The role of interleukin-22 in the mouse model of malaria infection, *Plasmodium chabaudi*

Victor Mensa Kouassi

The Francis Crick Institute
Mill Hill Laboratory

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I, Victor Mensa Kouassi confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Single nucleotide polymorphisms in the gene for the cytokine, interleukin-22, have been associated with severe malaria infection. This cytokine is known to have roles in protecting various tissues from the damaging effects of inflammation as well as by up-regulating the production of antimicrobial peptides. This thesis aims to investigate where IL-22 is produced in the mouse model of malaria infection, *Plasmodium chabaudi* in C57BL/6 mice, and whether this production provides protection from associated pathology.

IL-22 expression was analysed through qRT-PCR and the use of an IL-22 cre x R26R eYFP fate reporter mouse. In the liver, there were increases in mRNA until day 5, when they decreased, and a similar pattern was observed in eYFP+ cells. In the lungs, there was also a decrease in *il22* mRNA as well as eYFP+ cells after day 5 and this was shown to result from changes in the population of IL-22 producing γδ T cells. Decreases were observed in *il22* mRNA in the small intestine with no change in eYFP+ cells in the small or large intestine. These eYFP+ cells were mostly comprised of innate lymphoid cells and a possibility may have been that a proportion of them had switched to producing IFN-γ due to increased IL-12 expression in malaria infection.

C57BL/6 mice deficient in IL-22 (*il22*$^{-/-}$ mice) that were infected with *P. c. chabaudi AS* and *P. c. chabaudi CB* showed a decreased survival with increases in the liver enzymes. However changes in histopathology were not observed in the lungs, liver and intestine in the absence of IL-22 in malaria infection, suggesting the role of this cytokine may be subtle. Future work would need to further investigate whether the importance of IL-22 in malaria infection is due to its roles in protecting the integrity of the epithelia especially within the intestine and lung or its influences on the intestine microbiota.

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1. Introduction

1.1 The impact of malaria

Transmitted by mosquitoes of the *Anopheles* genus and caused by the intracellular apicomplexan parasite *Plasmodium*, malaria threatens approximately 3.3 billion of the world's population. In 2013, there were an estimated 207 million cases of this disease globally leading to around 600,000 deaths most of which were in children under the age of 5 (1-3). The economic burden of this disease is also estimated to be significant, with gross domestic product (GDP) known to be reduced in malaria endemic countries (4).

Over 100 species of *Plasmodium* are known to exist, infecting many vertebrate hosts such as birds, reptiles and mammals (3). Five of these species are able to cause pathology in humans. These include *Plasmodium vivax, P. malariae and P. ovale,* as well as *P. knowlesi,* a malaria parasite known to infect primates such as macaques which has more recently been shown to affect humans as well. Of the malaria parasites that infect humans*, P. falciparum* is known to cause the most deaths and severe complications observed in this disease (5).

1.2 The life cycle of *Plasmodium* in mammals

Once bitten by a *Plasmodium*-infected mosquito, sporozoites residing within the salivary glands are transmitted into the dermis of the host's skin, as the mosquito probes for a blood vessel (6). As shown in figure 1.1, these parasites then move into the bloodstream where they travel to the liver and infect hepatocytes (7). The liver stage of malaria is asymptomatic and lasts until the sporozoites produce thousands of merozoites within the hepatocyte. In *Plasmodium ovale and Plasmodium vivax*, the parasite may also form into hypnozoites, which remain dormant in the liver of

the host for months to years and resurface causing pathology in a future relapse (3, 8-10).

The merozoites occupy vesicles called merosomes within the hepatocytes, which pass into the bloodstream (12) . In the erythrocytic stage of disease, merozoites then invade erythrocytes, dividing asexually within them progressively through rings, trophozoite and multinucleate schizonts stages, and then egressing to invade more erythrocytes. This egress is known to require activity of proteases (13). Some species of *Plasmodium (P)* such as *P. vivax*, preferentially invade reticulocytes where as others such as *P. falciparum* invade erythrocytes at all stages of development (3, 5, 14).

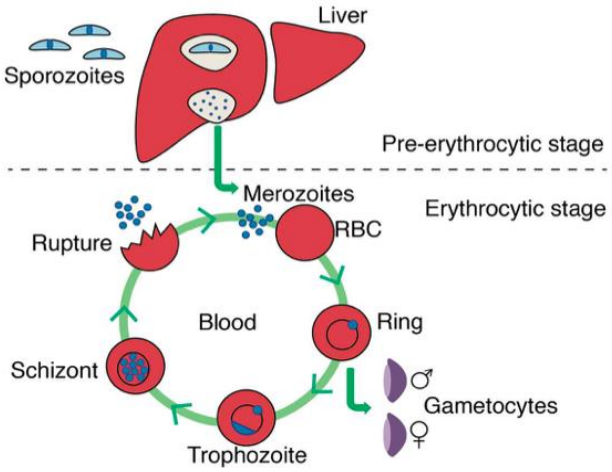


Figure 1.1 from Perez-Mazliah & Langhorne (11) shows the life cycle of the malaria parasite, *Plasmodium*, which occurs in two main stages the pre-erythrocytic or liver-stage and the erythrocytic or blood stage of infection.

The rupture of infected erythrocytes in the erythrocytic stage cycle of division and egress is associated with the symptomatic stage of malaria, and symptoms can include fever, headaches and vomiting in humans (15).

After invasion of the red blood cell, some merozoites develop into male and female gametocytes, this fate has been linked to expression levels of the DNA binding protein PfAP2-G within the parasites (16). Mature gametocytes circulate within the blood, enabling them to be taken into the mosquito on a subsequent bite of the host. Within the mosquito, gametocytes fuse to form oocysts in the mid-gut, which release sporozoites that travel to the salivary glands thus continuing the cycle.

1.3 Pathological complications in malaria

The symptoms of pathology in malaria arise during the erythrocytic stage of infection. In humans, this begins within 6 to 8 days after being bitten by a mosquito. (2,5). In *P. falciparum* infection, these symptoms occur with defined periodicity; coinciding with the rupture of the schizont-infected erythrocytes. In most infections these symptoms resolve with treatment. However, in approximately 10% of infections with *P. falciparum*, complications can arise during malaria infection leading to severe symptoms of this disease, in which organs such as the brain, lungs, liver as well as the placenta in pregnant women can be affected (17, 18).

Severe symptoms may arise from a feature of *P. falciparum* infection known as sequestration in which infected erythrocytes adhere to vascular endothelium enabling the parasite to avoid destruction by the spleen (2,6). *P. falciparum* expresses the protein, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), on the surface membrane of the infected erythrocyte. This protein, encoded by the *var* multigene family (19), is able to bind receptors on the host endothelium such as CD36, chondroitin sulphate A (CSA) and ICAM-1 (5).

Cerebral malaria, the most serious complication that can result from malaria infection, can lead to coma, lifelong disability, or even death. The exact mechanisms of pathogenesis in cerebral malaria remain under debate (20, 21). Some argue that sequestration of the malaria parasite within blood vessels of the brain contributes to this syndrome since sequestered parasites can be observed in histological sections of organs of malarial patients who died of cerebral malaria. However, this is still observed in patients who die of malaria complications other than cerebral malaria, albeit to a lesser degree (22). Evidence also points to proinflammatory cytokines

inducing this complication, with Tumour Necrosis Factor (TNF) reported to play a role (23, 24) .

Severe anaemia is another serious complication of this disease that can result from the destruction of infected erythrocytes by the immune system as well as haemolysis and the suppression of erythrocyte development by proinflammatory cytokines (25-27). It can also be caused by the increased destruction of uninfected erythrocytes (28).

Pregnant women infected with malaria are at an increased risk of miscarriages, low birth weight and severe disease as a result of placental malaria. Here, malaria parasites are able to bind to factors such as chondroitin sulfate A (CSA) allowing them to sequester within the placenta (15).

Lung pathology can also arise in malaria associated acute respiratory distress syndrome (MA-ARDS) in which inflammatory cells and mediators infiltrate the lung tissues (29-31). In studies using mouse models, this has been also associated with parasite sequestration and may also present with proteins in the broncheoalveolar lavage during malaria infection, signalling a decreased integrity of the lung epithelia (29, 32). This syndrome differs from metabolic acidosis, another complication of malaria, which occurs when the tissues starved of oxygen because of sequestered parasites in blood vessels undergo anaerobic respiration (33). Malaria infections have also been known to cause acute renal failure (34) as well as acute liver failure (35).

Secondary bacterial coinfections and increased endotoxaemia have also been associated with severe malaria infections, with non-typhoidal salmonella being the most commonly reported (36-38). Studies have found that this bacteraemia can triple the risk of mortality (39). Malaria infection has been shown to prime the inflammatory response to bacterial products such as lipopolysaccharides resulting in

exaggerated production of the cytokine IL-1β leading to dangerously high levels of inflammatory responses (40, 41).

Increased endotoxaemia and bacterial coinfection in malaria have been suggested to stem from perturbations to epithelial barriers in the gastrointestinal tract allowing translocation of bacteria (42). The involvement of the gastrointestinal tract has been suggested in studies examining this organ in mouse models of malaria infection. Using *P. yoelii* infection, Chau *et al.* (43) have suggested a mechanism in which reduction of nitric oxide in the intestine, due to its increased use to kill malaria parasites, could activate the degranulation of mast cells. These cells would then damage the integrity of the epithelial tight junctions and allow the dissemination of bacteria such as *Salmonella typhimurium*. Mooney *et al.* (44) propose that increased production of the anti-inflammatory cytokine IL-10 during malaria infection in the intestine could lead to blunting of inflammatory responses to bacteria, allowing their dissemination.

1.4 Mouse models of malaria infection

There are many questions that need to be answered in order to minimise the burden of this disease. These include the nature of immune responses that are generated against the parasite during infection, how these immune responses can lead to development of immunological memory and whether a vaccine can be used to induce this. How exactly severe pathology ensues, and how the parasite can be potentially pharmacologically inhibited. An invaluable way of answering these questions is by using mouse models of malaria infection.

Infecting inbred strains of laboratory mice such as BALB/c and C57BL/6 with rodent-specific species of the parasite *Plasmodium* has proven to provide an

invaluable opportunity to decipher the complex interactions that occur between the parasite and its host during malaria infection (42, 45). Through generating mice deficient in genes such as those for immune mediators and cytokines, it is also possible to elucidate the role of these factors through observing alterations in pathology (2, 3, 46, 47).

Most mouse models of malaria infection have been developed using parasites isolated from African thicket rats (e.g. *Grammomy rutilans and Grammomys surdaster)* these include *P. berghei, P. yoelii, P. c. chabaudi, P. c. adami* and *P. vinckei* (48). These different species and subspecies vary in their characteristics such as virulence, synchronicity and pathological complications. Even within a species such as *P. c. chabaudi* there are subspecies and strains of differing virulence (47, 48). The ability to also manipulate the genome of *P. berghei, P. yoelii* and *P. c. chabaudi* to tag, knockout and knock-in genes gives opportunities to investigate processes *in vivo* such as sequestration and invasion, which would not be possible in humans (49-53).

1.4 *Plasmodium chabaudi chabaudi*

Plasmodium chabaudi chabaudi is a useful model of malaria infection because of the similarities it shares with some of the human infecting malaria parasites, including *P. falciparum*. Like this debilitating human infecting parasite, *P. c. chabaudi* is also able to establish a chronic and synchronous infection and also undergoes sequestration (54). The severity of malaria infection in the mouse can depend upon the strain of *P. c. chabaudi* as well as the strain of mouse. The strains, *P. c. chabaudi chabaudi AS* and *P. c. chabaudi adami* cause infections which are usually eliminated by inbred mouse strains such as C57BL/6 but have a high mortality rate in other strains such as A/J (48).

P. c. chabaudi chabaudi AS in C57BL/6 mice establishes a non-lethal but chronic infection with sequestration, although, unlike *P. falciparum* this occurs in the liver and lungs but not in the brain (32). As this infection is non-lethal and chronic, the immune responses of the host, which can clear the parasite, can be studied in depth. Genetic engineering of this parasite is also possible, allowing *P. c. chabaudi* to be transformed to express fluorescent proteins such as mCherry (50). Through *in-vivo* imaging of infected mice, processes such as sequestration in the host can be analysed in further detail (32). More virulent strains of *P. c. chabaudi* exist such as *P. c. chabaudi chabaudi CB*, which can cause a higher rate of mortality in infected C57BL/6 mice.

Mice are usually injected intraperitoneally with 10,000 serially blood-passaged infected erythrocytes. Analysis of parasitaemia typically reveals a peak at around days 7 to 9, which is associated with weight loss, anaemia and hypothermia. This stage of the infection is associated with a Th1 response induced by IL-12 involving the proinflammatory cytokines IFN-γ and TNF-α, which can activate macrophages to destroy the parasites (55-58). After this peak, antibody responses are important for maintaining the clearance of parasites. In the chronic stage, recrudescence of parasites can also appear which may be associated with antigenic variation (59, 60).

Brugat *et al.* (32) have shown that organs in which mature *P. c. chabaudi* sequester, can develop pathology. In the lung, sequestration was linked to the production of IFN-γ, which accompanies the acute stage of this infection (32). Damage was also observed to the epithelium indicated by increased proteins in the broncheoalveolar lavage fluid. Alveolar septae cellularity, a histopathological measure of influx of inflammatory cells, was also observed to increase here.

Sequestration of *P. c. chabaudi* in C57BL/6 was associated with signs of tissue damage as shown by histopathology and by detection of liver enzymes in the plasma, such as alanine transaminase (ALT) (32). Seixas *et al.* (61) showed a TNF dependent mechanism for liver damage in *P. c. chabaudi* infection in BALB/c mice. Haemozoin is a pigment released by malaria parasites as a result of breakdown of haemoglobin and it may induce the release of proinflammatory cytokines(62). Brain pathology has not been observed in *P. c. chabaudi* AS infection, although, *il10*$^{-/-}$ mice have been shown to develop some breakdown of the blood brain barrier and some neurological symptoms during infection (63).

Since infection initiated via intraperitoneal injection of infected erythrocytes is different from the natural mode of transmission of malaria, a protocol has been optimised in which *P. c. chabaudi* can be successfully transmitted to mice via the bite of the mosquito, *Anopheles stephensi* (64). It has been found that after mosquito transmission, there is a lower peak of parasitaemia, with reduced virulence (65). This has been observed to be associated with changes in gene expression of the *cir* multigene family (66).

1.6 Immune responses to the liver-stage of *Plasmodium*

Although the stages of *Plasmodium* infection in which sporozoites infect hepatocytes within the liver are clinically silent, the host is still able to raise immune responses in efforts to clear the parasite. Injection of sporozoites attenuated by radiation leads to protective immunity in mice and humans (66, 68). *In vitro* experiments have shown that antibodies against *Plasmodium berghei* sporozoites can block invasion of hepatocytes (69). Studies in mice by Schofield *et al.* (70) showed that as well as antibodies against sporozoites attenuated by radiation, important immune

responses at this stage also included the release of interferon-gamma and CD8+ T cells. These cells have been shown through in vivo imaging of the infected mouse liver to form clusters around infected hepatocytes to eliminate them, in mice that were pre-immunized by previous infection with radiation attenuated sporozoites (71). Work by Hill *et al.* (72) which showed the major histocompatibility complex (MHC) class I allele, HLA B53 that is recognised by CD8+ T cells is associated with resistance to severe malaria, strongly suggesting that this CD8+ T cell response is also important in humans.

In addition to adaptive immunity mediated by antibodies and T cells, studies by Liehl *et al.* (73) demonstrated in *P. berghei* infected mice that there is also a hepatocyte intrinsic innate response where infection of these cells with sporozoites leads to their secretion of type 1 interferon. This cytokine may then act in an autocrine manner, inducing the hepatocytes to initiate transcription of interferon-stimulated genes (ISGs), which include chemokines, attracting innate immune cells that can kill infected hepatocytes.

1.7 Immune responses to the erythrocytic-stage of *Plasmodium*

Innate immune responses towards the erythrocytic stage of *Plasmodium* can be initiated by the activation of receptors on cells of the innate immune system such as macrophages and dendritic cells by Pathogen-Associated Molecular Patterns (PAMPS). *Plasmodium* PAMPS include substances such as haemozoin, a breakdown product of haem which can activate inflammatory pathways through NLRP3 resulting in the production of proinflammatory cytokine, interleukin-1β (IL-1β) (49, 51). Glycosylphosphatidylinositol (GPI) from *P. falciparum* has also been shown to activate TLR2 leading to inflammatory pathways through Myd88 (2, 3, 74, 75). These

signalling pathways may lead to the activation of the transcription factor, NF-κB which can induce production of inflammatory cytokines (3, 47, 76).

Through activation of these pathways, dendritic cells and macrophages can secrete cytokines such as TNF-α, IL-6, IL-12 and IL-18 which can go on to activate natural killer (NK) cells to secrete cytokines such as IFN-γ and TNF-α this has been shown in studies in mice and humans (5, 77). Human NK cells have also been shown *in vitro* to secrete IFN-γ rapidly in response to live *P. falciparum* infected erythrocytes (78). Studies have shown that *P. c. chabaudi* infected mice in which NK cells are depleted have a significantly higher parasitaemia and a 100% mortality (79) demonstrating the importance of these cells in control of parasites.

Activated antigen presenting cells (APCs) such as dendritic cells, take up, process and present peptides within major histocompatibility complex (MHC) class II proteins to CD4+ T cells. These cells then undergo cell division and differentiate into T cells which can act as effector cells through their production of cytokines or which develop into helper cells for B cells produce antibodies, resulting in the adaptive immune response (3, 5, 14, 80). CD4+ T cells, in particular, are able to differentiate, through the expression of master regulator transcription factors and induce various T helper (Th) responses in infection, depending on the cytokine milieu (81). These include Th1 cells, which express the master regulator T-bet, and the cytokine IFN-γ. Th2 cells which express GATA-3 and secrete IL-4. Th17 cells which express RORγT and express IL-17 and IL-22. They can also differentiate into follicular helper T cells (Tfh cells), which express IL-21 or induced Regulatory T cells (Tregs) that possess the master regulator FOXP3 and express the cytokine TGF-β (81-83).

IL-12 from antigen-presenting cells can induce a Type 1 helper response (Th1) in CD4+ T cells stimulating them to secrete IFN-γ. (2, 3, 76, 84). In-vitro experiments

by Pombo *et al.* (85)have shown large amounts of nitric oxide synthase activity during erythrocytic stage infection in humans, suggesting that nitric oxide induced by IFN-γ may be important for killing the malaria parasite. However, studies in mice have shown that nitric oxide is dispensable for controlling malaria infection (21, 86, 87).

Antibodies, produced by plasma cells, are known to have an important role in parasite clearance in the chronic phase of infection. Earlier studies have shown that passive transfer of antibodies from hyperimmune adult humans can result in reduced parasitaemia (88). Other studies have also shown an association between antibodies against the malaria parasite and protection or a reduced incidence of malaria infection in humans (89, 90).

Mice deficient in IL-4, an important Th2 cytokine are known to still mediate appropriate antibodies against *P. c. chabaudi* showing that this cytokine does not have an important role in the protective immune response to this parasite (59). Recently published studies by Pérez-Mazliah *et al.* (91) have revealed that interleukin-21, produced by CD4+ follicular T helper (Tfh) cells, are more important in orchestrating the B-cell response in germinal centres to control the chronic phase of *P. c. chabaudi* in C57BL/6 mice.

Despite immune responses raised against the malaria parasite, humans acquire long lasting protective immunity only after several years of exposure and never acquire sterile protection (47). This has been suggested to be due to immune evasion through antigenic variation in *Plasmodium* (32, 92). Antigens such as PfEMP1 that are expressed on the surface of infected erythrocytes are known as variant surface antigens (VSAs) and are encoded by *P falciparum* multigene families such as the *var* gene family encoding PfEMP1 in *P. falciparum*. The parasite can induce

expression of variants of these proteins to avoid recognition by the immune system whilst still allowing them to function (25-27, 93, 94).

The *Plasmodium* interspersed repeat *(pir)* multigene family has also been found in all species of *Plasmodium* so far sequenced such as P. vivax *(vir)*, P. yoelii *(yir)* and *P. c. chabaudi (cir)* and is related to the *rifin* family of *P. falciparum*. In *P. c. chabaudi*, 196 *cir* genes have been identified (28). However the role(s) of these genes in the immune response have not yet been established.

An immune response involving proinflammatory cytokines such as Tumour Necrosis factor (TNF) and Interferon-gamma (IFN-γ) is important in the clearance of the parasite from the host by activating macrophages to kill infected red blood cells (47). However over-exuberant responses are also known to lead to severe pathology. An overproduction of proinflammatory cytokines such as TNF, interleukin-1β (IL-1β) and IFN-γ is often observed in the pathology of severe malaria. These cytokines can induce large amounts of mediators such as nitric oxide, which can also cause pathology (15, 95). Larger amounts of TNF in the plasma have been shown to correlate with severe malaria (29, 30, 96), with higher concentrations of this cytokine being associated with cerebral malaria in children (97). This cytokine has been hypothesised to intensify sequestration by up-regulating adhesion molecules such as ICAM-1 and CD36 on the endothelium within blood vessels (98). Administration of antibodies against IFN-γ in mice infected with P. falciparum have also been shown to be able to prevent cerebral malaria and this was also associated with a down-regulation of Tumor necrosis factor (99, 100).

