CHAPTER 1

INTRODUCTION

1. Introduction

1.1 History of *Toxoplasma gondii*

Toxoplasma gondii was first discovered in a North African rodent (*Ctenodactylus gondi*) in 1908 by the French Pastorian, Charles Nicolle and his colleague Louis Manceaux whilst working in North Africa, and was later isolated in a rabbit by Splendore whilst working in Brazil (Dubey, Miller et al. 1970). The species designation originated from the North African rodent, *Ctenodactylus gondi*, from which the parasite was first isolated. The genus name refers to the crescent shape of the organism and is derived from the Greek term *toxon*, which means bow (Black and Boothroyd 2000).

Taxonomy

T. gondii is an obligate intracellular coccidian protozoan parasite belonging to the phylum Apicomplexa. This phylum consists of parasites that have a characteristically polarized cell structure and a complex arrangement of the cytoskeleton and organelles at their apical end. Other member pathogens include *Plasmodium*, which causes malaria, and *Cryptosporidium*.

1.2 Life cycle

T. gondii is a ubiquitous parasite that is able to replicate within almost any nucleated mammalian or avian cell and hence can infect humans, birds and many different species of mammals (Dubey, Miller et al. 1970). The parasite exists in three forms: tachyzoites, bradyzoites (tissue cysts) and oocysts. Tachyzoites are the free forms of the parasite

which are actively and rapidly dividing in the cells of acutely infected tissues. Tachyzoites differentiate into the much slower dividing bradyzoites 7-10 days post-infection and form tissue cysts which reside within the brain and muscle tissue of chronically infected hosts. Oocysts refer to the form of the parasites which are excreted in the faeces of cats infected with *T. gondii*.

Infections are usually transmitted by the ingestion of oocysts in the faeces of cats or eating undercooked meat, food or water that is contaminated with infective oocysts. Other possible modes of infection include transplantation of infected organs and accidental inoculation. The parasite is globally distributed; it is estimated that 5×10^8 people worldwide are infected with *T. gondii*, with high prevalence observed in Western Europe and in tropical countries, possibly due to high consumption of raw or undercooked meat (Evering and Weiss 2006; Subauste 2006).

The life cycle of *T. gondii* is divided between feline and non-feline infections, which are correlated with sexual and asexual replication respectively. Although the parasite infects a wide variety of mammalian species, its sexual stages are highly specific and occur exclusively in the feline species (the definitive hosts). The sexual cycle is initiated when a member of the feline species ingests the infective form of the parasite, oocysts or bradyzoites cysts (Fig.1). The sexual stages of the parasite occur within the gut epithelial cells of the cat where the ingested parasites undergo differentiation into male and female gametes. The products of the gamete fusion, the oocysts, are subsequently shed in the cat faeces (Dubey, Miller et al. 1970; Denkers and Gazzinelli 1998). The oocysts undergo

maturation once they are excreted from the cat intestines and are activated to become highly infectious sporozoites. These oocysts containing infective sporozoites can survive for extended periods (months and even years) in the environment. The asexual cycle of *T. gondii* occurs when any mammalian host (the intermediate host) ingests these highly infectious oocysts/sporozoites deposited in the cat faeces.

Following infection of the intermediate host, the mature oocysts differentiate back into tachyzoites, which proliferate rapidly within intracellular parasitophorous vacuoles. Following the rapid proliferation and accumulation of tachyzoites (usually 64 to 128 parasites per cell), the host cells eventually rupture and release the parasites into the extracellular milieu. The infection is subsequently spread throughout the host tissues when the free tachyzoites go on to infect neighbouring cells and almost any nucleated cell they encounter. This stage represents the rapidly replicating form of the parasite found during the acute phase of toxoplasmosis. Following the acute phase of the infection, the immune response is activated and tachyzoites differentiate into the slowly multiplying, essentially dormant form of the parasite, bradyzoites, which persist within tissue cysts. The tissue cysts are enclosed within the cyst wall, which is composed mainly of host tissue-derived products and therefore enables the bradyzoites to evade the host's immune response. Bradyzoites are infective to the definitive host (feline species) and the intermediate host (mammalian host) and are largely responsible for the transmission of the parasite to different species of mammalian and avian hosts. The tissue cysts are found predominantly in the central nervous system and in the muscle tissue where they are thought to persist for the life of the host.

This defines the chronic phase of the asexual cycle of the parasite (Denkers and Gazzinelli 1998). Upon ingestion of tissue cysts by consuming contaminated tissue from chronically infected subjects, the bradyzoites will be released from the tissue cysts as they pass through the digestive tract and infect the intestinal epithelial cells of the subsequent host where they will transform back to the rapidly multiplying tachyzoite form to complete the asexual stage of the life cycle. However, if the ingesting animal is a cat, the bradyzoites can differentiate into the sexual stages and can go on to complete the full life cycle.

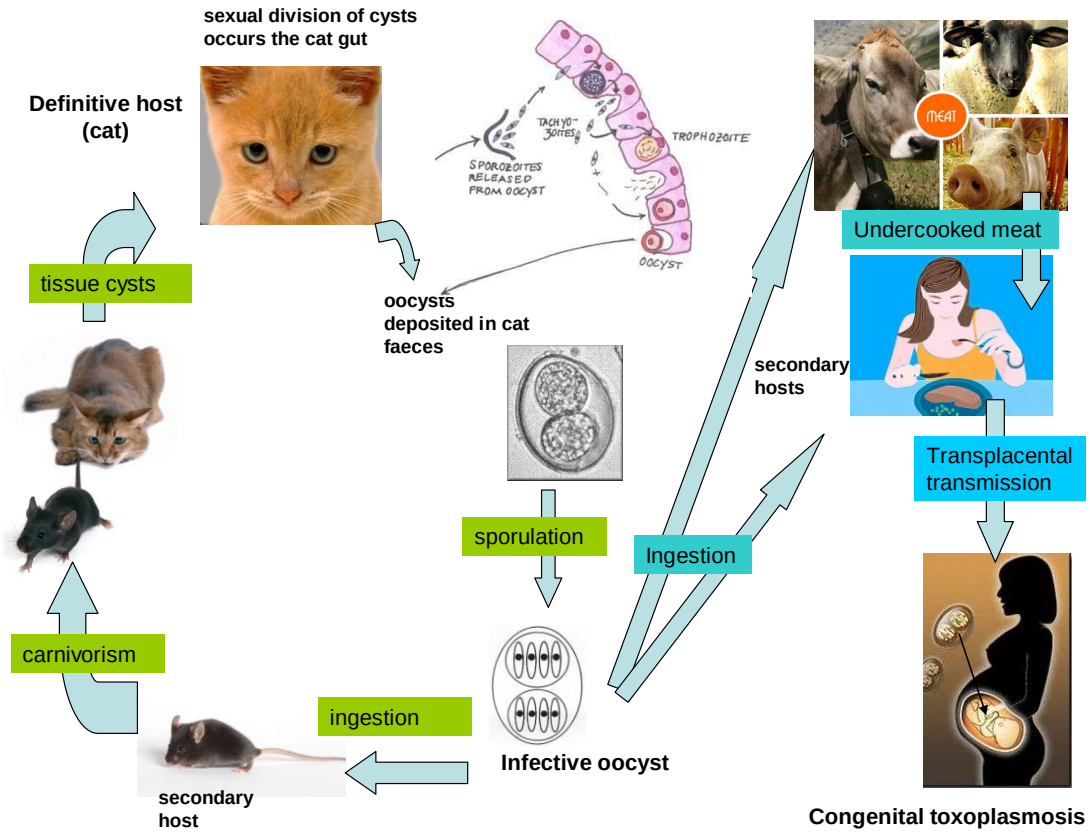


Fig. 1 *Toxoplasma gondii* infects all warm blooded vertebrates including humans and domestic animals. The feline species are the definitive hosts and they become infected by ingesting meat containing the cyst stage of the parasite. The sexual division of the cysts occurs within the enteroepithelial cells in the gut of the cat resulting in formation of oocysts which are then deposited into the environment by defaecation. The deposited oocysts take 2-3 days to mature into sporozoites which are infective to secondary hosts (eg. humans, cattle, mice). The parasite is commonly transmitted to humans by ingestion of under-cooked meat that is contaminated with oocysts. Once ingested, the oocysts rapidly differentiate into tachyzoites. In pregnant individuals, the infection can be transmitted to foetuses via the placenta, causing congenital toxoplasmosis.

1.3 Pathogenesis

Toxoplasmosis in immuno-competent hosts is classified as acquired (from ingestion of contaminated meat, food or water), congenital, or ocular. Most cases of toxoplasmosis are generally self limiting in immuno-competent hosts. The acute phase of infection is benign and it evolves to the asymptomatic chronic phase within a few weeks of infection (Denkers and Gazzinelli 1998).The most severe symptoms of the disease are generally observed in the congenitally acquired form of the infection, and in immuno-compromised patients (Denkers and Gazzinelli 1998; Sciammarella and Gaeta 2002). However acquired toxoplasmosis can range from subclinical lymphadenopathy to fatal disease in immunocompromised hosts. The most commonly observed clinical manifestations of the disease are however lymphadenopathy and fever.

Congenital transmission of *Toxoplasma* occurs when a pregnant female who is undergoing the acute phase of primary infection transmits the parasites to the foetus via the placenta (Fig.1). If the pregnant mother had acquired the infection prior to the pregnancy, very minimal risks of foetal transmission exist. The severity of the disease in the infant depends on the trimester during which the infection was contracted, with infection during the first trimester being the most severe. Pathological consequences can vary from spontaneous abortion of the pregnancy, stillbirth or poor foetal development resulting in hydrocephalus and neurological disorders such as blindness and mental disabilities (Black and Boothroyd 2000; Subauste 2006).

Ocular toxoplasmosis is another disease manifestation which occurs from activation of

cysts deposited inside or near the retina of the eye. Most cases of ocular toxoplasmosis are attributable to congenital infection; however it also occurs in normally acquired infection. In this form of toxoplasmosis, disease symptoms manifest late during chronic infection. The characteristic clinical presentation of ocular toxoplasmosis is the formation of focal necrotizing lesions in the retina, which have been reported to be caused by tissue damage due to parasite proliferation (Denkers and Gazzinelli 1998; Sciammarella and Gaeta 2002).

The most severe form of the toxoplasmosis occurs in immuno-compromised hosts. This includes patients with Acquired Immuno Deficiency Syndrome (AIDS) and patients receiving chemotherapy. In fact, it has been reported that forty percent (40%) of AIDS patients have clinical toxoplasmosis (Sciammarella and Gaeta 2002). The most common clinical manifestation of the disease in immuno-compromised hosts is toxoplasmic encephalitis, which is usually caused by multiple intracerebral focal brain lesions which develop over the course of days to weeks post-infection. The disease symptoms range from non-specific fever, to more focal neurological disorders such as headaches, speech and motor impairment, psychiatric abnormalities and seizures (Sciammarella and Gaeta 2002; Subauste 2006). Pulmonary toxoplasmosis is the second most common disease manifestation. Patients exhibit severe respiratory symptoms, which are difficult to distinguish from tuberculosis or pneumonia caused by infection with *Pneumocytis jiroveci* or *P. carinii* (Black and Boothroyd 2000; Sciammarella and Gaeta 2002). In immunocompromised individuals, toxoplasmic encephalitis is consistently fatal if left untreated; hence it is a serious concern in patients who suffer from AIDS.

1.4 Treatment and prevention strategies

The effective treatment for toxoplasmosis involves the combination of a sulphonamide antibiotic, usually sulphadiazine or sulphamerazine with pyrimethamine, which is also an anti-malarial drug. These drugs act synergistically by blocking the metabolic pathway involving p-aminobenzoic acid and the folic-folinic acid cycle of the tachyzoite stage parasite. This treatment regimen therefore inhibits tachyzoite replication during the acute phase of infection, but is not effective against the bradyzoites within tissue cysts (Dalgıç 2008).

Treatment of pregnant women involves administration of the antibiotic Spiramycin, which reduces the chance of congenital infection by 60%. Although this drug is routinely used in France, it is still considered an experimental drug in the United States and is not yet approved by the FDA (Chang and Pechere 1988; Russo and Galanti 1990).

Patients living with HIV/AIDS are also treated with a combination of sulphadiazine and pyrimethamine together with folic acid. An alternative treatment regimen is pyrimethanin with clindamycin. Usually these medications need to be taken for life in immunocompromised patients, and therefore may have severe side effects. There is no killed vaccine available yet to reduce or prevent infection in both humans and animals. However, a live vaccine using a non-persistant *T. gondii* strain is available in Europe and New Zealand to reduce abortion in sheep.

Preventive measures to avoid acquiring *T. gondii* infection include wearing gloves when

handling soil, washing hands thoroughly after handling soil that may contain cat faeces. Pregnant women are especially advised to wear gloves when handling the cat litter box, or avoid such work altogether. It is also advised to avoid eating raw and uncooked meat, or drinking unpasteurized milk. Meat and dairy products should be properly cooked and vegetables and fruits washed before consumption.

1.5 Immunology

1.5.1 Immunity to *T. gondii* infection

Infection with *T. gondii* elicits a strong and long lasting protective immunity (Alexander and Hunter 1998; Denkers and Gazzinelli 1998). Following infection of the intestinal epithelial cells (enterocytes) of the intermediate host, the infective bradyzoites transform into tachyzoites which rapidly proliferate within intracellular parasitophorous vacuoles (PV). Host cell invasion by the tachyzoites is an active, parasite-driven event which allows inclusion of the host cell plasma membrane but selectively excludes host cell membrane proteins. This results in the PV resisting phagosome-lysosome fusion, and subsequently not being acidified, which leads to the parasite surviving within the PV and escaping the host's immune response (Lang, Gross et al. 2007). When the host cells become full of tachyzoites they rupture and release the free tachyzoites which subsequently infect neighbouring cells, hence disseminating the infection. In immuno-competent hosts, the immune response will be triggered to effectively limit and control the infection without causing pathology, hence the asymptomatic nature of the disease. The enterocytes will release a milieu of pro-inflammatory cytokines and chemokines that attract polymorphonuclear leukocytes (PMNs), macrophages and dendritic cells among other immune cells (Fig.2). The tachyzoite stage is usually cleared from host tissues by the immune response, but the slow-growing bradyzoites persist to cause chronic infection as they are enclosed within a cyst wall which is able to evade the host immune response because it is mainly composed of host-tissue derived products (Denkers and Gazzinelli 1998).

Infection with *T. gondii* elicits both a humoral and cell mediated immune response. Experiments using B-cell deficient mice have showed that B-cells play an important role through the production of specific antibodies and prevent TE in mice (Suzuki 2002). The protective humoral response includes production of *T. gondii* specific antibodies such as IgA, IgM, IgG and IgE (Darcy, Deslee et al. 1988; Gross, Roos et al. 1992). The antibodies bind to the parasite surface and activate the complement pathway, which results in lysis of the parasite or alternatively the parasites are engulfed by macrophages via Fc receptors and subsequently killed by macrophage effector mechanisms (Fuhrman and Joiner 1989). The host's humoral response, however, does not play a significant role in eliminating the parasite as *T. gondii* resides within intracellular vacuoles; hence the antibodies have limited access to the parasites. *T. gondii* parasites' intracellular lifestyle therefore protects them from attack by antibodies and complement (Lang, Gross et al. 2007).

The strong, persistent T-cell mediated response appears significantly more important for the development of protective immunity during infection. The cell-mediated immune response controls parasite multiplication and actively induces transformation of the more virulent tachyzoites to the dormant less harmful bradyzoite form, and maintains the parasite in this form (Gazzinelli, Amichay et al. 1996).

1.5.2 Cell mediated response

Resistance to *T. gondii* mediated by T-cell independent mechanisms involves the activation of anti-microbial activities of multiple cell types (Hunter, Abrams et al. 1993).

T. gondii tachyzoites initiate activation and IL-12 production by cells of the innate immune system such as macrophages, neutrophils and dendritic cells. Neutrophils (Bliss, Zhang et al. 1999) and macrophages are the first line of defense against the parasite during the initial phases of infection (Sher, Oswald et al. 1993). IL-12, which is produced by macrophages, neutrophils and dendritic cells, is important for inducing the differentiation of naïve Th0 cells to IFN-γ producing Th1 cells (Fig. 3). Macrophages become activated by $CD8^+$ T-cell-produced IFN-γ to kill intracellular tachyzoites following infection. Parasite proliferation is inhibited by various IFN-γ mediated pathways including mechanisms involving NO production, tryptophan catabolism (Shrestha, Tomita et al. 2006) (Fig. 3) and limiting available iron for the parasite (Dimier and Bout 1998). Macrophages and other professional phagocytes are synergistically activated by IFN-γ and TNF-α, which leads to induction of the iNOS gene expression and subsequent production of anti-parasitic reactive nitrogen intermediates (RNI) and nitric oxide (NO) which in turn inhibit parasite growth (Langermans, Van der Hulst et al. 1992). However, experiments using murine models have shown that TNF-α and iNOS only play a partial role in parasite control during acute phase of infection (Suzuki 2002). NO production in response to synergistic macrophage activation by TNF-α and IFN-γ is also partially inhibited by the parasite (Schluter, Deckert-Schluter et al. 1999). Therefore additional pathways need to be in place to control the infection.

Dendritic cells have been demonstrated to be the primary source of the initial IL-12 that

activates NK cells to produce the first burst of IFN-γ as they are able to produce large amounts of IL-12 in the absence of IFN-γ following *T. gondii* exposure (Reis e Sousa, Hieny et al. 1997). Dendritic cells play a very essential role as the interface between the innate response and the development of adaptive immunity and resistance against *T. gondii* infection (Makala, Reyes et al. 2003) (Fig. 2). They are primarily involved as antigen presenting cells (APC) for development of parasite specific T-cell responses such as $CD4^+$ T cell activation (Kasper, Courret et al. 2004), and most importantly, are the major source of IL-12 (Fischer, Bonifas et al. 2000; Liu, Fan et al. 2006). Activated dendritic cells express elevated levels of co-stimulatory molecules such as CD40, CD80 and CD86 (Grewal and Flavell 1998). CD40 ligates with CD40L on T-cell surfaces and this interaction promotes IL-12 production by dendritic cells and is optimal for clonal expansion and Th1 differentiation of T-cells (Macatonia, Hosken et al. 1995; Heufler, Koch et al. 1996). CD80 and CD86 (which make up the B7 complex) also ligate with CD28 for efficient proliferation of T-cells and production of IFN-γ by T-cells. Dendritic cells therefore play an important role in the initiation of adaptive responses by facilitating the interaction with T-cells via CD40/C40L ligation and CD28-B7 interactions, thereby promoting IFN-γ production by T-cells which in turn activates parasite killing mechanisms.

In addition, neutrophils are also essential for development of immunity against *T. gondii* during the initial phase of infection. Studies using neutrophil deficient mice have shown that in absence of neutrophils, mice infected with *T. gondii* had reduced IL-12, TNF-α,

and IFN-γ responses, and displayed lethal systemic pathology which was associated with increased parasite proliferation (Bliss, Gavrilescu et al. 2001). Neutrophils are an important source of IL-12 and have been shown to be important in shaping the protective type-1 response during infections with various parasites (Romani, Mencacci et al. 1997; Romani, Mencacci et al. 1997; Tateda, Moore et al. 2001).

The macrophage populations in the brain, microglial cells and astrocytes are some of the cell populations that mediate protective responses in the central nervous system during *T. gondii* infection. Following infection, microglia are activated and produce TNF-α. In humans microglial cell activation involves TNF-α and IL-6 (Chao, Hu et al. 1993). Murine studies reveal IFN-γ mediated activation, and NO mediates the inhibitory effect of activated microglia on intracellular replication of tachyzoites (Chao, Hu et al. 1993). $CD8^+$ T-cells are important for the regulation of cytokine production by microglial cells during *T. gondii* infection. Granulocyte-macrophage colony stimulating factor (GM-CSF) and transforming growth factor (TGF) -β are other cytokines that are involved in the effector functions of microglial cells (Suzuki 2002). These cells are important during both acute and chronic murine toxoplasmosis. Astrocytes also appear to inhibit tachyzoite proliferation in the brains of infected animals (Peterson, Gekker et al. 1995). Following infection, astrocytes are activated to produce IL-1 and IL-6 which induce cellular infiltration into the brain (Fischer, Nitzgen et al. 1997). Inhibition of parasite growth in human brains involves TNF-α and IFN-γ and is mediated by NO. Nevertheless the inhibitory effects of murine astrocytes are independent of NO

(Peterson, Gekker et al. 1995; Halonen, Chiu et al. 1998).

The basis of macrophages, neutrophils and dendritic cells representing the first line of defense in the acute phase of *T. gondii* infection is primarily because these cell types express pattern recognition receptors which are involved in immune recognition of diverse parasitic antigens and rapid activation of the host defense (Iwasaki and Medzhitov 2004). *T. gondii* infection triggers pathways leading to IL-12 production and involves signaling through Toll-like receptors (TLR's) expressed on macrophages, dendritic cells and neutrophils. Signaling through these receptors is centrally mediated through the common adaptor molecule Myeloid Differentiation factor 88 (MyD88). MyD88 deficient mice have been shown to be highly susceptible to *T. gondii* infection and this was associated with diminished IL-12 responses (Scanga, Aliberti et al. 2002). MyD88-dependent signaling pathways in *T. gondii* are initiated primarily through TLR11. TLR11 plays a major role in IL-12 dependent control of *T. gondii* infection as studies using TLR11 deficient mice showed that mice lacking TLR11 had dramatically decreased levels of IL-12 and also developed more brain cysts during the chronic phase of infection compared with their WT counterparts (Denkers, Butcher et al. 2004; Yarovinsky, Zhang et al. 2005). TLR2 and TLR4 have also been shown to be involved in the recognition process of *T. gondii* antigens (Tomavo, Schwarz et al. 1989), but are not involved in initiating inflammatory responses as TLR2 deficient and TLR4 deficient mice showed normal IL-12 production *in vitro* and *in vivo* (Scanga, Aliberti et al. 2002). In other studies however, when a higher inoculum size was used to challenge the mice, TLR2 was shown to play a protective role where TLR2 deficient mice were shown to be

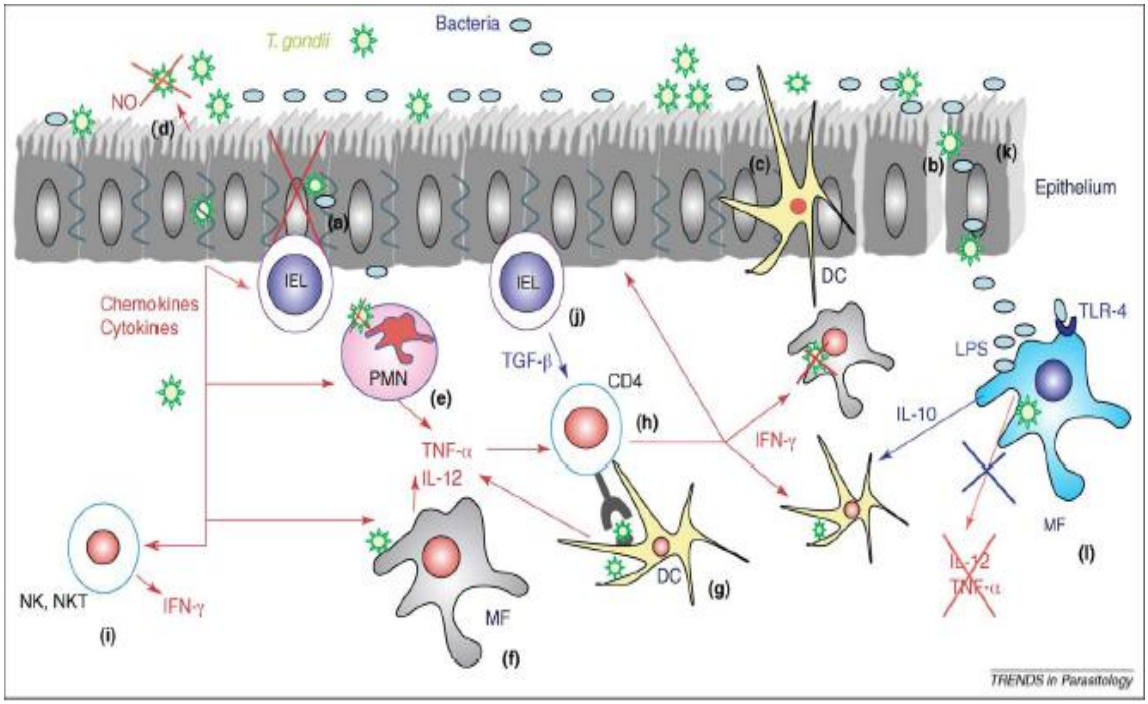
susceptible to infection and this was associated with impaired production of IL-12 and NO (Mun, Aosai et al. 2003). An additional pathway that has been implicated in IL-12 production involves the parasite antigens binding to the host cell chemokine receptor, CCR5. CCR5 deficient mice have been shown to be susceptible to *T. gondii* infection (Aliberti, Reis e Sousa et al. 2000). In summary, *T. gondii* infection activates pathways leading to IL-12 release by neutrophils, macrophages and dendritic cells which in turn induce a strong polarized type-1 response and activates anti-toxoplasmic effector mechanisms (Fig. 3).

In addition to the strong non-specific T-cell-independent immune response to infection, *T. gondii* infection elicits a potent parasite-specific T-cell-mediated type-1 immunity (Denkers and Gazzinelli 1998). Activated T-cells are crucial for the control of acute and chronic toxoplasmosis (Suzuki and Remington 1988; Gazzinelli, Hakim et al. 1991; Gazzinelli, Xu et al. 1992); athymic nude mice which lack functional T-cells were shown to be extremely susceptible to both virulent and avirulent *T. gondii* strains (Lindberg and Frenkel 1977; Gazzinelli, Xu et al. 1992). T-cells are a critical source of IFN-γ during chronic infection (Denkers 2003). The specific T-cell response is crucial for the development of parasite-specific T-cells, whilst the non-specific response limits co-infection by non-related pathogens (Fig. 2) (Hibbs, Lambert et al. 1971).

Natural killer (NK) cells are involved in limiting parasite growth during the early stages of *T. gondii* infection. They have been shown to play a central role in producing the

initial IFN-γ which activates the key effector mechanisms and initiates protective T-cell response that restrict intracellular *T. gondii* growth during acute infection prior to generation of adaptive immunity (Denkers 2003). NK cells do not, however, play an important role in prevention of toxoplasmic encephalitis during chronic infection as it was shown that NK cell deficient mice developed resistance towards TE despite the absence of NK cells (Kang and Suzuki 2001). While NK cells provide the initial IFN-γ required for optimum IL-12 production by dendritic cells during the early stages of infection, these NK cells require IL-12 to initiate the IFN-γ production. IL-12 plays the crucial role of inducing IFN-γ production by NK cells (Suzuki 2002).

$CD4^+$ T-cells are crucial for regulating the immune response by helping $CD8^+$ T-cells to produce IL-2, whilst $CD8^+$ T-cells play a major role as effector lymphocytes that result in microbicidal effects against *T. gondii*. The cytotoxic $CD8^+$ T-cells are capable of lysing *T. gondii* infected cells, thereby limiting parasite proliferation (Hakim, Gazzinelli et al. 1991; Subauste, Dawson et al. 1992). The protective activity of both $CD4^+$ and $CD8^+$ T cells is predominantly regulated by IFN-γ as activated T-cells have been shown to be a major source of IFN-γ during the acute stage of infection (Suzuki, Orellana et al. 1988). $CD4^+$ and $CD8^+$ T-cells act synergistically through their production of IFN-γ to prevent toxoplasmic encephalitis (TE) and the $CD8^+$ T cells regulate the number of *T. gondii* cysts in the brains of mice (Suzuki 2002). The early burst of IFN-γ from NK cells is critical in shaping the adaptive immune response following *T. gondii* infection.



(Buzoni-Gatel and Werts 2006)

Fig. 2 *Toxoplasma* invades the gut epithelium of the intermediate host and subsequently infects the gastro-intestinal epithelial cells, enterocytes (a), or directly invades the epithelium through the tight junctions that bind the enterocytes (b). The tachyzoites can alternatively be phagocytosed by dendritic cells upon infection of the gut epithelial cells (c). Infection of enterocytes with the parasites results in morphological and physiological changes within the cells which lead to secretion of cytotoxic molecules such as nitric oxide which subsequently destroy the parasites (d). Enterocytes also secrete chemotactic cytokines and chemokines that recruit polymorphonuclear leukocytes (PMNs) (e), macrophages (Mø) (f), dendritic cells (DCs) (g). These cells from the innate immune system are then activated to release cytokines such as IL-12 which trigger the adaptive CD4 immune response (h). Activated T-cells, NK and NKT cells (i) secrete IFN-γ which activates Mø, DC's and enterocytes for parasite clearance. IEL (j) are cytotoxic for infected enterocytes and produce TGF-β which inhibits IFN-γ production.

A persistent and unregulated production of pro-inflammatory cytokines (IFN-γ, IL-12, TNF-α) triggered by the parasite can lead to destruction of the host tissue. Immuno-pathology during *Toxoplasma* infection is commonly characterized by destruction of the liver and the small intestine, but depends on the mode of infection. There are different homeostatic mechanisms that are employed by the host to regulate the immune response. $CD4^+$ and $CD8^+$ T-cells are both involved in maintaining homeostasis within the primary site of infection, the gut epithelium (Kasper, Courret et al. 2004). Interstinal intraepithelial cells (IELs) are a distinct population of immune cells that also mediate anti-inflammatory mechanisms to limit severe immuno-pathology (Fig.2) (Kasper, Courret et al. 2004). It is evident that although IFN-γ is central for resistance to *T. gondii*, excessive levels of this cytokine are lethal and an anti-inflammatory response is necessary to counter the effects of the vigorous type-1 cell mediated response.

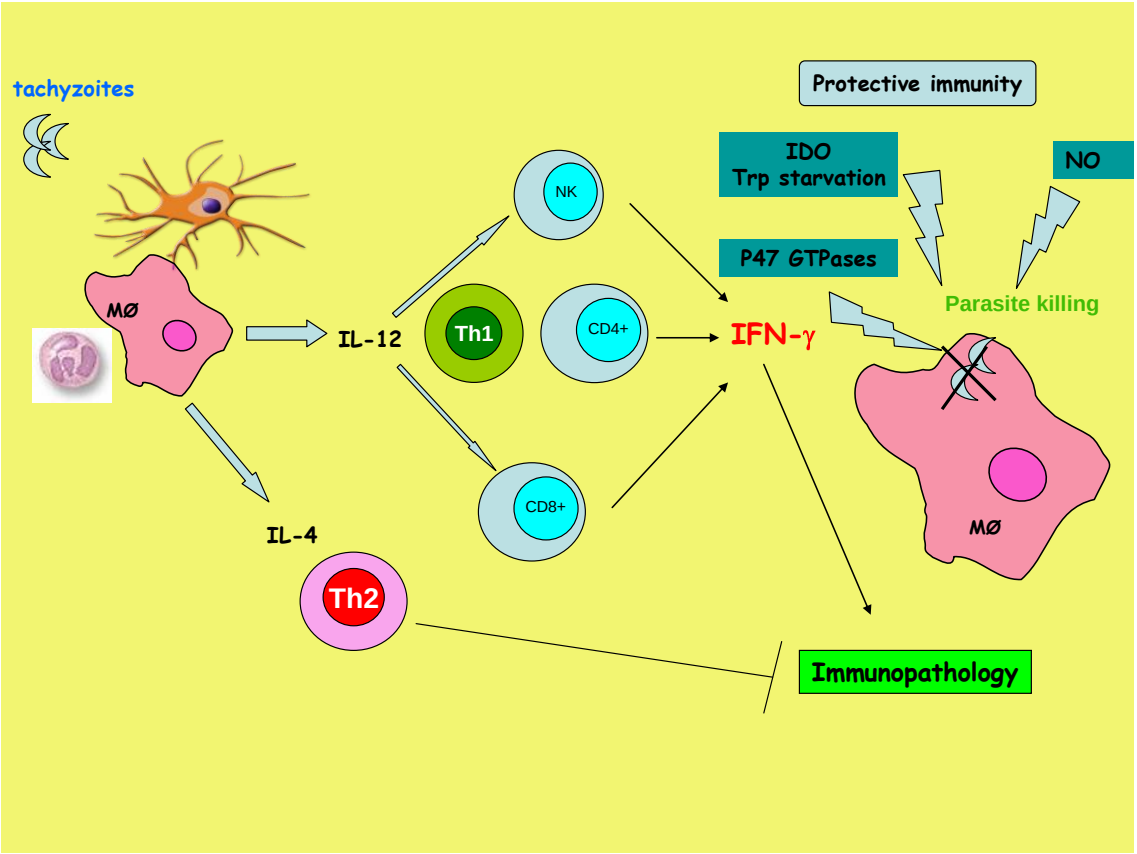


Fig. 3 Infection with *T. gondii* tachyzoites triggers a strong type-1 response by host innate immune cells. Dendritic cells are the major antigen presenting cells and the main source of IL-12 following exposure to *T. gondii* antigens. Macrophages and neutrophils also engulf the parasites and are activated to release IL-12. IL-12 produced by these innate immune cells induces the early production of IFN-γ from NK cells during the early phase of infection, and later $CD4^+$ and $CD8^+$ T-cells following the induction of adaptive responses. IFN-γ activates several macrophage effector mechanisms, which limit parasite proliferation: release of NO and RNI species; induction of IDO which leads to tryptophan depletion and subsequent parasite starvation; and the more recently discovered p47 GTPase proteins whose mechanisms are still unclear. Unregulated type-1 responses can be detrimental to the host and cause immunopathology. Hence the host macrophages can also produce type-2 cytokines such as IL-4

which suppress for example NO production thus limiting immunopathology.

1.5.3 The role of cytokines during *T. gondii* infection

The differentiation and development of naïve $CD4^+$ T-cells into effector cell populations which produce either T-helper-type 1 (Th1) [IFN-γ] or Th2 [IL-4, IL-5,IL-9, IL-10, IL-13 and TGF-β] is largely influenced by the cytokine environment in which the T-cell priming occurs (Murphy and Reiner 2002). As mentioned previously, the T-cell mediated response in toxoplasmosis is characterized and dependent on the production of type-1 cytokines such as IL-2, IL-12 and IFN-γ. Mice deficient in IFN-γ and IL-12 fail to develop a protective immunity and succumb early during acute infection (Scharton-Kersten, Wynn et al. 1996; Yap, Pesin et al. 2000). The presence of these cytokines drives the differentiation of naïve $CD4^+$ and $CD8^+$ T-cells into type-1 effector cells which are characterized by high IFN-γ production and activation of anti-microbial mechanisms.

Type-2 cytokines including IL-4, IL-10 also confer protection by down regulating the potentially detrimental inflammatory response (Suzuki, Yang et al. 1996; Suzuki, Sher et al. 2000). IL-4 is also involved in alternative macrophage activation which will be discussed later.

1.5.3.1 Type-1 cytokines

During early acute infection, macrophages, NK cells and dendritic cells produce a milieu of pro-inflammatory type-1 cytokines. Studies using IFN-γ knock out mice have shown that, IFN-γ is the main cytokine involved in parasite control and shaping the adaptive

response during the acute and chronic phase of murine toxoplasmosis (Suzuki, Orellana et al. 1988). It activates both haematopoietic and non-haemapoietic effector cells to restrict intracellular proliferation (Yap and Sher 1999). IFN-γ triggers the synthesis of chemokines that are essential for T-lymphocyte and other IFN-γ-producing lymphocyte recruitment (Gazzinelli, Amichay et al. 1996). In addition, IFN-γ synergizes with IL-12 in driving the differentiation of Th0 cells toward the Th1 phenotype, which is essentially a pro-inflammatory state. Another important function of IFN-γ is the induction of TNF-α which is produced by many cell populations. It has been widely reported that the anti-toxoplasmic activity of cells of macrophage lineage, including macrophages and microglial cells depends on TNF (Langermans, Van der Hulst et al. 1992; Chao, Hu et al. 1993). TNF-α and IFN-γ synergise and act on macrophages to induce expression of the inducible NO synthase (iNOS) gene which results in NO production. NO is critical in conferring resistance during the chronic phase of infection by preventing parasite replication in the brain (Suzuki 2002).

1.5.3.2 Type-2 cytokines

Although necessary to control parasite multiplication, the robust type-1 response induced by tachyzoites needs to be regulated in order to prevent toxoplasmic encephalitis and damage to the host's tissue. To prevent TE, down-regulatory cytokines, including IL-4, and IL-13, are released during infection and their presence promotes the differentiation of naïve Th0 cells towards a type-2 phenotype. Th2 cells predominantly secrete IL-4, IL-5, IL-6, IL-10 and IL-13. These cells are key mediators of humoral

immunity and they are involved in the activation of mast cells and eosinophils (Munder, Eichmann et al. 1998). IL-10 is an important immuno-regulatory cytokine. It is produced by a range of cells including T-regulatory lymphocytes, Th2 lymphocytes, macrophages, B-cells and mast cells (Gazzinelli, Wysocka et al. 1996). IL-10 deficient mice infected with *T. gondii* have been shown to be more susceptible to the infection than their wildtype counterparts, with severe immunopathology caused by excessive pro-inflammatory cytokine production being the cause of early mortality as opposed to uncontrolled parasite proliferation (Gazzinelli, Wysocka et al. 1996; Suzuki, Sher et al. 2000). IL-10 has been shown to antagonize many of the effects of IFN-γ (Howard, O'Garra et al. 1992), and has also been implicated in the inhibition of IFN-γ production by NK cells mainly through inhibiting IL-12 synthesis by macrophages (Denkers and Gazzinelli 1998). Furthermore, IL-10 downregulates the production of IL-1β and TNF by macrophages (Fiorentino, Zlotnik et al. 1991). IL-10 therefore plays a vital role in disease control by limiting inflammation induced by the robust pro-inflammatory type-1 response.

TGF-β production is also triggered by *T. gondii* infection (Bermudez, Covaro et al. 1993). It is important in down-regulating macrophage effector functions and also inhibits production of IFN-γ by NK cells (Hunter, Bermudez et al. 1995). TGF-β is produced by activated IELs and infected macrophages and is involved in the down-regulation of type-1 responses through altering expression of T-cell co-stimulatory molecules. In particular CD40/CD154 interaction that is critical for regulation of IL-12

production by macrophages (Takeuchi, Alard et al. 1998). Hence TGF-β antagonizes IL-12-induced IFN-γ production by NK cells. On the other hand, TGF-β also down-regulates the expression of TNF receptor on macrophages thereby facilitating intracellular survival of the parasite (Bermudez, Covaro et al. 1993).

IL-5 is another cytokine that is predominantly produced by type-2 lymphocytes and mast cells. It has been shown to play a role in mucosal immunity and it induces recruitment of eosinophils. IL-5 primarily induces a type-2 response through the ability of eosinophils to produce IL-4 during the early stages of infection (Sabin, Kopf et al. 1996). Furthermore, IL-5 synergizes with IL-2, TGF-β and IL-4 to induce antibody production by B-cells (Mack and McLeod 1992). In *T. gondii* infection, IL-5 has been reported to have a protective role during chronic disease but not during the acute phase (Zhang and Denkers 1999). Additional reports show that IL-5 has a counter protective role in the acute stage infection following oral *T. gondii* infection and this is associated with increased intestinal pathology and reduced IL-12 and IFN-γ in the peripheral blood (Nickdel, Roberts et al. 2001).

IL-4 plays a major role in the development of type-2 responses. It is produced by T-cells, mast cells, basophils, eosinophils and alveolar macrophages (Pouliot, Turmel et al. 2005). IL-13 has some overlapping biological activities with IL-4 as these two cytokines signal through a common receptor chain; IL-4Rα. Signaling through this receptor chain involves STAT-6 and GATA-3, and is key for the development of a type-2 phenotype

(Fig. 4). IL-4 and IL-13 are therefore central mediators of type-2 dominant responses and exhibit a multitude of functional activities in various disease models (Chiaramonte, Mentink-Kane et al. 2003). In toxoplasmosis, IL-13 has been shown to have antagonistic effects toward IFN-γ-induced effector functions. It has been shown to inhibit tryptophan degradation, which is IFN-γ mediated mechanism that is involved in controlling intracellular *T. gondii* proliferation in human fibroblasts (Chaves, Ceravolo et al. 2001). Studies have shown that IL-4 is a major factor required for the differentiation of naïve $CD4^+$ T-cells into mature IL-4-secreting Th2 type cells (Seder and Paul 1994) although under certain conditions IL-4 can be generated independently of IL-4Rα signaling (Noben-Trauth, Shultz et al. 1997; Brewer, Conacher et al. 1999). In the absence of IL-4, naïve $CD4^+$ T-cells preferentially produce IFN-γ (Paul 1997). IL-4 down-regulates the type-1 response by inhibiting the priming of macrophages for enhanced superoxide production induced by IFN-γ through inhibition of IFN-γ and IL-12R expression (Swain, Weinberg et al. 1990; Szabo, Dighe et al. 1997; Dimier and Bout 1998). In contrast, some reports have shown that IL-4 knock-out mice have increased expression of IFN-γ although the mechanism was not clear (Denkers and Gazzinelli 1998).

In addition, IL-4 has been shown to inhibit certain macrophage effector functions and it also potentiates the effect of IL-10 on macrophages (Oswald, Gazzinelli et al. 1992; Sher, Gazzinelli et al. 1992). These down-regulatory effects confer IL-4 a protective function during toxoplasmosis as they prevent development of toxoplasmic encephalitis (Suzuki, Yang et al. 1996). In addition to limiting immuno-pathology, IL-4 has been

shown to prevent TE by preventing the formation of cysts and proliferation of tachyzoites in the brain (Roberts, Ferguson et al. 1996). However, other reports from experiments using *in vivo* models suggest that IL-4 also plays an exacerbatory role during disease as IL-4 deficient mice were found to be more resistant to infection as compared to their wild-type counterparts (Villard, Candolfi et al. 1995; Nickdel, Lyons et al. 2004). It is important to note that there are other factors that influence the outcome of IL-4 mediated responses in experiments using animals, such as the genetic background of the experimental animals, their sex, the route of infection and the parasite strain (Alexander, Jebbari et al. 1998). Long-standing effects of IL-4 can also be detrimental to the host because IL-4 inhibits the anti-parasitic pro-inflammatory responses. Therefore IL-4 may have a protective role in maintaining a healthy type-1/type-2 balance during chronic stages of the disease but can also exacerbate disease during acute phase of *T. gondii* infection where the type-1 response is necessary for protection.

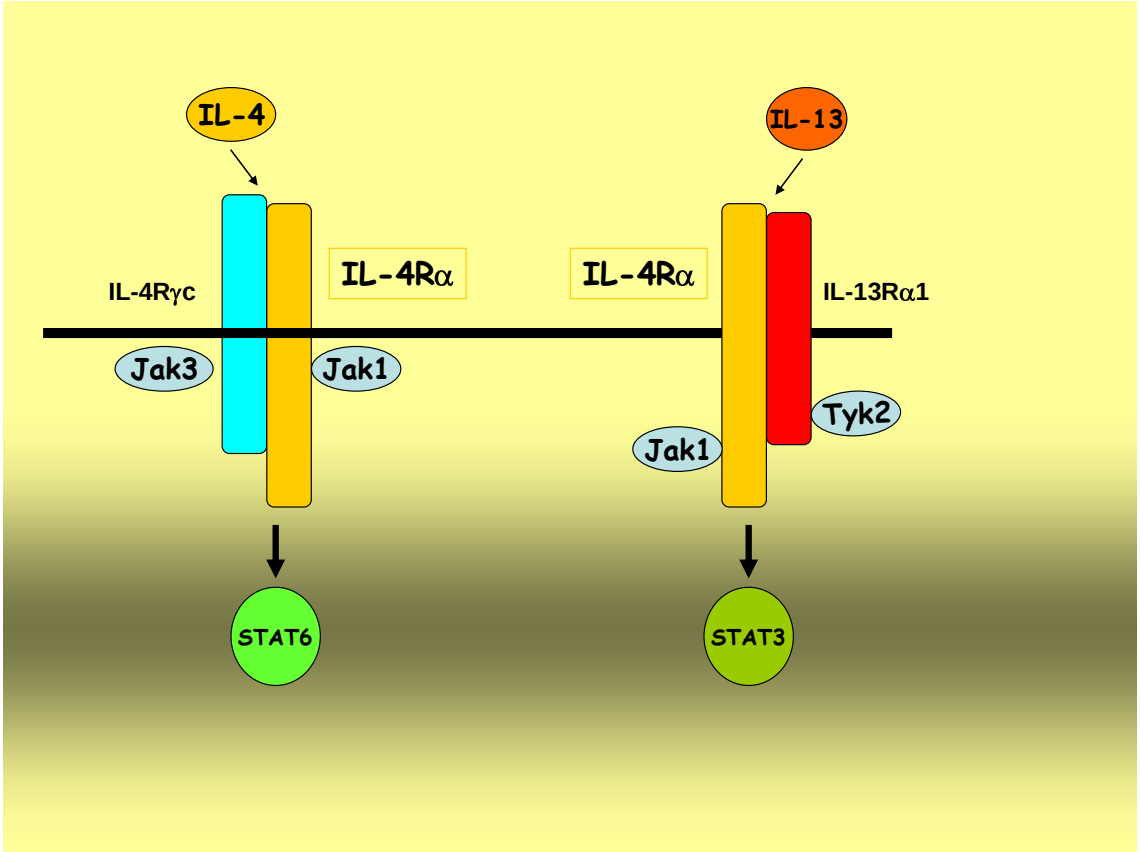


Fig. 4 A schematic representation of the IL-4/IL-13 signaling complex. IL-4 and IL-13 signal through the same IL-4Rα chain. IL-4/IL-13 bind to the IL-4Rα chain and signal through the JAK3/STAT6 pathway thereby inducing down-stream effector responses which include the production of type-2 cytokines such as IL-14 and IL-13.

1.5.3.3 Novel IFN-γ related pathways of *T. gondii* growth inhibition

As mentioned above, IFN-γ is reportedly the major effector cytokine mediating resistance during *T. gondii* infection. The mechanisms of IFN-γ mediated anti-toxoplasma activity are not yet clear. Several IFN-γ-regulated genes iNOS, IDO, and more recently, p47 GTPases, have been implicated to play a significant role in mediating these protective responses.

IFN-γ synergizes with TNF-α to activate macrophages and induce the expression of inducible nitric oxide synthase (iNOS) and reactive nitrogen intermediates (RNI's). iNOS is an enzyme which catalyzes the formation of nitric oxide (NO) from L-Arginine. It has been reported that NO can directly kill the parasites (Gazzinelli, Eltoum et al. 1993; Jun, Kim et al. 1993). Some studies have also shown that NO promotes tachyzoites conversion to the much slower dividing bradyzoite form of the parasite through inhibition of mitochondrial and nuclear enzymes essential for parasite respiration (Bohne, Heesemann et al. 1993). Although iNOS seems to be the predominant pathway used by classically activated macrophages to control *T. gondii* proliferation in tissue culture, the role of NO during *in vivo* infection is less clear. Studies using iNOS-deficient mice have shown that mice lacking iNOS are able to survive and control tachyzoite growth during the acute stage of infection and only succumb during chronic toxoplasmosis (Scharton-Kersten, Yap et al. 1997). Death was associated with uncontrolled proliferation of tachyzoites in the brain, suggesting that the protective anti-toxoplasmic effect in the brain is iNOS-dependent (Khan, Schwartzman

et al. 1997; Scharton-Kersten, Yap et al. 1997). Nevertheless, the observation that iNOS deficient mice are able to survive acute infection in an IFN-γ mediated manner suggest that there are alternative pathways other than NO production mediating anti-toxoplasma resistance *in vivo* (Khan, Matsuura et al. 1998).

