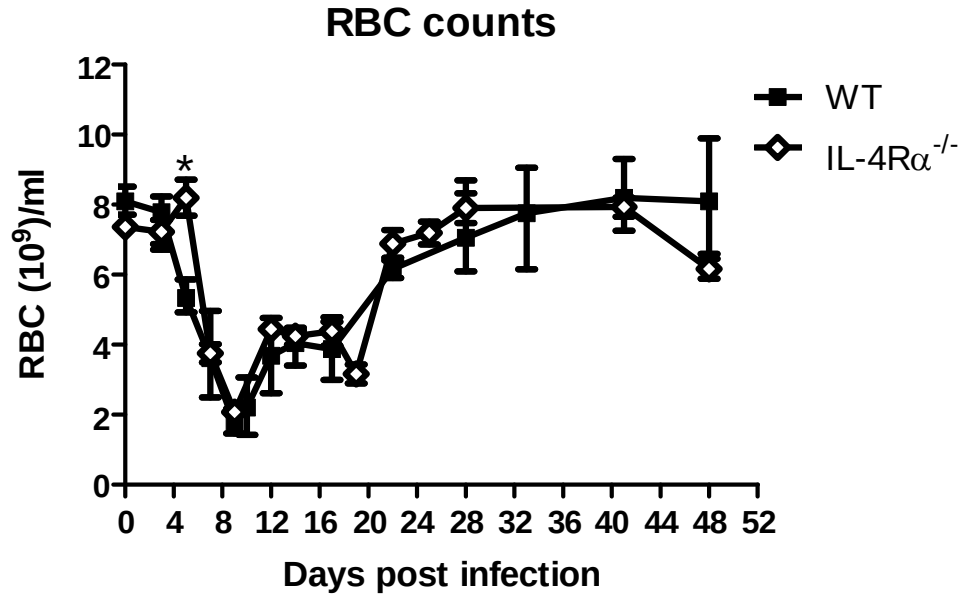
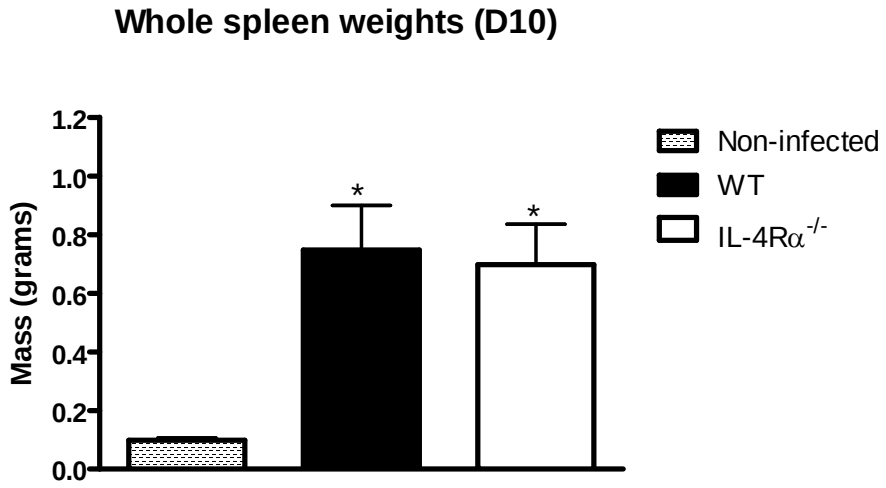


Figure 4.6: Comparison of **(A)** weight loss and **(B)** malaria induced anaemia during the course of *P. chabaudi* AS infection in WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Data are representative of two independent studies, n=14. * denotes $p<0.05$.

4.3.3 The influence of chronic disease on splenomegaly in wild-type (WT) and global IL-4Rα-deficient (IL-4Rα$^{-/-}$) female mice infected with *Plasmodium chabaudi* AS.

Whole spleen weights of non-infected and infected WT and IL-4Rα$^{-/-}$ mice were measured to determine the severity of splenomegaly during the course of *P. chabaudi* AS infection between the respective groups. It is clear that *P. chabaudi* AS parasites directly cause splenomegaly in the infected groups compared to no spleen enlargement observed in non-infected mice throughout the duration of the disease (Figure 4.7). Furthermore, a slight decrease but not significant difference in spleen weight was observed in the IL-4Rα$^{-/-}$ mice when compared to the WT mice on day 10 (Figure 4.7, A) and day 17 (Figure 4.7, B). Similarly, no significant difference in the severity of splenomegaly was observed between the WT and IL-4Rα$^{-/-}$ mice at day 48 post-infection (Figure 4.7, C).

A.



B.

C.

Figure 4.7: Comparison of the whole spleen tissue weights of non-infected and *P. chabaudi* AS infected WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. Data are representative of two independent studies, n=5-6. * denotes $p<0.05$.

4.3.4 Comparison of the splenic cytokine production in wild-type (WT) and global IL-4Rα-deficient (IL-4Rα$^{-/-}$) female mice following *Plasmodium chabaudi* AS infection.

IFN-γ production by WT derived splenocytes was significantly lower than IL-4Rα$^{-/-}$ derived splenocytes following ConA stimulation from day 10 infected mice while IFN-γ production was comparable following stimulation with antigen (Figure 4.8, A). IL-12 production by WT and IL-4Rα$^{-/-}$ derived splenocytes was comparable following all *in vitro* stimulation methods (Figure 4.8, B). In contrast, a significantly elevated IL-10 response was observed following *P. chabaudi* AS antigen (200μg) stimulation by IL-4Rα$^{-/-}$ derived splenocytes compared with WT derived splenocytes (Figure 4.8, C). Peak IL-4 production was induced by ConA stimulation in both groups (Figure 4.8, D).

At day 17, IFN-γ production was significantly reduced by IL-4Rα$^{-/-}$ derived splenocytes following ConA stimulation compared to WT derived splenocytes (Figure 4.9, A). IFN-γ production was not witnessed for any of the other stimulation methods. Furthermore, IL-12 production was below the sensitivity of the ELISA in the IL-4Rα$^{-/-}$ derived splenocytes compared to the WT derived splenocytes (Figure 4.9, B). A significantly reduced IL-10 response by IL-4Rα$^{-/-}$ derived splenocytes compared with WT derived splenocytes was observed following ConA stimulation. No IL-10 production was witnessed following any of the other stimulation methods (Figure 4.9, C). No significant differences in IL-4 production were obtained in WT and IL-4Rα$^{-/-}$ derived splenocytes following ConA stimulation. No IL-4 production was witnessed following any of the other stimulation methods in the IL-4Rα$^{-/-}$ derived splenocytes (Figure 4.9, D).

At day 48, no significant differences in IFN-γ production was observed between the WT and IL-4Rα$^{-/-}$ derived splenocytes following stimulation with any of the stimulation methods (Figure 4.10, A). IL-12 production by WT and IL-4Rα$^{-/-}$ derived splenocytes was comparable following *in vitro* stimulation (Figure 4.10, B). IL-10 production was significantly reduced in IL-4Rα$^{-/-}$ derived splenocytes following ConA stimulation when compared to WT derived splenocytes while IL-10 production was comparable following the other stimulation methods (Figure 4.10, C). IL-4 production was significantly reduced in IL-4Rα$^{-/-}$ derived splenocytes following ConA stimulation but showed significantly increased IL-4 production following *P. chabaudi* AS antigen stimulation when compared to WT derived splenocytes (Figure 4.10, D).

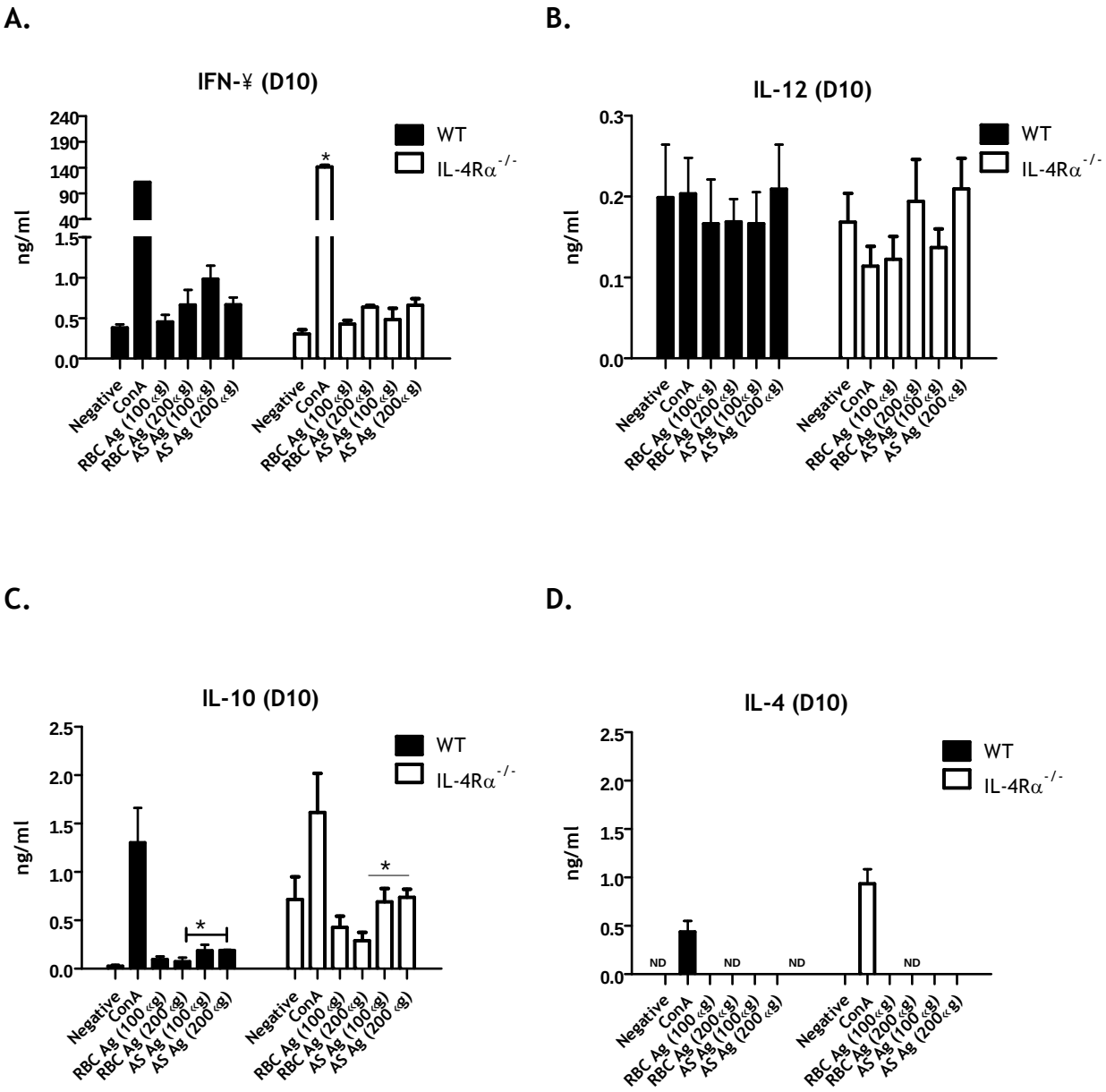


Figure 4.8: Comparison of day 10 splenic **(A)** IFN-γ, **(B)** IL-12,**(C)** IL-10 and **(D)** IL-4 production in *P. chabaudi* AS infected WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Data are representative of two independent studies, n=3-5. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

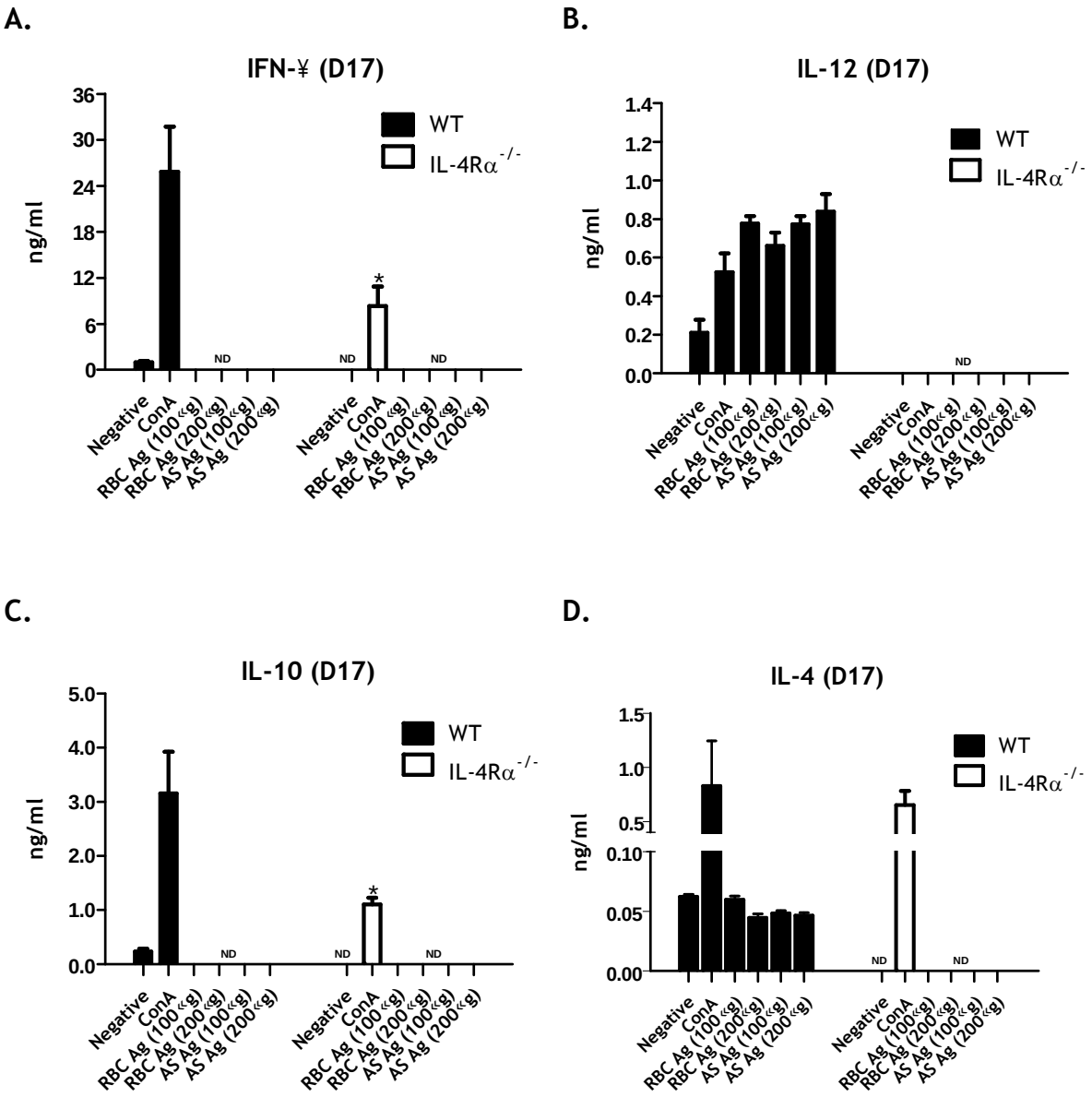


Figure 4.9: Comparison of day 17 splenic **(A)** IFN-γ, **(B)** IL-12, **(C)** IL-10 and **(D)** IL-4 production in *P. chabaudi* AS infected WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Data are representative of two independent studies, n=3-5. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

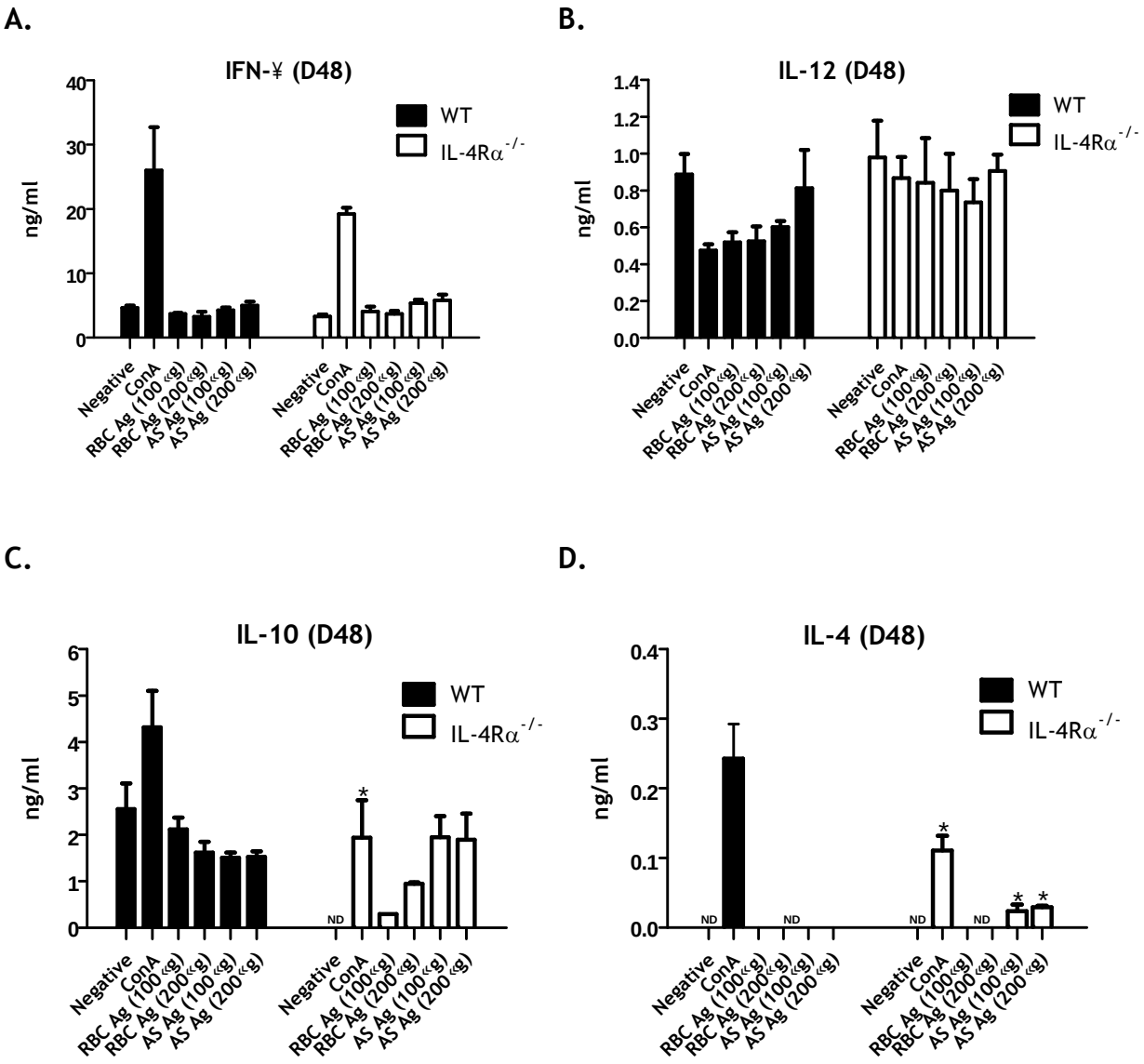


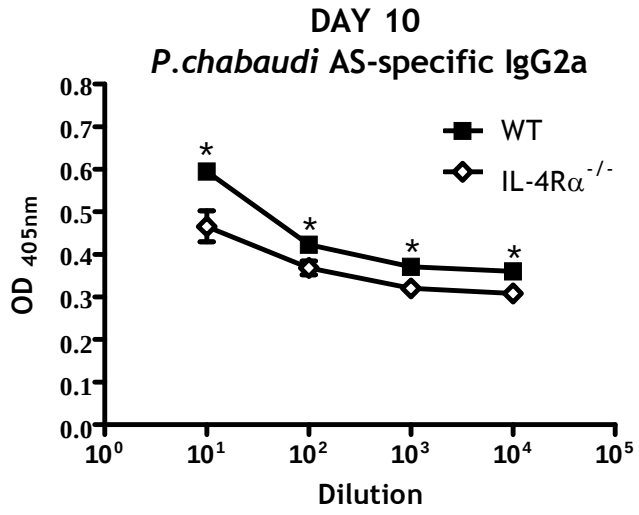
Figure 4.10: Comparison of day 48 splenic **(A)** IFN-γ, **(B)** IL-12, **(C)** IL-10 and **(D)** IL-4 production in *P. chabaudi* AS infected WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Data are representative of two independent studies, n=3-6. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

4.3.5 Comparison of the IgG2a and IgG1 antibody responses in wild-type (WT) and global IL-4Rα-deficient (IL-4Rα$^{-/-}$) mice following *Plasmodium chabaudi* AS infection.

Significantly increased IgG2a antibody responses were observed from day 10 WT infected mice compared to the IL-4Rα$^{-/-}$ mice (Figure 4.11, A). At day 17 post infection, comparable IgG2a antibody responses were observed between both groups (Figure 4.11, B). However, IgG2a antibody responses at day 48 were then significantly increased in the IL-4Rα$^{-/-}$ mice compared to their WT counterparts (Figure 4.11, C).

IgG1 antibody responses were significantly increased in the WT infected mice compared to the IL-4Rα$^{-/-}$ mice on days 10 and 17 (Figure 4.12, A, B). IgG1 antibody responses at day 48 were significantly reduced in the IL-4Rα$^{-/-}$ mice compared to their WT counterparts (Figure 4.12, C).

A.



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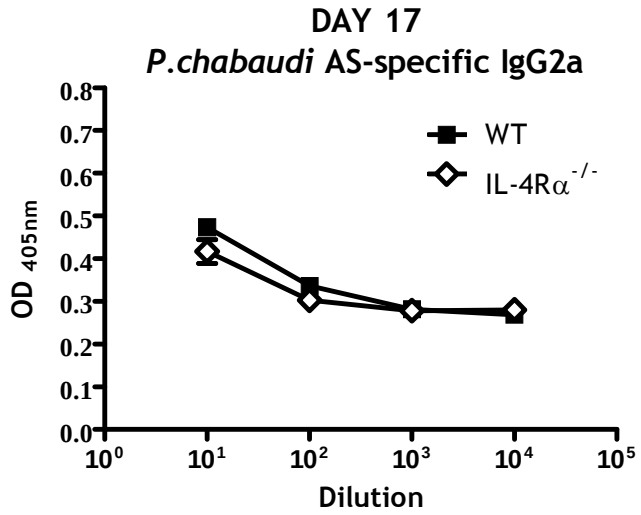

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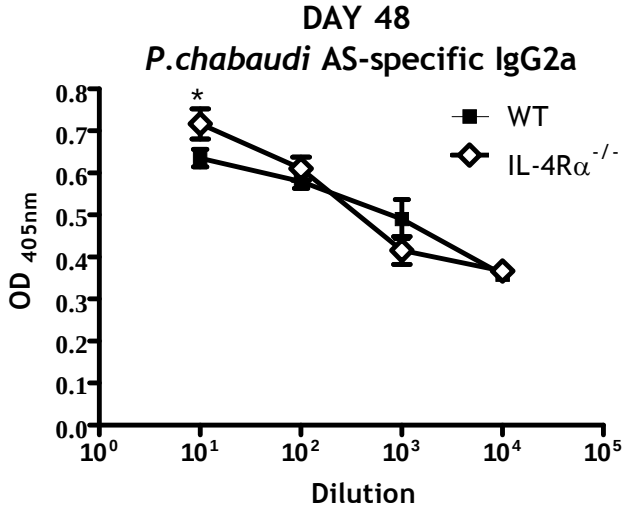
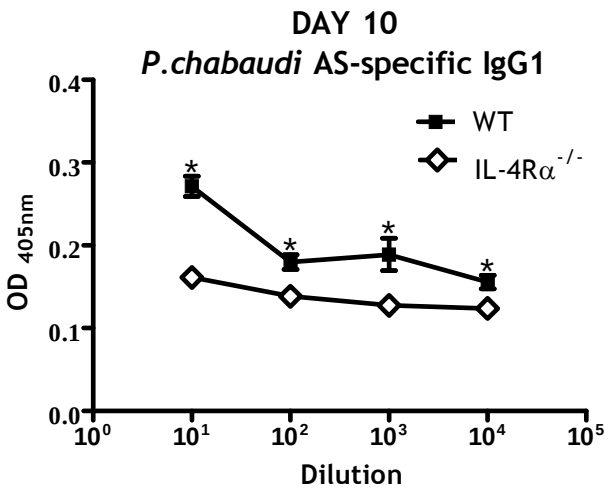


Figure 4.11: Comparison of the IgG2a antibody responses of *P. chabaudi* AS infected WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. Data are representative of two independent studies, n=4-6. * denotes $p<0.05$.

A.



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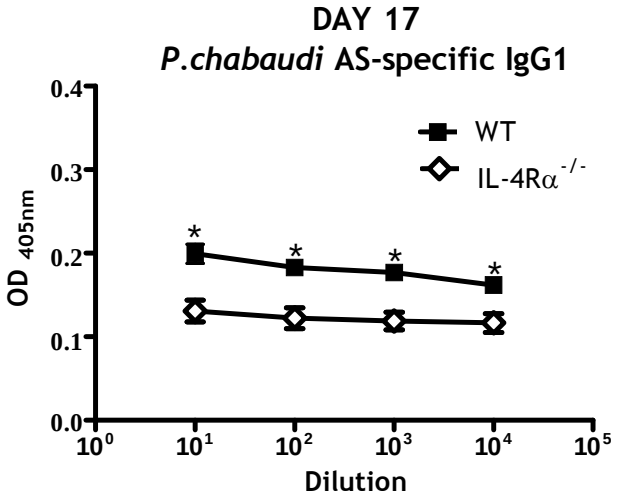

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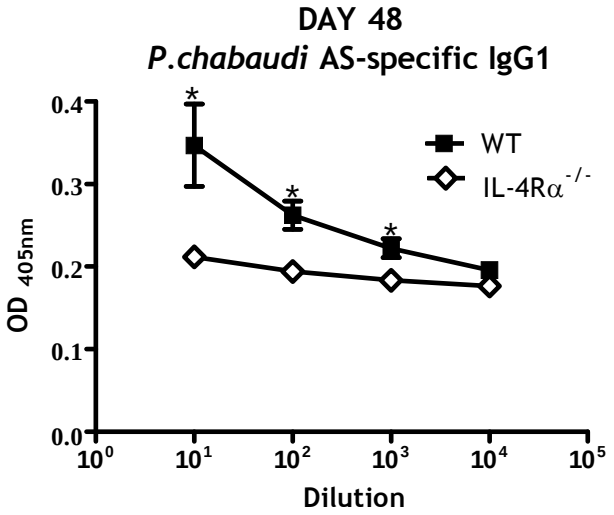


Figure 4.12: Comparison of the IgG1 antibody responses of *P. chabaudi* AS infected WT and IL-4Rα$^{-/-}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. Data are representative of two independent studies, n=4-6. * denotes $p<0.05$.

4.4 Discussion

In this study, male IL-4Rα$^{-/-}$ mice presented higher fatalities to *P. chabaudi* AS infection than the WT control and could not survive the duration of the experiment. However, no significant phenotypic differences were observed in parasitaemia levels, RBC counts and weight loss in the IL-4Rα$^{-/-}$ mice compared to the WT controls. There was evidence of an enhanced Th_1 immune response at day 10 in the IL-4Rα$^{-/-}$ mice as measured by increased ConA stimulated splenic IFN-γ production but IgG1 and IgG2a Ab titres remain comparable. It was evident that the magnitude of the immunological response in the infected male mice was greater than what had previously been found in female mice (chapter 3 and 4). An explanation for this finding was attributed to the fact that the immunomodulatory effects of testosterone, a male hormone, may underlie increased susceptibility to *Plasmodium* infection in males compared to females. It has been shown before in a *P. chabaudi* model that exposure of adult female mice to testosterone reduces Ab production, decreases MHC II cells in the spleen and increases $CD8^+$ T-cells in the spleen (Benten *et al.*, 1997) thereby increasing susceptibility to infection. Another study showed that it was possible for testosterone to modulate Th_1/Th_2 function in the protection against *P. chabaudi chabaudi* AS infection (Zhang *et al.*, 2000). IFN-γ$^{-/-}$ and IL-4$^{-/-}$ mice reveal that the effects of IFN-γ and IL-4 on survival were more pronounced in males than females. Male IFN-γ$^{-/-}$ and IL-4$^{-/-}$ mice have shorter survival times than their male WT counterparts. In contrast, no differences were obtained between female IFN-γ$^{-/-}$ and IL-4$^{-/-}$ mice and WT counterparts (Zhang *et al.*, 2000).

As a result of the extreme susceptibility of male BALB/c mice and particularly male IL-4Rα$^{-/-}$ mice to infection with *P. chabaudi* AS, female mice were used in all subsequent studies. Female IL-4Rα$^{-/-}$ mice infected with *P. chabaudi* AS did not display elevated peak parasitaemia compared to the WT control so the lack of IL-4/IL-13 function does not affect iRBC clearance. However, the IL-4Rα$^{-/-}$ mice displayed reduced parasitaemia levels at day 5 compared to the WT control indicating that Th_1 mechanisms and parasite control were possibly enhanced in the absence of IL-4/IL-13. A significantly enhanced recrudescent episode was displayed in the IL-4Rα$^{-/-}$ mice compared with the WT control indicating that the absence of IL-4/IL-13 function resulted in an impaired Th_2 protective immune

response during the chronic stage of the disease. Furthermore, there was evidence of early Th_1 associated cytokine control in the IL-4Rα$^{-/-}$ mice compared to the WT control as measured by increased IFN-γ production from ConA stimulated spleens. Following the declining parasitaemia, an impaired Th_2 immune response was characterized by reduced IL-10 and IL-4 production from ConA stimulated spleens and reduced IgG1 Ab titres from IL-4Rα$^{-/-}$ mice in comparison to the WT control indicating that adequate protection was compromised in the IL-4Rα$^{-/-}$ mice.

The results of the female *P. chabaudi* AS infection model are in agreement with previous findings obtained using *P. chabaudi* AS strain from our group (Couper, 2003) and with that obtained in chapter 3 using *P. chabaudi* AJ strain. It is evident that a significant role for IL-4Rα exists in the protective response against *P. chabaudi* AS infection that requires further investigation. IL-4 and IL-13 are Th2 cytokines whose biological functions are induced through a common IL-4Rα chain. The IL-4 receptor consists of two chains, the IL-4Rα chain and the IL-2Rγ chain (Hart *et al.*, 1999). The IL-13 receptor is a complex of the IL-4Rα chain and the IL-13Rα1 chain. Effective IL-13 signalling occurs only through the IL-4Rα chain and IL-13Rα1 chain. Thus, IL-4Rα is a common component of the receptor complexes for IL-4 and IL-13. Accordingly, IL-4 and IL-13 have many functional properties in common including the modulation of Th2 cell development, type 2 Ig class switching in B cells and inflammatory responses due to the regulation of macrophage functions (Brombacher, 2000). Of note is that murine lymphocytes do not have IL-13 receptors.

In conclusion, we have shown that a role for IL-4 and IL-13 exists for protection against *P. chabaudi* AS infection in male and female BALB/c mice. However, male mice succumbed to infection at a greater extent than the female mice due to possible immunomodulatory effects of sex hormones. Although no major differences were found between WT and IL-4Rα$^{-/-}$ mice at peak parasitaemia, there was a significant early delay in the onset of peak parasitaemia in the IL-4Rα$^{-/-}$ compared to the WT control mice. However, in comparison to the WT control, recrudescence was only evident in the IL-4Rα$^{-/-}$ mice, which indicated impairment in the Th_2 immune response, which was demonstrated by reduced

serum IgG1 antibody responses and discussed in more depth in the previous chapter.

IL-4 and IL-13 are pleotrophic cytokines and numerous cell types are responsive to signalling via IL-4Rα. Recently, Brombacher and colleagues have generated a number of tissue specific deficient mice (Bryson et al., 2011, Mc Farlane *et al.*, 2011, Brombacher *et al.*, 2009, Dewals *et al.*, 2009, Keating *et al.*, 2009, Michels *et al.*, 2009, Horsnell *et al.*, 2007, Radwanska *et al.*, 2007, Leeto *et al.*, 2006, Herbert *et al.*, 2004). This is achieved by the use of tissue specific promoters and a *Cre*/*loxP*-specific site-specific recombination technique (see Methodology, Chapter 2). Using such mice will ultimately allow dissection of the cell types responding to IL-4/IL-13 that mediate protection/susceptibility to *P. chabaudi* infection.

In chapter five, I shall examine whether the protective modulatory capacity of IL-4/IL-13 functions via IL-4Rα signalling on macrophages/neutrophils by utilization of macrophage/neutrophil-specific IL-4Rα gene deficient mice ($LysM^{cre}$IL-$4R\alpha^{-/lox}$).

Chapter Five

The role of IL-4/IL-13 responsiveness by macrophages and neutrophils during *Plasmodium chabaudi* AS erythrocyte infection in female mice.

5.1 Abstract

Differently activated macrophages display distinct biological features. Previous investigations have shown that IL-4, IL-13, IL-10, TGF-ß, immune complexes and apoptotic cells elicited during protozoan infections induce alternative activation states of macrophages thereby affecting the disease outcome. Disease outcome is affected on the one hand, by promoting parasite survival and proliferation and on the other hand, by limiting collateral tissue damage because of excessive Th_1-type inflammation. Thus, modulation of macrophage activation may be instrumental in allowing parasite persistence and long-term host survival. In the present study, macrophage/neutrophil-specific IL-4Rα$^{-/-}$ (LysM^{cre}IL-4Rα$^{-/flox}$) female mice were generated to understand the role of IL-4/IL-13 mediated functions on macrophages/neutrophils to determine whether the alternate activation of these cells provides long-term protective immunity in a *P. chabaudi* AS infection model. Our data showed that LysM^{cre}IL-4Rα$^{-/flox}$ mice developed protective immunity against *Plasmodium chabaudi* AS infection. Wild-type (WT) and LysM^{cre}IL-4Rα$^{-/flox}$ mice demonstrated similar disease profiles. The LysM^{cre}IL-4Rα$^{-/flox}$ mice showed no mortality, no recrudescent parasitaemias and recovered from infection more effectively compared with the IL-4Rα$^{-/-}$ mice. Furthermore, induced Th_1 (IFN-γ) and Th_2 (IL-10 and IL-4) spleen cytokine production, upon antigenic stimulation, and the induction of serum IgG2a and IgG1 antibody responses were similar to wild-type mice and presumably contributed to control of parasitaemia and long lasting protective immunity in the LysM^{cre}IL-4Rα$^{-/flox}$ mice. In contrast, IL-4Rα$^{-/-}$ mice were extremely susceptible to *P. chabaudi* AS infection with greater mortality and recrudescent parasitaemia observed. Additionally, Th_2 cytokines and antibody responses in the IL-4Rα$^{-/-}$ mice were impaired during the chronic stage of infection. Taken together, our results indicate that the alternate activation of macrophages/neutrophils by IL-4 and IL-13 are not essential for long-term protective immunity towards *P. chabaudi* AS infected mice.

5.2 Introduction

The Th_2 cytokines IL-4 and IL13 are closely related cytokines that share common biological properties with overlapping, yet distinct, functions. Consequently, IL-4 and IL-13 are important regulators of disease. The functional similarities are explained by both cytokines sharing the same IL-4Rα chain (Brombacher, 2000, Zurawski and de Vries, 1994). Signalling for both cytokines is dependent upon the IL-4Rα chain in association with either the common γ chain as well as via the type 2 receptor, a heterodimer between the IL-4R chain and the IL-13Rα1 chain. The type 2 receptor is the only functional IL-13R. IL-4Rα responsiveness therefore provides an effective strategy for protection against parasites such as *Nippostrongylus brasiliensis*, *Schistosoma mansoni*, *Trichuris muris*, and *Trichinella spiralis*. However, in allergic disease, IL-4 and IL-13 activation can have fatal consequences for the host (Brombacher, 2000). In *Plasmodium* studies, the effect of IL-4Rα signalling on macrophages/neutrophils for long-term protection is not well understood. Taken together, IL-4Rα signalling can confer protection or promote inflammation and tissue injury depending on the antigenic stimulus and the type of cell expressing the receptor.

Depending on the type of antigenic stimuli or cytokine environment, activation of macrophages can develop into two distinct pathways (Mantovani *et al.*, 2002, Goerdt and Orfanos, 1999; Munder *et al.*, 1988). Classically activated macrophages (caMø) (activated by Th1-type signals such as IFN- γ) serve a vital role in response to bacterial stimuli such as LPS (Brombacher *et al.*, 2009, Gordon, 2003). caMø produce Reactive Nitrogen Intermediates (RNI) and proinflammatory cytokines such as TNF and IL-12. Thus, caMø are indispendable effector cells of protective immunity against intracellular pathogens including *Mycobacterium tuberculosis* (M.Tb) and *Leishmania major* (Brombacher *et al.*,2009, Gordan, 2003, Holscher *et al.*, 2001, Louis *et al.*, 1998). Although caMø provide a beneficial role in host defense, proinflammatory responses can be detrimental to the host tissue if persistant escalation of inflammation occurs and can result in immunopathology. Thus, an alternate pathway of Mø activation has been proposed as a mechanism for attenuation of excessive inflammation (Brombacher *et al.*, 2009, Gordan, 2003, Goerdt and Orfanos, 1999). Alternate activation of macrophages (aaMø) are induced by the Th_2 cytokines, namely,

IL-4 and IL-13 via the shared IL-4Rα chain (Brombacher *et al.*, 2009, Gordon, 2003). aaMø attenuates excessive inflammation and secretes anti-inflammatory mediators to resolve inflammation (Brombacher *et al.*, 2009, Gordon, 2003; Goerdt and Orfanos, 1999). IL-10 is often co-induced with the Th_2 cytokines in the course of an immune response. It is not appropriate to classify IL-10 together with IL-4 and IL-13 as an alternative activator of macrophages. IL-10 acts on a distinct plasma-membrane receptor (IL-10R consisting of the ligand-binding subunit, IL-$10R_1$ and the accessory subunit, IL-$10R_2$) (Moore *et al.*, 2001) to those for IL-4 and IL-13. Its effects on macrophage gene expression are different, involving a more profound inhibition of a range of antigen-presenting and effector functions, together with the activation of selected genes or functions. IL-10 clearly has actions on macrophages that are distinct from those of IL-4 and IL-13 (Gordon, 2003).

It is known that immunity to *P. chabaudi* is complex and involves both innate and adaptive cellular and humoral responses (Namazi and Phillips, 2010, Couper *et al.*, 2005; Phillips *et al.*, 1997; Mohan *et al.*, 1997; Taylor-Robinson, 1995). $CD4^+$ Th_1 and Th_2 cells play important protective, but functionally different roles during malaria infection (Namazi and Phillips, 2010, Smith and Taylor-Robinson, 2003, Taylor-Robinson, 1995, Langhorne, 1989). Reports have shown that initial control against *P. chabaudi* infection is by innate cellular (such as macrophages and NK cells) and Th_1 responses whereby protection is associated with IFN-γ, TNF-α and NO production (Couper *et al.*, 2005; Taylor-Robinson *et al.*, 1993; Taylor-Robinson and Phillips, 1992; Langhorne *et al.*, 1989). Th_2 response rises when the infection becomes chronic and protection is largely dependent on B-cell and antibody production (Couper *et al.*, 2005; Smith and Taylor-Robinson *et al.*, 2003; Langhorne *et al.*, 1989) and could play a role in the elimination of the infection (Namazi and Phillips, 2010, McDonald and Phillips, 1980). While IL-4 is an important cytokine driving the Th_2 response, little is known about the cellular mechanisms orchestrating the Th_1-Th_2 switch and the involvement of alternative macrophage activation during *Plasmodium* infection. Therefore, the emphasis of the present study was to elucidate whether alternative activation of macrophages/neutrophils via IL-4Rα signalling was necessary for long-term host protection during *P. chabaudi* AS infection.

Functional studies on the role of aaMø in experimental mouse models of human diseases have been aided by the use of macrophage/neutrophil-specific IL-4Rα$^{-/-}$ mice established by Brombacher and colleagues (McFarlane *et al.*, 2011, Michels *et al.*, 2009, Keating *et al.*, 2009, Brombacher *et al.*, 2009, Herbert *et al.*, 2004). To determine the role of IL-4Rα signalling in macrophages/neutrophils during *P. chabaudi* AS infection, mice were engineered with a *loxP*-flanked IL-4Rα allele and Cre-recombinase expression under control of the regulatory region for the lysozyme M gene (LysM^{Cre} mice) (Clausen *et al.*, 1999) and thereby restricting Cre-mediated *loxP* recombination to only macrophages and neutrophils (McFarlane *et al.*, 2011, Michels *et al.*, 2009, Keating *et al.*, 2009, Brombacher *et al.*, 2009, Herbert *et al.*, 2004). We report here that the role for IL-4/IL-13 activated macrophages/neutrophils during Th_2 responses of *P. chabaudi* AS infected LysM^{cre}IL-4Rα$^{-/lox}$ mice was not critical for host survival or disease susceptibility.

5.3 Results

5.3.1 Comparison of the survival rates and disease phenotypes of wild-type (WT), global IL-4Rα gene deficient (IL-4Rα$^{-/-}$) and macrophage/neutrophil-specific IL-4Rα-deficient (LysM^{cre}IL-4Rα$^{-/lox}$) female mice following *Plasmodium chabaudi* AS infection.

WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice survived the acute stage infection with no mortalities observed whereas the IL-4Rα$^{-/-}$ mice displayed an enhanced mortality rate of 17% on day 12 (Figure 5.1). During the chronic-stage infection, 25% mortality was observed in the WT strain on day 22 while the majority of deaths, at 33% mortality, was observed in the IL-4Rα$^{-/-}$ mice at day 36 (Figure 5.1). In contrast, no deaths were seen in the LysM^{cre}IL-4Rα$^{-/lox}$ mice during the course of infection (Figure 5.1).

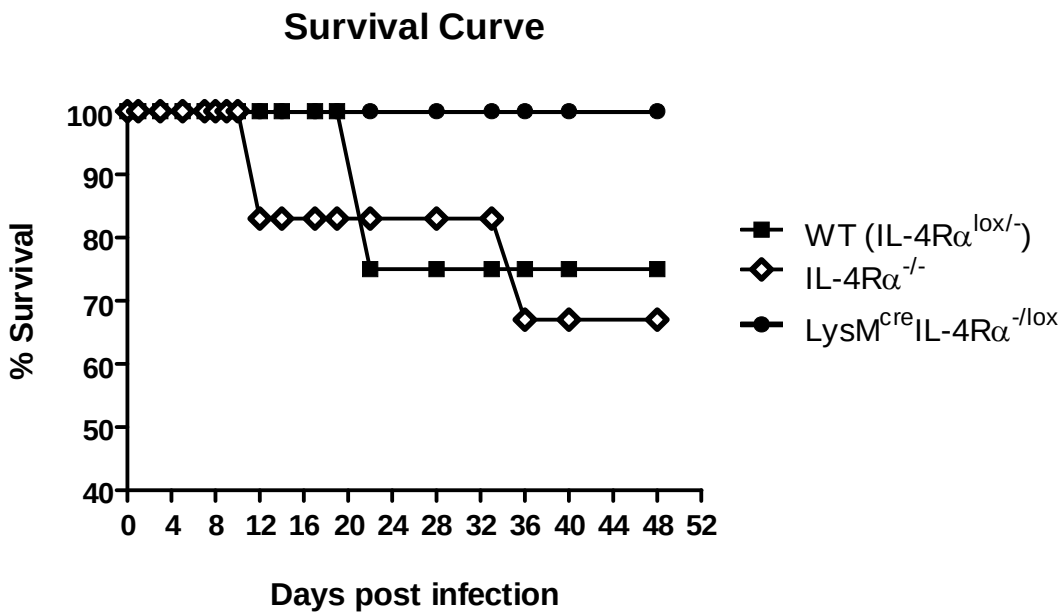


Figure 5.1: Comparison of the survival rates of *P. chabaudi* AS infection in WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. n=6. Data are representative of two independent studies.

Parasite burden around peak infection (day 7) showed no significant differences between the groups (Figure 5.2). Furthermore, no significant differences occurred between the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5.2). The IL-4Rα$^{-/-}$ mice demonstrated significantly greater recrudescent parasitaemia on days 19, 22, 28 and 33 post infection compared to the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5.2).