Regulatory T cells (Tregs) have been shown to be induced in malaria infection, with studies in humans showing larger numbers of this cells in adults during times of increased malaria transmission in sub-Saharan Africa (101). As reviewed by Finney *et*

al. (102), these cells are not able to quell the large inflammation induced during the blood stage of malaria infection.

The cytokine, IL-10 is known to have anti-inflammatory roles (34, 103) and mice deficient in this cytokine and infected with *P. c. chabaudi* have been shown to exhibit symptoms of cerebral malaria such as haemorrhage in the brain and oedema (35, 63). Studies in mice by Li *et al.* (104) revealed that administering antibodies against TNF in *P. c. chabaudi* infected IL-10 knockout mice decreased symptoms of increased pathology, demonstrating the ability of proinflammatory cytokines to cause pathology. IL-10 is produced by most types of differentiated CD4+ T helper cells including Th1 and Th17 cells, as well as other immune cells such as macrophages and dendritic cells to regulate cytokine production (105). Production of IL-10 by CD4+ T cells has been shown to be induced in *P. c. chabaudi* infection by IL-27 and provide protection from immunopathology (106). CD4+ T cells have also been found to produce IL-10 within *P. yoellii* infection (107). Further experiments also showed increased pathology when infected mice were treated with antibodies against another anti-inflammatory cytokine, Transforming Growth Factor β (TGF-β) (108). In humans, IL-10 is produced at higher levels during malaria infection. T cells producing both IFN-γ and IL-10 are increasingly observed in children exposed to malaria (109).

CD4+ T cells are also able to differentiate into Th17 cells in the presence of IL-6 and TGF-β, and these express the cytokines IL-17A, IL-17F and IL-22 (110). These cells have been shown to have roles combating infections such as *Citrobacter rodentium* and can also have pathogenic roles in inducing autoimmunity in experimental autoimmune encephalitis (111). Mastelic *et al.* (112) investigated whether these cells play a role in malaria infection. Although increases in IL-17A and IL-17F were observed in the liver during *P. c. chabaudi* infection in C57BL/6 mice, no

significant differences were observed in pathology due to the loss of IL-17 in *P. c. chabaudi* infection. In mice lacking IL-22, however, severe pathology was observed during *P. c. chabaudi* infection. IL-17 producing CD4+ T cells have been seen to be activated in *P. berghei* and *P. yoellii* infections but their role has not been identified (113).

1.7 Interleukin-22

A study examining the effect of polymorphisms in the *IFNG* gene and its neighbours, which include *IL22* and *IL26*, in resistance and susceptibility to severe malaria found two haplotypes within the *IL22* gene that were associated with resistance and susceptibility to severe malaria (114). This provided the first suggestion that this cytokine may play a role in protection from severe disease in malaria infection. Mastelic *et al.* (112) also showed that mice deficient in IL-22 backcrossed for 3-5 generations on C57Bl/6 (NIMR), developed a severe infection with 50% mortality, and significantly greater weight and temperature loss when infected with *P. c. chabaudi* AS. These were unexpected findings given what is known about this cytokine.

The cytokine, interleukin-22 is a member of the IL-10 family of cytokines, which also includes IL-19, IL-20, IL-24 and IL-26 (115). Originally known as Interleukin-10 related T Cell-Derived Inducible Factor (IL-TIF), it was first discovered to activate the transcription factors, signal transducer and activator of transcription factors 1 and 3 (STAT-1 and STAT-3) in mesangial and neuronal cell lines (116). In humans, the gene for this cytokine is located on chromosome 12 adjacent to the IFNG gene. In mice it is also located next to the IFNG gene but on chromosome 10, in the C57BL/6 and 129 strains of mice this gene has been observed to be duplicated (117).

Unlike most cytokines, the membrane-bound receptor for IL-22 is only expressed on non-haematopoietic cells such as hepatocytes and other epithelial cells in the lungs, kidney and gut (118). This receptor, known as IL22RA1, is a heterodimer of IL-10R2 and IL-22R1 (119, 120). IL-22 has a high affinity for the IL-22R1 subunit but a very low affinity for the IL-10R2 subunit. The IL-10R2 subunit, although, has an affinity for the IL-22 and IL-22R1 complex suggesting that the IL-22 first binds to IL-22R1 and then to IL-10R2 forming the complete activated functional receptor (121-123).

The activation of the IL-22 receptor leads to activation of the JAK-STAT signalling pathway in which the tyrosine kinases Janus Kinase 1 (JAK1) and tyrosine kinase 2 (tyk2) phosphorylate and activate each other and go on to phosphorylate and activate other proteins particularly signal transducer and activation of transcription 3 (STAT3). IL-22 has also been shown to activate the MAP Kinase pathway (124).

1.8 Cellular sources of Interleukin-22

Various lymphocytes are able to produce IL-22, as summarised in figure 1.1. Amongst T cells, Th1 cells have been shown to secrete this cytokine in response to IL-12 stimulation (125). Th17 cells were later revealed to express IL-22 in larger amounts (126). The transcription factor, aryl hydrocarbon receptor (AHR), is required for the expression of IL-22 in these cells (127). AHR has been also demonstrated to be important for IL-22 production in other cells such as group 3 innate lymphoid cells (128)

IL-23, secreted by dendritic cells is able to stimulate T cells and innate lymphoid cells to secrete IL-22 (126, 129, 130). The expression of the IL-23 receptor

is induced in cells such as Th17 cells by the cytokines TGF-β and IL-6 enabling the development of Th17 cells (131-133). The cytokine TGF-β is able to inhibit the production of IL-22 in Th17 cells through the activation of *c-maf* (134). The transcription factor, BATF has also been shown by ChIP analysis to bind to the IL-22 promoter (135).

Apart from Th17 cells, Th22 cells are also known to express IL-22, but unlike Th17 cells do not express IL-17 or IFN-γ or possess the transcription factor, RORγT (136). In mice, γδ T cells are also known to express IL-22 with IL-17A and IL-21, similar to Th17 cells they also express RORγT (137, 138). CD8+ T cells can also secrete IL-22 and this occurs in a subset now known as Tc22 as well as Tc17 cells (139). Natural Killer T (NKT) cells have also been shown to secrete IL-22 in the skin, intestine and lung (140). Innate lymphoid cells positive for RORγT and AHR (Group 3 ILCs) are also able to express IL-22. Myeloid cell populations such as neutrophils have been reported to produce this cytokine during colitis (141).

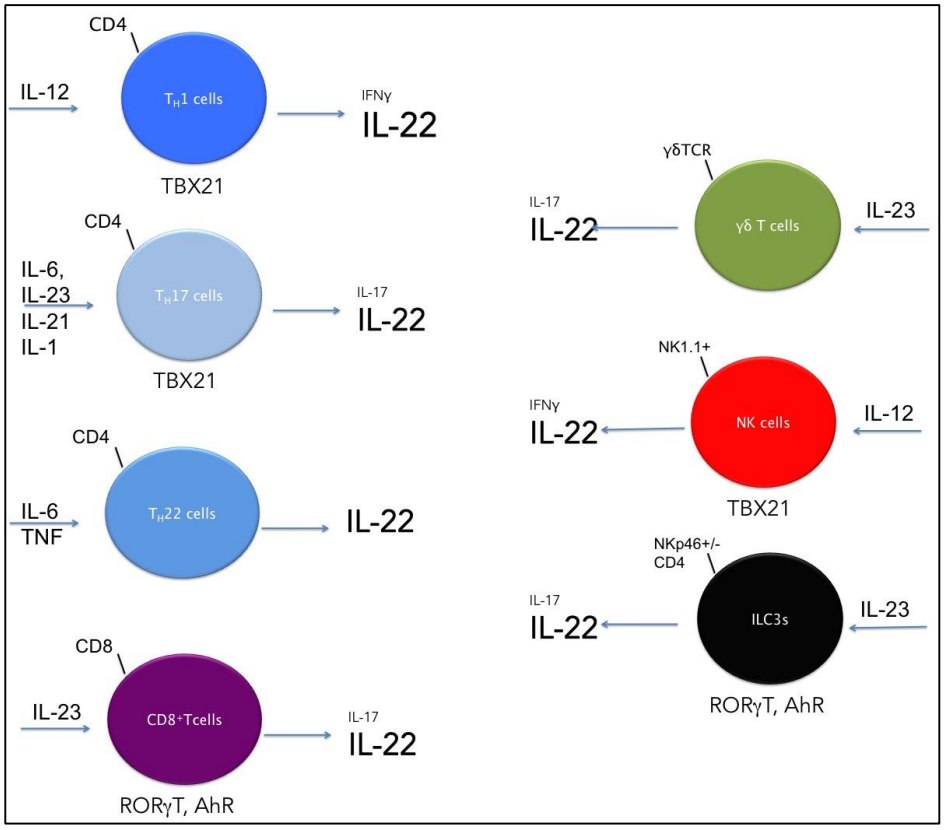


Figure 1.2 The various cells that produce the cytokine IL-22 and the cytokines which induce this production, adapted from Sabat *et al.* (108).

Through IL-22RA1, the functions of this cytokine are exerted and these include recruitment of proinflammatory cells, secretion of antimicrobial peptides and producing mediators to co-ordinate protection and repair of tissues in the epithelia (142). This cytokine also has a soluble receptor known as IL-22RA2 or IL-22 binding protein (IL-22BP), which antagonises and prevents tumorigenic effects this cytokine may have (143). It is able to bind IL-22 with a four-fold higher affinity than IL-22RA1 (144). It has been shown to be produced by a subset of dendritic cells, with the largest amounts being produced in the spleen (145). In the colon, it is constitutively expressed at normal conditions to prevent colorectal cancer. When the epithelia at this site are damaged during inflammation, the expression of this binding protein is down-regulated via IL-18, thus allowing IL-22 to mediate its effects (146). More recent studies by Martin *et al.* (147) have demonstrated that expression of IL-22BP in

the intestine can increase significantly during inflammatory bowel disease, due to increased production by eosinophils, strongly suggesting that the protective role that IL-22 may play could be hindered due to the increased presence of this factor in this case.

1.9 Known functions of Interleukin-22

IL-22 can have proinflammatory roles by inducing release of chemokines and proinflammatory mediators from epithelial cells. It also stimulates release of antimicrobial peptides from tissues. IL-22 induces release of anti-apoptotic factors such as BCL-xL and BCL-2 which inhibit apoptosis (148). These actions are mediated through the activation of STAT3 and result in tissue protection and repair.

IL-22 appears to play no role in infection models such as *Mycobacterium tuberculosis* and the helminth parasite, *Schistosoma mansoni* as no difference was observed in the clearance of pathogen or in pathology on the loss of this cytokine (149, 150). It could be argued that this is not surprising as this cytokine has not been shown to target immune cells thus is not like to be directly involved in the immune response against this pathogens. However, IL-22 does exert protective or pathogenic effects in various other models of infection and organ damage through exerting other roles in different organs, as summarised in table 1.1.

In the liver, IL-22 has protective roles in various models of induced acute hepatitis with agents such as Concanavalin A, carbon tetrachloride and liver injury with paracetamol overdose (151-154) as well as in regenerating liver tissue after partial hepatectomy (155). IL-22 also protects the liver during infections such as with Dengue virus (156). This protection may be exerted by the induction of anti-apoptotic proteins such Bcl-2 through the activation of STAT3 (157). Although, IL-22 can also

have pathogenic roles through these same actions by promoting cell survival in hepatocellular carcinoma (158).

In the intestine, Turner *et al.* (159), have recently shown that IL-22 protects the epithelia and aids expulsion of the helminth parasite, *Nippostrongylus brasiliensis* by mediating goblet cell hyperplasia. Observing elevated expression of mucins and decreased goblet cell staining in histological sections of IL-22 knockout mice revealed this. IL-22 ameliorates inflammation induced in the mouse colon by dextran sodium sulphate (DSS) through the activation of STAT3 leading to the expression of mucins from colonic epithelial cells (160).

IL-22 can, however, have pathogenic actions in the intestine during infection with oral cysts of *Toxoplasma gondii* in mice. It was discovered that *il22*$^{-/-}$ mice had significantly decreased small intestinal necrosis during the infection and this was associated with reduced levels of matrix metalloproteinase 2 (MMP2), an endopeptidase that regulates the extracellular matrix which is associated with colitis (161). This cytokine can induce the production of interleukin-18 from intestinal epithelial cells in this infection, contributing to defence against this parasite as well as increasing damaging inflammation (162).

IL-22 is able to influence the microbiota within the intestine. Zenewicz *et al.* (163) demonstrated that through losing the production of antimicrobial peptides such as RegIII**γ,** *il22*$^{-/-}$ mice have been revealed to have an increased susceptibility to colitis. This was associated, through the use of 16S rRNA gene-pyrosequencing, revealing alterations in the gut microbiota that were pathogenic when transmitted to wild type mice. IL-22 also regulates the fucosylation of intestinal epithelial cells by inducing production of the enzyme, Fut2 in epithelial cells, this can prevent the infection of tissue by bacteria such as *Salmonella typhimurium* (164, 165).

In psoriasis, IL-22 plays a pathogenic role (166). Through STAT3 activation, IL-22 upregulates production of **β**-defensins from keratinocytes (167) and recruits inflammatory cells through the chemokine, CXCL5 (168, 169). More recently, this cytokine has been revealed to also have beneficial roles in the skin. For example in fibroblast mediated wound repair. *Il22*$^{-/-}$ mouse were found to have abnormal wound healing due to reduced extracellular matrix production (170). IL-22 also plays a protective role in a mouse model of Leishmania infection by protecting epithelial cells in the skin. Infected *il22*$^{-/-}$ mice were demonstrated here to have reduced wound healing (171).

The epithelium of the lung is also protected by IL-22 during influenza infection (172, 173). It is also able to protect mucosal epithelial barriers in the lung from damage during infections such as with *Klebsiella pneumoniae* by inducing the expression of lipocalin-2, a protein which can limit bacterial growth (174). IL-22 provided by γδ T cells can prevent harmful lung fibrosis after inflammation induced by infection with *Bacillus subtilis* (137) or by administration of bleomycin (175) by slowing down the rate of collagen deposition. However, IL-22 can have pathogenic proinflammatory roles in the airway after bleomycin-induced inflammation, this can be dependent on the presence of IL-17A (179).

	Protective	Pathogenic
Liver	Acute Hepatitis, Dengue virus (151-154, 156, 157)	Hepatocellular carcinoma (176)
Skin	Wound repair (170)	Psoriasis-like inflammation (130, 177)
Lung	Influenza (172, 173)	
	Airway inflammation (+/- IL-17A) (178, 179)	
Gut	Helminth infections (159) (Turner *et al.* , 2013)	*Toxoplasma gondii* (150, 161) (Munoz *et al.* , 2009; Wilson *et al.* , 2010)
	Intestinal inflammation (160)	Colorectal cancer (146, 158, 180)
Pancreas	Acute pancreatitis (181, 182)	
Brain		West Nile Encephalitis (183), EAE (184)

Table 1.1 summarises the protective and pathogenic functions mediated by IL-22 in various organs in different conditions.

Acinar cells of the pancreas also express the membrane bound IL-22 receptor, IL-22R1. This cytokine has been shown to be able to inhibit autophagy in the pancreas induced by cerulein (182). In the brain, IL-22 is involved in the pathogenesis of west Nile encephalitis through increasing recruitment of neutrophils by inducing expression of the chemokines, CXCL1 and CXCL5 from endothelial cells (183). It is also shown, along with IL-17A, able to disrupt the blood brain barrier allowing diffusion of proteins into the brain in experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (184). Although this cytokine does not have a role in the development of EAE, since *il22*$^{-/-}$ mice still succumb to illness (185).

1.10 The role of Interleukin-22 in malaria infection

A study examining the effect of polymorphisms in the *IFNG* gene and its neighbours, which include *IL22* and *IL26*, in resistance and susceptibility to severe malaria found two haplotypes in the *IL22* gene that were associated with resistance and susceptibility to severe malaria (114). This provided the first suggestion that this

cytokine may play an important role in protection from severe disease in malaria infection.

Mastelic *et al.* (112) revealed further evidence of a role for this cytokine in malaria by infecting *il22*$^{-/-}$ mice on a C57BL/6 background with *P. c. chabaudi AS*. Although these mice were able to control parasitaemia, they progressed onto severe pathology shown by significant weight and temperature loss as well as 50% mortality. *Il22*$^{-/-}$ mice were shown to have a statistically significant increase of alanine transaminase (ALT) in the plasma, an enzyme whose increase in the plasma is a signal of liver damage.

1.11 Aims of this thesis

The lack of interleukin-22 has been shown to result in increased pathology during *P. c. chabaudi AS* infection whilst not significantly affecting parasitaemia (112). Markers of liver damage being increased in this infection on the loss of IL-22 suggested that this might stem from an important role in this cytokine in protecting hepatocytes vulnerable during malaria infection.

Characterisation of the actions of the cytokine IL-22 detailed within this introduction reveal that IL-22 also has crucial functions at the epithelial surfaces of the lungs and in the intestine, where it is known to be expressed at higher levels constitutively (186). It is therefore possible that the lack of IL-22 in these sites leads to increased pathology in *P. c. chabaudi* infections. This thesis aims to:

1) Understand how IL-22 could protect against severe pathology in the erythrocytic stage of malaria infection by analysing its production in different organs and cells during *P. c. chabaudi* infection. This shall be done by:

a) Analysing expression of *il22* mRNA in the spleen, liver, lungs and small and large intestine during *P. c. chabaudi* infection.

b) The cells producing IL-22 in spleen, liver, lung and gut will be identified using an Il-22^{cre} x R26R^{eYFP} fate reporter mouse (186). By employing the use of this mouse through the use of flow cytometry in the relevant organs, increases in IL-22 producing cell populations can be documented.

2) The effects of the lack of IL-22 in *P. c. chabaudi* infection shall be ascertained through the use of *il22*$^{-/-}$ mice that have been backcrossed onto C57BL/6 (NIMR) at least 8 times, and using appropriate NIMR C57BL/6 controls.

3) A significant increase in ALT in *il22*$^{-/-}$ mice suggests this increase in mortality of *il22*$^{-/-}$ mice after infection with *P. c. chabaudi* may be due to increased liver damage (112).

Histopathology and other measurements of pathology in the liver, lungs and intestine will be carried out to determine whether there are obvious changes in histopathology resulting from a lack of IL-22 in *P. c. chabaudi* infected mice.

2. Materials and Methods

2.1 Buffers

FACS buffer

Phosphate buffered saline (PBS)	pH 7.2	
Fetal calf serum (FCS)	2%	PAA Laboratories, GmBH
Sodium azide (NaN3)	0.05%	

Complete Iscove's medium

Iscove's modified Dulbecco's medium (IMDM)		Sigma
Foetal Bovine Serum	10%	PAA
β-mercaptoethanol	0.05mM	Gibco
L-glutamine	2mM	Gibco
Sodium Pyruvate	0.5 M	Sigma
HEPEs Buffer	6mM	Gibco
Streptomycin	100 µg ml−1	Gibco

Magnetic-activated cell sorting (MACS) buffer

Phosphate buffered saline (PBS)	pH 7.2	Gibco, Invitrogen
Foetal calf serum (FCS)	2%	PAA Laboratories, GmBH

ELISA Blocking buffer

Phosphate buffered saline (PBS)		
BSA	1%	Sigma-Aldrich
Tween-20	0.05%	Sigma-Aldrich
Sodium azide (NaN3)	0.05%	

ELISA Washing buffer

Phosphate buffered saline (PBS)	
Tween-20	0.05%

ELISA Detection buffer

Phoshphate buffered saline (PBS)		
BSA	1%	Sigma
Sodium azide (NaN3)	0.05%	

ELISA Diethanolamine buffer

Phosphate buffered saline (PBS)	
Diethanolamine	5%
MgCl2	2mM
Sodium azide (NaN3)	0.05%

Giemsa staining buffer

Na2HPO4	30g	(VWR)
KH2PO4	6g	(VWR)
Distilled Water	1L	

2.2 Antibodies

Marker	Fluorochrome	Clone	Source
CD45.2	APC-Cy7	104	Biolegend
TCRβ	APC	H57-597	
	APC-Cy7	H57-597	
γδTCR	BV421 (Brilliant violet 421)	GL3	
CD4	PerCP	RM4-5	
CD8	PE-Cy7	53.67	
NK1.1	PE (phycoerythrin)	PK136	
IL-22	APC (Allophycocyanin)	IL22JOP	eBioscience

Table 2.1 shows the antibodies used for flow cytometry analysis of eYFP+ cells in the fate reporter mouse and for intracellular cytokine staining of IL-22.

2.3 Mice

Animal experiments were conducted according to Home Office regulations. All mice were bred under specific pathogen free conditions at the Francis Crick Institute, Mill Hill Laboratory, London. Interleukin-22 knockout (*il22*$^{-/-}$) mice on a C57BL/6 background were originally provided by Jean-Christophe Renauld (Ludwig Institute for Cancer Research) were generated by targeting of the exons 1-3 with a neomycin-resistant gene (185). These *il22*$^{-/-}$ mice have been backcrossed onto NIMR C57BL/6 mice across more than eight generations. Littermate wild type controls were used alongside *il22*$^{-/-}$ mice. IL-22 fate reporter mice (IL-22$^{cre+/-}$ x Rosa26R^{eYFP}) mice (187, 188) that allow the observation of cells, which have expressed IL-22 were provided by Brigitta Stockinger (The Francis Crick Institute). These mice were generated by crossing mice heterozygous for having cre recombinase at the IL-22 gene locus with mice that had eYFP at the Rosa26 promoter.