A well known mechanism, induction of the IFN-γ inducible gene indoleamine 2,3 dioxygenase (IDO), has also been implicated in mediating some of the IFN-γ dependent anti-toxoplasma activity (Pfefferkorn 1984). IDO is an enzyme that catalyzes the degradation of the essential amino acid L-tryptophan through the kynurenine pathway, thereby compromising metabolic processes of the parasite (Fujigaki, Saito et al. 2002). Tryptophan is an essential amino acid that is required for protein synthesis; hence the depletion of tryptophan results in inhibition of parasite growth (Ball, Sanchez-Perez et al. 2007). IDO is found in almost all tissues and various cell types (Hayashi, Beck et al. 2004). Pfefferkorn 1984 first showed that treating human fibroblasts with IFN-γ induced IDO, which subsequently inhibited the growth of intracellular *T. gondii* by degrading tryptophan, which resulted in the parasite starving (Pfefferkorn and Guyre 1984). Murray et al., 1989 also showed that *T. gondii* was killed in IFN-γ activated, tryptophan-degrading human macrophages (Murray, Szuro-Sudol et al. 1989). In addition, an *in vivo* study using IFN-γ deficient mice demonstrated that IDO had an important role in IFN-γ induced anti-toxoplasma activity in various tissues. This study showed that IDO mRNA levels were significantly increased in the lungs and brains of infected C57BL/6J mice as compared to their IFN-γ deficient counterparts (Fujigaki, Saito et al. 2002). Tryptophan

concentrations were also dramatically decreased in the WT C57BL/6J mice in comparison with the IFN-γ deficient mice. In the same study, it was shown that although iNOS was highly upregulated in the lungs of IFN-γ deficient mice, there was increased proliferation of tachyzoite-specific mRNA in the lungs compared to the WT counterparts which expressed IDO in high amounts at the same time point (Fujigaki, Saito et al. 2002).

Few studies have reported on how IL-4 regulates the IFN-γ induced expression of IDO. IL-4 has been shown to strongly inhibit IFN-γ induced IDO mRNA expression in cultured human monocytes (Musso, Gusella et al. 1994) and in human fibroblasts (Chaves, Ceravolo et al. 2001). In contrast, a more recent study has shown that IL-4 increases IFN-γ induced IDO expression in mouse microglia (Yadav, Burudi et al. 2007). To date there have been limited reports on the effect of IL-4 on the regulation of IDO expression during *T. gondii* infection *in vivo*.

More recently p47 GTPases have emerged as potent effectors of microbial killing in different cells such as macrophages for various intracellular pathogens including *Mycobacterium avium* (Feng, Collazo-Custodio et al. 2004) *Leishmania major*, *Listeria monocytogenes* (Taylor, Feng et al. 2004) and *T. gondii* (Taylor, Collazo et al. 2000; Collazo, Yap et al. 2001). Murine astrocytes were shown to have the ability to kill intracellular *T. gondii* after activation with IFN-γ independently of iNOS and IDO. This IFN-γ mediated anti-toxoplasma activity however required the presence of IFN-γ

inducible p47 GTPase, Igtp (Halonen, Chiu et al. 1998; Halonen, Taylor et al. 2001; Melzer, Duffy et al. 2008)

The p47 GTPase family is a group of 47-48 kDa proteins which are potently induced in response to IFNs. Presently the family consists of 6 mouse proteins Igtp, LRG47, Irg47, Tgtp/Mg21, Iigp and Gtpi which are all intracellular membrane-bound proteins that have inherent GTPase activity, that is, they can bind and hydrolyse GTP (Taylor, Feng et al. 2004). Thus far, three of these proteins have been shown to be crucial in resistance to *T. gondii* infection.

Igtp deficient (Taylor, Collazo et al. 2000) and LRG47 deficient mice (Collazo, Yap et al. 2001) both displayed increased susceptibility to *T. gondii* infection compared to their WT counterparts. These mice succumb to infection with similar kinetics as that observed in IFN-γ deficient and STAT1-deficient mice. They reportedly die within 8-10 days post-infection, despite a robust IL-12 and IFN-γ production. The high mortalities were associated with unrestrained parasitemia. Both Igtp and LRG47 are therefore evidently critical for resistance against *T. gondii* infection during acute infection. On the other hand, IRG47 deficient mice survived during the acute phase of infection, although they displayed partial loss of resistance during the chronic phase of infection (Martens, Parvanova et al. 2005; Hunn, Koenen-Waisman et al. 2008).

Although the mechanisms through which p47 GTPases confer resistance to *T. gondii*

infection have not been determined, a model has been proposed. In *M. tuberculosis*, LRG47 has been found to localize exclusively to the Golgi body in *M. tuberculosis* infected macrophages (Butcher, Greene et al. 2005). LRG47 has been shown to promote the acidification and maturation of the bacterial phagosome in macrophages, thereby promoting bacterial clearance (MacMicking, Taylor et al. 2003). Igtp categorically localizes in the endoplasmic reticulum (Taylor, Stauber et al. 1997). Furthermore, p47 GTPases have also been shown to accumulate on the parasitophorous vacuole shortly after infection and the PV membrane exhibited signs of damage shortly thereafter. It is therefore proposed that p47 GTPases may be critical in mediating leakage of the *T. gondii* PV membrane, thereby exposing the parasites to the host cytosol and subsequent destruction (Martens, Parvanova et al. 2005; Zhao, Khaminets et al. 2009). Although this model is supported by studies using *M. tuberculosis*; neither Igtp nor LRG47 were found to localize to vacuoles containing live *T. gondii* parasites in IFN-γ activated macrophages (Butcher, Greene et al. 2005). Therefore, more studies need to be done to determine the mode of action through which p47 GTPases mediate protection during *in vivo T. gondii* infection.

Overall, there are evidently several non-redundant mechanisms involved in controlling parasite growth and mediating protection during *T. gondii* infection. NO has been shown to play a pivotal role during chronic infection by limiting the proliferation of cysts in the CNS. In addition, parasite growth can be inhibited by over-expressing IDO, a tryptophan degrading enzyme, thus starving the parasite of the essential amino acid and

compromising its growth. Lastly, although the mechanism of the recently discovered p47 GTPases is yet to be elucidated, they have been demonstrated to play a crucial role in resistance to *T. gondii* infection in an NO and IDO independent manner during both the acute and chronic phase of infection.

1.6 Macrophage activation

1.6.1 Classical macrophage activation.

Macrophages play a crucial role in the innate and adaptive immune responses to pathogens. They are involved in a range of essential processes and, depending on the cytokine environment and activating stimuli, can differentiate into distinct subsets that perform specific immunological roles (Noel, Raes et al. 2004). There are two stages of macrophage activation; first they are primed by NK cell or $CD4^+$ T-cell- produced IFN-γ to enhance expression of MHC class II molecules on the surface and antigen presentation. The primed macrophages then respond to secondary stimuli such as LPS to become fully activated. Macrophage effector functions influence the degree and duration of most inflammatory responses.

Macrophage populations induced by type-1 cytokines (IFN-γ, TNF-α) and microbial or parasitic antigens (such as LPS) exhibit pro-inflammatory properties and are considered "classically" activated macrophages (M1) (Fig. 5). Differentiation of classically activated macrophages requires priming by IFN-γ, through the IFN-γ-receptor (Dalton, Pitts-Meek et al. 1993; Huang, Hendriks et al. 1993). Classically activated macrophages can be characterized by a pro-inflammatory cytokine production phenotype, their ability to produce nitric oxide (NO), and increased expression of MHC Class II and CD86 expression (Noel, Raes et al. 2004). They have generally been shown to be very efficient in killing intracellular pathogens, are anti-proliferative (in cancerous tumors) and have

cytotoxic properties (Raes, De Baetselier et al. 2002). Immunopathology and tissue destruction is concomitant with a persistent type-1 response; however the ability of classically activated macrophages to produce matrix degrading proteases may also play a role.

1.6.2 Alternative macrophage activation

Differentiation of macrophages in the presence of IL-4 or IL-13 results in "alternatively" activated macrophages (M2) (Stein, Keshav et al. 1992; Gordon 2003) (Fig. 5). This activation programme is dependent on IL-4Rα signaling (Raes, Brys et al. 2005). Unlike classically activated macrophages, the differentiation of alternatively activated macrophages does not require any priming (Harding, Ramachandra et al. 2003). Alternatively activated macrophages display molecular and biological characteristics that differ from those of classically activated macrophages (Becker and Daniel 1990; te Velde, Rousset et al. 1990). They mainly release Th2 associated cytokines, which antagonize the effects of Th1 cytokines like IFN-γ. Macrophages that arise in the presence of factors such as IL-10, glucocorticoids and transforming growth factor (TGF)-β develop a phenotype which partially overlaps with alternatively activated macrophages (M2), they antagonize classically activated macrophages (M1) and contribute to the heterogeneity of type-2 associated cells (M2). These macrophages have been termed "regulatory macrophages" (M3) and they are characterized by high expression of regulatory cytokines such as TGF-β, IL-10 (Fig. 5), whilst the term "alternative activation" is largely limited to the effects of IL-4 and IL-13 (Mosser and

Edwards 2008). Alternatively activated macrophages (M2) have also been shown to promote cell growth, collagen deposition and have been implicated in the control of fibrogenesis (Misson, van den Brule et al. 2004). Furthermore, they release molecules which promote tissue repair and are often termed 'wound healing macrophages' (Mosser and Edwards 2008). These cells have also been reported to exhibit high endocytotic and phagocytotic capacity (Goerdt, Politz et al. 1999). They have an elevated level of macrophage mannose receptor activity and reduced pro-inflammatory cytokine expression (Stein, Keshav et al. 1992).

Alternatively activated macrophages are defective in antigen presentation to T-cells, produce minimal type-1 cytokines and are generally less efficient at killing intracellular parasites. In addition, the production of NO and L-citrulline from arginine by inducible NO synthase (iNOS) is suppressed in alternatively activated macrophages compared with classically activated macrophages. Instead arginase is upregulated in alternatively activated macrophages and it is involved in proline and polyamine biosynthesis (Munder, Eichmann et al. 1998; Yeramian, Martin et al. 2006). Proline promotes extracellular matrix (ECM) whilst polyamine is involved in cell proliferation.

Murine alternatively activated macrophages are characterized by increased expression of "found in inflammatory zone 1" (FIZZ1, also known as Resistin-like molecule (RELM)-α), and the secretory lectin chitinase3-like protein YM1 which are both associated with tissue remodeling (Holcomb, Kabakoff et al. 2000; Ling and Recklies 2004). Although

the function of these genes has not yet been fully elucidated, FIZZ1 and YM1 have been shown to be strongly induced in alternatively activated macrophages compared with classically activated macrophages. The *in vivo* induction of both these genes in macrophages is dependent on IL-4/IL-13 and the effects of IL-4 on FIZZ1 and YM1 expression in macrophages has been shown to be antagonized by IFN-γ (Raes, De Baetselier et al. 2002). FIZZ1, YM1 and arginase are therefore novel markers for alternative macrophage activation (Raes, Noel et al. 2002; Nair, Cochrane et al. 2003).

The activation state of macrophages can establish whether infection is resolved successfully or progresses to chronic state (Reiner and Locksley 1995). IL4Rα mediated alternatively activated macrophages have been shown to play a role in various parasitic infections including leishmaniasis (Rodriguez, Chang et al. 2004; Holscher, Arendse et al. 2006), schistosomiasis (Herbert, Holscher et al. 2004), trypanosomiasis (Raes, De Baetselier et al. 2002) and *Brugia Malayi* infections (Lawrence, Allen et al. 1995). Alternatively activated macrophages were shown to be essential and played a protective role during schistosomiasis whilst the contrary was observed during cutaneous leishmaniasis where alternatively activated macrophages were shown to exacerbate disease.

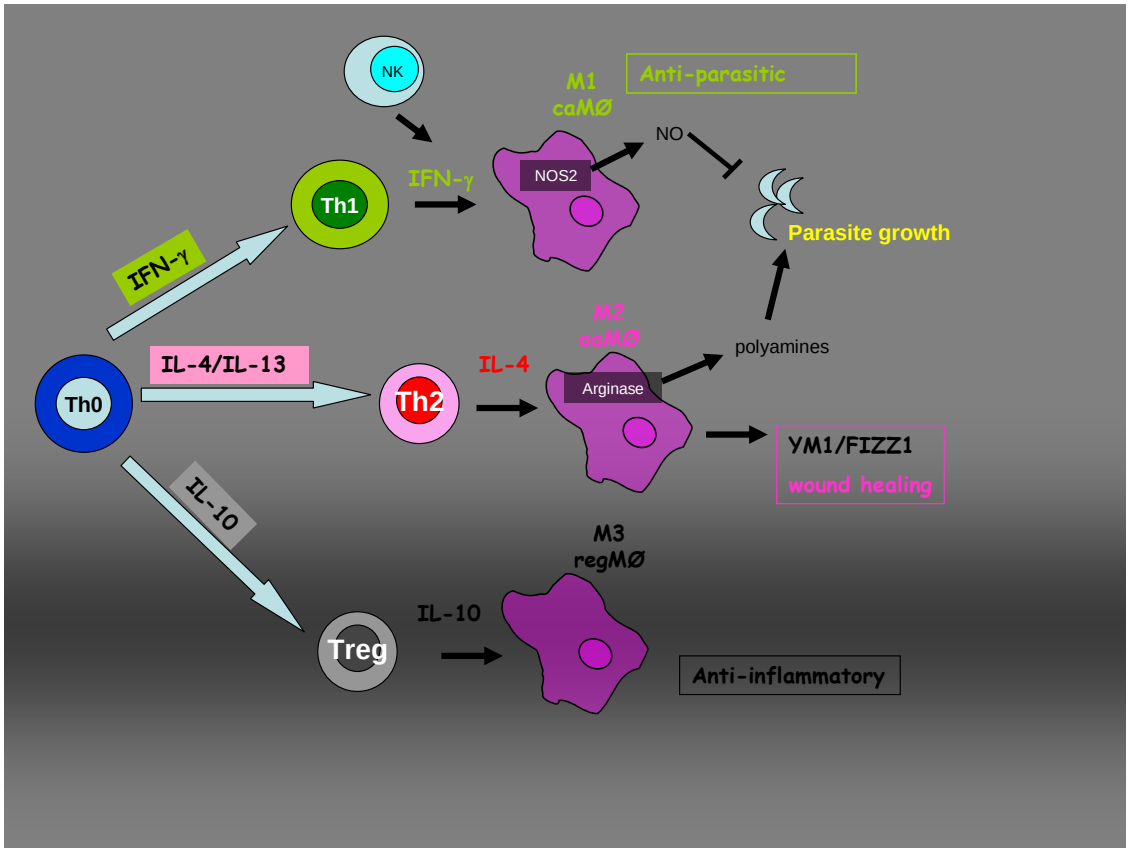


Fig.5 Cytokines that are released by immune cells can generate macrophages with distinct phenotypes. In an environment where there is high levels of IFN-γ being produced by Th-1 cells, macrophages undergo classical activation triggering the development of 'classically activated' macrophages (M1) which are characterized by production of high levels of NO and RNIs and are thus anti-parasitic as they limit parasite growth. Type-2 cytokines IL-4/IL-13 induce 'alternatively activated' macrophages (M2). IL-4 stimulates arginase activity and release of polyamines in these macrophages, which supports parasite proliferation. M2 are generally less phagocytic and they have been shown to characteristically produce large amounts of chitinase-like molecules such as YM1 and FIZZ1 which has been implicated in tissue repair. A third type of activated macrophages is termed regulatory macrophages (M3) and they arise when macrophages are exposed to IL-10. These macrophages produce high levels of IL-10 and

suppress pro-inflammatory immune responses.

1.7 Aims and Objectives

Preliminary studies as yet unpublished in our laboratory using BALB/c mice deficient in IL-4 and IL-4Rα have shown that IL-4 signaling through IL-4Rα plays a disease protective role during *T. gondii* infection. In the absence of IL-4 or signaling via IL-4Rα, female BALB/c mice infected by the peritoneal route were shown to be more susceptible than wild-type mice to *T. gondii* infection as measured by mortality. The mortalities were associated with increased pathology in the lungs (see appendix) and increased type-1 cytokine production by stimulated splenocytes. A similar but less severe phenotype was displayed by male IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice BALB/c mice infected by this route compared with wild-type controls and this was consistent with the well documented susceptibility bias of females to *T. gondii* (Roberts, Cruickshank et al. 1995; Roberts, Ferguson et al. 1996).

The purpose of the studies presented in this thesis was to further characterize the role of IL-4/IL-13 in the murine response against *T. gondii* using recently available cell specific IL-4Rα deficient BALB/c mice. The outcome of *T. gondii* infection in 3 tissue-specific IL-4Rα$^{-/-}$ BALB/c mice was investigated: macrophage/neutrophils (Herbert, Holscher et al. 2004), CD4^{+} T-cells (Radwanska, Cutler et al. 2007) and CD4^{+}/CD8^{+} T-cells (Dewals, Hoving et al. 2009) and compared with IL-4Rα intact mice and global IL-4Rα$^{-/-}$ mice. In particular pathology, parasite burden and immunological responses were measured and in addition where appropriate the effect of gender on disease outcome was

also measured. Our ultimate aim was to identify the cellular target(s) of the IL-4 or IL-4/IL-13 mediated activity resulting in the protection afforded signaling via IL-4Rα as well as identifying the immunological mechanisms associated with protection or pathology.

CHAPTER 2

GENERAL

MATERIALS AND METHODS

2. Methods

2.1 Mice

Macrophage/neutrophil-specific IL-4Rα deficient ($LysM^{cre}IL\text{-}4R^{-/lox}$), $CD4^{+}$T-cell-specific IL-4Rα deficient ($Lck^{cre}IL\text{-}4R\alpha^{-/lox}$) and $CD4^{+}CD8^{+}$ T-cell specific IL-4Rα deficient ($NLck^{cre}IL\text{-}4R\alpha^{-/lox}$) mice were generated, as previously described (Herbert, Holscher et al. 2004; Radwanska, Cutler et al. 2007; Dewals, Hoving et al. 2009). Male and female mice of all strains used were bred and maintained under specific pathogen free (SPF) conditions at the University of Strathclyde animal housing facility. Age (8-12 weeks old), and sex matched trans-gene negative littermates (IL-4Rα intact) were used as wild-type controls where stated.

2.1.1 Genotyping

DNA extraction

DNA was prepared from blood samples from experimental mice collected in DNAse/RNAse free microcentrifuges for use in PCR reactions. Briefly, 1ml of Boyle's solution (1:9 0.17M Tris: 0.16M ammonium chloride) was added to each blood sample to lyse erythrocytes followed by a brief vortex and 5 minutes incubation at room temperature. The blood samples were then centrifuged for 3 minutes at 13 000 rpm. The supernatant was then decanted, and the pellet resuspended in 100μl elution buffer (Qiagen). The pellets were then placed at -70°C until frozen, then taken out to thaw at room temperature. The samples were then mixed by a quick vortex and were kept at 4°C until used in PCR reactions.

Cre and lox-p site and IL-4Rα deletion determination by PCR

To verify the genotype of the cell specific IL-4Rα knock out mice PCR reactions were performed as previously described (Herbert, Holscher et al. 2004; Radwanska, Cutler et al. 2007; Dewals, Hoving et al. 2009). The sequences for the specific PCR primer pairs that were used to detect IL-4Rα deletion, Lck promoter for IL-4Rα deletion on $CD4^+$ T-cell populations, LysM promoter for macrophage/neutrophil-specific IL-4Rα deletion and, NLck promoter for $CD4^+$/$CD8^+$-specific IL-4Rα, LoxP site insertion are listed on Table 1. The PCR reactions were carried out in two steps; first the reaction to test for cre-recombinase expression and the second reaction to test for lox-p site insertion and IL4Rα deletion. The PCR reactions (Table 1.1) were performed in a total volume of 13μl: 6ul Reddymix Mastermix (ABgene, Epsom, UK), 0.5μl of the relevant primer pairs (Table 1) (Invitrogen, Paisley, UK), and molecular grade water (Sigma Aldrich, Poole, UK) to make up the volume. 1μl of DNA sample was used for each reaction. The PCR products were subsequently electrophoresed on a 1.6% agarose gel (Bioline, London, UK) made up in 0.5×TBE buffer (10.8g Tris, 5.5g Boric acid, 7.54g EDTA, make up to 1ml with dH_2O) and stained with 0.03% ethidium bromide (Sigma Aldrich, Poole, UK).

Table 1 A list of primer pairs and sequences used for genotypic analysis of the cell specific IL-4Rα knock out mice

Primer	**Primer sequence**
Lck-cre Forward	5'-AACCTGGGAAGTTGTG-3'
Lck-cre Reverse	5'-CACAGTTCCATCTGGTAT-3'
LysMcre Forward	5'-CTT GGG CTG CCA GAA TTT CTC-3'
LysMcre Reverse	5'-CCC AGA AAT GCC AGA TTA CG-3'
NLckcre Forward	5'-GAG GGT GGA ATG AAA CTC TCG GT-3'
NLckcre Reverse	5'-CAG GTA TGC TCA GAA AAC GCC TGG-3'
Flox IL-4R Intron 6-2	5'-CCCTTCCTGGCCCTGAATTT-3'
Flox IL-4R Intron 6 Reverse	5'-GTTTCCTCCTACCGCTGATT-3'
IL-4RKO P2	5'-CCT TTG AGA ACT GCG GGC T-3'
GAPDH Forward	5'-AGA TTG TTG CCA TCA AAC GAC-3'
GAPDH Reverse	5'-ATG ACA AGC TTC CAT TTC TTC-3'

Table 1.1 PCR programme for genotyping

Cycle number	**Duration of cycle**	**Temperature**
1	1 minute	94ºC
40	30 seconds	94ºC
	30 seconds	Annealing temperature 60ºC
	1minute	72ºC
1	5 minutes	72ºC
Hold		4ºC

Maintenance of parasites

T. gondii cysts were maintained in CD1 albino mice. Brains were harvested from mice that had been infected 17-21 weeks previously and homogenized in 1×PBS (pH7.4) by several passages using a 21G needle (BD Biosciences). Tissue cysts were enumerated and the stock mice were infected with 10 cysts intraperitoneally.

2.1.2 *In vivo Toxoplasma gondii* infections

Brains were harvested from a CD1 mouse infected with the moderately virulent *T. gondii* Beverly strain 17-21 weeks previously and subsequently homogenized in 1×PBS (pH 7.4) by several passages through a 21-gauge needle. Thereafter, 15μL of the brain suspension was spread on a glass microscope slide (BDH) and mounted with a cover slip (Menzel-Gläser, Braunshweig, Germany). The entire preparation was scanned and cyst numbers were counted under a microscope at 40× magnification. Appropriate dilutions were made and the inoculum was prepared in PBS (pH 7.4). Mice were then infected intraperitoneally with 200μl brain suspension diluted containing 10 cysts of *T. gondii*. Mice were monitored and weighed daily over a period of 9, 12 or 35 days depending on the experiment.

2.2 Preparation of Toxoplasma lysate antigen (TLA)

T. gondii RH strain tachyzoites were harvested from the peritoneum of previously infected mice using sterile PBS (pH 7.4). The peritoneal fluid containing tachyzoites was centrifuged at 1200 rpm for 5 minutes at 4ºC and the supernatant decanted. The

pellet was washed once with PBS and subsequently resuspended in the appropriate volume of PBS. The suspension was passaged 10 times through a 25-gauge needle and was disrupted by freezing in liquid nitrogen and thawing at 37ºC 5 times. The resulting lysate was filtered through a 0.22μm pore size filter, and the protein concentration was determined by Bradford assay. The TLA was then stored at -20ºC until used.

2.3 Splenocyte proliferation assay

For ex-vivo cytokine analyses, spleen cell suspensions were prepared from *T. gondii* infected mice. Spleens from infected mice were removed aseptically and placed in 5ml of complete RPMI-1640 medium (Lonza, Belgium) supplemented with 10% heat-inactivated fetal calf serum (Sigma Aldrich, Poole, UK), 2mM L-glutamine, 1% 100 IU/ml Penicillin and 1% 100μg/ml Streptomycin (PAA Laboratories, GmbH, Austria). Cell suspensions were prepared by gently teasing the spleens apart with sterile forceps and nitex (monofilament filter nylon cloth 100mm, Cadisch Precision Meshes, London, UK), and subsequently centrifuged at 1000 rpm at 4ºC for 5 minutes. The supernatants were decanted, and to lyse erythrocytes, the pellets were resuspended in 3ml of filter-sterilised Boyle's solution (1:9 0.17 M Tris: 0.16 M ammonium chloride). The cell suspension was subsequently centrifuged for 5 minutes at 4ºC. Pellets were then washed twice in 5ml complete medium by centrifuging at 1000rpm for 5 minutes. Following the washes, pellets were resuspended in between 5ml complete medium or more depending on the size of the pellet. Viable cells were counted by trypan blue exclusion using a haemocytometer (Assistant, Germany). The cell numbers were adjusted to 5×10^6 cells

per ml, and 100μl aliquots containing 5×10^5 cells were added to the wells of 96-well flat-bottomed tissue culture plates (Iwaki, Japan) which contained either 100μl complete medium alone, TLA at concentrations of 10 and 2μg/ml, or ConA (10μg/ml). Thus the final concentrations used to stimulate cell cultures were 1μg/ml and 5μg/ml and 5μg/ml ConA, unless otherwise stated. Cell cultures were incubated at 37ºC with 5% CO_2 for 72 hours. Following incubation, plates were stored at -20ºC until the conditioned media was collected to measure cytokine levels using sandwich ELISA.

2.4 Serum preparation for cytokine analysis

Mice were sacrificed by terminal anaesthesia and blood was collected into microtubes by cardiac puncture. Blood samples were left at 4ºC overnight to co-agulate and were afterwards centrifuged at 13 000 rpm for 10 minutes. The pellets were discarded and the serum transferred into clean microtubes and stored at -20ºC until used to measure cytokine levels by sandwich ELISA.

2.4.1 Measurement of NO by Griess method

Nitrite, as a measure of Nitric oxide (NO), was measured in spleen cell supernatants using the Griess method as previously described (Green, Wagner et al. 1982). The Griess reagents were prepared by making up two solutions: 2% sulphanilamide (Sigma) dissolved in 5% phosphoric acid (Sigma) and 0.2% naphthylenediamine (Sigma) dissolved in sterile H_2O. To prepare the reagent for the assay, 1 part of 2% sulphanilamide was added to 1 part of 0.2% naphthylenediamine and mixed. 30μl of the

splenocyte supernatants was added to the wells of a 96 well microplate, and a 30μl of doubling (1:2) Potassium nitrite (Sigma) standards ranging from 200μM to 0μM were added to the wells in duplicate. 50μl of the Griess reagent was subsequently added to each well and the plate left to develop for 15 minutes at room temperature. The absorbance was then read at 540nm to quantitate the amount of NO in the samples.

2.4.2 Cytokine analysis by ELISA

IFN-γ, IL-12p40/70, IL-4 and IL-10 levels were quantified in serum samples and splenocyte supernatants using sandwich ELISA. 96 well microtitre plates (Greiner Bio-One, Germany) were coated with 50μl/well of appropriate rat anti-mouse capture antibody overnight at 4ºC (IFN-γ at 2μg/ml; IL-12p40/70 at 2μg/ml; IL-4 at 500ng/ml in PBS (pH9.0) and IL-10 at 500ng/ml in PBS (pH 6.0)). The plates were then washed in PBS (pH 7.4) containing 0.05% Tween (Sigma, Poole, UK) before blocking for 1 hour at 37ºC with 200μl/well of blocking buffer made up of PBS (pH 7.4) containing 10% fetal calf serum (Sigma Aldrich, Poole, UK). The blocking buffer was then washed off 3 times and 30μl of either serum or cell supernatant samples were added to each well. A 1:2 dilution series of recombinant mouse IFN-γ, IL-12p40/70, IL-4 or IL-10 ranging from 20ng/ml to 0 (IFN-γ and IL-12p40/70) and 10ng/ml to 0ng/ml (IL-4 and IL-10) was set up on each plate in duplicate as a standard. The plates were subsequently incubated at 37ºC for 2 hours to allow the samples to bind to the plate-bound capture antibody. Following incubation, the plates were washed 4 times and to each well 50μl of biotinylated rat anti-mouse IFN-γ (1:500), IL-12p40/70 (1:1000), IL-4 (1:1000) or IL-10

(1:500) monoclonal antibody diluted in blocking buffer added. The plates were subsequently incubated at 37ºC for 1 hour followed by 5 washes. 50µl/well of conjugate streptavidin alkaline phosphotase (PharMingen, supplied by Insight Biotechnolgy, Wembley, UK) diluted 1/2000 (IFN-γ, IL-12p40/70 and IL-10) or conjugate streptavidin horseradish peroxidase diluted 1/4000 (IL-4) in blocking buffer. This was followed by 45 minute incubation at 37ºC and 6 washes thereafter. Finally respective substrates were added to the wells, 100µl/well of 1mg/ml p-nitrophenylphosphate (Sigma-Aldrich, Poole, UK) in glycine buffer (pH10.4) for IFN-γ, IL-12p40/70 and IL-10 or tetramethylbenzidine (6mg/ml in dimethyl sulfoxide) in 0.1M sodium acetate buffer (pH5.5) containing 0.0003% hydrogen peroxide for IL-4 (BDH, Poole, UK). The plates were then incubated in the dark at room temperature to allow appropriate colour development. For IFN-γ, IL-12p40/70 and IL-10, the plates were read at an absorbance of 405nm and 450nm for IL-4 using the SoftMax Pro software (Molecular devices, California, USA) on the spectrophotometer (SPECTRAmax 190 microtitre spectrophotometer).

2.4.3 Measurement of anti-*T. gondii*-specific IgG1 and IgG2a antibodies by ELISA

Blood samples were obtained by cardiac puncture on day 35 and serum samples prepared as previously described and stored at −20ºC until used. ELISA was adapted from (Voller, Bartlett et al. 1976) and performed as previously described by (Roberts and Alexander 1992). 96 well microtitre plates (Greiner Bio-One, Germany) were coated with 100µl/well of 5µg/ml TLA in PBS (pH9) overnight at 4ºC. Plates were then washed

3 times in PBS pH 7.4 containing 0.05% Tween before blocking for 1 hour at 37ºC with 200µl/well of wash buffer containg 5% dried milk (Marvel). The blocking buffer was then washed off 3 times, 100µl/well of serum samples (diluted 1:100 in wash buffer) added to the plate and serially diluted 1:3 down the plate and subsequently incubated for 1 hour at 37ºC. All samples were tested in duplicate and a negative control of unifected serum included on each plate. Following incubation, plates were washed 4 times and then 100µl/well of conjugate diluted in PBS containing 25% sheep serum added and the plates were incubated for 1 hour at 37ºC. Anti-mouse IgG1 and IgG2a horseradish peroxidase conjugates were used at 1:20 000 and 1:10 000 respectively. Plates were washed 5 times thereafter, and 100µl/well of TMB substrate [tetramethylbenzidine (6mg/ml in dimethyl sulfoxide) in 0.1M sodium acetate buffer (pH5.5) containing 0.0003% hydrogen peroxide] (BDH, Poole, UK) was added followed by 15 minutes incubation in the dark at room temperature. The reaction was then stopped by adding 50µl/well of 10% sulphuric acid. Absorbances were read at 450 nm using the SoftMax Pro software (Molecular devices, California, USA) on the spectrophotometer (SPECTRAmax 190 microtitre spectrophotometer). The readings obtained are expressed as means ±standard errors (SE) reciprocal end-point titers.

2.5 Histopatological analysis

For experiments that were terminated at days 9 and 12 post-infection, lung and liver tissues were kept for histopathological assessment. For experiments that were terminated at day 35 post-infection, lung, liver and brain tissues were kept for histopathological

analysis. Tissue samples were fixed in neutral buffered formalin (0.025M sodium phosphate monobasic monohydrate, 0.034M sodium phosphate dibasic anhydrous, 10% Formaldehyde at the time of sacrifice and later placed in histocassettes (Thermo Electron Corporation, Waltham, USA). The tissues were processed and embedded in wax. Sections were cut from wax-embedded sections and stained with Haematoxylin and Eosin.

The extent of inflammation observed in lung, liver and brain sections was scored using the following scale: 0, absent; 1, minimal; 2, mild; 3, moderate and 4, severe damage to the organ. The pathology was determined by examining interstitial inflammation and peribronchial pneumonitis in the lungs; portal tract and acini inflammation in the liver whilst brain tissues were examined for pneumonitis meningitis, perivascular cuffing, MG nodules and parasite cyst numbers. The number of necrotic lesions was also assessed. The raw histological scores are presented in a table format, and the average scores are presented graphically (dot-plots, each dot represents 1 mouse) as mean ± SE using the Prism GraphPad software.

2.6 RNA extractions

Total mRNA was extracted from lung tissues using the Trizol® reagent (Invitrogen, UK) using a protocol adapted from (Chomczynski and Sacchi 1987). Tissues were homogenized in 1ml Trizol® reagent using an electrical homogenizer. Homogenized samples were passaged through a 21 gauge needle 10 times and incubated at room temperature for 5 minutes before adding 500µl of chloroform. The homogenate was then

shaken vigorously for 15 seconds to mix before centrifuging for 15 minutes at 10,000g at 4ºC. Following centrifugation, the clear aqueous layer was carefully removed and transferred into new tubes. 600µL of ice-cold isopropanol was subsequently added to the aqueous layer and the mixture shaken vigorously for a few seconds to precipitate the RNA. The samples were then incubated at room temperature for 10 minutes, followed by centrifugation for 15 minutes at 10,000g and 4ºC. The supernatants were gently removed and discarded, and the RNA pellet retained. The pellets were then washed with ice-cold 75% ethanol, air-dried briefly at room temperature and finally re-suspended in 150µl RNase/DNAse-free water at 55ºC for 10 minutes. RNA was tested for contaminating genomic DNA prior to cDNA synthesis. Briefly, a PCR reaction was run on the RNA using TATA-box binding protein (Tbp) primers (house-keeping gene), and the presence of a PCR product indicated genomic DNA contamination. Contaminated RNA was re-extracted and/or treated with DNAse I (Invitrogen, UK) until the genomic DNA was removed. RNA quality was checked by running the samples on an ethidium bromide-stained 1.6 % agarose gel and finally quantified by optical density preparing 1:100 dilutions in RNase/DNAse-free water and reading absorbances at 260 and 280 nm using a spectrophotometer (GeneQuant pro). The RNA samples were then stored at -20°C until used to make cDNA for use in PCR.

2.6.1 Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was synthesized using Affinity Script Multiple Temperature Reverse Transcriptase (STRATAGENE, UK) according to the

manufacturer's protocol. In a total reaction volume 14.2µL, 1µg of RNA was added with RNase-free water, 500ng Oligo dinucleotides (Promega, Madison, USA) and 300ng random primers (Invitrogen). The reaction mixture was incubated at 65°C for 5 minutes then cooled down to room temperature to allow primers to anneal to RNA. The following were then added in this specific order, 2µl of 10× AffinityScript™ RT buffer (STRATAGENE, UK), 2µl of 100mM DTT (Invitrogen), 0.8µL of 100mM dNTP mix (Promega, Madison, USA) and lastly 1µl of AffinityScript™ Multiple Temperature Reverse Transcriptase (STRATAGENE). The final reaction volume of 20µL was mixed gently and incubated at 25°C for 10 minutes, followed by incubation at 55°C for 1 hour and then inactivated at 70°C for 15 minutes. All incubations were carried out in the PCR machine (Progene, Techne Thermocycler). The resultant cDNA was tested by PCR using Tbp primers. PCR products were run on a 1.6% agarose gel. cDNA samples were subsequently stored at -20°C until used as a template in Real-time PCR.

2.6.2 Quantitative Real Time Polymerase chain reaction (qRT-PCR)

RT PCR using SYBR® Green JumpStart^{TM} (Sigma, UK) was used to quantify the expression of FIZZ1, YM1, Arginase1, IL-12p40, NOS2, IFN-γ, IDO, LRG47 and Igtp GTPase mRNA transcripts using cDNA made from lung tissues from infected and non-infected mice. To allow gene copy number to be quantified in the experimental samples, standard curves were prepared using dilution series of plasmid (pDRIVE) containing the cloned gene of interest at 3000,000 copies, 300,000 copies, 30,000 copies, 3000 copies, 300 copies and 30 copies. Data was analysed using the 'standard curve method'

(Applied Biosystems) using Tata-binding protein (Tbp) as a housekeeping gene. To prevent cross contamination with genomic DNA, all equipment and plastic ware used for RT-PCR was pre-treated under UV light for 20 minutes before use. The primer pairs that were used and the programme used are as listed on Table 2 and Table 2.1 respectively.

Table 2 Primer sequences for primer pairs used in quatitative RT-PCR experiments.

Primer name	**Primer sequence (5'-3')**
TBP Forward	AAC AGC AGC AGC AAC AAC AGC AGG
TBP Reverse	TGA TAG GGG GTC ATA GGA GTC ATT GG
IFN-γ Forward	GGA ACT GGC AAA AGG ATG GTG AC
IFN-γ Reverse	GCT GGA CCT GTG GGT TGT TGA C
NOS2 Forward	GGT CTT TGA CGC TCG GAA CTG TAG
NOS2 Reverse	CAC AAC TGG GTG AAC TCC AAG GTC
IL-12p40 Forward	CCT GGT TTG CCA TCG TTT TG
IL-12p40 Reverse	TCA GAG TCT CGC CTC CTT TGT G
FIZZ1 Forward	ACC TTT CCT GAG ATT CTG CCC C
FIZZ1 Reverse	CAG TGG TCC AGT CAA CGA GTA AGC
YM1 Forward	GGC TAC ACT GGA GAA AAT AGT CCC C
YM1 Reverse	CCA ACC CAC TCA TTA CCC TGA TAG
Arginase1 Forward	TGA CAT CAA CAC TCC CCT GAC AAC
Arginase1 Reverse	GCC TTT TCT TCC TTC CCA GCA G
IDO Forward	GGC ACT CAG TAA AAT ATC TCC TAC
IDO Reverse	CTC TCA GTC CGT CCG TGC TC
Igtp Forward	TCT TTG GTG TGG ATG ATG GGT C
Igtp Reverse	TGT GCC TCT GGT GTC TGA AGT AGC
LRG47 Forward	GGA ACT GGT CTA CGG AAT CAA GG
LRG47 Reverse	ACA TAG TCC TCT ACG GTT TGG GC

SAG1 Forward	CTG TCA AGT TGT CTG CGG AAG GAC
SAG1 Reverse	CGT TAG CGT GGC ACC ATT ATC ACT C
TgCyst Forward	CGT TTG GAG AAA TGG TGT CCC AG
TgCyst Reverse	CCG CCT GAG TAT CCG CTT TTA C

The reactions were prepared by adding the following reagents in order: 10μl SYBR® Green JumpStart™ Taq ReadyMix™, 0.5μl forward primer, 0.5μl reverse primer, 8μl molecular grade DNAse/RNAse-free water and 1μl template cDNA to give a total reaction volume of 20μl. Each reaction was set up in duplicate, and a negative control was included with tubes containing 1μl of water instead of cDNA to screen for contamination of reagents or false amplification. The reactions were kept on ice and protected from light as SYBR Green is light-sensitive. The reactions were gently mixed and centrifuged briefly before being placed in the Multiplex quantitative PCR system instrument (Mx3000p™, STRATAGENE). The standard amplification protocol used for the RT-PCR programme is shown in Table 2.2, and the appropriate annealing temperatures for the primer pairs were as follows: TBP, 64°C; YM1, 64°C; FIZZ1, 64°C; Arginase1, 61°C; NOS2, 64°C; IFN-γ, 64°C; IL-12p40, 62°C; LRG47, 60°C, Igtp, 60°C; TgCyst antigen, 58°C and finally SAG1, 64°C.

Table 2.1 Quantitative RT-PCR programme

Cycles	**Duration of cycle**	**Temperature**
1	10 minutes	95ºC
40	30 seconds	95ºC
	1 minute	Annealing temperature
	1minute	72ºC
1	1 minute	95ºC
1	30 seconds	55ºC
1	30 seconds	95ºC

2.6.3 Parasite quantification using RT-PCR

Parasite numbers were quantified by comparing the changes in expression of parasite specific genes in the wildtype strain relative to the other mouse strains being studied. The expression of the target genes was normalized to Tbp expression. The primer pairs used in Real time PCR to detect bradyzoites were TgCyst matrix antigen and to detect the tachyzoite stage of the parasite SAG1 primers were used, the primer sequences are shown on Table 2. Gene copy numbers were calculated using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen 2001). In this method, mRNA transcript levels for each sample were normalized to TBP mRNA transcript levels and then expressed as a relative copy number compared with levels in WT controls. The following calculations were used:

$$\Delta\Delta CT = (C_{T,Target} - C_{T,Tbp})_{sample} - (C_{T,Target} - C_{T,Tbp})_{control}$$

WT samples were used as a control; therefore the calibrator is the average of (CT Target – CT Tbp) of WT samples

Then finally to get the relative gene copy number the values the values were entered in the equation $2^{-\Delta\Delta C}{}_{T}$. Each sample was treated separately and the relative gene copy numbers were averaged after the $2^{-\Delta\Delta C}{}_{T}$ calculation. Results were graphed as means ± SEM.

2.7 Statistical analyses

Statistical analyses were performed using the GraphPad Prism 4 software. All data is presented as mean ± standard error of mean (SEM). P values were calculated with the unpaired one-tailed Mann Whitney U test.

CHAPTER 3

The role of IL-4Rα signaling through macrophages/neutrophils during *T. gondii* infection

Abstract

The protective immune response against *Toxoplasma gondii* is widely recognised as being type-1 mediated. Nevertheless, the overproduction of type-1 cytokines can induce severe pathology. The extent to which Th2 cytokines can modulate the disease protective versus disease exacerbating effects of type-1 cytokines remains to be resolved. We have therefore, compared the disease induced by *T. gondii* infection in IL-$4^{-/-}$, IL-4R$\alpha^{-/-}$, and wild-type BALB/c mice, a mouse strain normally resistant to this disease. Increased mortality and increased lung pathology were observed in the absence of IL-4 and signalling via IL-4Rα and splenocyte type 1 cytokine production was increased during early infection (day 12) (see appendix). IL-4 can modulate type-1 inflammatory responses by counter-regulating the effects of IFN-γ on $CD4^+$ T cells and/or macrophages. We, therefore, compared the outcome of *T. gondii* infection in macrophage/neutrophil specific IL-4R$\alpha^{-/-}$ BALB/c ($LysM^{cre}$IL-4R$\alpha^{-/lox}$) mice and their wild type (IL-4R$\alpha^{-/lox}$) littermates as well as global IL-4R$\alpha^{-/-}$ mice. The disease phenotype following *T. gondii* as measured by mortality and pathology displayed by macrophage/neutrophil specific IL-4R$\alpha^{-/-}$ mice was similar to that of global IL-4R$\alpha^{-/-}$ animals and pro-inflammatory cytokine production was enhanced. Lung pathology in infected macrophage/neutrophil specific IL-4R$\alpha^{-/-}$ BALB/c ($LysM^{cre}$IL-4R$\alpha^{-/lox}$) and global IL-4R$\alpha^{-/-}$ mice was similar and more severe than in wild-type mice. Tachyzoite to bradyzoite conversion was significantly greater in the lungs of infected macrophage/neutrophil specific IL-4R$\alpha^{-/-}$ BALB/c ($LysM^{cre}$IL-4R$\alpha^{-/lox}$) than wild-type mice. These results clearly indicate a role for IL-4Rα signaling via

macrophages/neutrophils in limiting mortality and pathology during *T. gondii* infection. Nevertheless a clear correlation between pathology and classical and alternative macrophage activation markers in infected lungs as measured by real time PCR was not obvious.

3. 1 INTRODUCTION

It is widely acknowledged that a protective immune response against infection with the protozoan parasite *Toxoplasma gondii* is dependent on the rapid acquisition of a potent type-1 response**.** This initially involves the rapid generation of an innate response and the sequential production of IL-12 from neutrophils, dendritic cells and macrophages that in turn stimulate NK cell IFN-γ production (Gazzinelli, Hieny et al. 1993). The innate response in turn drives a strong acquired type-1 response in which $CD4^+$ T cells, but primarily IFN-γ producing $CD8^+$ T cells play the major protective roles (Suzuki, Orellana et al. 1988; Suzuki and Remington 1988; Parker, Roberts et al. 1991; Subauste, Koniaris et al. 1991). A number of mechanisms including the generation of toxic oxygen or nitrogen metabolites or by tryptophan starvation via the induction of indolamine oxygenase have been identified by which IFN-γ may mediate protection both by itself or augmented by other cytokines such as TNF-α, IL-1β, IL-12, IFN-α or IFN-β or alternatively CD40/CD40 ligation (Langermans, Van der Hulst et al. 1992; Heufler, Koch et al. 1996). Accordingly early experiments using neutralising antibodies suggested a counter protective role for type-2 and anti-inflammatory regulatory cytokines such as IL-4 and IL-10 respectively (Villard, Candolfi et al. 1995; Deckert-Schluter, Buck et al. 1997). However, it is now widely acknowledged that overproduction of pro-inflammatory cytokines such as IFN-γ, IL-12, TNF-α as well as nitric oxide production can be detrimental to the host as clearly demonstrated by increased pathology and death in infected mice deficient in IL-10 (Gazzinelli, Wysocka et al. 1996; Neyer, Grunig et al. 1997). Experiments using mice deficient in IL-4, a

cytokine that classically counter-regulates a type-1 response are less clear, with both disease protective and exacerbating roles being identified (Villard, Candolfi et al. 1995; Roberts, Ferguson et al. 1996; Suzuki, Yang et al. 1996). Quite a number of recent studies may help rationalise these apparent contradictory observations. For example, although being the archetypal Th2 promoting cytokine quite a number of studies have now demonstrated that IL-4 can also play a crucial role in the induction of Th1 responses. It may do this in part by priming DCs for IL-12 production (Lutz, Schnare et al. 2002) or alternatively by inhibiting IL-10 production either through the inhibition of Treg expansion (Mantel, Kuipers et al. 2007) or regulating macrophage IL-10 production directly (Oswald, Gazzinelli et al. 1992).

Most studies to date on the role of IL-4 during *T. gondii* infection have utilised highly susceptible C57BL/6 or B6/129 mice, and although protection against *T. gondii* is largely IFN-γ dependent, paradoxically the most susceptible strains (McLeod, Eisenhauer et al. 1989) are those producing the highest levels of this cytokine. This would suggest that regulation of pro-inflammatory mediators is somehow defective in these strains and a number of publications have highlighted this possibility (Suzuki, Sher et al. 2000; Kasper, Courret et al. 2004). Consequently a more appropriate model to study the role of Th2 cytokines in regulating a type 1 response and excess pathology during *T. gondii* infections would be one that is relatively resistant to infection such as the BALB/c mouse. We have therefore followed the course of avirulent *T. gondii* infection in IL-$4^{-/-}$, IL-$4R\alpha^{-/-}$ and wild type BALB/c mice. This demonstrated a

significant role for IL-4 in reducing pro-inflammatory cytokine production and limiting pathology and mortality. In order to identify the cellular target(s) for IL-4 mediated activity the outcome of *T. gondii* infection in macrophage/neutrophil specific IL-4R$\alpha^{-/-}$ BALB/c (LysM^{cre}IL-4R$\alpha^{-/lox}$) mice and their wild type IL-4Rα intact (IL-4R$\alpha^{-/lox}$) littermates as well as global IL-4R$\alpha^{-/-}$ mice was compared. The results provide compelling evidence that IL-4Rα signaling via macrophages/neutrophils plays a significant protective role in early infection with *T. gondii.*

3. 2 Results

3.2.1 Survival and bodyweights of LysM^{cre}IL-4Rα$^{-/lox}$ mice following *T. gondii* infection

In preliminary studies, female LysM^{cre}IL-4Rα$^{-/lox}$, WT littermates and IL-4Rα$^{-/-}$ mice were infected with 10 cysts of *T. gondii* Beverly strain intra-peritoneally, and monitored for disease and mortality over a period of 35 days. Whilst WT littermates were relatively resistant to infection (80% survival), LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ female mice became severely ill during the acute phase of infection, and were highly susceptible with a ~60% mortality rate (Fig. 3.1). Hence subsequent experiments could not be continued to day 35 due to the severe phenotype displayed and restrictions identifying a humane endpoint as agreed under the Home office license. We therefore examined day 12 post-infection in all further analyses at which point the severe disease in the IL-4Rα$^{-/-}$ mice was becoming evident.