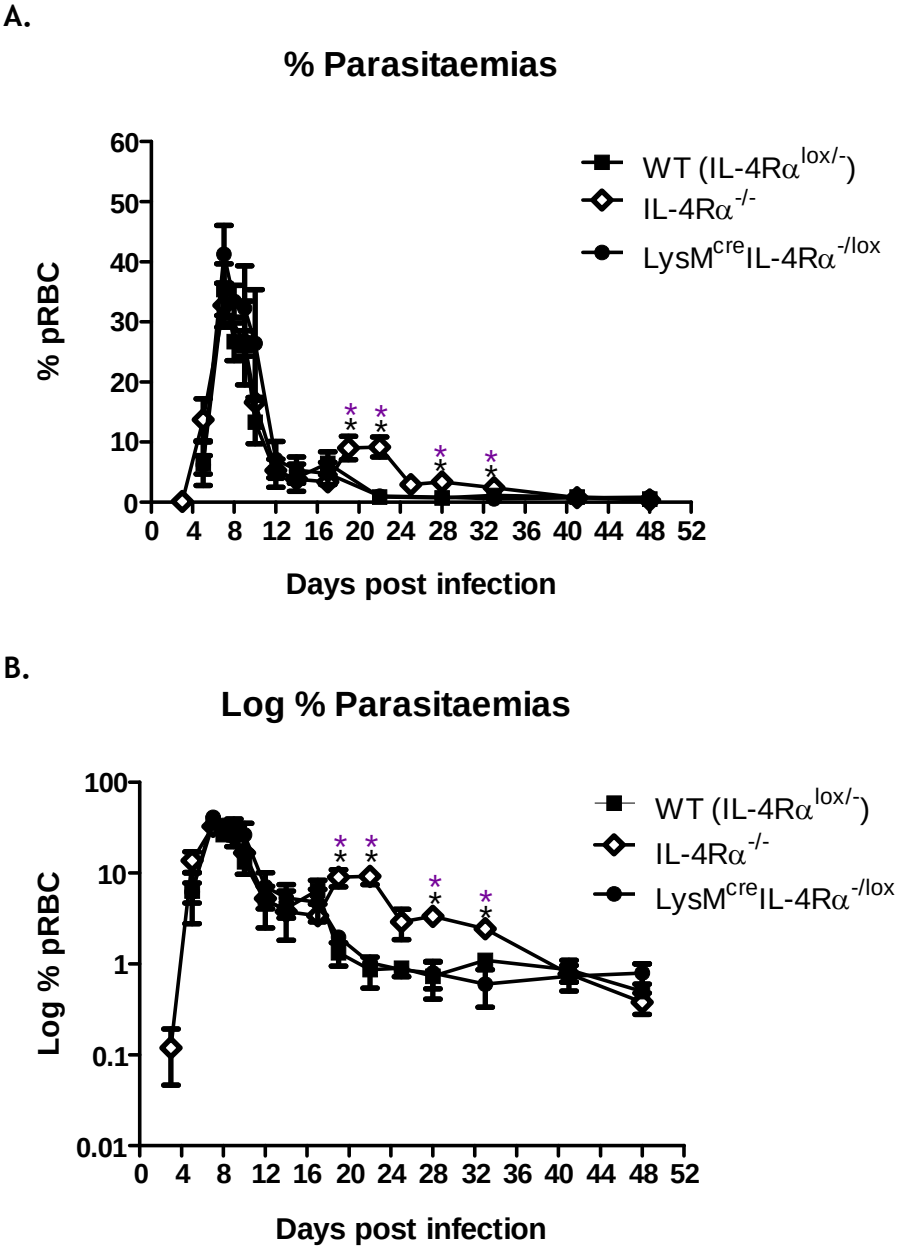
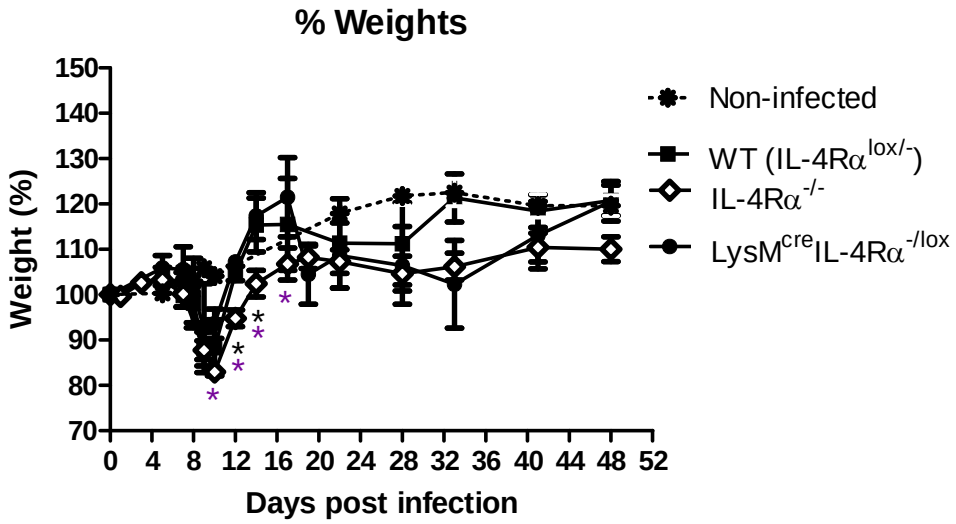


Figure 5.2: Comparison of the disease parasitaemias of *P. chabaudi* AS infection in WT (IL-4Rα$^{lox/-}$), LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ female mice on a BALB/c background. Results are displayed as a **(A)** % and as a **(B)** log % of parasitaemia levels in these groups. n=6; * and # denotes $p<0.05$ (* WT versus IL-4Rα$^{-/-}$ and * LysM^{cre}IL-4Rα$^{-/lox}$ versus IL-4Rα$^{-/-}$). Data are representative of two independent studies.

Contrary to infected mice, non-infected mice showed a steady weight gain over the 48 day period (Figure 5.3, A). Maximal weight loss was reached at day 10 of the peak infection but no significant differences occurred between the LysM^{cre}IL-4Rα$^{-/lox}$ mice and their WT counterparts (Figure 5.3, A). On the other hand, the IL-4Rα$^{-/-}$ mice showed a significant drop in weight on day 10 when compared to the LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5.3, A). Following recovery, all mice showed an increase in weight while a significant increase in weight was only observed in the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice when compared to the IL-4Rα$^{-/-}$ mice on days 12 and 14. Furthermore, weight gain was significantly lower in the IL-4Rα$^{-/-}$ mice on day 17 when compared to the LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5.3, A). LysM^{cre}IL-4Rα$^{-/lox}$ mice survived *Plasmodium chabaudi* AS infection. In contrast, WT and IL-4Rα$^{-/-}$ mice were more susceptible to infection as measured by mortality.

In contrast to non-infected mice, severe anaemia peaked in all groups at day 10 post infection with a significant drop in RBC counts (Figure 5.3, B) which corresponded to peak parasitaemia observed (Figure 5.2). At peak infection, no differences in RBC counts were observed between the groups. Following parasite control during the chronic stage of infection, a significant increase in RBC count was observed in the WT mice compared to the IL-4Rα$^{-/-}$ mice on day 14 while no significant differences occurred between the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5.3, B). RBC counts were significantly increased in the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice on days 19 when compared to the IL-4Rα$^{-/-}$ mice (Figure 5.3, B) and coincided with the reduced parasitaemia observed in these groups at the same time (Figure 5.2). In contrast, RBC counts in the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice were significantly lower at day 33 compared to IL-4Rα$^{-/-}$ mice but levelled off in all the groups as time progressed (Figure 5.2).

A.



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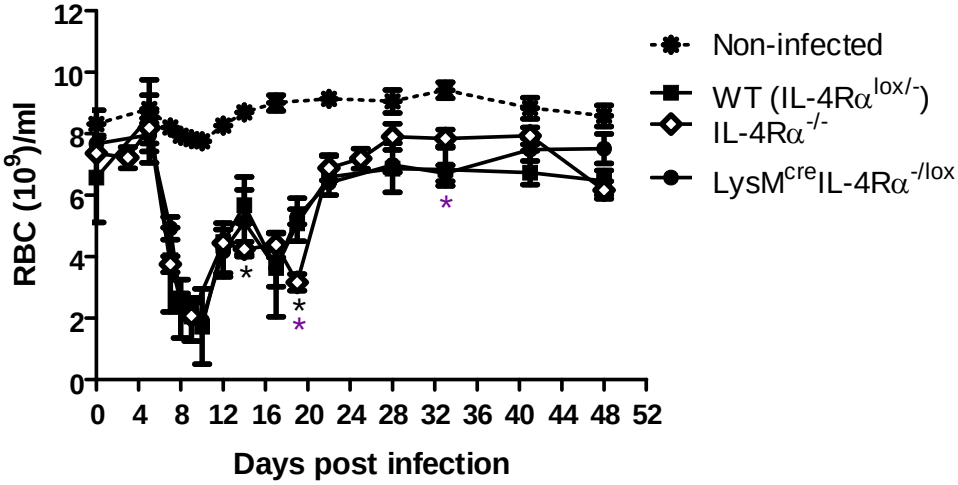


Figure 5.3: Comparison of **(A)** weight loss and **(B)** malaria induced anaemia during the course of *P. chabaudi* AS infection in WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/flox}$ female mice on a BALB/c background. n=6; * denotes $p<0.05$ (*WT versus IL-4Rα$^{-/-}$, *LysM^{cre}IL-4Rα$^{-/lox}$ versus IL-4Rα$^{-/-}$). Data are representative of two independent studies.

5.3.2 The influence of chronic disease on splenomegaly in macrophage/ neutrophil IL-4Rα-deficient (LysM^{cre}IL-4Rα$^{-/lox}$) female mice infected with *Plasmodium chabaudi* AS.

Whole spleen weights of non-infected and infected WT, IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice were measured to determine the severity of splenomegaly during the course of *P. chabaudi* AS infection between the respective groups. *P. chabaudi* AS parasite directly causes splenomegaly in the infected groups compared to no enlargement of spleens observed in non-infected mice throughout the duration of the disease (Figure 5.4). Furthermore, no significant decrease in spleen weight was observed in the infected IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice when compared to the infected WT mice on day 10 (Figure 5.4, A). Although no significant difference was seen between the infected WT and infected IL-4Rα$^{-/-}$ mice at day 17, a significant decrease in splenomegaly was observed in the infected LysM^{cre}IL-4Rα$^{-/lox}$ mice when compared to the infected WT control mice (Figure 5.4, B). Furthermore, no significant difference in the severity of splenomegaly was observed between the infected WT, IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ groups at day 48 post-infection (Figure 5.4, C).

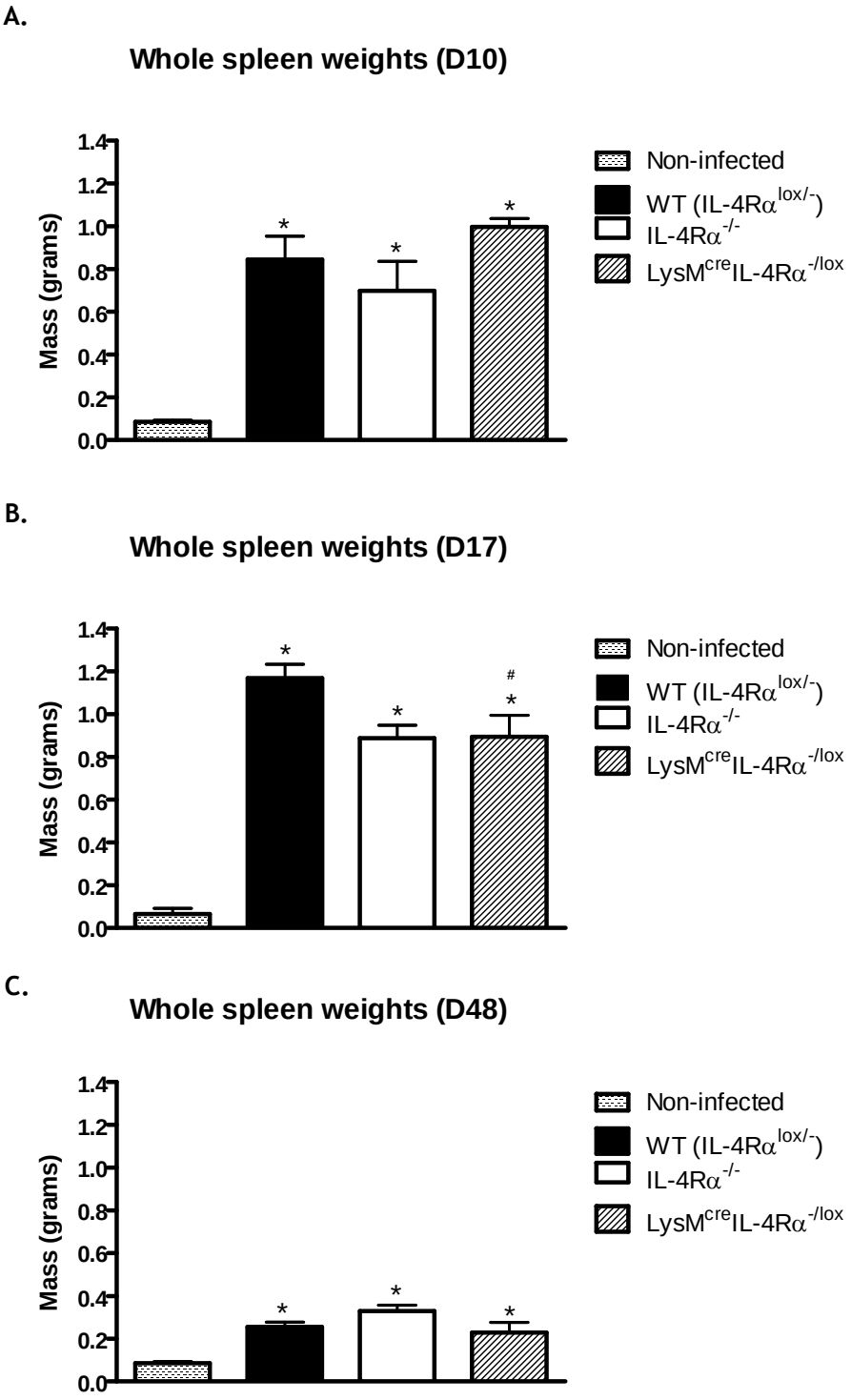


Figure 5.4: Comparison of the whole spleen tissue weights of non-infected and *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/flox}$ female mice on a BALB/c background. **(A)** Day 10 **(B)** Day 17 and **(C)** Day 48. n=5-6. * indicates non-infected versus the knock-out mice and $^{\#}$ indicates infected WT versus the infected LysM^{cre}IL-4Rα$^{-/lox}$ mice, and denotes $p<0.05$. Data are representative of two independent studies.

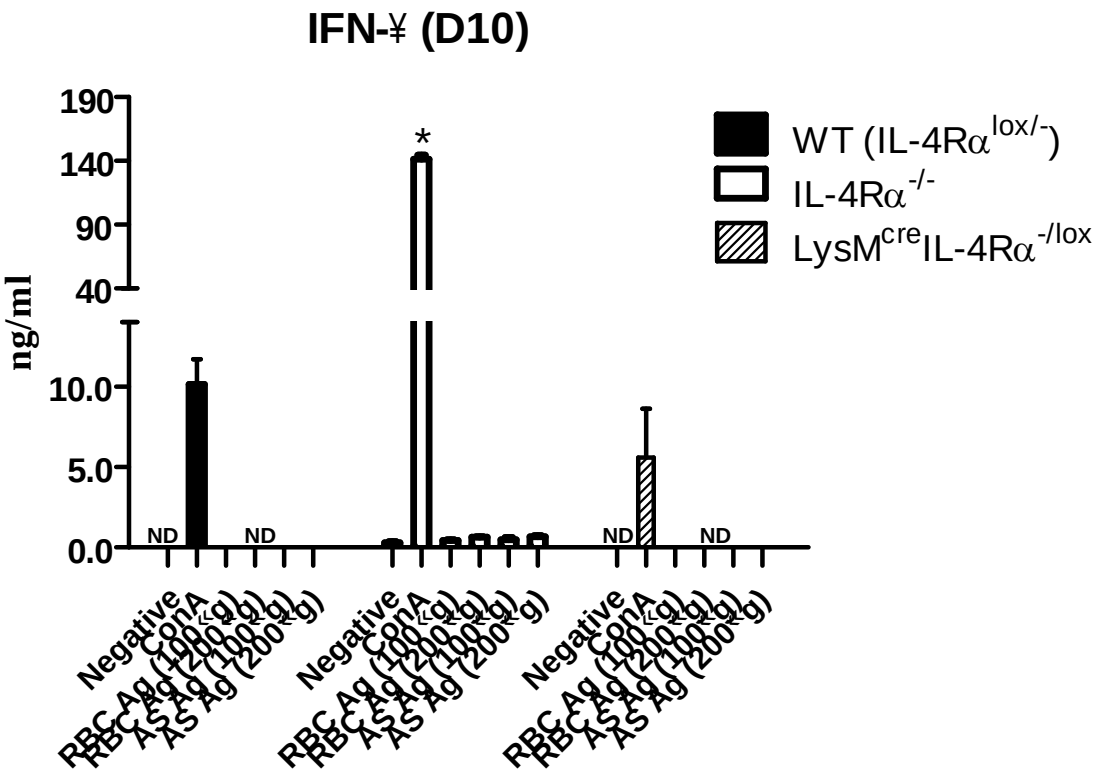
5.3.3 Comparison of the splenic cytokine production in wild-type (WT), Macrophage/Neutrophil-specific IL-4Rα-deficient (LysM^{cre}IL-4Rα$^{-/lox}$) and global IL-4Rα-deficient (IL-4Rα$^{-/-}$) female mice following *Plasmodium chabaudi* AS infection.

IFN-γ production by WT and LysM^{cre}IL-4Rα$^{-/lox}$ derived splenocytes were significantly lower than IL-4Rα$^{-/-}$ derived splenocytes following ConA stimulation from day 10 infected mice (Figure 5.5, A). IL-12 and IL-10 production by WT and LysM^{cre}IL-4Rα$^{-/lox}$ derived splenocytes could not be detected whilst no significant differences in IL-4 production by ConA stimulated splenocytes occurred between these groups (Figure 5.5, B). IL-4 production by ConA stimulated splenocytes was significantly increased in IL-4Rα$^{-/-}$ mice compared to the WT control and LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5.5, B).

IFN-γ production, at day 17, was significantly greater in the WT control and LysM^{cre}IL-4Rα$^{-/lox}$ ConA stimulated splenocytes but IFN-γ production from antigen-specific stimulated splenocytes was only significantly increased in LysM^{cre}IL-4Rα$^{-/lox}$ mice compared to WT and IL-4Rα$^{-/-}$ mice (Figure 5.6, A). IL-12 production was below the sensitivity of the ELISA in the IL-4Rα$^{-/-}$ derived splenocytes and IL-12 production was comparable with no differences observed in WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5.6, B). A significant increase in IL-10 production occurred in WT and LysM^{cre}IL-4Rα$^{-/lox}$ derived splenocytes upon ConA stimulation compared to IL-4Rα$^{-/-}$ derived stimulated splenocytes (Figure 5.7, A). Furthermore, IL-10 production from antigen-specific stimulated splenocytes from the LysM^{cre}IL-4Rα$^{-/lox}$ mice was significantly higher than the IL-4Rα$^{-/-}$ mice (Figure 5.7, A). Significantly, greater IL-4 production was obtained following ConA stimulation of IL-4Rα$^{-/-}$ derived splenocytes compared to the WT counterpart and LysM^{cre}IL-4Rα$^{-/lox}$ derived splenocytes (Figure 5.7, B). However, IL-4 production from antigen-specific stimulation of LysM^{cre}IL-4Rα$^{-/lox}$ derived splenocytes were significantly increased compared to the IL-4Rα$^{-/-}$ and WT mice (Figure 5.7, B).

At day 48, IL-4Rα$^{-/-}$ mice displayed a significantly reduced IFN-γ production from ConA stimulated splenocytes compared to the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice whilst IFN-γ production from antigen-specific stimulated IL-4Rα$^{-/-}$ derived splenocytes was significantly increased compared to the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5.8, A). IL-12 production was comparable with no differences observed in WT, IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5.8, B). WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice displayed comparable IL-10 production with significantly increased ConA and antigen-specific stimulation of splenocytes (Figure 5.9, A). IL-4 production was significantly reduced in the ConA stimulated splenocytes of IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/flox}$ mice compared to WT mice (Figure 5.9, B). In addition, IL-4 production by ConA stimulated splenocytes in LysM^{cre}IL-4Rα$^{-/lox}$ mice was also significantly reduced when compared to the IL-4Rα$^{-/-}$ mice (Figure 5.9, B). However, IL-4 production by antigen-specific stimulated IL-4Rα$^{-/-}$ derived splenocytes was significantly increased compared to the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5.9, B).

A.



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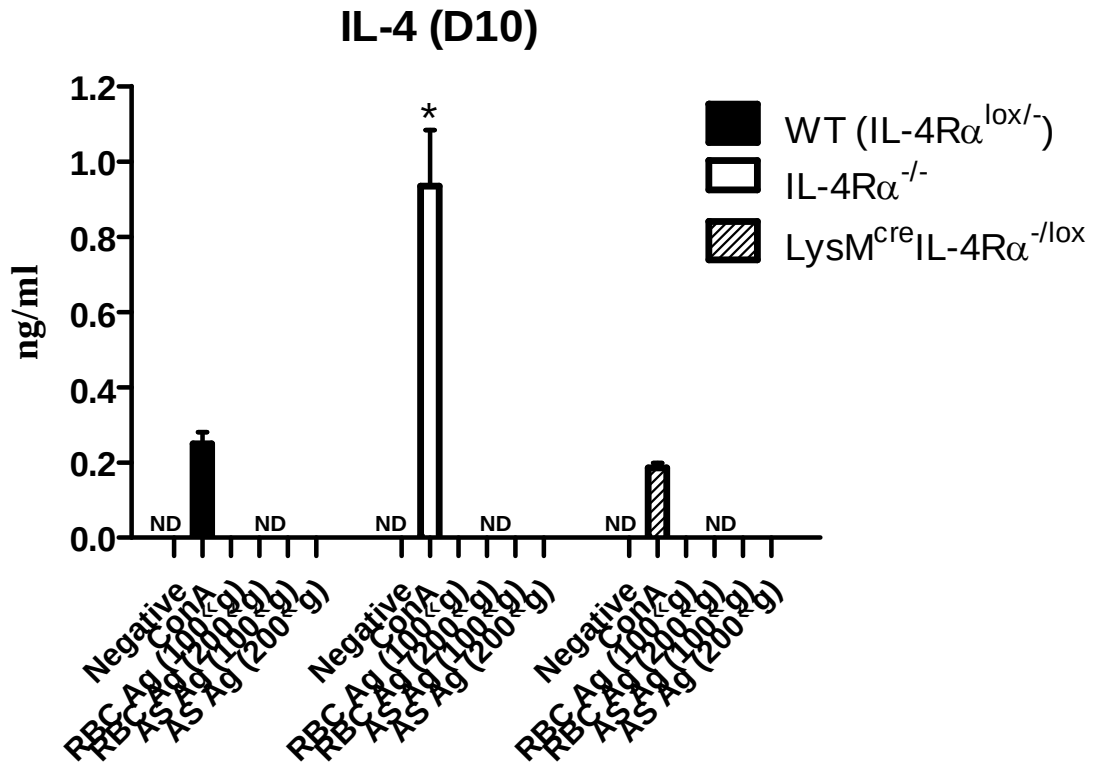
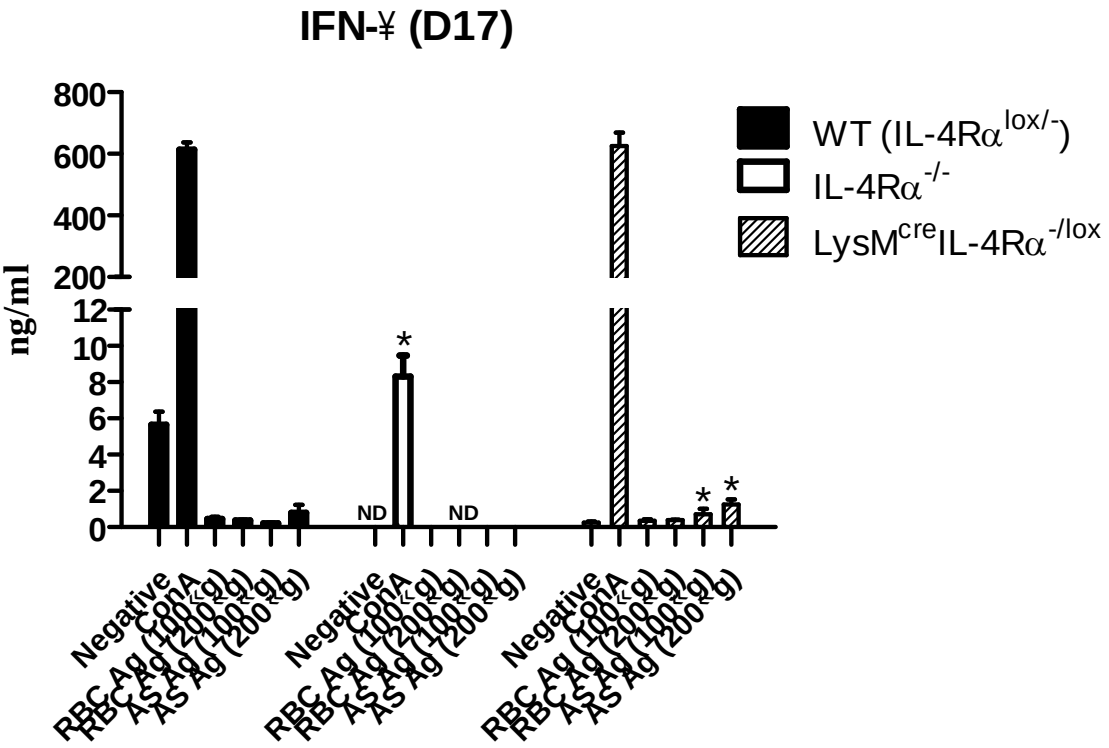


Figure 5.5: Comparison of day 10 splenic **(A)** IFN-γ and **(B)** IL-4 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. ND indicates not detected within the standard curve range of the ELISA. n=4-5 and * denotes $p<0.05$. Data are representative of two independent studies.

A.



B.

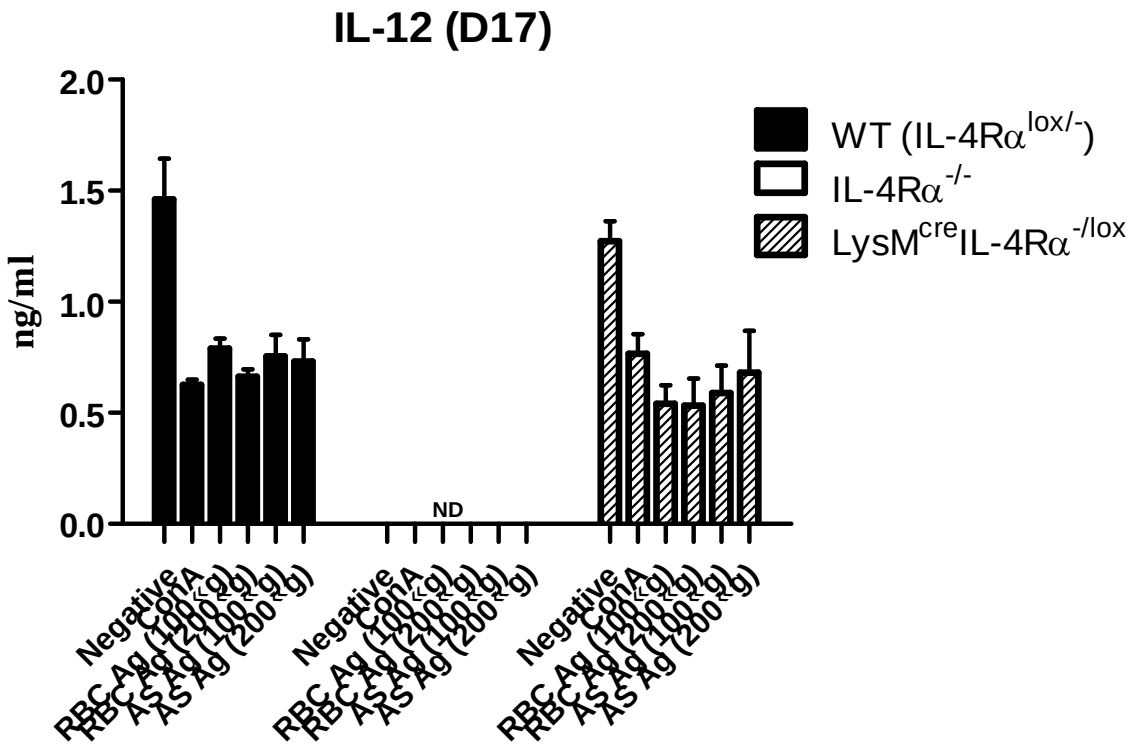
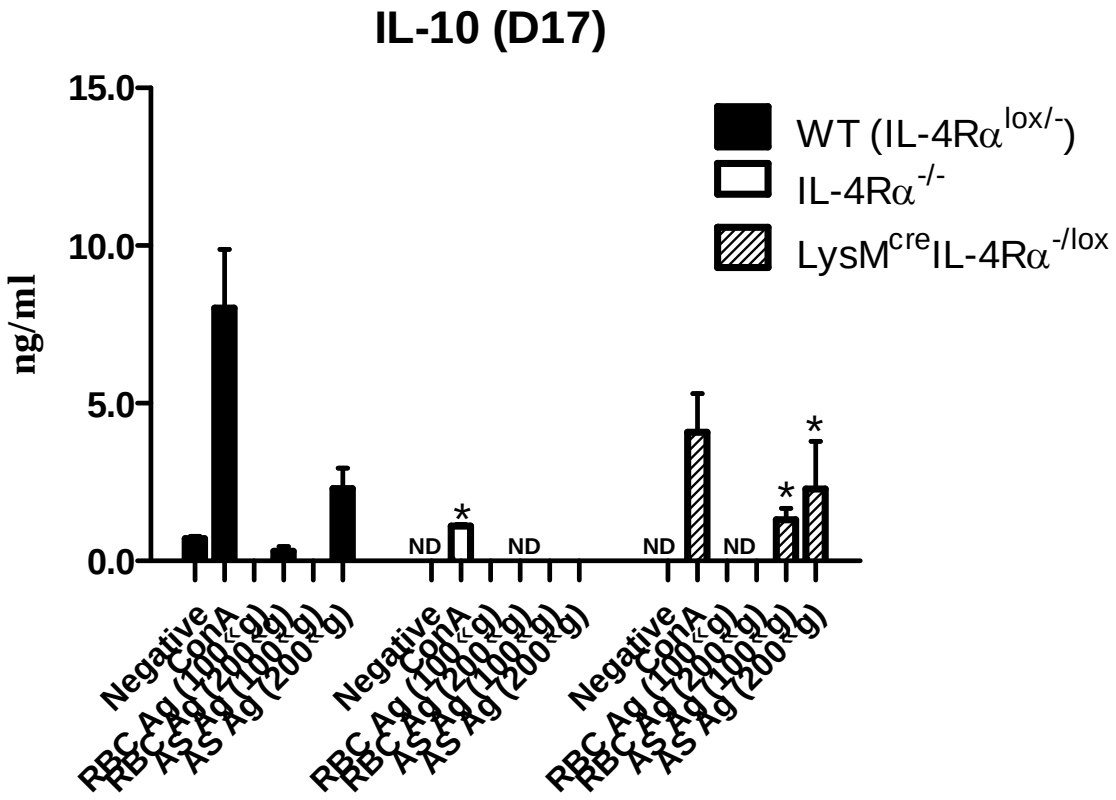


Figure 5.6: Comparison of day 17 splenic **(A)** IFN-γ and **(B)** IL-12 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. ND indicates not detected within the standard curve range of the ELISA and n=4-5. Data are representative of two independent studies.

A.



B.

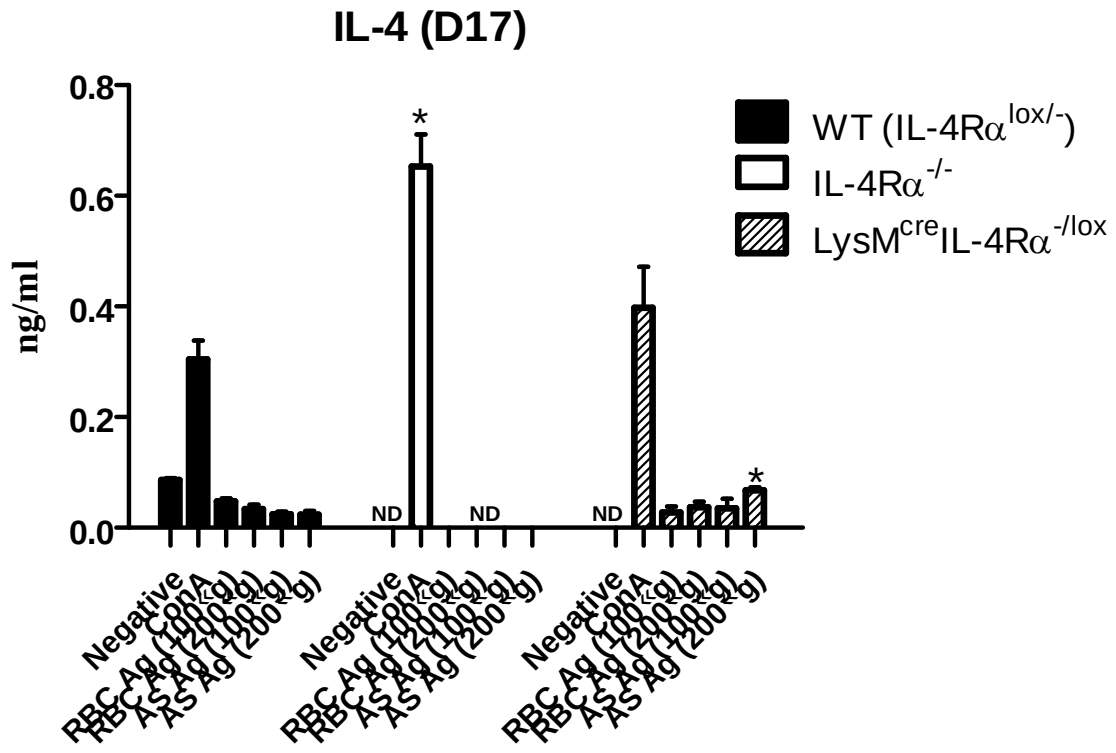


Figure 5.7: Comparison of day 17 splenic **(A)** IL-10 and **(B)** IL-4 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. n=4-5 and * denotes $p<0.05$. ND indicates not detected within the standard curve range of the ELISA. Data are representative of two independent studies.

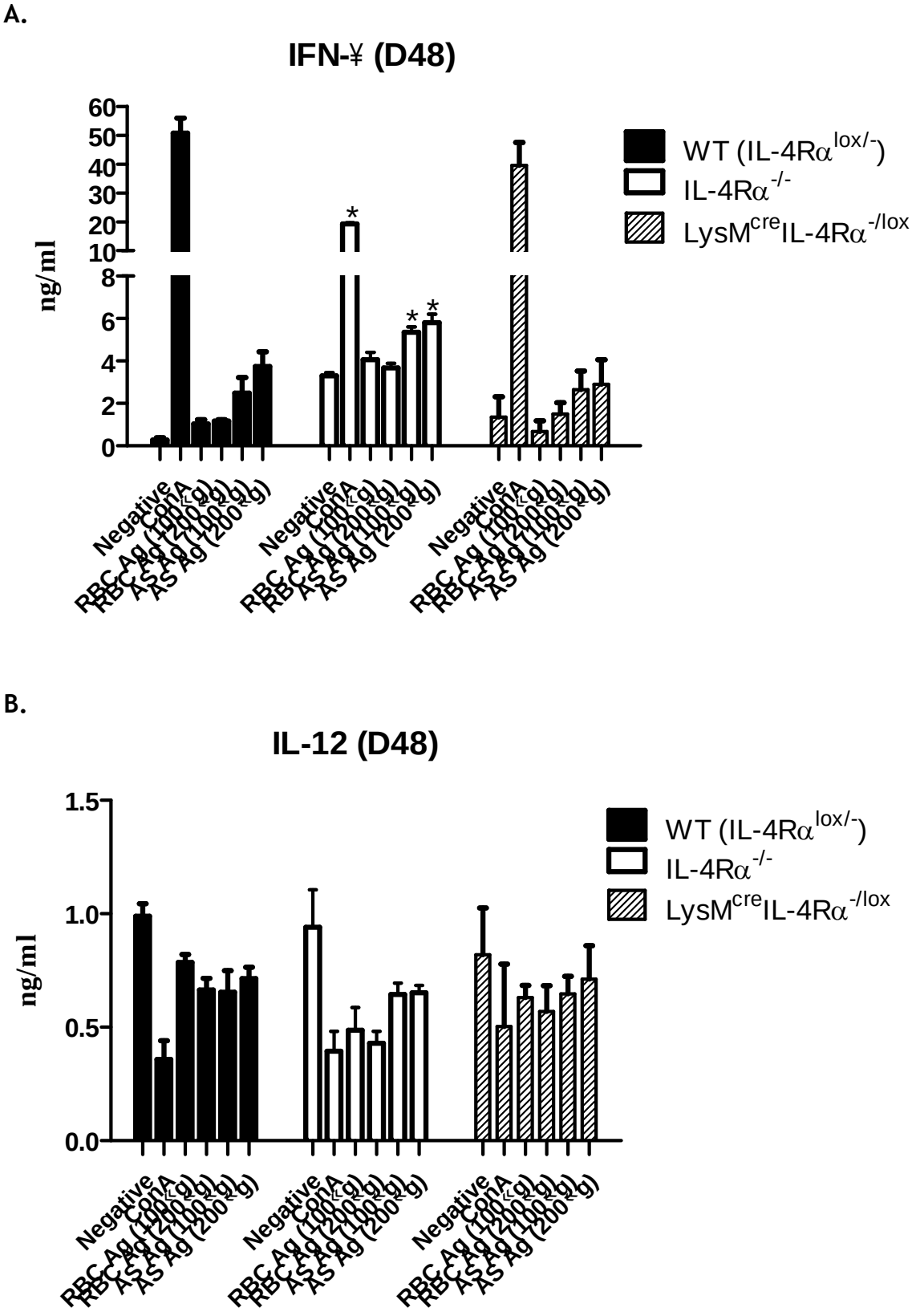
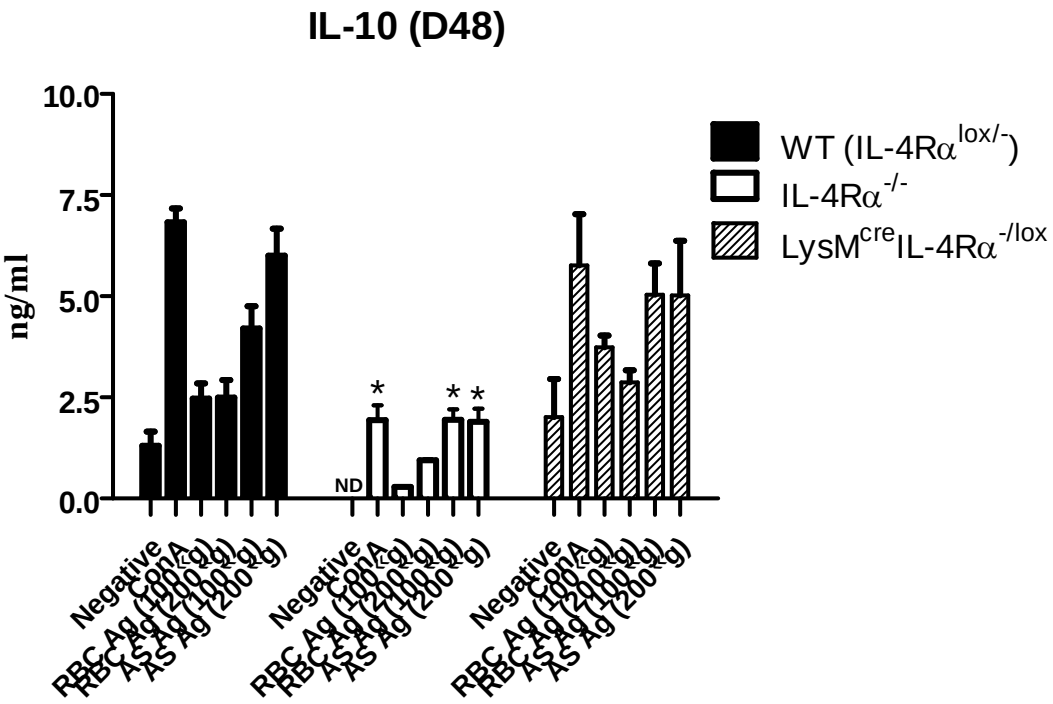


Figure 5.8: Comparison of day 48 splenic **(A)** IFN-γ and **(B)** IL-12 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. n=4-5. Data are representative of two independent studies.

A.



B.

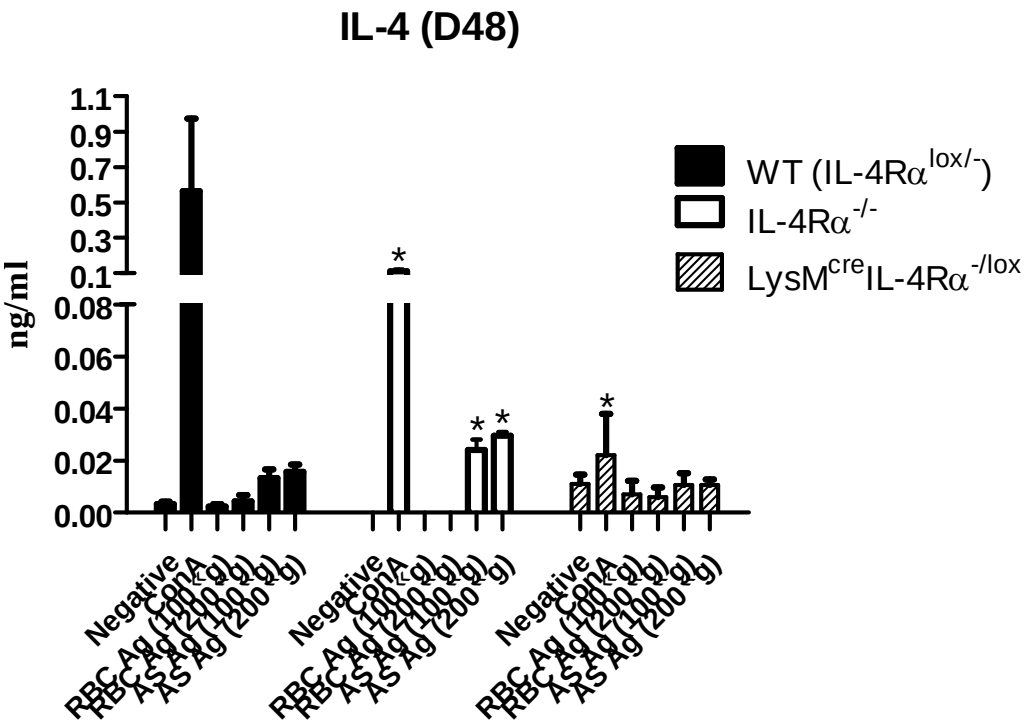


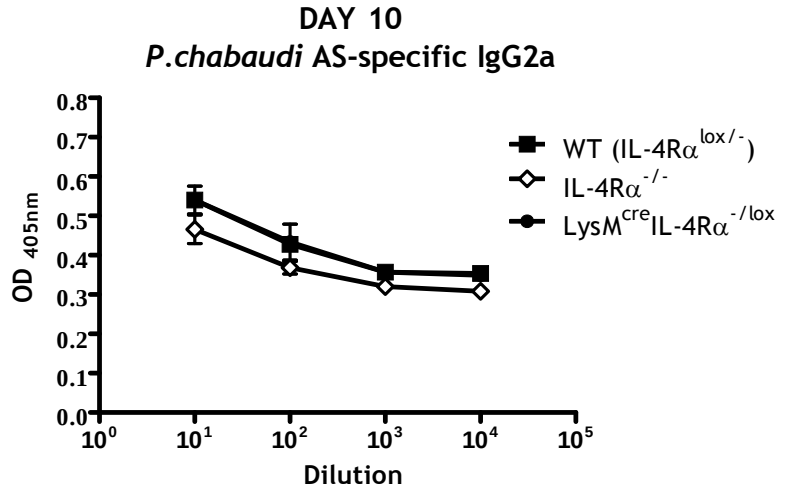
Figure 5.9: Comparison of day 48 splenic **(A)** IL-10 and **(B)** IL-4 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. n=4-5. Data are representative of two independent studies.