2.4 Infection with *Plasmodium chabaudi chabaudi*

Mice were infected with *Plasmodium chabaudi chabaudi AS (P.c.c. AS)* or *Plasmodium chabaudi chabaudi CB* (*P.c.c. CB*) by intraperitoneal injection. For injection of blood stage parasites, 10,000 infected red blood cells were injected into each mouse. Mice were also infected with *Plasmodium chabaudi* through the mosquito using a protocol optimised by Spence *et al.* (64). To do this, "feeder" mice were injected with 10,000 *P. c. chabaudi* infected erythrocytes intraperitoneally and after 14 days; mice with a suitable number of gametocytes (more than 0.1%) were fed to female *Anopheles stephensi* mosquitoes. Eight days later, oocysts were quantified in the midgut of the mosquito to monitor the progress of the parasite through its life cycle. At 15 days, sporozoites within the salivary glands of the mosquitoes were counted and the

infection was transmitted to experimental mice by allowing 20 mosquitoes to feed on each experimental mouse.

In all infections, parasitaemia was analysed by counting of thin blood smears by making a small nick at the tip of the tail and smearing a drop of blood on a microscope slide. After fixing with 100% methanol (BDH) these slides were stained with Giemsa solution (10% Giemsa stain (VWR) and 10% Giemsa staining buffer for 25 minutes. Parasitaemia was counted using bright-field microscopy with a 100x objective and calculated as the number of infected erythrocytes in an observed number of erythrocytes as a percentage.

Weight and temperature were monitored through the course of infections. Red blood cell counts were measured using a Vetscan HMII analyser (Abaxis). In order to do this 5 µl of blood was taken from the tip of the tail and diluted 1:10 in KGS containing heparin.

2.5 Analysis of markers of pathology in plasma

Under terminal anaesthesia by intraperitoneal injection of pentoject (Animal Care), blood samples were collected via cardiac puncture. Blood samples were also obtained at time points during the infection by tail vein bleed. These blood samples were then centrifuged twice and plasma was immediately frozen on dry ice after retrieval. Alanine Transaminase (ALT), Aspartate transaminase (AST), lactate and urea levels were measured using a cobas C111 chemistry analyser (Roche). Samples collected at day 0 and 5 of infection were diluted (1:10) in PBS (Gibco) and infected samples, collected at days 7 and 9, were similarly diluted in PBS (1:100).

2.6 Broncheoalveolar lavage fluid analysis

To retrieve broncheoalveolar lavage (BAL) fluid, mice were terminally anaesthetised, lungs were cannulated and 500µl of PBS (Gibco, invitrogen) was injected and retrieved. These samples were immediately placed on ice, centrifuged at 900g for 2 minutes at 4°C. Supernatants were obtained and subsequently frozen at -80°C.

IgM levels in the BAL fluid were quantified using ELISA. Maxisorp immunoplates (Nunc) were coated with a goat anti-mouse IgM unlabelled antibody (Southern Biotech) diluted to 1µg/mL in PBS and incubated overnight at 4°c. All incubations were performed with the plate within a humidity chamber. The plates were then blocked with blocking buffer for 2 hours at 37°c and washed with washing buffer. Samples of BAL fluid supernatants in duplicates were then serially diluted in blocking buffer, along with a standard of purified IgM (Sigma Aldrich) also serially diluted starting from 16 µg/mL. IgM was detected using a secondary goat anti-mouse IgM antibody labelled with alkaline phosphatase (Southern Biotech), diluted at 1µg/mL in detection buffer and incubated for 1 hour at room temperature. The substrate; para-nitrophenylphosphate (Sigma) was subsequently added and the plates were allowed to develop in the dark. Optical density (O.D.) was read using the Tecan Infinite M1000 spectrometer plate reader at a wavelength of 405nm. Concentrations were interpolated from the standard curve fitted to a four-parameter sigmoidal (logistic) model.

Protein levels in BAL fluid were measured using the Bradford assay. For each sample, 25 µl of BAL fluid was added to 125 µl PBS and a serial dilution was made of this with a dilution factor of 3. A standard was made serially diluting Bovine serum albumin (Sigma) in PBS starting from 133µg/mL. Bradford reagent (Bio-Rad) was

added to these dilutions and absorbance was measured using a Tecan Infinite M100 spectrometer at 595nm.

2.7 Histopathological analysis

After terminal anaesthesia, lungs were inflated by cannulation through the trachea and injection of 4% paraformaldehyde (VWR). They were then fixed in paraformaldehyde along with the liver, gut, spleen, kidney and brain. After fixation for at least 24 hours, these organs were then embedded in wax, sectioned and stained by the histology facility at the Francis Crick Institute, Mill Hill Laboratory. 5µm sections were taken and stained with haematoxylin and eosin (H&E). Sections of small intestines were also stained with Alcian Blue – Periodic Acid Schiff (AB-PAS) to identify goblet cells. Images were analysed with the use of a VS120 virtual slide scanning system (Olympus). Pathology was scored by a pathologist according to severity grading schemes described in Shackleford *et al.* (189). 0 means no pathology was observed, 1 denotes minimal pathology observed, 2 slight, 3 Moderate, 4 Moderately severe and 5 for severe. In the liver, this severity was defined by recording the level of focal necrosis, as well as numbers of kupffer cells and inflammatory cell foci were analysed. In the Lungs, the level of increased cellularity of the alveolar septae was defined.

2.8 Isolation of mononuclear cells.

To isolate mononuclear cells from the spleen, this organ was mashed through a cell strainer and washed with complete IMDM. Red blood cell lysis buffer (Sigma) was used to remove erythrocytes and after washing, the suspension of mononuclear cells was resuspended in complete IMDM. Whole livers and lungs were excised and

immediately placed into Iscove's modified Dulbecco's medium (IMDM) (Sigma) supplemented with 10% fetal bovine serum (PAA), 0.05 mM B-mercaptoethanol (Gibco), 2 mM L-glutamine (Gibco), 0.5 mM sodium pyruvate (sigma), 6mM Hepes buffer (Gibco) 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Gibco). After mincing and digestion for an hour in liberase (Roche), they were then mashed through a 70µm cell strainer and washed in medium. Liver mononuclear cells were extracted after suspension in 40% Percoll (GE Healthcare Life Sciences) and then washed again. With the use of red blood cell lysis buffer (Sigma), erythrocytes were removed from the single cell suspensions of lungs and livers. These cells were then washed again and counted using trypan blue dye (Sigma) to exclude dead cells.

To isolate mononuclear cells from the small and large intestine lamina propria, these organs were excised and placed in ice cooled PBS containing 10mM HEPES buffer. Faeces and mucus were removed and the tissue was cut into 2cm pieces. Tissue was placed in IMDM containing 10mM dithiotreitol, 1% FCS, penicillin, streptomycin, L- glutamine and 10mM HEPES buffer. To remove epithelial cells, tissue was placed in a 37°c incubator whilst shaking at 225rpm. The supernatant was then removed by passing tissue over a tea strainer. The tissue was then digested in IMDM containing DNase (5mg/mL) and Liberase TL (Roche) and a mixture of collagenases. Small intestine tissue was digested for 15 minutes and Large intestine for 30 minutes in a shaking incubator at 37°c and 225 rpm. The digested tissue was then mashed through a 100 µm cell strainer and washed with ice cold complete IMDM. This suspension was then resuspended in 40% percoll (GE Healthcare Life Sciences) and mononuclear cells were separated and resuspended in complete IMDM.

2.9 Positive selection of CD45+ lymphocytes by magnet-activated cell sorting (MACS)

Single cell suspensions of the liver and lung were counted and resuspended in MACS buffer. These were then incubated with CD45 microbeads (Miltenyi Biotec), Fc block (anti-CD16/32) (BD biosciences) and anti-CD45 PE-Cy7 (Biolegend) for 20 minutes. After washing and resuspension in MACS buffer, these cell suspensions were then transferred onto pre-wetted LS columns (Miltenyi Biotec). After the suspension was allowed to flow through, it was washed with MACS buffer and magnets were removed. CD45+ cells were then positively selected and frozen in TRI-Reagent for subsequent RNA extraction and qRT-PCR analysis. Through the use of flow cytometry, the correct positive selection of CD45+ cells was confirmed.

2.10 Analysis of eYFP+ lymphocytes by Flow Cytometry

To analyse eYFP positive lymphocytes in the IL-22 fate reporter mice, mononuclear cells were surface stained with fluorochrome-conjugated antibodies (all purchased from Biolegend) for the following markers; CD45.2 APC-Cy7, TCRβ APC, γδ TCR BV421, CD4 PerCP, CD8 PE-Cy7, NK1.1 PE. Dead cells were excluded from analysis using the Zombie Aqua BV510 viability dye (Biolegend).

Cells to be stained intracellularly were incubated for 4 hours in complete IMDM with the Brefeldin A containing solution, Golgi Plug (BD Biosciences) and stimulated with Phorbol 12-myristate 13-acetate (PMA) (Sigma) and ionomycin (Sigma). Fc block was used to prevent unspecific binding. To analyse populations of T-cells secreting IL-22, cells were stained with the following fluorochrome-conjugated antibodies; anti-TCRβ APC-Cy7, γδ anti-TCR BV421, anti-CD4 PerCP, anti-CD8 PE-Cy7, anti-NK1.1 PE (all from Biolegend). After fixation with 2% paraformaldehyde (Sigma) for 20 minutes and permeabilization with 0.1% octylphenyl-polyethylene glycol

(Sigma), cells were stained intracellularly with anti-IL-22 APC (Affymetrix Bioscience) and washed with FluoroFix buffer (Biolegend). Non-specific binding by this antibody against IL-22 was controlled for with the use of an isotype Rat IgG2a APC antibody (eBioscience). Samples were acquired using the BD FACS suite software on a BD verse flow cytometer (BD Biosciences) and data was analysed using Flowjo X software (Treestar Inc.)

2.11 Analysis of gene expression by qRT-PCR

To analyse gene expression through quantitative real time polymerase chain reaction (qRT-PCR), spleen, lungs, liver and small and large intestine were excised and immediately homogenised in TRI-Reagent (Life Technologies) using a Polytron System PT 1300 D (Kinematic AG). Aliquots of organ homogenates were made and then snap frozen. For the lungs and liver, mononuclear cells were also isolated as previously described and within these suspensions CD45+ lymphocytes were enriched through magnetic sorting (MACS) using CD45 microbeads (Miltenyi-Biotec) through manual cell separation using columns. CD45+ enriched cell populations were then snap frozen in TRI-Reagent.

RNA was extracted from samples using the RiboPure^{TM} kit (Life Technologies) according to the manufacturer's instructions. Concentration of RNA were measured using the NanoDrop 1000A spectrophotometer. Measuring the ratio of absorbance at 260 and 280 nm ensured purity. 2 µg of RNA was used to synthesise cDNA with the use of an Omniscript reverse transcription PCR kit (Qiagen).

Quantitative (q) RT-PCR was performed using the LightCycler Real-Time PCR assay (Roche). Probes from the Universal Probe Library were used which were designed to be suitable for the primers targeting *hprt, il22* and *il22bp*. The following primers (obtained from Sigma-Aldrich) were used *hprt* forward: 5'-

TCCTCCTAGACCGCTTTT-3' reverse: 5'-CCTGGTTCATCATCATCGCTAATC-3'; *il22* forward: 5'-TTTCCTGACCAAACTCAGCA-3' reverse: 5'-TCTGGATGTTCTGGTCGTCA-3'; *il22bp* forward: 5'-ACAACAGCATCTACTTTGTGCAG-3' reverse: 5'-CCCCCAGCAGTCAACTTTAT-3'. Expression of *il22* and *il22bp* was normalised to hprt and reactions were performed on a LightCycler 480 II (Roche).

In order to validate the *il22* primer set, the amplicon generated from qRT-PCR was analysed by gel electrophoresis using 2% metaphor agarose (Lonza) and confirmed to be of the expected size.

2.12 Statistical analysis

Data was analysed using GraphPad prism version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com. Differences in median were deemed statistically significant with a P value less than 0.05 and established using the Mann-Whitney U test or the Kruskal-Wallis test were more than two groups were analysed. Statistically significant values are denoted by (*) for less than 0.05, (**) for less than 0.01 and (***) for less than 0.001. Statistically insignificant differences are denoted by (ns).

3. Results - Analysing the expression of IL-22 in *Plasmodium chabaudi* infection

3.1 Expression of *il22* mRNA in *P. c. chabaudi* infection

In order to answer whether there is a role for IL-22 in this mouse model of malaria infection, it is important to observe if there are any changes in the expression of this cytokine at various time points, during the acute phase, of this infection. This analysis was first performed in the spleen, a lymphoid organ known to have important functions in malaria infection (190). Changes in IL-22 gene expression were also investigated in the liver, where previous studies in malaria infection suggested there is increased damage in mice that lack IL-22 (112), and where it is known that *P. c. chabaudi* parasites sequester (32). The lungs and the intestine are organs where IL-22 has been shown in various scenarios to have an important role (121), thus these organs were also included in order to investigate the role of this cytokine here.

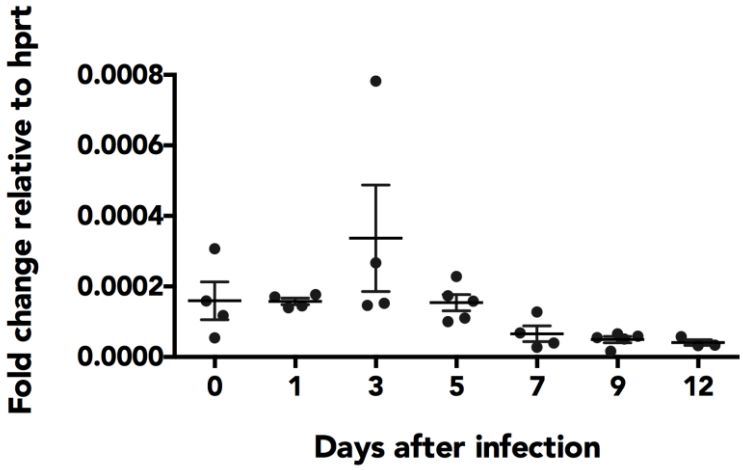


Figure 3.1 Levels of *il22* mRNA detected in the spleens of C57BL/6 mice during a time course of acute *P. c. chabaudi* infection, expressed as fold change relative to the housekeeping gene, *hprt*. Statistical significance analysed by the Kruskal-Wallis test (n=4). Lines show the median with the interquartile range. Representative of 1 experiment.

To detect *il22* mRNA in the spleen, whole organ homogenates were made throughout a time course of the acute *P. c. chabaudi chabaudi AS* infection in C57BL/6 mice. RNA was extracted and cDNA synthesised for use in qRT-PCR to detect IL-22 expression. As shown in figure 3.1, there is a slight trend for increase in *il22* mRNA expression at

day 3 of *P. c. chabaudi* infection, although the median level of expression did not change significantly. After day 5, the expression of this cytokine appears to decrease further.

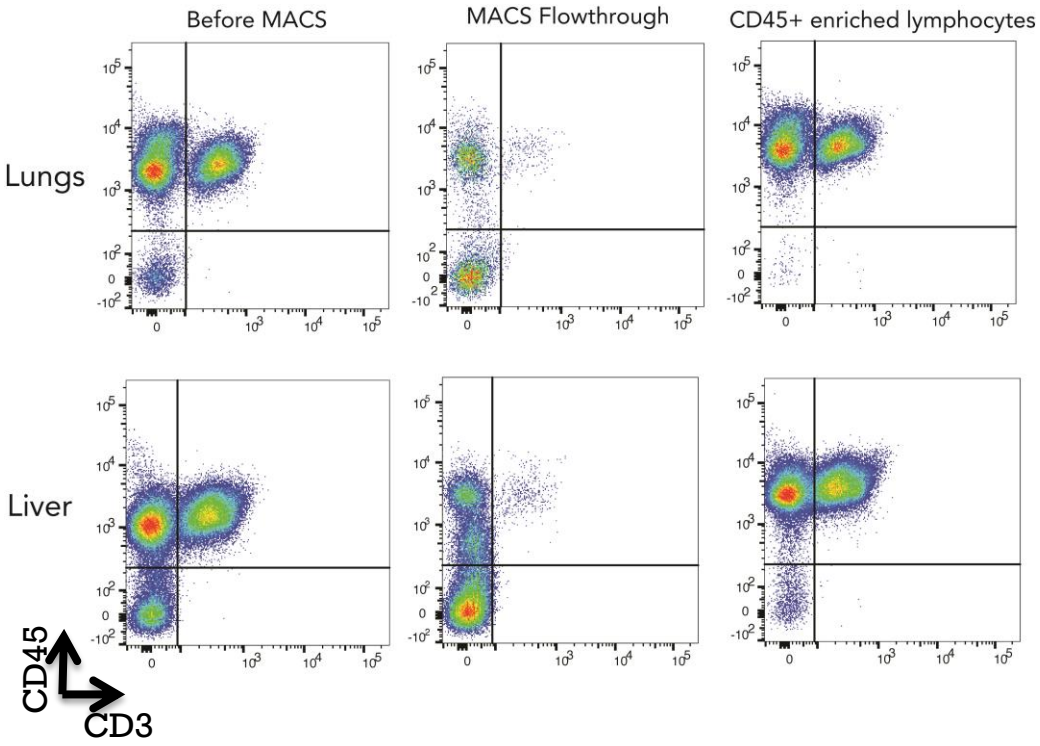


Figure 3.2 Flow cytometric analysis to validate enrichment of CD45+ lymphocytes through the use of MACS, performed on mononuclear cells isolated from the lungs and liver. Populations of CD45 positive and negative cells before and after MACS are revealed as well as cells in the flow through which were not positively selected.

In order to detect *il22* mRNA reliably in the lungs and liver, it was necessary to excise mononuclear cells from these organs and enrich CD45+ lymphocytes by positive selection using magnetic-associated cell sorting (MACS) before freezing these in TRI-Reagent for RNA extraction, cDNA synthesis and qRT-PCR analysis.

As can be observed in figure 3.2, at three stages during this process, cells were stained and analysed through flow cytometry to ensure correct and sufficient enrichment of CD45+ lymphocytes. Prior to mononuclear cells being sorted by MACS, it can be observed that there is a significant population of CD45- cells in the lungs and even more so in the liver. The MACS flow through shows the cells, which did not remain in the MACS columns, a large majority of these are shown to be the CD45-

cells. It can be observed in figure 3.2 that this process was able to enrich CD45+ lymphocytes successfully, increasing the possibility of measuring mRNA expression of *il22* by removing RNA from other cells such as epithelial cells, which would not be expressing this cytokine.

This process was carried out in a time course of acute *P. c. chabaudi AS* infection at days 0, 5, 7 and 10. As can be observed in figure 3.3 there is an increase in the total mononuclear cells as well as the CD45+ lymphocytes as the infection progressed in the liver and lung. In the liver between days 0 and 10, the number of total mononuclear cells almost triples. Although the number of CD45+ cells is similar between days 0 and 5 of infection, after this time point there is a sharp increase in the number of these cells. In the lungs it can also be observed that there is not much

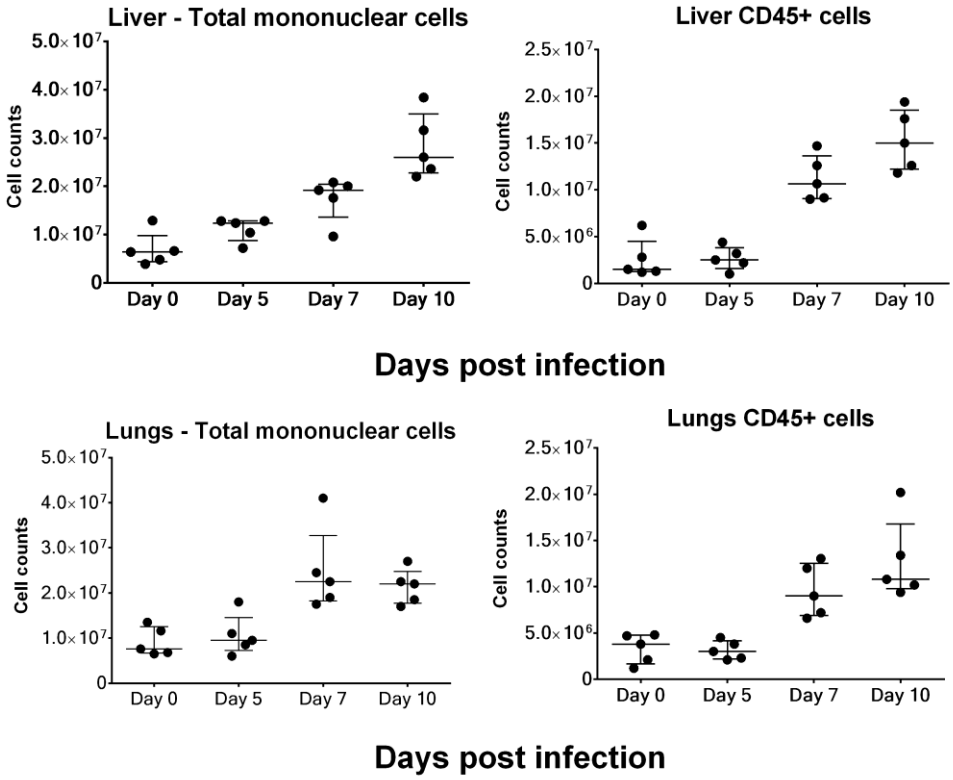


Figure 3.3 show numbers of total mononuclear cells as well as the numbers of CD45+ cells that were sorted from the lungs and liver by MACS. Lines represent the median with the interquartile range. (Statistical significance was measured by the Kruskal-Wallis test) (n =5). Representative of 1 experiment.

change in the total mononuclear cells or cells that are CD45+ between days 0 and 5. However after this time point, there is an increase in total mononuclear cells, with a

peak at day 7 and in CD45+ cells there is an increase until day 10 of infection. The Kruskal-Wallis test showed changes in the median in all 4 cases were statistically significant. In the Liver total mononononuclear cells (p=0.017) and CD45+ cells (p=0.0016) as well as in the lung total mononuclear cells (p=0.004) and CD45+ cells (p=0.002).