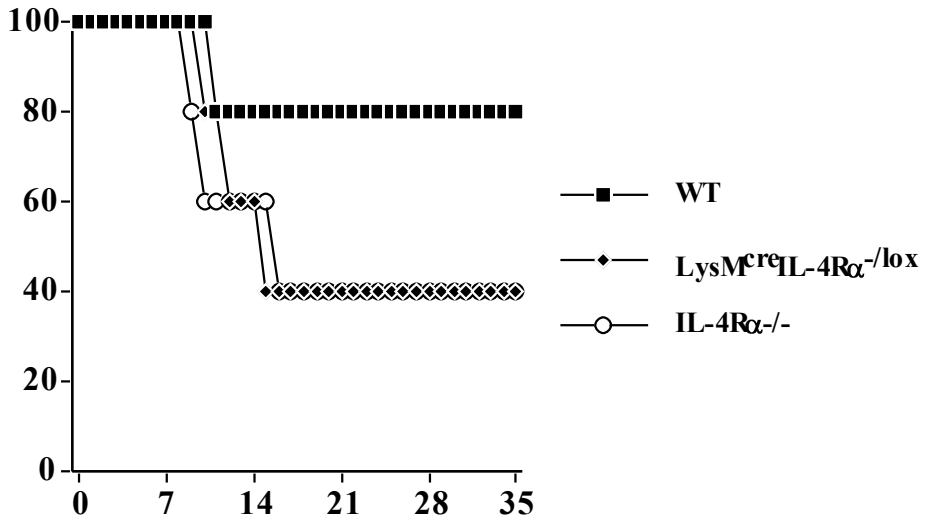


Fig.3.1 Percentage survival of female WT (solid square), LysM^{cre}IL-4R$\alpha^{-/lox}$ (solid diamond) and IL-4R$\alpha^{-/-}$ (open circle) mice infected with 10 *T. gondii* cysts intraperitoneally and monitored for survival over a period of 35 days. This data is representative of two independent experiments with similar results.

3.2.2 Histopathological analysis during acute *T. gondii* infection

To further investigate the increased susceptibility of LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice we examined the histopathology in the lungs 12 days post-infection as compared with wild-type littermates. There was a higher degree of interstitial pneumonitis in the lungs of LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice in comparison with WT littermates (Fig.3.2A), this was significant ($p<0.05$) only in LysM^{cre}IL-4Rα$^{-/lox}$ mice compared with WT controls. Macrophage-specific IL-4Rα deficient female mice displayed a similar degree of interstitial damage to the lung as global IL-4Rα deficient mice. There was significantly increased peribronchial inflammation in the lungs of IL-4Rα$^{-/-}$ mice compared with WT mice ($p<0.05$). Peribronchial inflammation was slightly increased in the lungs from LysM^{cre}IL-4Rα$^{-/lox}$ mice compared with WT mice, though the difference was not significant (Fig.3.2B). The livers from all 3 strains of mice displayed similar degrees of damage with minimal inflammation in the portal tracts (Fig3.2C) and mild to moderate degrees of inflammation in the acinar area (Fig.3.2D). Overall, macrophage-specific IL-4Rα deficient female mice displayed elevated degrees interstitial inflammation in the lungs which were similar to the levels observed in IL-4Rα$^{-/-}$ mice and moderately higher than WT mice and IL-4Rα$^{-/-}$ mice displayed significantly increased degrees of inflammation compared with lungs from *T. gondii* infected WT littermates.

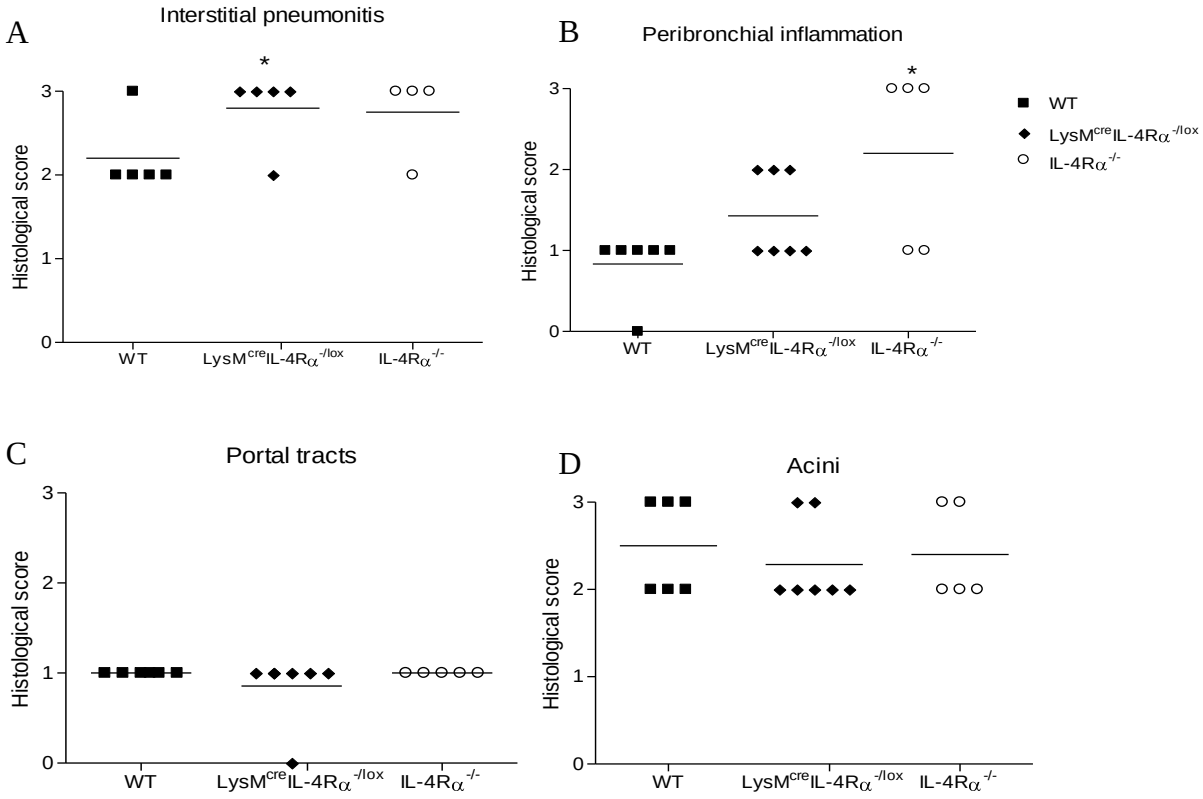


Fig. 3.2 Histopathological analysis of the lungs taken from female mice infected with *T. gondii* 12 days post-infection, lung interstitial (A), peribronchial (B) and liver portal tract (C) and acinar (D) inflammation. Data is representative of 3 independent experiments, *p<0.05 compared with WT control.

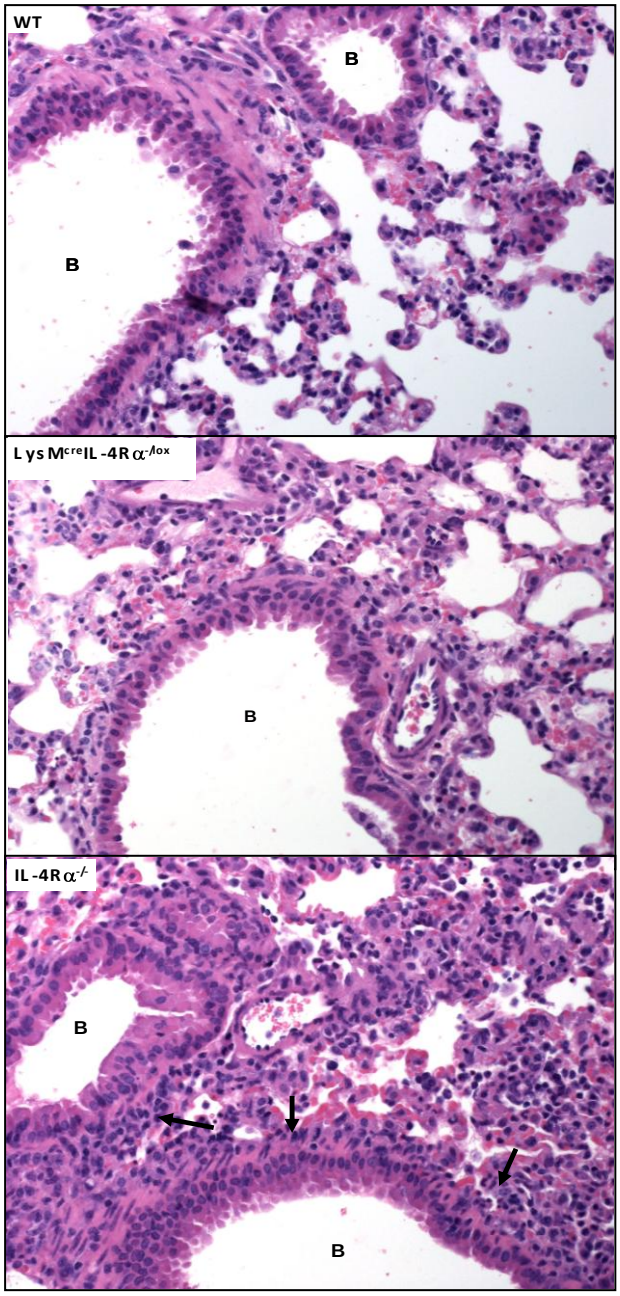


Fig. 3.2 B. Lung tissue sections depicting peribronchial inflammation (arrows, B= bronchiole) in female mice infected with *T. gondii* 12 days post-infection. IL-4Rα$^{-/-}$ mice displayed excessive degrees of peribronchial inflammation compared with WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice. LysM^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/-}$ mice displayed increased interstitial inflammation

compared to mild degrees observed in lungs from WT mice.

3.2.3 Serum cytokine levels in *T. gondii* infected female mice

The levels of cytokines circulating systemically were measured by capture ELISA. IL-12p40/70 levels were significantly lower in the serum of and IL-4Rα$^{-/-}$ mice compared with LysM^{cre}IL-4Rα$^{-/lox}$ and WT mice ($p<0.05$) (Fig.3.3B). Circulating IFN-γ and IL-10 levels were relatively low and there was no significant difference observed between the 3 mouse strains (Fig.3.3A, Fig.3.3C).

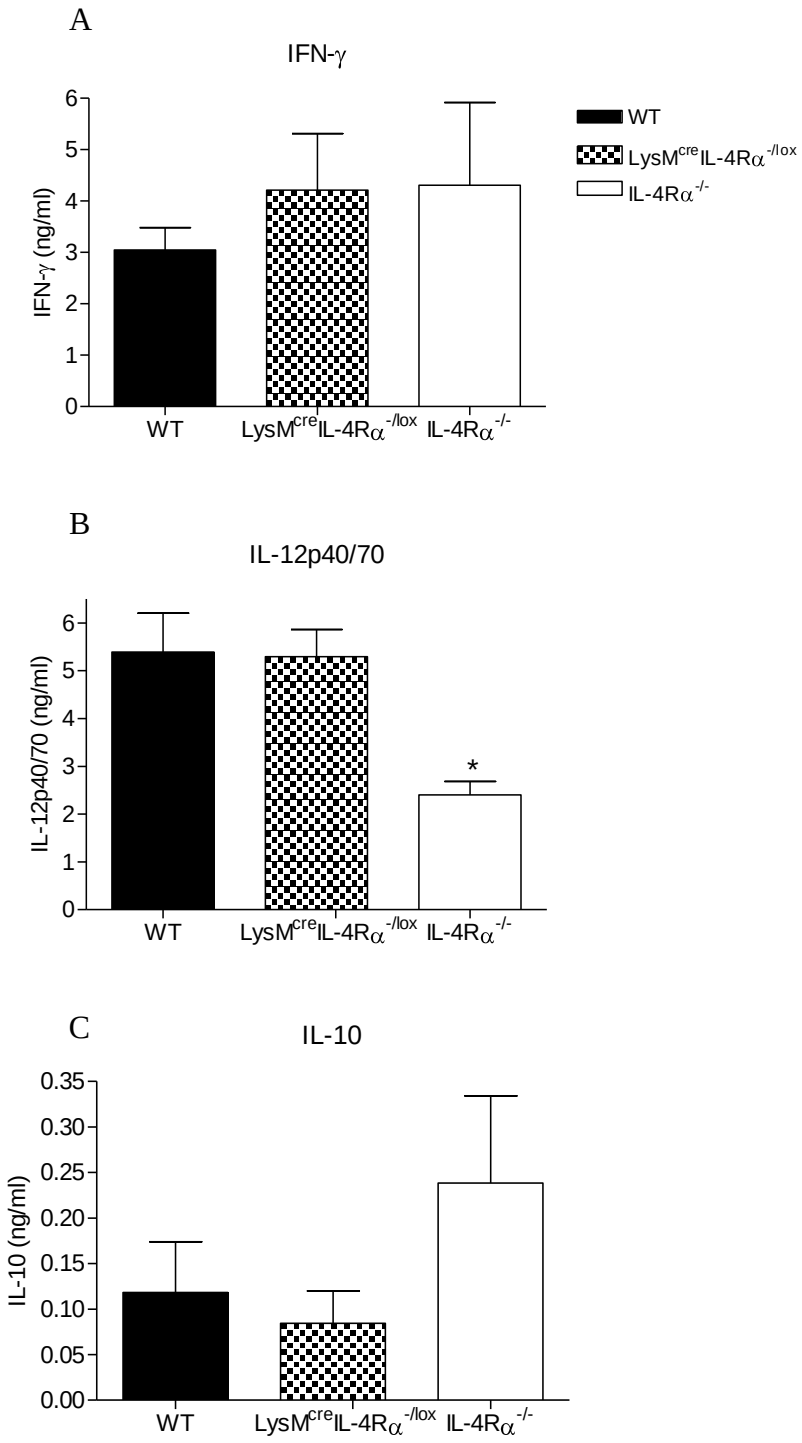


Fig.3.3 The level of serum IFN-γ (A), IL-12p40/70 (B) and IL-10 in the sera of female mice infected with *T. gondii* 12 days post-infection.*p<0.05

3.2.4 Analysis of *T. gondii*-specific splenocyte cytokine production

Splenocytes isolated from infected mice were stimulated with *T. gondii* lysate antigen (TLA) and cytokine production measured by capture ELISA. Splenocytes derived from $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ mice produced significantly increased levels of IFN-γ in a dose-dependent manner compared with the WT littermates when stimulated with TLA (Fig.3.4B). The type-1 response was therefore highly up-regulated in the knock-out strains compared to WT controls.

On the other hand, type-2 cytokines were produced at very low levels following stimulation with TLA. Splenocytes stimulated with multiple doses of TLA failed to produce measurable levels of IL-4 (results not shown). There were low levels of IL-10 detected following stimulation with TLA and it increased in a dose-dependent manner (Fig.3.5). Nevertheless, there was no significant difference observed between IL-4 and IL-10 production by splenocytes from WT, $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ mice.

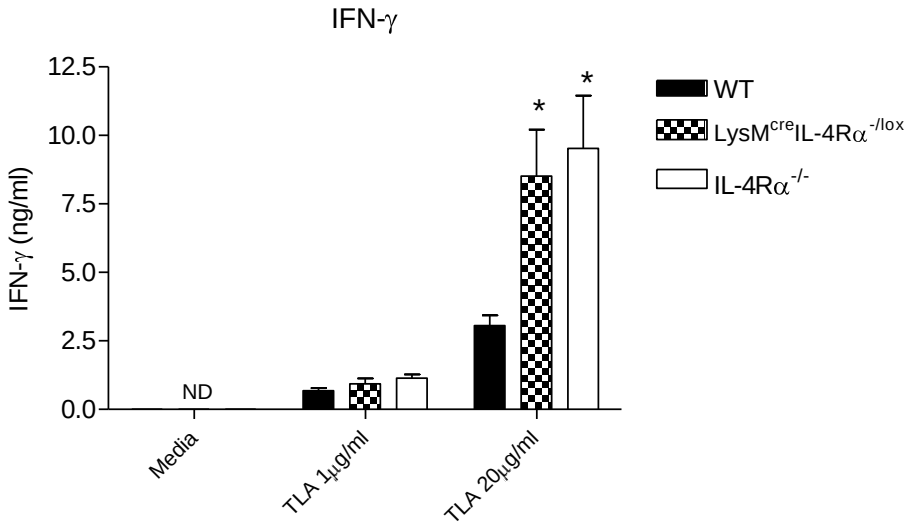


Fig.3.4 Splenocyte IFN-γ production following stimulation with various doses of *T. gondii* antigen.*p<0.05, compared with WT of corresponding stimulation dose

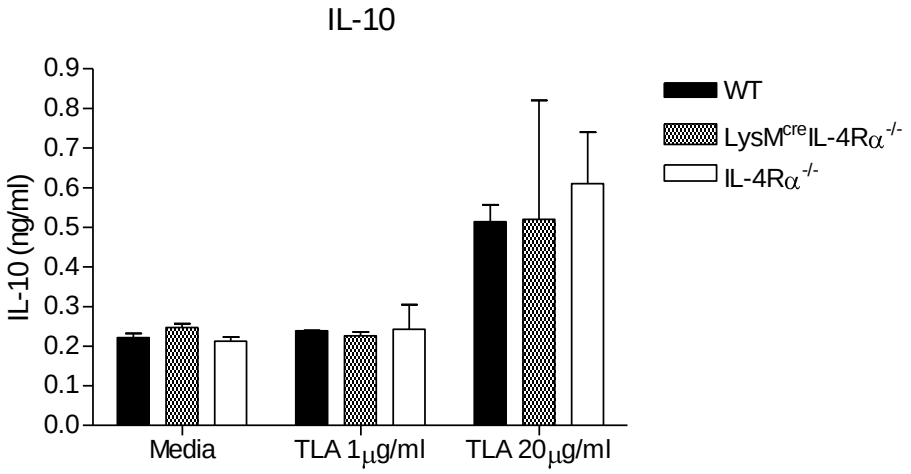


Fig.3.5 Splenocyte IL-10 production following stimulation with various doses of *T. gondii* lysate antigen.

3.2.5 Analyses of cytokine mRNA transcript expression and parasite burden in the lungs of *T. gondii* infected mice using RT-PCR.

Lung tissues from female mice infected with *T. gondii* were examined for cytokine mRNA transcript expression, markers for alternative macrophage activation and parasite burden 12 days post-infection. IFN-γ transcripts were highly upregulated in the lungs of WT, LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice (Fig.3.6A). IL-12p40 and NOS2 mRNA transcripts were also expressed in the lungs (Fig.3.6 B & C respectively), although both at a lower magnitude compared with IFN-γ. Overall, there was no significant difference observed in the mRNA transcript levels of IFN-γ, IL-12 and iNOS between the 3 mouse strains at day 12 post-infection with *T. gondii*.

To investigate whether alternative macrophage activation played a role in the increased mortality and pathology displayed in the lungs of LysM^{cre}IL-4Rα$^{-/lox}$ mice, the expression of markers of alternative macrophage activation were quantified in the lungs 12 days post-infection with *T. gondii*. Interestingly, FIZZ1 was markedly reduced in the lungs of the susceptible IL-4Rα$^{-/-}$ mice ($p<0.05$) as compared to LysM^{cre}IL-4Rα$^{-/lox}$ and WT mice (Fig.3.7 A). Both YM1 and Arginase1 transcripts were also up-regulated in the lungs; however there was no difference between the 3 strains (Fig.3.7B, Fig.3.7C).

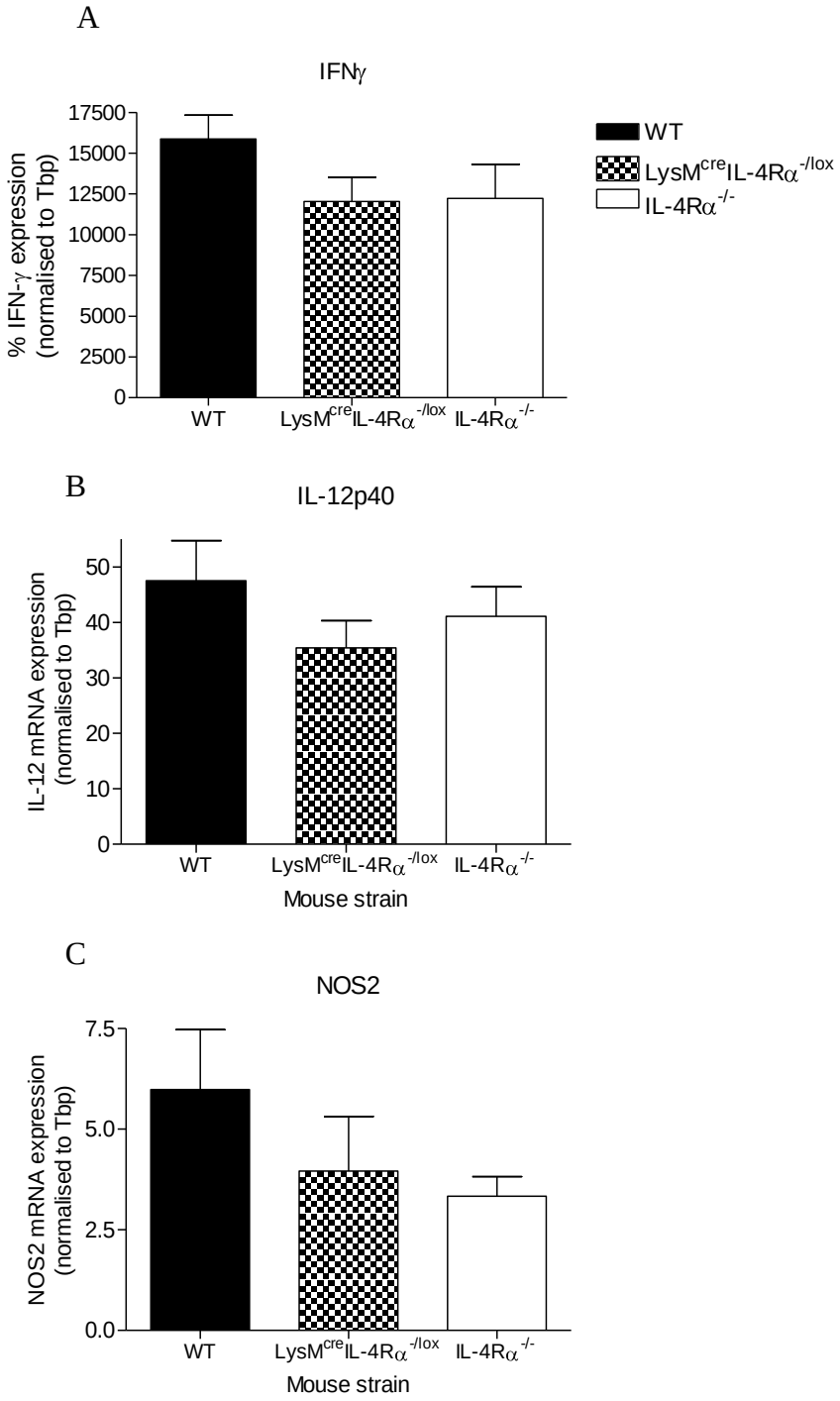


Fig.3.6 mRNA transcript expression in the lungs of female mice 12 days post-infection with *T. gondii*; IFN-γ (A), IL-12p40 (B) and NOS2 (C) transcript expression. No significant difference was observed between the 3 strains.

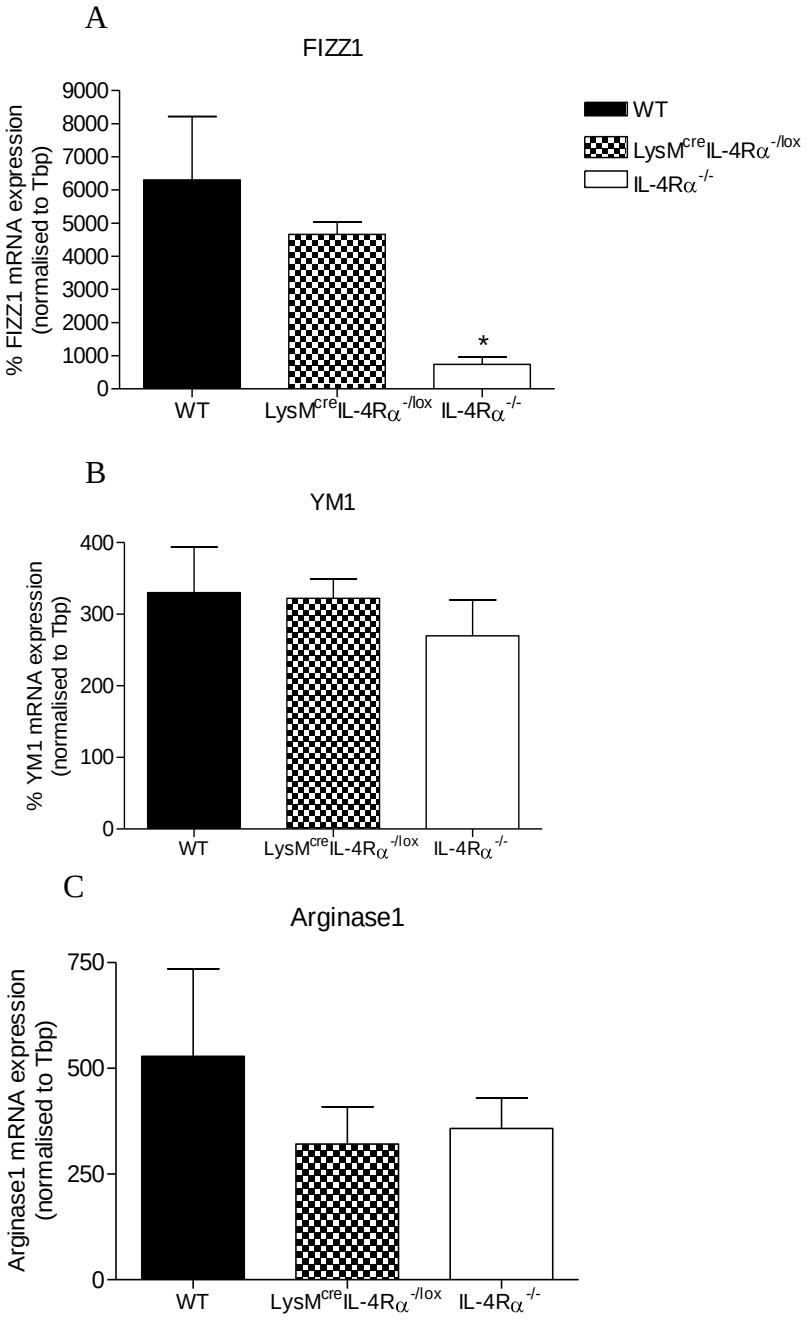


Fig.3.7 Expression mRNA transcripts of alternative macrophage markers, FIZZ1 (A), YM1 (B) and Arginase1 (C) in the lungs of *T. gondii* infected WT, LysM^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice 12 days post-infection as measured by Real time PCR. *$p<0.05$ compared with WT control.

Furthermore, to further investigate the pathology in the lungs and other possible signaling pathways and effectors involved in mediating the immunopathology, the level of IFN-γ-inducible genes IDO, and the p47 GTPases LRG47 and Igtp were quantified in the lungs of infected mice. IDO transcripts were significantly reduced ($p<0.05$) in the lungs of IL-4Rα$^{-/-}$ mice in comparison with LysM^{cre}IL-4Rα$^{-/lox}$ and WT mice (Fig.3.8A). Both LRG47 and Igtp GTPase transcripts were expressed in substantial amounts; however there was no difference between the 3 strains (Fig.3.8B, Fig.3.8C).

Parasite burden was analyzed in the lungs by measuring SAG1 transcripts (tachyzoite specific gene) and TgCyst antigen transcripts (bradyzoite specific gene) using quantitative RT-PCR. LysM^{cre}IL-4Rα$^{-/lox}$ mice had significantly lower levels of tachyzoite transcripts compared to WT littermates ($p<0.05$), whilst IL-4Rα$^{-/-}$mice had significantly increased bradyzoite transcripts in the lungs compared with both LysM^{cre}IL-4Rα$^{-/lox}$ and WT mice ($p<0.05$) (Fig.3. 9). Overall, there was an equivalent burden of tachyzoite and bradyzoite transcripts in the lungs of WT mice, whereas both LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$mice displayed an increased conversion of tachyzoite form of the parasite to the bradyzoite form. In other words, there were more bradyzoite than tachyzoite transcripts detected in macrophage-specific IL-4Rα deficient and in global IL-4Rα deficient female mice 12 days post *T. gondii* infection (Fig.3.9).

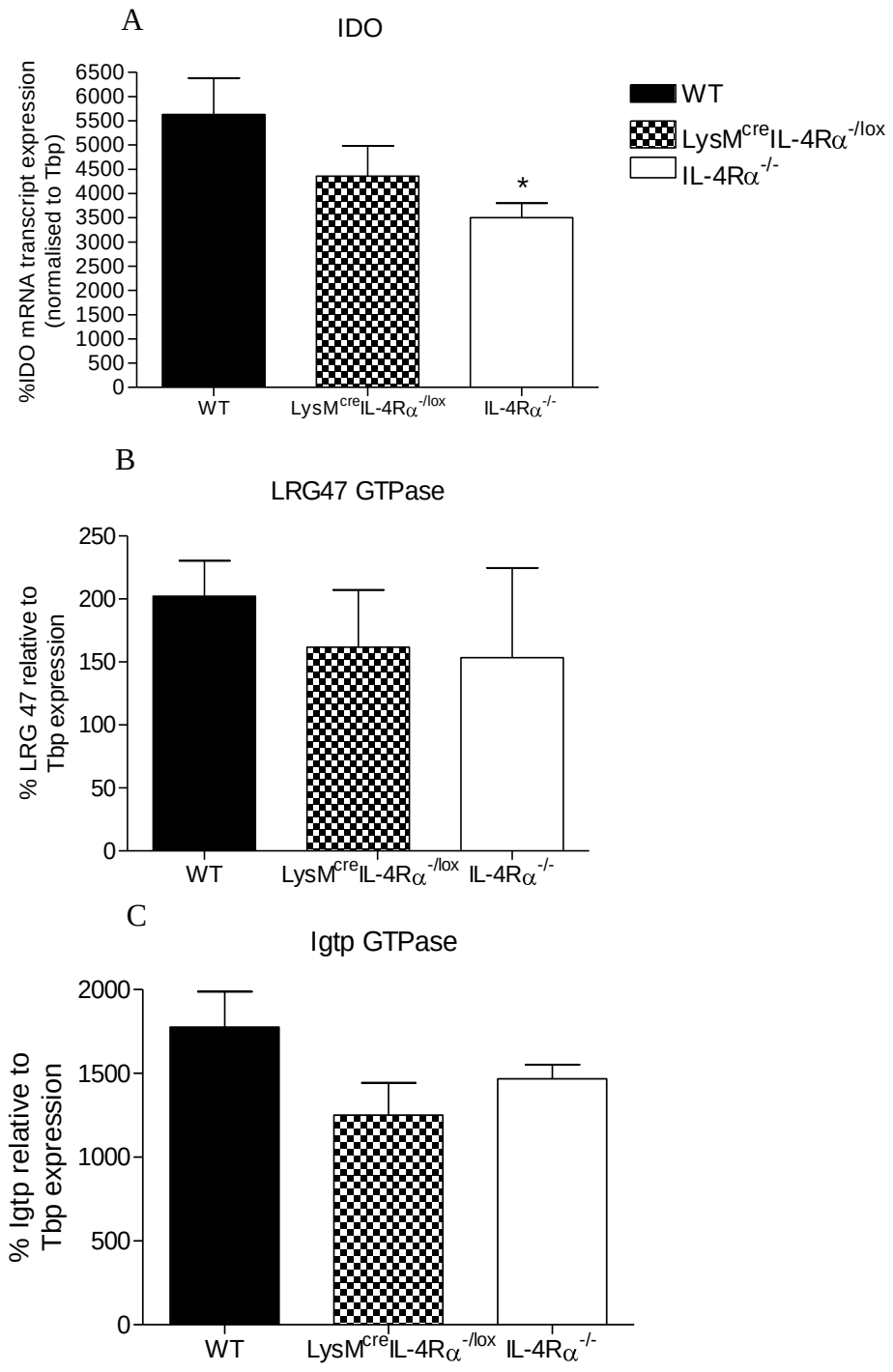


Fig.3.8 Expression of mRNA transcripts of IFN-γ-inducible genes IDO (A), LRG47 GTPase (B) and Igtp GTPase (C) in the lungs of WT, LysM^{cre}IL-4Rα$^{-/-}$ mice infected with *T. gondii* 12 days post infection.*$p<0.05$ compared with WT control

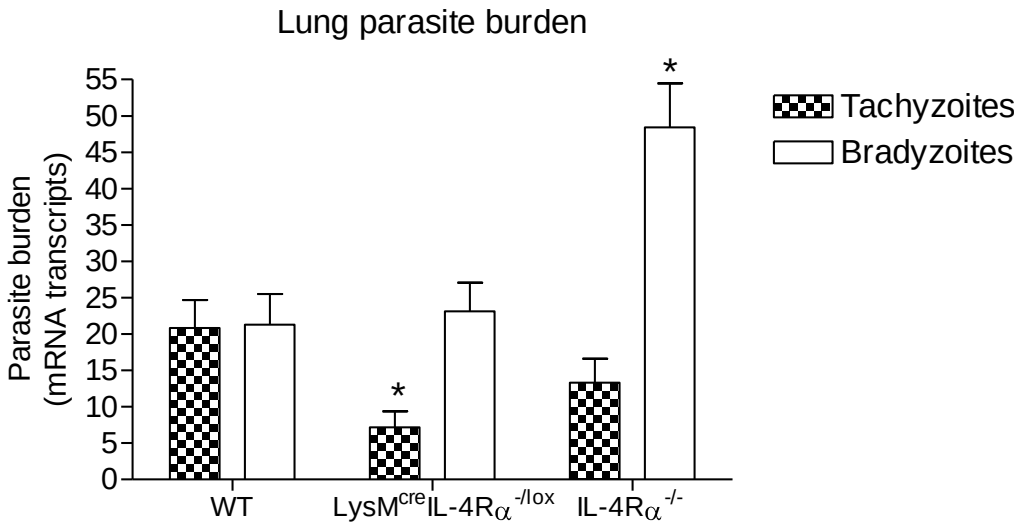


Fig.3.9 Parasite burden in the lungs of female mice 12 days post-infection. SAG1 (tachyzoite-specific gene) and TgCyst antigen (bradyzoite-specific gene) transcripts were quantified using RT-PCR. *$p<0.05$, compared with WT of corresponding parasite form

3.3 Discussion

Macrophages play a crucial role in the innate responses to many pathogens including *T. gondii* (Gazzinelli, Hieny et al. 1993) prior to the establishment of the adaptive immune responses, and it is well documented that cytokines can influence macrophage functional responses (Stout and Suttles, 1995, Stout et al., 2005). The role of IL-4 and IL-13 – dependent type-2 mediated immune responses in susceptibility to *T. gondii* has been reported in a few studies (Roberts, Ferguson et al. 1996; Suzuki, Yang et al. 1996), however the mechanisms have not yet been elucidated. Studies using IL-4 and IL-4Rα deficient mice infected with *T. gondii* Beverly also demonstrated that IL-4/IL-13 signaling is essential for survival of BALB/c mice during acute *T. gondii* infection (Nickdel et al., unpublished data). To evaluate the populations involved in IL-4/IL-13 mediated resistance to *T. gondii* infection, we infected female macrophage/neutrophil-specific IL-4Rα deficient mice ($LysM^{cre}IL\text{-}4R\alpha^{-/lox}$) with *T. gondii* Beverly intraperitoneally. WT littermates and global IL-4Rα deficient mice were used as controls. We show in this study that $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice are highly susceptible to *T. gondii* infection and they succumb to infection during the early stages of infection, with similar kinetics as $IL\text{-}4R\alpha^{-/-}$ mice.

The increased mortality was associated with severe pathology in the lungs. During early *T. gondii* infection, the parasites induce a potent IL-12 response that leads to IFN-γ mediated control of parasite proliferation (Gazzinelli, Wysocka et al. 1994). In this study, we observed a marked reduction of IL-12 in the serum of $IL\text{-}4R\alpha^{-/-}$ mice

compared with WT and LysM^{cre}IL-4R$\alpha^{-/lox}$ mice. More significantly, TLA-induced IFN-γ production was highly elevated in splenocytes from infected LysM^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice compared with WT controls. Although IFN-γ is critical to control parasite replication and dissemination during acute phase *T. gondii* infection, this early cytokine response must be regulated to prevent tissue damage. It is therefore possible that the lethal immunopathology observed in IL-4R$\alpha^{-/-}$ mice is associated with an unregulated IFN-γ dependent pro-inflammatory response.

It is well known that IL-4 and IL-13 signaling are critical for the development of a Th2 phenotype which largely antagonizes the effects of IFN-γ responses (Kuhn, Rajewsky et al. 1991; Kopf, Le Gros et al. 1993). IL-4/IL-13 mediated signals are also important inducers of alternatively activated macrophages (Gordon 2003). It is well established that IFN-γ-primed macrophages, which are classically activated, are involved in inhibiting parasite replication through various mechanisms (Pfefferkorn 1984; Adams, Hibbs et al. 1990; Chon, Hassanain et al. 1996; Butcher, Greene et al. 2005). However, although IL-4/IL-13 have been reported to be susceptibility factors in some protozoan infections (Noel, Hassanzadeh et al. 2002), very few studies have investigated the role of IL-4/IL-13 dependent alternatively activated macrophages in susceptibility to protozoan infections (Holscher, Arendse et al. 2006). As we observed excessive damage to the lungs of the susceptible IL-4R$\alpha^{-/-}$ mice following *T. gondii* infection in our study, we investigated the involvement of alternatively activated macrophages during *T. gondii* infection in the lung tissue. Interestingly, we found that IL-4R$\alpha^{-/-}$ mice infected with *T.*

gondii expressed significantly reduced levels of FIZZ1 mRNA transcripts in the lungs, compared with the WT counterparts. Although the exact function of FIZZ1 protein has not yet been elucidated, it is known that FIZZ1 expression is strongly induced by IL-4 and it has been associated with promoting deposition of extra-cellular matrix (Raes, Noel et al. 2002), thus may be involved in tissue repair.

The activation state or functional phenotype plays an important role at specific stages of the infection. In the initial stages, the classically activated macrophages are essential for the anti-parasitic properties (Reis e Sousa, Hieny et al. 1997; Bliss, Butcher et al. 2000), as the disease progresses the NO and RNI production by these macrophages may be detrimental and cause pathology (Candolfi, Hunter et al. 1994), and IL-4/IL-13 and even IL-10 (Wilson, Wille-Reece et al. 2005) may be involved in the anti-inflammatory and reparative stages of infection. Our data shows that there is excessive pathology in the lungs in absence of IL-4Rα signaling, and at the same time there is significantly reduced FIZZ1 expression compared with WT mice, which suggests a possible protective role of IL-4/IL-13 mediated signaling in wound healing during *T. gondii* infection. Although $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice were susceptible and succumbed to *T. gondii* infection as $IL\text{-}4R\alpha^{-/-}$ mice, there was no reduction of FIZZ1 or other markers of alternative macrophage activation markers measured. The mortality in these mice may therefore be mediated by other mechanisms. Other studies have shown that alternatively activated macrophages are essential for survival and preventing lethal pathology during *Schistosoma mansoni* infection (Herbert, Holscher et al. 2004). Recently published

studies have suggested that a mechanism through which these alternatively activated macrophages function during *Schistosoma* infection involves macrophage-specific arginase 1 and FIZZ1 inhibiting type-2 $CD4^+$ T-cell effector functions, thus limiting lethal immunopathology (Nair, Du et al. 2009; Pesce, Ramalingam et al. 2009; Pesce, Ramalingam et al. 2009).

In addition to the reduction of FIZZ1 mRNA transcript expression in the lungs of IL-4R$\alpha^{-/-}$ mice, we observed a marked reduction of the IFN-γ inducible gene indoleamine 2-3 dioxygenase (IDO) in IL-4R$\alpha^{-/-}$ mice but not in $LysM^{cre}$IL-4R$\alpha^{-/lox}$ mice. Interestingly, this reduction in IDO mRNA was concomitant with increased cyst mRNA burden in the lungs, which was not observed in the WT and $LysM^{cre}$IL-4R$\alpha^{-/lox}$ strains. IDO enzyme is expressed upon stimulation by IFN-γ in various antigen presenting cells, and it controls *T. gondii* growth by degradation of tryptophan thus limiting the amount of available tryptophan available for the parasite to survive intra-cellularly (Taylor and Feng 1991; Silva, Rodrigues et al. 2002). In fact, in human non-professional phagocytic cells, induction of IDO appears to be the main mechanism by which IFN-γ controls tachyzoite proliferation (Daubener, Pilz et al. 1993; Thomas, Garrity et al. 1993). Chaves *et al*. has shown in *in vitro* studies that IL-4 and IL-13 inhibit the transcription of IDO mRNA in 2C4 cells activated with IFN-γ, and consequently significantly inhibits IFN-γ mediated control of tachyzoite replication (Chaves, Ceravolo et al. 2001). In contrast, our data shows that in absence of IL-4R$\alpha^{-/-}$ signaling, IDO mRNA is markedly reduced in the lungs of *T. gondii* infected mice compared with WT counterparts. The

tachyzoite mRNA burden is however similar to that detected in WT mice. On the other hand, the cyst mRNA burden in the susceptible IL-4Rα$^{-/-}$ mice is significantly higher than in WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice. In addition, the rate of tachyzoite to bradyzoite conversion was increased in LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice but not in WT mice. This observation raises the possibility of the involvement of IDO in conversion of the tachyzoite form to the encysted form of the parasite. Although a clear correlation cannot be established at this point, it is evident that a reduction of IDO mRNA expression promotes cyst proliferation in absence of IL-4Rα signaling. Although a similar tachyzoite to bradyzoite conversion rate is observed in LysM^{cre}IL-4Rα$^{-/lox}$, the cyst burden is significantly less than in IL-4Rα$^{-/-}$ lungs, and in addition, the tachyzoite burden in LysM^{cre}IL-4Rα$^{-/lox}$ mice is significantly lower than in WT mice. Therefore, there may be other IL-4/IL-13-mediated mechanisms involved in inhibiting parasite replication, which are being potentiated on other cells in the lung other than macrophages/neutrophils. NO and RNI-mediated events are reportedly also involved in parasite control during the acute phase (Khan, Schwartzman et al. 1997) and also inhibition of cyst proliferation in the brain during chronic infection (Suzuki 2002). Furthermore, we looked at the expression of IFN-γ inducible genes, p47 GTPases LRG47 and Igtp, which are involved in parasite growth restriction during acute *T. gondii* infection (Taylor, Feng et al. 2004; Butcher, Greene et al. 2005; Martens, Parvanova et al. 2005) in the lungs, but there were no obvious differences in the expression of these genes between the 3 strains of mice.

In summary, our data shows that IL-4Rα signaling via macrophages/neutrophils is critical for survival of acute *T. gondii* infection in BALB/c mice, and the severity of disease in these mice was similar to that of IL-4Rα$^{-/-}$ mice, whereas WT mice survived. The increased mortality was associated with excessive pathology in the lungs which can be attributed to the exaggerated type-1 response. Furthermore, the susceptible of IL-4Rα$^{-/-}$ mice displayed reduced levels of FIZZ1, IDO mRNA, and a significantly increased burden of cyst mRNA (also increased conversion of tachyzoites to bradyzoites) in the lung, which may implicate the involvement of IL-4/IL-13 dependent alternatively macrophage activation and tryptophan catabolism in the resistance of acute *T. gondii* infection.

CHAPTER 4

The role of IL-4Rα signaling through $CD4^+$ T-cells during *T.gondii* infection

Abstract

The type-1 protective response against *Toxoplasma gondii* is widely reported to be dependent on both $CD4^+$ and $CD8^+$ T-cells that produce high levels of IFN-γ which activates parasite killing effector mechanisms. However, the overproduction of type-1 cytokines can lead to severe immunopathology. We have previously demonstrated the extent to which IL-4/IL-13 signaling can limit mortality and immunopathology using IL-4 and IL-4Rα deficient mice; whereby gene knock-out mice were more susceptible to infection compared with their wild-type BALB/c counterparts. Herein, we investigate whether $CD4^+$ T-cells are involved in mediating the protective response by using $CD4^+$ T-cell specific IL-4Rα deficient mice (Lck^{cre}IL-$4R\alpha^{-/lox}$). The disease phenotype during the acute phase *T. gondii* infection of the $CD4^+$ T-cell specific IL-4Rα deficient mice was similar to IL-4Rα intact (wildtype) mice as measured by mortality, systemic and local (lung) cytokine profile and immunopathology. There was also no difference in expression of markers for classical macrophage activation between either IL-4Rα deficient, $CD4^+$ T-cell specific IL-4Rα deficient mice or WT controls. However, during the chronic phase of infection, there was no lung pathology observed in mice lacking IL-4Rα on $CD4^+$ T-cells, which was significantly less than both WT and global IL-$4R\alpha^{-/-}$ mice. Overall the data suggests that IL-4 signaling through $CD4^+$ T-cells plays a disease exacerbatory role during the late stages of *T. gondii* infection.

4.1 Introduction

Interleukin-4 (IL-4) has been shown to have both disease protective (Roberts, Ferguson et al. 1996; Suzuki, Yang et al. 1996) and disease exarcebatory roles (Nickdel, Lyons et al. 2004) during *Toxoplasma gondii* infection, depending on the genetic background of the mouse and route of infection used. We have demonstrated in the previous chapter and in previous studies (appendix) that IL-4 and IL-4Rα deficient mice are more susceptible to *T. gondii* infection compared with their wildtype BALB/c littermates. The mortality is associated with an enhanced type-1 response characterized by high IFN-γ production, and increased lung immunopathology in both IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ than in WT mice.

Infection with *T. gondii* elicits a strong and persistent cell-mediated immunity, characterized by high levels of IL-12 and IFN-γ which result in parasite elimination and host protection (Denkers and Gazzinelli 1998). Acquired immunity induced by *T. gondii* is characterized by strong $CD4^+$ and $CD8^+$ T-cell activity. Athymic nude mice (lacking functional T-cells) were shown to be extremely susceptible to both virulent and avirulent strains of *T. gondii* (Gazzinelli, Hakim et al. 1991; Liesenfeld, Kosek et al. 1996). A few studies have examined the role of $CD4^+$ T-cells in the development of effector responses against *T. gondii*, and generally $CD8^+$ T-cells have been shown to play a more predominant effector role. Studies using CD4 deficient mice have shown different outcomes depending on the genetic background of the mice used. Depletion of $CD4^+$ T-cells in the susceptible C57BL/6 mice by antibody treatment exacerbated the infection

during the chronic phase (Johnson and Sayles 2002). In addition $CD4^{-/-}$ mice died more rapidly than their WT C57BL/6 counterparts and they had higher cyst burden in the brain and exhibited deficiency in IFN-γ production. In contrast, Liesenfield *et al* reported that BALB/c mice depleted of $CD4^+$ T-cells failed to develop necrosis in the ilea like the C57BL/6 mice, indicating that $CD4^+$ T-cells induce necrosis in the ilea of genetically susceptible mice and mediate mortality following oral infection (Liesenfeld, Kosek et al. 1996).

$CD4^+$ T-cells play an important synergistic role by helping maintain a robust $CD8^+$ T-cell response in most type-1 mediated infections (Denkers and Gazzinelli 1998). However, another study using C57BL/6 mice has shown that initiation of $CD8^+$ T-cell mediated immunity in $CD4^{-/-}$ mice is comparable to that of WT mice; these mice are able to resolve acute infection as efficiently as their WT counterparts, and they do not exhibit increased mortality (Combe, Curiel et al. 2005). $CD4^+$ and $CD8^+$ T-cells control parasites mainly by their production of IFN-γ (Schluter, Meyer et al. 2002). $CD4^+$ T-cells reportedly produced increased amounts of IFN-γ than $CD8^+$ T-cells (Miller, Wen et al. 2006). Nonetheless, when exposed to *Toxoplasma* antigen, $CD4^+$ T-cells also produce IL-2, IL-4 and IL-10, whereas $CD8^+$ T-cells produce predominantly IFN-γ and IL-2.