5.3.4 Comparison of the IgG2a and IgG1 antibody responses of macrophage/neutrophil IL-4Rα-deficient (LysM^{cre}IL-4Rα$^{-/lox}$) female mice following *Plasmodium chabaudi* AS infection.

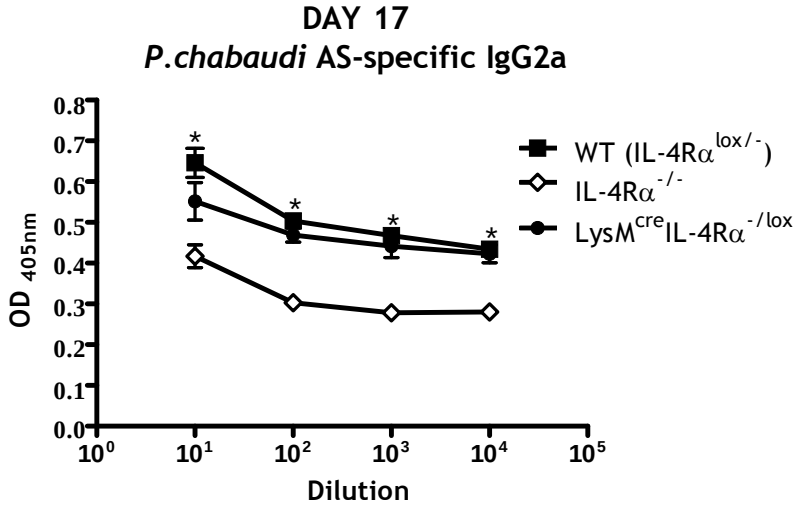
Peak parasitaemia levels demonstrated no significant differences in IgG2a antibody levels between the respective groups at day 10 of the infection (Figure 5.10, A). WT and LysM^{cre}IL-4Rα$^{-/lox}$ showed comparable IgG2a antibody responses, which were significantly greater than the IL-4Rα$^{-/-}$ mice at day 17 post infection (Figure 5.10, B). However, at day 48, IgG2a antibody responses showed no respective significant differences between the groups (Figure 5.10, C).

On days 10 and 17, IgG1 antibody levels were comparable between the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice but were significantly higher compared to the IL-4Rα$^{-/-}$ mice (Figure 5.11, A and B). On the contrary, no significant differences in IgG1 antibody levels were observed between the groups on day 48 (Figure 5.11, C).

A.



B.



C.

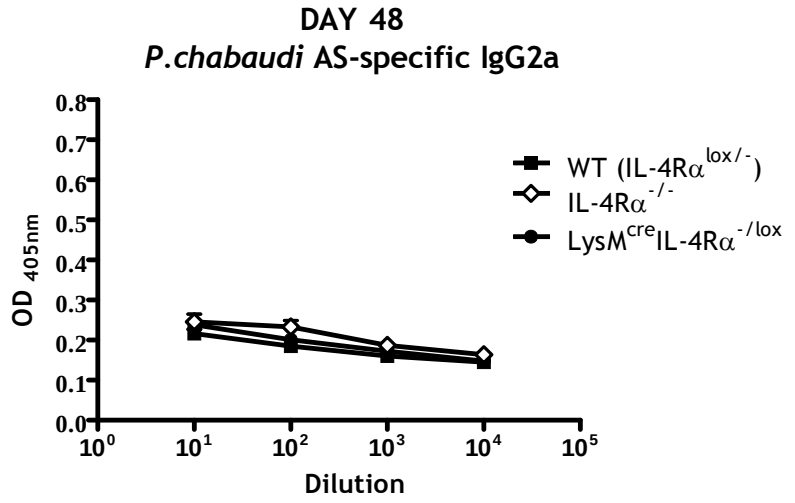
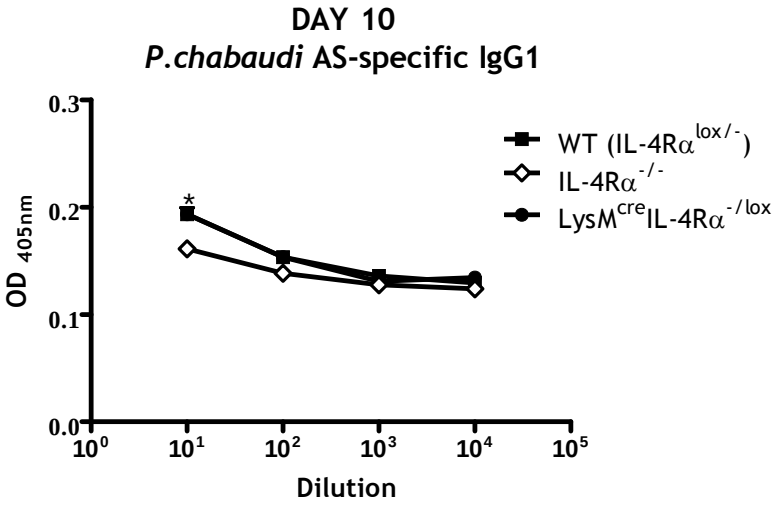
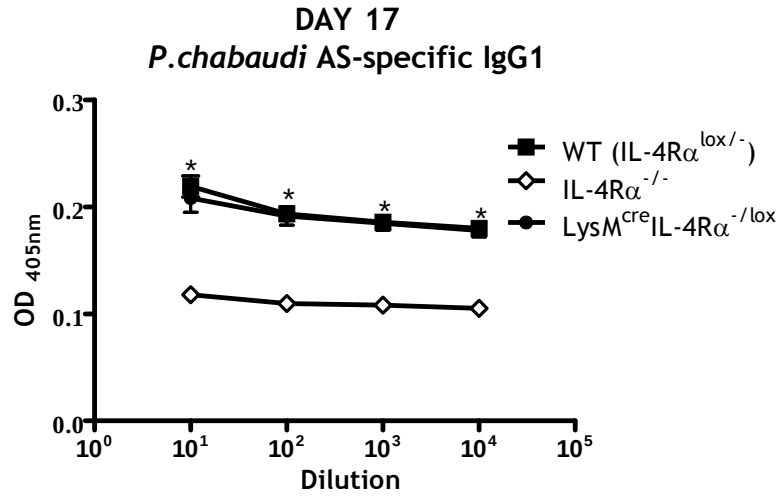


Figure 5.10: Comparison of the IgG2a antibody responses of *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. n=5-6 and * denotes $p<0.05$. Data are representative of two independent studies.

A.



B.



C.

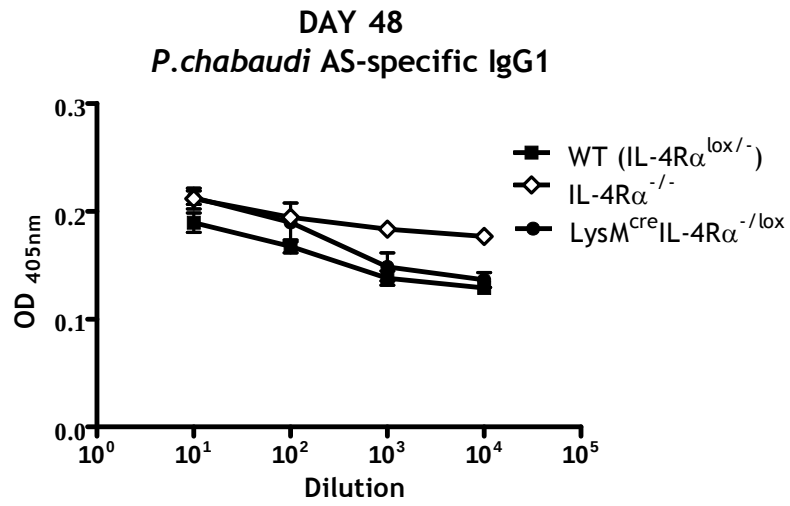


Figure 5.11: Comparison of the IgG1 antibody responses of *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. n=5-6 and * denotes $p<0.05$. Data are representative of two independent studies.

5.4 Discussion

We used the *Plasmodium chabaudi* AS disease model in which host protection or pathology was dependent on Th_1 and Th_2 responses to investigate the importance of alternate macrophage activation on disease outcome. This study provided evidence that long-term protection of LysM^{cre}IL-4Rα$^{-/flox}$ mice infected with *P. chabaudi* AS did not require the presence of IL-4 and IL-13 activation of macrophages. Thus, the increased susceptibility of IL-4Rα$^{-/-}$ mice compared with WT mice must depend on IL-4Rα signalling cell populations other than macrophages/neutrophils.

The disease profile of LysM^{cre}IL-4Rα$^{-/lox}$ mice was very similar to that of the WT control apart from the fact that the WT mice demonstrated only a 25% mortality rate at day 22 post infection. Acute parasitaemia in both these groups corresponded to the reduced RBC count and weight loss observed in the groups around this time. On the other hand, IL-4Rα$^{-/-}$ mice displayed greater mortality and greater recrudescent parasitaemia to the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice. Furthermore, peak infection correlated with the significantly reduced weight in IL-4Rα$^{-/-}$ mice compared to the WT control and LysM^{cre}IL-4Rα$^{-/lox}$ mice and following primary infection, weight loss and RBC counts were significantly lower in this group compared to the WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice. Taken together, these results show that in the absence of IL-4/IL-13 activation of macrophages, the LysM^{cre}IL-4Rα$^{-/lox}$ mice are able to survive the *P. chabaudi* AS infection and control chronic infection more effectively than the IL-4Rα$^{-/-}$ mice.

Cell-mediated responses during the acute stage (day 10) of infection demonstrated reduced IFN-γ production from ConA stimulated splenocytes in WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice compared with the IL-4Rα$^{-/-}$ mice. This may explain why no mortality was observed in the LysM^{cre}IL-4Rα$^{-/lox}$ mice due to no over-production of IFN-γ compared to IL-4Rα$^{-/-}$ mice. The detrimental effect of IFN-γ production in malaria was documented. Essentially, excessive production of a strong proinflammatory type I immune response and the resulting cytokines produced, namely IFN-γ, may directly contribute to severe disease such as severe anaemia, cerebral malaria and organ damage (Couper *et al.*, 2010, Good *et al.*, 2005, Schofield and Grau, 2005). IgG1 antibody levels around this time were significantly higher in the WT and LysM^{cre}IL-4Rα$^{-/flox}$ mice and may have

resulted from IL-4 stimulation of other cell types ($CD4^+$ Th_2 cells) which in turn activate B-cell differentiation into Th_2-type antibody production. Following recovery from primary infection (day 17), the cytokine profile in the WT and $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ mice showed a mixed Th_1-Th_2 phenotype. IFN-γ production from antigen-specific stimulated splenocytes in WT and $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ mice were significantly greater than $IL\text{-}4R\alpha^{-/-}$ mice and correlated to the increased IgG2a antibody levels at this time. In addition, IL-10 and IL-4 production from antigen-specific stimulated splenocytes were significantly greater than $IL\text{-}4R\alpha^{-/-}$ mice and correlated to the increased IgG1 antibody levels around this time. The presence of IL-12 was not detected in $IL\text{-}4R\alpha^{-/-}$ mice and not significant in WT and $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ mice. Previous studies in malarial anaemia have also shown that suppression of IL-12 decreases production of IFN-γ and IFN-α and the suppression of IL-12 appears to be a consequence of the induction of IL-10, which in turn is stimulated by infection (Ouma *et al*., 2008; Keller *et al* ., 2006). Following termination of the experiment at day 48, a Th2 phenotype was dominant in the WT and $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ mice with increased IL-10 and reduced IFN-γ production. Taken together these results indicate the presence of Th_1 and Th_2-type cytokine and antibody responses in $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ mice controlling *P.chabaudi* AS chronic infection in the absence of IL-4Rα signalling on macrophages/neutrophils.

Results from the $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ mice in the present study are in contrast to data found in previous work (Couper, 2003). These are the first two studies, which have utilized cell-specific gene deficient mice to provide data regarding IL-4Rα signalling via macrophages/neutrophils in a *P.chabaudi* AS infected mouse model. Couper showed that $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ mice displayed a similar disease course to IL- $4R\alpha^{-/-}$ mice with enhanced mortality and recrudescent infection and impaired Type 2 protective immunity to WT mice (data not shown). He concluded that IL-4 and IL-13 protection might be via regulation of macrophage/neutrophil function. An explanation for the differences observed between the two studies is the fact that Couper used male mice and the present study utilized female mice. Several studies have illustrated that immunological differences exist between the sexes and linked with circulating steroid hormones (Klein, 2004; Roberts *et al*., 2001; Klein, 2000; Zuk and McKean, 1996). Human studies have shown that increased susceptibility to infection is thought to be one

of the leading causes of increased death rates amoung men as compared with women (Klein, 2004; Owens, 2002; Klein, 2000). Rodent studies reveal that sex differences are present and may be mediated by endocrine-immune interactions. Castration of male mice reduces, whereas exogenous administration of testosterone increases mortality following *P. chabaudi* or *P. berghei* infection (Klein, 2004; Wunderlich *et al.*, 1991; Kamis and Ibrahim, 1989). In addition, male mice recover slower from *P. chabaudi*-induced weight loss, anaemia and hypothermia and have lower antibody responses than female mice (Klein, 2004). The phenotypic differences between the sexes in response to *P. chabaudi* infection may be mediated by sex differences in the expression of genes that modulate pro-inflammatory and Th_1 responses during infection. The immunomodulatory effects of testosterone may underlie increased susceptibility to *Plasmodium* infections in males compared with females. Exposure of adult female mice to testosterone reduces antibody production, decreases MHC II and increases $CD8^+$ T-cells in the spleen (Klein, 2004; Benten *et al.*, 1997). Another study aimed to determine why females were less susceptible than males and measured the effects of estrogen and progesterone responses in malaria infection (Klein *et al.*, 2008). Results of the *P. chabaudi* infected C57BL/6 female mice showed that the physiological levels of estrogen rather than progesterone enhanced immunity (IFN-γ and IL-10) and could possibly protect females from disease symptoms during malaria infection (Klein *et al.*, 2008). Furthermore, Bryson and colleagues (Bryson *et al.*, 2011) identified a dichotomy between the sexes in a disease model in an attempt to identify which cell types cause IL-4/IL-13 induction resulting in a non-healing *Leishmania mexicana* disease. They infected mice lacking IL-4Rα expression on $CD4^+$ T-cells (Lck^{Cre}IL-$4R\alpha^{-/lox}$) with *L. mexicana* and monitored disease progression. They found that the Lck^{Cre}IL-$4R\alpha^{-/lox}$ female mice developed small lesions, which subsequently healed, and a strong Th_1 response was manifested compared to the controls. In contrast, the male Lck^{Cre}IL-$4R\alpha^{-/lox}$ mice developed small lesions, which persisted, and a strong Th_1 response was elicited but IL-4 was also elevated independent of $CD4^+$ T-cell IL-4 responsiveness and not the case in the female mice (Bryson *et al.*, 2011). They suggest a significant effect of sex hormones on $CD4^+$ T-cell function whereby infected male but not female Lck^{Cre}IL-$4R\alpha^{-/lox}$ mice can drive IL-4 production independently of IL-4Rα

signalling. Hence, their finding also provides evidence for male and female immunological differences that can exist during similar parasitic infections.

In addition, the role of aaMø in various disease models utilizing LysM^{Cre}IL-4Rα$^{-/lox}$ mice show differences in disease susceptibility or protection. For example, the induction of IL-4/IL-13 by Mø's are required in a *Schistosoma mansoni* infection for protection against organ injury through douwnregulation of egg-induced inflammation (Herbert *et al.*, 2004). Similarly, IL-4Rα-responsive Mø's are essential promoters of protection in experimental autoimmune encephalomyelitis (EAE) (Keating *et al.*, 2009). Keating and colleagues also highlighted the importance of T-regs since the IL-4Rα$^{-/-}$ mice showed lower EAE incidence and LysM^{Cre}IL-4Rα$^{-/lox}$ mice were protected from EAE. They concluded that Mø activation in the absence of Th_2 cytokines can promote disease suppression by T-regs (Keating *et al.*, 2009). On the other hand, protection against infection can be independent of IL-4Rα responsive macrophages as is the case in the present study and similarly shown in other disease models of aaMø. For example, in *Trichinella spiralis* (Michels *et al.*, 2009), *Leishmania donovani* (McFarlane *et al.*, 2011) and *Leishmania mexicana* (Bryson *et al.*, 2011) infection studies utilizing LysM^{Cre}IL-4Rα$^{-/lox}$ mice, protection was shown to be independent of aaMø.

In conclusion, we have shown that a role for IL-4 and IL-13 alternate activation of macrophages/neutrophils is not crucial for host survival during chronic *P. chabaudi* AS infection in female BALB/c mice. IL-4 and IL-13 may therefore exert their protective functions via IL-4Rα signalling on different T-helper cell types such as CD4$^+$ and CD8$^+$ T-cells in *P. chabaudi* AS infection, which will be the focus of the next chapters.

Chapter Six

The role of IL-4/IL-4Rα signalling on $CD4^+$ T-cells during *Plasmodium chabaudi* AS erythrocyte infection in female BALB/c mice.

6.1 Abstract

A feature of *P. chabaudi* infection compared to other parasitic infections is that both Th_1 and Th_2 $CD4^+$ T-cells play an important protective role. $CD4^+$ Th_2-dependent control of *P. chabaudi* infection immediately following acute primary parasitaemia is known to involve IL-4 and antibody-dependent mechanisms. Further work in the previous chapters showed that mice deficient in the IL-4Rα, with abrogated IL-4 and IL-13 function, were more susceptible to infection during the chronic phase with impaired Th_2 protective responses. Additionally, our previous work on macrophage/neutrophil-specific IL-4Rα-deficient BALB/c mice demonstrated that these mice did not succumb to infection and were able to resolve infection as effectively as the WT controls. In the present study, $CD4^+$ T-cell-specific IL-4Rα deficient (Lck^{cre}IL-$4R\alpha^{-/lox}$) BALB/c mice were generated and characterized to elucidate the importance of IL-4/IL-13 responsive $CD4^+$ T-cells in a model of *P. chabaudi* AS infection. The data presented here, indicate that together with the WT control, Lck^{cre}IL-$4R\alpha^{-/lox}$ mice provided initial Th_1 control at day 10 as measured by splenic IFN-γ production and serum IgG2a antibody responses. Subsequent Th_1-Th_2 control around day 17 was demonstrated in the Lck^{cre}IL-$4R\alpha^{-/lox}$ mice as measured by increased splenic IFN-γ and IL-10 production and the induction of serum IgG1 antibodies. Following day 17, Lck^{cre}IL-$4R\alpha^{-/lox}$ mice experienced a recrudescent parasitaemia similar to the IL-$4R\alpha^{-/-}$ mice with reduced RBC count and increased mortality at days 36 and 40 while weight loss was not significant. Of note is that mortality was greater in the Lck^{cre}IL-$4R\alpha^{-/lox}$ than in the IL-$4R\alpha^{-/-}$ mice. Th_2 responses were down-regulated in the Lck^{cre}IL-$4R\alpha^{-/lox}$ mice as measured by reduced splenic IL-10 and IL-4 production and increased IFN-γ from ConA stimulated splenocytes accompanied by increased serum IgG2a antibody responses. Together, our results indicate that Lck^{cre}IL-$4R\alpha^{-/lox}$ and IL-$4R\alpha^{-/-}$ BALB/c mice were susceptible to chronic-stage *P. chabaudi* AS infection and that the effector functions of IL-4 responsiveness on $CD4^+$ T-cells are vital to clear and control the infection.

6.2 Introduction

Of the major T-cell subpopulations, the central involvement of $CD4^+$ T-cells are recognized as essential for immune protection against asexual erythrocytic-stages in both murine and human malaria. In *Plasmodium chabaudi* infection of mice, the importance of $CD4^+$ T-cells in parasite immunity originates from experiments with thymectomized mice (Leke *et al.*, 1981; McDonald and Phillips, 1975) and has been demonstrated by selective depletion of different lymphocyte subsets *in vivo* (Taylor-Robinson *et al.*, 1993; Podoba and Stevenson, 1991; Süss *et al.*, 1988) and by adoptive transfer of T-cells or $CD4^+$ T-cell lines and clones to immunodeficient hosts (Taylor-Robinson and Phillips, 1994; Taylor-Robinson and Phillips, 1993; Taylor-Robinson *et al.*, 1993; Meding and Langhorne, 1991). Purified $CD4^+$ T-cells or parasite-specific $CD4^+$ T-cell lines transferred to severe combined immunodeficiency or lethally irradiated mice, respectively, cleared the infection only in the presence of B cells (Taylor-Robinson and Phillips, 1993; Meding and Langhorne, 1991), indicating a requirement for B cells for final elimination of parasites. $CD4^+$ T-cells can be differentiated into two major subsets, namely, Th_1 and Th_2 cells. This differentiation is based upon the specific cytokine environment that will prime naïve T-cells into specific $CD4^+$ Th_1 or Th_2 cells, which in turn produce distinct cytokine profiles that indicate their function (Mosmann and Coffman, 1989, 1987). Th_1 cells produce IL-2, IFN-γ, and TNF-alpha and consequently activate macrophages and mediate delayed-type hypersensitivity responses and provide B-cell help through IgG2a Ab stimulation. Th_2 cells produce IL-4, IL-5, IL-6, and IL-10 and provide help for B-cell antibody responses (Brombacher, 2000; Mosmann and Coffman, 1989, 1987). Th_1 and Th_2 cells therefore mediate distinct immune functions, containing non-overlapping and often counter regulatory cell-mediated and humoral responses. One of the characteristics of *P. chabaudi* blood-stage infection of immunologically competent mice is that it generates a host protective immune response that is characterized by both $CD4^+$ Th_1 and Th_2 responses (Taylor-Robinson *et al.*, 1993; Langhorne, 1989). The levels of these two subsets of cells change during the course of infection (Taylor-Robinson and Phillips, 1992; Langhorne *et al.*, 1989) where Th_1 cells predominate during the acute phase and Th_2 cells predominate during the later phases of infection. The factors that regulate the differentiation of naïve T-cells into cells of either Th_1 or Th_2 phenotype following activation are

not clearly understood. These regulatory factors may include secreted cytokines (Abehsira-Amar *et al.*, 1992; Swain *et al.*, 1991), the nature of the antigen-presenting cell involved (Terrazas *et al.*, 2010; Quin and Langhorne, 2001; Dekruff *et al.*, 1992; Gajewski *et al.*, 1991; Schmitz *et al.*, 1993), the processing of the antigen for presentation to the T-cell (Terrazas *et al.*, 2010; Sponaas *et al.*, 2006; Quin and Langhorne, 2001; Soloway *et al.*, 1991), and the antigenic load (Abehsira-Amar *et al.*, 1992).

Studies have shown that IL-4 is necessary for Th_2 responses in malaria infection. IL-4 is an important cytokine as it drives differentiation of naïve $CD4^+$ T-cells into $CD4^+$ Th_2 T-cells that would subsequently eliminate the infection, while in its absence these cells preferentially become IFN-γ producing cells (Gordon 2003; Brombacher, 2000; Barner *et al*, 1998; Paul, 1997). *P. chabaudi* studies have previously shown that mice deficient in IL-4 production did not alter the outcome of the infection significantly but they did display significantly elevated peak parasitaemia compared to the WT control mice and extended recrudescenses with sustained Th_1 responses (Balmer *et al.*, 2000; Phillips *et al.*, 1997). Although these mice produced a delayed Th_2 response, it was not absent and protection was speculated to occur via IL-13 function. Further investigations lead to utilization of IL-4Rα$^{-/-}$ mice that effectively could not respond to IL-4 or IL-13, which proved that IL-13 does play a role in compensating for the absence of IL-4 to confer protection against *Leishmania* (Mohrs *et al.*, 1999). In our group we have also shown that *P. chabaudi* AS infected IL-4Rα$^{-/-}$ mice succumbed to infection more so than the WT control mice (Chapter 3). The different signalling pathways induced by IL-4 still remain controversial and are yet to be clarified in malaria infection. The intricacy of IL-4 and IL-13 responsiveness can further be attributed to the wide range of cell types expressing IL-4Rα (Nelms *et al.*, 1999); hence the need to address signalling of the receptor on specific cell types and their role in parasite immunity.

While, the specific functional role of IL-4/IL-13 responding $CD4^+$ T-cells during *P. chabaudi* infection remain poorly understood, the availability of $CD4^+$ cell specific IL-4Rα$^{-/-}$ mice will enable us to clarify this role. In other disease models for example, it has been shown that deletion of IL-4Rα on $CD4^+$ T-cells renders BALB/c mice resistant to *Leishmania mexicana* (Bryson *et al*., 2011), *Leishmania major* (Radwanska *et al*, 2007) infection and therefore indicate a major role for IL-4/IL-13 function via $CD4^+$ T-cells in rendering these mice susceptible to infection. The effect of IL-4Rα signalling via $CD4^+$ T-cells in *P. chabaudi* infections is the focus of the present study.

We have thus generated Lck^{cre}IL-4Rα$^{-/lox}$ mice to determine what effector functions IL-4/IL-13 have on $CD4^+$ T-cell function during *P. chabaudi* AS infection and whether the IL-4Rα signalling cascade results in long-term protection. Our finding, which is novel regarding the use of $CD4^+$ T-cell IL-4Rα$^{-/-}$ mice in malaria, illustrated that the absence of IL-4 signalling via IL-4Rα on these cell types rendered mice more susceptible to infection and are therefore important effectors in the second phase of *P. chabaudi* AS infection.

6.3 Results

6.3.1 Comparison of the survival rates and disease phenotypes of wild-type (WT), global IL-4Rα-deficient (IL-4Rα$^{-/-}$) and CD4^{+} T-cell IL-4Rα-deficient (Lck^{cre}IL-4Rα$^{-/lox}$) female mice following *Plasmodium chabaudi* AS infection.

WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice survived the acute stage infection with no mortalities observed whereas the IL-4Rα$^{-/-}$ mice displayed an enhanced mortality rate of 17% at day 12 (Figure 6.1). During the chronic-stage infection, mortality was observed in the IL-4Rα$^{-/-}$ mice at day 36 with 33% mortality and with the majority of deaths observed in the Lck^{cre}IL-4Rα$^{-/lox}$ mice at day 36, 25% mortality and day 40, 50% mortality (Figure 6.1).

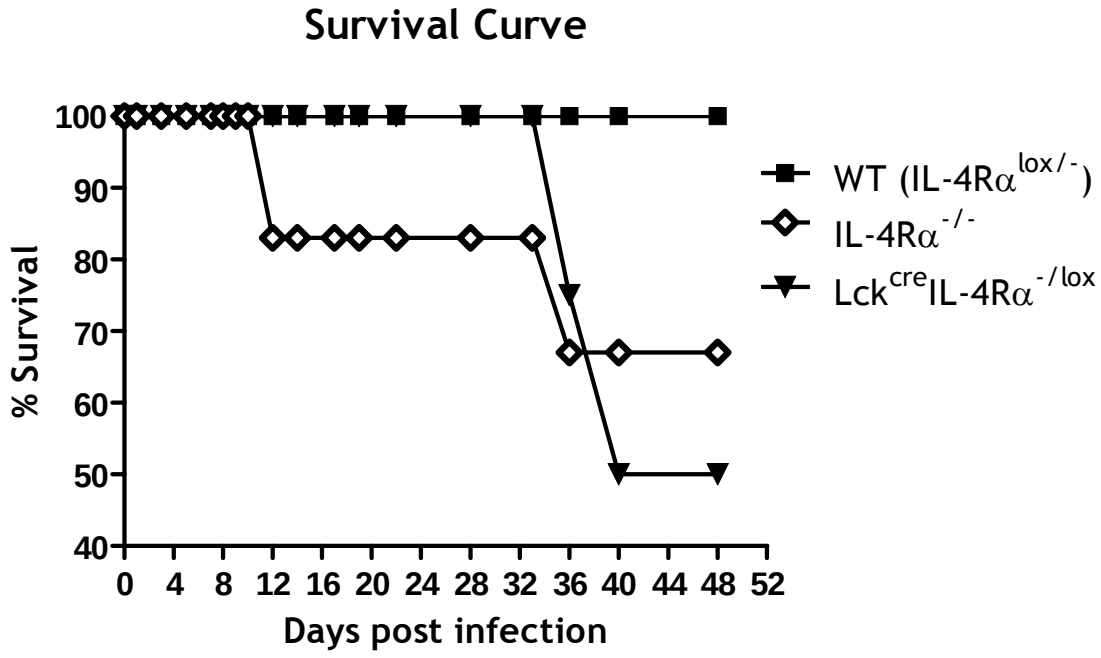


Figure 6.1: Comparison of the survival rates of *P. chabaudi* AS infection in **(A)** WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. WT n=14, IL-4Rα$^{-/-}$ n=18 and Lck^{cre}IL-4Rα$^{-/lox}$ n=13 and. Data are representative of two independent studies.

Parasite burdens were significantly higher in the Lck^{cre}IL-4Rα$^{-/lox}$ mice on days 22 and 28 compared to their WT counterparts during chronic-stage infection while no significant differences occurred around peak parasitaemia between the groups (Figure 6.2).

At day 3 of the early stage infection, parasite burdens were significantly lower in the IL-4Rα$^{-/-}$ mice compared to the Lck^{cre}IL-4Rα$^{-/lox}$ and WT mice. Furthermore, the IL-4Rα$^{-/-}$ mice demonstrated recrudescent parasitaemia on days 17, 22 and 28 post infection while no episode of recrudescence occurred in the WT mice (Figure 6.2).

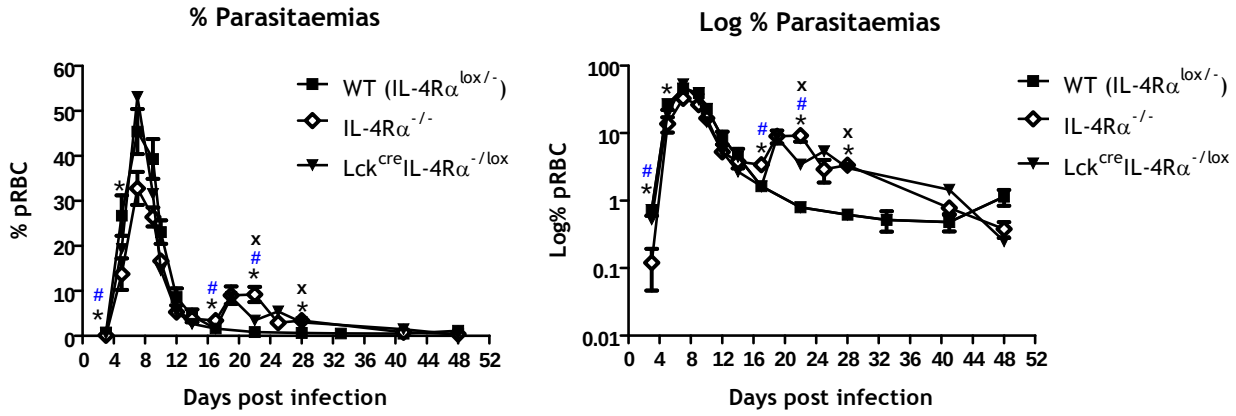
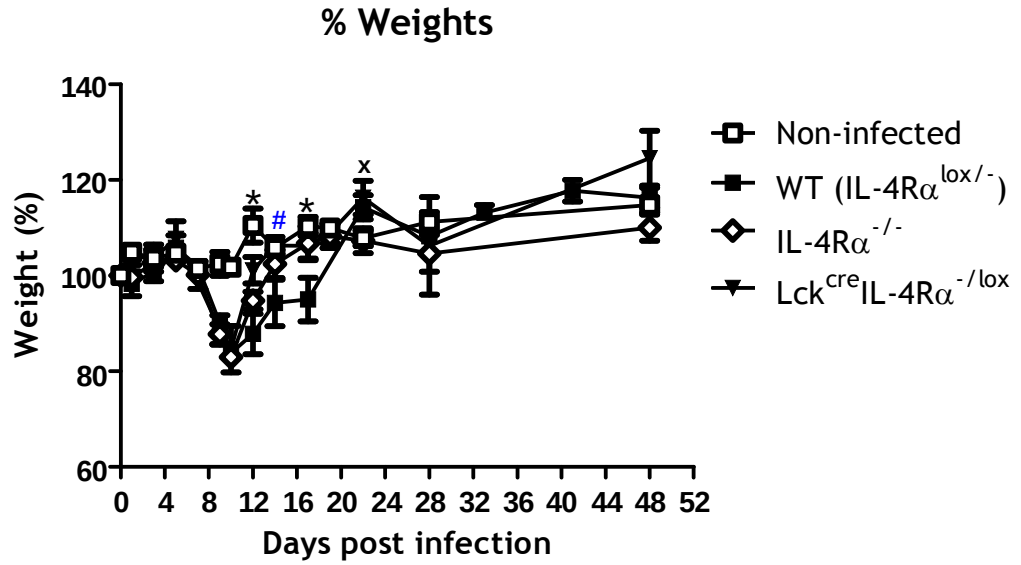


Figure 6.2: Comparison of the disease parasitaemias of *P. chabaudi* AS infection in WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. WT n=14, IL-4Rα$^{-/-}$ n=14 and Lck^{cre}IL-4Rα$^{-/lox}$, n=12. Results are displayed as a % and as a log % of parasitaemia levels in these groups. Data are representative of two independent studies. * (WT vs IL-4Rα$^{-/-}$), $^{\#}$ (IL-4Rα$^{-/-}$ vs Lck^{cre}IL-4Rα$^{-/lox}$) and ^{x} (WT vs Lck^{cre}IL-4Rα$^{-/lox}$) denotes $p<0.05$.

No significant differences in weight loss occurred around peak parasitaemia between the groups: maximal weight loss occurred on day 10 (Figure 6.3, A). Following recovery, a significant increase in weight occurred in the Lck^{cre}IL-4R$\alpha^{-/lox}$ mice on days 12, 14 and 17 post infection when compared to its WT counterpart (Figure 6.3, A). This increase in weight was also observed in the IL-4R$\alpha^{-/-}$ mice on day 17 when compared to the WT mice (Figure 6.3, A). Furthermore, no significant differences in weight loss were observed from day 17 in all groups.

At day 5 post infection, RBC counts were significantly higher in the IL-4R$\alpha^{-/-}$ mice when compared to the WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ mice (Figure 6.3, B) corresponding to the decreased parasite burdens observed in the IL-4R$\alpha^{-/-}$ mice at this time (Figure 6.2). Severe anaemia was observed in both groups at day 10 post infection (Figure 6.3, B) corresponding to peak infection (Figure 6.2). Following parasite control, a significant increase in RBC counts occurred in the Lck^{cre}IL-4R$\alpha^{-/lox}$ mice when compared to their WT counterparts and the IL-4R$\alpha^{-/-}$ mice (Figure 6.3, B). In contrast, RBC counts were significantly decreased in the Lck^{cre}IL-4R$\alpha^{-/lox}$ mice on day 22 when compared to the WT and IL-4R$\alpha^{-/-}$ mice (Figure 6.3, B), corresponding to the recrudescent parasitaemia observed in this group at that time (Figure 6.2).

A.



B.

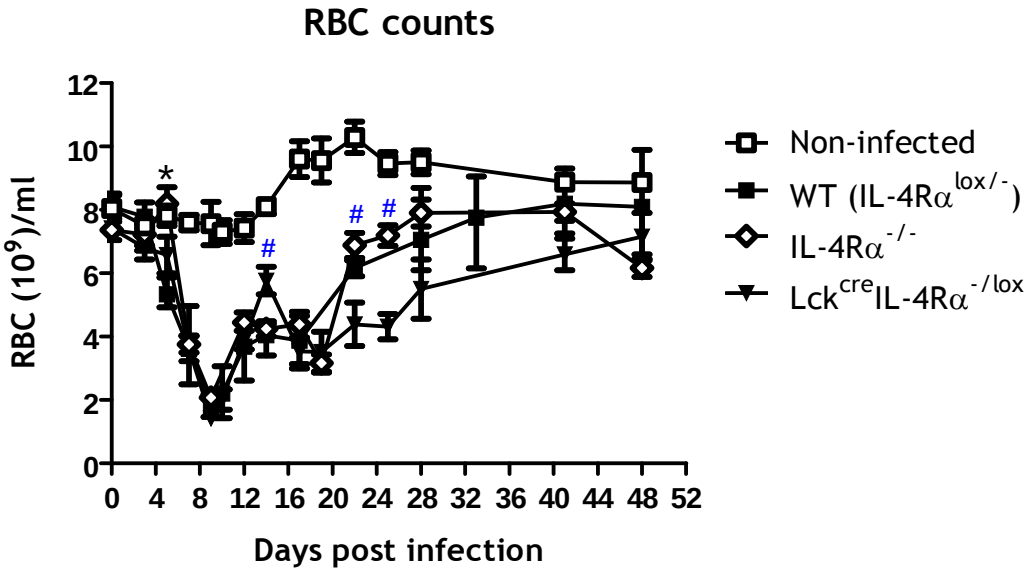


Figure 6.3: Comparison of **(A)** weight loss and **(B)** malaria induced anaemia during the course of *P. chabaudi* AS infection in WT(IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. WT(IL-4Rα$^{lox/-}$) n=14, IL-4Rα$^{-/-}$ n=14 and Lck^{cre}IL-4Rα$^{-/lox}$ n=12. **(A)** * (WT vs IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$), $^{\#}$ (WT vs Lck^{cre}IL-4Rα$^{-/lox}$) and ^{x} (WT and Lck^{cre}IL-4Rα$^{-/lox}$ vs IL-4Rα$^{-/-}$) **(B)** * (WT vs IL-4Rα$^{-/-}$) and $^{\#}$ (Lck^{cre}IL-4Rα$^{-/lox}$ vs IL-4Rα$^{-/-}$ and WT) denotes $p<0.05$. Data are representative of two independent studies.

6.3.2 The influence of chronic disease on splenomegaly in $CD4^+$ IL-4Rα-deficient (Lck^{cre}IL-4Rα$^{-/lox}$) female mice infected with *Plasmodium chabaudi* AS.

During the course of *P. chabaudi* AS infection, whole spleen weights of non-infected and infected WT as well as gene-deficient mice were measured to determine the severity of splenomegaly between the respective groups. Splenomegaly is a direct cause of *P. chabaudi* AS infection as was evident in the gene-deficient mice compared to no enlarged spleens observed in non-infected mice throughout the duration of the disease (Figure 6.4). Furthermore, no significant increase in spleen weights of the gene-deficient mice were observed in comparison to the WT control throughout the course of the infection and up to the termination of the experiment at day 48 (Figure 6.4).

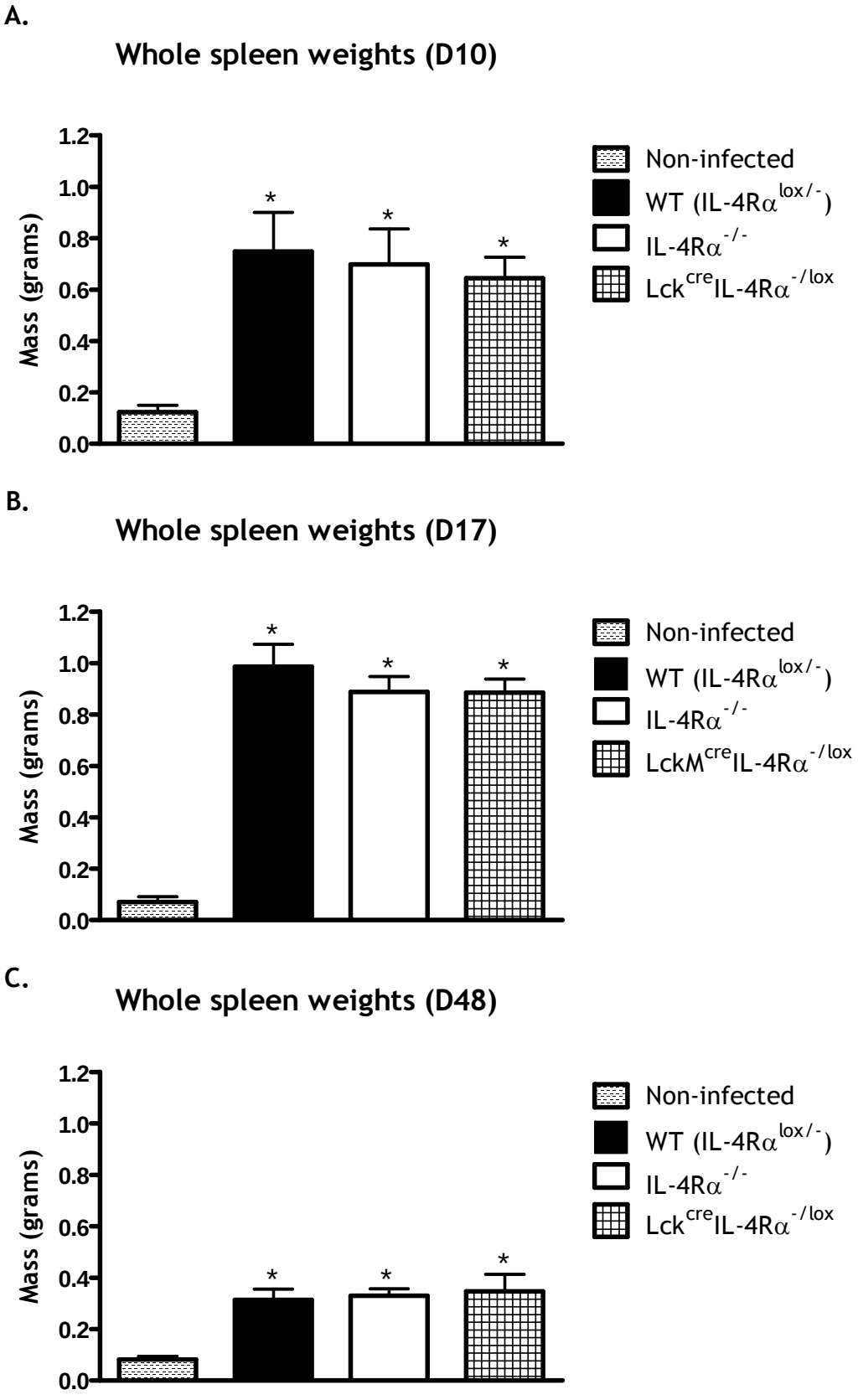


Figure 6.4: Comparison of the whole spleen tissue weights of non-infected and *P. chabaudi* AS infected WT(IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. Data are representative of two independent studies, n= 4-6. * denotes $p<0.05$.

6.3.3 Comparison of the splenic cytokine production in wild-type (WT), global IL-4Rα-deficient (IL-4Rα$^{-/-}$) and CD4^{+} T-cell IL-4Rα-deficient (Lck^{cre}IL-4Rα$^{-/lox}$) female mice following *Plasmodium chabaudi* AS infection.