The isolated CD45+ lymphocytes were frozen in TRI-Reagent for RNA extraction to analyse the expression of *il22* mRNA at these time points. As can be observed in figure 3.4, in the lungs there was no significant change in the median expression of *il22* mRNA from day 0 to day 5 of *P. c. chabaudi AS* infection. However, after day 5, there was a decrease in *il22* mRNA. This decreased level of expression maintained at day 10. The Kruskal-Wallis test found this change to be significant (p=0.0025). In the liver, also shown in figure 3.4, *il22* mRNA increased significantly by day 5 of infection. After this point, levels of expression were found to decrease by day 7, lower than the original levels detected in the naive mice, and this remained as low at day 10. These change were also found to be significant by the Kruskal-Wallis test (p=0.025).

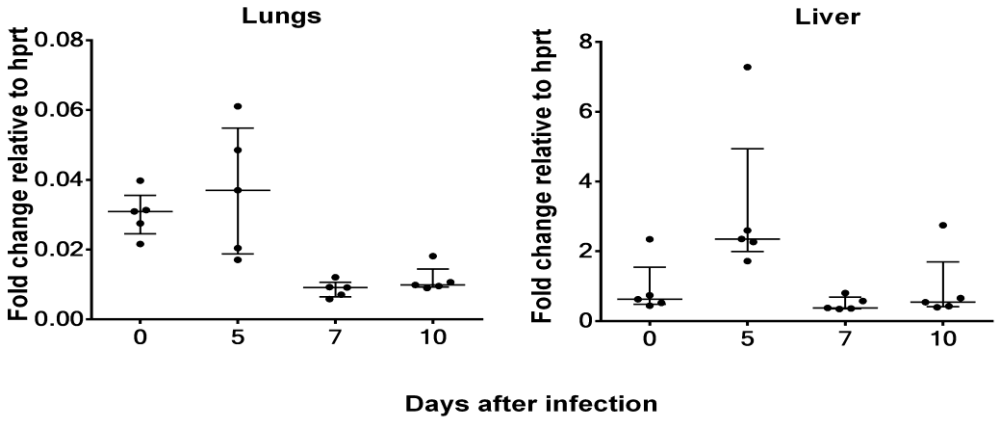


Figure 3.4 show levels of *il22* mRNA detected in the lungs and liver during 10 days of *P. c. chabaudi AS* infection. Lines show the median with the interquartile range. Statistical significance was measured using the Kruskal-Wallis test (n=5). Representative of 1 experiment.

Analysis of the expression of *il22* mRNA was also undertaken in the intestine during *P. c. chabaudi AS* infection. Total RNA was extracted from whole organ homogenates of the duodenum of the small intestine as well as the colon, which were snap frozen in Tri-Reagent (Ambion). Figure 3.5 shows the results of this analysis in these organs. As can be observed, in the small intestine there is no significant change in the median expression of *il22* mRNA until day 7. After this time point the expression of *il22* decreases significantly with a slight trend for increase by day 12 of the infection. These changes were shown to be significant (P=0.03, Kruskal-Wallis test). In the large intestine, there was a slight trend for increase between day 0 and day 5 of *P. c. chabaudi AS* infection. After day 5 of the infection the median level of *il22* mRNA expression was slightly reduced till day 12 of infection. These changes in the median were not shown to be significant (p=0.22, Kruskal-Wallis test).

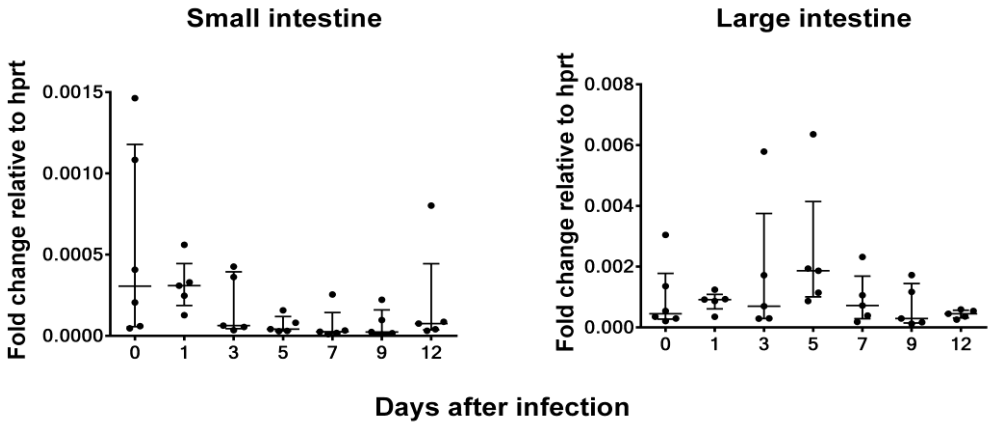


Figure 3.5 shows analysis of il22 mRNA in the small and large intestine during 12 days of *P. c. chabaudi AS* infection. Values expressed as fold change relative to *hprt*. Bars represent the median level of expression at each time point. Statistical significance analysed by Kruskal-Wallis test. (n =5). Representative of 1 experiment.

3.2 Analysing the mRNA expression of IL-22 Binding protein (IL-22BP)

Apart from its membrane-bound receptor, IL-22R1, IL-22 is known to have a second receptor known as IL-22 binding protein, which acts as a soluble antagonist. Studies

in other infection models such as influenza have found expression of this factor can change, impacting the ability of IL-22 to perform its functions (173).

To investigate whether the expression of IL-22BP is modulated within *P. c. chabaudi* infection to allow the cytokine to perform its actions, qRT-PCR was performed in cDNA synthesised from RNA isolated from whole organ homogenates of the spleen, lungs, liver and colon through a time course of acute *P. c. chabaudi* infection. Figure 3.6 shows the results of this analysis. As can be observed in the spleen, there is an early trend for increase by day 1 of infection, however this was not significant. After day 1, the median level of *il22bp* mRNA is reduced by day 5 of *P. c. chabaudi* infection with levels remaining low till day 12. This change was shown to be statistically significant by the Kruskal-Wallis test (p=0.0001). In the liver (p=0.78), lungs (p=0.78) and colon (p=0.38), the median level of *il22bp* expression can be observed to not change significantly in acute *P. c. chabaudi* infection.

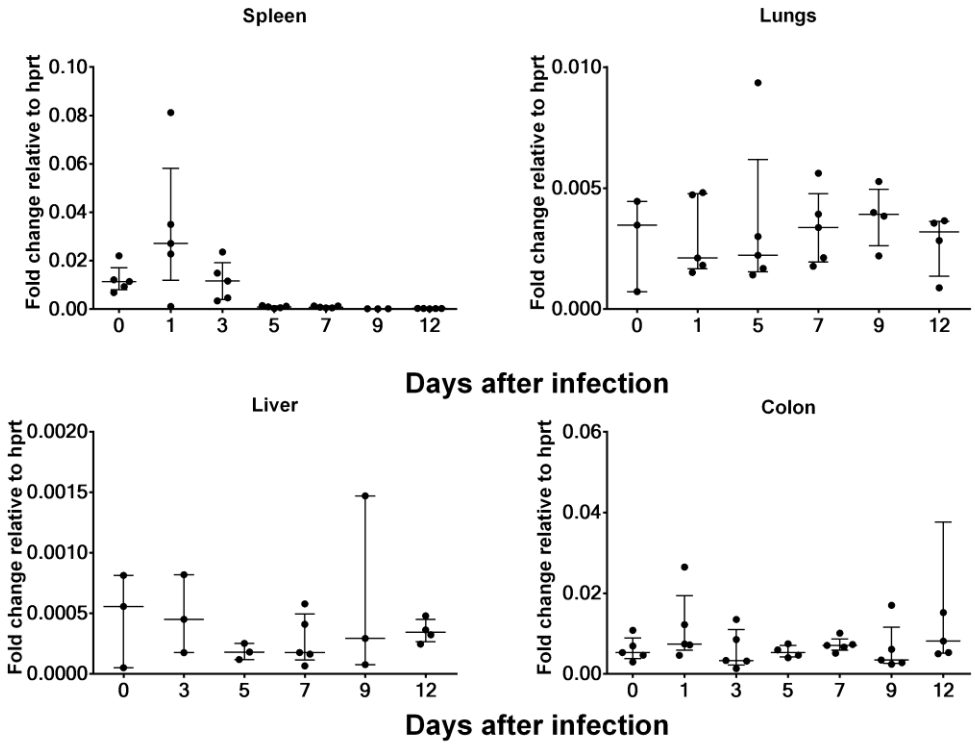


Figure 3.6 shows analysis of *il22bp* mRNA in whole organ homogenates of the spleen, lungs, liver and colon. Lines show the median level of *il22bp* mRNA expression with the interquartile range (Statistical significance was measured by the Kruskal-Wallis test) (n=5). Representative of 1 experiment.

3.3 Analysing expression of IL-22 through Intracellular cytokine staining in *P. c. chabaudi* infection

As well as analysing the mRNA expression of the cytokine, IL-22, another important aim was to analyse its cellular production during this infection. In order to deduce which cells produce this cytokine and in which organ or tissue, flow cytometry in combination with intracellular cytokine staining was used. This was attempted in the liver, an organ strongly suggested by work of Mastelic *et al.* (112) to be a site where IL-22 may play a role during malaria infection.

Mononuclear cells were isolated from the liver by density centrifugation in 37% percoll after mashing through a cell strainer and performing a red blood cell lysis. This was done in naive C57BL/6 mice as well as at day 7 of *P. c. chabaudi AS* infection. Cells were either not stimulated or stimulated with PMA and ionomycin. To ensure that signals revealing the presence of IL-22 were due to the intracellular staining of this cytokine, two negative controls were used. A "fluorescence-minus-one" (FMO) control, which contained all other fluorochrome-conjugated antibodies in the staining panel except the antibody against IL-22, was also used. An isotype control that was a fluorochrome-conjugated antibody with the same fluorochrome used to label IL-22 but did not have specificity for mouse protein was also employed. As can be observed in the dot plots within figure 3.7, in the FMO and isotype controls there was no discernible positive staining in these two negative controls. However, in the stimulated as well as the non-stimulated lymphocytes from the liver of naive C57BL/6 mice there were a very few cells that were clearly producing IL-22.

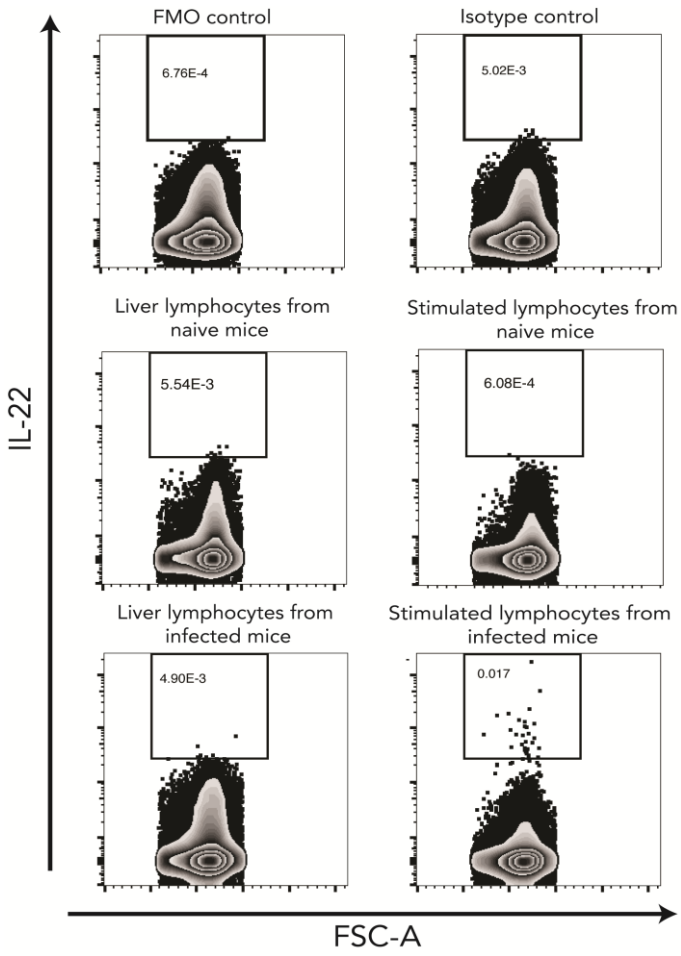


Figure 3.7 shows dot plots from the intracellular cytokine staining for IL-22 in liver lymphocytes from C57BL/6 isolated whilst naive and at day 7 of *P. c. chabaudi AS* infection. These lymphocytes were stained after stimulation with PMA and ionomycin or without any stimulation. Fluorescence minus one (FMO) and isotype controls are also shown.

Very few cells were observed to be positive for IL-22 through intracellular staining for iL-22 in the liver unless the staining was performed on lymphocytes from the liver of infected mice and this staining was also not very distinct. This suggested that it might prove difficult to analyse cytokine-producing cells in the relevant organs through a full time course of acute *P. c. chabaudi* infection by intracellular cytokine staining. Thus the use of IL-22 fate reporter mouse was employed, and analysed by flow cytometry.

3.4 Analysing expression of IL-22 in *P. c. chabaudi* infection with the use of an IL-22 fate reporter mouse

As efforts with intracellular cytokine staining proved difficult, an IL-22$^{+/-cre}$ x R26R^{eYFP} fate reporter mouse was infected with *P. c. chabaudi* and the eYFP positive cells were analysed by flow cytometry. Ahlfors *et al.* (186) generated this mouse which has the gene for cre recombinase at one allele of the IL-22 gene locus. Thus when the IL-22 gene is switched on, cre recombinase is expressed and this enzyme targets a floxed STOP cassette in front of the Rosa26 promoter, allowing the expression of the gene for eYFP which is located here. This eYFP expression is permanent in these cells, even after they may have stopped expressing IL-22. However, this mouse can contribute vital details into whether IL-22 is increasingly expressed during *P. c. chabaudi* infection, which cells produce it and in which organs.

These IL-22 fate reporter mice were infected with *P. c. chabaudi AS* and at various time points mononuclear cells were isolated from the spleen, liver, lung as well as the small and large intestines. Flow cytometry was used to analyse eYFP+ lymphocytes, which were also stained with fluorochrome-conjugated antibodies in order to characterise which lymphocytes had produced or were producing IL-22. As shown in figure 3.8, through the use of flow cytometry, single live lymphocytes were gated on and analysed for their expression of eYFP. Single cells were selected by gating on the edge of the population shown when forward scatter height (FSC-H) was placed against forward scatter area (FSC-A). Live cells were selected by gating on the cells that did not stain positive for dead cell staining and within this population, lymphocytes were selected. Within these cells, those positive for CD45 were gated on and their expression of eYFP was then analysed.

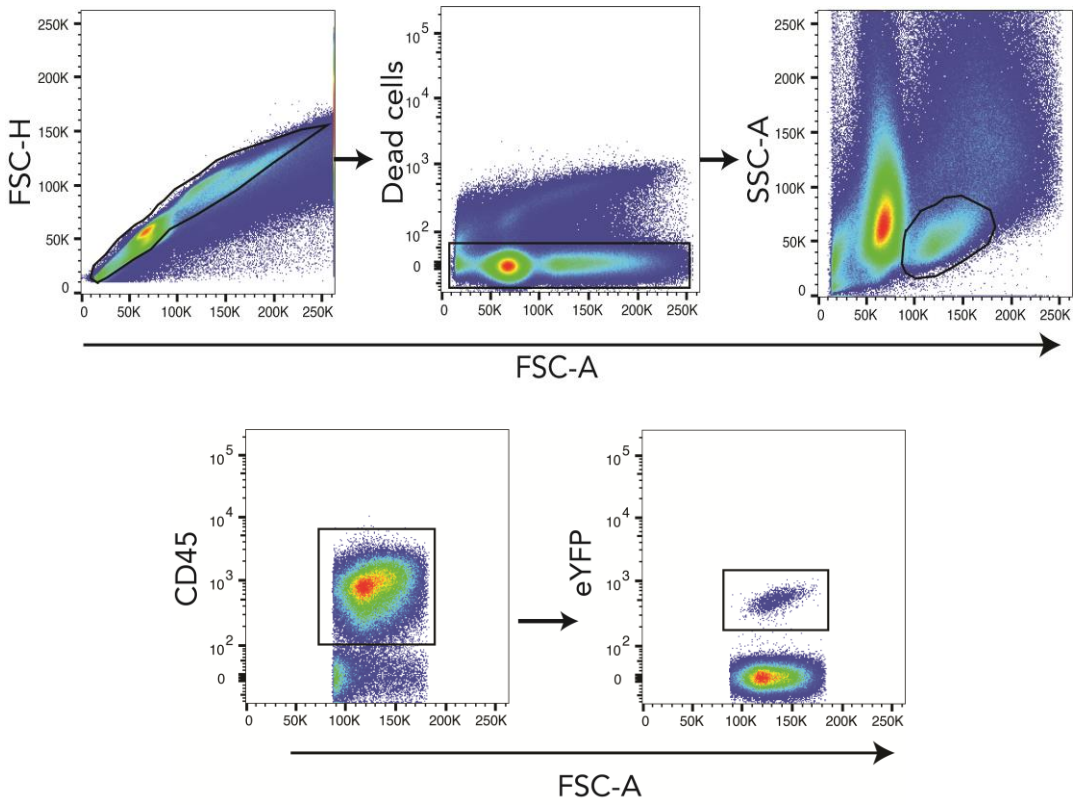


Figure 3.8 shows the gating strategy used to select single live lymphocytes that were eYFP+ for analysis through flow cytometry performed on mononuclear cells isolated from the small intestine of an IL-22 fate reporter mouse.

In the spleen it was observed that, in naive mice, there was already a very small proportion of lymphocytes that were positive for eYFP. As can be seen in figure 3.9, eYFP+ cells were only 0.003% of the lymphocyte population. While the proportion eYFP+ cells appeared to decrease significantly over days 5 and 7 ($p=0.005$, Kruskal-Wallis test), the total numbers of these cells in the spleen did not change significantly through the three time points ($p=0.34$, Kruskal-Wallis test).

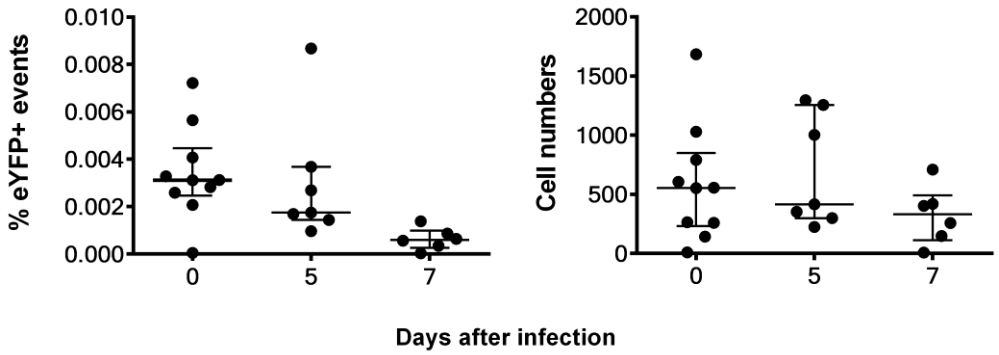


Figure 3.9 shows the proportion and total cell numbers of lymphocytes found to be positive for eYFP in the spleen whilst naive and at day 5 and 7 of *P. c. chabaudi AS* infection. Lines show the median with the interquartile range. Statistical significance measured using the Kruskal-Wallis test (n>5) Representative of 1 experiment.

A higher proportion and number of lymphocytes in the liver were found to be positive for eYFP than in the spleen. As shown in figure 3.10, the median percentage of lymphocytes from naive mice was 0.015% and this did not change at day 5. Although at day 7, the percentage of eYFP+ lymphocytes dropped and remained low at day 10 of *P. c. chabaudi* infection. These changes in the median were not found to be significant (p=0.08, Kruskal-Wallis test). There were no significant changes in the median total numbers of these cells in the liver (p=0.48, Kruskal-Wallis test).