IL-4 polarizes naive $CD4^+$ T-cells into a T-helper type-2 phenotype, as opposed to a type-1 cytokine secreting profile acquired in the presence of IFN-γ. The type-2 differentiation is achieved by signaling through the IL-4Rα, STAT-6 and GATA-3

(Cunningham, Serre et al. 2004). The role of IL-4 is generally recognized to promote the expansion of Th2 cell numbers, and to suppress the emergence of a type-1 phenotype. Although a highly polarized type-1 response confers protection against death and resistance to *T. gondii* infection by activating macrophage effector mechanisms, if unregulated it can lead to development of severe immunopathology. Thus, mechanisms that act to limit the intensity and duration of the type-1 response need to be activated by the host cells.

We have shown that in the absence of IL-4/IL-4Rα signaling mice infected with *T. gondii* develop more severe lung pathology and increased mortality compared with their WT or BALB/c littermates. Furthermore, we have shown that mice lacking IL-4Rα signaling on macrophage/neutrophil populations are highly susceptible to infection with *T. gondii*, with the mortality kinetics similar to global IL-4Rα deficient mice. Based on these results, we further investigated cell specific IL-4 responses using $CD4^+$ T-cell specific IL-4Rα deficient mice to investigate whether any protective response associated with IL-4 during *T. gondii* infection is mediated by $CD4^+$ T-cells.

4.2. Results

4.2.1 Survival of Lck^{cre}IL-4Rα$^{-/lox}$ mice during acute and chronic *T. gondii* infection compared with global IL-4Rα$^{-/-}$ and WT controls.

Both male and female mice from WT, Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ groups were infected with 10 cysts of *T. gondii* (RRA) Beverly strain intra-peritoneally and were monitored for disease by daily weighing and mortality throughout the duration of the experiments. All the infected male mice from all mouse strains survived for the duration of the experiment (35 days) (Fig4.1A). However the female knock-out strains were more susceptible to infection, with 86% survival rate in Lck^{cre}IL-4Rα$^{-/lox}$ and 59% survival rate in IL-4Rα$^{-/-}$ compared with 100% survival rates in female WT littermates (Fig4.1B). Mortalities were recorded during early acute phase, starting 9 days post-infection and were more frequent between day 9 and day 14 post-infection. Although the female CD4^{+} T-cell specific IL-4Rα deficient mice appeared to be somewhat more ill than the WT littermates as they displayed more signs of lethargy and had increased bodyweight loss, the difference in survival rates was not significantly different.

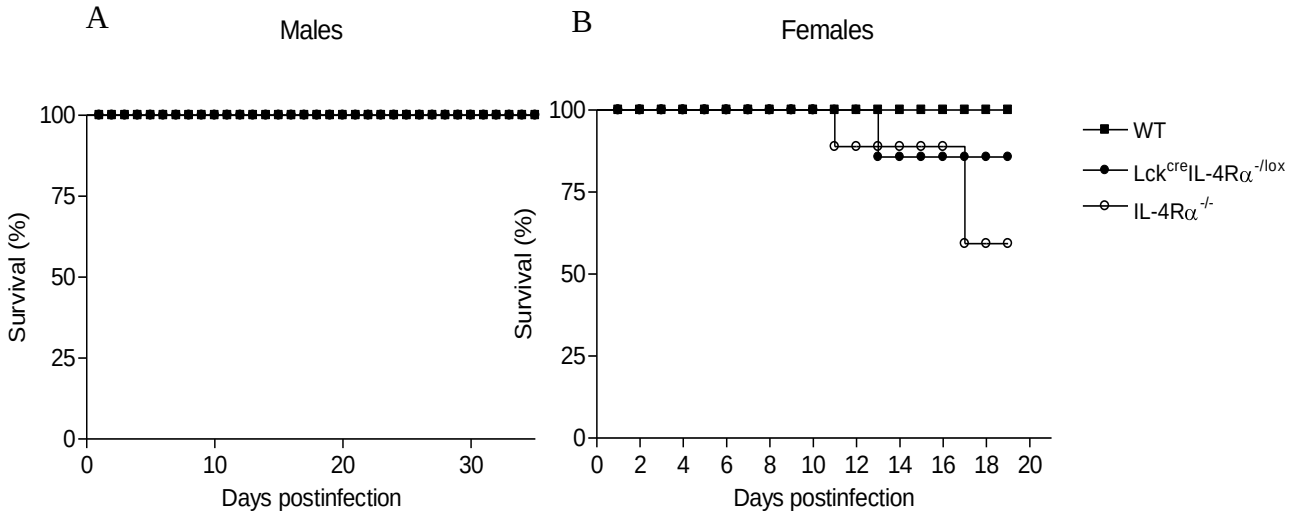


Fig.4.1 Male and female mice of the following strains, WT littermates, Lck^{cre}IL-4R$\alpha^{-/lox}$ and global IL-4R$\alpha^{-/-}$ were infected with 10 cysts of *T. gondii* Beverly strain intraperitoneally and monitored for disease over a period of 35 days. Although the male mice became very sick, no mortalities were observed for the duration of the experimental period (A). As with other studies, mortality was higher in female IL-4R$\alpha^{-/-}$ mice than their WT counterparts (B). Female mice lacking IL-4Rα signaling on $CD4^+$ T-cells were slightly more susceptible to infection compared to WT mice as measured by mortality; but over 2 experiments this was not significant.

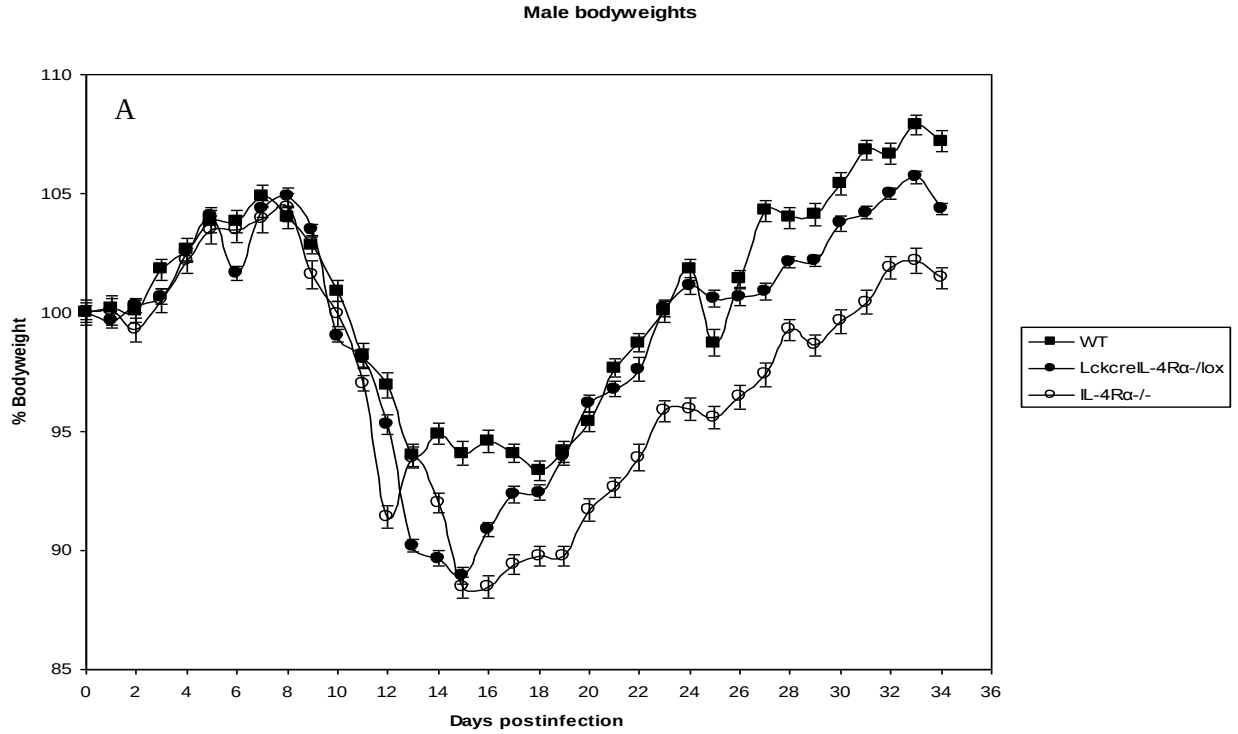
4.2.2 Bodyweights of Lck^{cre}IL-4Rα$^{-/lox}$ during acute and chronic *T. gondii* infection compared with global IL-4Rα$^{-/-}$ and WT controls.

Male mice from all three groups started displaying signs of illness during the acute phase of infection on day 8. This included unkempt pilo-erected fur, hunched backs, reduced movement, and semi-closed eyelids. Although the mice became very ill with progressive and considerable weight loss during the acute phase of infection, there were no mortalities in any of the strains (Fig.4.1A). The global IL-4Rα$^{-/-}$ mice had significantly lower bodyweights than the WT and CD4^{+} T-cell specific IL-4Rα$^{-/-}$ mice between days 9 and 15 with 12% bodyweight loss (Fig. 4.2A). IL-4Rα$^{-/-}$ mice had lower weights than CD4^{+} T-cell specific IL-4Rα$^{-/-}$ mice which had lower weights than WT mice between day 9 and 18, after which all groups of mice continued to regain their bodyweights with the CD4^{+} T-cell specific IL-4Rα$^{-/-}$ mice having a very similar weight profile to WT mice between day 18 and day 25, thereafter CD4^{+} T-cell specific IL-4Rα$^{-/-}$ mice maintained slightly lower weights than WT mice. Having lost the 12% of their bodyweight during the critical acute phase of infection, IL-4Rα$^{-/-}$ mice also regained approximately 13% of the lost weight although their average weights still remained significantly lower than those of the WT ($p=0.005$) and CD4^{+} T-cell specific IL-4Rα$^{-/-}$ up until termination of the experiment on day 35 (Fig.4.2A).

Female WT, CD4^{+} T-cell specific IL-4Rα$^{-/-}$ and global IL-4Rα$^{-/-}$ mice maintained very similar weight profiles throughout the infection (Fig. 4.2 B), although, between day13 and 16, the mean bodyweight of global IL-4Rα$^{-/-}$ mice was significantly lower than that

of WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice ($p<0.01$). There was no difference observed between the bodyweights of WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice.

Overall, male mice from all 3 strains were resistant to infection as measured by mortality with a 100% survival rate. Conversely with female mice, WT controls were resistant, but Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were more susceptible to infection with 86% and 59% survival rates recorded respectively. On the other hand, following infection male mice generally lost more bodyweights compared with female mice with exception of the WT mice which had very similar bodyweight profiles throughout infection. Male Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice lost slightly more bodyweights than female mice of the same strains. Although both male and female mice eventually recovered the weight, female mice had a more rapid recovery and there was no difference between the 3 strains at late stages of infection until termination of the experiment. Male IL-4Rα$^{-/-}$ mice on the other hand, had a delayed weight recovery relative to WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice ($p<0.01$), and the weight difference between the groups was maintained in the late time-points until the experiment was ended.


Male bodyweights
A
% Bodyweight
Days postinfection
WT
LckcrelL-4Rα-/lox
IL-4Rα-/-

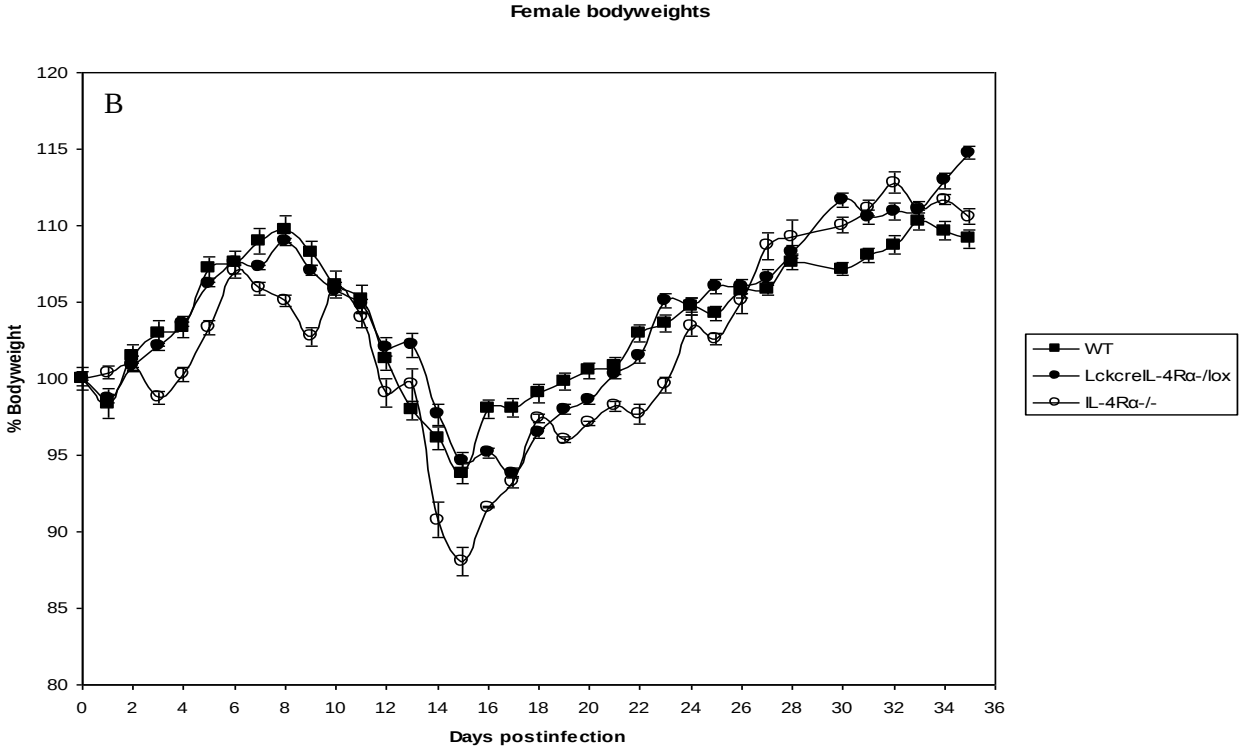

Female bodyweights
B
% Bodyweight
Days postinfection
WT
LckcrelL-4Rα-/lox
IL-4Rα-/-

Fig.4.2 Bodyweights of male and female mice infected with *T. gondii* Beverly strain intra-peritoneally with 10 cysts. During chronic phase infection in male mice, there was no significant difference between WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ mice (A). However, IL-4R$\alpha^{-/-}$ bodyweights were significantly lower than WT male mice, p=0.0029 (A). During acute infection in female mice (B), IL-4R$\alpha^{-/-}$ mice lost significantly more weight compared to WT and Lck^{cre}IL-4R$\alpha^{-/lox}$, with the lowest weight recorded at day 15, after which mice started to recover their bodyweight to match those of their WT counterparts.

4.2.3 Histopathological analysis during acute *T. gondii* infection

To further study the disease phenotype, the lung and liver tissues of *T. gondii* infected mice from all three strains were examined for histopathology. For male mice, two time points were examined, day 9, which represents a time point when the mice start to show severe signs of illness and day 35, a chronic phase time point where all mice had recovered the significant bodyweight, and appeared healthy although the weight of IL-4Rα$^{-/-}$ mice remained significantly less than the other two strains. For female mice, two early phase time points were examined, day 9 which represents the stage where mice started showing severe signs of illness and day 12 which was the stage when female mice were gravely ill and deaths occurred. We were unable to examine female mice at the chronic phase of infection as generally numbers were too few at this stage (n=2) due to early deaths.

The lung, liver and brain tissue of *T. gondii* infected WT, Lck^{cre}IL-4Rα$^{-/lox}$ and global IL-4Rα$^{-/-}$ male mice were removed from mice and tissue sections were analyzed and scored for histopathology. At day 9 post-infection, all three groups displayed mild to moderate degrees of inflammation in the lungs. Global IL-4Rα deficient mice exhibited significantly increased peribronchial inflammation (p=0.0325) compared to the absent to minimal inflammation observed in CD4^{+} T-cell specific IL-4Rα deficient mice and WT littermates (Table 4.1) (Fig. 4.1A). There were high degrees of interstitial pneumonitis in all 3 strains, although there was no significant difference in the severity in WT, CD4^{+} T-cell specific IL-4Rα deficient and global IL-4Rα deficient mice at day 9 post-infection

(Fig.4.1B). The pathological changes in the liver were more enhanced than the lungs at this time with moderate and severe inflammation of portal tracts and acini noted respectively. There was, however, no significant difference in the severity of hepatic inflammation between WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and global IL-4R$\alpha^{-/-}$ at this time point (Fig.4.1C, D) (Table 4.1).

Table 4.1 Histopathology in the lungs and livers of male mice following *T. gondii* infection at day 9 post infection

	Histopathological scores for:			
	Lungs		Liver	
Mouse strain	Peribronchial inflammation	Interstitial pneumonitis	Portal tracts	Acini
Wildtype	1	3	3	3
	0	2	2	3
	1	3	2	4
	0	2	2	4
	1	3	2	4
	1	3	1	4
$Lck^{cre}IL\text{-}4R\alpha^{-/lox}$	1	2	2	3
	1	3	2	3
	1	3	2	3
	0	3	2	3
	0	3	2	3
	1	3	2	4
$IL\text{-}4R\alpha^{-/-}$	1	2	1	3
	2	3	2	4
	1	3	2	3
	2	3	2	4
	1	2	2	4
	2	3	2	4

Table 4.1 Male mice were killed 9 days post-infection and examined for interstitial and peribronchial inflammation in the lungs and portal tract and acini inflammation in the liver. $IL\text{-}4R\alpha^{-/-}$ mice had significantly increased peribronchial inflammation ($p=0.021$) compared to the WT and $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice. There was no significant difference in hepatic pathology between all three strains at this time point. Both the lung and liver tissues had comparable severity of inflammation between the 3 strains.

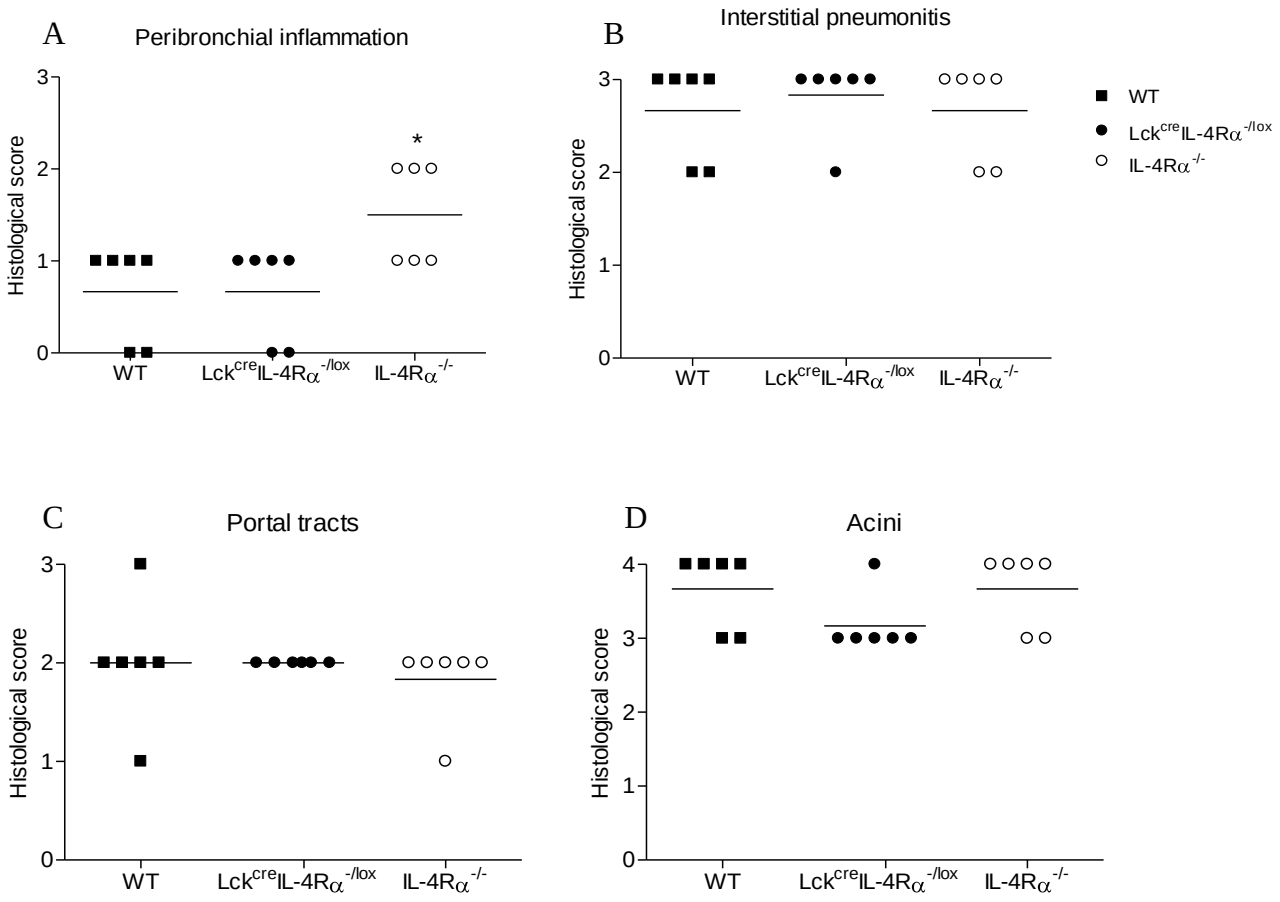


Fig.4.1 Histopathology of lungs and liver sections from male mice 9 days post-infection (graphical representation of Table 4.1). Lung peribronchial (A), and interstitial (B) inflammation and liver inflammation in the portal tracts (C) and acinar (D) at day 9 post-infection with *T. gondii*. Results are representative of two independent experiments with similar results.* $p<0.05$ compared with WT and $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$.

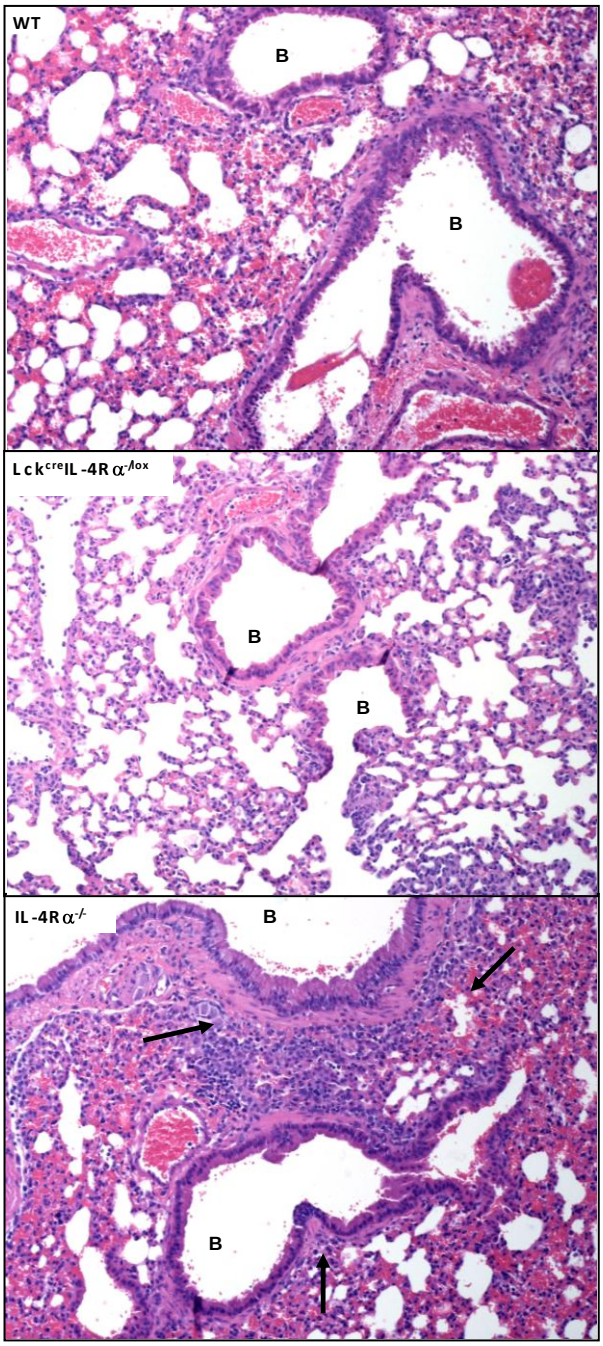


Fig.4.1A Lung tissue sections taken from male WT (top), Lck^{cre}IL-4Rα$^{-/lox}$ (middle) and IL-4Rα$^{-/-}$ (bottom) mice infected with *T. gondii* 9 days post-infection. WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice displayed minimal inflammation, whilst lungs from IL-4Rα$^{-/-}$ mice displayed significantly higher degrees of peribronchial inflammation following infection (arrows, B= bronchiole).

Female mice infected with *T. gondii* displayed comparable levels of lung and hepatic inflammation to the male mice 9 days post-infection. Lungs from infected female WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ mice had absent to minimal degrees of peribronchial inflammation; there was no significant difference between the 3 strains (Fig.4.2A). Similarly, there was an equivalent mild to moderate degree of interstitial pneumonitis in the lungs of mice from all three strains at day 9 post-infection(Fig.4.2B) (Table 4.2). In addition, there was moderate degrees hepatic inflammation as measured by portal tract and acini inflammation (Fig.4.2C, D). There were no significant differences in pathology of the lungs and livers from WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ female mice infected with *T. gondii* 9 days post infection (Table 4.2).

Overall, at day 9 post-infection WT and $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ male mice displayed minimal peribronchial inflammation whilst $IL\text{-}4R\alpha^{-/-}$ male mice had significantly increased peribronchial inflammation. With female mice, there was the same degree of peribronchial inflammation between the 3 strains, all lung tissues displayed minimal degrees of peribronchial inflammation at 9 days post-infections. In addition, there was no difference observed between male and female mice in terms of interstitial lung inflammation, hepatic portal and acinar inflammation.

At day 12 post infection, *T. gondii* infected female mice displayed similar degrees of inflammation in the lungs and in the liver to that observed at day 9 post infection (Table 4.3). There was minimal inflammation to the peribronchium (Fig.4.3A) and moderate

degrees of interstitial pneumonitis in the lungs of WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice (Fig.4.3B). Furthermore the portal tracts in the liver displayed mild degrees of inflammation (Fig.4.3C) whilst there was moderate acinar inflammation (Fig.4.3D). Overall, there was no difference in the degrees of inflammation to the lungs and liver between *T. gondii* infected female WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice at day 12 post infection, and in addition the degree of inflammation observed in WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ female mice at this time point was comparable to that observed at 9 days post infection.

Table 4.2 Histopathology in the lungs and livers of female mice following *T. gondii* infection at day 9 postinfection

	Histopathological scores for:			
	Lungs		Liver	
Mouse strain	Peribronchial inflammation	Interstitial pneumonitis	Portal tracts	Acini
Wildtype	0	2	1	2
	0	1	2	3
	1	3	2	4
	0	1	2	3
	0	2	2	2
	1	3	2	4
	1	2	2	3
$Lck^{cre}IL\text{-}4R\alpha^{-/lox}$	0	1	2	3
	0	1	2	4
	0	1	2	3
	0	1	2	3
	1	3	2	4
	1	3	2	4
	1	3	2	3
$IL\text{-}4R\alpha^{-/-}$				
	1	3	2	3
	1	3	2	3
	1	3	2	4
	0	2	2	3
	0	1	2	2

Table 4.2 Sections of lungs and liver from female mice were analyzed for pulmonary and hepatic pathology 9 days post-infection with *T. gondii*. There was mild peribronchial inflammation and moderate interstial pneumonitis in all 3 groups of mice. In the liver, the portal tracts displayed moderate to severe levels of inflammation, whilst there was severe inflammation of the acini. However there was no significant difference between WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ lung and liver immunopathology.

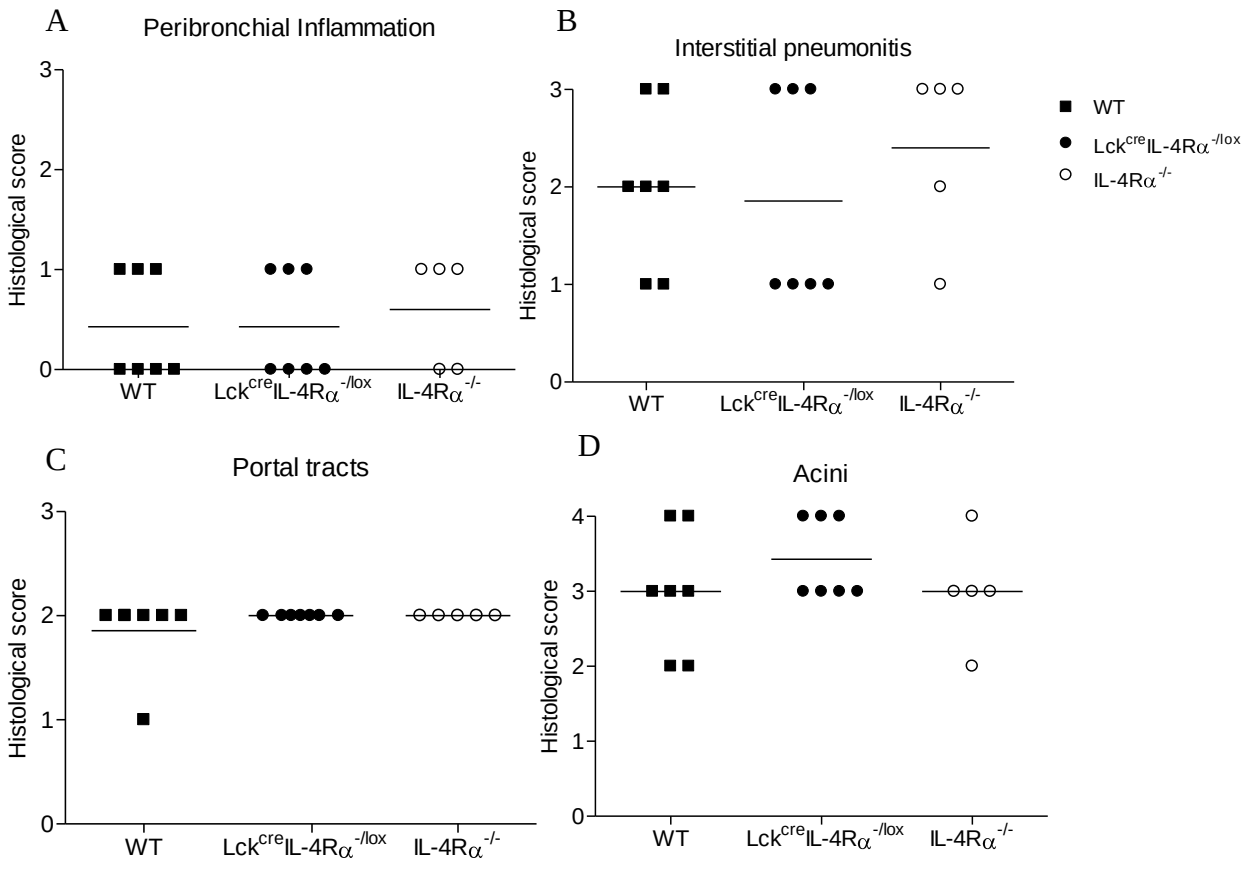


Fig.4.2 Histopathological score for lung and liver sections from *T. gondii* infected female mice 9 days post-infection (Table 3.2), peribronchial inflammation (A), interstitial pneumonitis (B), portal tract (C) and acinar (D) inflammation. Results are representative of two experiments with similar results. There was no significant difference between the 3 mouse strains.

Table 4.3 Histopathology in the lungs and livers of female mice following *T. gondii* infection at day 12 post-infection

	Histopathological scores for:			
	Lungs		Liver	
Mouse strain	Peribronchial inflammation	Interstitial pneumonitis	Portal tracts	Acini
Wildtype	1	3	1	3
	1	3	1	3
	1	3	1	3
	1	3	1	3
	1	3	1	3
	1	3	1	3
$Lck^{cre}IL\text{-}4R\alpha^{-/lox}$	0	2	1	3
	1	3	1	3
	1	3	1	3
	0	2	1	3
$IL\text{-}4R\alpha^{-/-}$	1	3	1	3
	1	3	1	2
	1	3	1	3
	0	2	1	3
	1	3	1	4

Table 4.3 Sections of lungs and livers from female mice were analyzed for pulmonary and hepatic pathology 12 days post-infection with *T. gondii*.

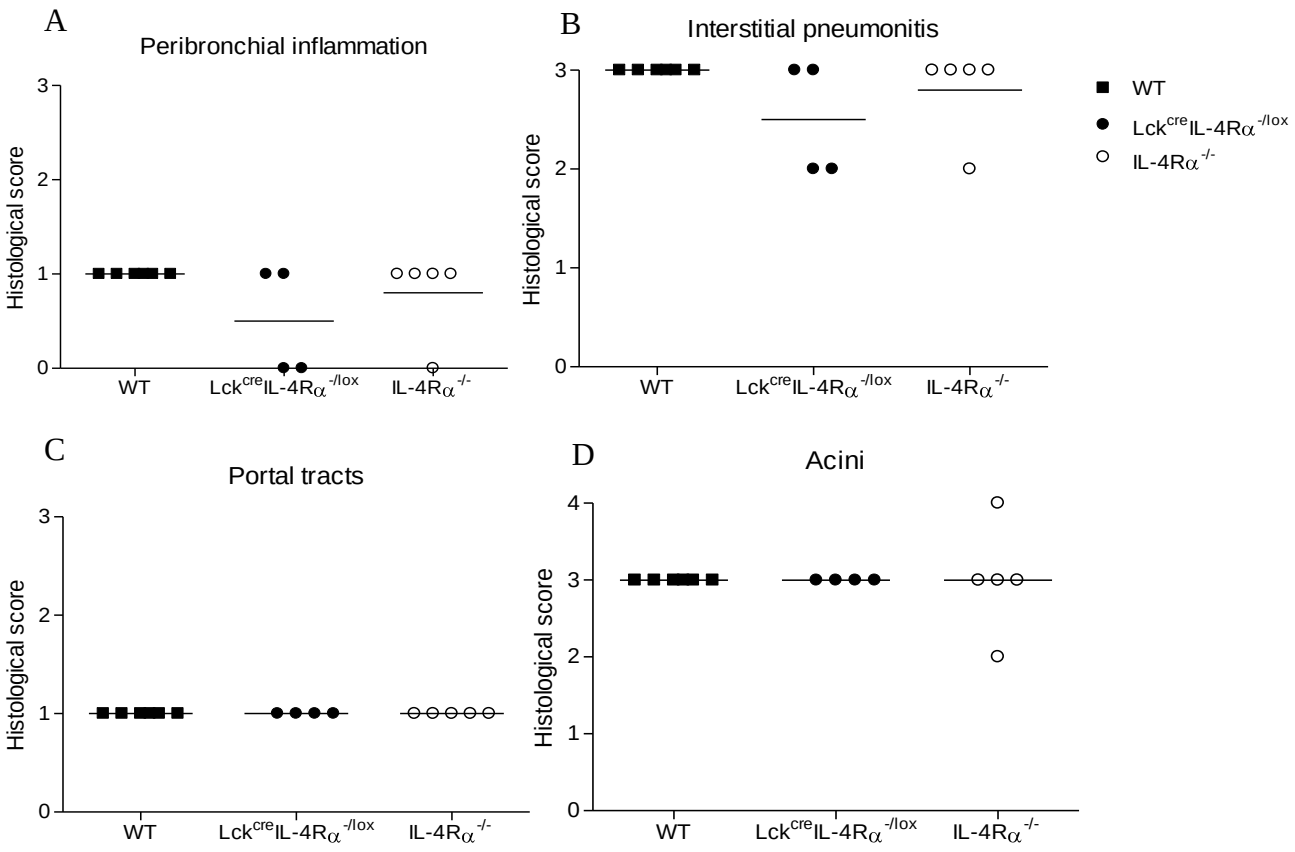


Fig.4.3 Histopathology of lung and liver sections from female WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ mice12 days post-infection with 10 cysts of *T. gondii* intraperitoneally; lung peribronchial (A) and interstitial (B) inflammation and liver portal tract (C) and acinar inflammation (D). Results are representative of two experiments with similar results. There was no significant difference observed between the 3 strains.

4.2.4 Systemic cytokine levels in *T. gondii* infected male and female mice

Blood was collected by cardiac puncture to measure systemic levels of type-1 and type-2 cytokines in the sera by using sandwich ELISA. Cytokine production was examined at day 9 post-infection for male mice, and at day 9 and 12 post-infection for female mice. At day 9 post-infection, IFN-γ production was highly up-regulated in the sera of all infected WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ male (Fig.4.4A) and female (Fig.4.4B) mice, compared with the uninfected controls, but there was no difference between the 3 strains in either males or female at day 9 post-infection with *T. gondii*. Female mice had significantly higher levels of systemic IFN-γ (approximately 30-fold higher) compared with IFN-γ levels of male mice at 9 days post-infection. At day 12 post-infection, systemic IFN-γ levels in female mice were reduced over 30-fold compared with the levels observed in infected female mice at day 9 post-infection (Fig.4.4C). IL-4R$\alpha^{-/-}$ had significantly elevated levels of IFN-γ compared with WT mice ($p=0.026$) but not with Lck^{cre}IL-4R$\alpha^{-/lox}$ mice ($p=0.45$). There was also no significant difference in the level of systemic IFN-γ between Lck^{cre}IL-4R$\alpha^{-/lox}$ and WT mice at this time point ($p=0.057$). Overall, females have a stronger systemic IFN-γ response at day 9 post-infection compared with male mice at this time point, there was no significant difference in systemic IFN-γ between WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ in either male or female mice at day 9 post-infection. At day 12 the systemic IFN-γ response in female mice is down-regulated, and IL-4R$\alpha^{-/-}$ mice have significantly increased levels of IFN-γ compared with WT mice (Fig.4.4).

There was no significant difference in IL-12p40/70 levels between WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ male mice at day 9 post-infection (Fig.4.5A). On the other hand, female $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice had significantly increased systemic IL-12 levels compared with WT mice ($p<0.05$) but not with $IL\text{-}4R\alpha^{-/-}$ mice at day 9 post-infection (Fig.4.5B) Furthermore, systemic IL-12p40/70 levels were reduced at day 12 post-infection (Fig.4.5C) compared with at day 9 post-infection in female mice but there was no difference between the 3 strains nonetheless.

IL-4 was not detected in the sera of WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ male mice at day 9 post-infection (Fig.4.6A), and also not in female WT and $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice,however there was a low but measurable amount of IL-4 detected in $IL\text{-}4R\alpha^{-/-}$ female mice 9 days post-infection (Fig.4.6B). At day 12 post-infection there was no IL-4 detected in the sera of female mice of all 3 strains (Fig.4.6C). Similarly, IL-10 was not detected in the sera of both male and female mice of all strains after 9 and 12 days post-infection with *T. gondii* (Fig.4.6D, E and F).

Overall, in female mice, circulating type-1 cytokine levels were considerably higher at day 9 post-infection compared to that detected at day 12 post-infection, as well as compared to the levels detected in male mice at day 9 post-infection. Although there was no significant difference in IFN-γ concentrations between all 3 strains of mice at day 9 post-infection in both male and female mice, IFN-γ levels were significantly higher in female $IL\text{-}4R\alpha^{-/-}$ mice in comparison with WT and $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice at day 12

post-infection. In addition, at day 12 post-infection, production of type1 cytokines in *T. gondii* infected female mice was markedly reduced compared with day 9 post-infection.

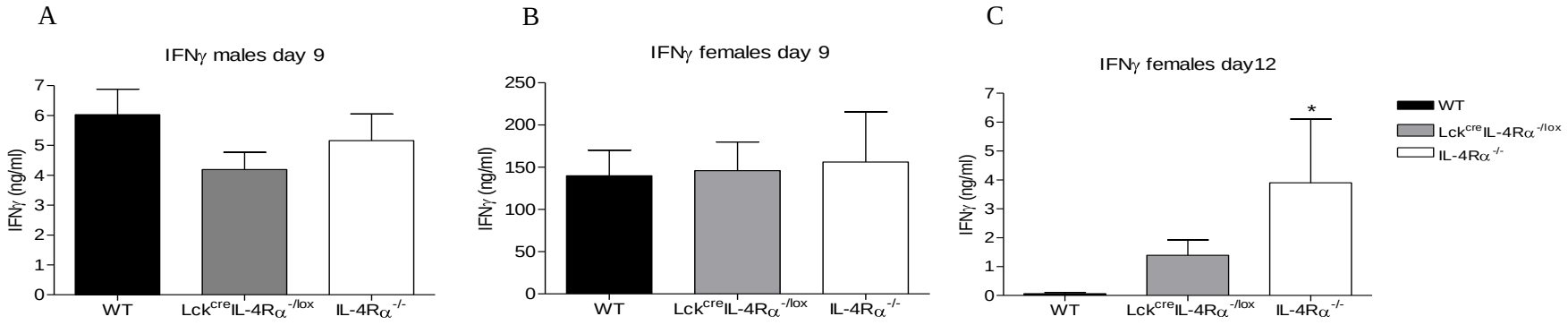


Fig.4.4 IFN-γ levels in sera from male mice 9 days post-infection (A), female mice 9 days post-infection (B) and female mice 12 days post-infection (C) with *T. gondii*. *p<0.05 compared with WT controls.

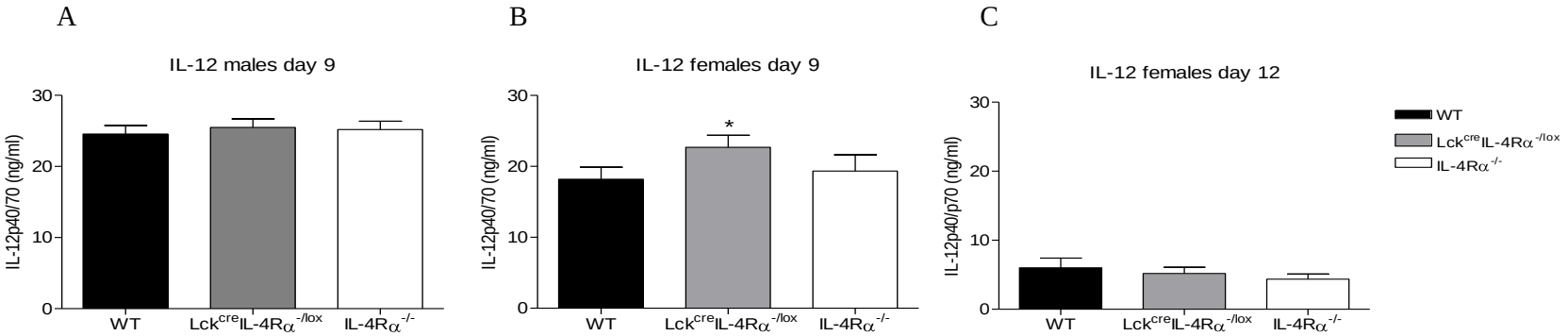


Fig.4.5 IL-12p40/70 levels in sera from male mice 9 days post-infection (A) female mice 9 days post-infection (B) and female mice 12 days post-infection (C) with *T. gondii*. *$p<0.05$ compared with WT controls.

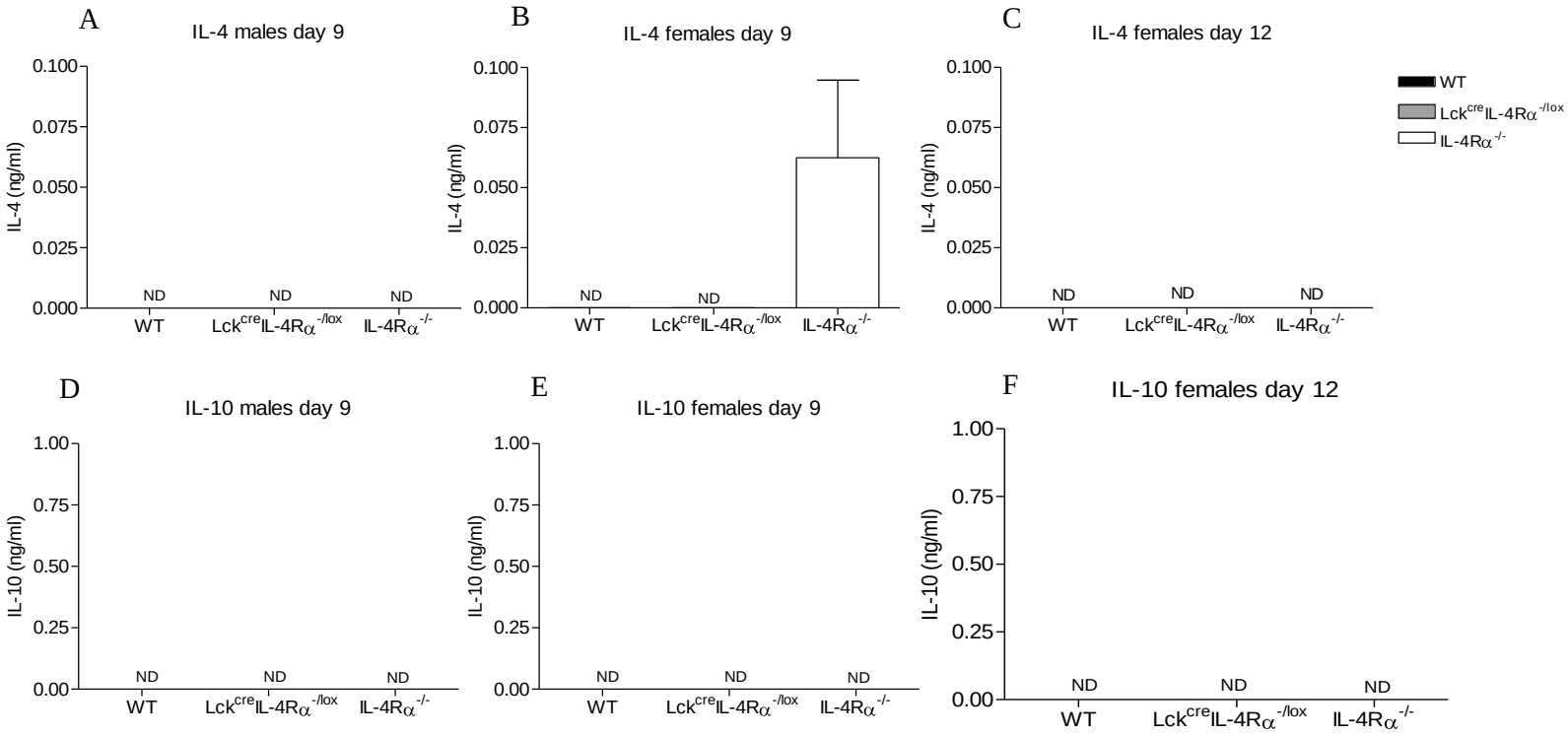


Fig. 4.6 Serum IL-4 and IL-10 levels in male and female mice 9 and 12 days post-infection with *T. gondii*. Male serum IL-4 9 days post-infection (A), female IL-4 after 9 days (B) and 12 days (C) post-infection. Serum IL-10 in male mice 9 days post-infection (D), female serum IL-10 9 days (E) and 12 days (F) post-infection. ND: not detected.

4.2.5 Analysis of *T. gondii*-induced splenocyte cytokine production

To measure *T. gondii* specific cytokine production by splenic T-cells, spleens from infected mice were removed from uninfected and infected WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ at day 9 for male mice and days 9 and 12 post-infection for female mice. Splenocytes were cultured *in vitro* and stimulated with *Toxoplasma* lysate antigen (TLA). The culture supernatants were subsequently assayed for nitric oxide (NO) by Griess reaction and IFN-γ, IL-12p40/p70, IL-10 and IL-4 production by ELISA.

At 9 days post-infection, splenocytes from infected male mice produced NO as measured by nitrite levels whether they were unstimulated, stimulated with TLA or ConA (Fig.4.7A). NO production was reduced in the IL-4Rα knock-out strains compared with their WT counterparts. Splenocytes from male Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice produced significantly lower NO compared with WT mice ($p=0.0022$ and $p=0.001$ respectively). In addition, splenocytes from global IL-4R$\alpha^{-/-}$ mice produced significantly lower NO than Lck^{cre}IL-4R$\alpha^{-/lox}$ mice ($p=0.0014$).