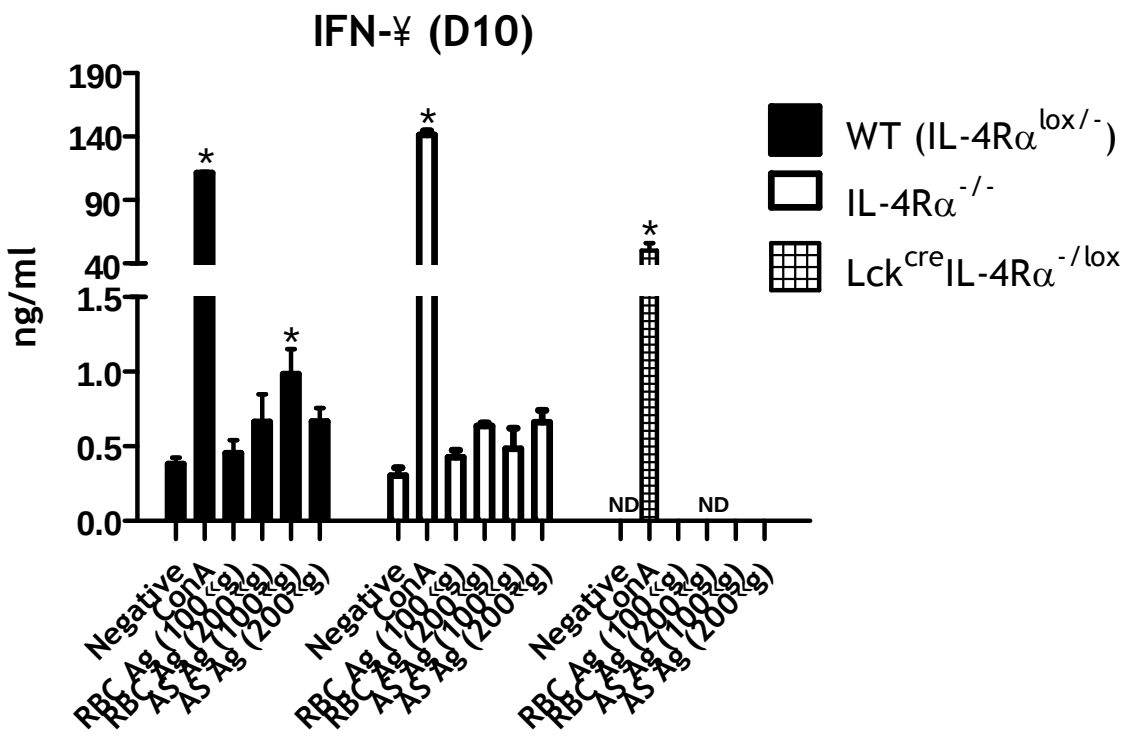
Th_1-type associated cytokine, IFN-γ, was significantly reduced in WT and Lck^{cre}IL-4Rα$^{-/lox}$ derived splenocytes following ConA stimulation from day 10 infected mice compared to IL-4Rα$^{-/-}$ derived splenocytes (Figure 6.5, A). IL-12 production was comparable between the groups with no striking differences (Figure 6.5, B). Th_2-type associated IL-10 cytokine levels were significantly greater in WT and IL-4Rα$^{-/-}$ derived splenocytes stimulated with specific *P. chabaudi* AS antigen compared to Lck^{cre}IL-4Rα$^{-/lox}$ derived splenocytes (Figure 6.6, A). Furthermore, IL-4 production was only significantly increased in the IL-4Rα$^{-/-}$ derived splenocytes following ConA stimulation (Figure 6.6, B).

On day 17 post-infection, IFN-γ production was significantly greater in the Lck^{cre}IL-4Rα$^{-/lox}$ ConA and antigen-specific stimulated splenocytes compared to WT and IL-4Rα$^{-/-}$ derived splenocytes (Figure 6.7, A). IL-12 production was only observed in the WT stimulated splenocytes: IL-12 levels were below the sensitivity of the ELISA in the Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ derived splenocytes (Figure 6.7, B). A significant increase in IL-10 production, seen in ConA stimulated splenocytes, occurred in WT mice compared to Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ derived splenocytes (Figure 6.8, A). However, Lck^{cre}IL-4Rα$^{-/lox}$ ConA stimulated splenocytes produced greater IL-10 cytokine levels than IL-4Rα$^{-/-}$ derived splenocytes at this time (Figure 6.8, A). In addition, significantly greater IL-4 production occurred in WT and IL-4Rα$^{-/-}$ derived splenocytes stimulated with ConA compared to Lck^{cre}IL-4Rα$^{-/lox}$ derived splenocytes (Figure 6.8, B).

At day 48, Lck^{cre}IL-4R$\alpha^{-/lox}$ mice displayed significantly increased IFN-γ production from ConA stimulated splenocytes compared to the WT and IL-4R$\alpha^{-/-}$ mice whilst IFN-γ production from antigen-specific stimulated WT and IL-4R$\alpha^{-/-}$ derived splenocytes was significantly increased compared to the Lck^{cre}IL-4R$\alpha^{-/lox}$ derived splenocytes (Figure 6.9, A). IL-12 production was comparable with no significant differences observed between the groups (Figure 6.9, B).

$LckM^{cre}$IL-4R$\alpha^{-/lox}$ mice displayed comparable IL-10 production with IL-4R$\alpha^{-/-}$ derived splenocytes following ConA stimulation but IL-10 production in these groups was significantly lower compared to WT derived splenocytes stimulated with ConA (Figure 6.10, A). IL-4 production was significantly reduced in the ConA stimulated splenocytes of $LckM^{cre}$IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice compared to the WT control (Figure 6.10, B). On the other hand, IL-4 production by antigen-specific stimulated Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ derived splenocytes was significantly increased compared to the WT mice (Figure 6.10, B).

A.



B.

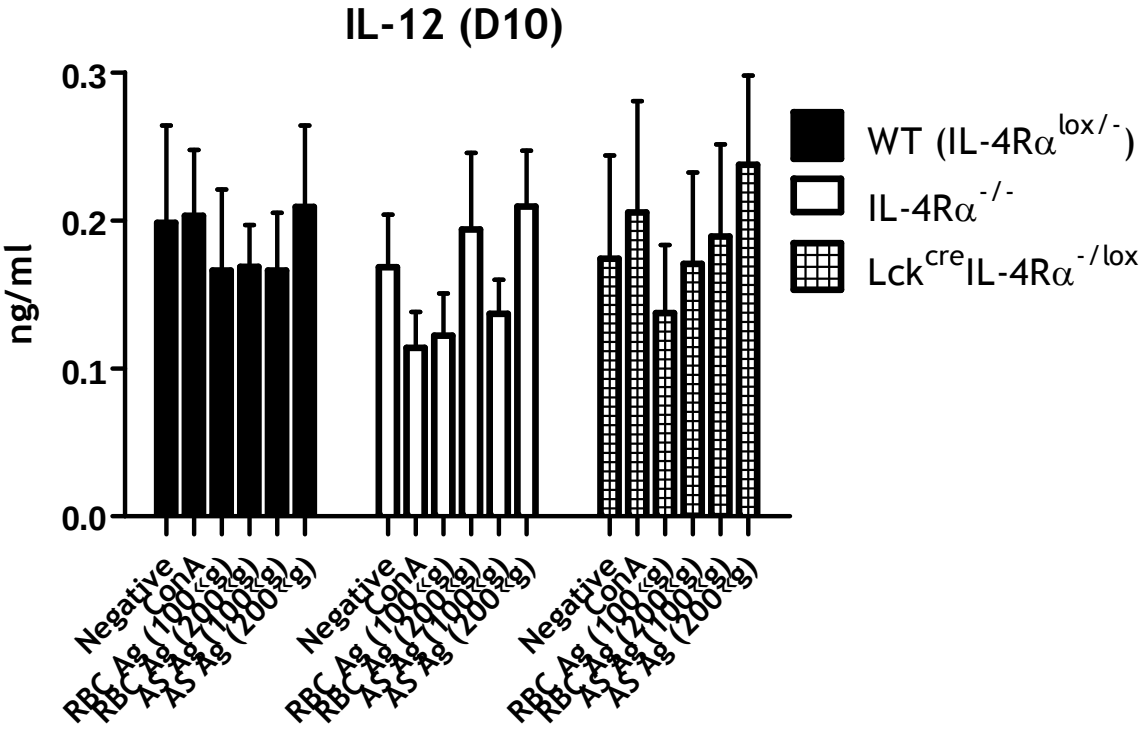


Figure 6.5: Comparison of day 10 splenic **(A)** IFN-γ and **(B)** IL-12 production in *P. chabaudi* AS infected WT(IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. Data are representative of two independent studies, n=4-5. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

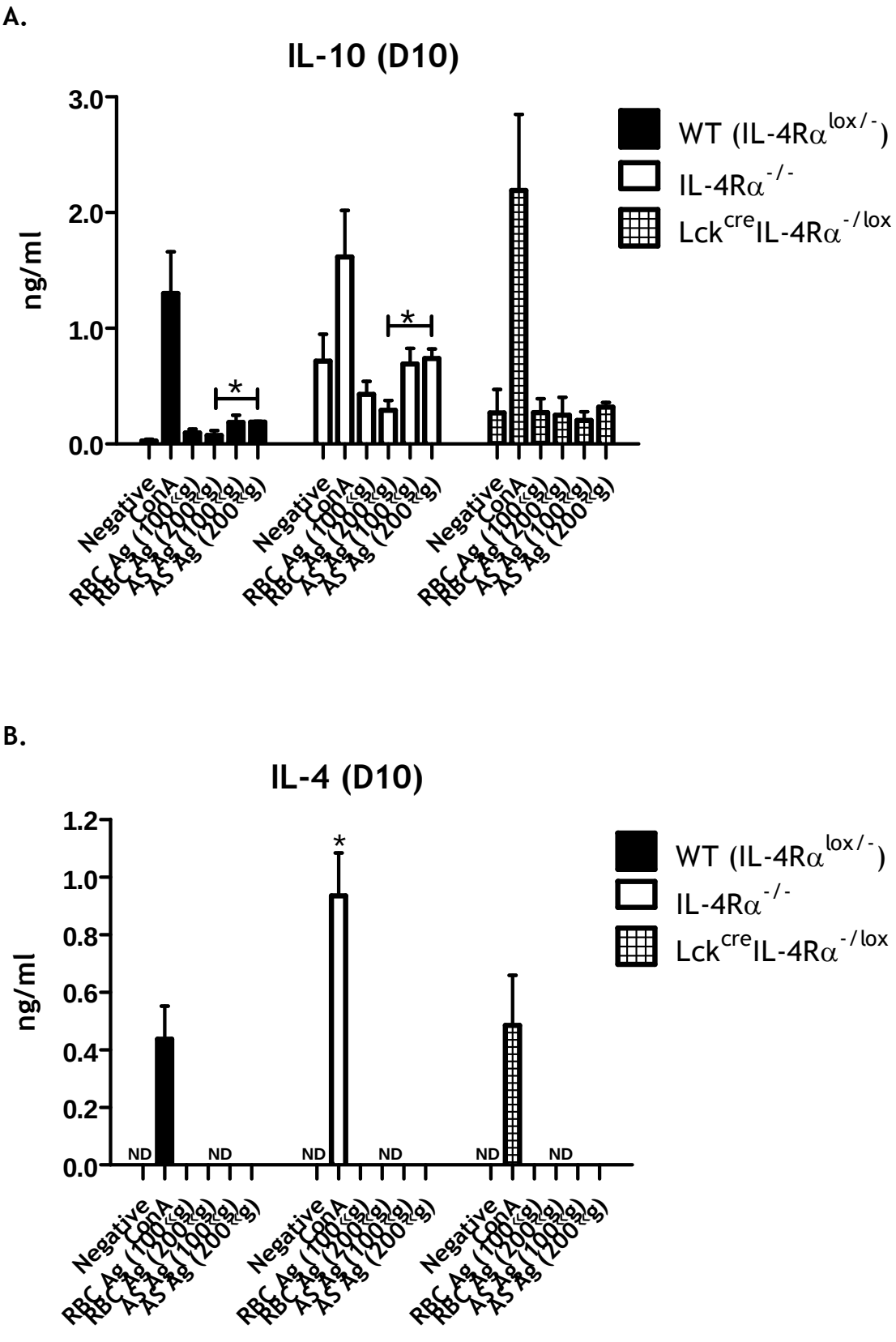


Figure 6.6: Comparison of day 10 splenic **(A)** IL-10 and **(B)** IL-4 production in *P. chabaudi* AS infected WT(IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. Data are representative of two independent studies, n=4-5. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

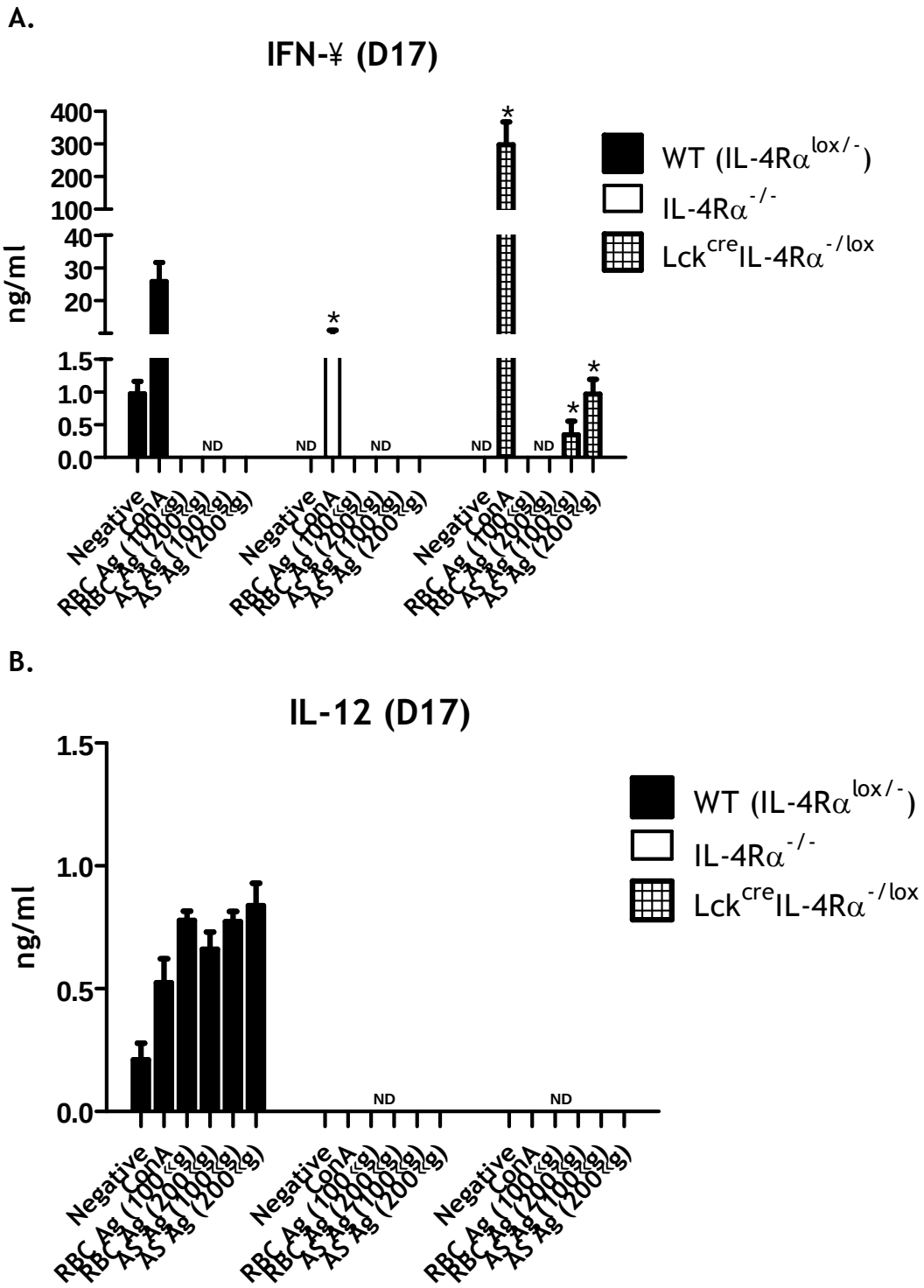


Figure 6.7: Comparison of day 17 splenic **(A)** IFN-γ and **(B)** IL-12 production in *P. chabaudi* AS infected WT(IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. Data are representative of two independent studies, n=4-5. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

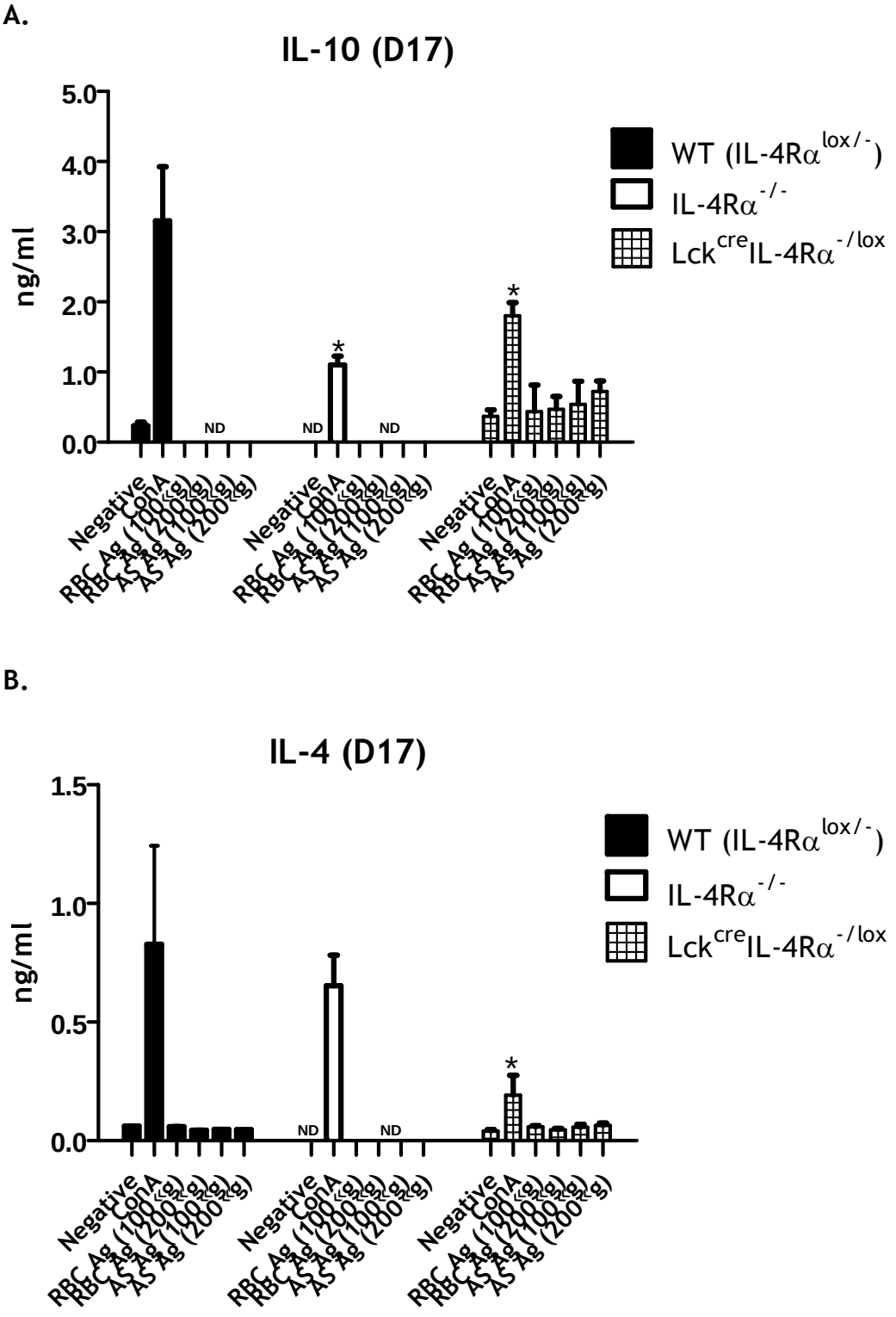


Figure 6.8: Comparison of day 17 splenic **(A)** IL-10 and **(B)** IL-4 production in *P. chabaudi* AS infected WT(IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. Data are representative of two independent studies, n=4-5. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

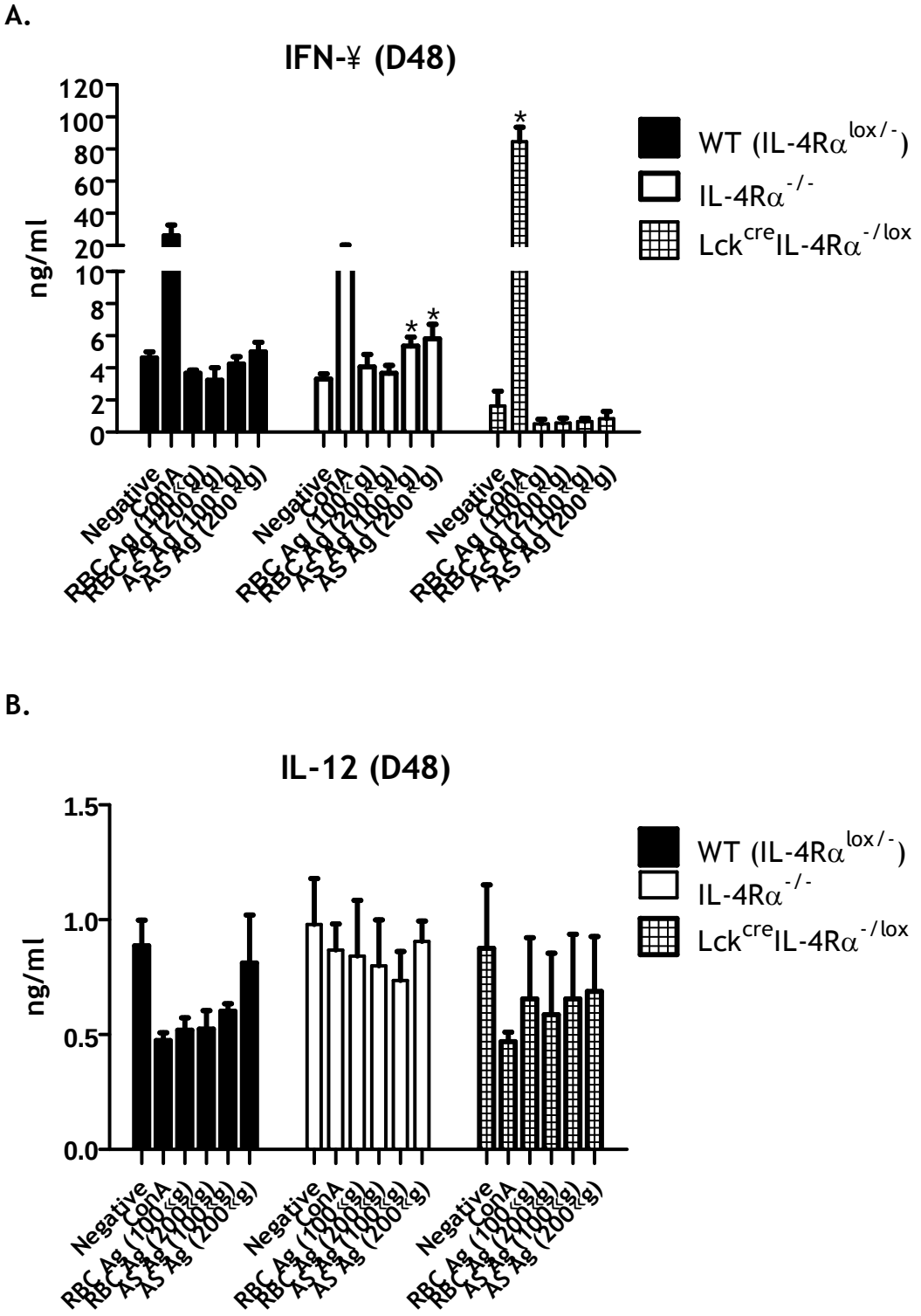


Figure 6.9: Comparison of day 48 splenic **(A)** IFN-γ and **(B)** IL-12 production in *P. chabaudi* AS infected WT(IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. Data are representative of two independent studies, n=4-5. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

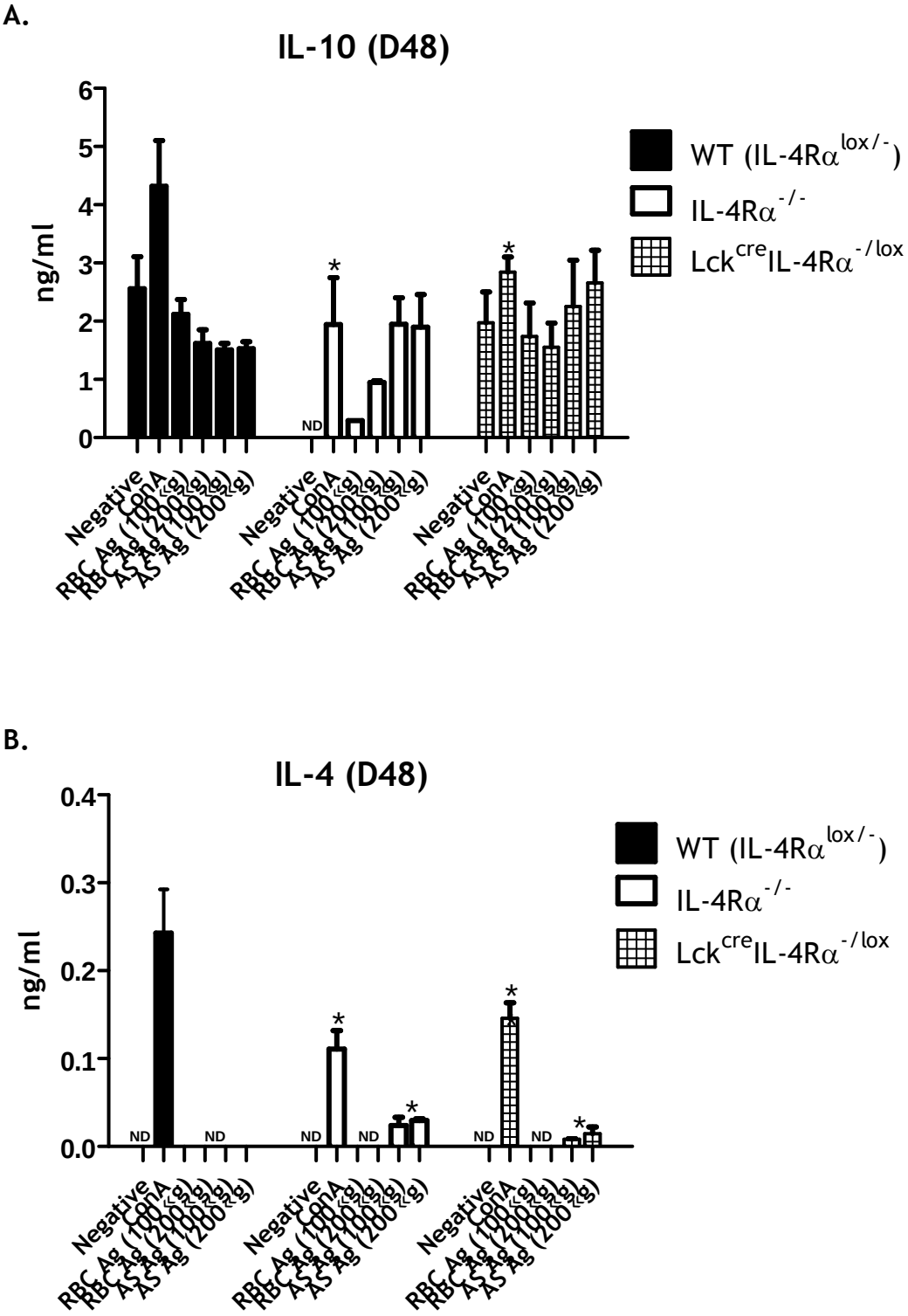


Figure 6.10: Comparison of day 48 splenic **(A)** IL-10 and **(B)** IL-4 production in *P. chabaudi* AS infected WT(IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. Data are representative of two independent studies, n=4-5. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

6.3.4 Comparison of the IgG2a and IgG1 antibody responses of $CD4^+$ IL-4Rα-deficient (Lck^{cre}IL-4Rα$^{-/lox}$) female mice following *Plasmodium chabaudi* AS infection.

Primary parasitaemia and peak infection demonstrated a significant increase in Th_1-type (IgG2a) antibody levels in WT and Lck^{cre}IL-4Rα$^{-/lox}$ compared to IL-4Rα$^{-/-}$ mice (Figure 6.11, A). All groups showed comparable IgG2a antibody responses at day 17 post infection (Figure 6.11, B). However, at day 48, IgG2a antibody levels showed a significant decrease in IgG2a levels in WT and Lck^{cre}IL-4Rα$^{-/lox}$ compared to IL-4Rα$^{-/-}$ mice (Figure 6.11, C).

Th_2-type antibody (IgG1) levels on days 10 and 17 were significantly increased in the WT and Lck^{cre}IL-4Rα$^{-/lox}$ compared to IL-4Rα$^{-/-}$ mice (Figure 6.12, A and B). In contrast, IgG1 antibody levels were subsequently significantly reduced in the gene-deficient mice compared to the WT control on day 48 of the infection (Figure 6.12, C).

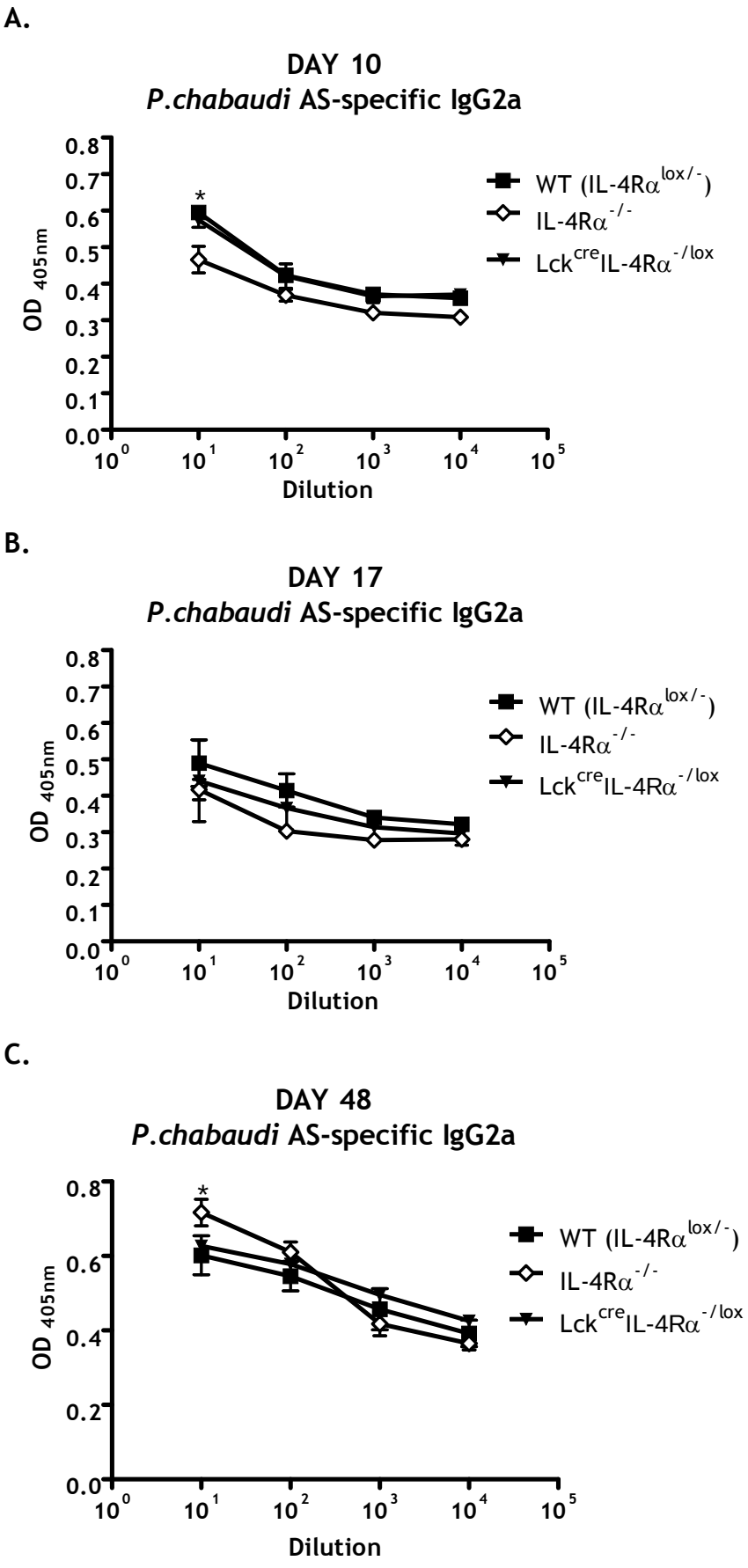


Figure 6.11: Comparison of the IgG2a antibody responses of *P. chabaudi* AS infected WT(IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/flox}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. Data are representative of two independent studies, n=4-6. * denotes $p<0.05$.

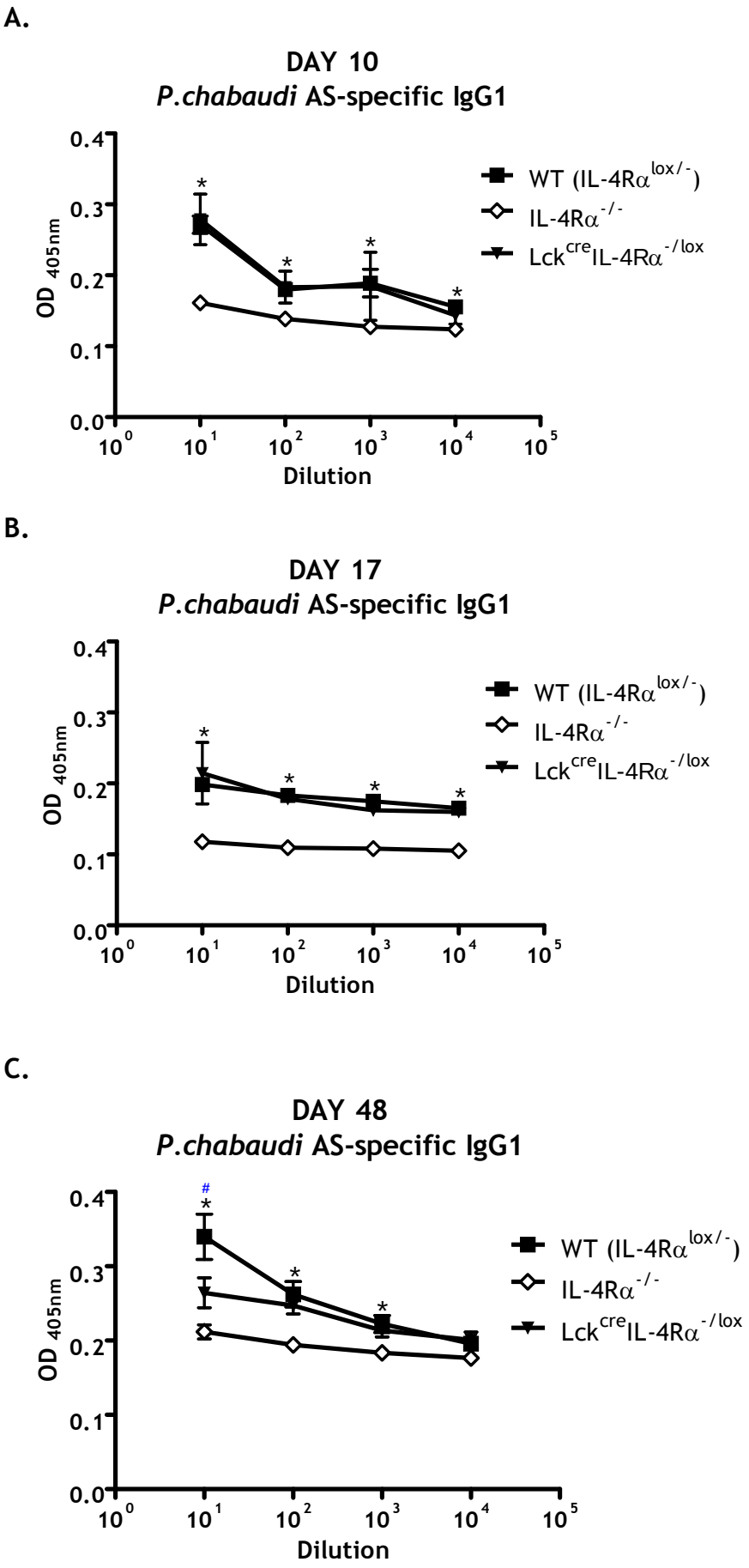


Figure 6.12: Comparison of the IgG1 antibody responses of *P. chabaudi* AS infected WT(IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/flox}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. Data are representative of two independent studies, n=4-6. * and $^{\#}$ denotes $p<0.05$.

6.4 Discussion

$CD4^+$ T-cells play a central role in protective immunity to *Plasmodium* infection (Stephens *et al.*, 2005, Langhorne *et al.*, 2002). Although signal transduction triggered by IL-4 has been extensively studied on $CD4^+$ T-cells in various disease models, the exact signalling events induced by IL-4/IL-13 via IL-4Rα specifically on $CD4^+$ T-cells have not yet been characterized in a model of murine malaria. To our knowledge, this report constitutes the first comprehensive analysis of the immunological events that shape a $CD4^+$ T-cell response to IL-4 and IL-13 during chronic *P. chabaudi* AS infection. Our findings specifically demonstrate that the abrogation of IL-4Rα signalling of $CD4^+$ T-cells resulted in the suppression of protective responses during chronic-stage disease. $CD4^+$ T-cell-specific IL-4Rα$^{-/-}$ (Lck^{cre}IL-4Rα$^{-/lox}$) mice generated, have a null mutation of IL-4Rα on $CD4^+$ T-cells, an incomplete deletion on $CD8^+$ T-cells and other T-cell subpopulations, and normal expression on non-T-cells (Bryson *et al.*, 2011, Michels *et al.*, 2009, Radwanska *et al.*, 2007, Leeto *et al.*, 2006). Our hypothesis was confirmed when the Lck^{cre}IL-4Rα$^{-/lox}$ mice, in response to *P. chabaudi* AS infection, were unable to induce an effective chronic protective Th_2-type immune response that was observed within the WT mice. Protection was therefore mainly dependent on $CD4^+$ T-cells responsiveness to IL-4.

The disease phenotype of the acute-stage infection in the Lck^{cre}IL-4Rα$^{-/lox}$ mice was comparable to that of the WT control. However, the profile changed following day 17 when the Lck^{cre}IL-4Rα$^{-/lox}$ mice demonstrated greater mortality on days 36 and 40 and recrudescence was evident on days 17, 22 and 28 corresponding to the reduced RBC count at day 22. Weight loss was not a significant factor at this point but these mice did however, gain weight faster than the WT control mice (days 12, 14, 17). The IL-4Rα$^{-/-}$ mice induced an impaired Th_2 immune response reminiscent of studies consistently shown by ourselves and others (Couper, 2003). The absence of the IL-4Rα subunit was hallmarked by observations of recrudescent parasitaemia, increased mortality and impaired Th_2-type immunity during the chronic stage. *P. chabaudi* blood-stage infection in the Lck^{cre}IL-4Rα$^{-/lox}$ in comparison to the IL-4Rα$^{-/-}$ mice were characterized by significantly elevated parasitaemia at day 3 and greater number of deaths during the chronic-stage of the disease. Anaemia is one of the major presentations of hyperreactive malarial splenomegaly while splenomegaly

is defined as reticuloendothelial and lymphoid hyperplasia due to chronic malaria infection (del Portillo *et al.*, 2011, Bryceson *et al.*, 1983). Splenomegaly between the groups was comparable throughout the course of infection suggesting that parasitaemia alone does not account for the increase in spleen size but does demonstrate the crucial importance of the spleen in parasite clearance (del Portillo *et al.*, 2011). This was similarly shown in human *P. falciparum* infections where splenectomised patients invariably showed an increase in parasitaemia during infection regardless of anti-malarial agents used (del Portillo *et al.*, 2011, Bachmann *et al.*, 2009). Taken together, the IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice seemed to control the initial acute-stage infection adequately but struggled to maintain protection and were susceptible to long-term disease, evidenced with increased mortality, compared to the WT mice. The complete absence of IL-4Rα responsiveness versus cell-specific deletion of IL-4Rα on CD4^{+} T-cells suggested that non-CD4^{+} T-cell IL-4Rα-dependent responses were insufficient to induce protection to chronic *P. chabaudi AS infection*.

At peak infection (day 10 post-infection), IFN-γ production from ConA stimulated splenocytes, although significantly lower than the WT control, correlated with IgG2a antibody responses from stimulated B-cells. Accumulating evidence indicates that activated CD4^{+} T cells release factors, including IFN-γ, which induce downstream mechanisms to kill parasites (Stephens *et al.*, 2005, Langhorne *et al.*, 2002, Li and Langhorne, 2000; Troye-Blomberg *et al.*, 1999; Mohan and Stevenson, 1998). IFN-γ is produced by natural killer (NK) cells, γδ T-cells, and CD4^{+} T-cells (Seixas *et al.*, 2002; Mohan *et al.*, 1997), all of which may play some role in controlling parasitemia, but it is the CD4^{+} T-cells together with B-cells that are crucial for the development of protective immunity (Stephens *et al.*, 2005, Langhorne *et al.*, 2002, Langhorne *et al.*, 1998; von der Weid *et al.*, 1996). The results seen in our experiments give further evidence that it is via IL-4Rα signalling on CD4^{+} T-cells that is crucial for the development of protective *P. chabaudi* AS immunity. Much of the pathogenesis of the acute blood-stage malaria infection in this model has been attributed to pro-inflammatory responses induced in part by IFN-γ from Th_1 CD4^{+} T-cells (Li *et al.*, 2001).

Following the acute infection and declining parasitaemia, at day 17, $CD4^+$ T-cell responses where characterized by a marked IFN-γ increase following ConA and antigen-specific stimulated splenocytes in the Lck^{cre}IL-4Rα$^{-/lox}$ mice compared to the WT and IL-4Rα$^{-/-}$ mice. It may be suggested that the presence of non-$CD4^+$ T-cells responding to IL-4Rα signalling is responsible for the induction of an elevated IFN-γ response in the Lck^{cre}IL-4Rα$^{-/lox}$ mice compared to the WT and IL-4Rα$^{-/-}$ mice since it has been established that IL-4/IL-13 can not only counter-regulate Th_1 responses but can also actually drive or facilitate the promotion of Th_1 responses (Alexander and McFarlane, 2008). Compared to the WT control, reduced IL-10 production of ConA stimulated splenocytes was evident in the gene deficient mice but IL-10 was significantly greater in the Lck^{cre}IL-4Rα$^{-/lox}$ mice compared to the IL-4Rα$^{-/-}$ mice at this stage. The elevated IL-10 levels in the Lck mice compared to the IL-4Rα$^{-/-}$ mice may have occurred as a result of the significantly elevated IFN-γ response observed at that time which required a greater suppressive action of the proinflammatory responses. IL-10 is known as an anti-inflammatory cytokine that plays an important role in regulating proinflammatory responses in malaria (Niikura *et al.*, 2011). In contrast to the WT and IL-4Rα$^{-/-}$ groups, Lck^{cre}IL-4Rα$^{-/lox}$ mice demonstrated reduced IL-4 cytokine production upon ConA stimulation but IgG1 antibody levels were still significantly higher in this group. These findings suggest an alternative route of IL-4 production from non-$CD4^+$ Th_2 cells resulting in B-cell activation of IgG1 humoral responses. Furthermore, another explanation for the presence of a mild Th_2 antibody response is the role of $CD8^-$ DCs. These DCs can induce the proliferation of MSP-1 specific transgenic $CD4^+$ T-cells and thereby induce IL-4 and IL-10 production as investigated previously (Sponaas *et al.*, 2006).

Upon termination of the experiment, at day 48, the immunological response in the gene-deficient mice yielded a greater Th_1-type response. A marked IFN-γ increase following ConA stimulated splenocytes in the Lck^{cre}IL-4Rα$^{-/lox}$ mice was still evident compared to the WT control and IL-4Rα$^{-/-}$ mice. However, this was reduced upon antigen-specific stimulation. Th_2-type responses in the gene-deficient mice were significantly impaired characterized by reduced IL-10 and IL-4 production following ConA stimulated splenocytes corresponding to significantly reduced IgG1 antibody levels. The importance of IL-10 as a regulatory cytokine due to its anti-inflammatory capabilites as mentioned before

(Niikura *et al*., 2011) again provides additional evidence that the reduced IL-10 levels observed coincided with the increased IFN-γ response. As a result of the abrogation of IL-4Rα signalling in IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice, the immunological response was biased toward a Th_1 phenotype but which failed to adequately provide complete protection in these gene-deficient mice. Together, these findings indicate that due to a dysregulated Th_2 protective immune response, the IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice are increasingly susceptible to the infection. In conclusion, we demonstrate that a major role exists for CD4$^+$ T-cell responsiveness to IL-4 during chronic murine malaria disease.

Findings in the present study have for the first time, provided evidence for the importance of IL-4Rα signalling on CD4$^+$ T-cells during chronic murine malaria disease. We show that initial control of acute infection relies on proinflammatory Th_1-type responses, which have been well documented and supported by our data. However, during the chronic stage, it is clear from the data presented here that Th_2-type responses are necessary for adequate protection but mainly dependent on IL-4Rα activated CD4$^+$ T-cells. In various infectious disease models, the effects of IL-4 responsive CD4$^+$ T-cells do not always provide resistance to infection but rather increases susceptibility. For example, in a more recent study by Bryson and colleagues where they infected Lck^{cre}IL-4Rα$^{-/lox}$ mice with *L. mexicana*, it was demonstrated that these mice developed small lesions that subsequently healed indicating that progressive infection was dependent on CD4$^+$ T-cell responsive to IL-4 (Bryson *et al*., 2011). Similarly, during *L. major* infection of Lck^{cre}IL-4Rα$^{-/lox}$ mice, mice were completely resistant to infection indicating no role for protection from CD4$^+$ T-cells responsive to IL-4 (Dewals *et al*., 2009, Radwanska *et al*., 2007).