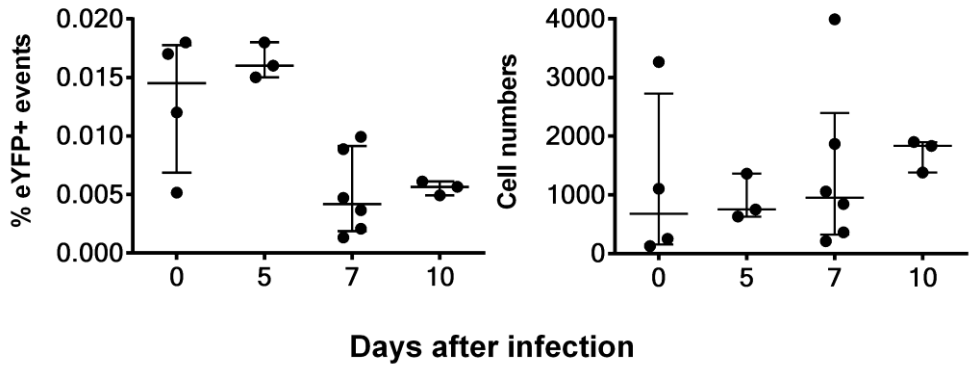


Figure 3.10 shows the percentages as well as total numbers of lymphocytes found to be positive for eYFP in the Liver whilst naive and at days 5, 7 and 10 of *P. c. chabaudi* infection Lines represent the median level of expression with the interquartile range (n>3) Statistical significance was measured by the Kruskal-Wallis test. Representative of 2 experiments.

Figure 3.11 shows that there were a small number of eYFP+ cells already in the lungs of naïve mice. At day 5 of infection, this cell population increased both in percentages and total numbers. However after this time point, the levels of eYFP+ lymphocytes decreased significantly by the peak of *P. c. chabaudi* infection at day 7 and this was reflected in both the percentages and the total cell numbers. Both in the percentages (p=0.02) and total numbers (p=0.01), the changes in the median were found to be significant by the Kruskal-Wallis test.

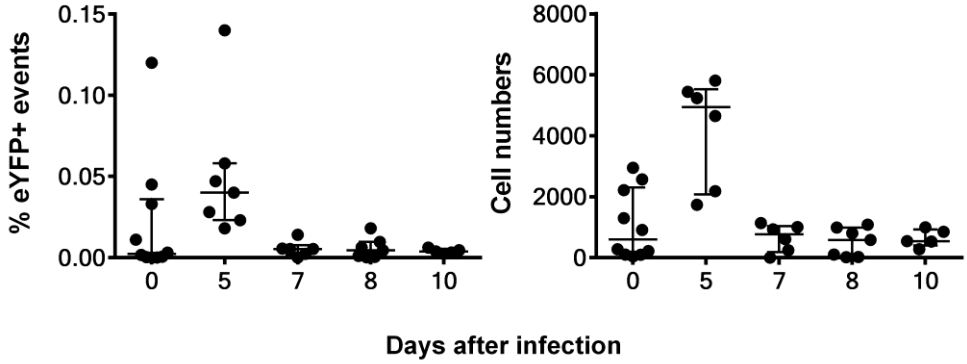


Figure 3.11 shows the percentages and total numbers of eYFP+ lymphocytes in the lungs whilst naive and at days 5, 7, 8 and 10 of *P. c. chabaudi* infection. Lines represent the median level of expression with the interquartile range (Statistical significance was measured by the Kruskal-Wallis test) (n>5). Representative of 1 experiment.

In the study described by Ahlfors *et al.* (186), the lamina propria of the small and large intestine were found to contain the largest numbers of eYFP+ lymphocytes in

the naive fate reporter mice. Studies here found similar numbers of eYFP+ lymphocytes in the naive mice. As can be observed in figure 3.12, a similar percentage of lymphocytes that were eYFP+ were found in both these organs, but there were no significant changes in the median percentages of the eYFP lymphocytes during acute infection in the small (p=0.77) and large (0.67) intestine by the Kruskal-Wallis test. There was an increase in the median cell number in the small intestine and this change was found to be significant (p=0.01, Kruskal-Wallis test).

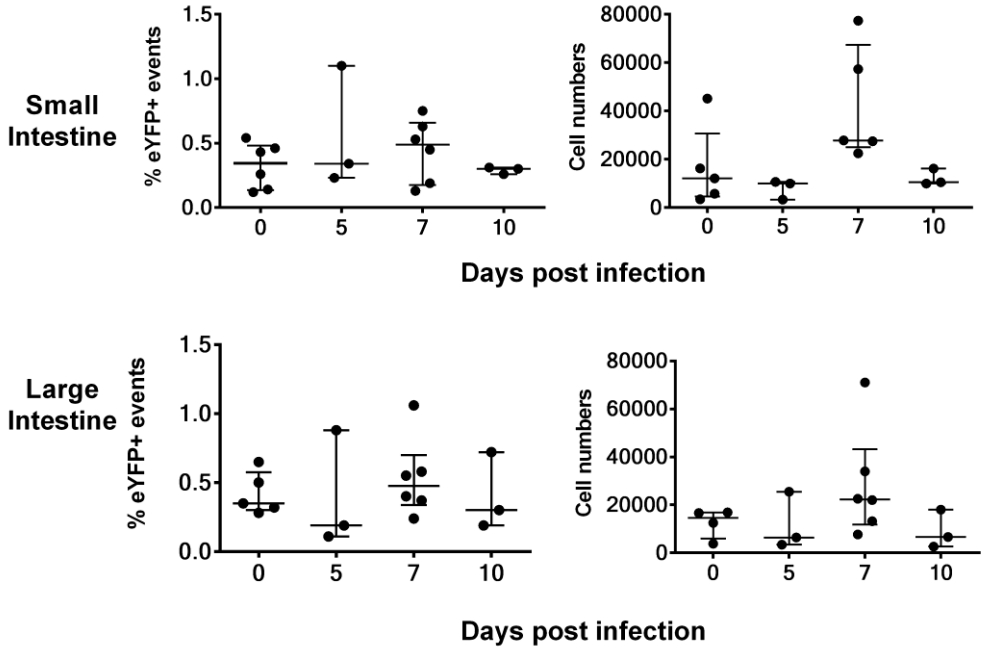


Figure 3.12 shows the percentage as well as total numbers of lymphocytes that were observed to be eYFP+ whilst naive and at days 5, 7 and 10 of *P. c. chabaudi* infection. Lines represent the median level of expression with the interquartile range (Statistical significance was measured by the Kruskal-Wallis test) (n>3) Representative of 1 experiment.

3.4 Characterising eYFP+ cells in the IL-22 fate reporter mouse in *P. c. chabaudi* infection

After analysing levels of eYFP+ lymphocytes in the various organs during *P. c. chabaudi* infection, a pertinent question to ask was what identities made up these cells. Whether these were cells normally associated with IL-22 production such as T cells and innate lymphoid cells and how they may change during infection. In figure

3.13, the gating strategy used to investigate which cell populations made up the eYFP+ lymphocytes is shown. After selecting on the eYFP+ lymphocytes, they were then plotted based on their expression of TCRβ. Of the TCRβ+ eYFP+ lymphocytes the proportion of these that were CD4+ or CD8+ was also recorded. Within the TCRβ- eYFP+ lymphocytes, the proportion of these that were positive for γδ TCR was surveyed and for those negative for this receptor, the expression of NK1.1, a marker for Natural Killer cells was also recorded. Cells that were eYFP+ and negative for both T cell receptors as well as NK1.1 were also measured.

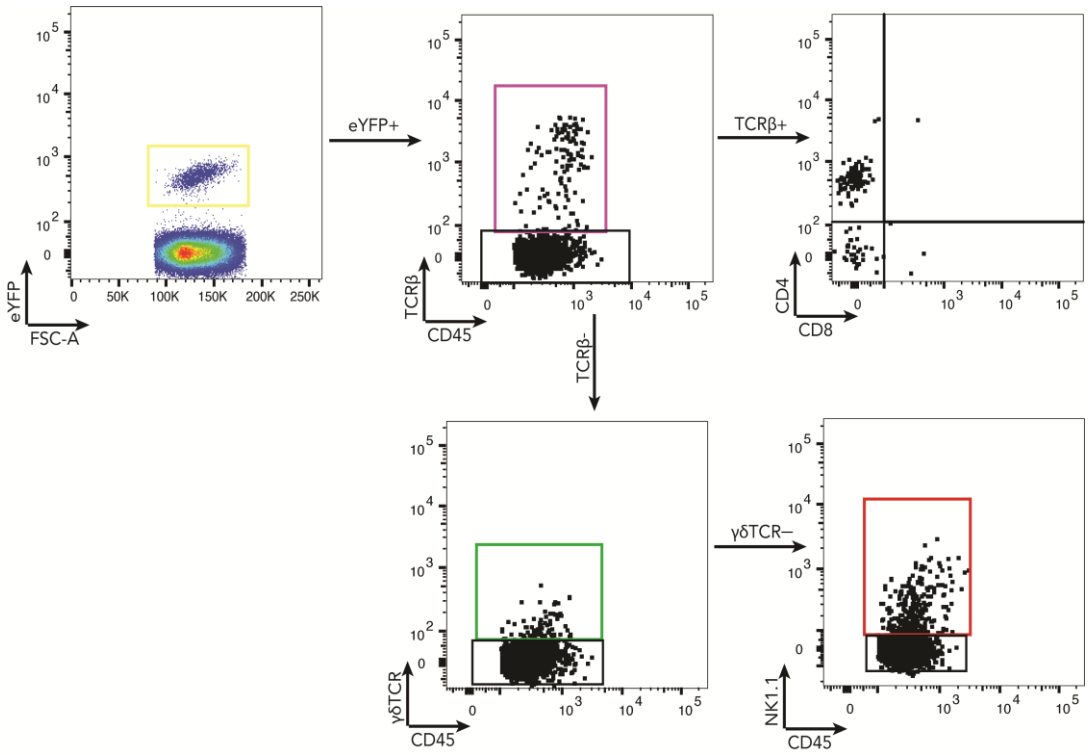


Figure 3.13 The gating strategy used to characterise cells found to eYFP+ through Flow cytometry. Cells shown here are from mononuclear cells isolated from the small intestine lamina propria.

Figure 3.14 shows the summarised data from characterisation of the eYFP+ lymphocytes in the spleen. From the pie charts (Fig 3.14A), It can be observed that the relatively few cells making up the eYFP+ lymphocyte population within the spleen were mostly CD4+ T cells and slightly smaller proportion of these were γδ T cells. Very

few CD8+ T cells, or cells not expressing T cell receptor or NK1.1 (TCR-NK1.1- cells) were positive for eYFP. At day 5, a very small population of eYFP+TCR-NK1.1- cells were observed, with the number of CD4+ T cells decreasing in this organ from a median of 480 cells at day 0 to 156 at day 5. At day 7 of *P. c. chabaudi AS* infection, this population of lymphocytes not expressing T cell receptor or NK1.1 was not observed with CD4+ T cells and γδ T cells once again forming the largest proportion of eYFP+ lymphocytes.

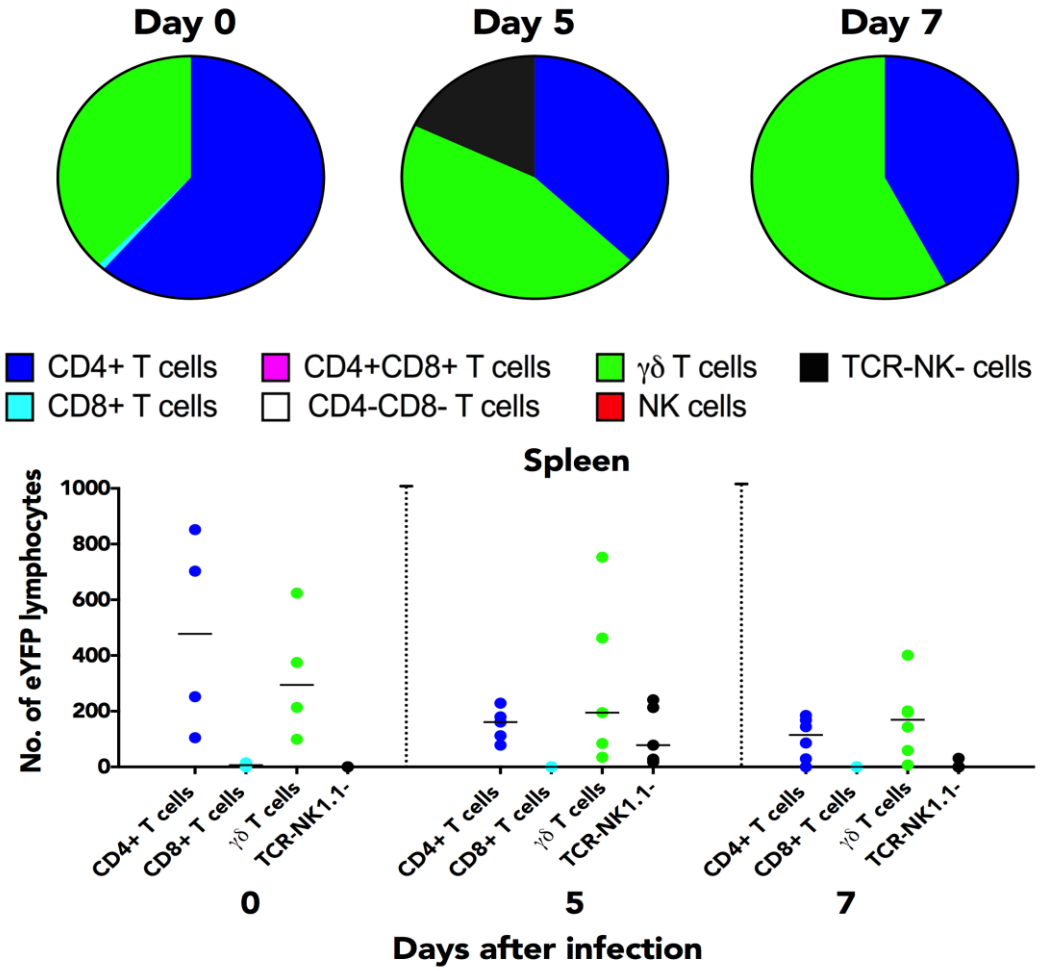


Figure 3.14 The Pie charts represent the proportions of each type of lymphocyte found to be positive for eYFP in the spleen as well as numbers of these different cell types. Lines represent the median level of expression (n=4). Representative of 1 experiment.

In the liver, the composition of the eYFP cell population was very different from the spleen. As shown in figure 3.15 in naïve mice, CD4+ T cells and cells negative for TCR

and NK1.1 formed the majority of eYFP+ lymphocytes with both cell types showing a similar median number of cells. A small population of T cells that were negative for both CD4 and CD8 were also found to be positive for eYFP. At day 5, the median number of the TCR-NK1.1- cells slightly increased, forming the large majority of eYFP+ lymphocytes at this time point. Day 7 of infection saw a more diverse range of eYFP+ lymphocytes as small numbers of CD4+ T cells as well as NK cells, γδ T cells, CD4-CD8- T cells and TCR-NK1.1- cells were all observed to be eYFP+. At day 10, again CD4+ T cells and cells negative for TCR and NK1.1 formed the small number of eYFP positive cells found in the liver at this stage of infection.

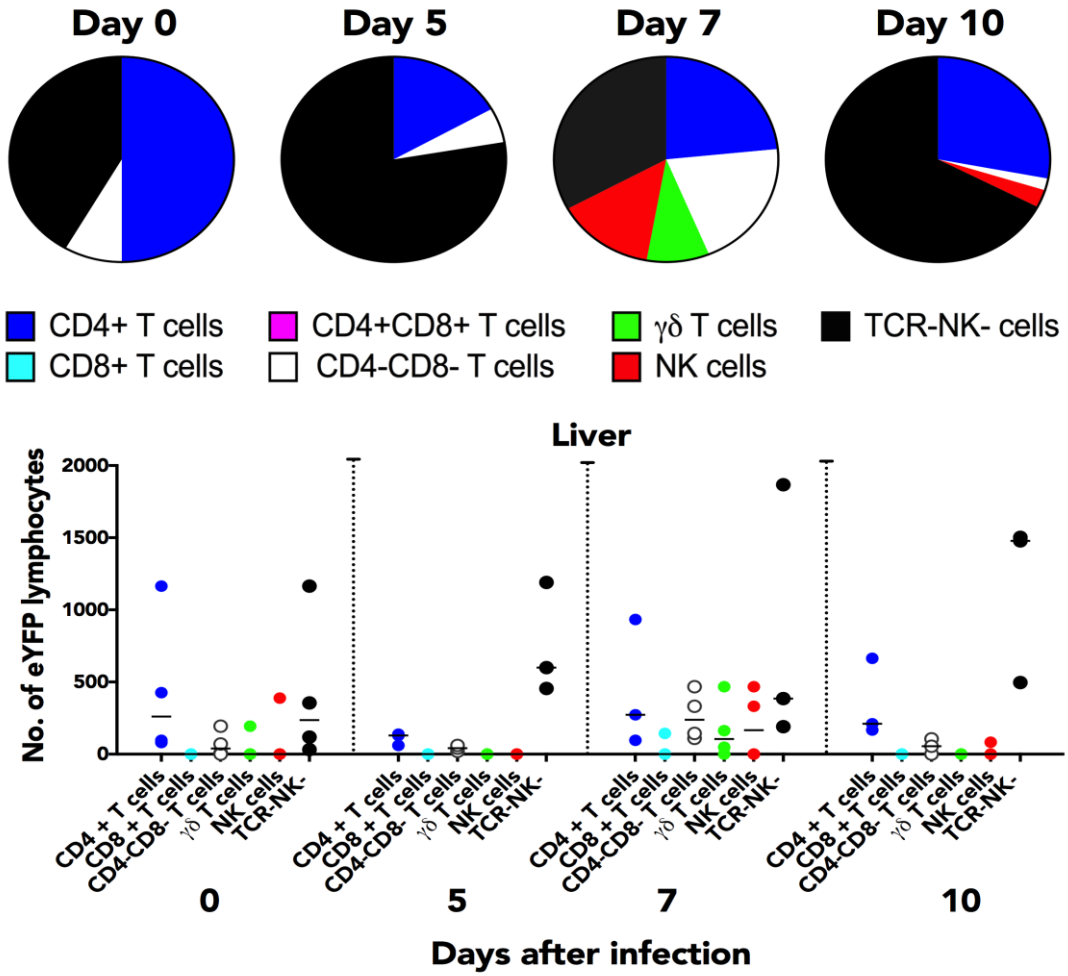


Figure 3.15 The Pie charts represent the proportions of each type of lymphocyte found to be positive for eYFP in the Liver as well as numbers of these different cell types. Lines represent the median level of expression (n=3). Representative of 1 experiment.

Within the lungs, where a larger but still small population of eYFP+ lymphocytes was observed in the naive mice, it was found that this was largely made up of γδ T cells as can be seen in Figure 3.16, with a median number of 978 eYFP+ γδ T cells being observed. CD4+ T cells, TCR-NK1.1- cells as well as a small proportion of CD4-CD8- T cells were also found to be eYFP in the lungs of IL-22 fate reporter mice in the steady state. At day 5 of *P. c. chabaudi* infection, where there was a significant increase in eYFP+ lymphocytes in the lungs, this increase was found to be due to an increase in the total numbers of γδ T cells to a median of 4,427 cells (p=0.0571, Mann-Whitney U-test). The pie chart at day 5 also reveals that the proportion of eYFP+ that were γδ T cells also increased from 45% at day 0 to 70% at day 5. As shown in figure 3.11, after day 5 the number of eYFP+ lymphocytes decreased to levels similar to that seen in the naive mice. This change in eYFP+ lymphocytes can be observed to be associated with changes in this population of γδ T cells. As the numbers of these cells decreased after this time point to levels similar to the naive mouse (p=0.0571, Mann-Whitney U-test). The proportion of these cells that were eYFP+ was also similar to that seen in the naive with CD4+ T cells and TCR- NK1.1- cells becoming more represented again at day 7. At this time point CD4-CD8- T cells were also observed in a larger proportion as they increased to a median of 839 cells at this time point. By day 10, these T cells reduced in number and the large proportion of the eYFP+ lymphocytes were made up of cells negative for TCR and NK1.1.