NO production by splenocytes from female mice was generally lower compared to comparative male cultures. However, similar to male mice, splenocytes from female Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice produced significantly lower levels of NO compared to WT mice (Fig.4.7 B). At day 12 post-infection, splenocytes from *T. gondii* infected mice had a more pronounced NO response. Similarly to day 9 post-infection in female mice, NO production was markedly reduced ($p=0.002$) in IL-4R$\alpha^{-/-}$ splenocytes

compared to their WT counterparts after 12 days post-infection in female mice (Fig.4.7C). NO production in female Lck^{cre}IL-4R$\alpha^{-/lox}$ mice was equivalent to the levels measured in WT splenocyte at day 12 post-infection (Fig.4.7)

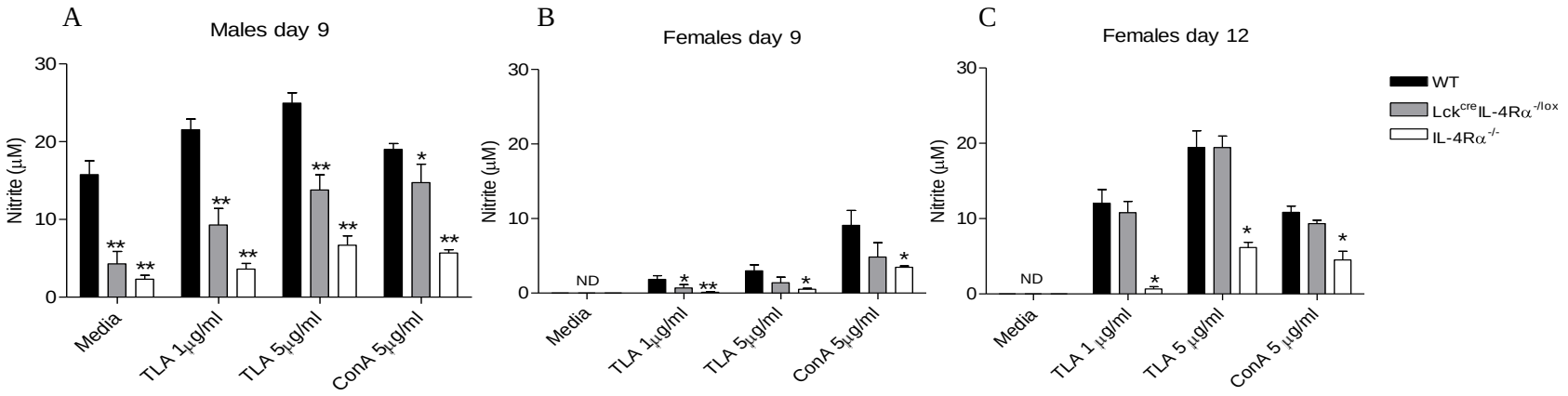


Fig.4.7 Splenocyte NO production 9 days post-infection in (A) males, (B) females and in female mice 12 days post-infection (C). Splenocyte NO production was significantly reduced in mice lacking IL-4Rα on $CD4^+$ T-cells (p=0.002) and in global IL-4Rα deficient mice compared with WT (p=0.001) for male mice. At day 9 post-infection TLA-stimulated splenocytes from female Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4Rα mice had significantly lower NO levels compared with WT mice (B), however at day 12 post-infection (C) splenocytes from female Lck^{cre}IL-4R$\alpha^{-/lox}$ mice produced similar levels of NO as WT mice, whereas IL-4R$\alpha^{-/-}$ mice had significantly reduced NO levels compared with both WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ mice at this time point.*p<0.05,**p<0.01 compared with corresponding WT

There was IFN-γ production in unstimulated, TLA stimulated and ConA stimulated spleen cells from infected male mice from all 3 strains 9 days post-infection (Fig. 4.8A). Non-stimulated splenic T-cells from IL-4Rα$^{-/-}$ male mice produced significantly higher amounts of IFN-γ compared with their WT and Lck^{cre}IL-4Rα$^{-/lox}$ counterparts (p=0.015). There was no difference in IFN-γ production in TLA stimulated splenocytes between the 3 strains; however splenocytes from IL-4Rα$^{-/-}$ male mice produced significantly increased IFN-γ compared with WT and Lck^{cre}IL-4Rα$^{-/lox}$ splenocytes in response to ConA stimulation (p=0.002). In addition there was a dose-dependent specific increase in antigen-induced IFN-γ compared with media alone. Splenocytes from female mice did not produce any IFN-γ at day 9 when unstimulated and in response to TLA stimulation at 1μg/ml (Fig. 4.8 B). There was IFN-γ production by splenocytes from all 3 strains when stimulated with 5μg/ml of TLA (about 3-fold lower compared with male splenocytes stimulated with the same TLA concentration). ConA stimulated cultures from female mice produced high IFN-γ levels, comparable with that of male mice; however there was no difference in IFN-γ production between the 3 strains in females as observed in male mice. IFN-γ production was more enhanced in WT, Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ in response to TLA stimulation at day 12 post-infection compared with day 9 post-infection (Fig. 4.8 C). There was however no significant difference in IFN-γ production at day 12 post-infection between the 3 strains. Overall at 9 days post-infection male splenocytes produce significantly more IFN-γ than their female counterparts irrespective of IL-4Rα deficiency. Female mice have a minimal response to

TLA at day 9 and produce higher amounts of IFN-γ equivalent to male mice by day 12 post-infection (Fig. 4.8).

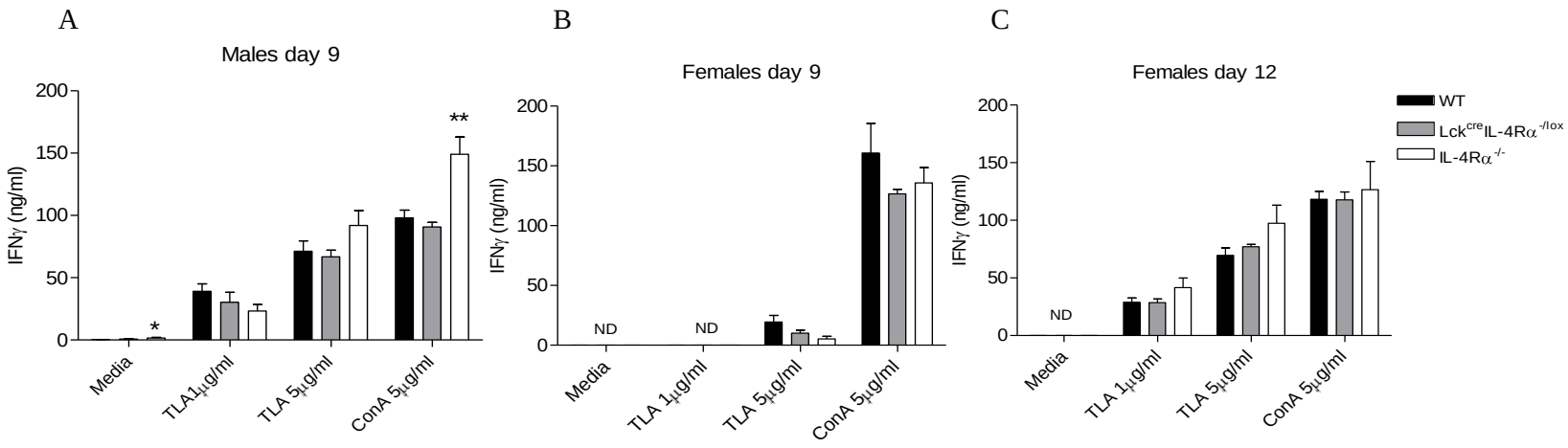


Fig.4.8 Splenocyte IFN-γ production in male mice 9 days (A) female mice 9 days (B) and female mice 12 days (C) post-infection with *T. gondii*. Splenocyte from WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ male (A) and female (B) mice were either unstimulated or treated with 1μg/ml or 5μg/ml TLA, or ConA at 5μg/ml for 72 hours, and IFN-γ production measured thereafter. Splenocytes from male amd female mice display a dose-dependent increase in TLA-induced IFN-γ production. *$p<0.05$, **$p<0.01$ compared with corresponding WT

IL-12p40/p70 production was highly upregulated in non-stimulated, TLA and ConA stimulated IL-4R$\alpha^{-/-}$ splenocytes compared with WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ splenocytes from male mice (p=0.0022) 9 days post-infection (Fig.4.9 A). WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ splenocytes produced lower and similar levels of IL-12 at this time point. In contrast, non-stimulated and TLA stimulated splenocytes derived from female IL-4R$\alpha^{-/-}$ mice produced significantly reduced levels of IL-12p40/70 ($p<0.01$) compared with WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ mice at this time point (Fig.4.9B). At day 12 post-infection, antigen induced IL-12p40/70 production was significantly higher in WT splenocyte compared to the low levels of Lck^{cre}IL-4R$\alpha^{-/lox}$ (p=0.0079) and IL-4R$\alpha^{-/-}$ (p=0.031) splenocytes in response to high dose antigen (TLA 5μg/ml) stimulation (Fig.4.9C). At this time point, non-stimulated, and TLA (1μg/ml) and ConA (5μg/ml) stimulated splenocytes produced very low (approximately 10-fold less) IL-12p40/70 compared with the slightly elevated levels at day 9 post-infection. Generally, IL-12p40/70 levels were markedly reduced at both at day 9 and day 12 post-infection in splenocytes from female IL-4R$\alpha^{-/-}$ mice compared with the level measured in male IL-4R$\alpha^{-/-}$ splenocytes at day 9 post-infection (Fig.4.9).

Overall, at day 9 post-infection there was a more pronounced, antigen-specific type-1 response in *T. gondii* infected male mice than in female mice as measured by IFN-γ production. Female splenocytes responded very weakly to TLA at day 9 post-infection and only show an antigen-specific type-1 response by day 12. Spleens from infected male mice generally produced more NO and IL-12p40/70 in response to TLA compared

with the levels detected in splenocytes from female mice.

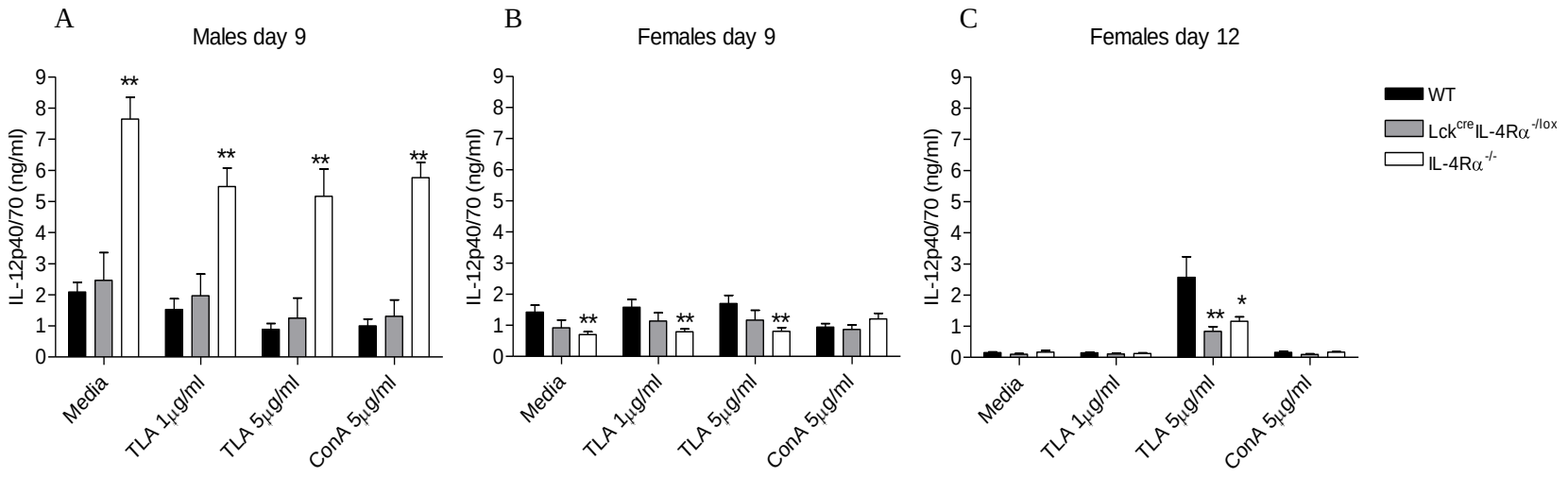


Fig.4.9 IL-12p40/70 production by splenocyte derived from spleens of male (A) and in female mice (B) 9 days post-infection with *T. gondii* and in female mice 12 days post-infection (C). *p<0.05, **p<0.01 compared with corresponding WT

Splenocytes from infected male IL-4Rα$^{-/-}$ mice produced significantly higher levels of IL-4 produced compared with WT and Lck^{cre}IL-4Rα$^{-/lox}$ male mice in non-stimulated (p=0.0043), TLA stimulated and ConA stimulated splenocytes (p=0.0011) at 9 days post-infection (Fig.4.10A). In contrast, splenocytes from infected female mice did not respond to stimulation with various doses of TLA, but responded to ConA stimulation, with regard to IL-4 production (Fig.4.10B). IL-4 production was increased in ConA stimulated splenocytes from WT and global IL-4Rα$^{-/-}$ female mice, but was markedly reduced in splenocytes derived from CD4^{+} T-cell specific IL-4Rα deficient mice (p=0.032) at day 9 post-infection (Fig.4.10B). At day 12 post-infection, splenocytes from female mice did not produce IL-4 when stimulated with various doses of TLA but with only with ConA similarly to the observation at day 9 post-infection (Fig.4.10C). However, IL-4 levels were significantly higher in IL-4Rα deficient mice (p=0.0087) compared to WT and Lck^{cre}IL-4Rα$^{-/lox}$ splenocytes at this time point (Fig.4.10C).

Finally, in infected male mice, IL-10 production was comparable in all three mouse strains in TLA stimulated splenocytes. However, for splenocytes stimulated with ConA, WT produced significantly higher levels of IL-10 compared with Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ splenocyte cultures 9 days post-infection (p=0.0043) (Fig.4.11A). There is also a dose-dependent increase in TLA-induced IL-10 production by splenocytes derived from male mice but not females. Splenocytes derived from infected female mice 9 days post-infection produced lower levels of IL-10 compared with male mice when stimulated with TLA and ConA (Fig.4.11B), and there was no difference between all 3

strains for non-stimulated or stimulated splenocytes from infected female mice. At day 12 post-infection, IL-10 production by splenocytes from all 3 mouse strains remained low, and there was no significant difference between WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice at this time point (Fig.4.11C). In summary, splenocytes from male mice have a slightly more pronounced antigen-specific type-2 cytokine response compared with female mice. Overall males express a higher type-2 or regulatory response than females including an antigen-specific IL-10 induction.

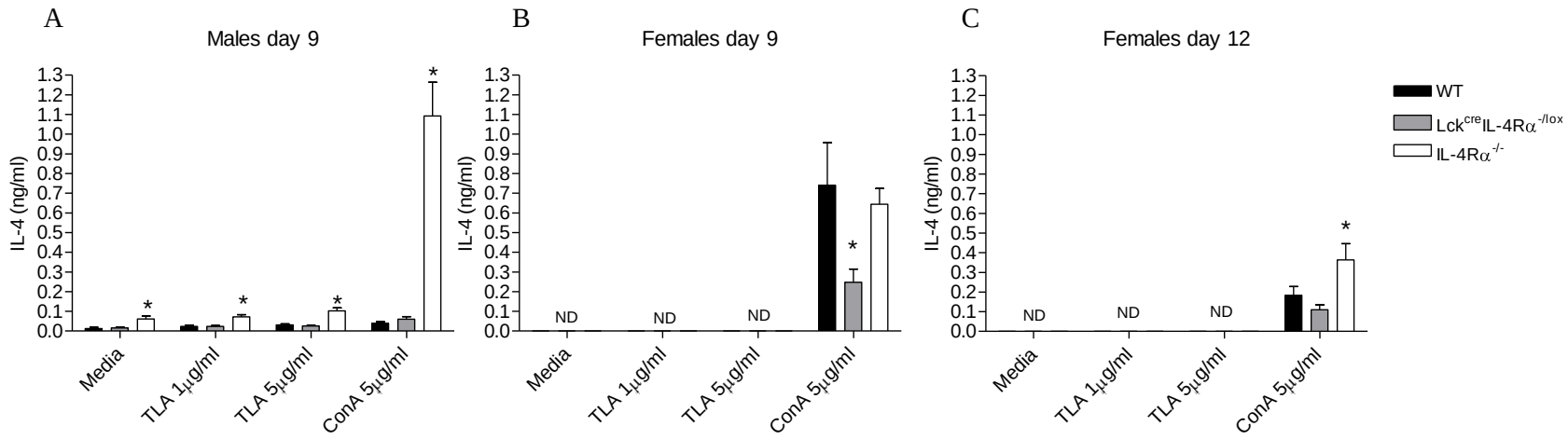


Fig.4.10 Splenocyte IL-4 production after 9 days post-infection in WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ male (A) and female (B) splenocytes and in female mice 12 days post-infection (C). *$p<0.05$ compared with corresponding WT

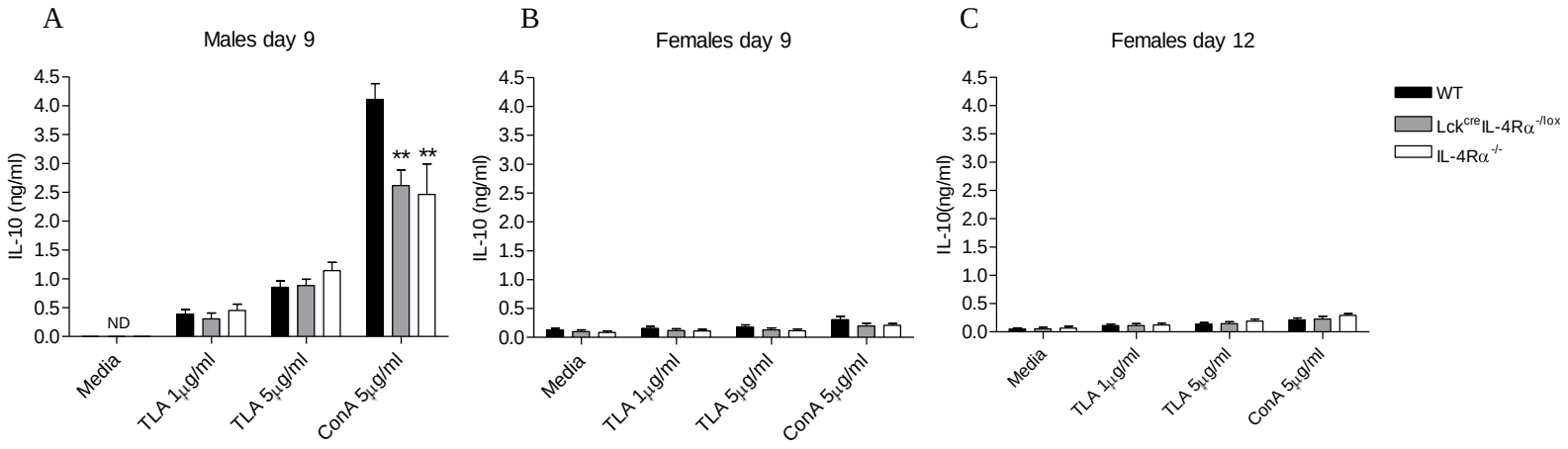


Fig. 4.11 IL-10 production by splenocytes derived from WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and IL-4Rα male (A) and female mice (B) 9 days post-infection and female mice 12 days post-infection (C) with *T. gondii*. **p<0.01 compared with corresponding WT

4.2.6 Measurement of cytokine and parasite expression in tissues using Real Time PCR

As our previous studies had implicated the lungs as the major tissue site involved in increased pathology in IL-4Rα$^{-/-}$ mice, we examined these tissues in further detail although increased pathology was only observed in *T. gondii* infected male mice in this study. In particular, we measured markers of alternative activation which reportedly require signaling via IL-4Rα as well as markers and inducers of classical activation all of which have been implicated in modulating *T. gondii* infection. Accordingly, lungs from infected mice were harvested and used to extract mRNA, which was subsequently used to make cDNA, and classical and alternative activation were measured using specific markers for the different activation states by means of quantitative Real time PCR.

The following cytokines and molecules were quantified in the lungs of *T. gondii* infected mice, IFN-γ, IL-12p40, NOS2, YM1, FIZZ1, Arginase1, at day 9 post-infection for male mice, and days 9 and 12 post-infection for female mice. At day 9 post-infection, IL-4Rα$^{-/-}$ male mice had significantly reduced levels of IFN-γ in the lungs relative to WT mice ($p=0.02$), whereas Lck^{cre}IL-4Rα$^{-/lox}$ mice had similar levels of IFN-γ mRNA transcripts as WT mice (Fig.4.12A). At the same time point, the levels of expression of IFN-γ mRNA transcripts were much lower (~3-fold magnitude) in the lungs of *T. gondii* infected female mice (Fig.4.12B) compared with male mice 9 days post-infection, and

there was also no difference between the 3 strains (Fig.4.12B). IFN-γ mRNA transcripts were increased 4-fold in the lungs of *T. gondii* infected female mice at day 12 post-infection compared with female mice at day 9 post-infection (Fig.4.12C). However, there was no difference in the level of transcript expression between WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ mice at this time point as well.

IL-12p40 mRNA expression was highly up-regulated in the lungs of infected male mice 9 days post-infection, however there was no difference between the 3 mouse strains (Fig.4.12D). In female mice IL-12p40 mRNA transcript expression at day 9 post-infection appeared to be less than the levels in male mice at the same time point, although there was no significant difference in expression in the lungs of female WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ mice (Fig.4.12E). IL-12p40 mRNA transcripts were upregulated over 10-fold in the lungs of female mice at day 12 post-infection in comparison with day 9 post-infection, but there was no difference between the 3 strains at this time point (Fig.4.12F).

Arginase and NOS compete for the same substrate, arginine, and are both induced in macrophages depending on the type-1/type-2 cytokine balance. Thus, we compared the levels of NOS2 and Arginase 1 mRNA transcripts and the other markers of alternative macrophage activation YM1 and FIZZ1 expression in the lungs of male and female mice at day 9 and day 12 post-infection with *T. gondii*. NOS2 mRNA expression was up-regulated in the lungs of male and female WT, $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ mice at

day 9 post-infection (Fig.4.13A, B), however there was no difference between the 3 strains. At day 12 post-infection, NOS2 mRNA transcripts were upregulated approximately 10-fold in the lungs of female mice in comparison with day 9 post-infection, but there was no difference between the 3 strains (Fig.4.13C).

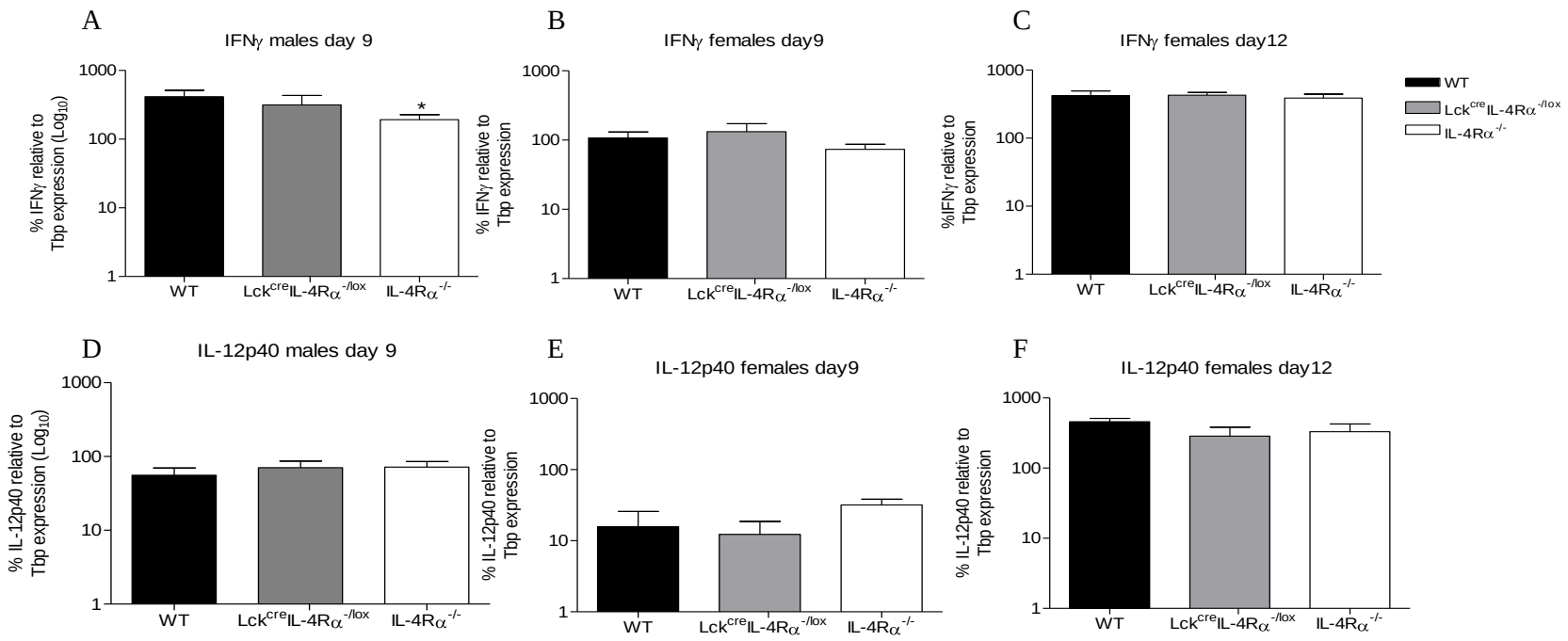


Fig.4.12 Expression of lung IFN-γ mRNA transcripts in male mice (A), and female mice (B) 9 days post-infection, and in female mice 12 days post-infection (C). Lung IL-12p40 transcript levels in male mice (D) and female mice (E) 9 days post-infection and in female mice 12 days post-infection (F), as measured by Real time PCR. *$p<0.05$ compared with corresponding WT

Lung arginase 1 mRNA levels were generally higher in *T. gondii* infected male and female mice irrespective of IL-4Rα deficiency compared with the NOS2 mRNA levels in the lung. Nevertheless, similarly to NOS2 mRNA transcript expression, there was no significant difference in the expression levels of Arginase1 mRNA transcripts in the lungs of *T. gondii* infected male mice at day 9 post-infection (Fig.4.13D) or in female mice after 9 days (Fig.4.13E) or even 12 days (Fig.4.13F) post-infection.

YM1 mRNA transcript expression was more abundant in male mice than in female mice at day 9 post-infection. Furthermore, YM1 mRNA transcripts were significantly increased ($p=0.026$) in the lungs of infected Lck^{cre}IL-4R$\alpha^{-/lox}$ male mice compared with WT controls at day 9 post-infection (Fig.4.14A), whereas in contrast, YM1 mRNA transcripts were significantly reduced in the lungs of female Lck^{cre}IL-4R$\alpha^{-/lox}$ mice compared with female WT ($p=0.025$) and IL-4R$\alpha^{-/-}$ mice ($p=0.005$) (Fig.4.14B) at day 9 post-infection. By day 12 post-infection lung YM1 mRNA transcript levels in female mice were increased (~100-fold) compared with day 9 post-infection, however there was no difference observed between the 3 mouse strains at this time point (Fig.4.14C).

In addition, male expression of lung FIZZ1 mRNA transcripts was generally more abundant than female mice at day 9 post-infection. Nevertheless there was no difference observed in the level of lung FIZZ1 mRNA transcripts between WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ in either male mice (Fig.4.14D) or female mice (Fig.4.14E) at day 9 post infection. By day 12 post-infection, FIZZ1 mRNA transcript levels were increased

(~100-fold) in the lungs of infected female mice compared with day 9 post-infection, however there was no difference observed between the 3 mouse strains (Fig.4.14F). Overall, the expression of mRNA transcripts of markers associated with alternative activation of macrophages, Arginase1, YM1 and FIZZ1 appear to be expressed in higher levels in the lungs of male mice than in female mice 9 days following infection with *T. gondii*. There was no difference in the expression of FIZZ1 and Arginase1 mRNA between the 3 mouse strains in both male and female mice at day 9 and day 12 post-infection. However, male $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice expressed significantly increased lung YM1 mRNA whereas female mice have significantly reduced levels of lung YM1 mRNA compared with their respective WT controls at day 9 post-infection. Finally the mRNA transcript expression of all 3 markers, Arginase1, YM1 and FIZZ1 was markedly increased in the lungs of female mice at day 12 post-infection relative to day 9 post-infection.

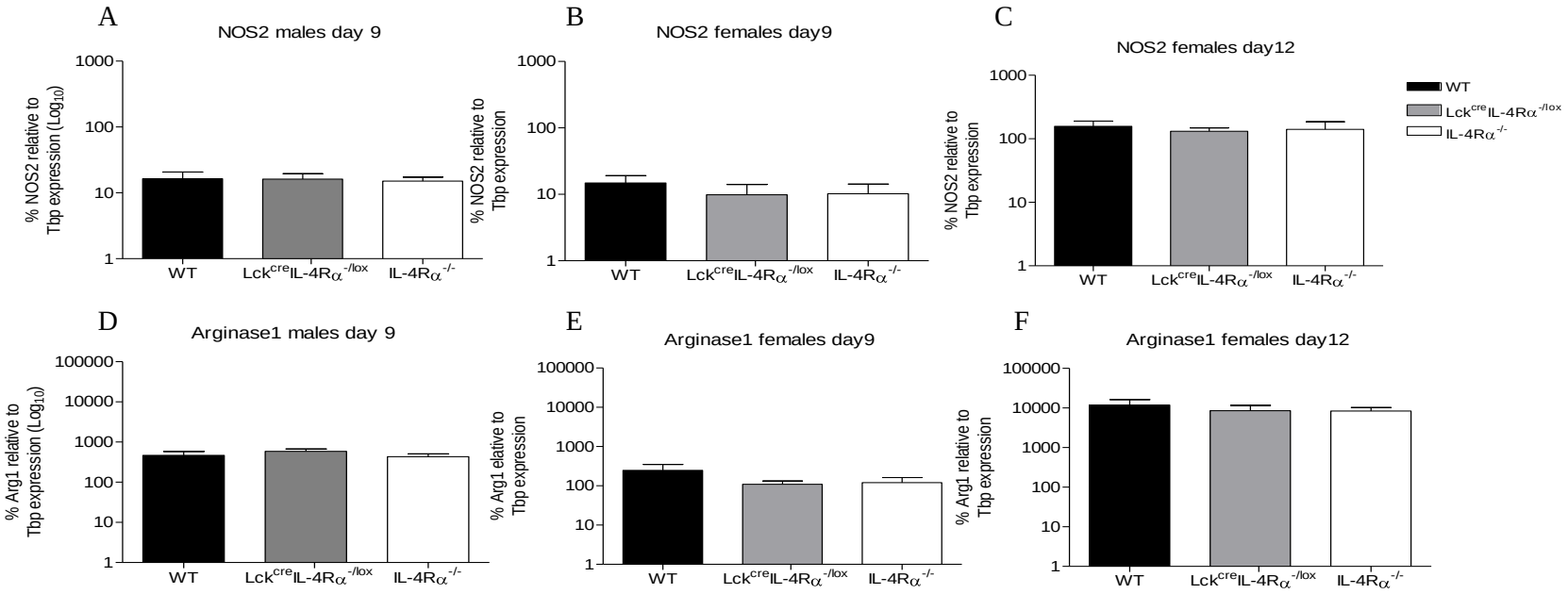


Fig.4.13 NOS2 and Arginase1 mRNA transcript expression in the lungs of *T.gondii* infected male (A, D) and female mice (B,E) 9 days post infection, and in female mice 12 days post-infection (C, F) as measured by RT-PCR

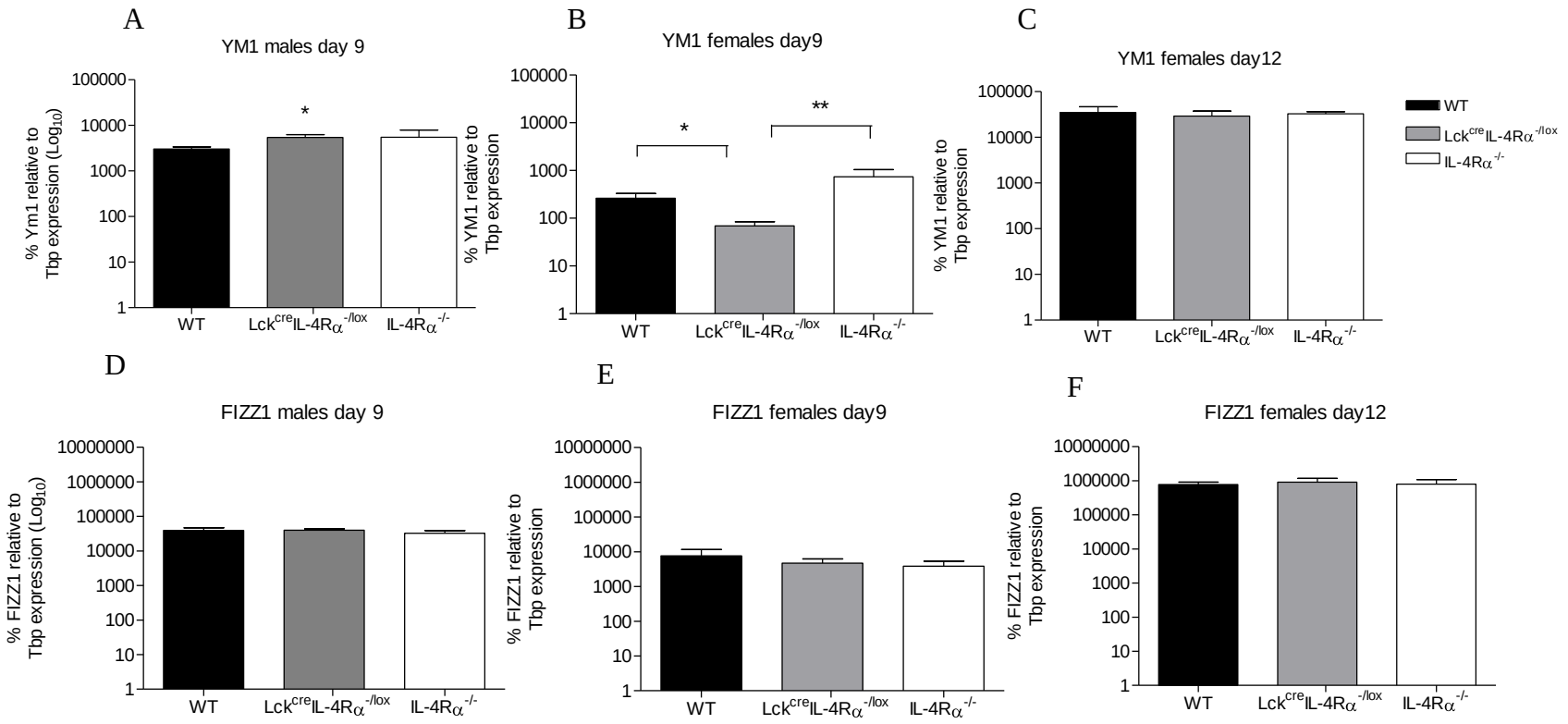


Fig. 4.14 Alternative macrophage activation markers YM1 and FIZZ1 mRNA transcript levels in the lungs of male (A, D) and female mice (B, E) 9 days post-infection, and in female mice 12 days post-infection (C, F).All levels of alternative macrophage activation markers measured with significantly higher than uninfected controls (not shown) *p<0.05, **p<0.01 compared with WT or $Lck^{cre}IL4R\alpha^{-/lox}$ controls

To further investigate the increased pathology observed in the lungs of infected male IL-4Rα$^{-/-}$ mice in this study, the expression of IFN-γ induced gene transcripts and the parasite burden were quantified in the lungs of *T. gondii* infected mice using RT-PCR. Lung LRG47 GTPase mRNA transcripts were more up-regulated in male mice than in female mice at day 9 post-infection. There was however no difference in LRG47 mRNA levels between WT, Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ male mice at day 9 post-infection (Fig.4.15A) of all 3 strains (Fig.4.15A). On the other hand, LRG47 GTPase mRNA transcript levels were significantly upregulated in the lungs of *T. gondii* infected Lck^{cre}IL-4Rα$^{-/lox}$ female mice compared with WT controls (p=0.035) (Fig.4.15B). Although LRG47 GTPase transcripts were upregulated in the lungs of female IL-4Rα$^{-/-}$ mice at this time point, the difference was not significantly different compared with WT controls (p=0.057). LRG47 mRNA transcript levels were markedly increased in the lungs of infected female mice at day 12 post-infection relative to the levels measured at day 9, however there were no significant difference between WT, Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ female mice at this time point (Fig.4.15C).

Similar to LRG47 transcript expression, Igtp GTPase mRNA transcript levels were higher in male lungs than in females at day 9 post-infection with *T. gondii*. Igtp GTPase mRNA transcript levels were significantly increased in the lungs of IL-4Rα$^{-/-}$ male mice compared with the WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice at day 9 post-infection (p=0.046) (Fig.4.15D). In female mice, there was no difference in Igtp transcript levels between the 3 mouse strains at day 9 post-infection (Fig.4.15E). Similarly, there was no significant

difference observed in the expression of lung Igtp GTPase between the 3 strains of mice at day 12 post-infection in female mice, although the magnitude of transcript expression was markedly increased (~100-fold) in female mice at this time point compared with day 9 post-infection (Fig.4.15F).

IDO mRNA transcripts levels were higher in the lungs of *T. gondii* infected male mice than in female mice at day 9 post-infection, however there was no difference in the level of expression in either male or female WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice at this time point (Fig.4.16,A & B respectively). At day 12 post-infection, there was a significantly increased level of expression of IDO transcripts in the lungs of *T. gondii* infected female mice relative to day 9 post-infection in all 3 mouse strains. Nevertheless, lung IDO mRNA transcript levels were significantly reduced in Lck^{cre}IL-4R$\alpha^{-/lox}$ ($p=0.0079$) and IL-4R$\alpha^{-/-}$ (0.004) female mice at day 12 post-infection compared with WT controls (Fig.4.16C).

Lastly, we assessed parasite burden in the lungs of *T. gondii* infected male and female mice at day 9 and day 12 post-infection by quantifying SAG-1 (a tachyzoite specific gene transcript) and TgCyst matrix antigen (a bradyzoite specific gene transcript) mRNA levels using RT-PCR. There were no significant differences between the levels of both tachyzoite and bradyzoite specific mRNA transcripts between WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ male mice at day 9 post-infection (Fig.4.17A). In addition the level of tachyzoite mRNA transcripts was comparable to that of bradyzoite mRNA

levels in all 3 mouse strains in male mice at this time point. With female mice, although there was no significant difference observed in the levels of either tachyzoite or bradyzoite-specific mRNA transcript levels between the 3 strains (Fig.4.17B). The ratio of bradyzoite to tachyzoite transcripts in $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice at day 9 post-infection was suggestive of increased tachyzoite to bradyzoite conversion (Fig.4.17B). Furthermore, female $IL\text{-}4R\alpha^{-/-}$ mice appear to have lower cyst burdens in the lungs compared with WT and $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice at day 9 post-infection, although the difference is not significant (Fig.4.17B). At day 12 post-infection, tachyzoite and bradyzoite specific mRNA transcripts were present in an equivalent ratio in the lungs of female WT mice; there was slightly more tachyzoite mRNA transcripts than bradyzoites in $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice, and slightly more bradyzoites than tachyzoite mRNA transcripts detected in $IL\text{-}4R\alpha^{-/-}$ mice, however these differences were not significant (Fig.4.17C). Overall, there were no significant differences in both tachyzoite and cyst burden in the lungs of both male and female mice at day 9 and day 12 post-infection.

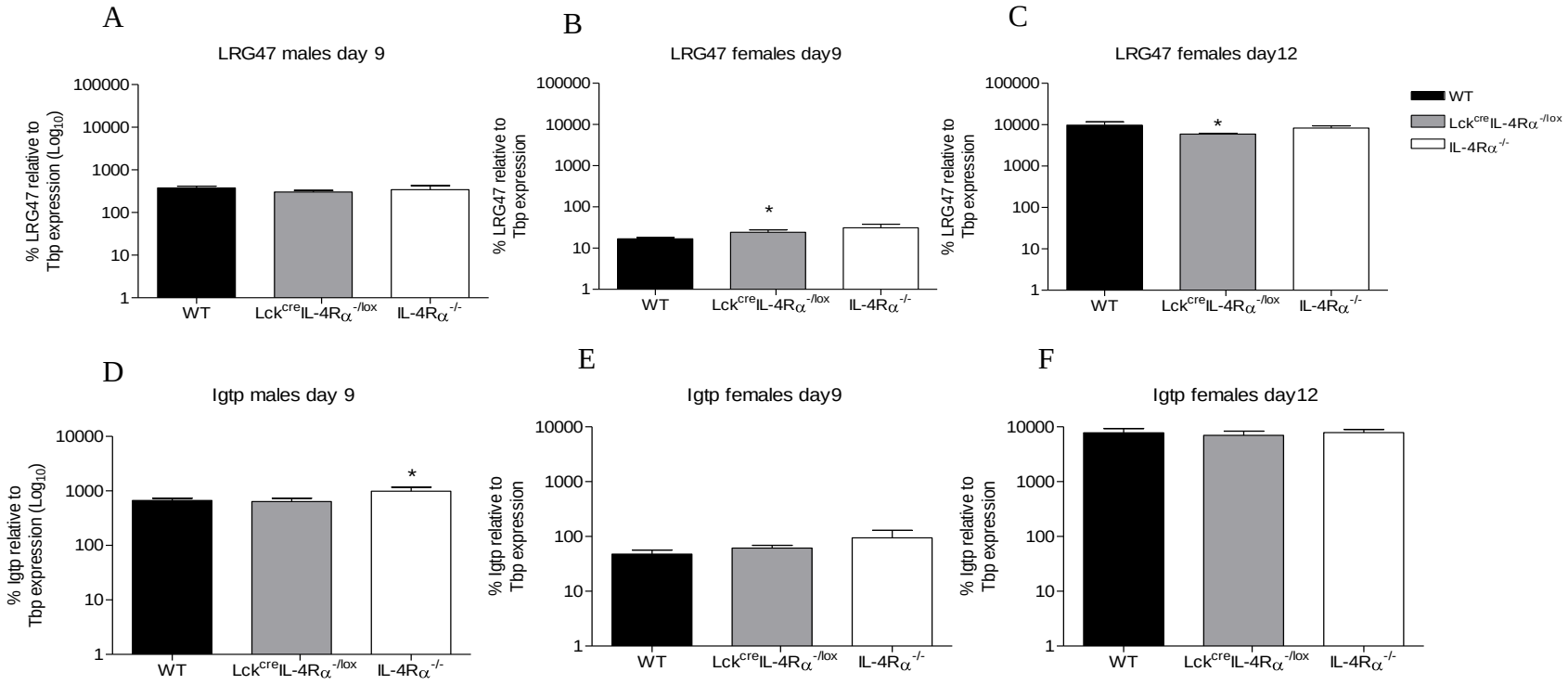


Fig. 4.15 mRNA transcript expression of IFN-γ inducible genes LRG47 and Igtp GTPase in the lungs of *T. gondii* infected male mice (A, D) and female mice (B, E) 9 days post infection, and in female mice 12 days post-infection (C, F). *p<0.05 compared with WT control

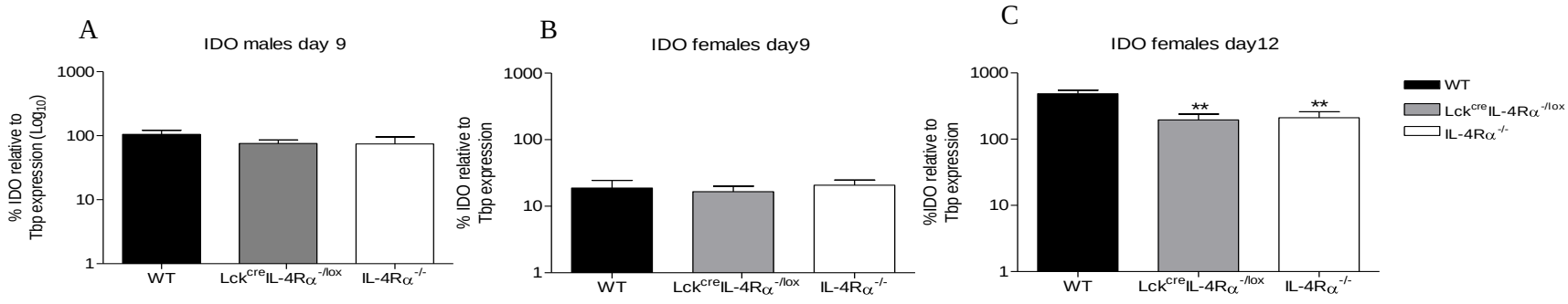


Fig.4.16 mRNA transcript expression of IFN-γ-inducible gene IDO in the lungs of male mice (A), and female mice(B), 9 days post-infection, and in female mice 12 days (C) post-infection with *T. gondii* . **p<0.01 compared with WT controls

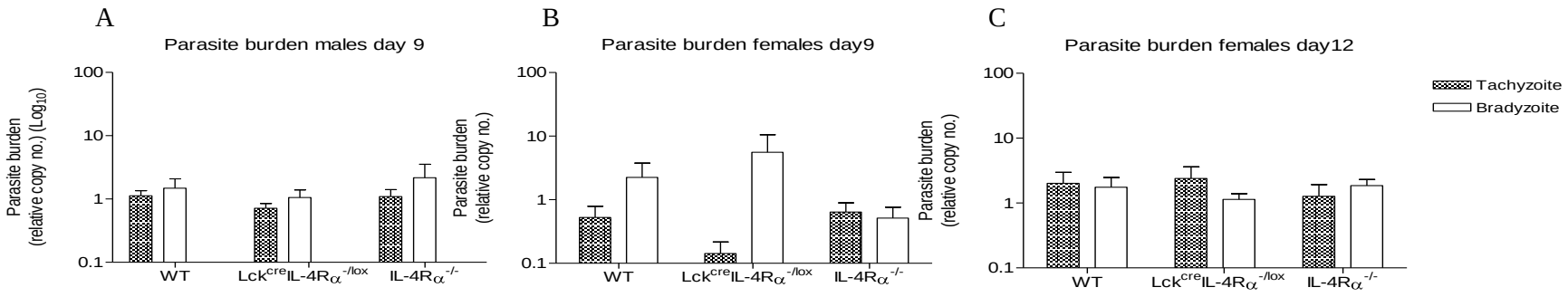


Fig. 4.17 Parasite burden measured by SAG1 (tachyzoite specific gene) and TgCyst antigen (bradyzoite specific gene) levels in the lungs of *T. gondii* infected male mice 9 days post-infection (A), female mice 9 days post-infection (B) and female mice 12 days post-infection (C).

4.2.7 Histopathological analysis during chronic *T. gondii* infection

During chronic infection differences observed in the lung pathology between WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ male mice were more pronounced (Fig.4.18). There was a significantly higher degrees of peribronchial and interstitial inflammation in the lungs of IL-4R$\alpha^{-/-}$ mice compared to WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ mice 35 days post-infection (Fig.4.18 A, B). There was mild interstitial inflammation in the lungs of WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ mice at this time point while inflammation was significantly higher and rated moderate in IL-4Rα deficient mice. Interestingly, $CD4^+$ T-cell specific IL-4Rα deficient mice displayed absent peribronchial inflammation in all infected mice (n=5) at day 35 post-infection, which was significantly lower than the mild and moderate degrees of peribronchial inflammation observed in WT ($p<0.05$) and global IL-4R$\alpha^{-/-}$ ($p<0.001$) mice respectively at this time-point (Fig.4.18A). Furthermore, the liver pathological scores of male mice were lower in the chronic phase of infection at day 35 compared with the acute phase, day 9 (Fig. 4.1). Nonetheless, there was no significant difference in portal tract and acini inflammation in male mice between the 3 mouse strains at day 35 post infection (Fig.4.18 C, D).