These novel findings within a murine model of malaria infection have provided some insight into the mechanisms governing Th_2 immunity during chronic-stage *P. chabaudi* AS infection. We next (Chapter 7) wanted to extend our investigation further by looking at the effects of abrogation of IL-4Rα signalling on all T-cell populations in our model. With the availability of CD4$^+$CD8$^+$ T-cell IL-4Rα-deficient (iLck^{cre}IL-4Rα$^{-/lox}$) mice, we were able to determine whether IL-4Rα–dependent pathways of CD4$^+$CD8$^+$ T-cell activation was required for protective immunity during the chronic stage of *P. chabaudi* AS infection.

Chapter Seven

The role of IL-4Rα signalling on $CD4^+$ and $CD8^+$ T-cells during *Plasmodium chabaudi* AS erythrocyte infection in BALB/c female mice.

7.1 Abstract

The role of IL-4Rα signalling via $CD4^+$ and $CD8^+$ T-cell populations during *P. chabaudi* AS infection was determined using a novel mouse model lacking the IL-4Rα expression specifically on all T-cells ($iLck^{cre}IL\text{-}4R\alpha^{-/lox}$). Here, we demonstrate that $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice infected with *P. chabaudi* AS were not as susceptible to infection as $IL\text{-}4R\alpha^{-/-}$ mice as measured by survival and recrudescent infections during the course of the experiment. In addition, $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice had comparable parasitaemia levels to the WT control throughout the infection. Significant weight loss was obtained in the $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice at day 22 with increased anaemia observed at days 17 and 22 compared to the $IL\text{-}4R\alpha^{-/-}$ mice. Interestingly, splenomegaly was significantly lower in the $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice at days 10 and 17 but then significantly increased at day 48 compared to the WT and $IL\text{-}4R\alpha^{-/-}$ mice. The immunology of infection in $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice was characterized by an early Th_1 phenotype as measured by splenic IFN-γ and increased serum IgG2a antibody titres, similarly found in the $IL\text{-}4R\alpha^{-/-}$ mice. Subsequently, during the latter part of the infection, Th_2 responses were impaired with significantly reduced splenic IL-4 and IL-10 cytokine production in both gene-deficient mice compared to the WT control. Although, no mortalities were observed in the $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice, there was some evidence of a delayed impaired Th_2-type immune response in these mice. In conclusion, the results indicated that $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice were more resistant to infection compared to the $IL\text{-}4R\alpha^{-/-}$ mice but still displayed a diminished Th_2 protective immune response compared with WT mice later in infection.

7.2 Introduction

It is well established that immunity against the pre-erythrocytic stages of *Plasmodium* infection depends on $CD8^+$ T-cell responses (Lundie *et al.*, 2008, Good and Doolan, 1999). On the contrary, immunity to the erythrocytic stages is largely dependent on $CD4^+$ T-cell and humoral responses. Although $CD8^+$ T-cells were shown to not protect against erythrocytic stages of infection, evidence in murine models have indicated that $CD8^+$ T-cells contribute to the pathology of experimental cerebal malaria (ECM) (Lundie *et al.*, 2008, Renia *et al.*, 2006).

IL-4 is a crucial mediator of $CD4^+$ Th_2 cell differentiation and suppression of IFN-γ producing $CD4^+$ Th_1 cells (de Sa Pinheiro *et al.*, 2007, Nelms *et al.*, 1999). This cytokine also plays a pivotal role in the differentiation of B-cells controlling the specificity of IgG class switching and the development of memory B-cells. Several studies have also indicated that IL-4 helps to sustain the growth and prolongs survival of $CD4^+$ T and B-cells (Nelms *et al.*, 1999). Furthermore, IL-4 modulates important functions of $CD8^+$ T-cells, which compared with $CD4^+$ T-cells undergo distinct differentiation pathways and exhibit vastly different functional properties. Previous *in vitro* studies have described a number of effects of IL-4 on $CD8^+$ T-cells including the induction of IL-4 secretion and enhancement of IL-2 induced proliferation (Kienzle *et al.*, 2002, Miller *et al.*, 1990, Acres *et al.*, 1988). Further studies have suggested a role for IL-4 in the *in vitro* development of cytotoxic T-cells (Trenn *et al.*, 1988, Palacious *et al.*, 1987) and some studies indicated that IL-4 could decrease cytolytic activity *in vitro* (Falchetti *et al.*, 1996). IL-4 was also shown to be necessary for the *in vitro* generation of memory $CD8^+$ T-cells (Huang *et al.*, 2000). de Sa Pinheiro and colleagues have revealed a role for IL-4 in the generation of memory $CD8^+$ T-cells against the pre-erythrocytic stages of the parasite in a *P. yoelii* mouse model using parasite-specific transgenic $CD8^+$ T-cells (de Sa Pinheiro *et al.*, 2007). Using IL-$4R^{-/-}$ mice, they demonstrated that IL-4 acts directly on activated anti-parasitic $CD8^+$ T-cells through the IL-4R and promote the survival of memory $CD8^+$ T-cells (de Sa Pinheiro *et al.*, 2007).

The generation of macrophage/neutrophil IL-4Rα deficient ($LysM^{cre}$IL-$4R\alpha^{-/lox}$) mice in chapter 5 has shown that the alternate activation of macrophages was not a requirement for chronic protective immunity during *P. chabaudi* AS infection. Furthermore, the utilization of $CD4^+$ T-cell specific IL-$4R\alpha^{-/-}$ mice has demonstrated a significant role for IL-4 signalling via $CD4^+$ T-cells in limiting recrudescent episodes in chronic infections. However, $CD8^+$ T-cells were still able to respond to IL-4 in these mice. In order to determine whether IL-4 signalling via $CD8^+$ T-cells also played a role in control, we studied the course of *P. chabaudi* AS infection by utilizing $iLck^{cre}$IL-$4R\alpha^{-/lox}$ mice (Bryson *et al.*, 2011, Dewels *et al.*, 2009) that had a pan T-cell deletion of IL-4Rα.

7.3 Results

7.3.1 Comparison of the survival rates and disease phenotypes of wild-type (WT), $CD4^+CD8^+$ T-cell IL-4Rα-deficient ($iLck^{cre}IL\text{-}4R\alpha^{-/flox}$) and global IL-4Rα-deficient ($IL\text{-}4R\alpha^{-/-}$) female mice following *Plasmodium chabaudi* AS infection.

WT and $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice survived the acute stage infection with no mortalities observed whereas the $IL\text{-}4R\alpha^{-/-}$ mice displayed an enhanced mortality rate of 17% at day 12 post infection, as shown previously in chapter 4, (Figure 4.1). During the chronic-stage infection, 17% mortality was observed in the WT mice on day 33 and again the majority of deaths were observed in the $IL\text{-}4R\alpha^{-/-}$ mice at day 36, 33% mortality (Figure 7.1). In contrast, no fatalities were observed in the $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice during the course of the study (Figure 7.1).

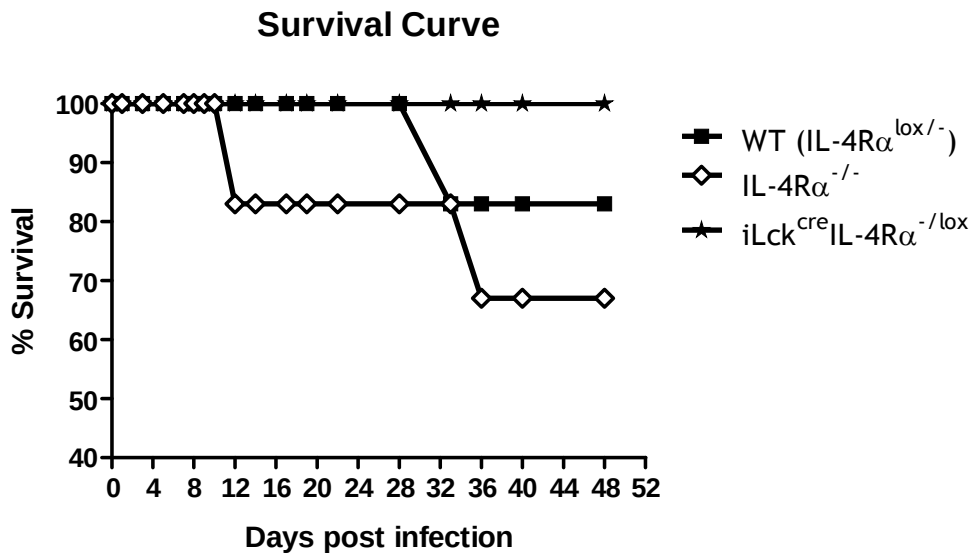


Figure 7.1: Comparison of the survival rates of *P. chabaudi* AS infection in WT ($IL\text{-}4R\alpha^{lox/-}$), $IL\text{-}4R\alpha^{-/-}$ and $iLckM^{cre}IL\text{-}4R\alpha^{-/flox}$ female mice on a BALB/c background. WT ($IL\text{-}4R\alpha^{lox/-}$) n=9, $IL\text{-}4R\alpha^{-/-}$ n=6 and $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ n=7.

Parasite burden at day 3 was significantly higher in the WT and iLck^{cre}IL-4Rα$^{-/lox}$ mice compared to IL-4Rα$^{-/-}$ mice (Figure 7.2) while peak infection (day 7) showed no significant differences between the groups (Figure 6.2). Furthermore, no significant differences in parasite load occurred between the WT and iLck^{cre}IL-4Rα$^{-/lox}$ mice throughout the duration of the disease. Only the IL-4Rα$^{-/-}$ mice demonstrated significantly greater recrudescent parasitaemias during the course of the disease (Figure 7.2).

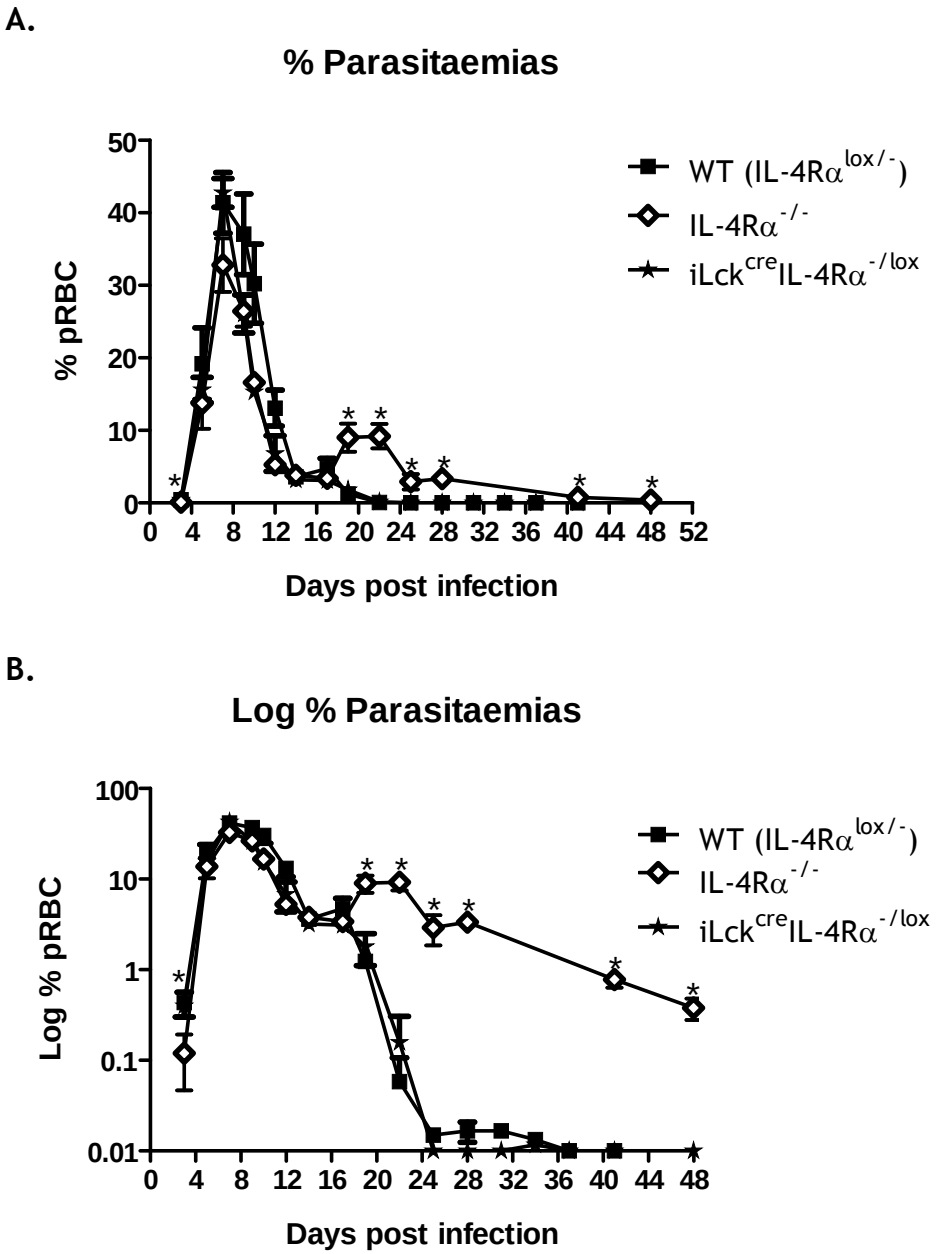
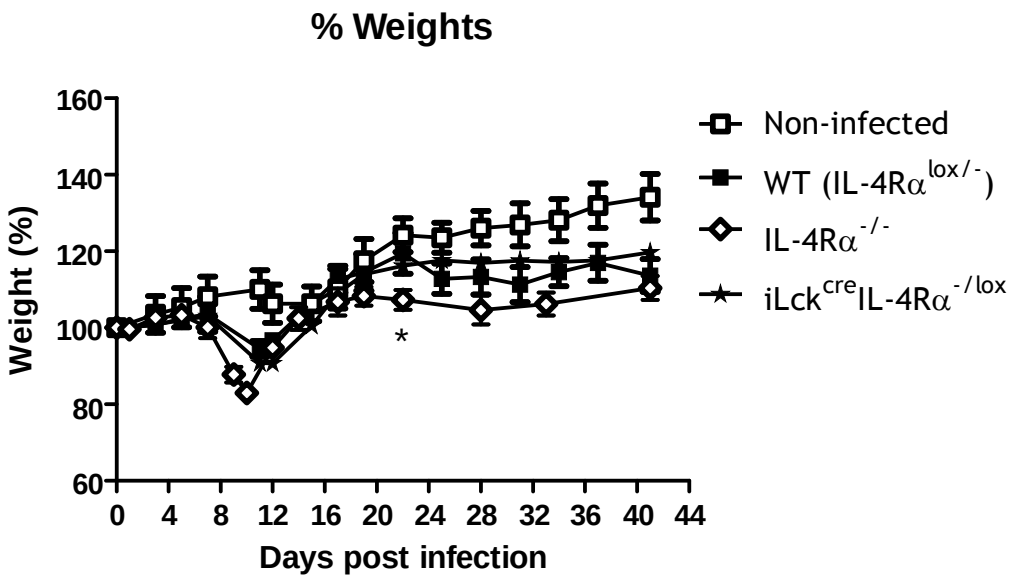


Figure 7.2: Comparison of the disease parasitaemias of *P. chabaudi* AS infection in WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. Results are displayed as a **(A)** % and as a **(B)** log % of parasitaemia levels in these groups. n=6. * denotes $p<0.05$.

Non-infected mice showed a steady weight gain over the 48 day period (Figure 7.3, A). Maximal weight loss in the infected IL-4Rα$^{-/-}$ mice was reached earlier at day 10 compared to the infected WT and iLck^{cre}IL-4Rα$^{-/lox}$ mice which demonstrated maximal weight loss at day 12 (Figure 7.3, A). Following recovery, all mice showed an increase in weight while a significant increase in weight was only observed in the WT and iLck^{cre}IL-4Rα$^{-/lox}$ mice when compared to the IL-4Rα$^{-/-}$ mice at day 22 (Figure 7.3, A).

Contrary to non-infected mice, severe anaemia was reached in all groups at day 10 post-infection with a significant drop in RBC counts (Figure 7.3, B). At day 10 of the peak infection, no differences in RBC counts were observed between the groups. However, at day 12, RBC counts were significantly reduced in the iLck^{cre}IL-4Rα$^{-/lox}$ mice compared to the WT control and IL-4Rα$^{-/-}$ mice (Figure 7.3, B). Following parasite control during the latter stage of the infection, a significant increase in RBC count was observed in the iLck^{cre}IL-4Rα$^{-/lox}$ mice compared to the IL-4Rα$^{-/-}$ mice on day 14 while no significant differences occurred between the WT and iLck^{cre}IL-4Rα$^{-/lox}$ mice (Figure 7.3, B). Furthermore, the RBC count then dropped in the iLck^{cre}IL-4Rα$^{-/lox}$ mice on days 17 and 22 compared to the IL-4Rα$^{-/-}$ mice (Figure 7.3, B) but seemed to level off in all the groups as time progressed (Figure 7.3, B).

A.



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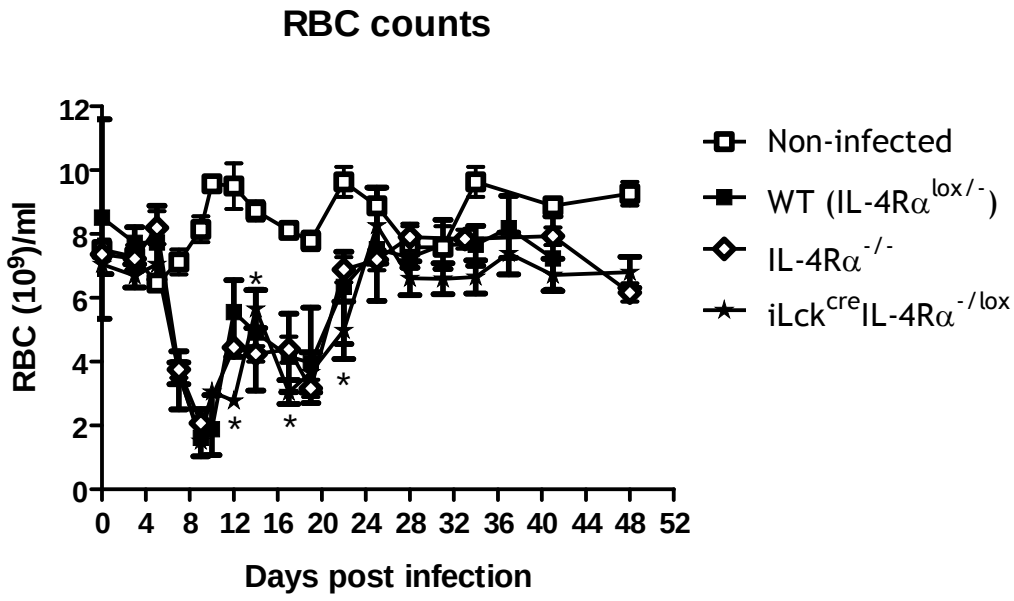


Figure 7.3: Comparison of **(A)** weight loss and **(B)** malaria induced anaemia during the course of *P. chabaudi* AS infection in WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/flox}$ female mice on a BALB/c background. WT (IL-4Rα$^{lox/-}$) n=5-9, IL-4Rα$^{-/-}$ n=6 and iLck^{cre}IL-4Rα$^{-/flox}$ n=7. Results are a representative of two separate experiments. * denotes $p<0.05$.

7.3.2 The influence of chronic disease on splenomegaly in $CD4^+CD8^+$ T-cell IL-4Rα-deficient ($iLck^{cre}IL\text{-}4R\alpha^{-/lox}$) female BALB/c mice infected with *Plasmodium chabaudi* AS.

Whole spleen weights of non-infected and infected WT, $IL\text{-}4R\alpha^{-/-}$ and $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice were measured to determine the severity of splenomegaly during the course of *P. chabaudi* AS infection between the respective groups. It was apparent that *P. chabaudi* AS parasite directly causes splenomegaly in the infected WT and $IL\text{-}4R\alpha^{-/-}$ groups compared to non-enlarged spleens observed in the non-infected mice throughout the duration of the disease (Figure 7.4). Interestingly, no increase in whole spleen weight was observed in the infected $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice compared to the non-infected group at day 10 (Figure 7.4, A) whilst a significant increase was seen in the infected WT and $IL\text{-}4R\alpha^{-/-}$ groups. As the infection progressed, all infected groups demonstrated splenomegaly compared to non-infected mice at day 17 post-infection (Figure 7.4, B). However, $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice again showed a significant decrease in whole spleen weight when compared to the infected WT and $IL\text{-}4R\alpha^{-/-}$ mice (Figure 7.4, B). Furthermore, at day 48, no significant difference in the severity of splenomegaly was observed between the infected WT control and $IL\text{-}4R\alpha^{-/-}$ mice but a striking increase was observed in the $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ group at this time (Figure 7.4, C).

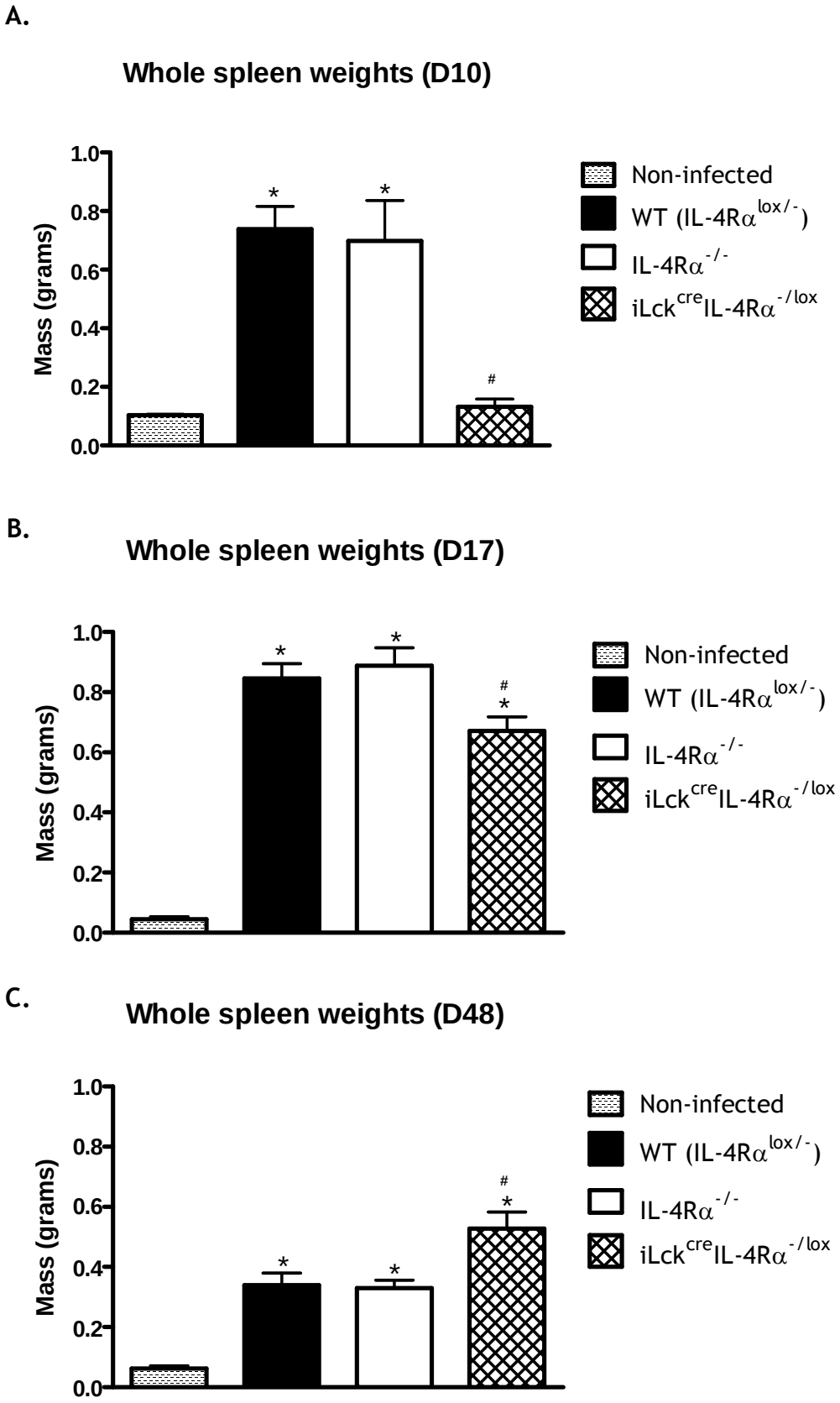


Figure 7.4: Comparison of the whole spleen tissue weights of non-infected and *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. n=5-8. * Infected vs non-infected mice and $^{\#}$ iLck^{cre}IL-4Rα$^{-/flox}$ vs WT (IL-4Rα$^{lox/-}$) and IL-4Rα$^{-/-}$ mice. *and $^{\#}$ denotes $p<0.05$.

7.3.3 Comparison of the splenic cytokine production in wild-type (WT), global IL-4Rα-deficient (IL-4Rα$^{-/-}$) and CD4^{+}CD8^{+} T-cell IL-4Rα-deficient (iLck^{cre}IL-4Rα$^{-/lox}$) female BALB/c mice following *Plasmodium chabaudi* AS infection.

IFN-γ production, was significantly reduced by WT derived splenocytes following ConA stimulation from day 10 infected mice compared to the gene-deficient mice (Figure 7.5, A). On the other hand, IFN-γ production from ConA stimulated splenocytes was significantly lower in the iLck^{cre}IL-4Rα$^{-/lox}$ group compared to the IL-4Rα$^{-/-}$ mice (Figure 7.5, A). IL-12 production was comparable between the groups with no striking differences (Figure 7.5, B). Th2 biased IL-10 cytokine levels were significantly greater in the WT derived splenocytes stimulated with ConA compared to gene-deficient mice derived splenocytes (Figure 7.6, A) while IL-4 production demonstrated no significant differences between the groups (Figure 7.6, B).

IFN-γ production, at day 17 post-infection, was significantly greater in the WT and iLck^{cre}IL-4Rα$^{-/lox}$ ConA and antigen-specific stimulated splenocytes compared to the IL-4Rα$^{-/-}$ derived splenocytes (Figure 7.7, A). IL-12 production was only observed in the WT and iLck^{cre}IL-4Rα$^{-/lox}$ stimulated splenocytes and was below the sensitivity of the sensitivity of the ELISA in the IL-4Rα$^{-/-}$ derived splenocytes (Figure 7.7, B). No significant differences in IL-10 production was observed between the groups (Figure 7.8, A) while IL-4 production from ConA stimulated splenocytes of IL-4Rα$^{-/-}$ mice were significantly greater than the WT and iLck^{cre}IL-4Rα$^{-/lox}$ stimulated splenocytes (Figure 7.8, B).

At day 48, WT and iLck^{cre}IL-4Rα$^{-/lox}$ mice displayed significantly increased IFN-γ production from ConA stimulated splenocytes compared to the IL-4Rα$^{-/-}$ mice whilst IFN-γ production from antigen-specific stimulated IL-4Rα$^{-/-}$ derived splenocytes was significantly increased compared to the WT and iLck^{cre}IL-4Rα$^{-/lox}$ derived splenocytes (Figure 7.9, A). IL-12 production was not significant although it seemed greater in the IL-4Rα$^{-/-}$ mice (Figure 7.9, B). WT and gene-deficient mice displayed comparable IL-10 production following ConA stimulation but IL-10 production were significantly greater in the WT and IL-4Rα$^{-/-}$ derived splenocytes stimulated with antigen compared to the iLck^{cre}IL-4Rα$^{-/lox}$ derived splenocytes

(Figure 7.10, A). Furthermore, IL-4 production from ConA stimulated splenocytes was significantly reduced in the gene-deficient mice compared to the WT control (Figure 7.10, B). In addition, IL-4 was significantly elevated in the iLck^{cre}IL-4Rα$^{-/lox}$ derived splenocytes following ConA and antigen-specific stimulation compared to the IL-4Rα$^{-/-}$ mice (Figure 7.10, B).

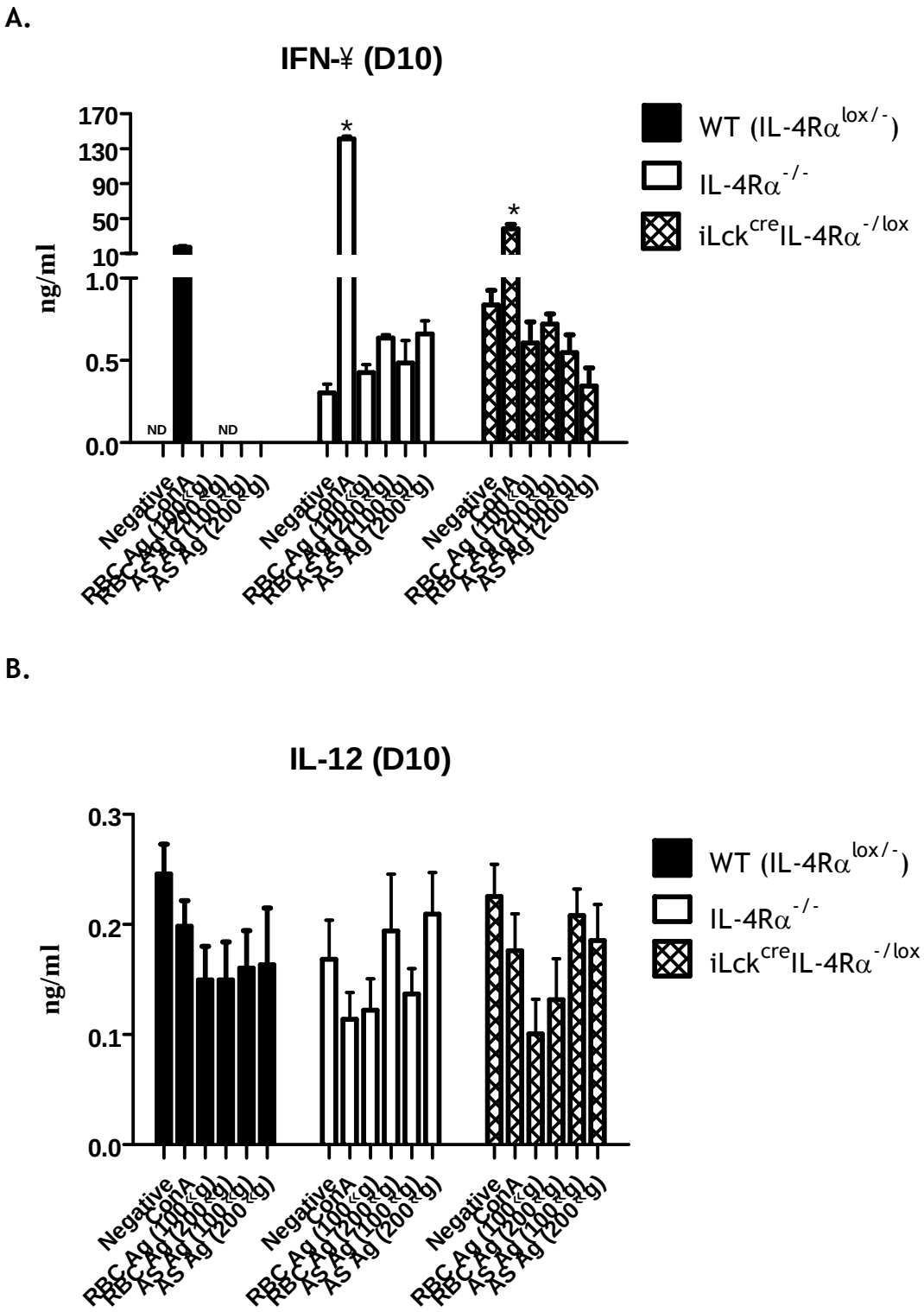


Figure 7.5: Comparison of day 10 splenic **(A)** IFN-γ and **(B)** IL-12 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/flox}$ female mice on a BALB/c background. n=4-5. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

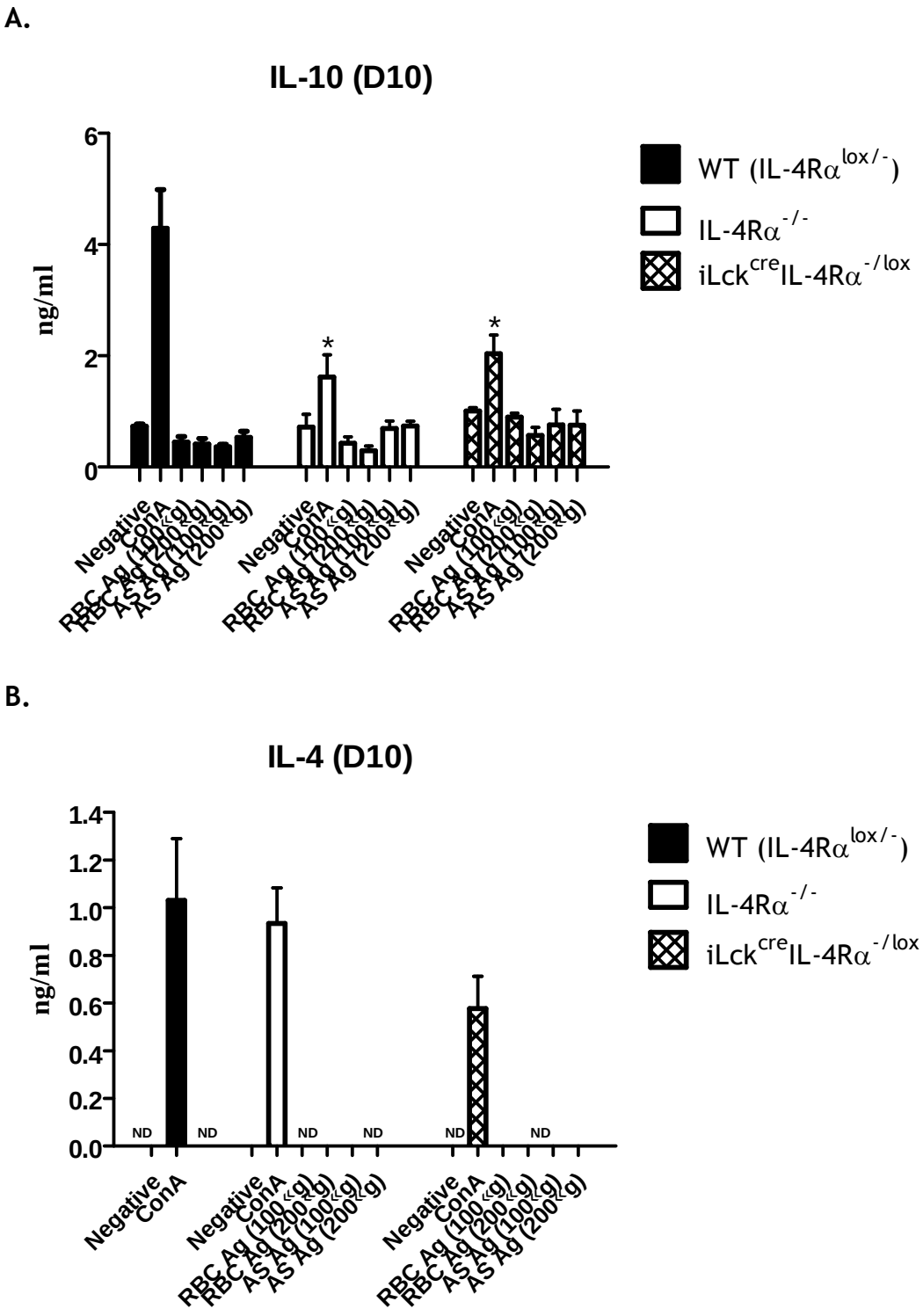
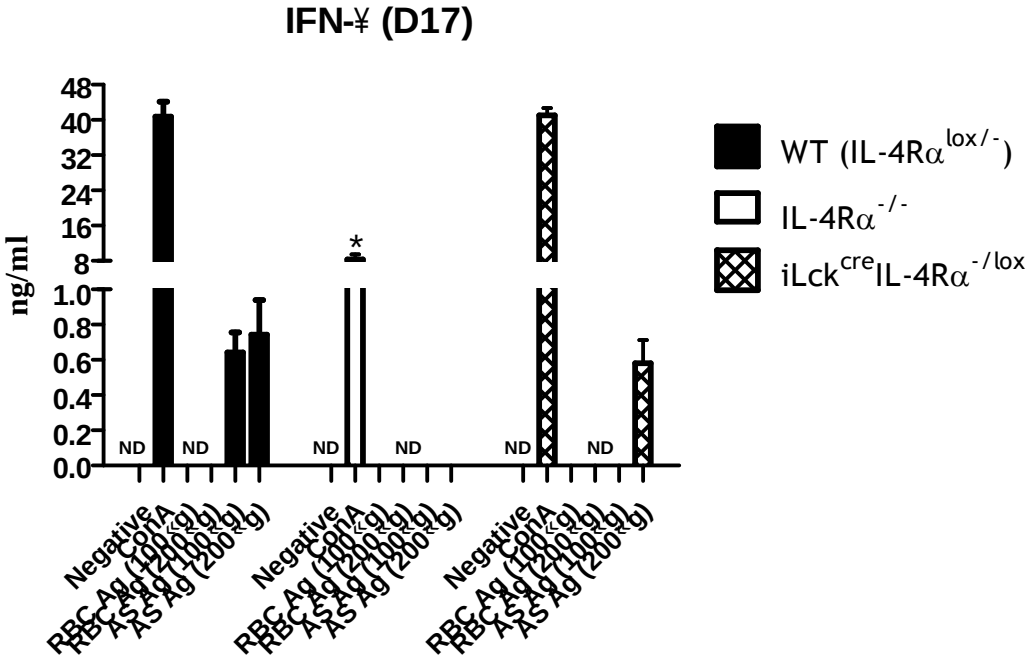


Figure 7.6: Comparison of day 10 splenic **(A)** IL-10 and **(B)** IL-4 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/flox}$ female mice on a BALB/c background. Results are a representative of two separate experiments, n=5-9. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

A.



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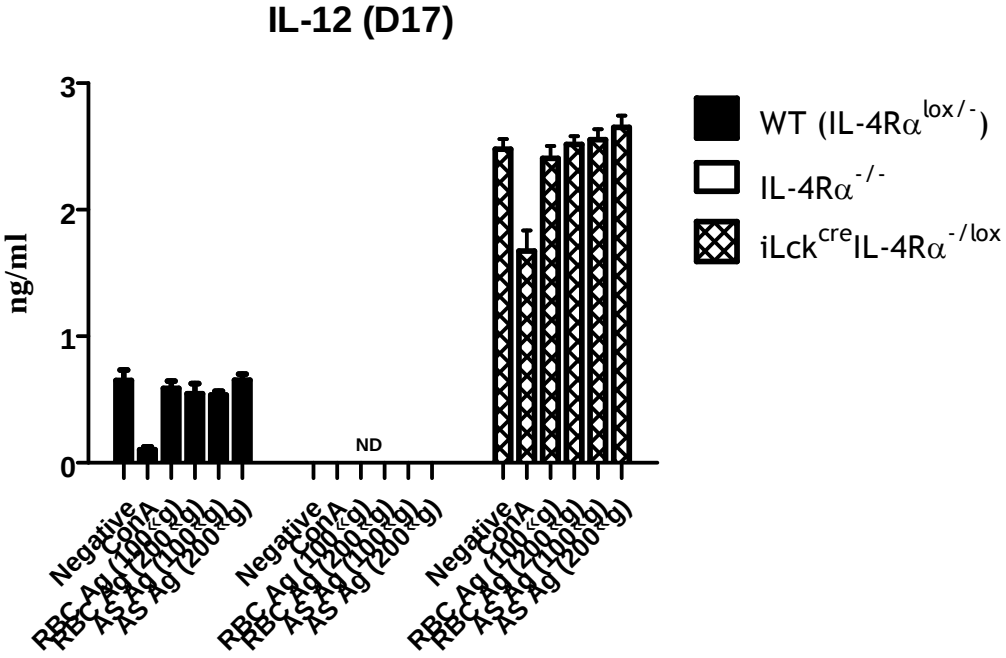
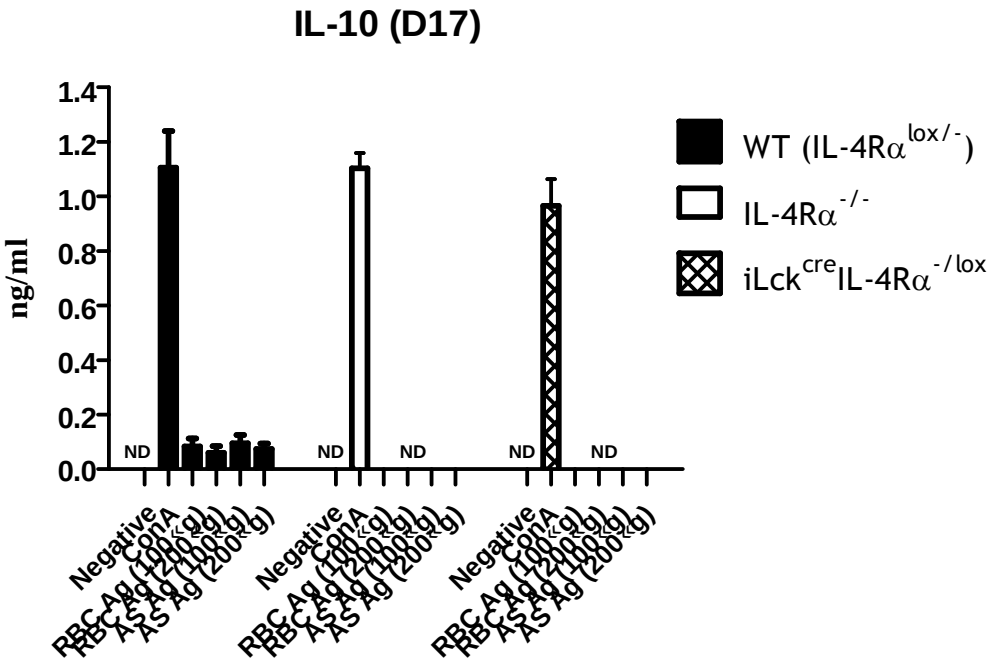


Figure 7.7: Comparison of day 17 splenic **(A)** IFN-γ and **(B)** IL-12 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/flox}$ female mice on a BALB/c background. n=5-9. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

A.



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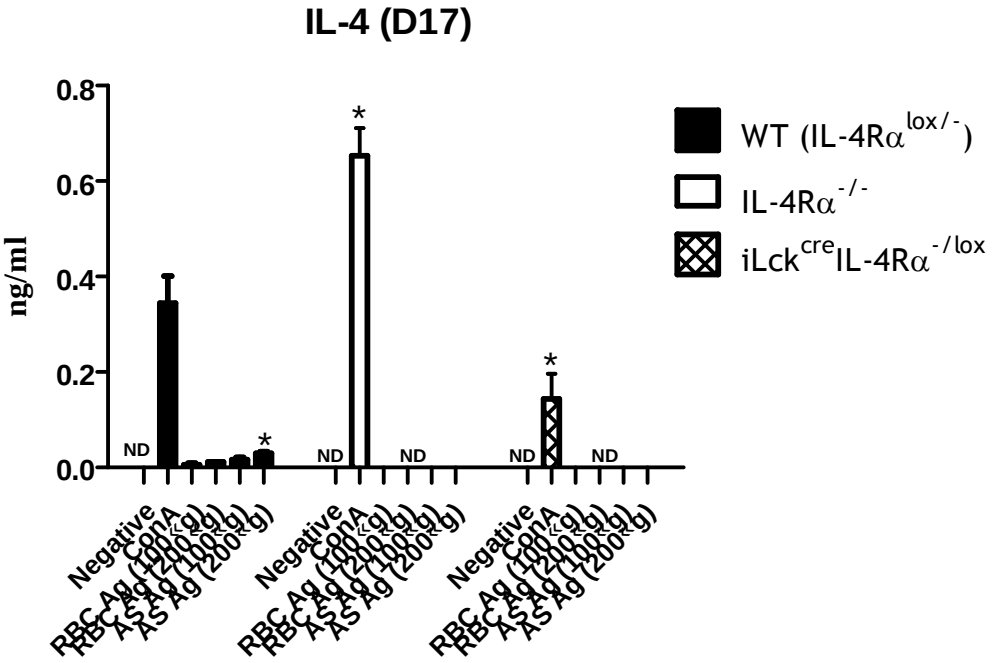
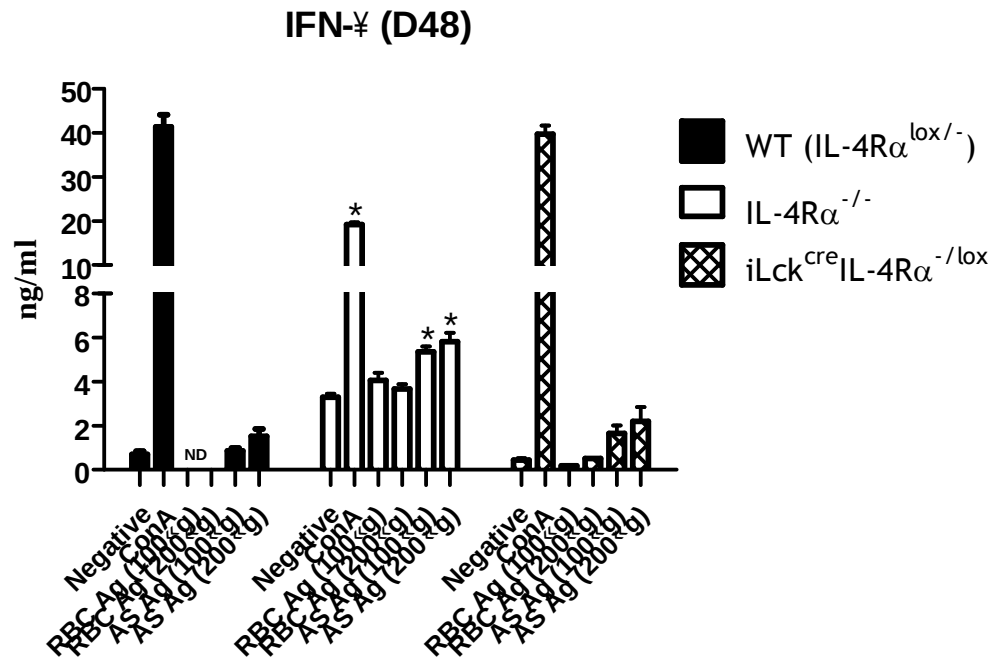


Figure 7.8 Comparison of day 17 splenic **(A)** IL-10 and **(B)** IL-4 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. n=5-9. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

A.