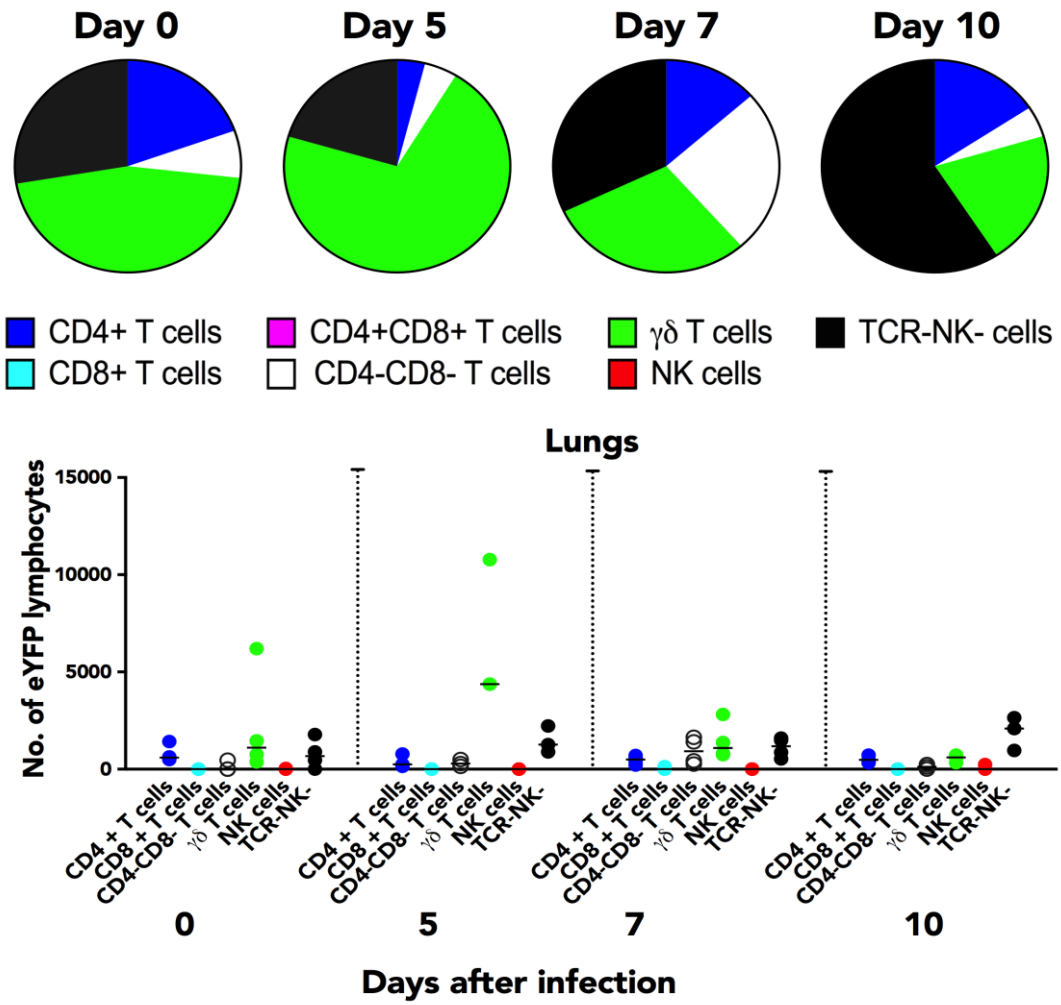


Figure 3.16 The Pie charts represent the proportions of each type of lymphocyte found to be positive for eYFP in the lungs as well as numbers of these different cell types Lines represent the median level of expression (n=3). Representative of 1 experiment.

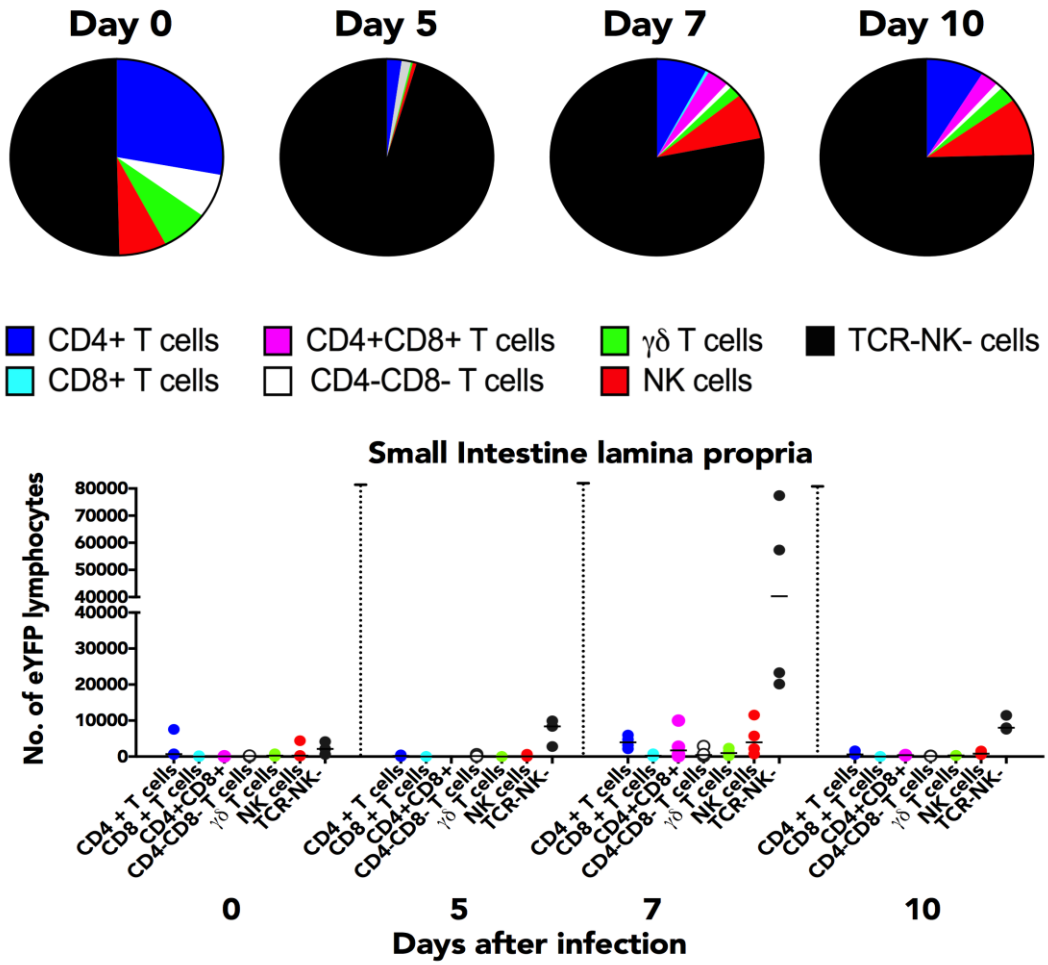


Figure 3.17 The Pie charts represent the proportions of each type of lymphocyte found to be positive for eYFP in the small intestine lamina propria as well as numbers of these different cell types (n=4). Lines represent the median level of expression. Representative of 1 experiment.

In the lamina propria of the small intestine, it can be observed from the pie charts (fig 3.17) that at day 0, most of the eYFP+ lymphocytes, around 50%, were negative for the TCR and NK.1.1 markers. CD4+ cells made up the next largest proportion of these cells. At day 5, a slight increase in the numbers of TCR-NK1.1- eYFP+ lymphocytes was observed and these cells also formed a larger proportion of this population. At day 7, the number of these cells negative for TCR and NK1.1 increased further to a median of 41,000 cells. Other cell types were also found to be eYFP+ at this time point with CD4+ T cells, CD4+CD8+ T cells and NK cells also being represented. At day 10, these TCR-NK1.1- lymphocytes had decreased in number but still formed the largest proportions of eYFP lymphocytes.

In Naïve mice, in the lamina propria of the large intestine (Figure 3.18), the TCR-NK1.1- lymphocytes were the large majority of eYFP+ lymphocytes, around 75%, with a median of around 6600 cells. This number of cells did not change significantly at day 5 but the TCR-NK1.1- lymphocytes formed a slightly higher share of the eYFP+ lymphocytes. At day 7, although their numbers grew, the TCR-NK1.1- levels formed a slightly lower proportion as the NK cells were also observed to increase in frequency. Finally at day 10, the distribution of different lymphocytes resembled that in day 0, with the numbers of NK and TCR-NK1.1- cells that were positive for eYFP falling.

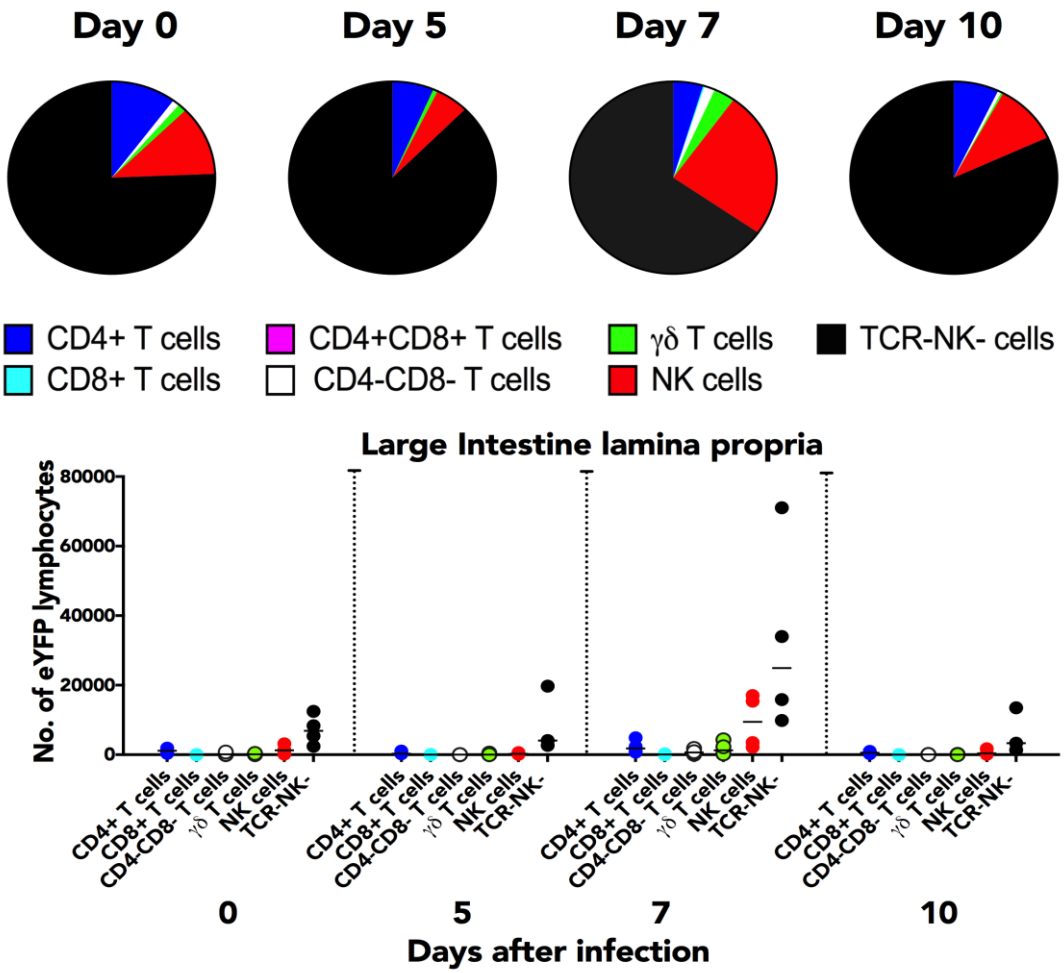


Figure 3.18 The Pie charts represent the proportions of each type of lymphocyte found to be positive for eYFP in the large intestine lamina propria as well as numbers of these different cell types Lines represent the median level of expression (n=4). Representative of 1 experiment.

3.5 Summary of results chapter 1

In analysing *il22* mRNA the spleen, there were no significant changes were observed in the median and only very small percentages and numbers of eYFP+ lymphocytes observed, strongly suggesting that IL-22 was not produced in this organ during *P. c. chabaudi* infection.

Significant increases were observed in the median number of CD45+ lymphocytes in the liver and lung during *P. c. chabaudi* infection. In the liver, there were significant increases in *il22* mRNA by day 5 and then subsequent decreases. Small levels of eYFP+ lymphocytes were observed in this organ, which decreased after day 5 of *P. c. chabaudi* infection. In the lungs, significant decreases were found after day 5 of infection. The IL-22 fate reporter mouse showed significant increases in eYFP+ lymphocytes by day 5 which then significantly dropped by the peak of infection. This transient change was shown to result from the γδ T cell eYFP+ population; it would be interesting to find out the cause of this sudden change in γδ T cells, whether it could be, for example, as a result of increased apoptosis during infection.

Il22 mRNA analysis in the small intestine revealed significant decreases between day 0 and 7 of *P. c. chabaudi* infection. However, in the fate reporter mouse, trends for increases were observed at the peak of infection. This discrepancy may have been due to the permanent labelling of eYFP in IL-22 producing cells. In the large intestine, trends for increases in il22 mRNA were observed which were not significant and no significant changes in the median level of eYFP lymphocytes. Based on these changes in expression of this cytokine, the next question was what would be the impact of the loss of this IL-22 on the progression of *P. c. chabaudi* infection, whether there would be any consequences on the control of the infection or pathology.

4. Results - Analysing the consequences of the lack of IL-22 in *Plasmodium chabaudi* infection

4.1 The loss of IL-22 results in decreased survival of *Plasmodium chabaudi* infection

In order to investigate the role of interleukin-22 in malaria infection, interleukin-22 knockout (*il22*$^{-/-}$) mice on a C57BL/6 background were infected with *Plasmodium chabaudi* and markers of pathology were analysed and compared with the wild type. Mice were infected with two strains of *Plasmodium chabaudi chabaudi:* AS and *CB.* As shown in the survival curves in figure 4.1, *il22*$^{-/-}$ mice were observed to have a decreased survival during this infection as compared to that in the wild-type mice in which mortality was not observed as a result of *P. chabaudi* infection. In *P. c. chabaudi AS* infection, 43% mortality was observed in *il22*$^{-/-}$ mice between days 9 and 12 of infection. Just after the peak of parasitaemia, which occurs at day 7 of infection. No deaths were observed in the infected wild type mice and this difference was found to be significant (p=0.0049, Mantel-cox test). In *P. c. chabaudi CB* infection, *il22*$^{-/-}$ mice showed 50% mortality between days 9 and 10. Again no deaths were reported in the

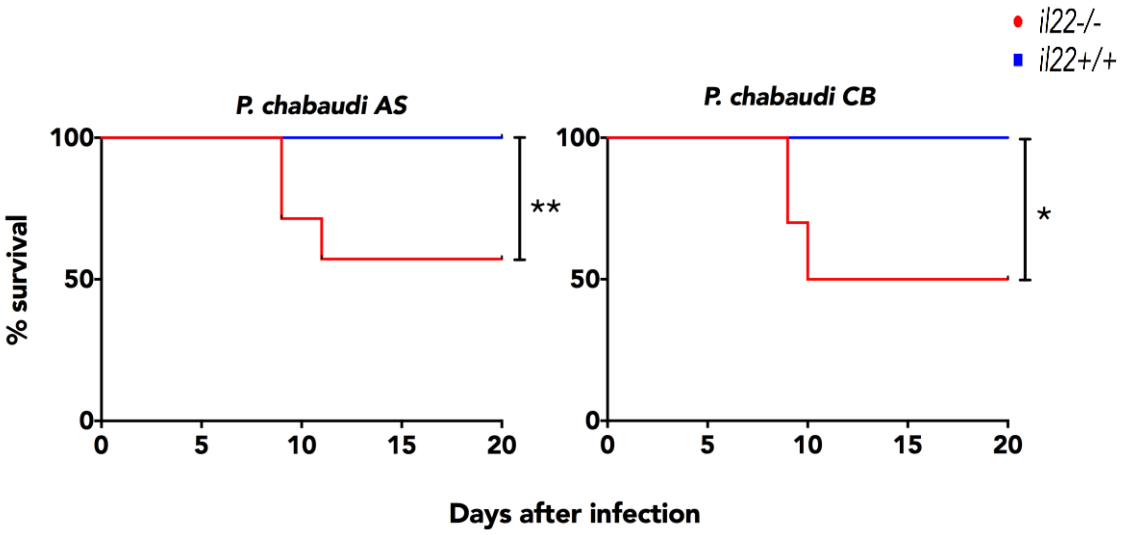


Figure 4.1 shows the survival curves of *il22*$^{-/-}$ and wild type infected with *P. c. chabaudi AS* (n=14) and *P. c. chabaudi CB* (n=10). (* p<0.05, ** p<0.01, horizontal bars show time points between which statistical significance was measured using the Gehan-Breslow-Wilcoxon test). Representative of 2 experiments for *P. chabaudi. A.S.* and 1 experiment for *P. chabaudi C.B.*

infected wild-type mice, and this difference was significant (p = 0.0163, Mantel-cox test).

4.2 Analysis of increased pathology in *Plasmodium chabaudi chabaudi* AS infection in *il22*$^{-/-}$ *mice*

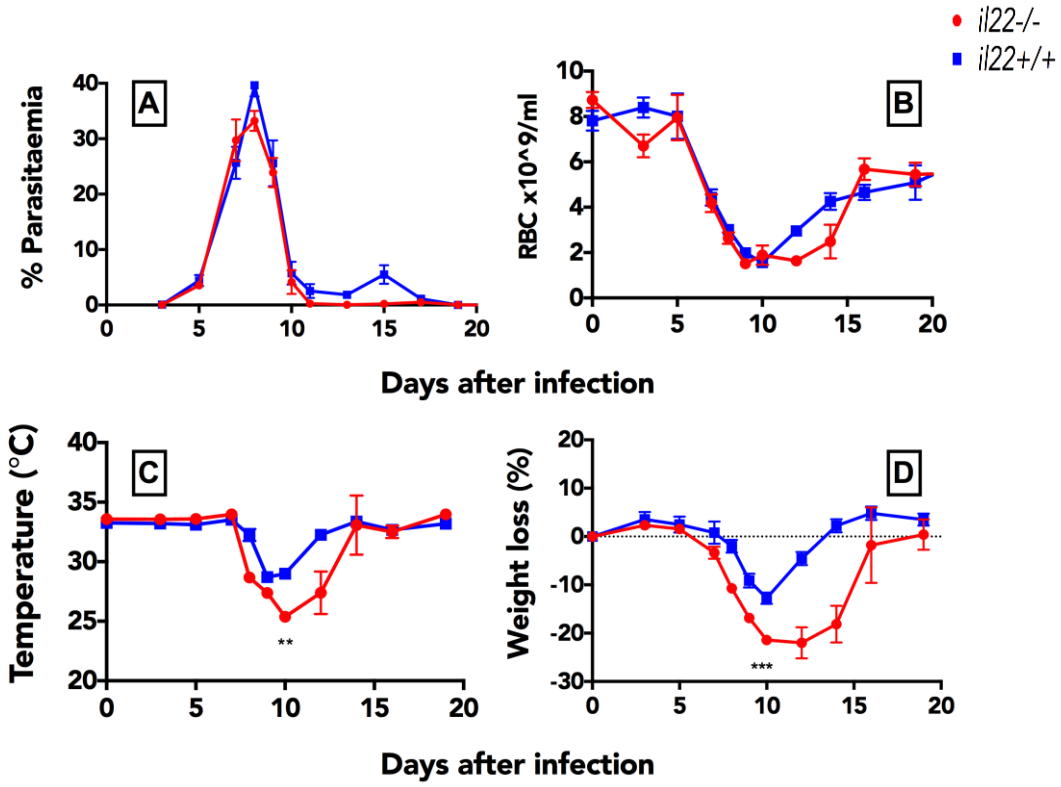


Figure 4.2 The development of parasitaemia (A), anaemia (B), hypothermia (C) and cachexia (D) in *il22*$^{-/-}$ and *il22*$^{+/+}$ injected with 10,000 red blood cells infected with *P. c. chabaudi AS.* Points show the median with the interquartile range (n=7) (* p<0.05, ** p<0.01, *** p<0.001, Statistical significance measured using the Mann-Whitney U-test). Representative of 2 experiments.

When *il22*$^{-/-}$ and wild type mice were infected with 10^5 *P. c. chabaudi AS,* they developed a similar courses of parasitaemia, as shown in figure 4.2A, although the levels of recrudescence observed at day 15 in *il22*$^{+/+}$ mice was absent in *il22*$^{-/-}$ mice. Although *il22*$^{-/-}$ mice mostly had a similar development of anaemia to that in wild type mice, there was a slower rate of recovery in these mice between days 10 and 15 (figure 4.2B). In figure 4.2C, it can be seen that just after the peak of infection, at day 10, *il22*$^{-/-}$ mice had a significantly greater loss of temperature and a significantly

greater weight loss at day 9 (figure 4.2D). This shows that despite the reduced survival seen in *P. c. chabaudi* infections when IL-22 is absent, approximately 50% of mice were still able to control the infection, but all mice exhibited increased pathology.

The detection of increased levels of the enzymes alanine transaminase (ALT) and aspartate transaminase (AST) in the plasma is common indicator of damage to the liver. As can be observed in figure 4.3, plasma levels of these enzymes were similarly low in both groups of naïve mice. However during the infection, levels of these enzymes increase and the increase is greater in the *il22*$^{-/-}$ mice. Levels of ALT were

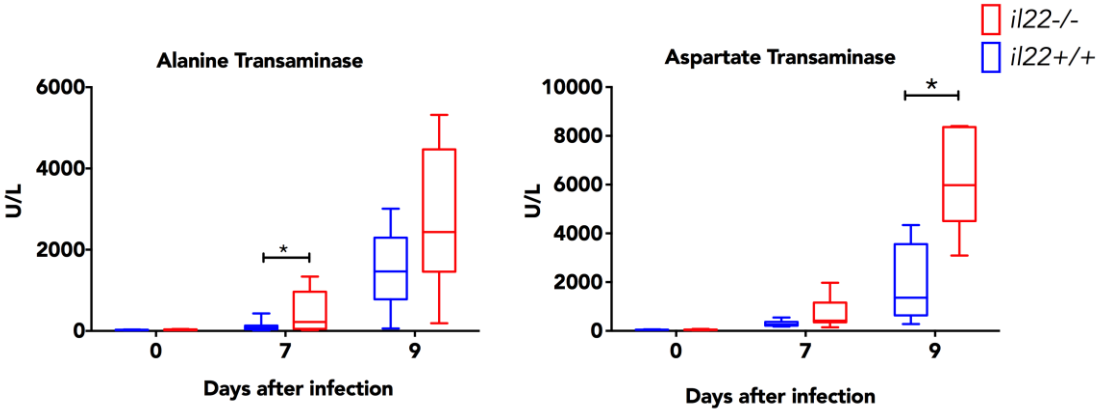


Figure 4.3 The levels of liver enzymes in *il22*$^{-/-}$ and *il22*$^{+/+}$ at day 0, 7 and 9 of *P. c. chabaudi AS* infection (n=14) (* p<0.05, horizontal bars show time points between which statistical significance was measured using the Mann-Whitney U-test). Box and whisker plots show the median with the interquartile range. Representative of 2 experiments.

significantly greater in *il22*$^{-/-}$ mice than the wild type at day 7 of *P. c. chabaudi AS* infection (p=0.02) and levels of AST were significantly greater at day 9 (p=0.01).