Analysis of brain tissue from *T. gondii* infected male mice showed mild to moderate degrees of meningitis (Fig.4.19 A), mild perivascular cuffing (Fig.4.19 B) and comparable numbers of small MG nodules (Fig.4.19C) at day 35 post infection, irrespective of the mouse strain. IL-4R$\alpha^{-/-}$ mice had higher cyst burden in the brain

(Fig.4.19D) and calcified necrotic lesions were noted in some of these mice, however the overall severity of pathology in the brains of IL-4R$\alpha^{-/-}$ mice was not significantly different compared with that of WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ mice at this time point.

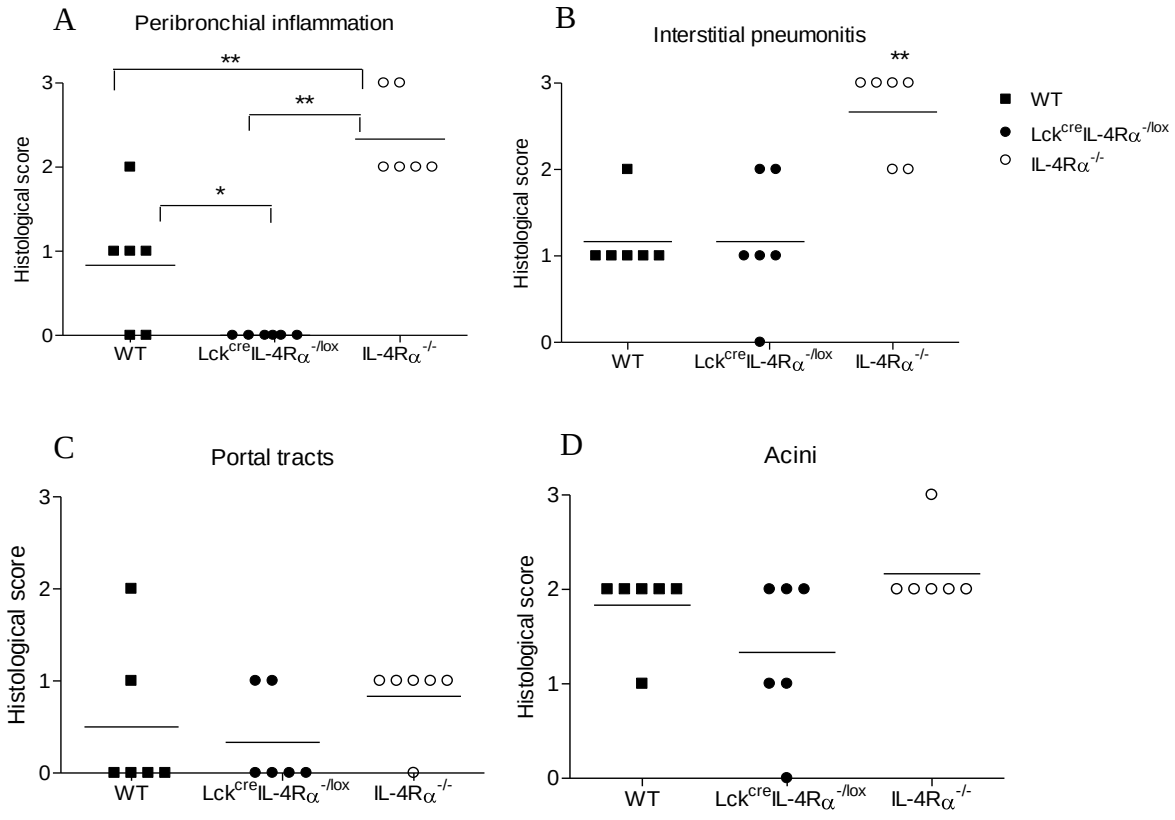


Fig.4.18 Lung (A, B) and liver (C, D) histopathology in male mice during chronic infection, day35 post infection. There was no peribronchial inflammation observed in the lungs of $CD4^+$ IL-4Rα deficient mice compared to the minimal and moderate inflammation observed in WT and IL-4Rα$^{-/-}$ respectively (A). There was increased interstitial pneumonitis in the lungs of IL-4Rα$^{-/-}$ compared with WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice (B). No difference was observed in the liver inflammation between all 3 groups (C, D).*$p<0.05$ compared with WT, **$p<0.01$ compared with Lck^{cre}IL-4Rα$^{-/lox}$ and WT

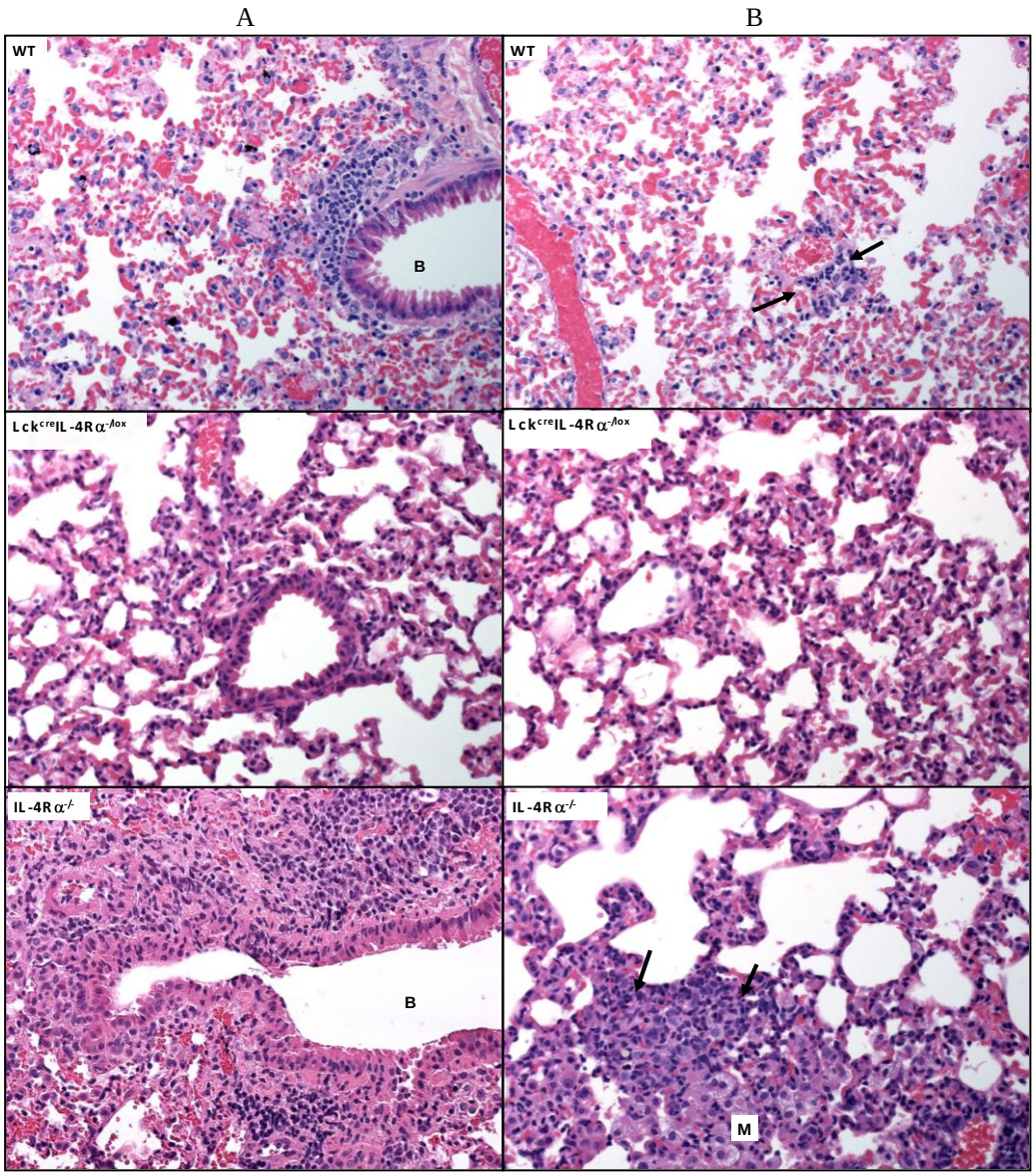


Fig.4.18A, B. Lung sections showing peribronchial (panel A) and interstitial (panel B) inflammation in WT (top), Lck^{cre}IL-4Rα$^{-/lox}$ (middle) and IL-4Rα$^{-/-}$ (bottom) male mice 35 days post-infection with *T. gondii*. WT mice displayed minimal inflammation, Lck^{cre}IL-4Rα$^{-/lox}$ had minimal interstitial and lacked peribronchiole inflammation, whilst IL-4Rα$^{-/-}$ lungs had high degrees of interstial inflammatory cells and foamy macrophages (M) and elevated peribronchial inflammation.

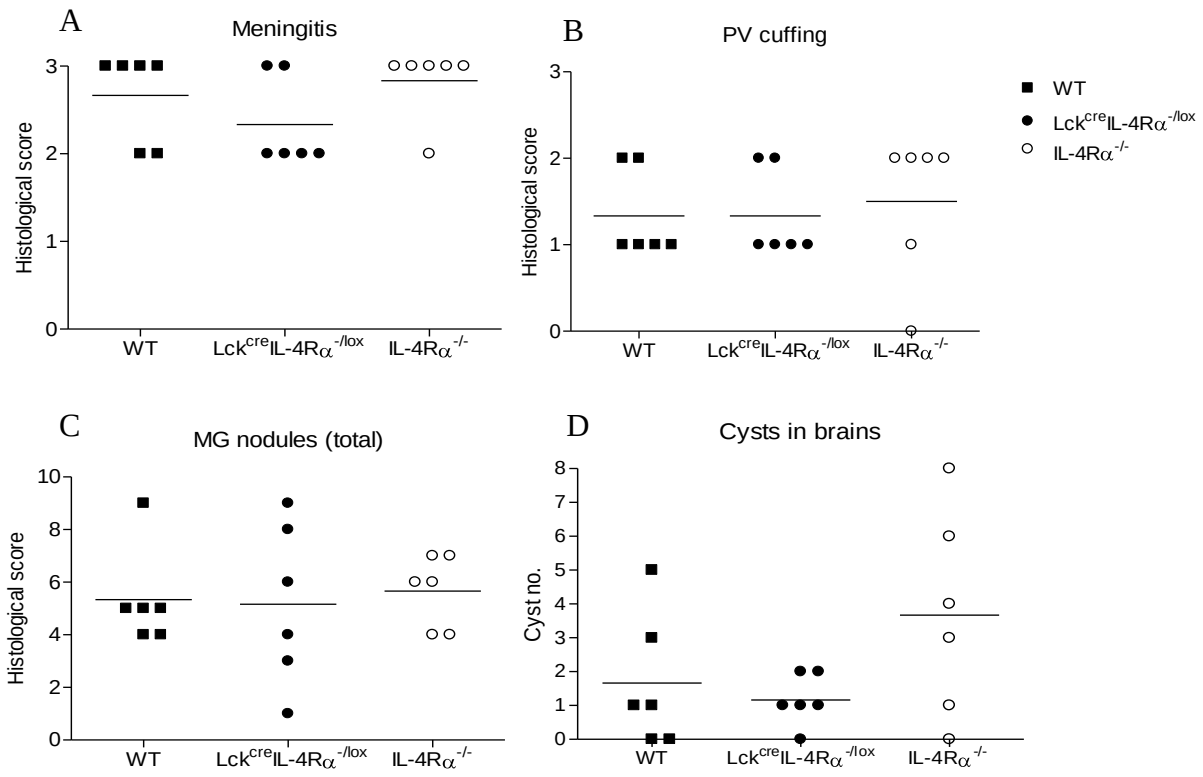


Fig.4.19 Histopathological scores for inflammation and parasite burden in the brains of male mice 35 days post-infection. The meninges (A) and perivascular areas (B) had an equivalent inflammatory response in the 3 groups of mice. There were also no differences in MG nodules numbers between the three groups (C). There were more cysts counted in the brains of IL-4Rα$^{-/-}$ mice (D), however the numbers were not significantly different to WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice.

4.3 Discussion

Interleukin- 4 (IL-4) has been previously described by our group and others to be a susceptibility factor during *T. gondii* infection in resistant BALB/c mice (Roberts, Ferguson et al. 1996; Suzuki, Yang et al. 1996). IL-4 deficient and IL-4Rα deficient mice were shown to be more susceptible to infection compared to their WT counterparts, and mortality was associated with severe type-1 mediated immunopathology in the lungs (Nickdel, unpublished data, appendix). In this chapter, we used $CD4^+$ T-cell-specific IL-4Rα deficient mice on the resistant BALB/c background to further investigate the cell populations that are involved in the observed IL-4/IL-13-mediated protective responses to *T. gondii* infection.

$CD4^+$ T-cells play a central role in regulating the cell-mediated immune response to various infections. In acute and chronic *T. gondii* infection, large numbers of both $CD4^+$ and $CD8^+$ T-cells are recruited to the site of infection and their ability to produce IFN-γ is critical for parasite destruction and host protection against the infection. Studies using antibody depletion of $CD4^+$ T-cells in mice chronically infected with the ME-49 strain of *T. gondii* resulted in exarcebation of disease (Gazzinelli, Xu et al. 1992) and reactivation of CNS toxoplasmosis leading to severe disease (Vollmer, Waldor et al. 1987). The protective response against *T. gondii* infection is dependent on an early vigorous innate response involving production of IL-12 by neutrophils, dendritic cells and macrophages that in turn stimulate T-cells and NK cells to produce IFN-γ. Naïve $CD4^+$ T-cells differentiate into type-1 cytokine (IFN-γ) producing effector cells in this

environment. Conversely, IL-4 is a key cytokine in driving the differentiation of naïve $CD4^+$ T-cell in type-2 cytokine producing cells.

In this study, we have looked at the role of IL-4Rα signaling via $CD4^+$ T-cells in limiting immunopathology and mortality during intra-peritoneal *T. gondii* infection. We show that male mice lacking IL-4Rα on $CD4^+$ T-cells as well as global IL-4R$\alpha^{-/-}$ mice are resistant to *T. gondii* infection and they survived for the duration of the experiment in the same way as the WT controls with a 100% survival rate. On the other hand, female Lck^{cre}IL-4R$\alpha^{-/lox}$ mice were partially susceptible to infection compared with the male mice of the same strain; however the mortality rate was not significantly different compared with their WT counterparts. In general there were no significant differences between WT and $CD4^+$ T-cells specific IL-4Rα deficient mice in terms of mortality rates. Over a number of experiments approximately 50% of female IL-4R$\alpha^{-/-}$ mice died over the course of infection.

In addition to their role in host immune responses and defense, cytokines interact with factors implicated in bodyweight regulation and energy metabolism during some disease conditions (Hotamisligil, Shargill et al. 1993). We investigated the role of IL-4/IL-13 signaling in development of disease by looking at the bodyweight profiles in male and female mice over the course of *T. gondii* infection. We showed that all infected male and female WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4Rα mice lost a significant amount of bodyweight during the first 15 days of infection. However, although the susceptible female IL-4R$\alpha^{-/-}$

mice lost significantly more weight between day 13 and 16, they regained the lost weight quite rapidly, and all 3 strains continued to regain their lost bodyweights by the end of the experiment, with no significant difference observed between the strains throughout the chronic phase of infection. In contrast, the resistant male IL-4R$\alpha^{-/-}$ mice started to regain their lost bodyweight from day 15 as the other 2 strains. However, whereas WT and Lck^{cre}IL-4R$\alpha^{-/lox}$ mice regained their starting bodyweights, IL-4R$\alpha^{-/-}$ mice did not recover their lost weight and remained at their reduced bodyweight for the duration of the experiment. In addition, male Lck^{cre}IL-4R$\alpha^{-/lox}$ mice had similar weights as WT mice between day 18 and day 25, however thereafter maintained a reduced weight gain rate in the chronic phase compared to WT mice. It is surprising that the male IL-4R$\alpha^{-/-}$ mice, which have a 100% survival rate, fail to recover the lost bodyweights whereas the susceptible female IL-4R$\alpha^{-/-}$ mice recover their bodyweight fully following chronic *T. gondii* infection. The weightloss observed in mice during the acute phase of *T. gondii* infection has been associated with physical wasting and loss of muscle mass caused by disease (Arsenijevic, Girardier et al. 1997). In line with our observation, studies by Arsenijevic et al. have described a model in which female Swiss Webster mice infected with *T. gondii* lose 20-30% of their bodyweight during the first 14 days of infection, and of those surviving some regain weight (gainers) whereas other infected mice remain at their reduced bodyweight indefinitely (non-gainers) (Arsenijevic, Girardier et al. 1997). In this study it is suggested that the divergence in weight gain response to *T. gondii* infection is linked to different cytokine expression profiles between the gainers and the non-gainers once stabilization of weight has occurred. This

study shows that the mice that do not regain weight (non-gainers) have significantly higher levels of serum IFN-γ than the mice that regain the bodyweight after 30 days post-infection. Moreover, the non-gainers were also shown to have a distinct type-2 response shown by increased IL-4, IL-5, IL-6 and IL-10 mRNA in the brain and spleen in chronic infection (Arsenijevic, Girardier et al. 1997; Arsenijevic, Girardier et al. 1998). In our study we show that the difference in weight gain is between male and female IL-4Rα$^{-/-}$ mice, and we observed a higher systemic IFN-γ response in female mice compared with male mice at day 9 post-infection. Nonetheless, in later studies (chapter 5) we measured serum IFN-γ at day 35 post-infection and found no difference. During acute *T. gondii* infection (day 9), male global IL-4Rα deficient mice survive despite the increased pathology in the lung, whereas female IL-4Rα$^{-/-}$ mice die and there was no difference in the lung pathology compared with female WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice. The death of infected female IL-4Rα$^{-/-}$ mice can be correlated with higher serum levels of IFN-γ than in male mice. The lethal type-1 response is supported by previous studies in our group that IL-4$^{-/-}$ mice succumb to infection due to unregulated IFN-γ and IL-12 production during acute infection (Roberts, Ferguson et al. 1996). Nevertheless, there is not enough evidence at this stage to make a correlation between resistance to *T. gondii* infection and bodyweight regulation, although there is a clear difference in IL-4/IL-13 mediated regulation of bodyweight and energy metabolism during *T. gondii* infection in male and female mice and between WT and IL-4Rα$^{-/-}$ male mice.

Furthermore, male mice have a more robust parasite-speficic splenocyte type-1 response

(IFN-γ and IL-12) compared with female mice at day 9 post-infection irrespective of IL-4Rα deficiency. The inability of female mice to respond to antigen may play a role in the increased susceptibility of female IL-4Rα$^{-/-}$ mice compared to their more resistant male counterparts. Female mice only gained the ability to respond to antigen by producing IFN-γ at day 12 post-infection. Splenocytes from male IL-4Rα$^{-/-}$ mice also show significantly increased spontaneous and TLA-specific IL-12 production compared with spleens from WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice, whereas TLA and ConA stimulated splenocytes from female IL-4Rα$^{-/-}$ mice produce significantly lower levels of IL-12 compared with the WT and Lck^{cre}IL-4Rα$^{-/lox}$ spleens. This observation represents a very significant difference as IL-12 is critical for the initiatiation of the IFN-γ response in the early stages of infection which is required to control parasite proliferation and dissemination (Gazzinelli, Hieny et al. 1993). IL-4/IL-13 mediated effects are usually associated with anti-inflammatory and down-regulation of the type-1 responses. Thus in the absence of IL-4 signaling one would expect to have an exaggerated type-1 response, as observed in splenocyte cultures from male mice in the present study. Some studies have however demonstrated that IL-4 can play a crucial role in induction of Th1 responses by priming dendritic cells for IL-12 production (Lutz, Schnare et al. 2002), which may offer an explanation for our observation of reduced IL-12 production in absence of IL-4/IL-13 signaling. More importantly, our data demonstrates that this effect is sex-dependent as male mice deficient in IL-4Rα have augmented IL-12 production whereas in female mice this response is abrogated during early stages of *T. gondii* infection. Earlier studies by Walker *et al* have also demonstrated that male mice have a

more rapid and higher systemic IL-12 response compared with female mice following infection with *T. gondii* (Walker, Roberts et al. 1997). Overall, male mice appear to be resistant to *T. gondii* infection due to their ability to mount an early and effective parasite specific type-1 response, whereas female mice have a robust non-specific IFN-γ response,but a reduced and delayed parasite-specific type-1 response which may be contributing to female mice being more susceptible and succumbing to acute infection in absence of IL-4 signaling unlike the resistant male counterparts.

Furthermore, despite the absence of IL-4Rα signaling, splenocytes derived from both male and female Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice produced significantly reduced levels of nitrite at day 9 post-infection. Th2 cytokines including IL-4 can inhibit iNOS induction in macrophages by IFN-γ (Liew, Li et al. 1991). It is therefore interesting to observe a significant reduction of NO production in the absence of IL-4/IL-13 signaling. This observation also contradicts previous observations in our group where splenocytes from IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ produced significantly increased NO compared with WT mice. Although NO is an important effector molecule for defense during intracellular pathogens (Liew 1995; Khan, Matsuura et al. 1996; Akaike and Maeda 2000), it has also been implicated in mediating immunopathology in some conditions (Bogdan 2000). It is therefore important to have a balanced NO response, in fact a study by Niedbala has shown that NO is needed in low concentrations to induce protective Th1 responses, whereas high NO concentrations were generally cytotoxic (Niedbala, Wei et al. 1999; Niedbala, Wei et al. 2002). Therefore because we studied the NO response in the acute

phase where the immune response is very dynamic, various factors (such as the severity of disease, age of the mice and also the form of the parasite as this is the stage where tachyzoite would be converting to bradyzoites in response to the stress exerted by the host's immune response) may play a role in influencing the level of NO production in order to maintain this delicate balance. Production of reactive nitrogen intermediates (RNI) and NO by classically activated macrophages is an important effector mechanism that restricts replication of intracellular parasites such as *T. gondii* (Adams, Hibbs et al. 1990; Yap and Sher 1999). However, in some infection studies, RNI's have been shown to be involved in immunosuppression of T-cell proliferation in mice infected with *Trypanosoma brucei* (Sternberg and McGuigan 1992) and *Listeria monocytogenes* (Gregory, Wing et al. 1993). Furthermore, Candolfi *et al* has shown that RNI's are responsible for decreased proliferation of ConA and TLA-stimulated spleen T-cells from *T. gondii* infected BALB/c mice (Candolfi, Hunter et al. 1994). In addition neutralization of IFN-γ *in vitro* and *in vivo* reversed the suppression of T-cell proliferative responses to mitogen during acute *T. gondii* infection. Therefore there is a possibility that there is a negative feed-back mechanism whereby NO is selectively inhibiting its own production by inhibiting Th1 cells in absence of IL-4/IL-13 signaling. Interestingly, NO inhibition has also been associated with enhanced weight loss and decreased survival in mice undergoing graft-versus host disease after bone marrow transplantation (Drobyski, Keever et al. 1994). There may be a complex interplay between innate, adaptive and metabolic pathways which involve NO and IL-4 signaling which remains to be resolved.

NO production is closely associated with classically activated macrophages, as well as the interconversion between tachyzoites and bradyzoites of *T. gondii* parasites (Bohne, Heesemann et al. 1993; Bohne, Heesemann et al. 1994; Lyons, McLeod et al. 2002). We investigated the expression of macrophage activation markers and parasite burden in the lungs of male and female mice following *T. gondii* infection. During the acute stage, there was a significant reduction of IFN-γ mRNA transcripts in the lungs of male IL-4Rα$^{-/-}$ mice compared to WT controls. There was however no difference in the expression of alternative macrophage activation markers in these mice. Interestingly, male Lck^{cre}IL-4Rα$^{-/lox}$ mice expressed significantly increased lung YM1 at day 9 post-infection compared with WT mice, whereas on the other hand female mice expressed significantly reduced levels of lung YM1 compared with WT and IL-4Rα$^{-/-}$ mice at the same time point. YM1 is a chitinase-like lectin which can bind to extracellular matrix and has been implicated in wound healing (Marchesi, Minucci et al. 2006). Despite the fact that there was no difference observed in the degree of lung pathology between female WT, Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice at this time point (day 9), it is possible that the lack of IL-4 signaling on CD4^{+} T-cells in female Lck^{cre}IL-4Rα$^{-/lox}$ mice is delaying the induction of YM1 expression. Nevertheless, a clear correlation between expression of alternative macrophage markers and pathology in the lung is not obvious. Furthermore, although there was no significant difference in lung parasite burden between female WT, Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice at this time point (day 9), the rate of tachyzoite to bradyzoite conversion of the parasite was significantly higher in Lck^{cre}IL-4Rα$^{-/lox}$ mice compared with WT and IL-4Rα$^{-/-}$ mice. This effect was not seen

in male mice at the same time point. In addition female Lck^{cre}IL-4R$\alpha^{-/lox}$ mice express significantly increased levels of lung LRG47 GTPase mRNA compared with the other 2 strains at day 9 post-infection. The IFN-γ inducible p47 GTPase has been reported to be important for controlling parasite replication, although the mechanism through which it functions has not yet been described (Feng, Collazo-Custodio et al. 2004; Taylor, Feng et al. 2004; Martens, Parvanova et al. 2005). LRG47 may play a role in the regulation of parasite form interconversion; however we do not have sufficient evidence at this stage to make a direct correlation between tachyzoite to bradyzoite conversion and the expression of p47GTPases. Another of the IFN-γ inducible genes, IDO, which has been implicated in inhibition of *T. gondii* growth, was significantly reduced in the lungs of female Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice at day 12 post-infection. Again, this does not correlate with the parasite burden as there was a similar tachyzoite and bradyzoite burden observed in the lungs of the 3 strains of mice at this time point. The stage-specific expression of surface antigens by *T. gondii* is a known mechanism that facilitates parasite persistence (Kim and Boothroyd 2005). Tachyzoites stimulate a more robust type-1 cytokine production response by macrophages than bradyzoites both *in vitro* and *in vivo* (Schade and Fischer 2001). Therefore, the parasite stage/form will not only affect the macrophage effector functions and T-cell responses, it will also influence the IFN-γ and IL-4-dependent macrophage activation programmes that these cells undergo depending on the type-1/type-2 cytokine balance.

In chronically infected (day 35) male mice, mice lacking IL-4Rα on $CD4^+$ T-cells had

no inflammation in the lungs compared to the mild and moderate degrees of inflammation observed in the lungs of WT and global IL-4Rα deficient mice respectively. This implicates an immuno-pathogenic role for IL-4Rα signaling via $CD4^+$ T-cells during chronic *T. gondii* infection. Of significance, in the repeat study of infected male mice, male Lck^{cre}IL-4R$\alpha^{-/lox}$ mice showed no lung pathology compared with WT and IL-4R$\alpha^{-/-}$ mice as early as day 9 post-infection which suggests that this effect may be mediated as early as day 9 post-infection. Indeed, there have been reports that $CD4^+$ T-cells may play a significant role in promoting tissue inflammatory responses following *T. gondii* infection, though the mechanisms have never been identified. Israelski *et al*. reported that C3H/Hen mice depleted of CD4 T-cells by antibody depletion and chronically infected with *T. gondii* (ME49 strain) had reduced levels of CNS inflammation, which only appeared when the anti-CD4 antibody treatment was terminated (Israelski, Araujo et al. 1989). An immunopathogenic role for $CD4^+$ T-cells is further described in a study of ocular toxoplasmosis using CD4 and CD8-deficient mice, whereby inflammation in the eye was shown to be primarily mediated by $CD4^+$ T-cells, while $CD8^+$ T-cells were more important in limiting parasite replication (Lu, Huang et al. 2004). Furthermore, Liensenfeld *et al*. also showed that C57BL/6 mice died following oral infection with ME46 strain of *T. gondii*, and the mortality was mediated by increased necrosis in the small intestines, which was dependent on the presence of $CD4^+$ T-cells (Liesenfeld, Kosek et al. 1996). More recently, Stumhofer *et al*. also showed that depletion of $CD4^+$ T-cells rescues IL-27R deficient mice and reduces inflammation in the brain (Stumhofer, Laurence et al. 2006). These studies clearly show

the significance of $CD4^+$ T-cells in *T. gondii* induced immunopatholgy. Our study therefore further explains a possible mechanism through which $CD4^+$ T-cells may be mediating these immuno-pathogenic responses, through IL-4Rα signaling.

Histopathological analysis of the brain during chronic infection (day 35) showed no difference in brain pathology between WT and mice lacking IL-4Rα signaling. Although studies in our group and others (Roberts, Ferguson et al. 1996; Suzuki, Yang et al. 1996) using mouse strains other than BALB/c previously reported that IL-4 is important for formation of cysts and controls tachyzoite proliferation in the brain, we did not observe any significant difference in the number of parasite cysts in the brains of WT, Lck^{cre}IL-$4R\alpha^{-/lox}$ and IL-$4R\alpha^{-/-}$ male mice. This observation is not surprising, as $CD8^+$ and not $CD4^+$ T-cells reportedly play a more prominent role in parasite replication in the CNS during *T. gondii* infection (Gazzinelli, Hakim et al. 1991; Parker, Roberts et al. 1991; Suzuki 2002).

It is evident from our observation that IL-4Rα signaling through $CD4^+$ T-cells is not essential for protection during acute and chronic infection as mice lacking IL-4Rα on $CD4^+$ T-cell tend to survive with similar disease progression as WT mice in terms of survival and bodyweight regulation. $CD8^+$ T-cells have been reported to be important in regulating *T. gondii* cysts in the brains (Suzuki 2002), and typically require $CD4^+$ T-cells help through production of IFN-γ and IL-2. However, our observation suggests that $CD4^+$ T-cell help may not be essential in parasite regulation, and that the cytotoxic T-

cell may be getting a signal from cytokines produced by other cell types (non-T-cells) in the central nervous system. In addition, it has been shown that $CD8^+$ T-cell cytotoxicity is impaired in the absence of IL-4Rα signaling (Marsland, Schmitz et al. 2005). Consequently future experiments using mice lacking IL-4Rα signaling on both $CD4^+$ and $CD8^+$ T-cells will clearly indicate whether IL-4Rα signaling via $CD8^+$ T-cells is a susceptibility factor during chronic *T. gondii* infection.

CHAPTER 5

T. gondii infection in $CD4^+CD8^+$ T-cell specific IL-4R$\alpha^{-/-}$ mice

Abstract

In previous chapters, we have shown that macrophages/neutrophils, but not $CD4^+$ T-cell specific IL-4Rα-mediated signaling mediate host protection and survival during *T. gondii* infection. In fact, $CD4^+$ T-cells responding to IL-4 were shown to mediate immunopathology in the chronic stage of infection. In this study, $CD4^+/CD8^+$ T-cell specific IL-4Rα deficient mice ($NLck^{cre}IL\text{-}4R\alpha^{-/lox}$) were used to further dissect the role of IL-4 responsiveness on T-cell populations in male and female BALB/c mice during *Toxoplasma gondii* infection. Although the absence of IL-4Rα on $CD4^+$ and $CD8^+$ T-cells resulted in impaired *T. gondii* specific nitric oxide and IFN-γ production by infected spleen cells from male $NLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice, it was not essential for survival of male mice, whereas female $NLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice were partially susceptible and succumbed to infection during late stages of infection. We show that mortality was associated with an impaired antigen-specific type-1 response combined with a pronounced systemic production of IFN-γ and IL-12 during the early stages of infection in female mice. At day 9 post-infection, female $NLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice display a significantly increased parasite burden in the lungs compared with IL-4Rα intact (WT) and $IL\text{-}4R\alpha^{-/-}$ mice. However by day 12 the parasite burden was reduced and this was concomitant with a significant increase in NOS2 and the IFN-γ inducible genes LRG47 GTPase mRNA transcripts in the lungs.

5. Introduction

The protective immune response against *T. gondii* has been reported to be dependent on both $CD4^+$ and $CD8^+$ T-cells (Gazzinelli, Xu et al. 1992). $CD8^+$ T –cells are however widely recognized as the major cell population involved in the early type-1 response and resistance to acute *T. gondii* in mice (Parker, Roberts et al. 1991; Subauste, Koniaris et al. 1991; Shirahata, Yamashita et al. 1994). The role of IL-4Rα signaling via $CD4^+$ T-cells was studied in the previous chapter, where $CD4^+$ T-cell specific IL-4Rα deficient mice were relatively resistant to *T. gondii* infection and exhibited normal type-1 responses. Although NO production by splenocytes from infected mice lacking IL-4Rα on $CD4^+$ T-cells was impaired in comparison with splenocytes derived from WT counterparts, parasite burdens remained controlled in the lungs and brains of these mice during acute or chronic stages of infection. In fact, the presence of IL-4 signaling via $CD4^+$ T-cells seemed to be pathogenic during chronic (and possibly as early as day 9 post-infection) *T. gondii* infection as male $CD4^+$ T-cells-specific IL-4Rα$^{-/-}$ infected mice exhibited limited inflammation in the lungs compared with the higher degrees of inflammation observed in the lungs of WT and global IL-4Rα deficient mice.

Several infectious diseases require a coordinated interaction between $CD4^+$ and $CD8^+$ T-cell populations in order to resolve the infection. The protective immune response against the intracellular bacteria, *Listeria monocytogenes*, requires both $CD4^+$ and $CD8^+$ T-cells to be efficiently induced (Unanue 1997). Also, infections with the influenza virus (Riberdy, Christensen et al. 2000) and lymphocytic choriomeningitis virus (Matloubian,

Concepcion et al. 1994) require $CD4^+$ T-cells to sustain effective cytotoxic $CD8^+$ T-cell responses and clear the viruses during infection.

While $CD4^+$ T-cells play a critical synergistic role by helping maintain a robust $CD8^+$ T-cell response in some infections, for some they are not required for the development of efficient cytotoxic $CD8^+$ T-cell responses. For instance, *Encephalititozoon cuniculi* infections (Moretto, Casciotti et al. 2000) are able to mount normal $CD8^+$ T-cell immunity in absence of $CD4^+$ T-cells whereas in *Plasmodium yoelii* infections (Carvalho, Sano et al. 2002) $CD4^+$ T-cell help is critical. There are contrasting reports with regard to *T. gondii* infection. Gazinelli *et al* first reported that depletion of $CD4^+$ T-cells preceding vaccination did not protect mice against lethal *T. gondii* infection (Gazzinelli, Hakim et al. 1991). Conversely, a study using $CD4^{-/-}$ mice found that normal $CD8^+$ T-cell immunity could be induced in the absence of $CD4^+$ T-cells during *T. gondii* infection (Casciotti, Ely et al. 2002). In addition, Combe *et al* also showed that normal $CD8^+$ T-cell immunity can be induced in absence of $CD4^+$ T-cells during *T. gondii* infection using $CD4^{-/-}$ mice (Combe, Curiel et al. 2005).

$CD8^+$ T-cells are the primary T-cell population involved in the resolution of *T. gondii* infection. Mice depleted of $CD8^+$ T-cells were shown to be unable to control both acute and chronic toxoplasmosis (Suzuki and Remington 1988). *In vivo* studies have shown that IL-4 is critical for the development of protective $CD8^+$ T-cell memory responses against tumors and infection models. Cytotoxic T-cell mediated immune responses against mammary and colon carcinoma failed to be induced in the absence of IL-4

(Schuler, Qin et al. 1999). Studies using *Plasmodium yoelli* also demonstrated that IL-4 secreting $CD4^+$ T-cells are essential for induction of $CD8^+$ T-cell responses during malaria liver stages (Carvalho, Sano et al. 2002). Furthermore, Stager *et al* also showed that protective $CD8^+$ T-cell mediated immunity was dependent on IL-4 during *Leishmania donovani* infection (Stager, Alexander et al. 2003). The role of IL-4 in *T. gondii* $CD8^+$ T-cell mediated responses is not yet fully elucidated. More recently, a study using mice deficient in STAT-6 on $CD8^+$ T-cells displayed compromised IFN-γ production and subsequent increased cyst proliferation in the brains of *T. gondii* infected $STAT6^{-/-}$ mice as compared to the WT controls (Jin, Takamoto et al. 2009). Nevertheless, the mechanisms of IL-4 signalling in $CD8^+$ T-cell mediated activation are still unclear. In this study, we used $CD4^+$ and $CD8^+$ specific IL-4Rα deficient mice ($NLck^{cre}$IL-4R$\alpha^{-/lox}$) to investigate the role of IL-4Rα signaling through both $CD4^+$ and $CD8^+$ T-cells in the regulation of the immune response, immunopathology and induction of alternatively activated macrophages in various organs following *T. gondii* infection.

5.1 Results

5.1.1 Survival of NLck^{cre}IL-4Rα$^{-/lox}$ male and female mice following *T. gondii* infection

Both male and female mice from WT, NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ strains were infected i.p with 10 cysts of *T. gondii* (RRA) Beverley strain and monitored for disease and mortality on a daily basis over a period of 35 days. Male WT and NLck^{cre}IL-4Rα$^{-/lox}$ mice survived for the complete duration of the experiment with a 100% survival rate, whilst their IL-4Rα$^{-/-}$ counterparts succumbed to infection with mortalities recorded primarily during the later stages of infection. The first death from the IL-4Rα$^{-/-}$ strain occurred at day 10, followed by more deaths on days 20, 22 and 27 and an overall 74.4% survival rate (Fig. 5.1A). Conversely, in our studies in the previous chapter (chapter 4) there were no deaths recorded in IL-4Rα$^{-/-}$ mice over several (n=3) individual experiments in male mice. Female mice exhibited more signs of illness and were more susceptible to infection in comparison with their male counterparts, with most mortalities occurring during the early stages of infection between day 9 and 11 post-infection for IL-4Rα$^{-/-}$ (Fig5.1 B). NLck^{cre}IL-4Rα$^{-/lox}$ mice only started succumbing to infection from day 23. The survival rates recorded over a chronic experiment, which was only performed once over a period of 35 days, were 94%, 80% and 88% for WT, NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice respectively (Fig. 5.1B). Overall, NLck^{cre}IL-4Rα$^{-/lox}$ and Lck^{cre}IL-4Rα$^{-/lox}$ female mice were generally more resistant to *T. gondii* infection relative to the global IL-4Rα$^{-/-}$ counterparts, with NLck^{cre}IL-4Rα$^{-/lox}$ mice only showing partial susceptibility in the late stages of infection.

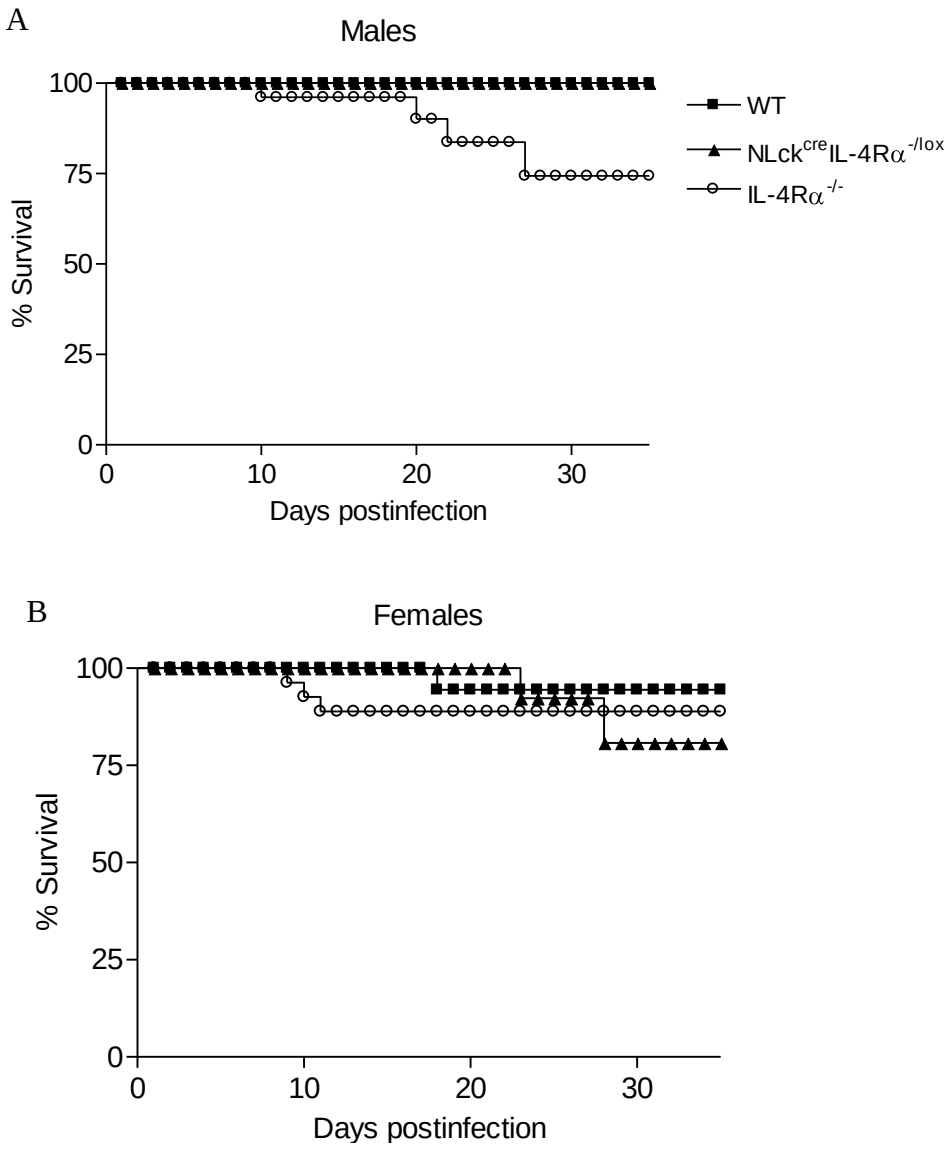


Fig.5.1 Survival curves for male (A) and female (B) WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice infected with 10 cysts of *T. gondii* Beverly intraperitoneally.

5.1.2 Bodyweights of NLck^{cre}IL-4Rα$^{-/lox}$ mice following *T. gondii* infection

Male and female mice infected with *T. gondii* intraperitoneally were weighed daily for a period of 35 days. Male IL-4Rα$^{-/-}$ mice started losing weight from day 9 post-infection, which was also a time point when they started showing signs of grave illness (Fig.5.2A). These mice progressively lost 13.7% of their starting bodyweight by day 17 post-infection, and thereafter started to recover the weight gradually but failed to fully regain the starting bodyweights, finishing with only 93.2% of their starting bodyweights by day 35 post-infection. On the other hand, male WT and NLck^{cre}IL-4Rα$^{-/lox}$ mice started losing later at day11 and the weight loss was minimal compared with IL-4Rα$^{-/-}$mice. The lowest weights recorded were on days 13 (5.48% loss) and 18 (4.5% loss) for NLck^{cre}IL-4Rα$^{-/lox}$ and WT mice respectively. Both strains of mice continued to regain their bodyweight progressively thereafter, attaining their starting bodyweights (≥100%) by day 26 and day 28 post-infection respectively (Fig.5.2A). The bodyweight profiles of male mice in this chapter are very similar to what we observed in previous studies (chapter 4) where infected male IL-4Rα$^{-/-}$ mice lost more bodyweight compared with WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice, and also failed to recover the lost bodyweight at the same rate as WT and Lck^{cre}IL-4Rα$^{-/lox}$ mice (Fig.4.2A).

Female mice showed slightly more rapid weight loss compared with male mice, starting at day 9 post-infection (Fig.5.2B). By day 13 all 3 groups of mice had lost significant amounts of weight, 17%, 14% and 16% bodyweight loss for WT, NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$mice respectively. WT mice continued to regain their starting bodyweight

faster than NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$mice, and had recovered by day 29, while the latter only recovered 100% of their bodyweight by day 32 post-infection. Between day 13 and 24, both NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$mice exhibited significantly lower bodyweights compared with WT mice. Nonetheless, all 3 groups of mice had recovered their full bodyweight by the end of the 35 day period with no significant difference observed between the 3 strains.

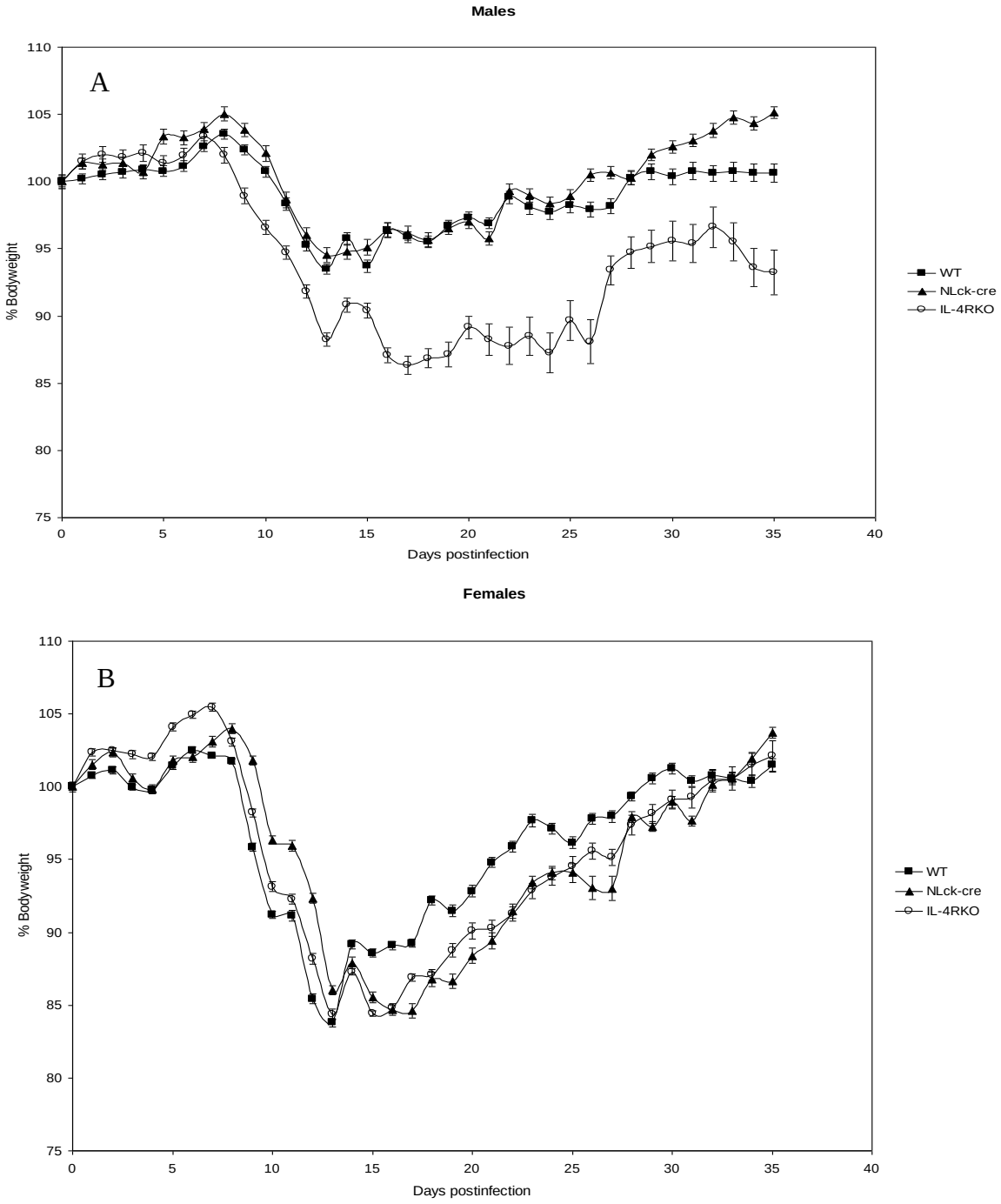


Fig.5.2 Bodyweights of male (A) and female (B) mice from WT (solid square), NLck^{cre}IL-4Rα$^{-/lox}$ (solid triangle) and IL-4Rα$^{-/-}$(open circle) infected with *T. gondii* intraperitoneally.