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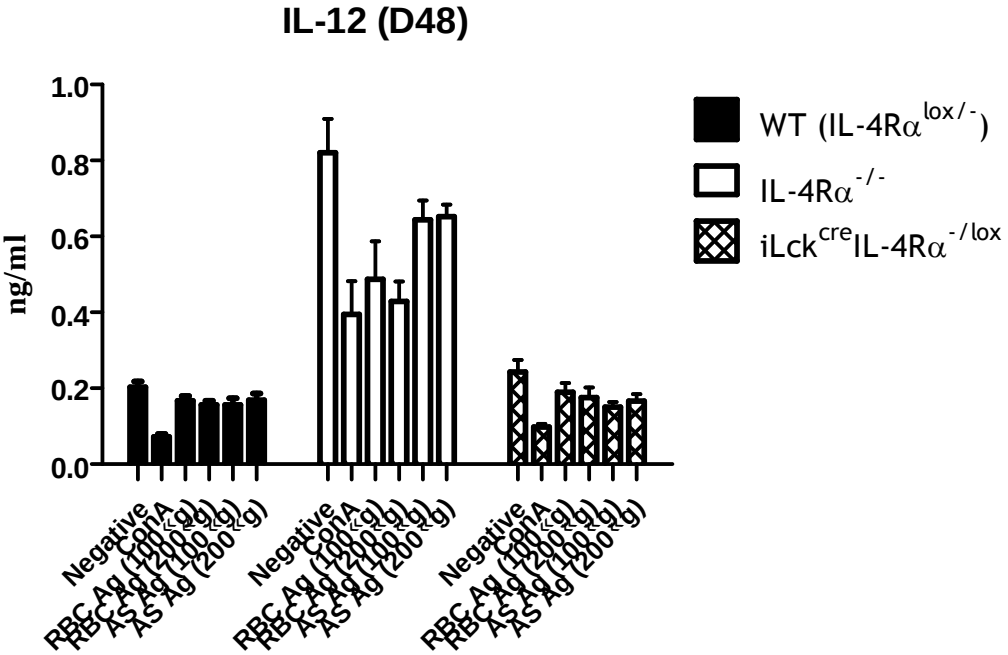
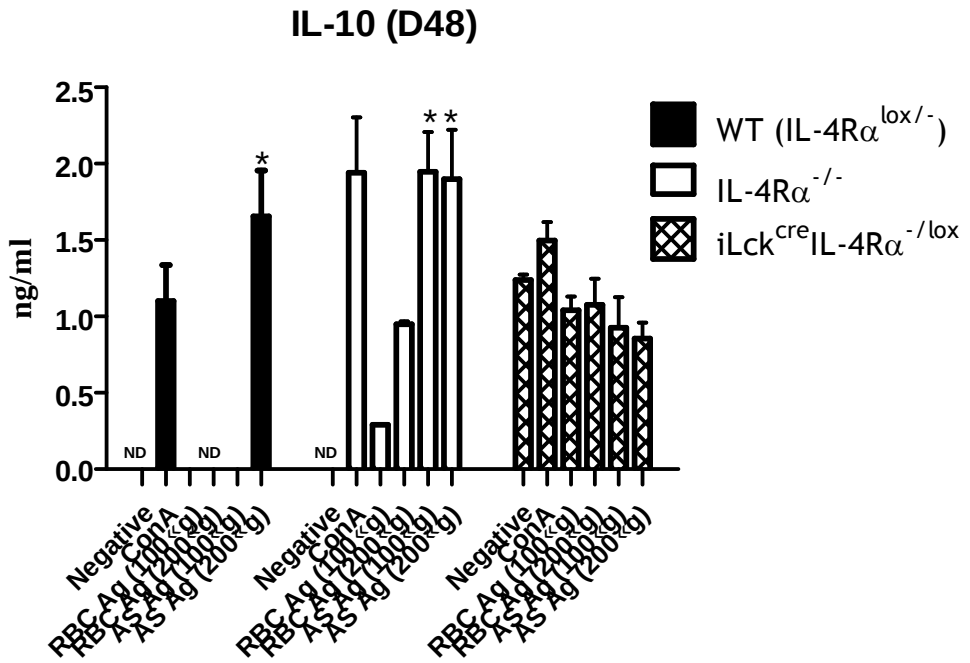


Figure 7.9 Comparison of day 48 splenic **(A)** IFN-γ and **(B)** IL-12 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. n=5-9. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

A.



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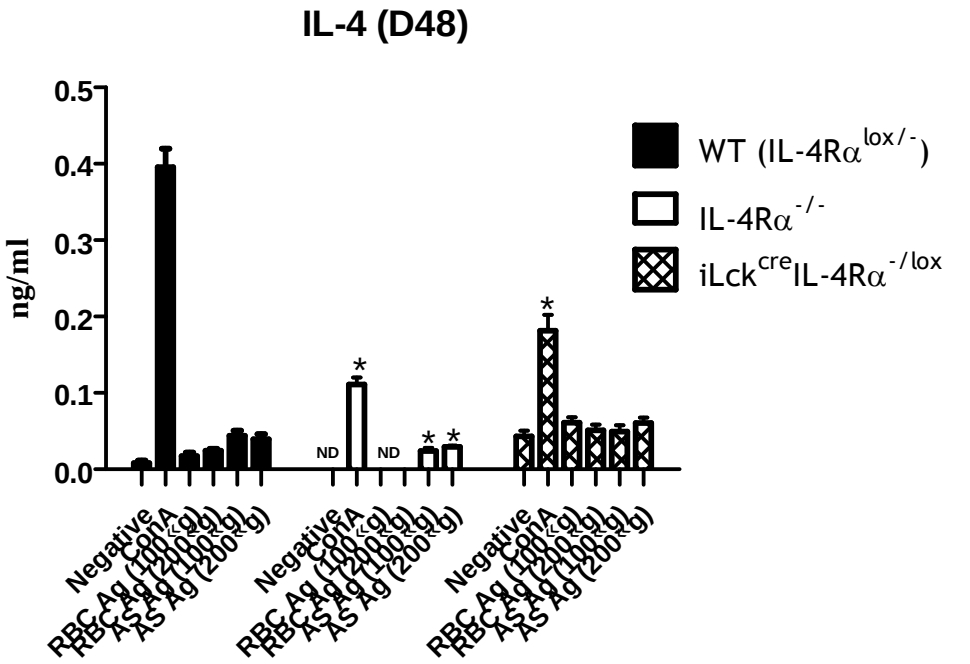


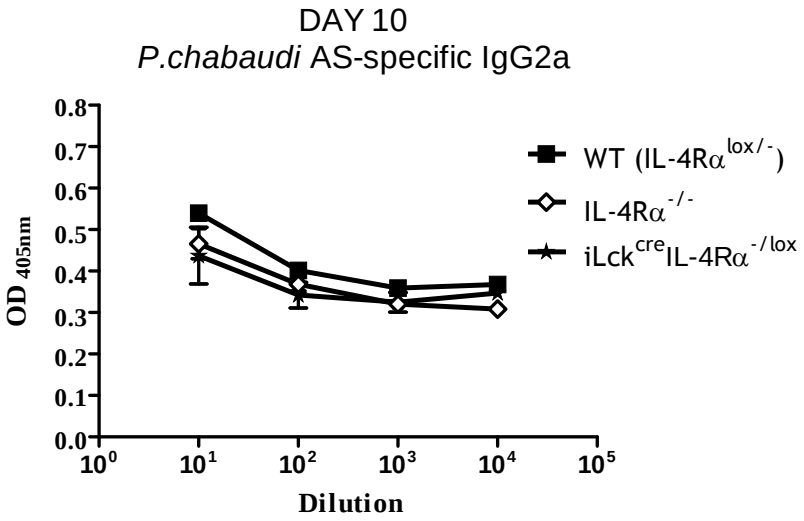
Figure 7.10: Comparison of day 48 splenic **(A)** IL-10 and **(B)** IL-4 production in *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background. n=5-9. ND indicates not detected within the standard curve range of the ELISA. * denotes $p<0.05$.

7.3.4 Comparison of the serum IgG2a and IgG1 antibody responses of $CD4^+CD8^+$ T-cell IL-4Rα-deficient ($iLck^{cre}IL\text{-}4R\alpha^{-/lox}$) female BALB/c mice following *Plasmodium chabaudi* AS infection.

Peak infection at day 10 demonstrated no significant differences in Th_1-type (IgG2a) antibody levels in the respective groups (Figure 7.11, A). A day 17 of the infection, IgG2a antibody levels were significantly increased in the WT and $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice compared to the $IL\text{-}4R\alpha^{-/-}$ mice (Figure 7.11, B). However, at day 48, the IgG2a antibody levels were significantly reduced in the WT and $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice compared to the $IL\text{-}4R\alpha^{-/-}$ mice (Figure 7.11, C).

Th_2 associated antibody levels, namely IgG1, at day 10 of the primary infection was comparable between the groups (Figure 7.12, A). At day 17 post infection, IgG1 antibody levels were significantly increased in the WT control compared to the gene-deficient mice and, $IL\text{-}4R\alpha^{-/-}$ mice demonstrated greater IgG1 levels compared to the $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice (Figure 7.12, B). Contrary to day 17, at day 48 of the infection, IgG1 antibody levels were then significantly increased in the WT and $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice compared to the $IL\text{-}4R\alpha^{-/-}$ mice (Figure 7.12, C).

A.



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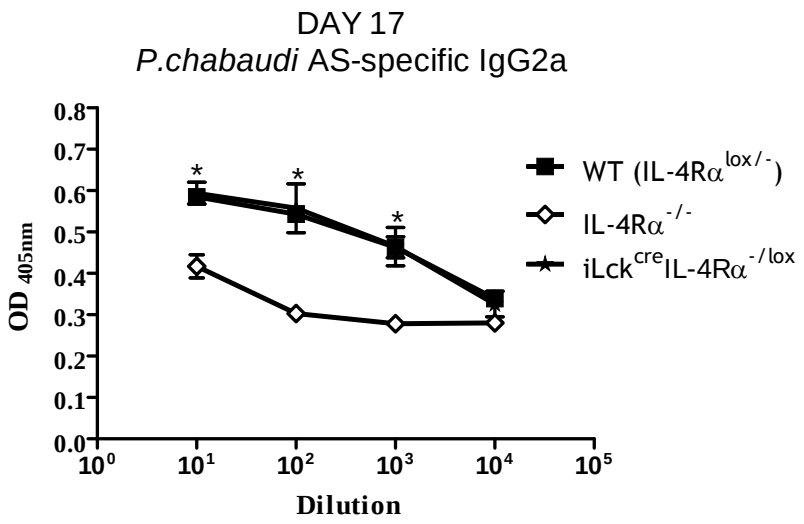

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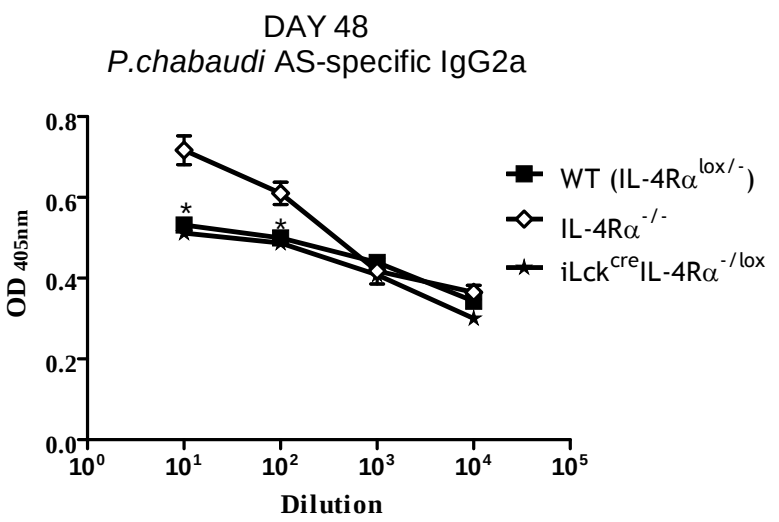
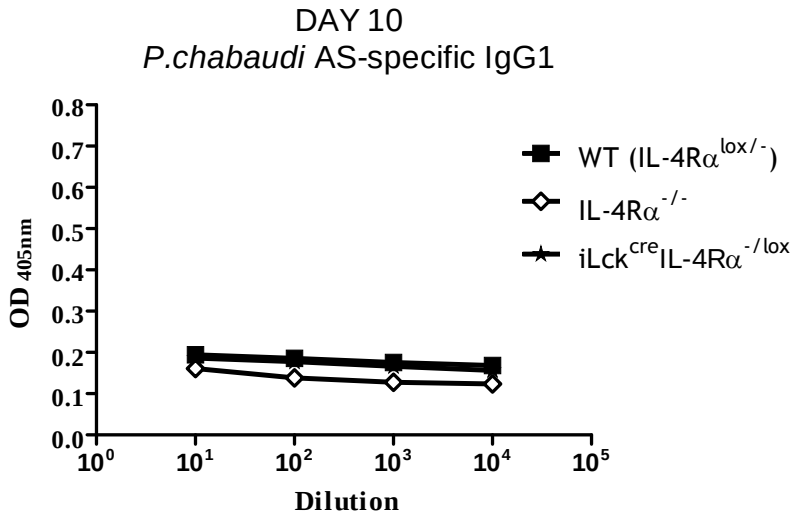


Figure 7.11: Comparison of the IgG2a antibody responses of *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. n=5-8. * denotes $p<0.05$.

A.



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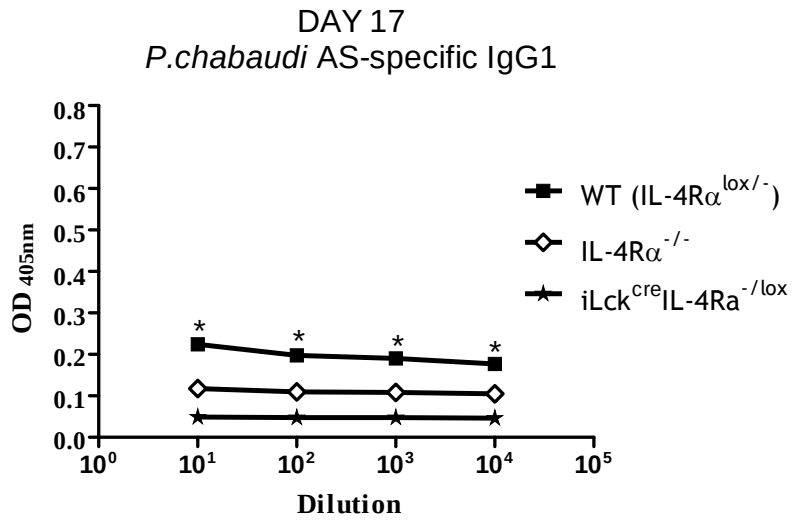


C.

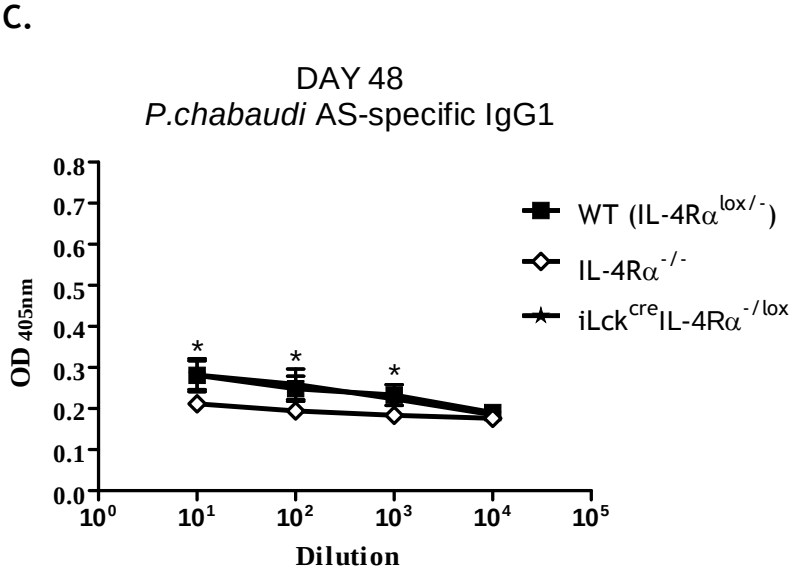


Figure 7.12: Comparison of the IgG1 antibody responses of *P. chabaudi* AS infected WT (IL-4Rα$^{lox/-}$), IL-4Rα$^{-/-}$ and iLck^{cre}IL-4Rα$^{-/lox}$ female mice on a BALB/c background on **(A)** day 10 **(B)** day 17 and **(C)** day 48. n=5-8. * denotes.

7.4 Discussion

Our understanding of how cell-specific IL-4Rα signalling co-ordinates Th_2 type immunity in malaria is relatively poor. Transgenic mouse models with the IL-4Rα expression disrupted in defined cell populations have provided a valuable tool to addressing our understanding of the signalling pathways and has highlighted key cellular players in the control of infections where Th_2 effector responses are involved (Dewals *et al.*, 2009). While IL-4 mediates multiple effects on T-cells, murine T and B cells do not respond to IL-13 (Mohrs *et al.*, 2000). We have shown in the first instance that cell specific IL-4Rα$^{-/-}$ mice demonstrated that aaMac were not crucial for protection against the long-term effects of *P. chabaudi* AS infected female BALB/c mice. Secondly, we provided evidence that CD4^{+} T-cells responsive to IL-4 played a significant protective role during chronic disease. We subsequently generated a mouse specifically impaired for IL-4Rα expression on all T-cell populations (iLck^{cre}IL-4Rα$^{-/lox}$) and these mice were compared with IL-4Rα$^{-/-}$ mice after *P. chabaudi* AS infection. The results demonstrated that the iLck^{cre}IL-4Rα$^{-/lox}$ mice were not as susceptible to the infection as the IL-4Rα$^{-/-}$ mice and displayed a similar disease phenotype to the WT controls. However, significant weight loss and reduced RBC counts were evident in the iLck^{cre}IL-4Rα$^{-/lox}$ mice compared to the IL-4Rα$^{-/-}$ mice following the patent parasitaemia suggesting a possible delayed response in this group.

The immunological profile at day 10 of the infection showed a typical Th_1 response in both gene-deficient mice which was significantly enhanced compared to the WT control as measured by increased splenic IFN-γ production. Collectively, these data support previous findings in which Th_1 responses are necessary for destroying patent parasitaemia (Hafalla *et al.*, 2011, Taylor-Robinson and Phillips, 1994). The significantly elevated splenic IFN-γ response in the gene-deficient mice could be a result of an elevated innate proinflammatory response. The significance of IFN-γ as a key role player in early protection against parasitaemia in humans was also demonstrated in an *in vitro* model exposing peripheral blood mononuclear cells (PBMCs) to *P. falciparum* infected RBC (Hafalla *et al.*, 2011, Artavanis-Tsakonas and Riley, 2002).

Progression of the infection maintained protective Th_1 responses in the iLck^{cre}IL-4Rα$^{-/lox}$ mice by day 17 with increased IgG2a Ab titres. Splenic IL-10 production was produced in all groups at this time and could have facilitated the balance between Th_1 pro-inflammatroy and anti-inflammatory responses. By day 48, Th_2 cytokine responses were impaired in both gene-deficient groups as measured by reduced IL-4 splenic cytokine production compared to the WT control. However, at day 48, IL-4Rα$^{-/-}$ mice demonstrated elevated levels of antigen-specific splenic IL-10 production compared to the iLck^{cre}IL-4Rα$^{-/lox}$ mice suggesting that downregulaton of pro-inflammatory responses could result in recurring parasitaemia, hence recrudescent infection observed in the IL-4Rα$^{-/-}$ mice. The role of anti-inflammatory cytokines (TGF-ß and IL-10) was shown to down-regulate the protective and potentially detrimental defence mechanisms once peripheral parasitemia has been brought under control. This delicate balance between pro-inflammatory and anti-inflammatory responses appears to be a major determinant of the clinical outcome of a *Plasmodium* infection. Studies in murine malaria suggest an important role for TGF-ß and IL-10 in regulating the overproduction of IFN-γ and TNF-α during erythrocytic infections (Hafalla *et al.*, 2011, Stevenson and Riley, 2004). Furthermore, in *P. chabaudi* infection, it has been shown that IL-10 production from Tr1 and Treg are associated with suppression of proinflammatory cytokine production and expansion of pathological Th_1 responses (Niikura *et al.*, 2011).

At day 48, in the iLck^{cre}IL-4Rα$^{-/lox}$ mice, increased IFN-γ, reduced IL-10 and increased IL-4 splenic cytokine production was observed compared to the IL-4Rα$^{-/-}$ mice. However, a Th_1 immune response was consistent in the gene-deficient mice, which resulted in detrimental effects in the IL-4Rα$^{-/-}$ mice such as increased mortality and recrudescence, not so in the iLck^{cre}IL-4Rα$^{-/lox}$ mice. Furthermore, the reduced IgG1 Ab titres at day 48 was only evident in the IL-4Rα$^{-/-}$ mice indicating that the increased IgG1 Ab titres seen in the iLck^{cre}IL-4Rα$^{-/lox}$ mice, comparable to the WT control, may be due to non-T-cell IL-4 dependent mechanism of B-cell stimulation. These novel findings indicate that non-T-cells responsive to IL-4/IL-13 activation could have facilitated chronic *P. chabaudi* AS protection in the iLck^{cre}IL-4Rα$^{-/lox}$ mice compared to the IL-4Rα$^{-/-}$ group independent of T-cell IL-4Rα signalling. In a more recent study by Dewals and colleagues utilizing pan-T-cell IL-4Rα-deficient mice, the role of non-T-cells

responsive to IL-4 was described and its differing effects on the outcome of parasitic infections (Dewals *et al.*, 2009). They showed that *L. major* infected Lck^{cre}IL-4Rα$^{-/lox}$ and iLck^{cre}IL-4Rα$^{-/lox}$ mice developed a healer phenotype thus implicating a role for IL-4Rα-responsive non-T-cells in providing protection (Dewals *et al.*, 2009). On the contrary, *S. mansoni* infected Lck^{cre}IL-4Rα$^{-/lox}$ mice, survived the infection while iLck^{cre}IL-4Rα$^{-/lox}$ mice succumbed to infection indicating that IL-4Rα-responsive non-$CD4^{+}$ T-cells contribute to protection (Dewals *et al.*, 2009).

An interesting observation occurred when measuring whole spleen organs for comparison since splenomegaly is a clinical sign of chronic malarial disease. The spleen consists of micro-anatomical zones and microcirculations adapted to performing different functions from the induction of adaptive immunity, recycling of iron and phagocytosis of erythrocytes as well as destruction of damaged or infected RBCs and pathogens including *Plasmodium* (del Portillo *et al.*, 2011). Spleen size has been used for many years as a tool to determine the intensity of malaria transmission in endemic regions (del Portillo *et al.*, 2011, Chaves *et al.*, 2011, Snow *et al.*, 1989, Neva *et al.*, 1970). During blood-stages of malaria infection, the spleen is the main organ involved in the development of the immune response and in elimination of iRBCs (del Portillo *et al.*, 2011, Engwerda *et al.*, 2005). Splenomegaly develops when the infection persists or when there is insufficient time to fully resolve the infection (Wilson *et al.*, 2009, Greenwood, 1987). The WT control and IL-4Rα$^{-/-}$ mice displayed comparable enlarged spleens while iLck^{cre}IL-4Rα$^{-/lox}$ mice initially displayed significantly smaller spleens than the WT and IL-4Rα$^{-/-}$ mice. However, upon termination of the experiment, iLck^{cre}IL-4Rα$^{-/lox}$ mice displayed significantly larger spleens than the WT and IL-4Rα$^{-/-}$ mice suggesting that splenomegaly was more pronounced in the iLck^{cre}IL-4Rα$^{-/lox}$ mice during long-term infection. It seems that in the absence of IL-4Rα signalling via T-cells, splenomegaly is delayed. However, this was not the case in the IL-4Rα$^{-/-}$ mice, which would suggest that non-T-cell populations responding to IL-4/IL-13 are involved in delaying this phenomenon. The role of the spleen in normal and pathological conditions caused by malaria infection is still to be elucidated but recent advances in bioengineering and microfluidics are paving the way to construct 3D organs-on-a chip, including the spleen (Baker, 2011, Deplaine *et al*, 2011, del Portillo *et al.*, 2011).

In conclusion, the results using pan T-cell gene-deficient mice were intriguing and require further investigation. Unfortunately, in the duplicate experiment there was an accident resulting in the loss of the tissue specific group. Due to time constraints and availability of the tissue-specific mice, this experiment could not be repeated. However, some of the observations were of great significance. For example, the role of non-T-cells responsive to IL-4/IL-13 in chronic *P. chabaudi* AS protection and the concept of delayed splenomegaly in the absence of IL-4Rα signalling on all T-cells require further investigation.

Chapter Eight

General Discussion

8. General Discussion

Early studies presented in this thesis demonstrated that *P. chabaudi* AJ infected female IL-4Rα$^{-/-}$ mice were more susceptible to infection than their WT counterparts as determined by greater recrudescent parasitaemia and severity of chronic infection. These results were similar to those previously obtained in our group in which male gene-deficient BALB/c *P. chabaudi* AS infected mice were used (Couper, 2003). Our findings indicated that the immunological response during chronic infection was not parasite strain dependent. In addition, similar results were observed in female mice to those previously using male mice suggesting that the enhanced recrudescence observed in the IL-4Rα$^{-/-}$ mice was also independent of host gender. With the availability of the *P. chabaudi* AS strain we were then able to investigate the role of IL-4Rα-signalling in the original model described by Couper (2003). That is, male BALB/c mice infected with *P. chabaudi* AS parasite. Consequently, our experiments indicated that IL-4Rα$^{-/-}$ male mice were extremely susceptible to the infection characterized by high mortality and such severe pathology that the experiment studies on these mice had to be terminated at day 14. Consequently, all further studies utilized the more resistant female BALB/c gene-deficient mice infected with *P. chabaudi* AS. The results obtained using the female *P. chabaudi* AS infection model were in agreement with previous findings (Couper, 2003) and with that obtained in using the AJ strain of *P. chabaudi*. Overall a significant role for IL-4Rα signalling in providing protective immunity against *P. chabaudi* AS chronic infection was confirmed, and required further investigation.

We have demonstrated that IL-4Rα plays a major protective role during chronic *P. chabaudi* AS infection in male and female BALB/c mice and that IL-4Rα$^{-/-}$ mice, unlike their WT counterparts, were unable to effectively clear and control chronic infection characterized by increased mortality, enhanced recrudescent parasitaemia and an impaired Th_2 immune response. However, male mice succumbed to infection at a greater extent than the female mice, infected with the same *P. chabaudi* AS parasite, possibly due to immunomodulatory effects of sex hormones. The influence of testosterone on disease susceptibility has been described before for malaria (Klein, 2008, 2004) and other parasitic infections (Bryson *et al.*, 2011). Collectively, the present data provided evidence that abrogation of IL-4Rα signalling on all cell types resulted in an impairment of the

protective Th_2 phenotype in the IL-4Rα$^{-/-}$ mice. Furthermore, a similar overall effect in the disease profile and immunological response was observed in the male and female IL-4Rα$^{-/-}$ mice infected with either *P. chabaudi* AS or AJ parasites respectively and therefore chronic disease was not strain-specific or host gender dependent.

Having established a suitable model, the effect of IL-4Rα signalling on certain cell types during chronic malaria disease was studied. Our understanding of how cell-specific IL-4Rα signalling coordinates Th_2 immunity in murine malaria is relatively poor and with the use of transgenic mouse models with IL-4Rα expression disrupted in defined cell populations we have been able to identify key cellular players involved in controlling parasite infections involving Th_2-type responses. These novel investigations described here for P. *chabaudi* AS infection will prove useful in our understanding of protective immune mediated mechanisms during chronic disease.

In the first instance, we wanted to investigate the possible role of IL-4Rα signalling on macrophages/neutrophils using macrophage/neutrophil-specific IL-4Rα$^{-/-}$ (LysM^{cre}IL-4Rα$^{-/lox}$) mice. We found that long-term protection of LysM^{cre}IL-4Rα$^{-/flox}$ mice infected with *P. chabaudi* AS did not require the presence of IL-4/IL-13 activated macrophages as a similar disease profile in these mice was shown compared with the WT control. Consequently, we suggested that the increased susceptibility of IL-4Rα$^{-/-}$ mice compared with WT mice must depend on IL-4Rα signalling on cell populations other than macrophages/neutrophils. However, these results were contrary to that shown by Couper (Couper, 2003). He showed that LysM^{cre}IL-4Rα$^{-/flox}$ male mice displayed a similar disease course to IL-4Rα$^{-/-}$ mice, namely an impaired Type 2 protective immune response, compared to WT mice. Couper hypothesized that IL-4/IL-13 protection might be via regulation of macrophage/neutrophil function. The major difference between the two studies was that Couper utilized male mice and the present study female mice. Previous studies have shown that susceptibility to infection and immunological differences between sexes can be influenced by gender differences (hormonal) when infected with the same parasite (Klein *et al.*, 2008, Klein, 2004). Immunomodulatory effects of testosterone was shown to increase susceptibility and mortality following *P. chabaudi* or *P. berghei* infection (Klein,

2004; Wunderlich *et al.*, 1991; Kamis and Ibrahim, 1989) and recovery is delayed in male mice compared to female mice (Klein, 2004). A recent study using cell-specific IL-4Rα$^{-/-}$ female mice infected with *L. mexicana* showed that these mice developed small lesions, which subsequently healed and was associated with a strong Th_1 phenotype while the male mice developed small lesions that persisted and associated with a strong Th_1 response but significantly elevated IL-4 production as well (Bryson *et al.*, 2011). IL-4 production independently of IL-4Rα signalling has been observed in a number of immunological studies previously (Alexander *et al.*, 2002, Brewer *et al.*, 1999, Mohrs *et al.*, 1999). The work by Bryson and colleagues represent for the first time a sex-associated influence on this ability (Bryson *et al.*, 2011). Our current data have consequently supported their findings regarding this sex associated influence and IL-4Rα signalling but in a model of *P. chabaudi* AS infection. Taken together, our LysM^{cre}IL-4Rα$^{-/lox}$ infection model demonstrated no significant role for IL-4Rα signalling via macrophages/neutrophils in providing significant protection during chronic *P. chabaudi* AS infection.

We then investigated the role of CD4^{+} T-cells responsive to IL-4Rα signalling in *P. chabaudi* AS infection since CD4^{+} T-cells have been shown to play a major role during innate and adaptive immunity to malaria infection (Stephens *et al.*, 2005, Langhorne *et al.*, 2002). We generated a mouse deficient in IL-4Rα function via CD4^{+} Tcells (Lck^{cre}IL-4Rα$^{-/flox}$) only. Our studies demonstrated that initial control in the Lck^{cre}IL-4Rα$^{-/lox}$ mice was associated with a Th_1 phenotype characterized by splenic IFN-γ production and serum IgG2a antibody responses. Subsequent Th_1-Th_2 control in the Lck^{cre}IL-4Rα$^{-/lox}$ mice around day 17 was demonstrated as measured by increased splenic IFN-γ and IL-10 production and the induction of serum IgG1 antibodies. During chronic infection, recrudescence was evident in the Lck^{cre}IL-4Rα$^{-/lox}$ mice similar to the IL-4Rα$^{-/-}$ mice accompanied by reduced RBC count and increased mortality at days 36 and 40. Th_2 responses were down-regulated in the Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice characterized by reduced splenic IL-10 and IL-4 production, increased IFN-γ from ConA stimulated splenocytes and increased serum IgG2a antibody responses. The importance of IL-10 as a regulatory cytokine with anti-inflammatory functions (Niikura *et al.*, 2011) provides additional evidence that the reduced IL-10 levels observed in the

gene-deficient mice coincided with the increased IFN-γ response observed. Consequently, due to abrogation of IL-4Rα signalling in IL-4Rα$^{-/-}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice, protective immunity was biased toward a Th_1 phenotype but which failed to adequately provide complete protection during the chronic phase. Strikingly, mortality was significantly greater in the Lck^{cre}IL-4Rα$^{-/lox}$ than in the IL-4Rα$^{-/-}$ mice. IL-4 responsive CD4$^+$ T-cells do not always provide protection but rather increases susceptibility to infection such as in *L. mexicana* infection in which non-healing progressively growing lesions are associated with a biased Th_2 response in BALB/c mice (Bryson *et al.*, 2011). Bryson and colleagues infected Lck^{cre}IL-4Rα$^{-/lox}$ mice with *L. mexicana* and demonstrated that these mice developed small lesions that subsequently healed indicating that progressive infection was dependent on CD4$^+$ T-cell responsive to IL-4 (Bryson *et al.*, 2011). Similarly, during *L. major* infection of Lck^{cre}IL-4Rα$^{-/lox}$ mice, resistance to infection was achieved implicating no role for protective immunity from CD4$^+$ T-cells responsive to IL-4 (Dewals *et al.*, 2009, Radwanska *et al.*, 2007). In summary, our findings present for the first time evidence that Lck^{cre}IL-4Rα$^{-/lox}$ together with IL-4Rα$^{-/-}$ BALB/c mice were susceptible to chronic-stage *P. chabaudi* AS infection and that the effector functions of IL-4 responsiveness on CD4$^+$ T-cells were critical for the clearance and control of the infection.

While a significant role for CD4$^+$ T-cells responsive to IL-4/IL-13 stimulation in chronic *P. chabaudi* AS infection was demonstrated, we wanted to determine whether this biological effect was reflected in CD4$^+$CD8$^+$ T-cell IL-4Rα$^{-/-}$ mice (iLck^{cre}IL-4Rα$^{-/lox}$). The role of IL-4 in induction of a wide-spectrum of intracellular signalling cascades in CD8$^+$ T-cells has been described before but only in a model of *P. yoelii* liver-stage infection (de Sa Pinheiro *et al.*, 2007, Carvalho *et al.*, 2002). Carvalho and colleagues have previously revealed a crucial role for IL-4 in the generation of memory CD8$^+$ T-cell responses against liver-stages of the parasite (Carvalho *et al.*, 2002). de Sa Pinheiro have subsequently extended their investigations and identified signalling pathways activated by IL-4 on CD8$^+$ T-cells and showed that IL-4 has a strong *in vivo* and *in vitro* anti-apoptotic effect on activated and resting CD8$^+$ T-cells (de Sa Pinheiro *et al.*, 2007). In the present study, contrary to the Lck^{cre}IL-4Rα$^{-/lox}$ mice, the iLck^{cre}IL-4Rα$^{-/lox}$ mice did not succumb to infection and displayed a similar

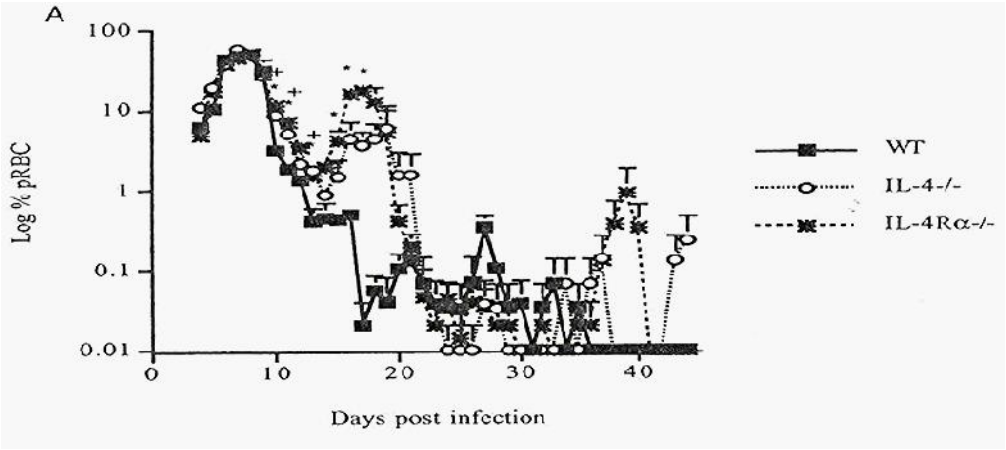
disease profile to the WT controls compared to the IL-4Rα$^{-/-}$ mice. However, Th_1 responses remained dominant during acute infection and declining parasitaemia but Th_2 responses were diminished during the latter part of infection. Interestingly, splenomegaly was delayed in the iLck^{cre}IL-4Rα$^{-/lox}$ mice compared to the WT and IL-4Rα$^{-/-}$ mice suggesting that non-T-cell populations responding to IL-4/IL-13 are involved in this delayed response. Overall, these findings suggest that non-T-cells responsive to IL-4/IL-13 activation could have facilitated protection in chronic *P. chabaudi* AS infection in the iLck^{cre}IL-4Rα$^{-/lox}$ mice compared to the IL-4Rα$^{-/-}$ group independent of T-cell IL-4Rα signalling. The role of non-T-cells in providing protection was suggested in parasitic studies. It is known that both IL-4 and IL-13 promote CD40L-induced IL-12 production by macrophages and DCs (Bullens *et al.*, 2001). This could indicate that DCs may be the IL-4/IL-13 responsive cells facilitating protection against, for example, *L.major* in the absence of IL-4Rα responsive CD4$^+$ T-cells in BALB/c mice (Radwanska *et al.*, 2007).

To conclude, utilizing cell-type specific IL-4Rα$^{-/-}$ mice we demonstrate that upon infection with *P. chabaudi* AS, initial control is dependent on innate type-1 responses followed by adaptive immunity. Progressive infection and control of recrudescent parasitaemia is dependent on Th_2 responses. Hence CD4$^+$ T-cells responsive to IL-4Rα signalling seem to be vital and the dominant role-player in providing protection during chronic disease. Furthermore, we present no role for aaMø in providing protection in our female model utilizing LysM^{cre}IL-4Rα$^{-/lox}$ mice. Intriguingly alternative macrophage activation may provide protection in male mice suggesting the effect is host gender determined suggesting a significant effect of sex hormones on macrophages/neutrophil function. The role for CD8$^+$ T-cells during blood-stage malaria infection is not well established as has been documented for pre-erythrocytic stage malaria infections (de Sa Pinheiro *et al.*, 2007, Carvalho *et al.*, 2002). Consequently, we could speculate that in the absence of IL-4Rα signalling on CD4$^+$ T-cells, IL-4 responsive CD8$^+$ T-cells may play a role in exacerbation of the disease. Interesting results from the CD4$^+$ and CD8$^+$ T-cell IL-4Rα$^{-/-}$ mice demonstrate a role for non-T-cells dependent on IL-4Rα responsiveness in providing protection in the infected mice and require further investigation.

Future studies focusing on the major role-players involved in chronic protection, namely $CD4^+$ T-cells would provide in depth understanding of the control of malarial infection. This can be achieved by focusing on IL-4Rα expression on $CD4^+$ T-cells, whether it is upregulated during infection using specialized techniques (FACs analysis) and whether upregulation is concomitant with the Th_1-Th_2 switch. Secondly, if this evidence is true for $CD4^+$ T-cells then we are able to manipulate the cytokine environment by upregulation of IL-4/IL-13 production from $CD4^+$ T-cells to facilitate protection and clearance of chronic blood-stage infection. Thirdly, establishing which non-T-cells responsive to IL-4/IL-13 are involved in providing protection during the chronic stage of malaria infection. Perhaps with the availability of non-T-cell IL-4Rα$^{-/-}$ mice (DC and B-cell IL-4Rα$^{-/-}$ mice) a better understanding of immune mechanisms mediating protective immunity in malaria infections will be accomplished.

ADDENDUM ONE

A.



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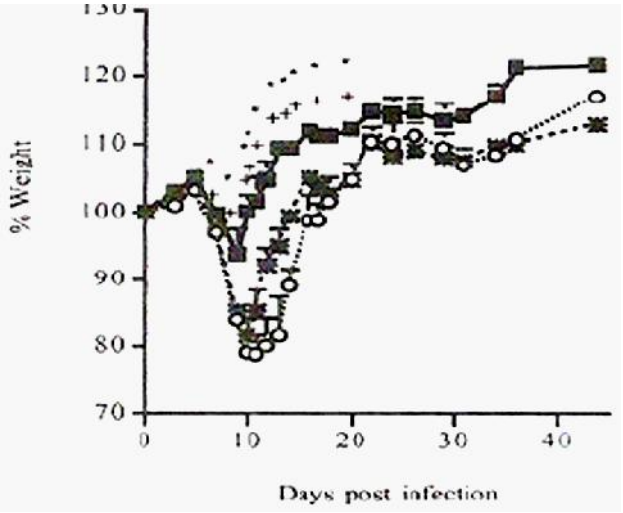

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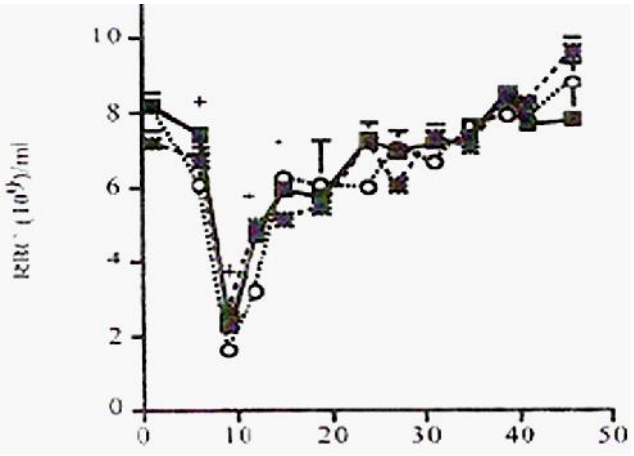

Addendum 1 Figure: (A) Comparison of the course of *P. chabaudi* AS erythrocyte infection in WT, IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ male mice. Comparison of **(B)** weight loss and **(C)** malaria induced anaemia in WT, IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ male mice during infection. Results are representative of **(A)** 3 and **(B +C)** 2 similar experiments.