Levels of the liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST) were also measured in mice infected with *P. c. chabaudi CB* at day 7 and 9 of infection and whilst naive. As can be observed in figure 4.4, at day 7, there is a trend for higher levels of the liver enzymes in *il22*$^{-/-}$ mice. At day 9 of infection the median concentration of both enzymes in the plasma can be observed to be significantly higher than in infections of the wild type animals. Surprisingly, the

levels of these liver enzymes measured in the wild type at these time points was lower than those observed in *P. c. chabaudi AS* infection.

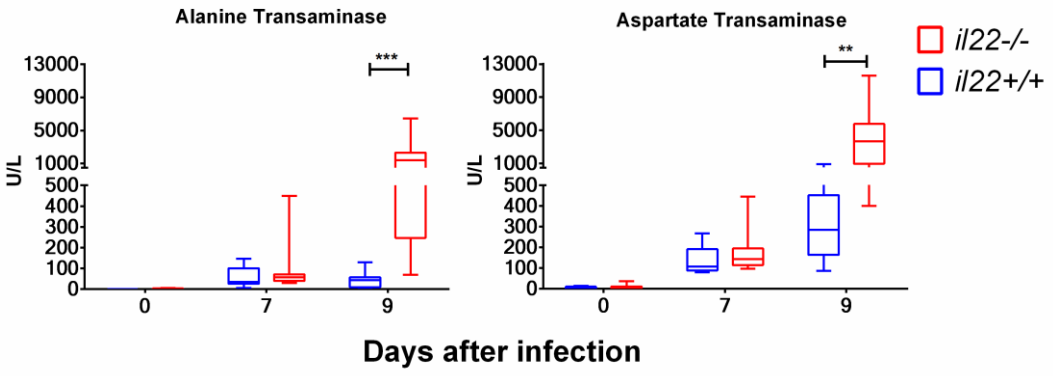


Figure 4.4 Levels of the liver enzymes; alanine transaminase (ALT) and aspartate transaminase (AST) detected in the plasma during *P. c. chabaudi CB* infection. Box and whisker plots show the median with the interquartile range. Analysed using Mann-Whitney U-test (n=10) (* p<0.05, ** p<0.01, *** p<0.001. Horizontal bars show time points between which significance was measured). Representative of 2 experiments.

Since this increase in liver enzymes suggested a significantly greater level of damage to the liver during *P. c. chabaudi* infection as a result of a lack of IL-22, the histopathological changes in this organ were then examined. As can be observed in figure 4.5, whilst naive both *il22*$^{-/-}$ and *il22*$^{+/+}$ show no obvious histopathological differences whereas at day 9 post-infection it can be observed that in both groups of mice, there is damage to the livers associated with this infection. Focal necrosis can be observed, (brighter pink areas of the images) as well as increased kupffer cells (stained brown). The combined histopathological scores which take into account the level of focal necrosis and amounts of Kupffer cells as well as other inflammatory cells revealed that, despite the differences observed in levels of liver enzymes, these histopathological changes observed in the liver due to *P. c. chabaudi* infection showed no difference based on the loss of IL-22.

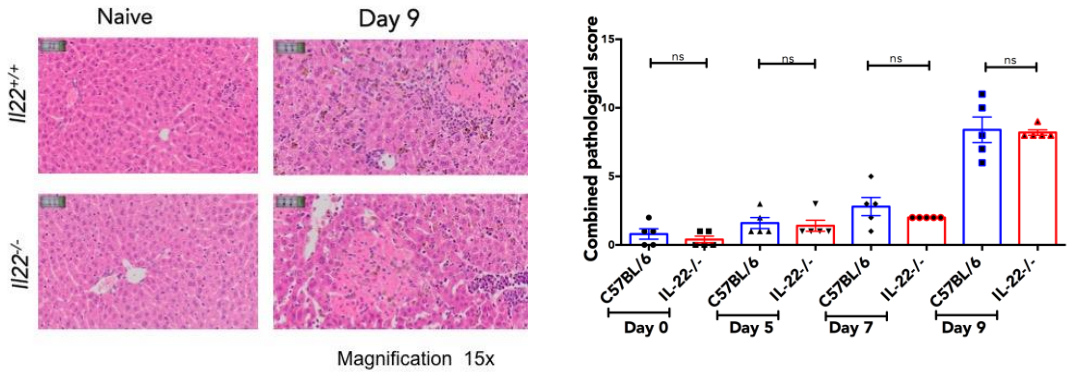


Figure 4.5 Histology of the liver in *il22-/-* and wild type mice whilst naive and at day 9 of *P. c. chabaudi AS* infection. Combined histopathological scores of the livers over a time course of *P. c. chabaudi* infection. Bars show the median with the interquartile range (ns= not statistically significant. Statistical significance measured using the Mann-Whitney U-test (n =5). Representative of 1 experiment.

Levels of lactate were also measured in the plasma of mice infected with *P. c. chabaudi CB* as a marker or possible metabolic acidosis. As can be observed in figure 4.6, the concentration of lactate was similarly low in both *il22*$^{-/-}$ and wild type mice whilst naive. However at day 9 of infection, lactate levels were significantly elevated in both groups of mice (wild type mice vs. naïve *p=0.0012*), with the levels observed in the *il22*$^{-/-}$ group being even more highly elevated than the wild type *(p=0.039)*

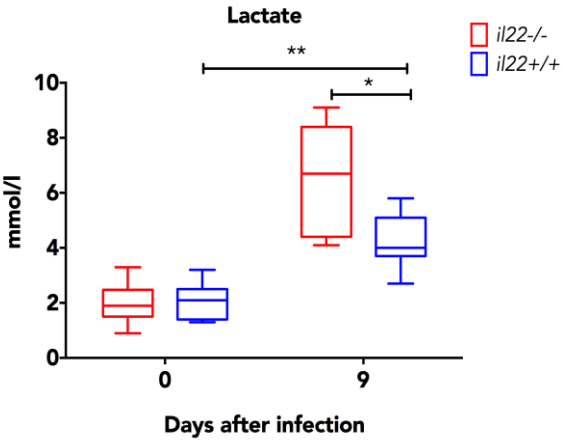


Figure 4.6 Lactate levels in the plasma whilst naive and at day 9 of *P. c. chabaudi CB* infection in *il22*$^{-/-}$ and wild type mice. Box and whisker plots represent the median and interquartile range. (n=10) (* p<0.05, ** p<0.01, horizontal bars show time points between which significance was measured by the Mann-Whitney U-test). Representative of 1 experiment.

IL-22 is known to also have important protective roles in the lungs and this organ is known to be vulnerable to damage during *P. c. chabaudi* infection (191). Histopathological analysis of this organ showed that there was an increase in alveolar septae cellularity after the peak of *P. c. chabaudi* infection as previously shown by Brugat *et al.* (32). This was not observed to occur at a significantly different level in the absence of functional IL-22 (figure 4.7).

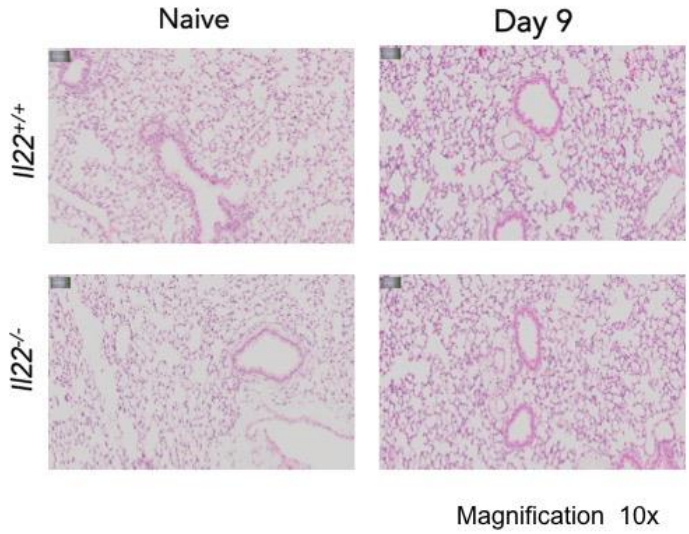


Figure 4.7 Histology of the lung of *il22*$^{-/-}$ and *il22*$^{+/+}$ mice whilst naive and at day 9 of *P. c. chabaudi AS* infection. Representative of 1 experiment.

Since no histopathological changes were observed in the lung as a result of the loss of IL-22, measurements of the contents of the broncheoalveolar lavage (BAL) fluid were performed to determine whether the integrity of the epithelia in this organ had been impaired. This was done by perfusing PBS through the tracheae of these mice and then retrieving it. Levels of total protein within this fluid was measured by the Bradford assay and quantities of IgM antibodies, were also detected by ELISA.

The results of this analysis are shown in figure 4.8. The median concentration of total protein in the BAL fluid was not significantly different in the *il22*$^{-/-}$ and *il22*$^{+/+}$ mice. Although the levels of total protein did not appear to change significantly in infection, at day 9, there was a trend for higher total protein in the *il22*$^{-/-}$ than the wild type (p=0.1296). Measurements of total IgM levels in the BAL fluid found similarly low concentrations in naive *il22*$^{-/-}$ and wild type mice. At day 9 of infection,

levels of this protein had risen in both groups of mice, with a trend for higher total IgM in the BAL fluid of *il22*$^{-/-}$ mice (p=0.2747).

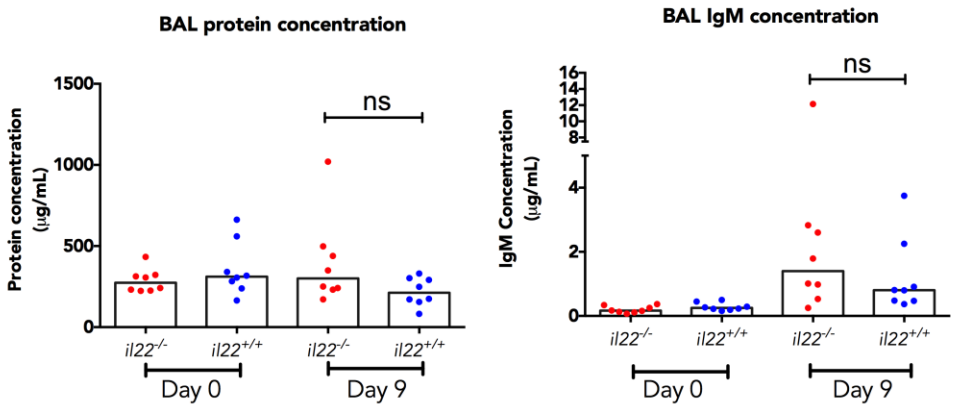
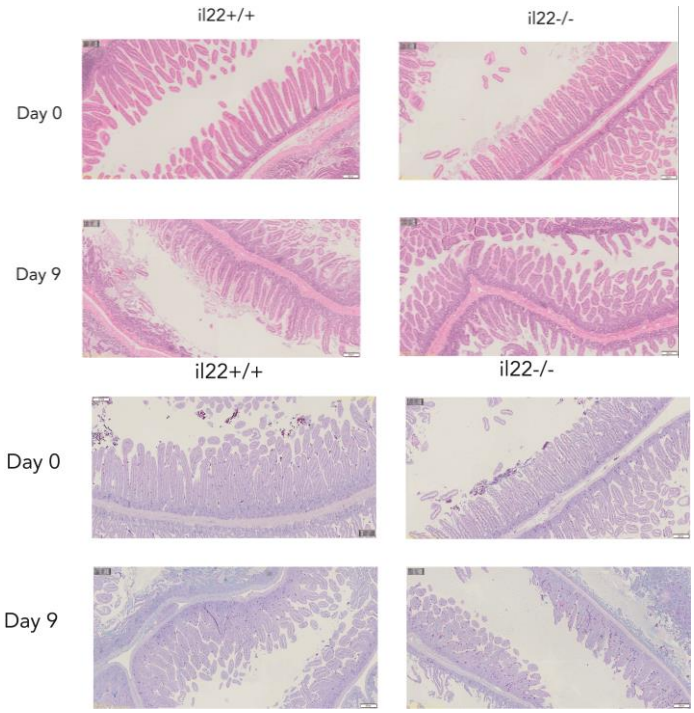


Figure 4.8 Total protein concentration and IgM concentration in the BAL fluid whilst naive and at day 9 of *P. c. chabaudi AS* infection in *il22*$^{-/-}$ and wild type mice. Bars express median level of expression. (n=8) (Horizontal bars show time points between which significance was measured using the Mann-Whitney U-test). Representative of 1 experiment.

IL-22 is known to have active roles in the intestine (115, 118). It has also been suggested that this organ may be affected by malaria infection (43, 44). In chapter 3 (Figure 3.5), levels of this cytokine's mRNA were observed to decrease in the small intestine. The intestine was also investigated for histopathological changes in *Plasmodium chabaudi* infection. Sections of the ileal tissue were examined for histopathological changes such as blunting of the villi, epithelial loss and neutrophil infiltration. As can be observed in images of sections of the ileum stained with haematoxylin & eosin (H&E) in figure 4.9A, there were no differences observed due to *P. c. chabaudi AS* infection or due to the lack of IL-22. As previous studies by Turner *et al.* (159) demonstrated a role for this cytokine in mediating goblet cell hyperplasia in helminthic infection, sections were also stained with Alcian Blue period-acid-schiff (AB-PAS) which exposes goblet cells as purple dots. However, counting these cells in 20 villi crypt units found there were no significant changes in numbers, due to *P. c. chabaudi AS* infection or due to the lack of IL-22 (Figure 4.10).



A
H&E staining in the ileum
Magnification 4x

B
AB-PAS staining in the ileum
Magnification 4x

Figure 4.9 Sections of the ileum of *il22*$^{-/-}$ and wild type mice stained with H&E (A) and AB-PAS (B), goblet cells staining as purple dots, whilst naive and at day 9 of *P. c. chabaudi AS* infection. Representative of 1 experiment.

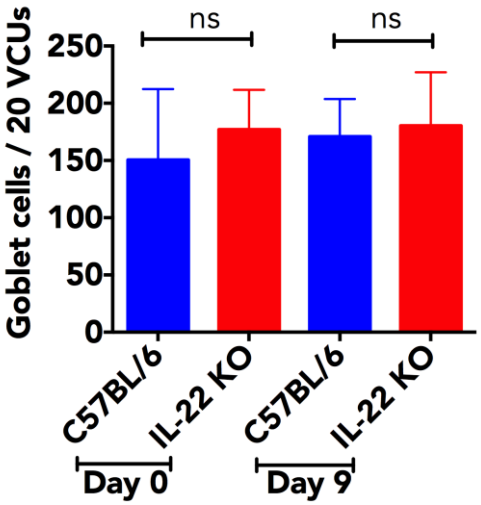


Figure 4.10 Goblet cell counts from 20 villi crypt units in the ileum of il22-/- and wild type mice whilst naive and at day 9 of *P. c. chabaudi AS* infection. Bars show median with interquartile range (horizontal bars show time points between which significance was measured using the Mann-Whitney U-test) ns = not statistically significant (n=5). Representative of 1 experiment.

4.3 Analysing pathology in mosquito transmitted *Plasmodium chabaudi chabaudi AS*

Il22$^{-/-}$ and wild type mice were also infected with *Plasmodium chabaudi chabaudi AS* via 20 mosquito bites using a protocol optimised by Spence *et al.* (64). As observed in figure 4.11, this mode of transmission resulted in an infection of reduced virulence in the wild type as well as *il22*$^{-/-}$, as both had a reduced parasitaemia compared to when infections were initiated via injection of infected red blood cells. Interestingly, at the peak of infection, which occurred at day 10, there was a trend for reduced parasitaemia in the infected *il22*$^{-/-}$ mice (p=0.093). Despite this reduced parasitaemia, both *il22*$^{-/-}$ and wild type mice suffered similar levels of anaemia during this infection.

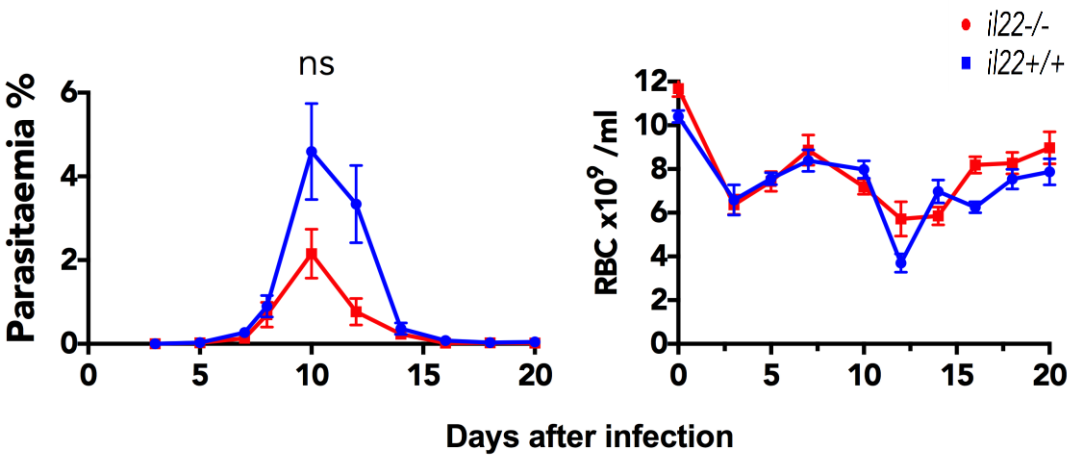


Figure 4.11 The resulting parasitaemia and anaemia after *il22*$^{-/-}$ and wild type mice are infected with P. c. chabaudi AS via 20 mosquito bites. Points and bars represent median with the interquartile range (n=15). Statistical significance measured using the Mann-Whitney U-test. ns = not statistically significant. Representative of 1 experiment.

When *P. c. chabaudi AS* is transmitted through the mosquito in *il22*$^{-/-}$ reduced survival is not observed. However, as shown in figure 4.12, at the peak of infection at day 10, infected *il22*$^{-/-}$ mice can still be observed to have significantly higher levels of ALT in this infection even in this less virulent infection.

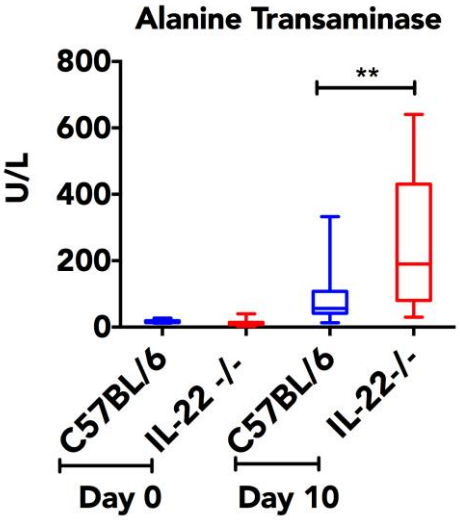


Figure 4.12 Alanine transaminase levels in the plasma whilst naive and at day 10 of mosquito transmitted *P. c. chabaudi AS* infection in il22-/- and wild type mice. Box and whisker plots represent the median and interquartile range. (n=15) (p<0.01, horizontal bars show time points between which significance was measured by the Mann-Whitney U-test). Representative of 1 experiment.**

4.5 Summary of Results Chapter 2

In *P. c. chabaudi AS* infection in il22-/- mice, a 43% mortality was observed with no mortality observed in the wild type. Although there was no difference in parasitaemia or anaemia in *P. c. chabaudi* infection compared to the wild type, due to the loss of IL-22, a significant loss of temperature and weight was observed. Furthermore, analysis of liver enzymes in the plasma found significant increases in ALT and AST. This was also observed in *P. c. chabaudi CB* infection, however, no significant difference in liver histopathology. No differences in the histopathology of the lungs was observed either, although a trend for increased IgM was observed in the broncheoalveolar lavage fluid of infected *il22*$^{-/-}$ mice suggesting the loss of IL-22 may have resulted in increased epithelial leakage in this organ. In the intestine there was also no discernible difference in histopathology due to *P. c. chabaudi AS* infection or due to the loss of IL-22. However only the gross histopathology of this organ was measured.

Mosquito transmission of *P. c. chabaudi AS* resulted in a trend for lower parasitaemia in infected *il22*$^{-/-}$ mice. Despite this, mice that lacked IL-22 still had an increased level of ALT in the plasma, suggesting that there was still liver damage despite the reduced virulence in this infection.

Results from these experiments suggest that IL-22 may have a protective role in *P. c. chabaudi* infection, with the lack of this cytokine resulting in the liver being more damaged and the lung epithelial integrity being reduced.

5. Discussion

Unlike most cytokines, interleukin-22 does not target other immune cells. It rather targets non-haematopoietic cells such as the epithelial cells of the lungs, intestine and skin, as well as hepatocytes in the liver. In these organs, as well as others such as the pancreas and skin it mediates its functions, which can be protective or pathogenic dependent on the context.