5.1.3 Histopathological analysis of tissues taken from *T. gondii* infected mice

In this study we carried out histopathological analysis on lung and liver tissue sections at day 9 post-infection for male mice and days 9 and 12 post-infection for female mice from mice deficient in IL-4Rα on both $CD4^+$ and $CD8^+$ T-cells compared with WT and global IL-4R$\alpha^{-/-}$ mice. There was minimal degree of peribronchial inflammation observed in the lungs of male WT and IL-4R$\alpha^{-/-}$ mice at day 9 post-infection but interestingly, there was no peribronchial inflammation observed in male NLck^{cre}IL-4R$\alpha^{-/lox}$ mice (n=7) at this early time point (Fig.5.3A) which was significantly lower than the level of damage observed in IL-4R$\alpha^{-/-}$ mice ($p<0.05$). This observation suggests that coordinated IL-4Rα signaling via $CD4^+$ and $CD8^+$ T-cells mediates immunopathology in the lungs during acute *T. gondii* infection whereas depletion of IL-4Rα on $CD4^+$ T-cells alone did not reduce peribronchial inflammation (relative to WT mice) in the lungs during acute phase infection (Table4.1/Fig.4.1) Nevertheless, the same minimal degree of interstitial inflammation was observed in the lungs of all 3 strains of mice at this time point (Fig.5.3B). There was also a mild degree of inflammation in the portal tracts (Fig.5.3C) and moderate inflammation in the acini (Fig.5.3D) at this time point, although there was no difference observed between the 3 strains.

Tissues from female mice were also examined and overall there was minimal to absent levels of peribronchial inflammation in the lungs of WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice (Fig.5.4A) and no significant difference between the 3 strains at day 9 post-

infection. In addition, the lungs from all 3 strains of mice similarly displayed minimal to moderate degrees of interstitial inflammation (Fig.5.4B). Furthermore, the pathology in the livers of female WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice was comparable as shown by minimal inflammation in the portal tracts (Fig.5.4C) and moderate levels of acinar inflammation (Fig.5.4D) observed in the 3 strains. This observation is consistent with the pathology observed in female WT, Lck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice studied in the previous chapter (Fig.4.1). At day 12 post-infection, female WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice display minimal peribronchial inflammation and mild interstitial pneumonitis in the lungs (Fig.5.5 A and B respectively), and there was no difference observed in lung pathology between the 3 strains at this time point. The liver portal tracts of WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice displayed minimal inflammation with no difference observed between the 3 strains (Fig.5.5C) and there was mild to moderate degrees of inflammation in the acini of WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ mice. Female IL-4R$\alpha^{-/-}$ mice displayed significantly increased levels of inflammation in the acini compared with WT mice at day 12 post infection ($p<0.05$) (Fig.5.5D).

Overall, female Lck^{cre}IL-4R$\alpha^{-/lox}$ and female NLck^{cre}IL-4R$\alpha^{-/lox}$ display very similar levels of lung and liver immunopathology relative to their respective WT and IL-4R$\alpha^{-/-}$ controls at day 9 post-infection. However, whereas male mice lacking IL-4Rα on CD4$^+$ T-cells display minimal degrees of lung peribronchial damage during acute infection, mice deficient in IL-4Rα on both CD4$^+$ and CD8$^+$ T-cell populations are devoid of any damage to the peribronchial area of the lung during acute *T. gondii* infection.

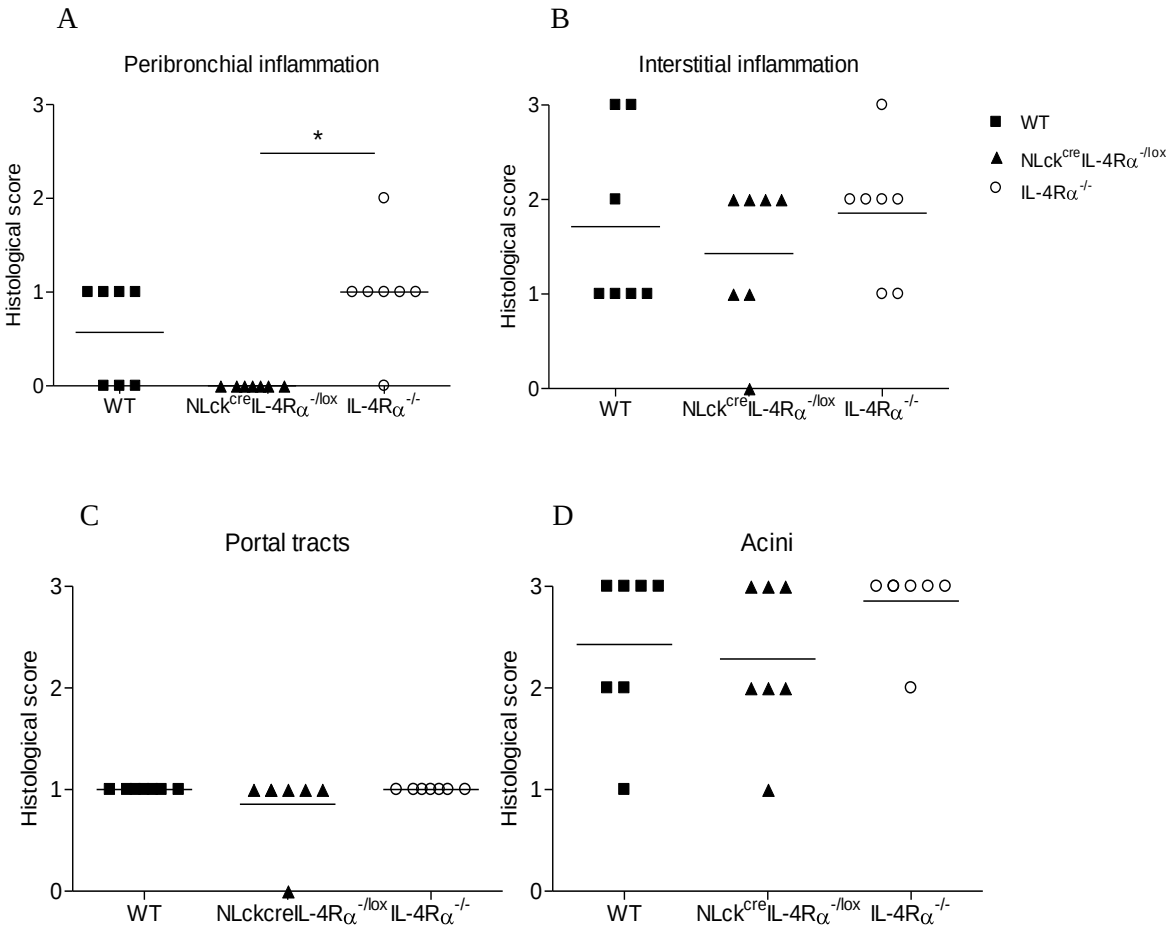


Fig.5.3 Histopathological analysis of lung tissues from WT (solid square), NLck^{cre}IL-4Rα$^{-/lox}$ (solid triangle) and IL-4Rα$^{-/-}$ male mice 9 days post-infection with *T. gondii*. Lung peribronchial inflammation (A), interstitial inflammation (B), and liver portal tracts (C) and acinar (D) inflammation. *$p<0.05$ NLck^{cre}IL-4Rα$^{-/lox}$ compared with IL-4Rα$^{-/-}$

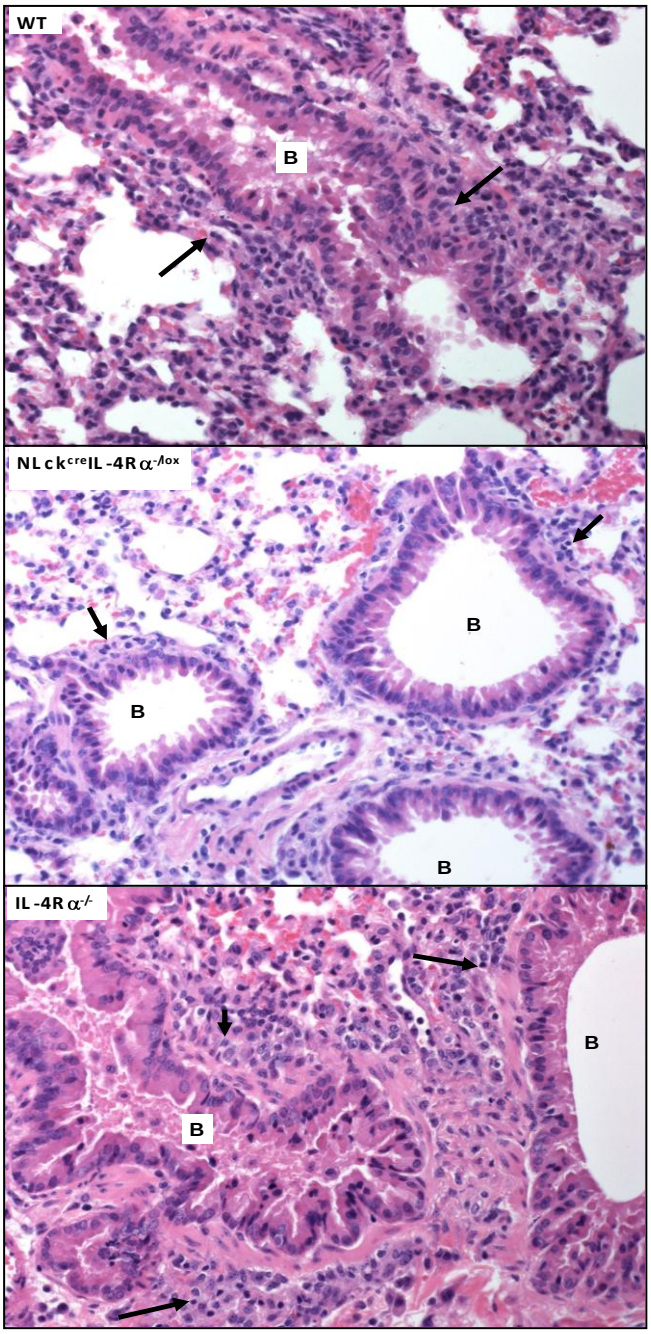


Fig.5.3A Lung tissue sections showing peribronchial inflammation (arrows) in WT (top), NLck^{cre}IL-4Rα$^{-/lox}$ (middle) and IL-4Rα$^{-/-}$ (bottom) male mice 9 days post-infection with *T. gondii*. WT and IL-4Rα$^{-/-}$ lungs displayed moderate peribronchial inflammation compared with the very minimal inflammation observed in NLck^{cre}IL-4Rα$^{-/lox}$ lungs.

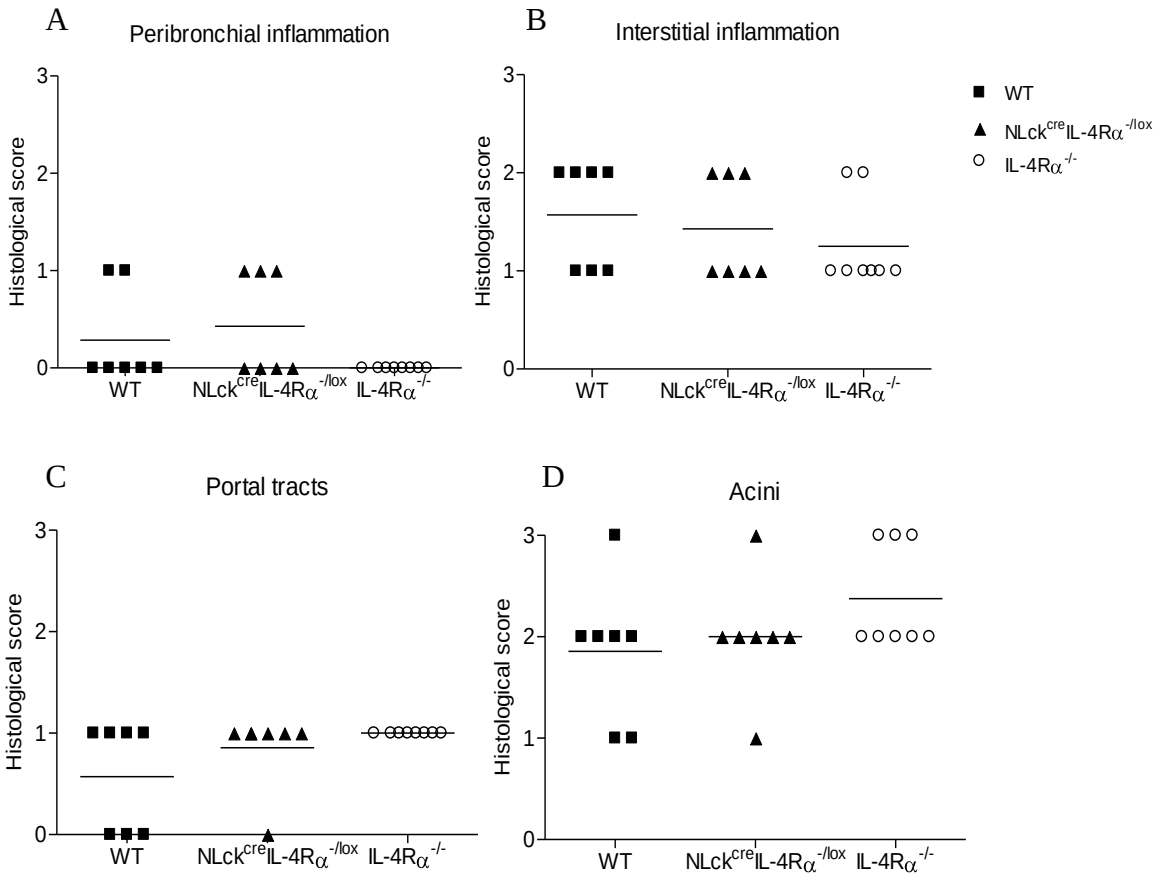


Fig.5.4 Female mice histopathology analysis 9 days post *T. gondii* infection. Lung peribronchial (A) and interstitial (B) inflammation and liver portal tract (C) and acinar (D) inflammation

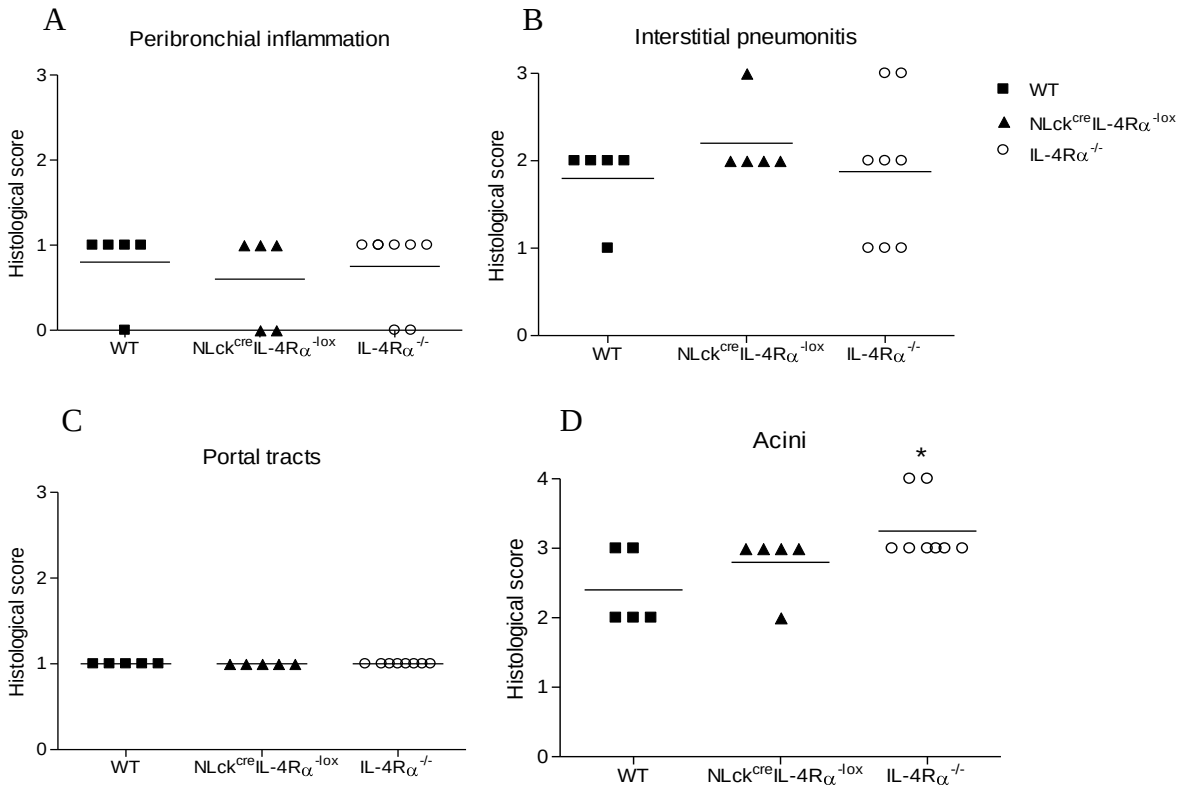


Fig.5.5 Histopathological analysis of lung and liver tissue from female mice 12 days post-infection with *T. gondii*. Lung peribronchial inflammation (A), lung interstitial pneumonitis (B), liver portal tract (C) and acinar (D) inflammation. *$p<0.05$ IL-4R$\alpha^{-/-}$ compared with WT

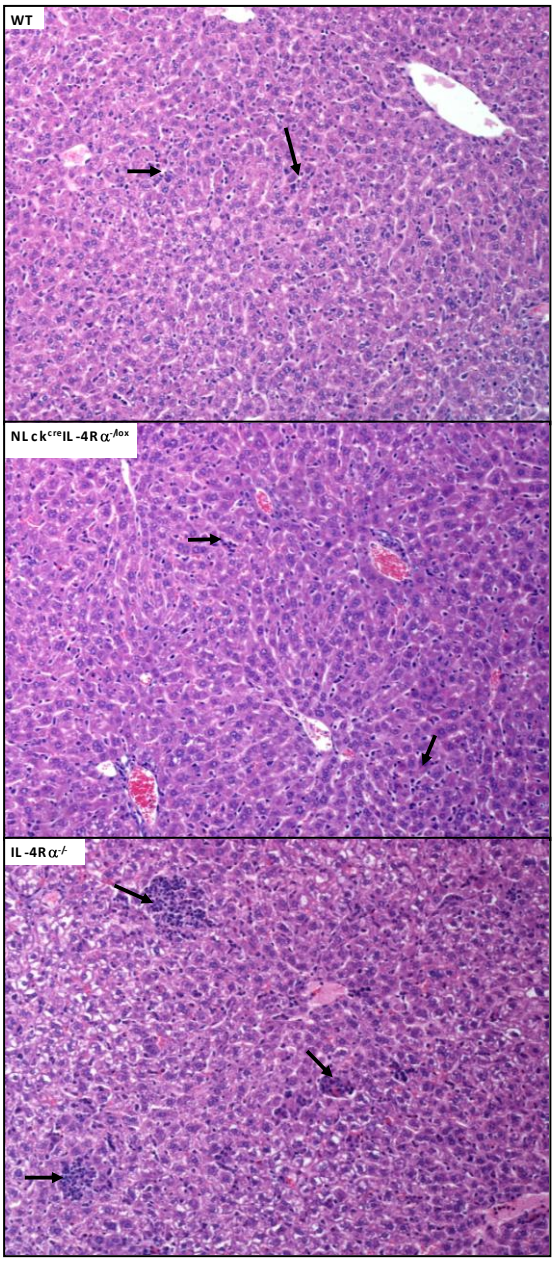


Fig.5.5D Liver tissue sections depicting acinar inflammation in WT (top), NLck^{cre}IL-4Rα$^{-/lox}$ (middle) and IL-4Rα$^{-/-}$ (bottom) female mice 12 days post-infection with *T. gondii*. IL-4Rα$^{-/-}$ mice displayed numerous foci of necrosis (arrows) whilst WT and NLck^{cre}IL-4Rα$^{-/lox}$ liver tissues had few and smaller foci of inflammation.

5.1.4 Systemic cytokine levels in male and female mice during *T. gondii* infection

Sera taken from *T. gondii* infected male and female mice was analysed for the level of circulating cytokines at day 9 for male mice, and days 9 and 12 post-infection for female mice. IFN-γ levels were high in the sera of male mice from all 3 groups (Fig.5.6A). Similarly for female mice, the levels of sera IFN-γ were increased in all 3 strains of mice at day 9 with no significant difference observed between WT, NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice (Fig.5.6B). While in this study systemic IFN-γ levels were comparable between males and females at day 9 post-infection, in another repeat study they were significantly greater in females. However, by day 12 post-infection, IFN-γ levels detected in the sera in female mice were reduced compared with day 9, which was consistent with the observation in the previous chapter where IFN-γ levels were lower at day 12 post-infection. As in the previous chapter, female IL-4Rα$^{-/-}$ mice displayed significantly different IFN-γ levels compared with WT and NLck^{cre}IL-4Rα$^{-/lox}$ mice at this time point (Fig.5.6C). However, in this study the levels were significantly reduced ($p<0.05$).

In male mice, sera IL-12p40/70 levels were similar for NLck^{cre}IL-4Rα$^{-/lox}$ and WT mice, but significantly higher ($p<0.01$) in IL-4Rα$^{-/-}$mice compared with WT mice at day 9 post-infection (Fig.5.6D). Similarly, in female mice serum IL-12p40/70 was significantly increased in IL-4Rα$^{-/-}$ ($p<0.01$) compared with WT, NLck^{cre}IL-4Rα$^{-/lox}$ mice at day 9 post-infection (Fig.5.6E). At day 12 post-infection, the level of IL-12p40/70 in sera from female NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice was significantly

increased in comparison with WT mice ($p<0.05$) (Fig.5.6F), whereas in the previous chapter there was no difference in serum IL-12 levels between WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice both at day 9 and at day 12 post-infection with *T. gondii* (Fig.4.6C, D).

Overall, at day 9 post-infection, both male and female mice displayed an increased systemic type-1 response with significantly higher IL-12p40/70 being detected in IL-4R$\alpha^{-/-}$mice compared with WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ mice. Female NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice maintained the robust systemic IL-12p40/40 production at day 12; although IFN-γ levels were markedly reduced in the more susceptible female IL-4R$\alpha^{-/-}$ mice at day 12 post-infection.

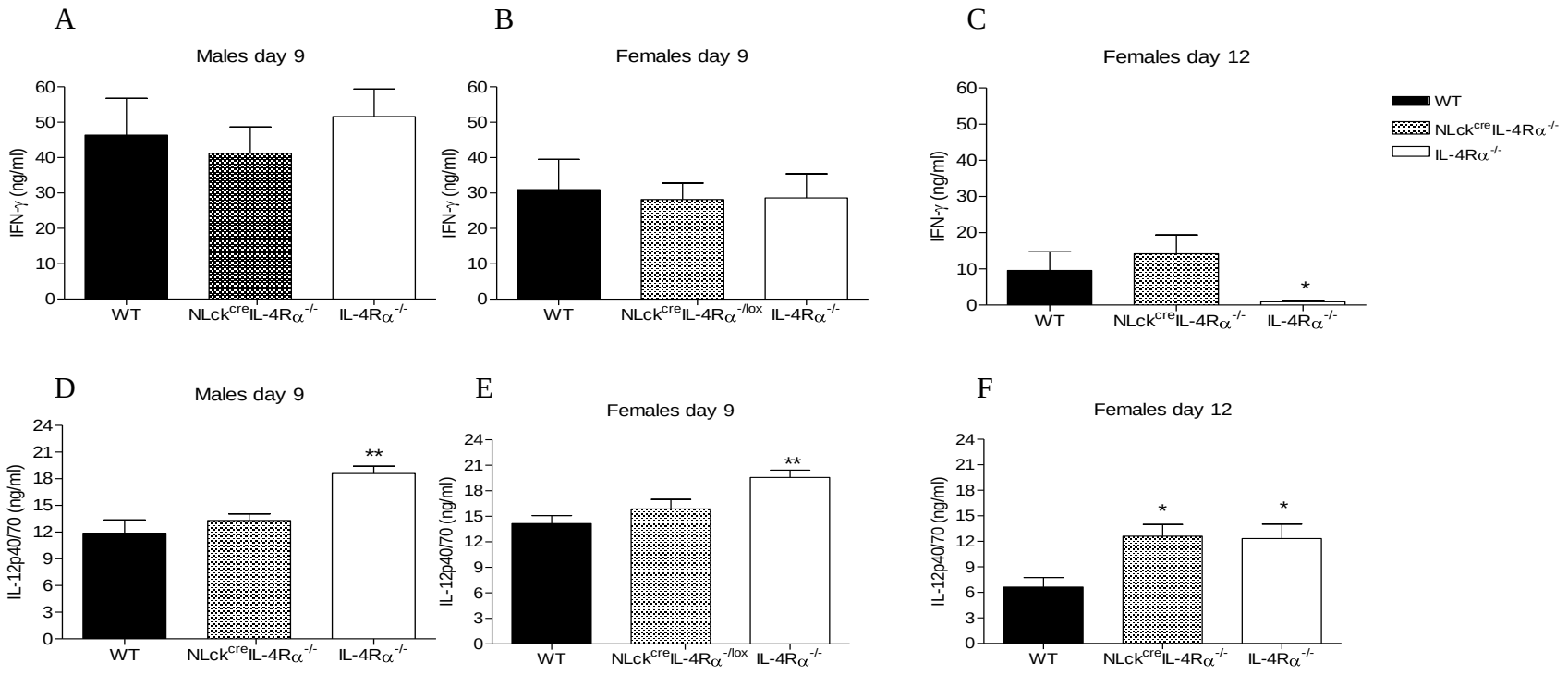


Fig.5.6 The level of systemic IFN-γ and IL-12p40/70 in male mice at day 9 post-infection (A, D respectively) and in female mice at day 9 (B, E) and day in female mice at day 12 (C, F) post-infection with *T. gondii*. **p<0.01 IL-4Rα$^{-/-}$ compared with WT and NLck^{cre}IL-4Rα$^{-/lox}$ mice

5.1.5 Analysis of *T. gondii*-induced splenocyte cytokine production

To further analyze the role of IL-4Rα signaling in the generation of antigen-specific type-1 and type-2 recall responses, spleen cells from *T. gondii* infected WT, NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were cultured with media only (non-stimulated control) or TLA (at concentrations 1μg/ml and 5μg/ml); or ConA (5μg/ml). Culture supernatants were subsequently analyzed for cytokine production by ELISA.

Similar to our observations in the previous chapter where splenocytes from *T. gondii* infected male Lck^{cre}IL-4Rα$^{-/lox}$ mice displayed reduced NO responses, *T. gondii* antigen-specific NO production as measured by nitrite concentration was significantly reduced in male NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice ($p<0.01$) compared with WT mice at day 9 post-infection (Fig.5.7A). Splenocyte derived from WT, NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ female mice failed to respond to antigen stimulation and nitrite was only detected in ConA stimulated cultures at this time point (Fig.5.7B). By day 12 post-infection, the capacity of splenocytes derived from female mice to respond to *T. gondii* antigen was evident. There was an increased level of antigen-specific NO production by splenocytes from female mice at day 12 post-infection, which was significantly reduced in IL-4Rα$^{-/-}$ mice compared with WT and NLck^{cre}IL-4Rα$^{-/lox}$ mice ($p<0.05$)

In addition, splenocytes from male mice produced IFN-γ in response to TLA stimulation, whereas splenocytes from female mice failed to produce IFN-γ when stimulated with various doses of TLA (Fig.5.8A, B), which is consistent with our

observations in the previous chapter (4). Moreover, TLA stimulated splenocytes from male NLck^{cre}IL-4R$\alpha^{-/lox}$ mice produced significantly lower levels of IFN-γ compared with WT and IL-4R$\alpha^{-/-}$ mice ($p<0.05$) at day 9 post-infection (Fig. 5.8A), whereas in chapter 4 mice deficient of IL-4Rα on CD4^{+} T-cells only (Lck^{cre}IL-4R$\alpha^{-/lox}$) were able to maintain antigen-specific splenocyte recall IFN-γ responses similar to that of their WT counterparts (Fig.4.8A). Splenocytes from female mice recovered their ability to respond to antigen and produce IFN-γ by day 12 post-infection, and IFN-γ levels were lower in NLck^{cre}IL-4R$\alpha^{-/lox}$ mice compared with WT and IL-4R$\alpha^{-/-}$ mice (Fig.5.8C).

Furthermore, IL-12p40/70 production was greater in TLA stimulated splenocytes from male mice compared with female mice at day 9 post-infection with *T. gondii*, nevertheless there was no significant difference observed in the levels of parasite-induced IL-12p40/70 between WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ in splenocyte cultures from both male and female mice at day 9 post-infection (Fig.5.9A,B). Furthermore, IL-12p40/70 production remained low in female mice at day 12 post-infection. In fact, female WT and NLck^{cre}IL-4R$\alpha^{-/-}$ mice displayed a significantly lower IL-12 production ($p<0.05$) in comparison with IL-4R$\alpha^{-/-}$ mice at day 12 post-infection (Fig.5.9C). As in the previous chapter, IL-12 production by male mice was significantly greater than in female mice.

Although female mice generally displayed an impaired antigen-specific type-1 response compared with male mice at day 9 post-infection, antigen-specific type-2 recall

responses were similar in male and female mice irrespective of IL-4Rα deficiency at this time point. IL-4 (Fig.5.10A, B) and IL-10 (Fig.5.11A, B) production was low in in TLA stimulated splenocytes from *T. gondii* infected male and female and there was no differences observed between WT, NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice at day 9 post-infection. In ConA stimulated cultures, male IL-4Rα$^{-/-}$ mice produced significantly increased levels of IL-4 compared with WT and NLck^{cre}IL-4Rα$^{-/lox}$ splenocytes, whereas in splenocytes from female mice there was no difference between the 3 strains in ConA stimulated cultures. At day 12 post-infection, IL-4 production was diminished in TLA stimulated splenocytes from all 3 strains of mice and was only detected in ConA stimulated splenocytes, where IL-4Rα$^{-/-}$ splenocyte cultures had significantly higher levels of IL-4 compared with WT and NLck^{cre}IL-4Rα$^{-/lox}$ cultures (Fig.5.10C). IL-10 production remained low in TLA-stimulated splenocytes from female mice of all 3 strains 12 days post-infection, and there was no difference between the strains (Fig.5.11C).

Overall, female mice appeared to have delayed TLA specific T-cell type-1 responses, whereas male mice had a strong type-1 response from an early time point. Of note, the antigen induced T-cell specific response of NLck^{cre}IL-4Rα$^{-/lox}$ mice was significantly less than that of either WT or IL-4Rα$^{-/-}$ mice and this was noted for both male and female mice. This effect was not evident in Lck^{cre}IL-4Rα$^{-/lox}$ mice as opposed to NLck^{cre}IL-4Rα$^{-/lox}$.

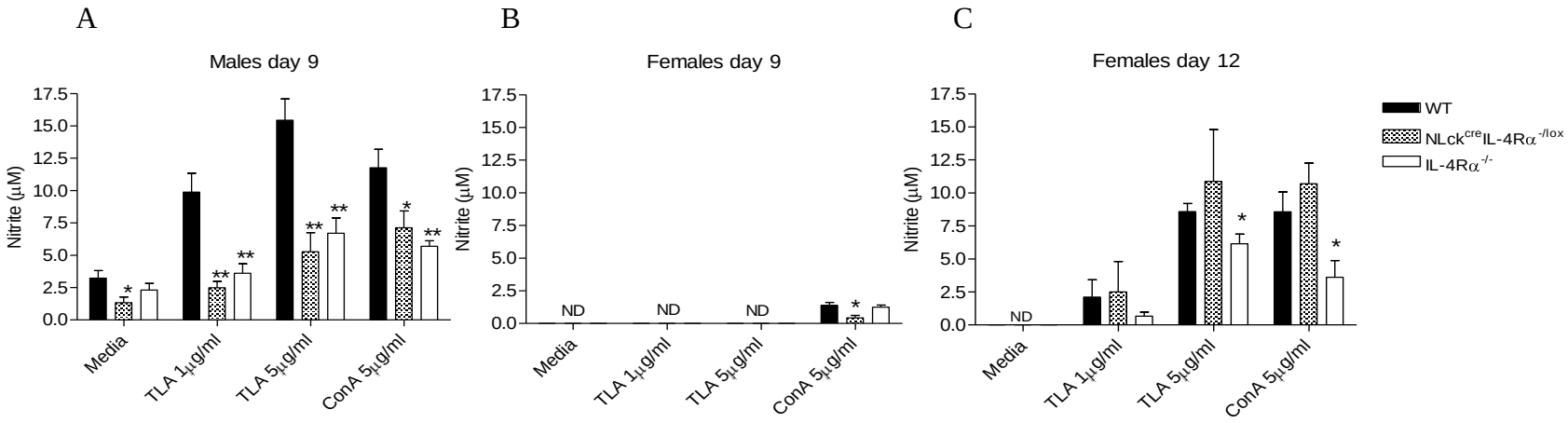


Fig. 5.7 NO production as measured by nitrite levels in splenocytes from *T. gondii* infected male (A) and female mice 9 days (B) and 12 days (C) post-infection. *p<0.05, **p<0.01 compared with corresponding WT

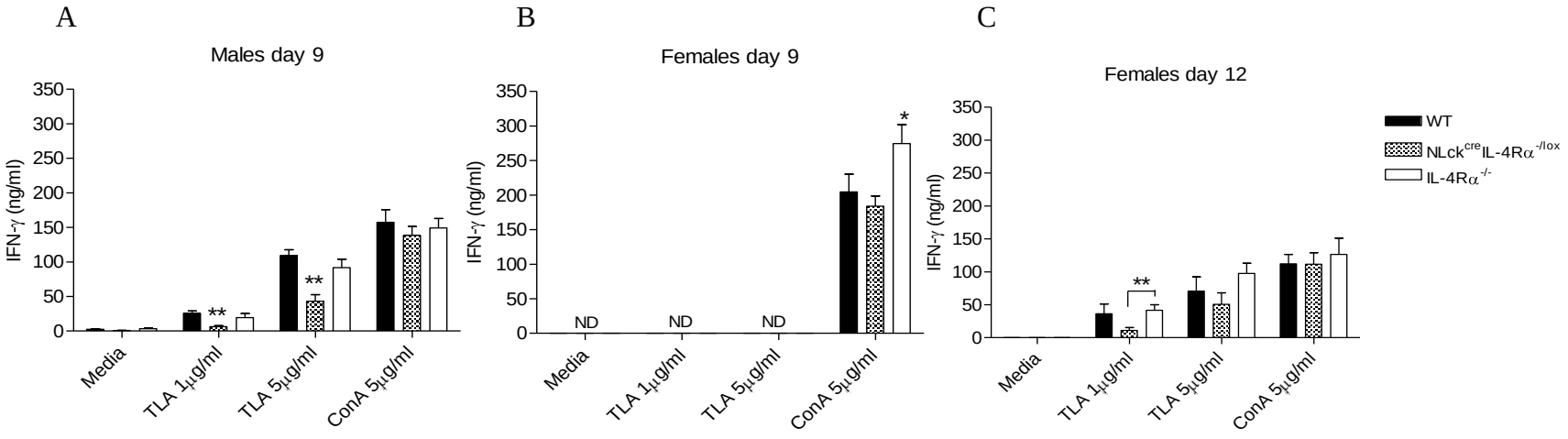


Fig. 5.8 IFN-γ production by splenocytes from *T. gondii* infected male mice at day 9 post-infection (A) and female mice at day 9 (B) and day 12 (C) post-infection.*p<0.05, **p<0.01 compared with WT of corresponding treatment

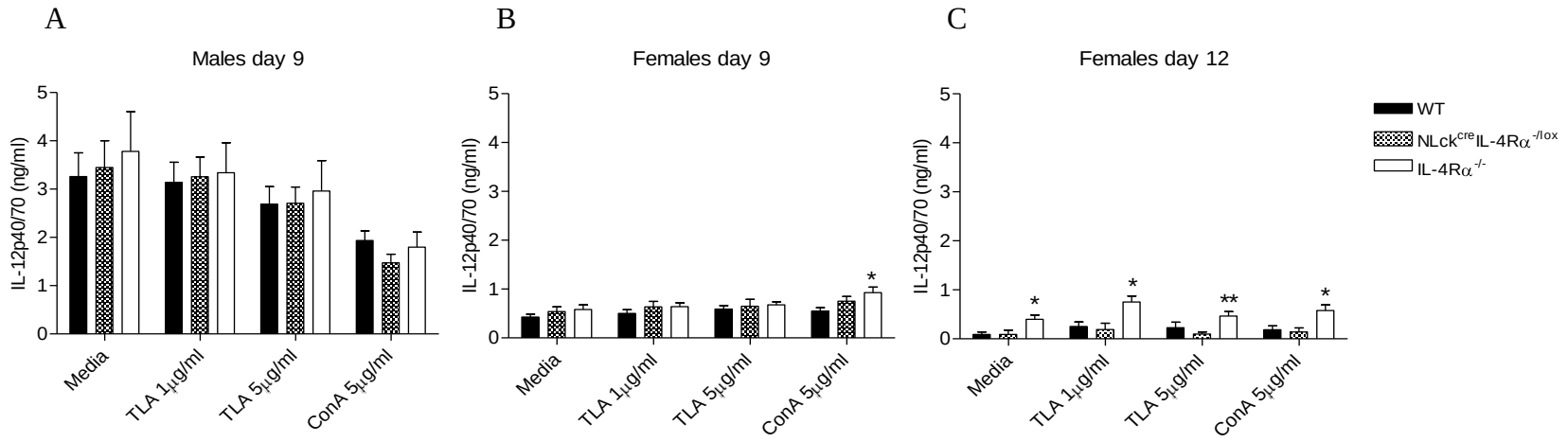


Fig. 5.9 IL-12p40/70 production by splenocytes derived from *T. gondii* infected male mice 9 days post-infection (A) and female mice 9 days (B) and 12 days (C) post-infection.*$p<0.05$, **$p<0.01$ compared with corresponding WT.

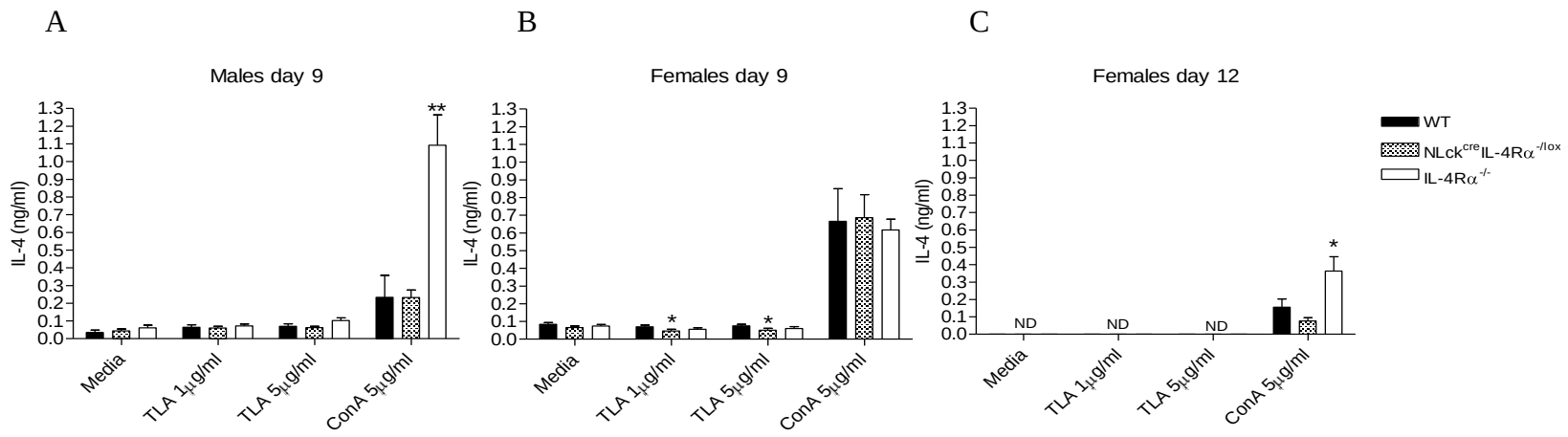


Fig. 5.10 IL-4 production by splenocytes from *T. gondii* infected male mice 9 days post-infection (A) and female mice 9 days (B) and 12 days (C) post-infection. *p<0.05, **p<0.01 compared with corresponding WT andNLck^{cre}IL-4R$\alpha^{-/lox}$ mice

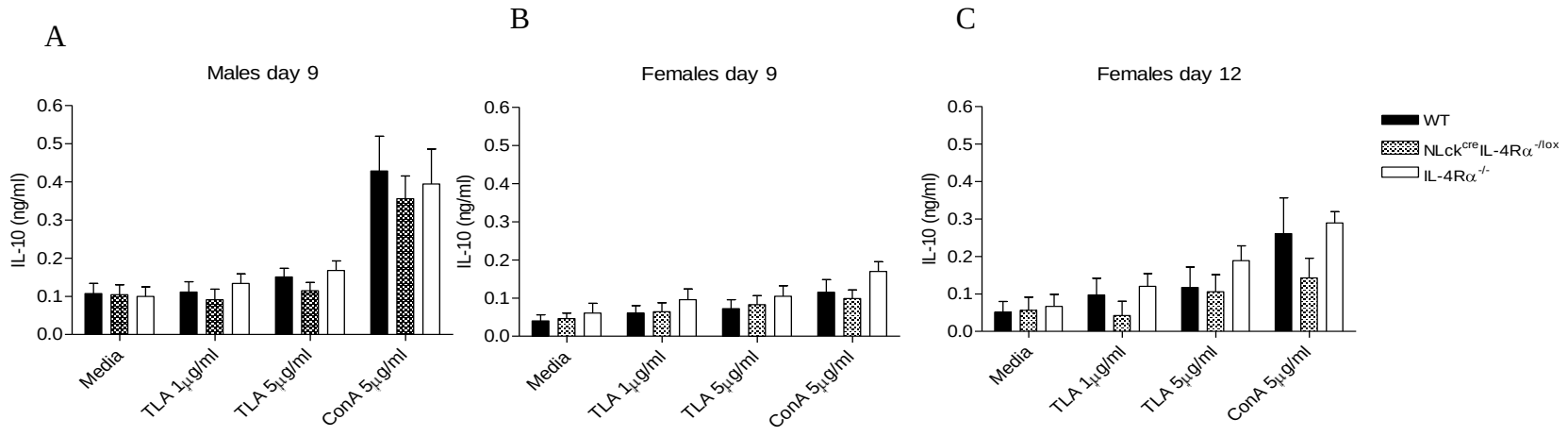


Fig. 5.11 IL-10 production by splenocytes from *T. gondii* infected male mice 9 days post-infection (A) and female mice 9 days (B) and 12 days (C) post-infection.

5.1.6 Analyses of cytokine mRNA levels and parasite burden in the lungs of *T. gondii* infected mice

Lung tissues from infected WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ male (day 9) and female (days 9 and 12) mice were examined for cytokine mRNA transcript expression using RT-PCR to further analyze the immune response in the lungs. At day 9 post-infection IFN-γ transcripts were significantly up-regulated in the lungs of male WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice but there was no difference observed between the 3 strains (Fig.5.12A). In female mice IFN-γ transcript levels in the lungs were slightly reduced at day 9 post-infection in comparison with the levels observed in male mice; nonetheless there was no difference in the level of expression between the 3 strains at day 9 (Fig.5.12 B) and day 12 (Fig.5.12C) post-infection.

Male mice had high levels of IL-12p40 transcript expression in the lungs at day 9 post-infection, and lungs from IL-4R$\alpha^{-/-}$mice had significantly reduced levels compared with their WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ counterparts ($p<0.05$) mice at this time point (Fig.5.12D). On the other hand, female mice displayed very low levels (~100-fold less) of IL-12p40 transcript levels in the lungs at day 9 post-infection in comparison with the levels observed in male mice at the same time point (Fig.5.12E), nonetheless there was no difference between the 3 strains. At day 12 post-infection, lung IL-12p40 transcript expression was upregulated approximately 10-fold over in female mice (Fig.512F), and IL-4R$\alpha^{-/-}$ mice had significantly increased IL-12p40 transcripts in the lungs compared

with lungs from infected female WT mice at this time point.

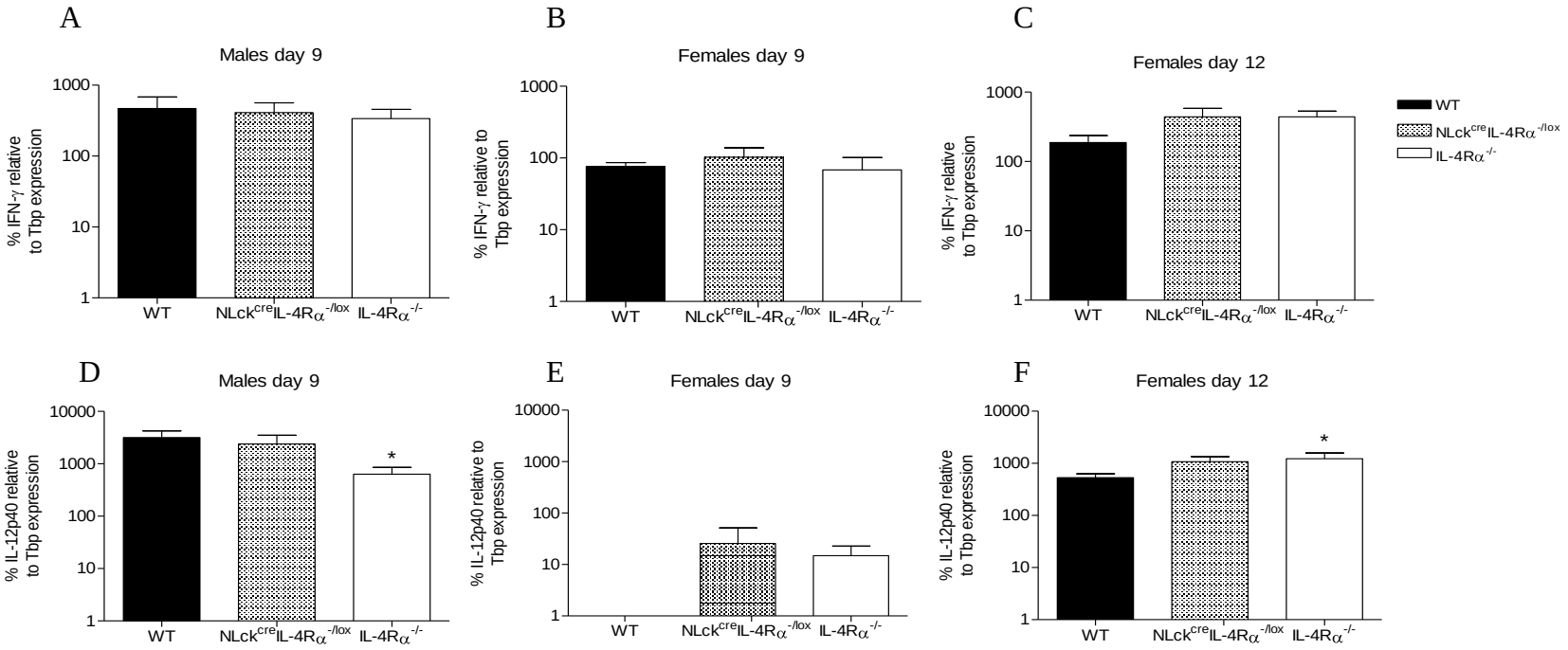


Fig.5.12 IFN-γ and IL-12p40 mRNA transcript expression in lungs from infected male and female mice 9 days post-infection with *T. gondii*, IFN-γ in males 9 days (A), in females 9 days (B), and in female mice 12 days (C) post-infection. IL-12p40 transcript expression in male mice 9 days (D), female mice 9 days (E) and female mice 12 days (post-infection with *T. gondii*. *$p<0.05$ compared with WT controls

NOS2 and Arginase 1 compete for the same substrate L-Arginine to produce either nitric oxide or alternatively L-ornithine and proline respectively. We compared expression of NOS2 and Arginase 1 mRNA transcripts in the lungs of infected male and female mice following *T. gondii* infection. NOS2 mRNA transcript levels were expressed in abundance in the lungs of *T. gondii* infected male mice at day 9 post-infection (Fig.5.13A) compared with lower levels observed in female mice (Fig.5.13B), nonetheless there was no difference between WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ in both male and female mice at this time point. At day 12 post-infection, lung NOS2 transcripts were significantly up-regulated in the lungs of infected female mice with NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice having significantly higher levels ($p<0.05$) compared with WT mice (Fig.5.13C). Similarly, male mice expressed higher levels (~1000-fold) of lung Arginase1 mRNA transcripts than female mice 9 days post-infection (Fig.5.13D, E), nevertheless there was no difference between the 3 strains in both male and female mice at day 9 post-infection. By day 12 post-infection, female mice had increased lung Arginase expression at least 10-fold than at day 9, and IL-4R$\alpha^{-/-}$ mice had significantly increased levels of Arginase1 mRNA transcripts compared with WT mice at this time point (Fig.5. 13F). In addition, the level of Arginase 1 mRNA transcripts appeared to be higher than NOS2 mRNA transcripts in both male and female mice at both time points investigated, suggesting that during acute *T. gondii* infection Arginase 1 expression is highly upregulated relative to NOS2 mRNA transcription.