ADDENDUM TWO

Table I. *Immunoregulatory roles of IL-10 during infection*[a]

Pathogen	Source of IL-10	Role of IL-10	Effect of Neutralization/Absence
Protozoa			
Toxoplasma gondii	Th1, DC, MΦ	Th1, APC suppression; inhibits IL-12, TNF-α, IFN-γ	↑ Immunopathology; ↑ TNF-α, IFN-γ
Leishmania spp.	nTreg, Tr-1 Th1, DC, MΦ	Th1 suppression, parasite persistence, memory development; reduced IL-2 and IFN-γ	↑ IFN-γ; ↑ parasite clearance; ↓ memory responses
Plasmodium spp.	nTreg, Th1/Tr1, DC, MΦ	Th1 suppression; reduced IL-12 and IFN-γ	↑ TNF-α, IFN-γ; ↑ parasite clearance; ↑ mortality; ↑ immune pathology/CM
Trypanosoma cruzi	CD4 T cells, MΦ	Inhibits NO, TNF-α, IL-12 and IFN-γ; reduces pathology; inhibits parasite killing	Severe pathology; ↑ TNF-α, IFN-g; ↑ parasite clearance
Bacteria			
Mycobacteria spp.	T cell, DC	Suppresses MΦ and DC IL-12, NO, TNF-α production	↑ Airway inflammation; ↑ IFN-γ; ↓ bacterial load
Listeria monocytogenes	MΦ	Inhibits MΦ bacteria killing	↓ Bacterial load; ↓ Ag specific T cells and memory
Helicobacter spp.	nTreg, Tr1	Suppresses Th1 IFN-γ	↓ bacterial load, ↑ colitis
Bordetella spp.	DC, Tr-1	Suppresses IL-12 from APC	↑ T cell proliferation; ↑ IFN-γ in vitro
Streptococcus pyogenes	Tr1	Suppresses IL-12; induces granzyme B	ND
Nematode			
Schistosoma mansoni	nTreg, innate cells	Th2 suppression; Th1 cytokine (IFN-γ) and proliferation suppression; increased CCR8 expression on CD4	↑ Immunopathology (granuloma) and mortality
Heligmosomoides polygyrus	DC, T cell (non-Treg)	Suppresses IFN-γ; increases IL-4	↑ IFN-γ; ↓ IL-4; no suppression of *Citrobacter rodentium*-induced colitis
Virus			
HIV	MΦ, CD8 T cell	Suppresses cytolysis and IL-2	↑ Monocyte IL-6, TNF-α and IFN-g
Hepatitis	MΦ, nTreg	Suppresses PBMC IFN-γ and mononuclear IL-12	↑ IFN-γ in vitro; ↓ lesion severity
HSV-1	nTreg	Suppresses IFN-γ and IL-2, T cell migration, and Ag-specific T cell numbers	ND
LCMV	nTreg, DC	Suppresses TNF-a, IL-2, CTL activity	↓ Lymphopenia; ↑ IFN-γ; ↑ $CD8^+$ T cells; ↑ viral clearance; ↑ memory
MCMV	Th1	Th1 suppression, down-regulation of MHC II	↓ Viral load; ↑ MHCII expression
Fungus			
Candida albicans	DC, nTreg	DC induce nTreg; control immunopathology and develop memory	↓ nTreg activation and expansion

Couper *et al.*, **2008**. *The Journal of Immunology*. 180: 5771-5777.

REFERENCES

Abehsira-Amar, O., Gibert, M., Joliy, M., Thèze, J. And Jankovic, D. L. (1992). IL-4 plays a dominat role in the differential development of Th0 into Th1 and Th2 cells. *J Immunol*, **148** (12), 3820-9.

Achidi, E. A., Apinjoh, T. O., Mbunwe, E., Besingi, R., Yafi, C., Wenjighe, A. N., Ajua, A. and Anchang, J. K. (2008). Febrile status, malarial parasitaemia and gastro-intestinal helminthiases in schoolchildren resident at different altitudes, in south-western Cameroon. *Ann Trop Med Parasitol*, **102** (2), 103-18.

Acres, R. B., Widmer, M. B., Grabstein, K. H. and Gillis, S. (1988). Regulation of human T-cell proliferation and CTL development by human recombinant interleukin-4. *Ann N Y Acad Sci*, **532**, 1-7.

Adachi, K., Tsutsui, H., Kashiwanura, S., Seki, E., Nakano, H., Takeuchi, O., Takeda, K., Okumura, K., Van Kaer, L., Okamura, H., Akira, S. and Nakanishi, K. (2001). *Plasmodium berghei* infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism. *J Immunol*, **167** (10), 5928-34.

Akpogheneta, O. J., Duah, N. O., Tetteh, K. K., Dunyo, S., Lanar, D.E., Pinder, M. and Conway, D. J. (2008). Duration of naturally acquired antibody responses to blood-stage *Plasmodium falciparum* is age dependent and antigen specific. *Infect Immun*, **76** (4), 1748-55.

Alexander, J., Carter, K. C., Al-Fasi, N., Satoskar, A. and Brombacher, F. (2000). Endogenous IL-4 is necessary for effective drug therapy against visceral leishmaniasis. *Eur J Immunol*. **30** (10), 2935-43.

Alexander, J., Brombacher, F., McGachy, H. A., McKenzie, A. N., Walker, W. and Carter, K. C. (2002). An essential role for IL-13 in maintaining a non-healing response following *Leishmania mexicana* infection. *Eur J Immunol*, **32** (10), 2923-33.

Alexander, J. and McFarlane, E. (2008). Can type-1 responses against intracellular pathogens be T helper 2 cytokine dependent? *Microbes and Infection*, **10**, 953-959.

Al-Yaman, F., Genton, B., Anders, R. F., Falk, M., Triglia, T., Lewis, D., Hii, J., Beck, H. P. and Alpers, M. P. (1994). Relationship between humoral response to *Plasmodium falciparum* merozoite surface antigen-2 and malaria morbidity in a highly endemic area of Papua New Guinea. *Am J Trop Med Hyg,* **51,** 593-602.

Amino, R., Giovannini, D., Thiberge, S., Gueirard, P., Boisson, B., Dubremetz, J. F., Prevost, M. C., Ishino, T., Yuda, M. and Menard, R. (2008). Host cell traversal is important for progression of the malaria parasite through the dermis to the liver. *Cell Host Microbe*, **3** (2), 88-96.

Arnot, D. E. and Jensen, A. T. (2011). Antigenic variation and the genetics and epigenetics of the PfEMP1 erythrocyte surface antigens in *Plasmodium falciparum* malaria. *Adv Appl Microbiol*, **74**, 77-96.

Artavanis-Tsakonas, K. and Riley, E. M. (2002). Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live *Plasmodium falciparum*-infected erythrocytes. *J Immunol,* **169,** 2956-2963.

Artavanis-Tsakonas, K., Tongren, J. E. and Riley, E. M. (2003). The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology. *Clin Exp Immunol*, **133** (2), 145-52.

Bachmann, A., Esser, C., Petter, M., Predehl, S., von Kalckreuth, V., Schmiedel, S., Bruchhaus, I. and Tannich, E. (2009). Absence of erythrocyte sequestration and lack of multicopy gen family expression in Plasmodium falciparum from a splenectomized malaria patient. *PLoS ONE*, **4**, e7459.

Baird, J. K. (1995). Host age as a determinant of naturally acquired immunity to *Plasmodium falciparum*. *Parasitol Today,* **11,** 105-111.

Baker, M. (2011). Tissue models: a living system on a chip. *Nature*, **471**, 661-665.

Balmer, P., Alexander, J. and Phillips, R. S. (2000). Protective immunity to erythrocytic *Plasmodium chabaudi* AS infection involves IFN gamma-mediated responses and a cellular infiltrate to the liver. *Parasitology,* **121 Pt 5,** 473-482.

Balmer, P., Phillips, H. M., Maestre, A. E., McMonagle, F. A. and Phillips, R. S. (2000b). The effect of nitric oxide on the growth of *Plasmodium falciparum*, *P. chabaudi* and *P. berghei* in vitro. *Parasite Immunol,* **22,** 97-106.

Banchereau, J. and Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature*, 1998, **392** (6673), 245-52.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B. and Palucka, K. (2000). Immunobiology of dendritic cells. *Annu Rev Immunol,* **18,** 767-811.

Bannister, L. and Mitchell, G. (2003). The ins, outs and roundabouts of malaria. *Trends Parasitol,* **19,** 209-213.

Barner, M., Mohrs, M., Brombacher, F. and Kopf, M. (1998). Differences between IL-4R alpha-deficient and IL-4-deficient mice reveal a role for IL-13 in the regulation of Th2 responses. *Curr Biol*, **8** (11), 669-72.

Baum, J., Richard, D., Healer, J., Rug, M., Krnajski, Z., Gilberger, T., Green, J. L., Holder, A. A. and Cowman, A. F. (2006). A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. *J Biol Chem*, **281** (8), 5197-5208.

Belnoue, E., Kayibanda, M., Vigario, A. M., Deschemin, J. C., van Rooijen, N., Viguier, M., Snounou, G. and Rénia, L. (2002). On the pathogenic role of brain-sequestered alphabeta CD8+ T cells in experimental cerebral malaria. *J Immunol*, **169** (11), 6369-75.

Belz, G. T., Shortman, K., Bevan, M. J. and Heath, W. R. (2005). CD8 alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J Immunol*, **175** (1), 196-200.

Benten, W. P., Ulrich, P., Kühn-Velten, W. N., Vohr, H. W. and Wunderlich, F. (1997). Testosterone-induced susceptibility to *Plasmodium chabaudi* malaria: persistence after withdrawal of testosterone. *J Endocrinol*, **153** (2), 275-81.

Bereczky, S., Montgomery, S. M., Troye-Blomberg, M., Rooth, I., Shaw, M. A. and Farnert, A. (2004). Elevated anti-malarial IgE in asymptomatic individuals is associated with reduced risk for subsequent clinical malaria. *Int J Parasitol,* **34,** 935-942.

Bernasconi, N. L., Traggiai, E. and Lanzavecchia, A. (2002). Maintenance of seroligical memory by polyclonal activation of human memory B cells. *Science*, **298**, 2199-2202.

Bernasconi, N. L., Onai, N. and Lanzavecchia, A. (2003). A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood*, **101**, 4500-4504.

Beutlar, B. and Cerami, A. (1988). Tumour necrosis cachexia shock and inflammation: a common mediator. *Ann Rev Biochem,* **57,** 505-518.

Biedermann, T., Zimmermann, S., Himmelrich, H., Gumy, A., Egeter, O., Sakrauski, A. K., Seegmüller, I., Voigt, H., Launois, P., Levine, A. D., Wagner, H., Heeg, K., Louis, J. A and Röcken, M. (2001). IL-4 instructs TH1 responses and resistance to Leishmania major in susceptible BALB/c mice. *Nat Immunol*, **2**, 1054-60.

Bogdan, C., Stenger, S., Rollinghoff, M. and Solbach, W. (1993). Cytokine interactions in experimental cutaneous leishmaniasis. Interleukin 4 synergizes with interferon-gamma to activate murine macrophages for killing of *Leishmania major* amastigotes. *Eur. J. Immunol*, **21**, 327-333.

Brewer, J., Conacher, M., Hunter, M. C., Mohrs, M., Brombacher, F. and Alexander, J. (1999). Aluminium hydroxide adjuvant initiates strong antigen specifc Th2 responses in the absence of IL-4 and IL-13 mediated signaling. *J. Immunol***, 163**, 6448-54.

Brombacher, F., Arendse, B., Peterson, R., Hölscher, A. and Hölscher, C. (2009). Analyzing classical and alternative macrophage activation in macrophage/neutrophil-specific IL-4 receptor-alpha-deficient mice. *Methods Mol Biol*, **531**, 225-52.

Brombacher, F. (2000). The role of interleukin-13 in infectious diseases and allergy. *Bioessays,* **22,** 646-656.

Brown, A. E., Webster, H. K., Teja-Isavadharm, P. and Keeratithakul, D. (1990). Macrophage activation in *falciparum* malaria as measured by neopterin and interferon-gamma. *Clin Exp Immunol,* **82,** 97-101.

Bryson, K.J., Millington, O. R., Mokgethi, T., McGachy, H. A., Brombacher, F. and Alexander, J. (2011). BALB/c mice deficient in $CD4^+$ T cell IL-4Rα expression control Leishmania mexicana load although female but not male mice develop a healer phenotype. *J PLoS Negl Trop Dis*, **5**(1), e930.

Bullens, D. M., Kasran, A., Thielemans, K., Bakkus, M. and Ceuppens, J. L. (2001). CD40L-induced IL-12 production is further enhanced by the Th2 cytokines IL-4 and IL-13. *Scand J Immunol*, **53**, 455-63.

Burns, J. M., Flaherty, P. R. and Nanavati, P. and Weidanz, W. P. (2004). Protection against Plasmodium chabaudi malaria induced by immunization with apical membrane antigen 1 and merozoite surface protein 1 in the absence of gamma interferon or interleukin-4. *Infect Immun*, **72** (10), 5605-5612.

Calle, J. M., Nardin, E. H., Clavijo, P., Boudin, C., Stuber, D., Takacs, B., Nussenzweig, R. S. and Cochrane, A. H. (1992). Recognition of different domains of the *Plasmodium falciparum* CS protein by the sera of naturally infected individuals compared with those of sporozoite-immunized volunteers. *J Immunol,* **149,** 2695-2701.

Cambridge, G., Leandro, M. J., Edwards, J. C., Ehrenstein, M. R., Salden, M., Bodman-Smith, M. and Webster, A. D. (2003). Serologic changes following B lymphocyte depletion therapy for rheumatoid arthritis. Arthritis Rheum, 48, 2146-2154.

Carvalho, L. H., Sano, G., Hafalla, J. C., Morrot, A., Curotto de Lafaille, M. A., Zavala, F. (2002) IL-4-secreting CD4+ T cells are crucial to the development of CD8+T-cell responses against malaria liver stages. *Nat Med*, 8, 166–170.

Cavacini, L. A., Parke, L. A. and Weidanz, W. P. (1990). Resolution of acute malarial infections by T cell-dependent non-antibody-mediated mechanisms of immunity. *Infect Immun,* **58,** 2946-2950.

Cavanagh, D. R., Elhassan, I. M., Roper, C., Robinson, V. J., Giha, H., Holder, A. A., Hviid, L., Theander, T. G., Arnot, D. E. and McBride, J. S. (1998). A longitudinal study of type-specific antibody responses to *Plasmodium falciparum* merozoite surface protein-1 in an area of unstable malaria in Sudan. *J Immunol*, **161** (1), 347-59.

Cavanagh, D. R., Dodoo, D., Hviid, L., Kurtzhals, J. A., Theander, T. G., Akanmori, B. D., Polley, S., Conway, D. J., Koram, K. and McBride, J. S. (2004). Antibodies to the N-terminal block 2 of Plasmodium falciparum merozoite surface protein 1 are associated with protection against clinical malaria. *Infect Immun*, **72** (11), 6492-502.

Cavinato, R. A., Bastos, K. R., Sardinha, L. R., Elias, R. M., Alvarez, J. M. and d'Império Lima, M. R. (2001). Susceptibility of the different developmental stages of the asexual (schizogonic) erythrocyte cycle of Plasmodium chabaudi chabaudi to hyperimmune serum, immunoglobulin (Ig)G1, IgG2a and F(ab')2 fragments. *Parasite Immunol*. **23** (11), 587-97.

Celada, A., Cruchaud, A. and Perrin, L. H. (1982). Opsonic activity of human immune serum on in vitro phagocytosis of *Plasmodium falciparum* infected red blood cells by monocytes. *Clin Exp Immunol,* **47,** 635-644.

Celada, A. (2009). Macrophage biology: The regulation of gene expression. *IRB Barcelona Scientific Report*. 70-74.

Chan, M. M., Evans, K. W., Moore, A. R. and Fong, D. (2010). Peroxisome Proliferator-Activated Receptor (PPAR): Balance for survival in parasitic infections. *J Biomed Biotechnol*, **2010**, 828951.

Charles, C. K., Parikh, S., Sun, J. C., Myrick, A., Lanier, L. L., Rosenthal, P. J. and DeRisi, J. L. (2008). Experimental malaria infection triggers early expansion of natural killer cells. *Infect Immun,* **76** (12), 5873-5882.

Chaves, L. F., Taleo, G., Kalkoa, M. and Kaneko, A. (2011). Spleen rates in children: an old and new surveillance tool for malaria elimination initiatives in island settings. *Trans R Soc of Trop Med and Hyg*, **105**, 226-231.

Choudhury, H. R., Sheikh, N. A., Bancroft, G. J., Katz, D. R. and De Souza, J. B. (2000). Early nonspecific immune responses and immunity to blood-stage nonlethal *Plasmodium yoelii* malaria. *Infect Immun,* **68,** 6127-6132.

Claasen, A., Lloberas, J. and Celada, A. (2009). Macrophage activation: classical versus alternative. *Methods Mol Biol*, **531**, 29-43.

Clausen, B. E., Burkhardt, C., Reith, W., Renkawitz, R. and Forster, I. (1999). Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res*, **8,** 265-77.

Coban, C., Ishii, K. J., Kawai, T., Hemmi, H., Sato, S., Uematsu, S., Yamamoto, M., Takeuchi, O., Itagaki, S., Kumar, N., Horii, T. and Akira, S. (2005). Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J Exp Med,* **201,** 19-25.

Coban, C., Ishii, K. J., Uematsu, S., Arisue, N., Sato, S., Yamamoto, M., Kawai, T., Takeuchi, O., Hisaeda, H., Horii, T. and Akira, S. (2006). Pathological role of Toll-like receptor signaling in cerebral malaria. *International Immunol*, **19** (1), 67-79.

Cohen, J., Nussenzweig, V., Nussenzweig, R., Vekemans, J. and Leach, A. (2010). From the circumsporozoite protein to the RTS, S/AS candidate vaccine. *Hum Vaccin*, **6** (1), 90-6.

Colonna, M., Pulendran, B. and Iwasaki, A. (2006). Dendritic cells at the host-pathogen interface. *Nat Immunol,* **7,** 117-120.

Conway, D. J., Cavanagh, D. R., Tanabe, K., Roper, C., Mikes, Z. S., Sakihama, N., Bojang, K. A., Oduola, A. M., Kremsner, P. G., Arnot, D. E., Greenwood, B. M. and McBride, J. S. (2000). A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses. *Nat Med*, **6** (6), 689-92.

Couper, K. N. Immunological studies on the related apicomplexan parasites *Toxoplasma gondii* and *Plasmodium chabaudi* AS. (PhD Thesis 2003). Pg 149-174.

Couper, K. N., Phillips, R. S., Brombacher, F. and Alexander J. (2005). Parasite-specific IgM plays a significant role in the protective immune response to asexual erythrocytic stage *Plasmodium chabaudi* AS infection. *Parasite Immunol*, **27**, 171-80.

Couper, K. N., Blount, D. G., de Souza, J. B., Suffia, I., Belkaid, Y. and Riley, E, M. (2007). Incomplete depletion and rapid regeneration of Foxp3+ regulatory T cells following anti-CD25 treatment in malaria-infected mice. *J Immunol*, **178**, 4136-4146.

Couper, K. N., Blount, D. G., Wilson, M. S., Hafalla, J. C., Belkaid, Y., Kamanaka, M., Flavell, R. A., de Souza, J. B. and Riley, E. M. (2008). IL-10 from $CD4^{+}CD25^{-}Foxp3^{-}CD127^{-}$ adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *PLoS Pathogens*, **4** (2), e1000004.

Couper, K. N., Barnes, T., Hafalla, J. C., Combes, V., Ryffel, B., Secher, T., Grau, G. E., Riley, E. M. and de Souza, J. B. (2010). Parasite-derived plasma microparticles contribute significantly to malaria infection-induced inflammation through potent macrophage stimulation. *PLoS Pathogens*, **6** (1), e1000744.

Cowman, A. F. and Crabb, B. S. (2006). Invasion of red blood cells by malaria parasites. *Cell,* **124,** 755-766.

Crompton, P. D., Mircetic, M., Weiss, G., Baughman, A., Huang, C. Y., Topham, D. J., Treanor, J. J., Sanz, I., Lee, F. E., Durbin, A. P., Miura, K., Narum, D. L., Ellis, R. D., Malkin, E., Mullen, G. E., Miller, L. H., Martin, L. B. and Pierce, S. K. (2009). The TLR9 ligand CpG promotes the acquisition of *Plasmodium falciparum*-specific memory B cells in malaria-naïve individuals. *J Immunol*, **182** (5), 3318-26.

Crompton, P. D., Pierce, S. K. and Miller, L. H. (2010). Advances and challenges in malaria vaccine development. *J Clin Invest*, **120** (12), 4168-78.

Cross, C. E. and Langhorne, J. (1998). *Plasmodium chabaudi chabaudi* (AS): inflammatory cytokines and pathology in an erythrocytic-stage infection in mice. *Exp Parasitol,* **90,** 220-229.

D'Andrea, A., Aste-Amezaga, M., Valiante, N. M., Ma, X., Kubin, M. and Trinchieri, G. (1993). Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med,* **178,** 1041-1048.

D'Ombrain, M. C., Voss, T. S., Maier, A. G., Pearce, J. A., Hansen, D. S., Cowman, A. F. and Schofield L. (2007). Plasmodium falciparum erythrocyte membrane protein-1 specifically suppresses early production of host interferon-gamma. *Cell Host Microbe*, **2** (2), 130-8.

D'Ombrain, M. C., Hansen, D. S., Simpson, K. M. and Schofield, L. (2007). gammadelta-T **cells** expressing **NK** receptors predominate over **NK cells** and conventional T **cells** in the innate IFN-gamma response to Plasmodium falciparum malaria. *Eur J Immunol*, 1864-73.

D'Ombrain, M. C., Robinson, L. J., Stanisic, D. I., Taraika, J., Bernard, N., Michon, P., Mueller, I. and Schofield, L. (2008). Association of early interferon-gamma production with immunity to clinical malaria: a longitudinal study among Papua New Guinean children. *Clin Infect Dis*, **47** (11), 1380-7.

D'Ombrain, M. C., Robinson, L. J., Mueller, I. and Schofield, L. (2009). Mechanisms underlying early interferon-gamma production in human Plasmodium falciparum malaria. *Clin Infect Dis*, **48** (10), 1482-3.

de Sa Pinheiro, A., Morrot, A., Chakravarty, S., Overstreet, M., Bream, J. H., Irusta, P. M. and Zavala, F. (2007). IL-4 induces a wide-spectrum intracellular signaling cascade in CD8+ T cells. *J Leukoc Biol*, **81** (4), 1102-10.

de Souza, J. B., Todd, J., Krishegowda, G, Gowda, D. C., Kwiatkowski, D. and Riley, E. M. (2002). Prevalence and boosting of antibodies to Plasmodium falciparum glycosylphophatidylinositols and evaluation of their association with protection from mild and severe clinical malaria. *Infect Immun*, 2002, **70**, 5045-5051.

Dechamps, S., Maynadier, M., Wein, S., Gannoun-Zaki, L., Maréchal, E. and Vial, H, J. (2010). Rodent and nonrodent malaria parasites differ in their phospholipid metabolic pathways. *J Lipid Res*, **51** (1), 81-96.

Defrance, T., Carayon, P., Billian, G., Guillemot, J. C., Minty, A., Caput, D. and Ferrara, P. (1994). Interleukin 13 is a B cell stimulating factor. *J Exp Med*, **179** (1), 135-43.

DeKruyff, R. H., Fang, Y. and Umetsu, D. T. (1992). IL-4 synthesis by in vivo primed keyhole limpet hemocyanin-specific CD4+ T cells. Influence of antigen concentration and antigen-presenting cell type. *J Immunol*, **149** (11), 3468-76.

del Portillo, H. A., Ferrer, M., Brugat, T., Martin-Jaular, L., Langhorne, J. and Lacerda, M. V. (2011). The role of the spleen in malaria. *Cell Microbiol*.

Deplaine, G., Safeukui, I., Jeddi, F., Lacoste, F., Brousse, V., Perrot, S., Biligui, S., Guillotte, M., Guitton, C., Dokmak, S., Aussilhou, B., Sauvanet, A., Cazals Hatem, D., Paye, F., Thellier, M., Mazier, D., Milon, G., Mohandas, N., Mercereau-Puijalon, O., David, P. H, Buffet, P. A. (2011). The sensing of poorly deformable red blood cells by the human spleen can be mimicked in vitro. *Blood*, **117** (8), e88-95.

Dewals, B., Hoving, J. C., Leeto, M., Marillier, R. G., Govender, U., Cutler, A. J., Horsnell, G. C. and Brombacher, F. (2009). IL-4Rα responsiveness of non-CD4 T cells contributes to resistance in *Schistosoma mansoni* infection investigated in pan-T cell-specific IL-4Rα-deficient mice. *Am J Pathol*, **175**, 706-716.

Diggs, C. L., Hines, F. and Wellde, B. T. (1995). *Plasmodium falciparum*: passive immunization of Aotus lemurinus griseimembra with immune serum. *Exp Parasitol*, **80**, 291-296.

Doolan, D. L., Sedegah, M., Hedstrom, R. C., Hobart, P., Charoenvit, Y. and Hoffman, S. L. (1996). Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8+ cell-, interferon gamma-, and nitric oxide-dependent immunity. *J Exp Med*, **183**, 1739-1746.

Doolan, D. L., Wizel, B. and Hoffman, S. L. (1996). Class I HLA-restricted cytotoxic T lymphocyte responses against malaria--elucidation on the basis of HLA peptide binding motifs. *Immunol Res*, **15** (4), 280-305.

Drakeley, C. J, Carneiro, I., Reyburn, H., Malima, R., Lusingu, J. P, Cox, J., Theander, T. G, Nkya, W. M., Lemnge, M. M. and Riley, E. M. (2005). Altitude-dependent and -independent variations in *Plasmodium falciparum* prevalence in northeastern Tanzania. *J Infect Dis*, **191**, 1589–1598.

Egan, A. F., Morris, J., Barnish, G., Allen, S., Greenwood, B. M., Kaslow, D. C., Holder, A. A. and Riley, E. M. (1996). Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19-kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1. *J Infect Dis,* **173,** 765-769.

Engwerda, C. R., Beattie, L. and Amante, F. H. (2005). The importance of the spleen in malaria. *Trends Parasitol*, **21**, 75-80.

Falanga, P. B., Franco da Silveira, J. F. and Pereira da Silva, L. (1984). Protective immune response to *Plasmodium chabaudi*, developed by mice after drug controlled infection or vaccination with parasite extracts: analysis of stage specific antigens from the asexual blood cycle. *Parasite Immunol,* **6,** 529-543.

Falchetti, R., Lanzilli, G., Casalinuovo, I. A., Gaziano, R., Palamara, A.T., Di Francesco, P., Ravagnan, G. and Garaci, E. (1996). Splenic CD4+ and CD8+ T cells from influenza immune mice concurrently produce in vitro IL2, IL4, and IFN-gamma. *Cell Immunol*. **170** (2), 222-9.

Favre, N., Ryffel, B., Bordmann, G. and Rudin, W. (1997). The course of *Plasmodium chabaudi chabaudi* infections in interferon-gamma receptor deficient mice. *Parasite Immunol,* **19,** 375-383.

Flesch, I.E., Wandersee, A. and Kaufmann, S. H. (1997). Effects of IL-13 on murine listeriosis. *Int Immunol*, **9**, 467-474.

Fort, M. M., Cheung, J., Yen, D., Li, J., Zurawski, S. M., Lo, S., Menon, S., Clifford, T., Hunte, B., Lesley, R., Muchamuel, T., Hurst, S. D, Zurawski, G., Leach, M. W., Gorman, D. M. and Rennick, D. M. (2001). IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity,* **15** (6), 985-95.

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

Franks, S., Koram, K. A., Wagner, G. E., Tetteh, K., McGuinness, D., Wheeler, J. G., Nkrumah, F., Ranford-Cartwright, L. and Riley, E. M. (2001). Frequent and persistent, asymptomatic *Plasmodium falciparum* infections in African infants, characterized by multilocus genotyping. *J Infect Dis,* **183,** 796-804.

Freire-De-Lima, C. G., Nascimento, D. O., Soares, M. B., Bozza, P. T., Castro-Faria-Neto, H. C., de Mello, F. G., DosReis, G. A. and Lopes, M. F. (2000). Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature*, **403** (6766), 199-203.

Freitas do Rosário, A. P., Muxel, S. M., Rodríguez-Málaga, S. M., Sardinha, L. R., Zago, C. A., Castillo-Méndez, S. I., Alvarez, J. M. and D'Império Lima, M. R. (2008). Gradual decline in malaria-specific memory T cell responses leads to failure to maintain long-term protective immunity to Plasmodium chabaudi AS despite persistence of B cell memory and circulating antibody. *J Immunol*, **181** (12), 8344-55.

Gajewski, T. F. and Fitch, F. W. (1991). Differential activation of murine Th1 and Th2 clones. *Res Immunol*, **142**, 19–23.

Gajewski, T. F, Pinnas, M., Wong, T., Fitch, F. W. (1991). Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen-presenting cell populations. *J Immunol*, **146**, 1750–8.

O' Garra, A. and Vieira, P. (2007). T_H1 cells control themselves by producing interleukin-10. *Nat Rev Immunol*, **7** (6), 425-8.

Garvin, A. M., Abraham, K. M., Forbush, K. A., Farr, A. G., Davison, B. L. and Perlmutter, R. M. (1990). Disruption of thymocyte development and lymphomagnesis induced by SV40 T-antigen. *Int Immunol*, **2**, 173-180.

Gatto, D., Martin, S.W., Bessa, J., Pellicioli, E., Saudan, P., Hinton, H. J. and Bachmann, M. F. (2007) Regulation of memory antibody levels: the role of persisting antigen versus plasma cell life span. *J Immunol*, **178**, 67–76.

Gazzinelli, R. T. and Denkers, E. Y. (2006). Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. *Nat Rev Immunol,* **6,** 895-906.

Goel, V. K., Li, X., Chen, H., Liu, S. C., Chishti, A. H. and Oh, S. S. (2003). Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. *Proc Natl Acad Sci U S A,* **100,** 5164-5169.

Goerdt, S. and Orfanos, C. E. (1999). Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity*, **10**, 137-142.

Good, M. F. and Doolan, D. L. (1999). Immune effector mechanisms in malaria. *Curr Opin Immunol,* **11,** 412-419.

Good, M. F. (2001). Towards a blood-stage vaccine for malaria: are we following all the leads? *Nat Rev Immunol,* **1,** 117-125.

Good, M. F., Stanisic, D., Xu, H., Elliott, S. and Wykes, M. (2004). The immunological challenge to developing a vaccine to the blood stages of malaria parasites. *Immunol Rev*, **201**, 254-67.

Good, M. F. (2005). Vaccine-induced immunity to malaria parasites and the need for novel strategies. *Trends Parasitol*, **21** (1), 29-34.

Gordon, S. (2003). Alternative Macrophage Activation. *Nature Reviews Immunology,* **3,** 23-35.

Greenwood, B. M. (1987). Asymptomatic malaria infections – do they matter? *Parasitol Today*, **3**, 206–214.

Grun, J. L. and Weidanz, W. P. (1981). Immunity to *Plasmodium chabaudi adami* in the B-cell-deficient mouse. *Nature,* **290,** 143-145.

Grünig, G., Corry, D. B, Leach, M. W, Seymour, B. W, Kurup, V. P. and Rennick, D. M. (1997). Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. *J Exp Med*, **185** (6), 1089-99.

Gu, H., Marth, J. D., Orban, P. C., Mossmann, H. and Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type specific gene targeting. *Science*, **265,**103-6.

Gueirard, P., Tavares, J., Thiberge, S., Bernex, F., Ishino, T., Milon, G., Franke -Fayard, B., Janse, C. J., Ménard, R. and Amino, R. Development of the malaria parasite in the skin of the mammalian host. *PNAS*, **107** (43), 18640-18645.

Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C. and Amigorena, S. (2002). Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol,* **20,** 621-667.

Hansen, S. G., Vieville, C., Whizin, N., Coyne-Johnson, L., Siess, D. C., Drummond, D. D., Legasse, A. W., Axthelm, M. K., Oswald, K., Trubey, C. M., Piatak, M. Jr., Lifson, J. D., Nelson, J. A., Jarvis, M. A. and Picker, L. J. (2009). Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat Med*, **15** (3), 293-9.

Hafalla, J. C., Silvie, O. and Matuschewski, K. (2011). Cell biology and immunology of malaria. *Immunol Rev*, **240**, 297-316.

Hänscheid, T., Egan, T. J. and Grobusch, M. P. (2007). Haemozoin: from melatonin pigment to drug target, diagnostic tool, and immune modulator. *Lancet Infect Dis*, **7** (10), 675-85.

Hart, P. H., Bonder, S. C., Balogh, J., Dickensheets, H. L., Donnelly, R. P. and Finlay-Jones, J. J. (1999). Differential responses of human monocytes and macrophages to IL-4 and IL-13. *J Leukoc Biol*, **66**, 575–578.

Heddini, A. (2002). Malaria pathogenesis: a jigsaw with an increasing number of pieces. *Int J Parasitol,* **32** (13), 1587-1598.

Herbert, D. R., Christoph Hölscher, C., Mohrs, M., Arendse, B., Schwegmann, A., Radwanska, M., Leeto M., Kirsch, R., Hall,P., Mossmann, H., Claussen, B., Förster, I. and Frank Brombacher. (2004). Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T Helper 1 responses and immunopathology. *Immunity*, **20**, 623-635.

Hermsen, C., van de Wiel, T., Mommers, E., Sauerwein, R. and Eling, W. (1997). Depletion of CD4+ or CD8+ T-cells prevents *Plasmodium berghei* induced cerebral malaria in end-stage disease. *Parasitology*, **114** (Pt 1), 7-12.

Herrera, S., Perlaza, B. L., Bonelo, A. and Arevalo-Herrera, M. (2002). Aotus monkeys: their great value for anti-malaria vaccines and drug testing. *Int J Parasitol*, 32 (13), 1625-35.

Hochrein, H., O'Keefe, M., Luft, T., Vandenabeele, S., Grumont, R. J., Maraskovsky, E. and Shortman, K. (2000). Interleukin (IL-4) is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *J Exp Med*, **192**, 823-833.

Hoffmann, K. F., Cheever, A. W. and Wynn, T. A. (2000). IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J Immunol*, **164** (12), 6406-16.

Hölscher, C., Atkinson, R. A., Arendse, B., Brown, N., Myburgh, E., Alber, G. and Brombacher, F. (2001). A protective and agonistic function of IL-12p40 in mycobacterial infection. *J Immunol*, **167**, 6957-6966.

Hölscher, C., Arendse, B., Schwegmann, A., Myburgh, E. and Brombacher, F. (2006). Impairment of alternative macrophage activation delays cutaneous leishmaniasis in non-healing BALB/c mice. *J Immunol*, **176**, 1115-21.

Horsnell, W. G., Cutler, A. J., Hoving, C. J., Mearns, H., Myburgh, E., Arendse, B., Finkelman, F. D., Owens, G. K., Erle, D. and Brombacher, F. (2007). Delayed goblet cell hyperplasia, acetylcholine receptor expression, and worm expulsion in SMC-specific IL-4Rα-deficient mice. *PLoS Pathog*, **3** (1), e1.

http://www.wehi.edu.au/education/wehitv/malaria_lifecycle_part_1_human_host/ (Drew Berry, Biomedical Animator, The Walter and Elisa Hall Institute of Medical Research, WEHI-Tv, 2008)

http://www.niaid.nih.gov/.../pages/lifecycle.aspx. National Institute of Allergy and Infectious Diseases (NIAID) website

Huang, L. R., Chen, F. L., Chen, Y. T., Lin, Y. M. and Kung, J. T. (2000). Potent induction of long-term CD8_ T cell memory by short-term IL-4 exposure during T cell receptor stimulation. *Proc Natl Acad Sci USA*, **97,** 3406–3411.

Hunt, N. H. and Grau, G. E. (2003). Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. *Trends Immunol,* **24,** 491-499.

Ing, R. and Stevenson, M. M. (2009). Dendritic cell and NK cell reciprocal cross talk promotes gamma interferon-dependent immunity to blood-stage Plasmodium chabaudi AS infection in mice. *Infect Immun,* **77** (2), 770-92.

John, C. C., Zickafoose, J. S., Sumba, P. O., King, C. L. and Kazura, J. W. (2003). Antibodies to the Plasmodium falciparum antigens circumsporozoite protein, thrombospondin-related adhesive protein, and liver-stage antigen 1 vary by ages of subjects and by season in a highland area of Kenya. *Infect Immun,* **71** (8), 4320-5.

Joss, A., Akdis, M., Faith, A., Blaser, K. and Akdis, C. A. (2000). IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. *Eur J Immunol,* **30** (6), 1683-90.

Jung, T., Schrader, N., Hellwig, M., Enssle, K. H. and Neumann, C. (1999). Soluble human interleukin-4 receptor is produced by activated T cells under the control of metalloproteinases. *Int Arch Allergy Immunol,* **119** (1), 23-30.

Jung, T., Wagner, K., Neumann, C. and Heusser, C. H. (1999). Enhancement of human IL-4 activity by soluble IL-4 receptors in vitro. *Eur J Immunol*, **29** (3), 864-71.

Kamis, A. B. and Ibrahim, J. B. (1989). Effects of testosterone on blood leukocytes in *Plasmodium berghei*-infected mice. *Parasitol Res*, **75** (8), 611-3.

Keating, P., O'Sullivan, D., Tierney, J. B., Kenwright, D., Miromoeini, S., Mawasse, L., Brombacher, F. and La Flamme, A. C. (2009). Protection from EAE by IL-4Ralpha(-/-) macrophages depends upon T regulatory cell involvement. *Immunol Cell Biol*, **87** (7), 534-45.

Keller CC, Yamo O, Ouma C, Ong'echa JM, Ounah D, Hittner JB, Vulule JM, Perkins DJ. (2006). Acquisition of hemozoin by monocytes down-regulates interleukin-12 p40 (IL-12p40) transcripts and circulating IL-12p70 through an IL-10-dependent mechanism: in vivo and in vitro findings in severe malarial anemia. *Infect Immun*, **74** (9), 5249-60.

Kienzle, N., Buttigieg, K., Groves, P., Kawula, T. and Kelso, A. (2002). A clonal culture system demonstrates that IL-4 induces a subpopulation of noncytolytic T cells with low CD8, perforin, and granzyme expression. *J. Immunol*. **168,** 1672–1681.

Kim, C. C., Parikh, S., Sun, J. C., Myrick, A., Lanier, L. L., Rosenthal, P. J. and DeRisi, J. L. (2008). Experimental malaria infection triggers early expansion of natural killer cells. *Infec Immun*, **76** (12), 5873-82.

Kinyanjui, S. M., Bull, P., Newbold, C. I. and Marsh, K. (2003). Kinetics of antibody responses to Plasmodium falciparum-infected erythrocyte variant surgace antigens. *J Infect Dis*, **187** (4), 667-74.

Kinyanjui, S. M., Conway, D. J., Lanar, D. E. and Marsh, K. (2007). IgG antibody responses to *Plasmodium falciparum* merozoite antigens in Kenyan children have a short half-life. *Malar J*, **6**, 82.

Kitaguchi, T., Nagoya, M., Amano, T., Suzuki, M. and Minami, M. (1996). Analysis of roles of natural killer cells in defense against Plasmodium chabaudi in mice. *Parasitol Res*, **82** (4), 352-7.

Klein, S. L. (2000). Hormones and mating system affect sex and species differences in immune function among vertebrates. *Behav Processes*, **51** (1-3), 149-166

Klein, S. L. (2000). The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci Biobehav Rev*, **24** (6), 627-38.

Klein, S. L. (2004). Hormonal and immunological mechanisms mediating sex differences in parasite infection. *Parasite Immunol*, **26**, 247-264.

Klein, P. W, Easterbrook, J. D, Lalime, E. N. and Klein, S. L. (2008). Estrogen and progesterone affect responses to malaria infection in female C57BL/6 mice. *Gend Med*, **5** (4), 423-33.

Kobayashi, M., Fitz, L., Ryan, M., Hewick, R., Clark, S., Chan, S., Loudon, R., Sherman, F. and Perussia, B. (1989). Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med,* **170,** 827-845.

Krishnegowda, G., Hajjar, A. M., Zhu, J., Douglass, E. J., Uematsu, S., Akira, S., Woods, A. S. and Gowda, D. C. (2005). Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. *J Biol Chem,* **280,** 8606-8616.

Kropf, P., Freudenberg, M. A., Modolell, M., Price, H. P., Herath, S., Antoniazi, S., Galanos, C., Smith, D. F. and Müller, I. (2004). Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite *Leishmania major*. *Infect Immun*, **72** (4), 1920-8.

Kropf, P., Fuentes, J. M., Fähnrich, E., Arpa, L., Herath, S., Weber, V., Soler, G., Celada, A., Modolell, M. and Müller, I. (2005). Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. *FASEB J*, **19**, 1000-2.

Kurtzhals, J. A., Adabayeri, V., Goka, B. Q., Akanmori, B. D., Oliver-Commey, J. O., Nkrumah, F. K., Behr, C. and Hviid, L. (1998). Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. *Lancet,* **351,** 1768-1772.

Kwiatkowski, D., Bate, C. A., Scragg, I. G., Beattie, P., Udalova, I. and Knight, J. C. (1997). The malarial fever response--pathogenesis, polymorphism and prospects for intervention. *Ann Trop Med Parasitol,* **91,** 533-542.

Langhorne, J. (1989). The role of CD4+ T-cells in the immune response to *Plasmodium chabaudi*. *Parasitol Today*, **5** (11), 362-4.

Langhorne, J., Meding, S. J., Eichmann, K. and Gillard, S. S. (1989). The response of CD4+ T cells to *Plasmodium chabaudi chabaudi*. *Immunol Rev*, **112**, 71-94.

Langhorne, J., Gillard, S., Simon, B., Slade, S. and Eichmann, K. (1989). Frequencies of CD4+ T cells reactive with *Plasmodium chabaudi chabaudi*: distinct response kinetics for cells with Th1 and Th2 characteristics during infection. *Int Immunol*, **1,** 416-424.

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

Langhorne, J., Quin, S. J. and Sanni, L. A. (2002). Mouse models of blood-stage malaria infections: immune responses and cytokines involved in protection and pathology. *Chem Immunol*, **80,** 204-228.

Langhorne, J., Albano, F. R., Hensmann, M., Sanni, L., Cadman, E., Voisine, C. and Sponaas, A. M. (2004). Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection. *Immunol Rev,* **201,** 35-47.

Langhorne, J., Ndungu, F. M, Sponaass, A. M. and Marsh, K. (2008). Immunity to malaria: more questions than answers. *Nat Immunol*, **9**, 725-732.

Lean, I. S., McDonald, S. A., Bajaj-Elliott, M., Pollok, R. C., Farthing, M. J., and McDonald, V. (2003). Interleukin-4 and transforming growth factor-beta have opposing regulatory effects on gamma interferon mediated inhibition of *Cryptosporidium parvum* reproduction, *Infect. Immun*, **71**, 4580-4585.

Leeto, M., Herbert, D. R., Marillier, R., Schwegmann, A., Fick, L. and Brombacher, F. (2006). TH1-dominant granulomatous pathology does not inhibit fibrosis or cause lethality during murine schistosomiasis. *Am J Pathol*, **169**, 1701–1712

Leisewitz, A. L., Rockett, K. A., Gumede, B., Jones, M., Urban, B. and Kwiatkowski, D. P. (2004). Response of the splenic dendritic cell population to malaria infection. *Infect Immun*, **72** (7), 4233-9.

Leke, R., Viens, P. and Davies, A. J. (1981). Interaction between Plasmodium chabaudi and C57Bl mice with particular reference to the immune response. *Clin Exp Immunol*, **45** (3), 627-32.

Li, C. and Langhorne, J. (2000). Tumor necrosis factor alpha p55 receptor is important for development of memory responses to blood-stage malaria infection. *Infect Immun*, **68** (10), 5724-30.

Li, C., E. Seixas, and J. Langhorne. 2001. Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite. Med Microbiol Immunol, **189**, 115–126

Li, C., Sanni, L. A., Omer, F., Riley, E. and Langhorne, J. (2003). Pathology of *Plasmodium chabaudi chabaudi* infection and mortality in interleukin-10-deficient mice are ameliorated by anti-tumor necrosis factor alpha and exacerbated by anti-transforming growth factor beta antibodies. *Infect Immun,* **71,** 4850-4856.

Liew, F. Y., Li, Y. and Millott, S. (1990). Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of Leishmania major through the induction of nitric oxide. *J Immunol,* **145,** 4306-4310.

Limbach, K., Aguiar, J., Gowda, K., Patterson, N., Abot, E., Sedegah, M., Sacci, J. and Richie, T. (2011). Identification of two new protective pre-erythrocytic malaria vaccine antigen candidates. *Malar J*, **10**, 65.

Louis, J., Himmelrich, H., Parra-Lopez, C., Tacchini-Cottier, F. and Launois, P. (1998). Regulation of protective immunity against Leishmania major in mice. *Curr Opin Immunol*, **10** (4), 459-64.