The aim of this thesis was to investigate the production and role of interleukin-22 in the *Plasmodium chabaudi* mouse model of malaria infection. To pursue this, the expression of this cytokine was analysed during infection in the spleen, liver as well as the lungs in addition to the small and large intestine. Through the use of qRT-PCR, the production of *il22* mRNA was analysed, and the production as well as characterisation of the cellular sources of this cytokine was performed with the use of an IL-22 fate reporter mouse. Another important question to ask was what the consequences of the lack of IL-22 in *P. c. chabaudi* infection were. This was addressed with experiments analysing pathology in $il22^{-/-}$ mice infected with *Plasmodium chabaudi*.

Since the spleen is a lymphoid organ known to have important roles in malaria infection (190), the expression of IL-22 was studied in this organ. Here, mRNA analysis found no significant changes in the median during *P. c. chabaudi AS* infection, although there was an early trend towards an increase, as was observed by Mastelic *et al.* (112). Studies examining eYFP+ cells in the fate reporter mice during this infection found very few lymphocytes positive for eYFP whilst naive and this did not change in infection. In combination, this strongly suggests that the spleen is not important in secreting IL-22 during *P. c. chabaudi* infection. Studies in the rat have found the spleen to be a site where IL-22 binding protein (IL-22BP) is highly expressed

by conventional dendritic cells (145). In these experiments, the spleen was the only site where a significant change in median IL-22BP mRNA expression was observed. It would be interesting to observe if this could play a role in *P. chabaudi* infection.

There was a significant increase in the number of CD45+ lymphocytes in the liver and lung during *P. c. chabaudi* infection. This agrees with previous studies, which have shown that recruitment of immune cells accompanies sequestration of the malaria parasite, in these organs, and is associated with in immunopathology (32).

In the liver, IL-22 mRNA was found to have increased significantly by day 5 of *P. c. chabaudi* infection and then subsequently decreased significantly by day 7. This pattern of expression is similar to that observed in previous experiments reported by Mastelic *et al.* (112). In the fate reporter mouse this decrease could also be observed by day 7 of infection in the percentages of eYFP+ lymphocytes, however it was not reflected in the cell numbers. This could be explained by the expansion of lymphocytes other than those that are eYFP+ at the peak of infection, since as aforementioned, total numbers of CD45+ lymphocytes are observed to increase significantly in this infection.

Intracellular cytokine staining was attempted for this cytokine in lymphocytes isolated from the liver, however the IL-22 fate reporter mouse was resorted to as an alternative since only very low numbers of lymphocytes could be found to be positive for IL-22 in the naive mouse. Analysis of data produced from flow cytometry using this mouse demonstrate that these issues with intracellular cytokine staining stemmed from the fact that IL-22 is not produced in large amounts in the liver, despite the important role of this cytokine in protection from liver damage, due to injury or to infection (153, 154).

Mastelic *et al.* (112) found that CD8+ T cells were the largest producers of IL-22 in *P. c. chabaudi* infection. These cells have been shown to produce IL-22 in other circumstances as well (136). However studies with the fate reporter mouse did not find significant production of this cytokine by this cell type. Rather, CD4+ T cells were shown here to be much larger producers of IL-22. It is unlikely that this discrepancy was caused by the different techniques used to assay for cellular production of IL-22.

The lungs were also found to have a decrease in il22 mRNA expression after day 5 of *P. c. chabaudi* infection. In the percentages and numbers of eYFP+ lymphocytes there was a strikingly similar result of an increase at day 5 of the infection and a subsequent decrease back to original levels. This change in the median was significant. Characterising these cells revealed that this was due to changes in the size of the eYFP+ γδ+ T cell population. Although the decreases were back to levels observed in the naive mouse, the fact that this decrease occurs at the peak of infection where there is a greater risk of inflammatory damage may mean that the protective effects of IL-22 in the lung seen in other infection models (137, 172, 192) are lost when epithelial cells are most vulnerable. It is possible that this may contribute to the pathology associated with sequestration seen in the lung at the peak of *P. c. chabaudi AS* infection in C57BL/6 as described by Brugat *et al.* (32).

For the small intestine, mRNA analysis found a significant decrease in *il22* during the time course of *P. c. chabaudi* infection, however no significant changes were observed in the median percentages of eYFP+ lymphocytes. The numbers of eYFP+ lymphocytes rather showed a trend for increase at day 7 of *P. c. chabaudi* infection. This discrepancy may be explained by the fact that the fate reporter mouse permanently labels IL-22 producing cells as eYFP+, recording cumulative expression over time, thus these eYFP positive cells may not necessarily be actively producing

this cytokine at this time. Further work could investigate this by employing the use of intracellular cytokine staining with the fate reporter mouse to confirm the production of IL-22 protein in these cells. In the large intestine, significant changes in il22 mRNA expression were not observed. There was a trend, however, for increased expression till day 5 of *P. c. chabaudi* infection. There was also no significant difference in the percentages of eYFP+ lymphocytes during this infection. In a lot of these analyses of eYFP total numbers and percentages statistical significance was not observed in differences. In future work it would be important to repeat these experiments with larger groups of mice for statistical power.

Of the cells that were eYFP+ in the small intestine whilst naive and during infection, a large majority were shown to be CD45+ and negative for T-cell or NK cell markers. There is a strong likelihood that these are innate lymphoid cells, as this population was also found to conform to the large majority of this organ's eYFP+ lymphocytes in the work of Ahlfors *et al.* (186). As reviewed by Spits *et al.* (193), innate lymphoid cells are important sources of cytokines that lack antigen receptors. As markers that are unique to innate lymphoid cells have not yet been identified, further analysis would require the use of a "dump channel" which contains markers for T, B and NK cells such as CD3, CD19 and DX5 in order to ensure that eYFP+ cells analysed are correctly identified as innate lymphoid cells (194). Developments in Flow cytometry technology allowing analysis of more than 12 parameters in the same experiment make this now feasible (195, 196).

Ahlfors *et al.* (186), commented on the lower than expected eYFP+ populations being observed in the IL-22 fate reporter mouse, since the expression of both eYFP+ and IL-22 was observed to be low as compared to studies done in the IL-17 fate reporter mouse (188). It was suggested that this may be due to the expression

of IL-22 being monoallelic like has been shown for IL-10 (197). Since, in the fate reporter mouse, one allele of IL-22 is disabled by the introduction of cre recombinase, the possibility of IL-22 expression being monoallelic would lead to reduced eYFP+ cells being observed.

The cytokine, interleukin-12 has been shown to be important for stimulating protective immune response in *P. c. chabaudi AS* infection (55). This cytokine has also been shown to be able to induce plasticity in group 3 innate lymphoid cells, stimulating production of IFN-γ by these cells, rather than IL-22 (198). It would be interesting to observe whether eYFP+ innate lymphoid cells observed in the intestine lamina propria switch to producing IFN-γ as a result of increased systemic levels of IL-12 in *P. c. chabaudi* infection. This may explain observations of decreased il22 mRNA in the studies detailed here in the small intestine lamina propria. As the IL-22 fate reporter mouse has all cells that have produced IL-22 permanently labelled with eYFP, cells that may have switched to expressing IFN-γ would have been included in analysis. This could be further investigated through the use of intracellular cytokine staining for both IL-22 and IFN-γ in lymphocytes isolated from the *P. c. chabaudi* infected IL-22 fate reporter mouse. By comparing the fractions of eYFP+ cells that are positive for IL-22 or IFN-γ at various time points in the acute infection, possible switching of cytokine production in these cells may become apparent.

In this study, the role of interleukin-22 in *P. c. chabaudi* infection was also investigated by examining the consequences on the development of the infection as well as the pathology in the absence of this cytokine. To do this, *il22*$^{-/-}$ mice were infected with *P. c. chabaudi AS* or *P. c. chabaudi CB* via injection of infected red blood cells. Il22$^{-/-}$ mice were also infected with *P. c. chabaudi AS* via mosquito bite.

In *P. c. chabaudi AS* infection, *il22*$^{-/-}$ mice were observed to have a decreased survival despite having similar levels of parasitaemia as the infected wild type mice. This strongly suggests a decreased tolerance of the pathology of infection as discussed by Ayres & Schneider (199). Weight and temperature loss were also significantly greater due to the loss of IL-22, around the peak of *P. c. chabaudi* infection. *P. c. chabaudi CB* also caused significant weight and temperature loss in infected il22$^{-/-}$ mice. Although, no significant difference was observed when compared to the wild type in infection, possibly because of the increased severity of this infection.

On infection with *P. c. chabaudi AS* through the injection of infected red blood cells, no differences were observed in parasitaemia due to the loss of IL-22. However, in a mosquito transmitted *P. c. chabaudi AS* infection, a trend for a lower peak of parasitaemia was observed in infected *il22*$^{-/-}$ mice. This infection differed from the direct injection of infected red blood cells in that the mosquito-bite transmitted sporozoites into the skin of the mice, which developed into merozoites within the liver. Although there are still increases in liver enzymes in this model of malaria infection and infected *il22*$^{-/-}$ mice still had a significantly higher level than the wild type. It may be interesting to investigate whether the liver, without IL-22, may provide a more difficult environment for sporozoites to grow possibly from the lack of survival factors that IL-22 induces in the liver in *il22*$^{-/-}$ mice (157) resulting in hepatocytes in these mice being less able to support the parasitic infection. However, the initial blood stage parasitaemia soon after egress from the liver in in *il22*$^{-/-}$ mice was be similar to the wild type, suggesting that the same number of merozoites may have emerged from the liver. This could be further investigated by measuring the magnitude of 18s RNA from sporozoites by qRT-PCR in the liver 42 hours after

mosquito bite as has been done by Nahrendorf *et al.* (200). Doing this in *il22*$^{-/-}$ mice as well as the wild type could show whether differences in the liver due to the loss of IL-22 affect the growth of sporozoites in the liver stage and whether this results in the differences observed in the parasitaemia of *il22*$^{-/-}$ mice.

In these investigations of the role of interleukin-22 in *P. c. chabaudi*, the possibility of this cytokine playing a role in the skin was not investigated. IL-22 has been shown to have important roles in the skin, the most documented case being psoriasis (201). Work in the naive IL-22 fate reporter mouse has shown the majority of eYFP+ cells in the skin were γδ+ T cells (186). Immune changes have been described in this organ during malaria infection but the possibility of a role of IL-22 here has not been investigated. This could be done by analysing changes in eYFP+ cells in fate reporter mice shortly after transmission of *P. c. chabaudi AS* via mosquito bite using the protocol of Spence *et al.* (64). Doing this analysis in both wild type and *il22*$^{-/-}$ mice could reveal whether the lack of IL-22 could reveal whether this cytokine could affect number of sporozoites reaching the liver by analysing the liver burden as well in these mice.

Analysis of liver enzymes in the plasma revealed that infected *il22*$^{-/-}$ mice had significantly higher levels than the wild type mice in both *P. c. chabaudi AS* and *P. c. chabaudi CB* infections. Interestingly, this was also observed in mosquito transmitted *P. c. chabaudi AS* infection even though there was a reduced level of virulence with no mortality being observed in infected *il22*$^{-/-}$ mice. However, no discernible differences were observed in the histopathology of the liver in *P. c. chabaudi AS* infection due to the loss of IL-22. This may mean that the difference in liver damage shown in ALT measurements between infected *il22*$^{-/-}$ mice and the infected wild type mice may be too subtle to be observed through histopathology since considerable

liver damage is a common feature for *P. c. chabaudi AS* infection. This strongly suggests that liver damage may not be the main cause of the loss of survival in infected *il22*$^{-/-}$ mice.

In studies of the lung during *P. c. chabaudi AS* infection, Brugat *et al.* (32) found an increased infiltration of IFN-γ producing lymphocytes as well as increased IgM in the broncheoalveolar lavage fluid, an indicator of epithelial damage leading to decreased integrity. In the experiments detailed here, IgM was found to increase in both infected *il22*$^{-/-}$ mice and, as expected, the wild type mice during infection. A trend was found for higher IgM in the infected *il22*$^{-/-}$ mice, with a higher median level found here. This suggests that the loss of IL-22 may slightly compromise the epithelial integrity within the lung further. However, experiments to analyse the total protein level in the BAL found no significant differences due to the infection. Although, infected *il22*$^{-/-}$ mice did again show a trend for higher protein levels in the BAL than the wild type. As with the liver, no obvious differences were observed in the histopathology of *P. c. chabaudi AS* infection due to the loss of IL-22, increased alveolar septae cellularity was observed to a similar degree. This may suggest in both these organs changes due to the loss of IL-22 in this model of malaria infection may only be very subtle. *Il22* mRNA analysis as well as studies in the fate reporter mouse showed decreases in IL-22 expression at the peak of *P. c. chabaudi* infection that were mediated by changes in the γδ+ T cell population.

In examination of the histopathology within the intestine, no changes were observed due to the loss of IL-22 or *P. c. chabaudi AS* infection. It was hypothesised that this cytokine may protect the epithelia of this organ and thus its loss may result in damage to the epithelia. Sections of this organ were examined with H&E as well as AB-PAS staining which showed little differences or change in goblet cell numbers. In

experiments by Chau *et al.* (43), intestine epithelial integrity during *Plasmodium yoellii* infection was examined by immunofluorescence staining of tight junction proteins. Using this technique could signal changes in malaria infection that could be influenced by the lack of IL-22.

Clearly, an important role for IL-22 in the intestine is its influence on the microbiota through regulating levels of antimicrobial peptides (202-204) and glycosylation of the epithelia (164, 165). IL-22 has a role in maintaining the integrity of the intestine epithelia preventing dissemination of bacteria. Work by Aujla *et al.* (174) found this cytokine was able to induce the antimicrobial peptides β-defensin 2 and β-defensin 3 to protect against the pathogen *Klebsiella pneumoniae* in the lung. This cytokine also induces RegIIIβ and RegIIIγ providing an innate immune response against *Citrobacter rodentium* infection in the intestine (205). In further work, it would be of interest to investigate whether the loss of IL-22 an important inducer of these antimicrobial peptides in the steady state, could result in decreased epithelial integrity and how this could explain increased pathology observed in *P. c. chabaudi* infected *il22*$^{-/-}$ mice. Antimicrobial peptides are unlikely to directly inhibit growth of malaria parasites, however, decreased production of antimicrobial peptides in the loss of IL-22 in *P. c. chabaudi* infection could result in decreased integrity of the gut epithelial barrier leading to the dissemination of bacteria or their products. Studies by Sonnenberg *et al.* (204) demonstrated the ability of this cytokine in protecting the epithelial integrity of the intestine by showing greater dissemination of intestinal bacteria to the spleen and liver in C57BL/6 *RAG*$^{-/-}$ mice who had their IL-22 producing ILCs depleted using an antibody against CD90.2.

Sequestration of *P. c. chabaudi* has been shown to occur in the intestine, although lower than at other sites such as the liver and lung. This was done through

imaging organs of mice infected with genetically engineered fluorescent parasites expressing the enzyme luciferase (32). This may well be associated with changes in immune and inflammatory cell populations, which could threaten the integrity of the epithelia.

Freudenberg *et al.* (40) showed that the lethal dose of LPS was lower in mice infected with *P. c. chabaudi*, strongly suggesting that malaria infection may prime responses. This has been recently supported by evidence showing that *P. c. chabaudi AS* infection can lead to excessive amounts of the proinflammatory cytokine, IL-1β being secreted leading to inflammatory damage (41).

This hypothesis of bacterial or endotoxin dissemination could explain observations made in measurements of liver enzymes in the plasma. Work by Balmer *et al.* (206) indicated that the liver can act as a firewall to clear commensal bacteria which disseminate from the intestine into the bloodstream. If increased dissemination of bacteria occurs in malaria infection, increased levels of LPS could induce further liver injury and further increases in ALT and AST. However if this was the case, it could be argued that the ensuing increased inflammatory response in *il22*$^{-/-}$ mice to LPS in *P. c. chabaudi* infection would be more clearly observed as a difference from the wild type e.g. induction of pulmonary oedema, a feature that increased endotoxaemia has been shown to induce, was not observed in these studies such as in histopathology of the lung (207).

Increased endotoxaemia as well as bacterial coinfections are known to be a feature of malaria infection (36, 37), it would be interesting to investigate further whether IL-22 may act to mitigate this. This phenomenon may, in part, explain variability observed in some results between experiments such as mortality. Mice in these experiments were housed in a specific pathogen free unit, which would surely

influence the gut microbiota, and possible bacterial or LPS translocation may vary due to this. Future investigations would need to take this into account; this could be done by analysing differences that could occur in the microbiota in *P. c. chabaudi* as well as on the loss of IL-22. Behnsen *et al.* have conducted studies such as this (208), where 16s rRNA sequencing was used to analyse the representation of different populations of bacteria within the microbiota. By using this technique, a key experiment could be done to answer whether changes in the microbiota within the intestine due to the loss of IL-22 make C57BL/6 more vulnerable to *P. chabaudi AS* infection. Experiments using "cohousing" of mice could also be performed to answer this question. This has been done in studies such as those by Zenewicz *et al.* (163) where they housed wild type mice with *il22*$^{-/-}$ mice in the same cages for 4-6 weeks when the altered microbiota of the *il22*$^{-/-}$ mice was transmitted to the wild type mice. After this, they induced colitis using dextran sodium sulfate (DSS) and found that wild type mice with microbiota similar to *il22*$^{-/-}$ mice exhibited the exacerbated pathology in DSS induced colitis usually associated with *il22*-/- mice. By cohousing *il22*$^{-/-}$ and wild type mice and then infecting them with *P. c. chabaudi AS* it would be possible to ask the question of whether the difference in microbiota in *il22*$^{-/-}$ mice could play a role in enhanced pathology when these mice are infected.

The mouse model of malaria infection, *Plasmodium chabaudi*, has important uses due to the similarities it shares with the most dangerous malaria parasite infecting humans, *Plasmodium falciparum* (54). This model of malaria infection was useful here in studying the role of IL-22 as it normally causes a low level of pathology in wild type mice. This allowed the possibility of clear differences in pathology of mice lacking IL-22 to be observed. Another species of malaria parasite that could be used to study pathology in malaria infection due to the lack of IL-22 through a mouse

model include *Plasmodium berghei.* Deroost et al have used the parasite, Plasmodium berghei NK65 to study lung pathology (191) and found a significantly higher level of protein in the BAL fluid in infection than with *P. c. chabaudi AS.* The protective role of IL-22 may be more easily observed in a malaria infection where there is a greater scale of pathology than *P. chabaudi.*

Koch *et al.* (114) identified two single nucleotide polymorphisms in the *IL22* gene that showed a weak association with resistance and susceptibility to severe malaria in West African children. In these studies, severe malaria was defined by the presence of cerebral malaria or severe anaemia. However, experiments detailed within this thesis did not find signs of these complications in *P. c. chabaudi AS* infected *il22*$^{-/-}$ mice. In further work, it would be of interest to investigate whether polymorphisms of the *il22* gene are associated with the increased endotoxaemia (36) as well as bacteraemia (37, 210) observed in malaria infected humans. Levels of LPS in the plasma, a marker of dissemination could be studied using the limulus amoebocyte assay as has been done in studies of microbial translocation from the intestine in SIV infected macaques (211). In these studies, the histopathology associated with microbial translocation was not examined.

If IL-22 does indeed have a protective role in malaria infection, the therapeutic opportunities of this cytokine, as reviewed by Sabat *et al.* (121) could be harnessed in the future for use in the field to treat patients found to be at greater risk of suffering from severe malaria. It is possible that individuals with mutations in this cytokine could be suffering from increased severity in malaria due to reduced integrity of the epithelia and lung. This could allow dissemination of bacteria or their products, therefore exacerbating an already increased inflammatory state.

6. Conclusions

This aim of this thesis was to investigate the production of interleukin-22 and the consequences of its lack, in the mouse model of malaria, *Plasmodium chabaudi*. This was examined by analysing the expression of this cytokine's mRNA as well as its cellular production during this infection. The pathological consequences that ensued when this cytokine was absent in *Plasmodium chabaudi* were also examined.

The spleen was concluded to not be an important site of IL-22 production in this infection. In the liver and lung, there were transient increases in *il22* mRNA. In the lung this was due to changes in the population of γδ T cells. The intestine, where IL-22 is expressed in the largest amounts, showed significant decreases in this cytokine's mRNA in the small intestine and no significant changes in the large intestine. A significant increase was observed in eYFP+ cell numbers in the small intestine lamina propria but no changes were observed in the large intestine. Further work would need to investigate the proportion of eYFP+ cells that switch to producing IFN-γ in *P. c. chabaudi* infection compared to those that remain IL-22 producers.

The stark mortality observed in *P. c. chabaudi AS* and *P. c. chabaudi CB* infected *il22*$^{-/-}$ mice strongly suggested that this cytokine plays an important role in malaria infection. This was associated in increased liver enzymes and lactate in the plasma. A trend for increased IgM in the BAL fluid of infected *il22*$^{-/-}$ mice strongly implied an enhanced reduction of epithelial integrity in *P. c. chabaudi* infection on the loss of IL-22. Histopathological changes were observed in infection due to the loss of IL-22 in the liver, lungs or the ileum. Later experiments encountered issues with reproducibility, showing that the role IL-22 might be subtler than previously envisaged.

To conclude the lungs and the intestine may be vulnerable to damage from an increased inflammatory state due to *P. c. chabaudi* infection. IL-22 may well have an important role in protecting these epithelia and preventing dissemination of bacterial products, further work will need to study this subtle action of this cytokine by analysing the epithelial integrity in the intestine such as by analysing antimicrobial peptide production and levels of LPS in the plasma.

7. References

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