Louis, J. A., Conceiçao-Silva, F., Himmelrich, H., Tacchini-Cottier, F. and Launois P. (1998). Anti-leishmania effector functions of CD4+ Th1 cells and early events instructing Th2 cell development and susceptibility to Leishmania major in BALB/c mice. *Adv Exp Med Biol*. **452**, 53-60.

Lundie, R. J., de Koning-Ward, T. F., Davey, G. M., Nie, C. Q., Hansen D. S., Lau, L. S., Mintern, J. D., Belz, G. T., Schofield, L., Carbone., F. R., Villadangos, J. A., Crabb, B. S. and Heath, W. R. (2008). Blood-stage *Plasmodium* infection induces CD8+ T lymphocytes to parasite-expressed antigens, largely regulated by CD8alpha+ dendritic cells. *Proc Natl Acad Sci USA*, **105** (38), 14509-14.

Luty, A. J., Lell, B., Schmidt-Ott, R., Lehman, L. G., Luckner, D., Greve, B., Matousek, P., Herbich, K., Schmid, D., Migot-Nabias, F., Deloron, P., Nussenzweig, R. S. and Kremsner PG. (1999). Interferon-gamma responses are associated with resistance to reinfection with Plasmodium falciparum in young African children. *J Infect Dis*, **179** (4), 980-8.

Mannetti, R., Parronchi, P., Guidizi, M., Piccinni, M., Maggi, E., Trinchieri, G. and Romangnani, S. (1993). Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med*, **177,** 1199-1204.

Mantovani, A., Sozzani, S., Locati,M., Allavena, P. and Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*, **23** (11), 549-55.

Manz, R. A. and Radbruch, A. (2002). Plasma cells for a lifetime? *Eur J Immunol*, **32**, 923-927.

Manz, R. A., Thiel, A., Radbruch, A. (1997). Lifetime of plasma cells in the bone marrow. *Nature*, **388**, 133–134.

Marsh, K. and Kinyanjui, S. (2006). Immune effector mechanisms in malaria. *Parasite Immunol*, **28,** 51-60.

Marsh, K., Otoo, L., Hayes, R. J., Carson, D. C. and Greenwood, B. M. (1989). Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. *Trans R Soc Trop Med Hyg*, **83,** 293-303.

Martinez, F. O., Helming, L. and Gordon, S. (2009). Alternative activation of macrophages: and immunologic functional perspective. *Annu Rev Immunol*, **27**, 451-83.

Mazier, D., Beaudoin, R. L., Mellouk, S., Druilhe, P., Texier, B., Trosper, J., Miltgen, F., Landau, I., Paul, C. and Brandicourt, O., et al. (1985). Complete development of hepatic stages of *Plasmodium falciparum* in vitro. *Science*, **227** (4685), 440-2.

McCaffrey, R. L., Fawcett, P., O'Riordan, M., Lee, K. D., Havell, E. A., Brown, P. O. and Portnoy, D. A. (2004). A specific gene expression program triggered by Gram-positive bacteria in the cytosol. *Proc Natl Acad Sci USA*, **101** (31), 11386-91.

McCall, M. B., Roestenberg, M., Ploemen, I., Teirlinck, A., Hopman, J., de Mast, Q., Dolo, A., Doumbo, O. K., Luty, A., van der Ven, A. J., Hermsen, C. C. and Sauerwein, R. W. (2010). Memory-like IFN-γ responses by NK cells following malaria infection reveals the crucial role of T-cells in NK cell activation by P. falciparum. *Eur J Immunol*, **40** (12), 3472-7.

McDonald, V. and Phillips, R. S. (1975). *Plasmodium chabaudi* in mice: adoptive transfer of immunity with enriched populations of spleen T and B lymphocytes. *Immunology*, **34**, 821-830.

McDonald, V. and Phillips, R. S. (1978). *Plasmodium chabaudi* in mice. Adoptive transfer of immunity with enriched populations of spleen T and B lymphocytes. *Immunology,* **34,** 821-830.

McDonald, V. and Phillips, R. S. (1980). *Plasmodium chabaudi*: Adoptive transfer of immunity with different spleen cell populations and development of protective activity in the serum of lethally irradiated recipient mice. *Exp Parasitol*, **49**, 26-33.

McDonald, S. A., O'Grady, J. E., Bajaj-Elliott, M., Notley, C. A., Alexander, J., Brombacher, F. and McDonald, V. (2004). IL-4 confers protection against the early acute phase of *Cryptosporidium parvum* infection by promoting Th1 cytokine expression. *J Inf. Dis*, **190**, 1019-25.

McFarlane, E., Carter, K. C., McKenzie, A., Kaye, P. M., Brombacher, F. and Alexander, J. (2011). Endogenous IL-13 plays a crucial role in liver granuloma maturation during *Leishmania donovani* infection via a macrophage independent mechanism. *Journal of Infectious Diseases*, **204**, 36-43.

McKenzie, A. N., Culpepper, J. A., de Waal Malefyt, R., Briere, F., Punnonen, J., Aversa, G., Sato, A., Dang, W., Cocks, B. G., Menon, S. and et al. (1993). Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc Natl Acad Sci U S A,* **90,** 3735-3739.

McLean, S. A., Pearson, C. D. and Phillips, R. S. (1982). *Plasmodium chabaudi*: antigenic variation during recrudescent parasitaemias in mice. *Exp Parasitol,* **54,** 296-302.

Meding, S. J., Cheng, S. C., Simon-Haarhaus, B. and Langhorne, J. (1990). Role of gamma interferon during infection with *Plasmodium chabaudi chabaudi*. *Infect Immun,* **58** (11), 3671-3678.

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

Melo, G. C., Reyes-Lecca, R. C., Vitor-Silva, S., Monteiro, W. M., Martins, M., Benzecry, S. G., Alecrim, M. and Lacerda, M. V. (2010). Concurrent helminth infection protects schoolchildren with *Plasmodium vivax* from anemia. *PLos ONE*, **5** (6), e11206.

Mencacci, A., Del Sero, G., Cenci, E., d'Ostiani, C. F., Bacci, A., Montagnoli, C., Kopf, M. and Romani, L. (1998). Endogenous interleukin 4 is required for development of protective CD4þ T helper type 1 responses to Candida albicans. *J Exp Med*, **187**, 307-317.

Ménard, R. (2001). Gliding motility and cell invasion by Apicomplexa: insights from the *Plasmodium* sporozoite. *Cell Microbiol*, **3** (2), 63-73.

Menzies, F. M., Henriquez, F. L., Alexander,J. and Roberts, C. W. (2010). Sequential expression of macrophage anti-microbial/inflammatory and wound healing markers following innate, alternative and classical activation. *Clin Exp Immunol*, **160** (3), 369-79.

Metzger, W. G., Okenu, D. M., Cavanagh, D. R., Robinson, J. V., Bojang, K. A., Weiss, H. A., McBride, J. S., Greenwood, B. M. and Conway, D. J. (2003). Serum IgG3 to the *Plasmodium falciparum* merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. *Parasite Immunol*, **25** (6), 307-12.

Michels, C. E., Scales, H. E., Saunders, K., McGowan, S., Brombacher, F., Alexander, J. and Lawrence, C. E. (2009). IL-4Rα expression on neither $CD4^+$ T cells, nor macrophages and neutrophils is required for protective immunity to *Trichinella spiralis*. *Immunology*, **128**, 385-94.

Miller, C. L., Hooton, J. W., Gillis, S. and Paetkau, V. (1990). IL-4 potentiates the IL-2-dependent proliferation of mouse cytotoxic T cells. *J Immunol,* **144,** 1331–1337.

Millington, O. R., Di Lorenzo, C., Phillips, R. S., Garside, P. and Brewer, J. M. (2006). Suppression of adaptive immunity to heterologous antigens during *Plasmodium* infection through hemozoin-induced failure of dendritic cell function. *J Biol,* **5,** 5.

Minty, A., Chalon, P., Derocq, J. M., Dumont, X., Guillemot, J. C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B. and et al. (1993). Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature,* **362,** 248-250.

Mitchell, G. H., Thomas, A. W., Margos, G., Dluzewski, A. R. and Bannister, L. H. (2004). Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells. *Infect Immun*, **72** (1), 154.

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

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

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

Mohrs, M., Ledermann, G., Kohler, A., Dorfmuller, A., Gessner, A. and Brombacher, F. (1999). Differences between IL-4- and IL-4 receptor alpha-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signaling. *J Immunol*, **162,** 7302-8.

Mohrs, M., Holscher, C. and Brombacher, F. (2000). Interleukin-4 Receptor Alpha-Deficient BALB/c Mice Show an Unimpaired T Helper 2 Polarization in Response to *Leishmania major* Infection. *Infection and Immunity*, **68** (4) 1773-1780.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*, **19**, 683–765.

Mosmann, T. R. and Coffman, R. L. (1987). Two types of mouse helper T-cell clone: implications for immune regulation. *Immunol Today*, **8**, 223-227.

Mosmann, T. R. and Coffman, R. L. (1989). Thl and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*, **7**, 145-173.

Mosser, D. M. (2003). The many faces of macrophage activation. *J Leukoc Biol*, **73** (2), 209-12.

Murray, H. M., Tsai, C. W., Liu, J. and Ma, X. (2006). Visceral *Leishmania donovani* infection in interleukin-$13^{-/-}$ mice. *Infect Immun*, **74**, 2487-2490.

Nacher, M., Gay, F., Singhasivanon, P., Krudsood, S., Treeprasertsuk, S., Mazier, D., Vouldoukis, I. and Looareesuwan, S. (2000). Ascaris lumbricoides infection is associated with protection from cerebral malaria. *Parasite Immunol*, **22** (3), 107-13.

Namazi, M. J. and Phillips, R. S. (2010). Immune responses of NIH mice infected with avirulent and virulent strains of Plasmodium chabaudi adami single and mixed infections. *Korean J Parasitol*, **48** (1), 23-33.

Nardin, E., Zavala, F., Nussenzweig, V. and Nussenzweig, R. S. (1999). Pre-erythrocytic malaria vaccine: mechanisms of protective immunity and human vaccine trials. *Parassitologia,* **41,** 397-402.

Narum, D. L. and Thomas, A. W. (1994). Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of *Plasmodium falciparum* merozoites. *Mol Biochem Parasitol,* **67,** 59-68.

Nau, G. J., Richmond, J. F., Schlesinger, A., Jennings, E. G., Lander, E. S. and Young, R. A. (2002). Human macrophage activation programs induced by bacterial pathogens. *Proc Natl Acad Sci USA*, **99** (3), 1503-8.

Ndungu, F. M., Cadman, E. T., Coulcher, J., Nduati, E., Couper, E., McDonald, D. W., Ng, D. and Langhorne, J. (2009). Functional memory B Cells and long-lived plasma cells are generated after a single *Plasmodium chabaudi* infection in mice. *PloS Pathogens,* **5** (12), e1000690.

Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J. and Paul, W. E. (1999). The IL-4 receptor: signaling mechanisms and biologic functions. *Annu Rev Immunol*, **17,** 701-38.

Neva, F. A., Sheagren, J. N., Shulman, N. R. and Canfield, C. J. (1970). Malaria: host-defense mechanisms and complications. *Ann Intern Med,* **73**, 295-306.

Newman, K. C., Korbel, D. S., Hafalla, J. C. and Riley, E. M. (2006). Cross-talk with myeloid accessory cells regulates human natural killer cell interferon gamma responses to malaria. *PLoS Pathog*, **2**, e118.

Niikura, M., Inoue, S. and Kobayashi, F. (2011). Role of interleukin-10 in malaria: focusing on coinfection with lethal and non-lethal murine malaria parasites. *J Biomed Biotechnol*, **2011**, 383962.

Noble, A. and Kemeny, D. M. (1995). Interleukin-4 enhances interferon-γ synthesis but inhibits development of interferon-γ-producing cells. *Immunology*, **85**, 357-363.

Noël, W., Raes, G., Hassanzadeh Ghassabeh, G., De Baetselier, P. and Beschin, A. (2004). Alternatively activated macrophages during parasite infections. *Trends Parasitol*, **20** (3), 126-33.

Nussenzweig, V. and Nussenzweig, R. S. (1985). Circumsporozoite proteins of malaria parasites. *Cell*, **42** (2), 401-3.

O'Donnell, R. A., Saul, A., Cowman, A. F. and Crabb, B. S. (2000). Functional conservation of the malaria vaccine antigen MSP-119across distantly related *Plasmodium* species. *Nat Med,* **6,** 91-95.

O'Donnell, R. A., de Koning-Ward, T. F., Burt, R. A., Bockarie, M., Reeder, J. C., Cowman, A. F. and Crabb, B. S. (2001). Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. *J Exp Med,* **193,** 1403-1412.

Omer, F. M. and Riley, E. M. (1998). Transforming growth factor beta production is inversely correlated with severity of murine malaria infection. *J Exp Med,* **188,** 39-48.

Omer, F. M., Kurtzhals, J. A. and Riley, E. M. (2000). Maintaining the immunological balance in parasitic infections: a role for TGF-beta? *Parasitol Today*, **16** (1), 18-23.

Omer, F. M., de Souza, J. B. and Riley, E. M. (2003). Differential induction of TGF-beta regulates proinflammatory cytokine production and determines the outcome of lethal and nonlethal *Plasmodium yoelii* infections. *J Immunol,* **171,** 5430-5436.

Ortaldo, J. R., Mason, A. T., O'Shea, J. J., Smyth, M. J., Falk, L. A., Kennedy, I. C., Longo, D. L. and Ruscetti, F. W. (1991). Mechanistic studies of transforming growth factor-beta inhibition of IL-2-dependent activation of CD3- large granular lymphocyte functions. Regulation of IL-2R beta (p75) signal transduction. *J Immunol*, **146,** 3791-3798.

Osier, F. H., Fegan, G., Polley, S. D., Murungi, L., Verra, F., Tetteh, K. K., Lowe, B., Mwangi, T., Bull, P. C., Thomas, A. W., Cavanagh, D. R., McBride, J. S., Lanar, D. E., Mackinnon, M. J., Conway, D. J. and Marsh, K. (2008). Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria. *Infect Immun*, 76 (5), 2240-8.

Ouma, C., Davenport, G. C., Were, T., Otieno, M. F., Hittner, J. B., Vulule, J. M., Martinson, J., Ong'echa, J. M., Ferrell, R. E. and Perkins, D. J. (2008). "Haplotypes of IL-10 promoter variants are associated with susceptibility to severe malarial anemia and functional changes in IL-10 production," *Human Genetics*, **124** (5), 515–524.

Owens, I. P. (2002). Ecology and evolution. Sex differences in mortality rate. *Science*, **297** (5589), 2008–2009.

Paaijmans, K. P., Read, A. F. and Thomas, M. B. (2009). Understanding the link between malaria risk and climate. *Proc Natl Acad Sci USA*, **106** (33), 13844-9.

Paaijmans, K. P., Blanford, S., Bell, A. S., Blanford, J. I., Read, A. F. and Thomas, M, B. (2010). *Proc Natl Acad Sci USA*, **107** (34), 15135-9.

Palacios, R., Sideras, P., Boehmer, H. (1987). Recombinant interleukin 4/BSF-1 promotes growth and differentiation of intrathymic T cell precursors from fetal mice in vitro. *EMBO J.* 6, 91–95.

Parham, P. E. and Michael, E. (2010). Modelling climate change and malaria transmission. *Adv Exp Med Biol*, **673**, 184-99.

Parham, P. E. and Michael, E. (2010). Modeling the effects of weather and climate change on malaria transmission. *Environ Health Perspect*, **118** (5), 620-6.

Pasquetto, V., Fidock, D. A., Gras, H., Badell, E., Eling, W., Ballou, W. R., Belghiti, J., Tartar, A. and Druilhe, P. (1997). *Plasmodium falciparum* sporozoite invasion is inhibited by naturally acquired or experimentally induced polyclonal antibodies to the STARP antigen. *Eur J Immunol,* **27,** 2502-2513.

Paul, W. E. (1997). Interleukin 4: signalling mechanisms and control of T cell differentiation. *Ciba Found Symposium*, **204**, 208-16.

Peluffo, G., Piacenza, L., Irigoin, F., Alvarez, M. N. and Radi, R. (2004). L-arginine metabolism during interaction of Trypanosoma cruzi with host cells. *Trends Parasitol*. **20** (8), 363-9.

Perlmann, H., Helmby, H., Hagstedt, M., Carlson, J., Larsson, P. H., Troye-Blomberg, M. and Perlmann, P. (1994). IgE elevation and IgE anti-malarial antibodies in *Plasmodium falciparum* malaria: association of high IgE levels with cerebral malaria. *Clin Exp Immunol,* **97,** 284-292.

Perlmann, P., Perlmann, H., Looareesuwan, S., Krudsood, S., Kano, S., Matsumoto, Y., Brittenham, G., Troye-Blomberg, M. and Aikawa, M. (2000). Contrasting functions of IgG and IgE antimalarial antibodies in uncomplicated and severe *Plasmodium falciparum* malaria. *Am J Trop Med Hyg,* **62,** 373-377.

Perry, J. A., Rush, A., Wilson, R. J., Olver, C. S. and Avery, A. C. (2004). Dendritic cells from malaria-infected mice are fully functional APC. *J Immunol*, **172**, 475–482.

Phillips, R. S., Mathers, K. E. and Taylor-Robinson, A. W. (1994). T cells in immunity to Plasmodium chabaudi chabaudi: operation and regulation of different pathways of protection. *Res Immunol*, **145** (6), 406-12.

Phillips, R. S., Brannan, L. R., Balmer, P. and Neuville, P. (1997). Antigenic variation during malaria infection--the contribution from the murine parasite *Plasmodium chabaudi*. *Parasite Immunol,* **19,** 427-434.

Phillips, R. S. (2001). Current status of malaria and potential for control. *Clin Microbiol Rev,* **14,** 208-226.

Pichyangkul, S., Saengkrai, P., Yongvanitchit, K., Stewart, A. and Heppner, D. G. (1997). Activation of gamma delta T cells in malaria: interaction of cytokines and a schizont-associated *Plasmodium falciparum* antigen. *J Infect Dis*, **176** (1), 233-41.

Pichyangkul, S., Yongvanitchit, K., Kum-arb, U., Hemmi, H., Akira, S., Krieg, A. M., Heppner, D. G., Stewart, V. A., Hasegawa, H., Looareesuwan, S., Shanks, G. D. and Miller, R. S. (2004). Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. *J Immunol*, **172** (8), 4926-33.

Plebanski, M. and Hill, A. V. (2000). The immunology of malaria infection. *Curr Opin Immunol*, *12* (4), 437-41.

Podoba, J. E. and Stevenson, M. M. (1991). $CD4^+$ and $CD8^+$ T lymphocytes both contribute to acquired immunity to blood-stage *Plasmodium chabaudi* AS. *Infect Immun*, **59** (1), 51-58.

Powrie, F., Menon, S. and Coffman, R. L. (1993). **Interleukin-4** and interleukin-10 synergize to inhibit cell-mediated immunity in vivo. *Eur J Immunol*, **23** (11), 3043-9.

Price, R. N., Tjitra, E., Guerra, C. A., Yeung, S., White, N. J. and Anstey, N. M. (2007). *Vivax* malaria: neglected and not benign. *Am J Trop Med Hyg*, **77**, 79-87.

Price, R. N., Douglas, N. M. and Anstey, N. M. (2009). New developments in Plasmodium **vivax** malaria: severe disease and the rise of chloroquine resistance. *Curr Opin Infect Dis*, **22** (5), 430-5.

Pulendran, B., Smith, J. L., Caspary, G., Brasel, K., Pettit, D., Maraskovsky, E. and Maliszewski, C. R. (1999). Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc Natl Acad Sci U S A,* **96,** 1036-1041.

Punnonen, J., Aversa, G., Cocks, B. G., McKenzie, A. N., Menon, S., Zurawski, G., de Waal Malefyt, R. and de Vries, J. E. (1993). Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci U S A,* **90,** 3730-3734.

Quin, S. J. and Langhorne, J. (2001). Different regions of the malaria merozoite surface protein 1 of *Plasmodium chabaudi* elicit distinct T-cell and antibody isotype responses. *Infect Immun*, **69** (4), 2245-2251.

Quin, S. J., Seixas, E. M., Cross, C. A., Berg, M., Lindo, V., Stockinger, B. and Langhorne, J. (2001). Low CD4(+) T cell responses to the C-terminal region of the malaria merozoite surface protein-1 may be attributed to processing within distinct MHC class II pathways. *Eur J Immunol*, **31,** 72-81.

Radwanska, M., Cutler, A. J., Hoving, J. C., Magez, S., Holscher, C., Bohms, A., Arendse, B., Kirsch, R., Hunig, T., Alexander, J., Kaye, P. and Brombacher, F. (2007). Deletion of IL-4Ralpha on CD4 T cells renders BALB/c mice resistant to *Leishmania major* infection. *PLoS Pathog* **3:**e68-79.

Raes, G., Beschin, A., Ghassabeh, G. H. and De Baetselier, P. (2007). Alternatively activated macrophages in protozoan infections. *Curr Opin Immunol*, **19** (4), 454-9.

Ramanathan, S., de Kosak, Y., Saoudi, A., Goureau, O., Van der Meide, P. H., Druet, P. and Bellon, B. (1996). Recombinant IL-4 aggravates experimental autoimmune uveoretinitis in rats. *J Immunol*, **157**, 2209-2215.

Renia, L., Potter, S. M., Mauduit, M., Rosa, D. S., Kayibanda, M., Deschemin, J. C., Snounou, G. and Grüner, A. C. (2006). Pathogenic T cells in cerebral malaria. *Int J Parasitol*, **36** (5), 547-54.

Reyes, J. L. and Terrazas, L. I. (2007). The divergent roles of alternatively activated macrophages in helminthic infections. *Parasite Immunol*, **29** (12), 609-19.

Riley, E. M., Morris-Jones, S., Blackman, M. J., Greenwood, B. M. and Holder, A. A. (1993). A longitudinal study of naturally acquired cellular and humoral immune responses to a merozoite surface protein (MSP1) of *Plasmodium falciparum* in an area of seasonal malaria transmission. *Parasite Immunol,* **15,** 513-524.

Riley, E. M. (1999). Is T-cell priming required for initiation of pathology in malaria infections? *Immunol Today,* **20,** 228-233.

Riley, E. M., Wahl, S., Perkins, D. J. and Schofield, L. (2006). Regulating immunity to malaria. *Parasite Immunol,* **28,** 35-49.

Roberts, D. W., Rank, R. G., Weidanz, W. P. and Finerty, J. F. (1977). Prevention of recrudescent malaria in nude mice by thymic grafting or by treatment with hyperimmune serum. *Infect Immun,* **16,** 821-826.

Roberts, C. W., Walker, W. and Alexander, J. (2001). Sex-associated hormones and immunity to protozoan parasites. *Clin Microbiol Rev*, **14** (3), 476-88.

Robinson, L. J., D'Ombrain, M. C., Stanisic, D. I., Taraika, J., Bernard, N., Richards, J. S., Beeson, J. G., Tavul, L., Michon, P., Mueller, I. and Schofield, L. (2009). Cellular tumor necrosis factor, gamma interferon, and interleukin-6 responses as correlates of immunity and risk of clinical Plasmodium falciparum malaria in children from Papua New Guinea. *Infect Immun*, **77** (7), 3033-43.

Sabbatani, S., Fiorino, S. and Manfredi, R. (2010). The emerging of the fifth malaria parasite (*Plasmodium knowlesi*). A public health concern? *J Infect Dis*, **14** (3), 299-309.

Sam, H. and Stevenson, M. M. (1999). Early IL-12 p70, but not p40, production by splenic macrophages correlates with host resistance to blood-stage *Plasmodium chabaudi* AS malaria. *Clin Exp Immunol,* **117,** 343-349.

Schandené L, Alonso-Vega C, Willems F, Gérard C, Delvaux A, Velu T, Devos R, de Boer M, Goldman M. (1994). B7/CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10. *J Immunol,* **152** (9), 4368-74.

Schmieg, J., Gonzalez-Aseguinolaza, G. and Tsuji, M. (2003). The role of natural killer T cells and other T cell subsets against infection by the pre-erythrocytic stages of malaria parasites. *Microbes Infect,* **5,** 499-506.

Schmitz, J., Assenmacher, M. and Radbruch, A. (1993). Regulation of T helper cell cytokine expression: functional dichotomy of antigen-presenting cells. *Eur J Immunol*, **23** (1), 191-9.

Schofield, L. and Grau, G. E. (2005). Immunological processes in malaria pathogenesis. *Nat Rev Immunol*, **5** (9), 722-35.

Sedegah, M., Charoenvit, Y., Minh, L., Belmonte, M., Majam, V. F., Abot, S, Ganeshan, H., Kumar, S., Bacon, D. J., Stowers, A., Narum, D. L., Carucci, D. J. and Rogers, W. O. (2004). Reduced immunogenicity of DNA vaccine plasmids in mixtures. *Gene Ther*, **11** (5), 448-56.

Seixas, E., Fonseca, L. and Langhorne, J. (2002). The influence of gamma delta T cells on the CD4+ T cell and antibody response during a primary *Plasmodium chabaudi chabaudi* infection in mice. *Parasite Immunol*, **24** (3), 131-40.

Shankar, A. H. (2000). Nutritional modulation of malaria morbidity and mortality. *J Infect Dis,* **182 Suppl 1,** S37-53.

Shear, H. L., Marino, M. W., Wanidworanun, C., Berman, J. W. and Nagel, R. L. (1998). Correlation of increased expression of intercellular adhesion molecule-1, but not high levels of tumor necrosis factor-alpha, with lethality of *Plasmodium yoelii* 17XL, a rodent model of cerebral malaria. *Am J Trop Med Hyg,* **59,** 852-858.

Sher, A., Pearce, E. and Kaye, P. (2003). Shaping the immune response to parasites: role of dendritic cells. *Curr Opin Immunol,* **15,** 421-429.

Silvie, O., Franetich, J. F., Charrin, S., Mueller, M. S., Siau, A., Bodescot, M., Rubinstein, E., Hannoun, L., Charoenvit, Y., Kocken, C. H., Thomas, A. W., Van Gemert, G. J., Sauerwein, R. W., Blackman, M. J., Anders, R. F., Pluschke, G. and Mazier, D. (2004). A role for apical membrane antigen 1 during invasion of hepatocytes by *Plasmodium falciparum* sporozoites. *J Biol Chem,* **279,** 9490-9496.

Sim, B. K., Chitnis, C. E., Wasniowska, K., Hadley, T. J. and Miller, L. H. (1994). Receptor and ligand domains for invasion of erythrocytes by *Plasmodium falciparum*. *Science,* **264,** 1941-1944.

Skamene, E., Stevenson, M. M. and Lemieux, S. (1983). Murine malaria: dissociation of natural killer (NK) cell activity and resistance to Plasmodium chabaudi. *Parasite Immunol*, **5** (6), 557-65.

Skeen, M. J., Miller, M. A., Shinnick, T. M. and Ziegler, H. K. (1996). Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. *J Immunol*, **156** (3), 1196-206.

Slifka, M. K., Matloubian, M. and Ahmed, R. (1995). Bone marrow is a major site of long-term antibody production after acute viral infection. *J Virol*, **69**, 1895–1902.

Slifka, M. K., Antia, R., Whitmire, J.K. and Ahmed, R. (1998). Humoral immunity due to long-lived plasma cells. Immunity 8: 363–372.

Smith, E. C and Taylor-Robinson, A. W. (2003). Parasite-specific immunoglobulin isotypes during lethal and non-lethal murine malaria infections. *Parasitol Res*, **89** (1), 26-33.

Smith, C. M., Belz, G. T., Wilson, N. S., Villadangos, J. A., Shortman, K., Carbone, F. R. and Heath, W. R. (2003). Cutting edge: conventional CD8α$^+$ dendritic cells are preferentially involved in CTL priming after footpad infection with herpes simplex virus-1. *J Immunol*, **170**, 4437–4440.

Snow, R. W., Shenton, F. C., Lindsay, S. W., Greenwood, B. M., Bennet, S., Wheeler, J., Del Giudice, G., Verdini, A. S. and Pessi, A. (1989). Sporozoite antibodies and malaria in children in a rural area of The Gambia. *Annals of tropical medicine and parasitology*, **83**, 559-568.

Soloway, P., Fish, S., Passmore, H., Gefter, M., Coffee, R. and Manser, T. (1991). Regulation of the immune response to peptide antigens: differential induction of immediate-type hypersensitivity and T cell proliferation due to changes in either peptide structure or major histocompatibility complex haplotype. *J Exp Med*, **174** (4), 847-58.

Soulard, V., Roland, J., Sellier, C., Gruner, A. C, Leite-de-Moraes, M., Franetich, J. F., Rénia, L., Cazenave, P. A. and Pied, S. (2007). Primary infection of C57BL/6 mice with *Plasmodium yoelii* induces a heterogeneous response of NKT cells. *Infect Immun*, **75** (5), 2511-22.

Sponaas, A. M., Cadman, E. T., Voisine, C., Harrison, V., Boonstra, A., O'Garra, A. and Langhorne, J. (2006). Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells. *JEM*, **203** (6), 1427-1433.

Stager, S., Alexander, J., Carter, K. C., Brombacher, F. and Kaye, P. M. (2003). Both interleukin-4 (IL-4) and IL-4 receptor alpha signaling contribute to the development of hepatic granulomas with optimal antileishmanial activity. *Infect Immun*, **71**, 4804-4807.

S. Stager, J. Alexander, A.C. Kirby, M. Botto, N.V. Rooijen, D.F. Smith, F. Brombacher, P.M. Kaye. (2003). Natural antibodies and complement are endogenous adjuvants for vaccine-induced CD8þ T-cell responses. *Nat Med*, **9** 1287-1292.

Stavnezer. J. (1996). Immunoglobulin class switching. *Curr Opin Immunol*, **8** (2), 199-205.

Stempin, C. C., Dulgerian L. R., Garrido, V. V. and Cerban F. M. (2010). Arginase in parasitic infecitons: Macrophage activation, immunosuppression, and intracellular signals. *J Biomed Biotechnol*, **2010**, 683485.

Stephens, R., Albano, F. R., Quin, S., Pascal, B. J., Harrison, V., Stockinger, B., Kioussis, D., Weltzien, H. and Langhorne, J. (2005). Malaria-specific transgenic $CD4^+$ T cells protect immunodeficient mice from lethal infection and demonstrate requirement for a protective threshold of antibody production for parasite clearance. *Blood*, **106** (5), 1676-1684.

Stephens, R. and Langhorne, J. (2006). Priming of CD4+ T cells and development of CD4+ T cell memory; lessons for malaria. *Parasite Immunol*, **28** (1-2), 25-30.

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

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

Stevenson, M. M. and Tam, M. F. (1993). Differential induction of helper T cell subsets during blood-stage *Plasmodium chabaudi* AS infection in resistant and susceptible mice. *Clin Exp Immunol,* **92,** 77-83.

Stevenson, M. M. and Zavala, F. (2006). Immunology of malaria infections. *Parasite Immunol,* **28,** 1-4.

Stevenson, M. M., Tam, M. F., Belosevic, M., van der Meide, P. H. and Podoba, J. E. (1990). Role of endogenous gamma interferon in host response to infection with blood-stage *Plasmodium chabaudi* AS. *Infect Immun,* **58,** 3225-3232.

Stevenson, M. M., Huang, D. Y., Podoba, J. E. and Nowotarski, M. E. (1992). Macrophage activation during *Plasmodium chabaudi* AS infection in resistant C57BL/6 and susceptible A/J mice. *Infect Immun,* **60,** 1193-1201.

Stevenson, M. M., Su, Z., Sam, H. and Mohan, K. (2001). Modulation of host responses to blood-stage malaria by interleukin-12: from therapy to adjuvant activity. *Microbes Infect,* **3,** 49-59.

Struik, S. S. and Riley, E. M. (2004). Does malaria suffer from lack of memory?. *Immunol Rev*, **201**, 268-90.

Su, Z. and Stevenson, M. M. (2002). IL-12 is required for antibody-mediated protective immunity against blood-stage *Plasmodium chabaudi* AS malaria infection in mice. *J Immunol,* **168,** 1348-1355.

Su, Z., Segura, M., Morgan, K., Loredo-Osti, J. C. and Stevenson, M. M. (2005). Impairment of protective immunity to blood-stage malaria by concurrent nematode infection. *Infect Immun,* **73,** 3531-3539.

Su, Z. and Stevenson, M. M. (2000). Central role of endogenous gamma interferon in protective immunity against blood-stage *Plasmodium chabaudi* AS infection. *Infect Immun,* **68,** 4399-4406.

Süss, G., Eichmann, K., Kury, E., Linke, A. and Langhorne, J. (1988) Roles of CD4- and CD8-bearing T lymphocytes in the immune response to the erythrocytic stages of *Plasmodium chabaudi*. *Infect Immun*, **56**, 3081–3088.

Suzuki, Y., Yang, Q., Yang, S., Nguyen, N., Lim, S., Liensenfeld, O., Kojima, T. and Remington, J. S. (1996). IL-4 is protective against development of toxoplasmic encephalitis. *J Immunol*, **157**, 2564-2569.

Swain, S. L, Bradley, L. M, Croft, M., Tonkonogy, S., Atkins, G., Weinberg, A. D., Duncan, D. D, Hedrick, S. M., Dutton, R. W. and Huston, G. (1991). Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development. *Immunol Rev*, **123**, 115-44.

Taniguchi, T., Tachikawa, S., Kanda, Y., Kawamura, T., Tomiyama-Miyaji, C., Li, C., Watanabe, H., Sekikawa, H. and Abo, T. (2007). Malaria protection in beta 2-microglobulin-deficient mice lacking major histocompatibility complex class I antigens: essential role of innate immunity, including gammadelta T cell. *Immunol*, **122** (4), 514-21.

Taylor, R. R., Egan, A., McGuinness, D., Jepson, A., Adair, R., Drakely, C. and Riley, E. (1996) Selective recognition of malaria antigens by human serum antibodies is not genetically determined but demonstrates some features of clonal imprinting. *Int Immunol*, **8**, 905–915.

Taylor-Robinson, A. W. and Phillips, R. S. (1992). Functional characterization of protective CD4+ T-cell clones reactive to the murine malaria parasite *Plasmodium chabaudi*. *Immunology,* **77,** 99-105.

Taylor-Robinson, A. W. and Phillips, R. S. (1993). Protective CD4+ T-cell lines raised against *Plasmodium chabaudi* show characteristics of either Th1 or Th2 cells. *Parasite Immunol*, **15,** 301–310.

Taylor-Robinson, A. W. and Phillips, R. S. (1994a). B cells are required for the switch from Th1- to Th2-regulated immune responses to *Plasmodium chabaudi chabaudi* infection. *Infect Immun,* **62**, 2490-2498.

Taylor-Robinson, A. W. and Phillips, R. S. (1994b). Th1 and Th2 CD4+ T cell clones specific for *Plasmodium chabaudi* but not for an unrelated antigen protect against blood stage *P. chabaudi* infection. *Eur J Immunol*, **24,** 158-164.

Taylor-Robinson, A. W., Phillips, R. S., Severn, A., Moncada, S. and Liew, F. Y. (1993). The role of TH1 and TH2 cells in a rodent malaria infection. *Science,* **260,** 1931-1934.

Taylor-Robinson, A. W. (1995). Regulation of immunity to malaria: valuable lessons learned from murine models. *Parasitol Today*, **11** (9), 334-42.

Terrazas, C. A., Terrazas, L. I. and Gómez-Garcia, L. (2010). Modulation of dendritic cell responses by parasites: A Common Strategy to Survive. *Journal of Biomed and Biotechnol*, **2010,** 357106.

Thawani, N., Tam, M. and Stevenson, M. M. (2009).STAT6-mediated suppression of erythropoiesis in an experimental model of malarial anemia. *Haematologica*, **94**, 195-204.

Trape, J. F., Pison, G., Spiegel, A., Enel, C. and Rogier, C. (2002). Combating malaria in Africa. *Trends Parasitol,* **18,** 224-230.

Trenn, G., Takayama, H., Hu-Li, J., Paul, W. E. and Sitkovsky, M. V. (1988). B cell stimulatory factor 1 (IL-4) enhances the development of cytotoxic T cells from Lyt-2_ resting murine T lymphocytes. *J. Immunol*, **140,** 1101–1106.

Troye-Blomberg, M., Worku, S., Tangteerawatana, P., Jamshaid, R., Söderström, K., Elghazali, G., Moretta, L., Hammarström, M. and Mincheva-Nilsson, L. (1999). Human gamma delta T cells that inhibit the in vitro growth of the asexual blood stages of the Plasmodium falciparum parasite express cytolytic and proinflammatory molecules. *Scand J Immunol*, **50** (6), 642-50.

Tsutsui, N. and Kamiyama, T. (1999). Transforming growth factor beta-induced failure of resistance to infection with blood-stage *Plasmodium chabaudi* in mice. *Infect Immun,* **67,** 2306-2311.

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

Uzonna, J. E., Wei, G., Yurkowski, D. and Bretscher, P. (2001). Immune elmination of *Leishmania major* in mice: implications for immune memory, vaccination, and reactivation disease. *J Immunol*, **167** (12), 6967-74.

van der Heyde, H. C., Pepper, B., Batchelder, J., Cigel, F. and Weidanz, W. P. (1997). The time course of selected malarial infections in cytokine-deficient mice. *Exp Parasitol,* **85,** 206-213.

van der Heyde, H. C., Batchelder, J. M., Sandor, M. and Weidanz, W. P. (2006). Splenic γδ T Cells regulated by $CD4^+$ T cells are required to control chronic *Plasmodium chabaudi* malaria in the B-Cell-deficient mouse. *Infect Immun*, **74** (5), 2717-2725.

van der Heyde, H. C., Pepper, B., Batchelder, J., Cigel, F. and Weidanz, W. P. (1997). The time course of selected malarial infections in cytokine-deficient mice. *Exp Parasitol*, **85** (2), 206-13.

von der Weid, T., Kitamura, D., Rajewsky, K. and Langhorne, J. (1994a). A dual role for B cells in *Plasmodium chabaudi chabaudi* (AS) infection? *Res Immunol,* **145,** 412-419.

von der Weid, T., Kopf, M., Kohler, G. and Langhorne, J. (1994b). The immune response to *Plasmodium chabaudi* malaria in interleukin-4-deficient mice. *Eur J Immunol,* **24,** 2285-2293.

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

Waki, S., Uehara, S., Kanbe, K., Nariuch, H. and Suzuki, M. (1995). Interferon-gamma and the induction of protective IgG2a antibodies in non-lethal *Plasmodium berghei* infections of mice. *Parasite Immunol,* **17,** 503-508.

Walther, M., Woodruff, J., Edele, F., Jeffries, D., Tongren, J. E., King, E., Andrews, L., Bejon, P., Gilbert, S. C., De Souza, J. B., Sinden, R., Hill, A. V. and Riley, E. M. (2006). Innate immune responses to human malaria: heterogeneous cytokine responses to blood-stage Plasmodium falciparum correlate with parasitological and clinical outcomes. *J Immunol*, **177** (8), 5736-45.

Weinbaum, F. I., Evans, C. B. and Tigelaar, R. E. (1976). Immunity to *Plasmodium Berghei yoelii* in mice. I. The course of infection in T cell and B cell deficient mice. *J Immunol*, **117,** 1999-2005.

Weindanz, W. P., LaFleur, G., Brown, A., Burns, J. M., Gramaglia, I. and van der Heyde, H. C. (2010). γδ T cells but not NK cells are essential for cell-mediated immunity against *Plasmodium chabaudi* malaria. *Infect Immun*, **78** (10), 4331-4340.

Weiss, W. R., Kumar, A., Jiang, G., Williams, J., Bostick, A., Conteh, S., Fryauff, D., Aguiar, J., Singh, M., O'Hagan, D., Ulmer, J. and Richie, T. (2007). Protection of rhesus monkeys by a DNA prime/poxvirus boost malaria vaccine depends on optimal DNA priming and inclusion of blood stage antigens. *PLoS One*, **2**, e1063.

White, W. I., Evans, C. B. and Taylor, D. W. (1991). Antimalarial antibodies of the immunoglobulin G2a isotype modulate parasitemias in mice infected with *Plasmodium yoelii*. *Infect Immun*, **59,** 3547-3554.

World Health Organization. World Malaria Report. (2008). Available online at http://whqlibdoc.who.int/publications/2008/9789241563697_eng.pdf

World Health Organization. Malaria. Fact sheet No 94. (2010). Available online at http://www.who.int/mediacentre/factsheets/fs094/en/index.html

Wilson, E. H., Wille-Reece, U., Dzierszinski, F. and Hunter, C. A. (2007). A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis. J *Neuroimmunol*, **165** (1-2), 63-74.

Wilson, S., Jones, F. M., Mwatha, J. K., Kimani, G., Booth, M., Kariuki, H. C., Vennervald, B. J., Ouma, J. H., Muchiri, E. and Dunne, D. W. (2009). Hepatosplenomegaly associated with chronic malaria exposure: evidence for a pro-inflammatory mechanism exacerbated by shistosomiasis. *Parasite Immunol*, **31** (2), 64-71.

Wunderlich, F., Marinovski, P., Benten, W. P., Schmitt-Wrede, H. P. and Mossmann, H. (1991). Testosterone and other gonadal factor(s) restrict the efficacy of genes controlling resistance to Plasmodium chabaudi malaria. *Parasite Immunol*, **13** (4), 357-67

Wykes, M. N., Zhou, Y. H., Liu, X. Q. and Good, M. F. (2005). *Plasmodium yoelii* can ablate vaccine-induced long-term protection in mice. *J Immunol*, **175** (4), 2510-6.

Xu, H., Wipasa, J., Yan, H., Zeng, M., Makobongo, M. O., Finkelman, F. D., Kelso, A. and Good, M. F. (2002). The mechanism and significance of deletion of parasite-specific CD4+ T cells in malaria infection. *J Exp Med*, **195** (7), 881-92.

Yanez, D. M., Manning, D. D., Cooley, A. J., Weidanz, W. P. and van der Heyde, H. C. (1996). Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. *J Immunol*, **157** (4), 1620-4.

Yoneto, T., Yoshimoto, T., Wang, C. R., Takahama, Y., Tsuji, M., Waki, S. and Nariuchi, H. (1999). Gamma interferon production is critical for protective immunity to infection with blood-stage Plasmodium berghei XAT but neither NO production nor NK cell activation is critical. *Infect Immun*, **67** (5), 2349-56.

Yoneto, T., Waki, S., Takai, T., Tagawa, Yi., Iwakura, Ylk Mizuguchi, J., Nariuchi, H. and Yoshimoto, T. (2001). A critical role of Fc receptor-mediated antibody-dependent phagocytosis in the host resistance to blood-stage *Plasmodium berghei* XAT infection. *J Immunol*, **166** (10), 6236-41.

Yu, K., Mitchell, C., Xing, Y., Magliozzo, R. S., Bloom, B. R. and Chan, J. (1999).Toxicity of nitrogen oxides and related oxidants on mycobacteria: *M. tuberculosis* is resistant to peroxynitrite anion. *Tubercle Lung Dis*, **79**, 191–198.

Zhang, S., Kim, C. C., Batra, S., McKerrow, J. H. and Loke, P. (2010). Delineation of diverse macrophage activation programs in response to intracellular parasites and cytokines. *PLoS Negl Trop Dis*, **4** (3), e648.

Zhang, J. G., Hilton, D. J., Willson, T. A., McFarlane, C., Roberts, B.A., Moritz, R.L., Simpson, R.J., Alexander, W. S., Metcalf, D. and Nicola, N. A. (1997). Identification, purification, and characterization of a soluble interleukin (IL)-13-binding protein. Evidence that it is distinct from the cloned Il-13 receptor and Il-4 receptor alpha-chains. *J Biol Chem*, **272**, 9474-9480.

Zhang, Z., Chen, L., Saito, S., Kanagawa, O. and Sendo, F. (2000). Possible modulation by male sex hormone of Th1/Th2 function in protection against *Plasmodium chabaudi chabaudi* AS infection in mice. *Exp Parasitol*, **96** (3), 121-9.

Zinkernagel, R. M. and Hengartner, H. (2006). Protective 'immunity' by pre-existent neutralizing antibody titers and preactivated T cells but not by so-called 'immunological memory'. *Immunol Rev*, **211**, 310–319.

Zuk, M and McKean, K. A. (1996). Sex differences in parasite infections: patterns and processes. *Int J Parasitol*, **26** (10), 1009-23.

Zurawski, G. and de Vries, J. E. (1994). Interleukin 13 elicits a subset of the activities of its close relative interleukin 4. *Stem Cells*, **12,** 169-174.

Zurawski, G. and de Vries, J. E. (1994). Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol Today*, **15,**19-26.