Furthermore, markers of alternative macrophage activation were also measured in the

lungs. Lung YM1 (Fig.5.14A) and FIZZ1 (Fig.5.14D) transcript expression in male mice was highly upregulated at day 9 post-infection compared with female mice at the same time point. In addition, although there was no difference observed in female YM1 transcripts between the 3 strains at day 9 (Fig.5.14B), at day 12 post-infection female IL-4R$\alpha^{-/-}$ mice had significantly increased levels of lung YM1 compared with WT mice ($p<0.05$) (Fig.5.14C). Furthermore, in female mice a significant reduction of FIZZ1 transcript expression was observed in the lungs of IL-4R$\alpha^{-/-}$ mice at day 9 post-infection ($p<0.05$), as compared with WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ mice (Fig.5.14E). There was however no difference observed in FIZZ1 mRNA transcript expression in female mice between the 3 strains at day 12 as observed at day 9 post-infection (Fig.5.14F).

Furthermore, we investigated the level of mRNA transcript expression of IFN-γ inducible genes IDO, LRG47 and Igtp GTPases, which have been reported to be involved in *T. gondii* eradication. IDO mRNA transcripts were highly upregulated in male mice at day 9 post-infection (Fig.5.15A) and in comparison much lower in female mice at day 9 post-infection (Fig.5.15B). However at day 12 post-infection IDO mRNA transcripts were increased in female lungs (Fig.5.15C). Nevertheless, there were no differences observed between WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$mice for both male and female.

The p47 GTPases transcripts on the other hand were highly up-regulated in the lungs of both male and female mice at day 9 post-infection, irrespective of IL-4Rα deficiency.

There was no significant difference in LRG47 (Fig.5.16A, D) and Igtp GTPase (Fig.5.16B, E) in male and female mice at 9 days post-infection. Howevever, at day 12 post-infection female NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice had significantly increased lung LRG47 transcripts ($p<0.05$) compared with WT mice (Fig.5.16C), and in addition Igtp transcripts were significantly increased in female IL-4R$\alpha^{-/-}$ mice ($p<0.05$) compared with WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ mice at this time point (Fig.5.16F).

Finally, to measure parasite burden in the lungs of *T. gondii* infected mice, SAG1 (a tachyzoite specific gene) and TgCyst antigen (a bradyzoite specific gene) transcripts were quantified by RT-PCR. There was no difference in tachyzoite and bradyzoite mRNA transcripts in the lungs of male WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice at day 9 post-infection (Fig.5.17A). In female mice on the other hand, NLck^{cre}IL-4R$\alpha^{-/lox}$ mice had significantly higher cyst burden in the lungs compared with WT mice at day 9 post-infection (Fig.5.17B) and there was also an increased conversion of tachyzoites to bradyzoite form of the parasite in these mice. At day 9 post-infection, female IL-4R$\alpha^{-/-}$ mice also appeared to have increased tachyzoite to bradyzoite conversion relative to WT mice, however it was to a lesser extent than the rate of conversion observed in the lungs of NLck^{cre}IL-4R$\alpha^{-/lox}$ mice. At day 12 post-infection, the bradyzoite burden in the lungs of IL-4R$\alpha^{-/-}$ mice was significantly higher compared WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ mice ($p<0.05$) at this time-point (Fig.5.17C).

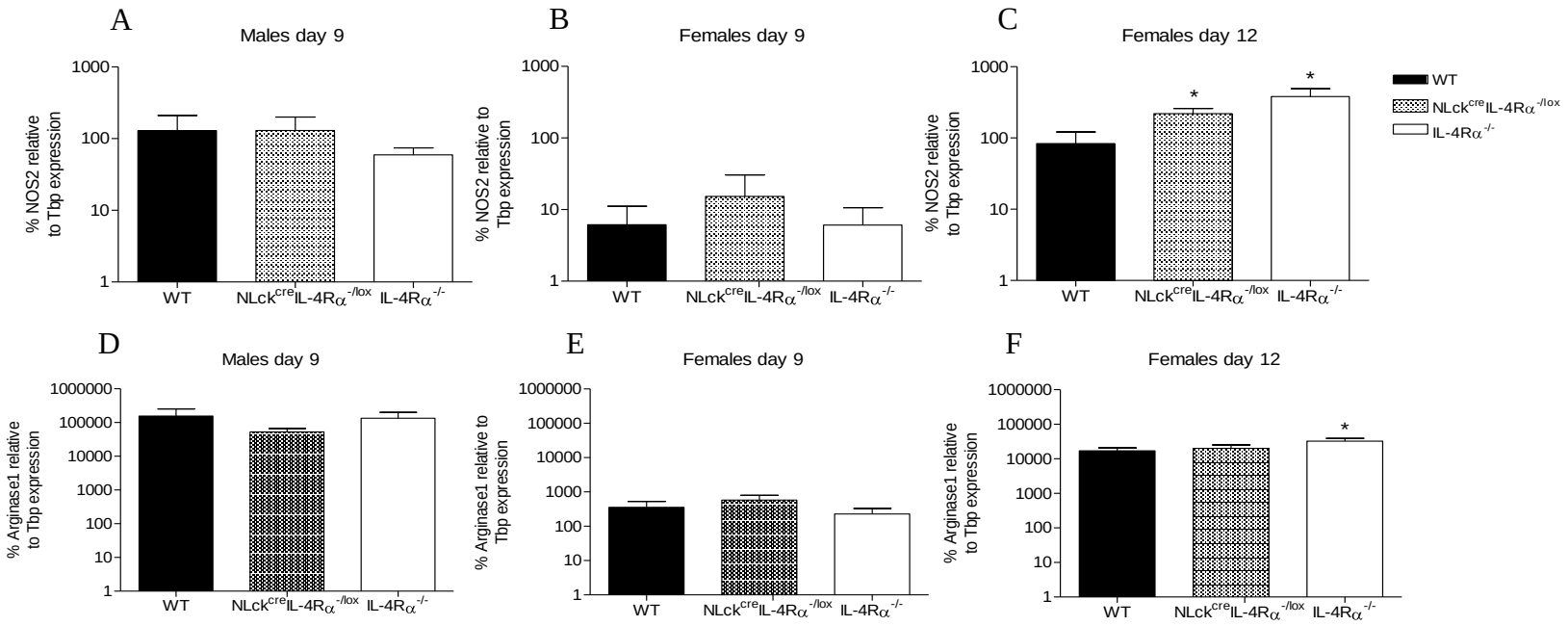


Fig.5.13 NOS2 and Arginase1 mRNA transcript expression in the lungs of male mice 9 days(A, D), female mice 9 days (B, E) and female mice 12 days (C, F) post-infection with *T. gondii*. *p<0.05 compared with WT control

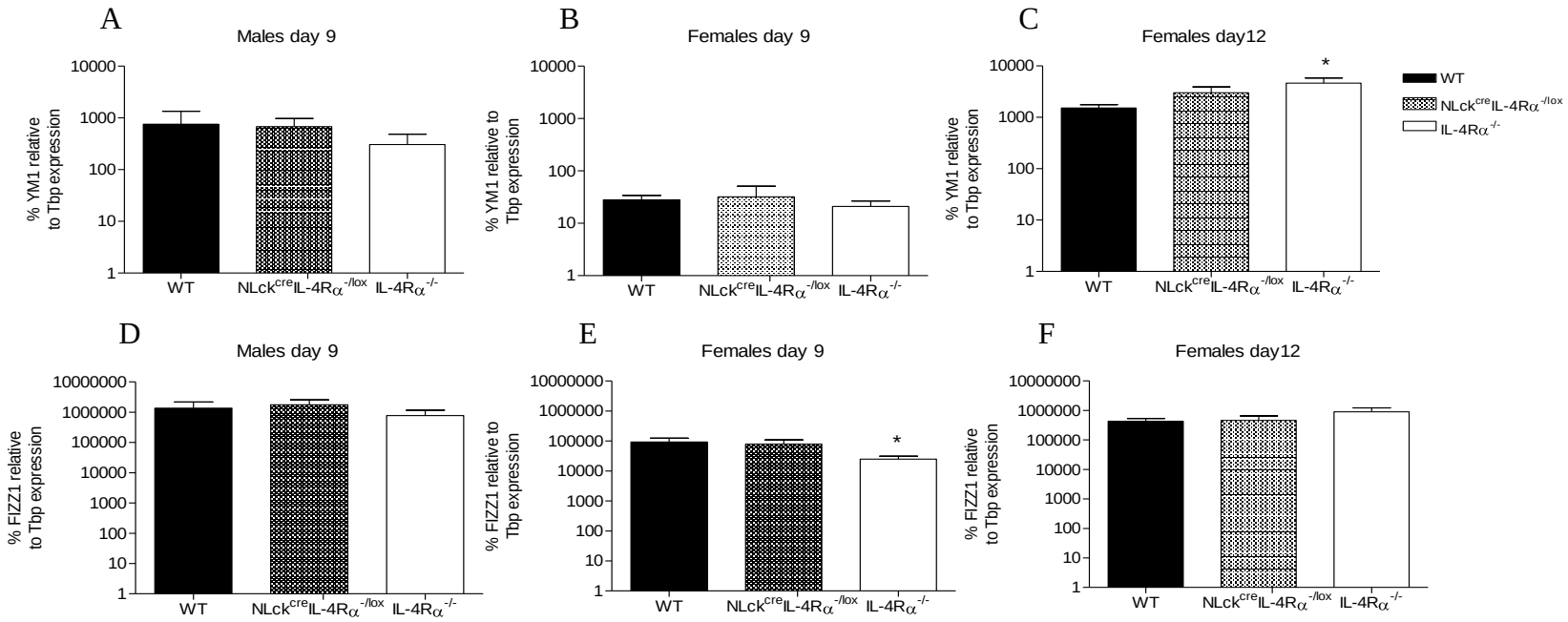


Fig. 5.14 YM1 and FIZZ1 mRNA transcript expression in the lungs of male mice 9 days post-infection (A, D), female mice 9 days (B, E) and female mice 12 days (C, F) post-infection with *T. gondii*. *p<0.05, compared with WT controls

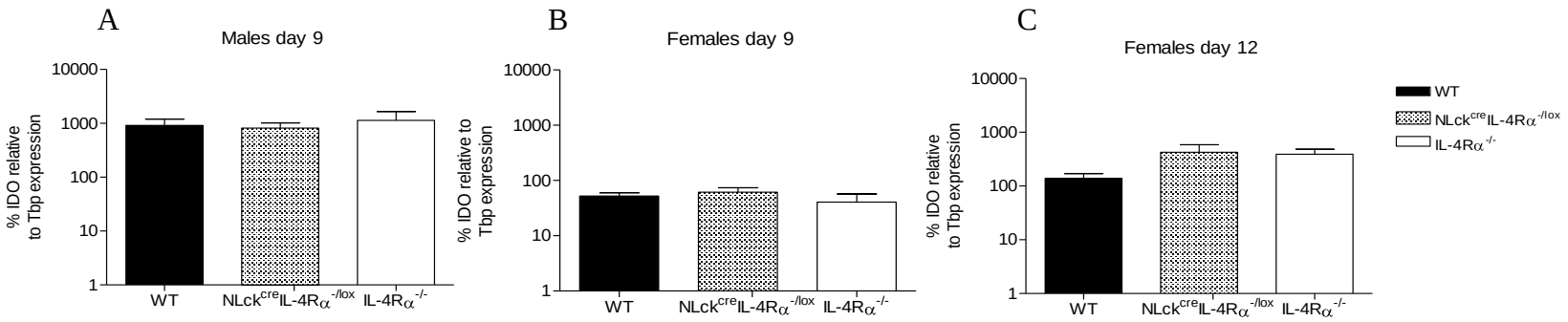


Fig.5.15 IDO mRNA transcript expression in the lungs of male mice 9 days post-infection (A), female mice 9 days post-infection (B) and female mice 12 days post-infection (C) with *T. gondii*.

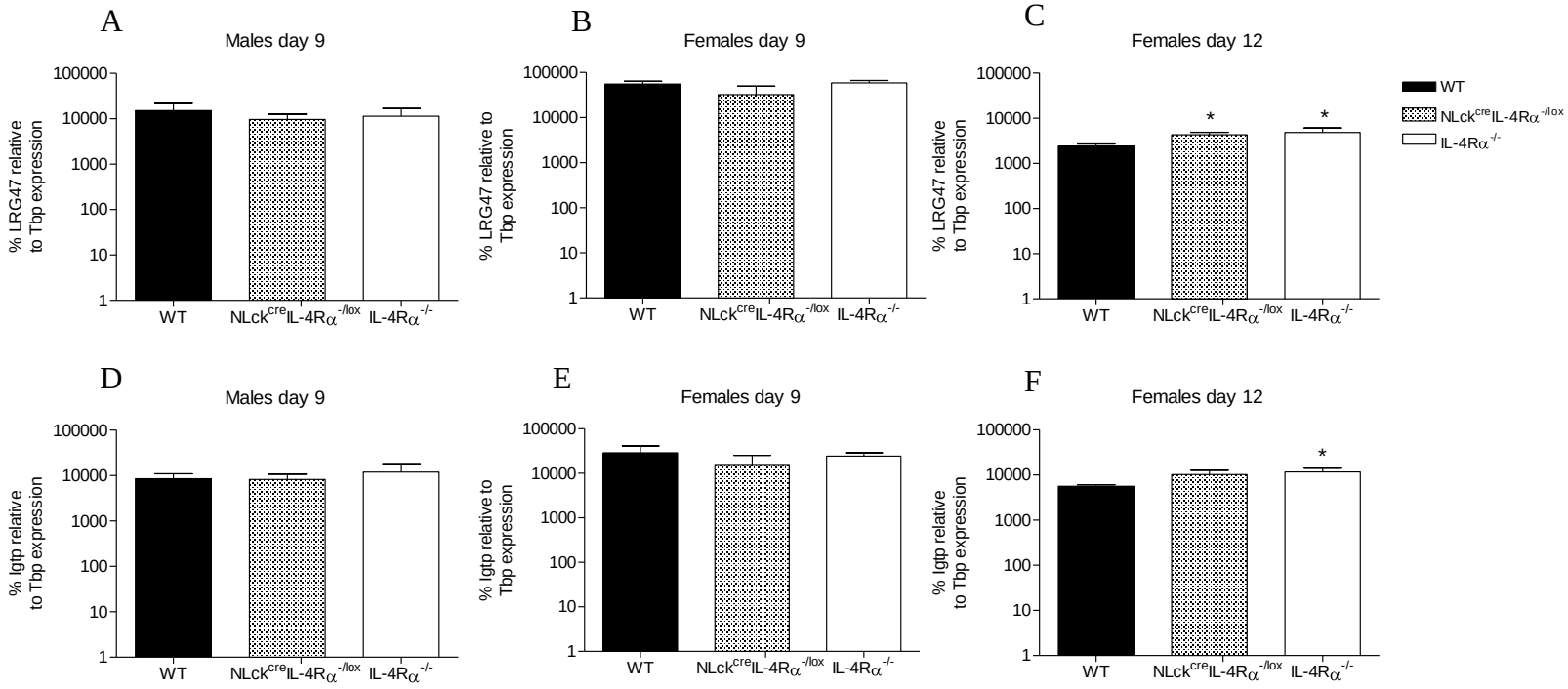


Fig.5.16 IFN-γ-inducible genes LRG47 and Igtp GTPase mRNA transcript expression in the lungs of male mice 9 days post-infection (A, D) female mice 9 days post-infection (B, E) and female mice 12 days with *T. gondii* (C, F). *p<0.05, compared with WT controls

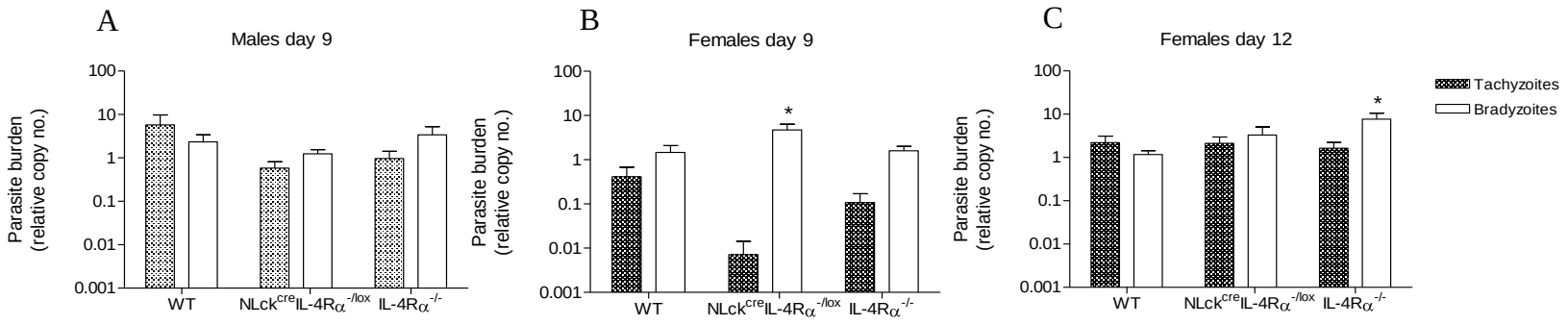


Fig. 5.17 Parasite burden in the lungs of male mice at day 9 (A) female mice at day9 (B) and female mice at day 12 (C) post-infection with T. gondii,as measured by tachyzoite (SAG1) and bradyzoite (TgCyst antigen) specific mRNA transcripts by RT-PCR. Bradyzoite mRNA was significantly higher in female NLck^{cre}IL-4R$\alpha^{-/lox}$ mice (p=0.0278) compared with WT and IL-4R$\alpha^{-/-}$ female mice at day 9 post-infection (B), whereas at day 12 post-infection female IL-4R$\alpha^{-/-}$ mice had significantly higher cyst burden in the lungs compared with WT mice (p=0.015) (C).*p<0.05 compared with corresponding parasite form in WT controls.

5.1.7 Histopathology analysis during chronic *T. gondii* infection

Lung, liver and brain tissue sections from *T. gondii* infected male mice were examined for pathology 35 days post-infection. There was mild to moderate peribronchial inflammation in WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ lungs, whilst IL-4R$\alpha^{-/-}$ mice displayed significantly reduced levels of peribronchial inflammation ($p<0.05$) compared to the latter strains at day 35 post-infection (Fig. 5.18A). Minimal interstitial inflammation was observed in the lungs of WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice but there was no significant difference between the 3 strains (Fig.5.18B).

The portal tracts of the liver sections from all 3 strains of mice displayed no pathology 35 days post-infection with *T. gondii* (Fig.5.18C). Furthermore, there was minimal acinar inflammation in the liver of mice from all 3 strains (Fig.5.18D). Overall, at day 35 post-infection there was mild to moderate damage in the lungs of WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ male mice, whilst the lungs from IL-4R$\alpha^{-/-}$ mice displayed significantly lower levels of inflammation. There was minimal damage to the liver and no difference between the 3 strains.

In the brains, the level of inflammation of the meninges (Fig.5.19A), and perivascular cuffing (Fig.5.19B) was minimal and comparable between the 3 groups 35 days post-infection. In addition there was equivalent parenchymal activity (Fig.5.19C) and similar numbers of cysts counted (Fig.5.19D) in the brains of the 3 strains at this time point. Essentially, there was minimal inflammation in the brains and low parasite cysts

observed in the brains of male mice from WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice 35 days post-infection with *T. gondii*.

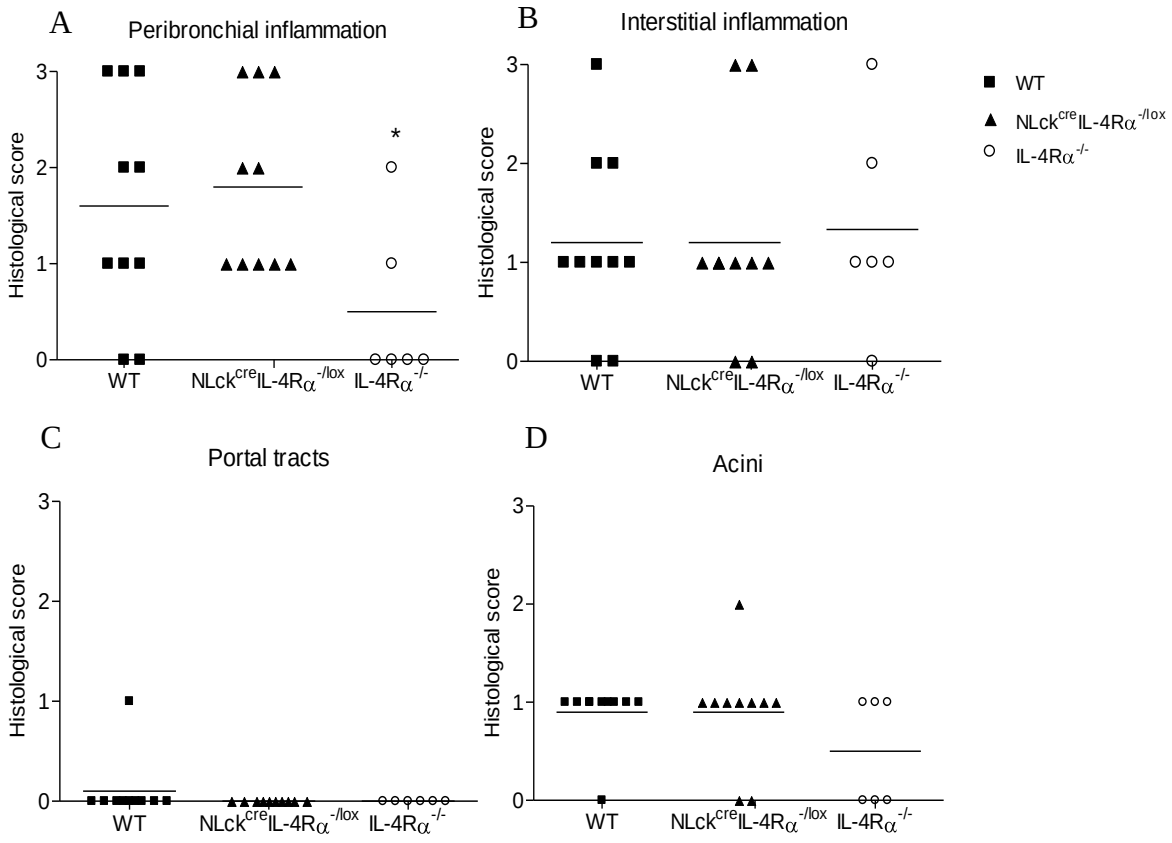


Fig. 5.18 Lung peribronchial (A) and interstitial inflammation (B) and liver portal tracts (C) and acinar inflammation (D) in male mice 35 days post infection with *T. gondii*.

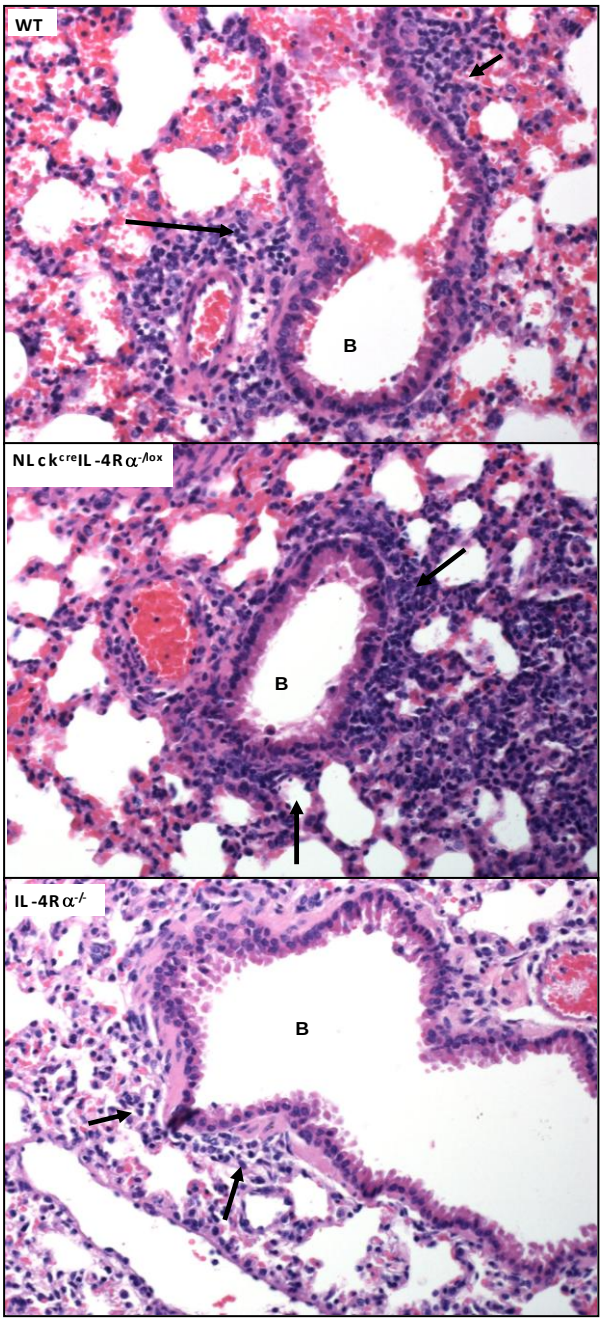


Fig.5.18A Lung tissue sections showing peribronchial inflammation in male WT (top), NLck^{cre}IL-4Rα$^{-/lox}$ (middle) and IL-4Rα$^{-/-}$ (bottom) mice 35 days post-infection with *T. gondii*. WT and NLck^{cre}IL-4Rα$^{-/lox}$ mice displayed mild degrees of inflammation compared with the minimal inflammation observed in IL-4Rα$^{-/-}$ lungs.

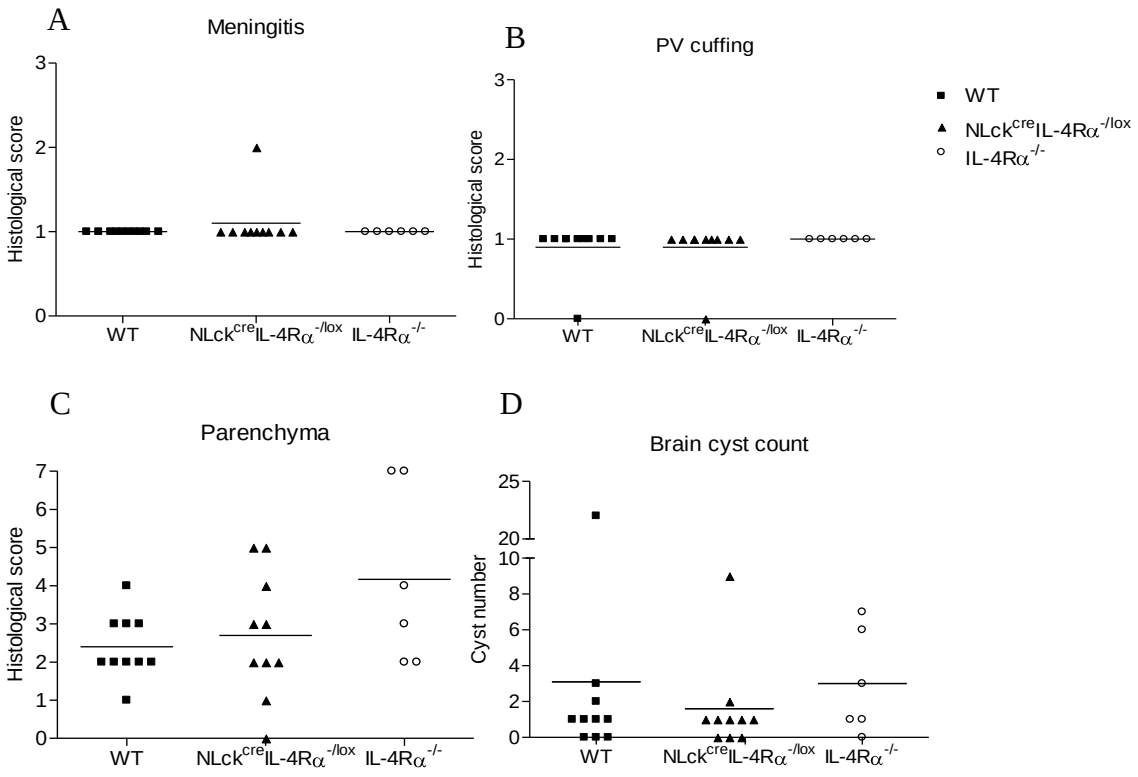


Fig.5.19 Pathological analysis of the brains of *T. gondii* infected WT (closed square), NLck^{cre}IL-4R$\alpha^{-/lox}$ (closed triagle) and IL-4R$\alpha^{-/-}$ (open circle) male mice 35 days post-infection. Meningitis pneumonitis (A), perivascular cuffing (B), parenchymal activity (C) and the number of parasite cysts counted in the brain (D). Data representative of one experiment (n=1).

Furthermore, we examined lung and brain histopathology in tissues from *T. gondii* infected female mice at day 35 post-infection. For the previous experiment, we were unable to examine this time point for female mice as the IL-4R$\alpha^{-/-}$ mice succumbed to infection quite early and hence numbers were too few (n=2) due to early deaths. However, in this chapter, we had more mice available to begin with and therefore were able to have enough mice per experimental group surviving to day 35. We observed mild peribronchial inflammation (Fig.5.20A) and mild to moderate interstitial inflammation (Fig.5.20B) in the lungs of female WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice, and there was no difference in the degree of pathology observed in the lungs between the 3 strains 35 days post-infection. There was minimal inflammation in the portal tracts of WT and IL-4R$\alpha^{-/-}$ livers which was absent in the NLck^{cre}IL-4R$\alpha^{-/lox}$ livers, in fact IL-4R$\alpha^{-/-}$ liver portal tracts had significantly higher degree of inflammation ($p<0.05$) compared with NLck^{cre}IL-4R$\alpha^{-/lox}$ mice (Fig.5.20C). In addition the liver acini of WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ mice displayed minimal inflammation, whereas IL-4R$\alpha^{-/-}$ mice had mild damage to the acinar which was significantly higher than WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ mice (Fig.5.20D).

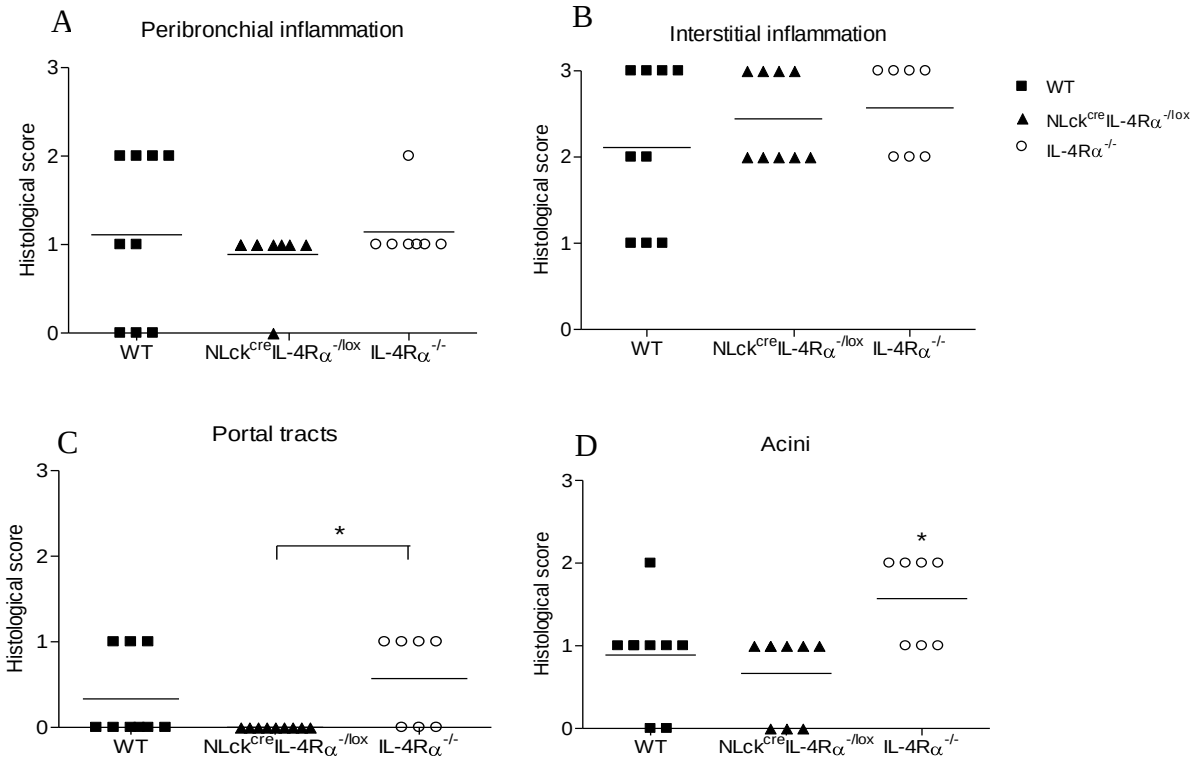
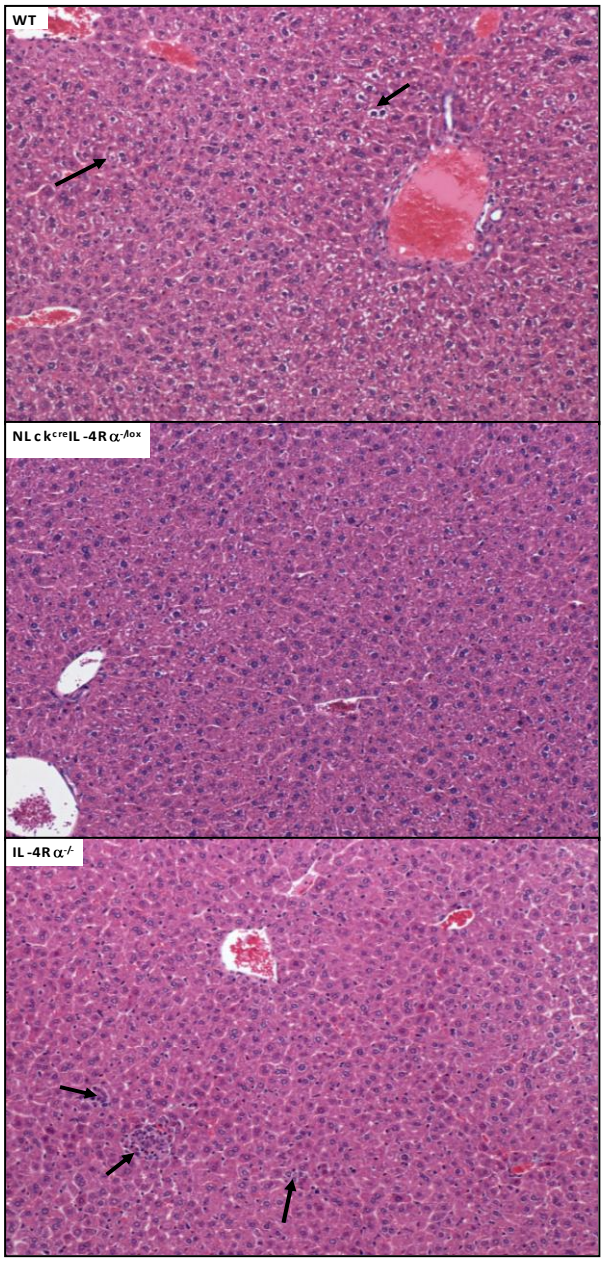


Fig.5.20 Lung peribronchial (A), and interstitial inflammation (B) and liver pathology in the portal tracts (C) and acini (D) in female mice 35 days post-infection with *T. gondii*. *p<0.05



5. 20C, D Liver sections of female WT (top), NLck^{cre}IL-4Rα$^{-/lox}$ (middle) and IL-4Rα$^{-/-}$ mice 35 days post-infection with *T. gondii*. WT livers displayed minimal degrees of inflammation with few collections of inflammatory cells (arrows); NLck^{cre}IL-4Rα$^{-/lox}$ mice had normal liver morphology with no inflammation whilst IL-4Rα$^{-/-}$ livers had mild inflammation with a few foci of necrosis (arrows).

Female mice displayed mild inflammation of the meninges in the brains (Fig.5.21A), which was slightly higher than the minimal degree observed in male mice at the same time point, as well as minimal perivascular cuffing (Fig.5.21B), which was similar in male mice (Fig.5.20B) and similarly to male mice, there was no difference between WT, NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice. However, there was significantly reduced parenchymal activity in the brains of female NLck^{cre}IL-4R$\alpha^{-/lox}$ mice compared with WT and IL-4R$\alpha^{-/-}$ female mice ($p<0.05$) (Fig.5.21C) which was not observed in male counterparts. In addition, there was a higher cyst burden in the brains of female IL-4R$\alpha^{-/-}$ mice compared with WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ mice, which again was not seen in male mice.

Overall, there was minimal brain pathology, very low to absent liver damage and minimal lung pathology in male mice at day 35 post-infection. In fact male IL-4R$\alpha^{-/-}$ displayed significantly lower degrees of lung inflammation compared with WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ mice. Whereas in female mice there was mild brain pathology, and unlike in male mice, female NLck^{cre}IL-4R$\alpha^{-/lox}$ mice had significantly reduced parenchymal activity compared with WT and IL-4R$\alpha^{-/-}$ mice and there was generally increased degrees of liver pathology and a higher cyst burden in the brain in female mice both of which were significantly higher in IL-4R$\alpha^{-/-}$ mice compared with WT and NLck^{cre}IL-4R$\alpha^{-/lox}$ mice.

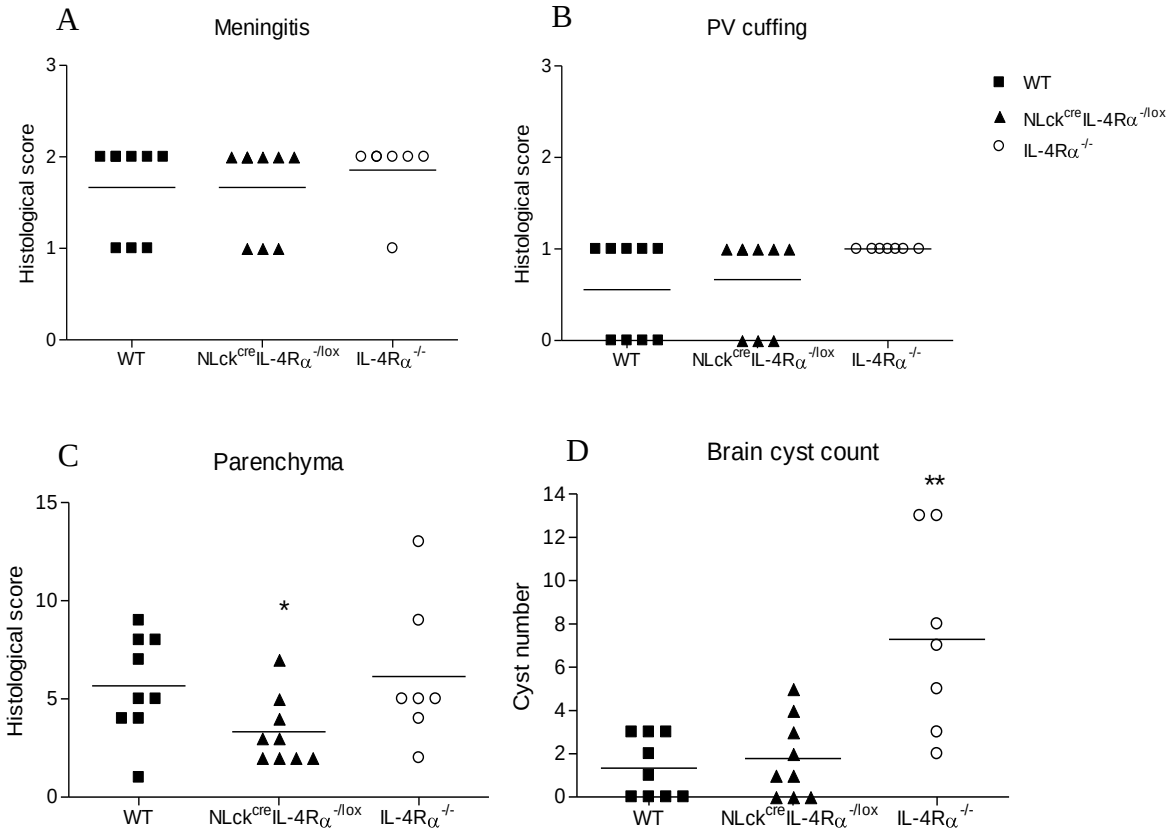


Fig.5.21 Pathological analysis of brain tissue from female mice infected with 10 cysts of *T. gondii* peritoneally 35 days post-infection. Meningitis (A), perivascular cuffing (B), parenchymal activity (C) and the number of cysts counted in the brain (D). *p<0.05, **p<0.01

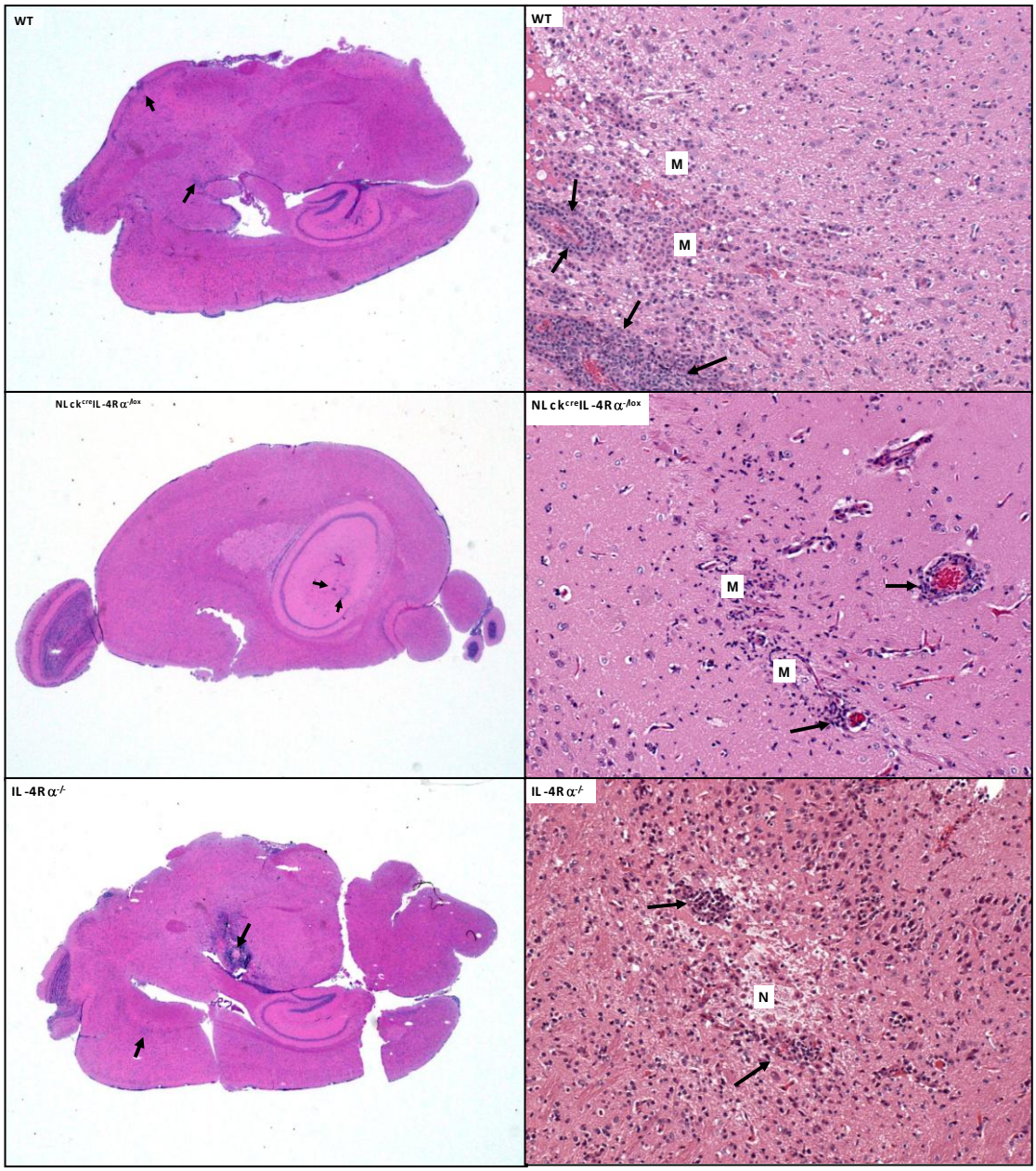


Fig. 5.21 Brain sections showing areas of inflammatory activity in female WT (top), NLck^{cre}IL-4Rα$^{-/lox}$ (middle) and IL-4Rα$^{-/-}$ (bottom) mice infected with *T. gondii* 35 days post-infection, in lower power (left panel) and high power resolution (right panel). WT (top) and NLck^{cre}IL-4Rα$^{-/lox}$ (middle) brains have some inflammatory foci (arrows) and meningitis with HP pictures

showing a small collection of microglia (M) and mild PV cuffing. IL-4R$\alpha^{-/-}$ (bottom) brains have a few areas of inflammatory activity and meningitis on the surface. HP picture shows areas of necrosis (N) with surrounding inflammation and PV cuffing by inflammatory cells (arrows).

5.1.8 Systemic cytokine and antibody response in mice chronically infected with *T. gondii*

The systemic levels of IFN-γ, IL-12p40/70 and parasite specific IgG1 and IgG2a antibodies were measured in the serum of male and female mice infected with *T. gondii* 35 days post-infection. IFN-γ was not detected in both male (Fig.5.22C) and female (Fig.5.22D) sera at this time point. However, there were similar levels of IL-12 detected in sera from both male (Fig.5.22A) and female (Fig.5.22B) WT, NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice at day 35 post-infection.

Furthermore, we examined *T. gondii* specific antibody responses by measuring *T. gondii* lysate antigen- specific IgG1 and IgG2a levels in the serum at day 35 post-infection in male and female mice. *T. gondii*-specific IgG1 was significantly higher ($p<0.05$) in sera of male IL-4Rα$^{-/-}$ mice compared with WT and NLck^{cre}IL-4Rα$^{-/lox}$ mice, and there was no significant difference between WT and NLck^{cre}IL-4Rα$^{-/lox}$ mice (Fig.5.23A). Conversely sera of female IL-4Rα$^{-/-}$ mice had significantly lower levels of IgG1 compared with WT and NLck^{cre}IL-4Rα$^{-/lox}$ mice ($p<0.05$) and there was no significant difference between NLck^{cre}IL-4Rα$^{-/lox}$ mice and WT littermates (Fig.5.23A). In addition, *T. gondii*-specific IgG2a levels were significantly reduced in sera of male NLck^{cre}IL-4Rα$^{-/lox}$ mice compared with WT littermates ($p<0.05$) and IL-4Rα$^{-/-}$ mice ($p<0.01$) (Fig.5.23B) and there was no difference observed between WT and IL-4Rα$^{-/-}$ male mice. On the other hand, female WT, NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice displayed significantly lower levels of *T. gondii*-specific IgG2a in their sera compared with their

male counterparts at day 35 post-infection. Nonetheless there was no significant difference in IgG2a levels between the 3 strains in female mice at this time point (Fig.5.23B). In general, the antigen specific type-2 (IgG1) antibody response was markedly higher in absence of IL-4Rα signaling in male mice but not in female mice. *T. gondii* specific type-1 antibody response (IgG2a) was significantly lower in male $NLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice compared with WT and $IL\text{-}4R\alpha^{-/-}$ mice, whereas in female mice there was no difference between the 3 starins and the levels were lower compared with male mice.

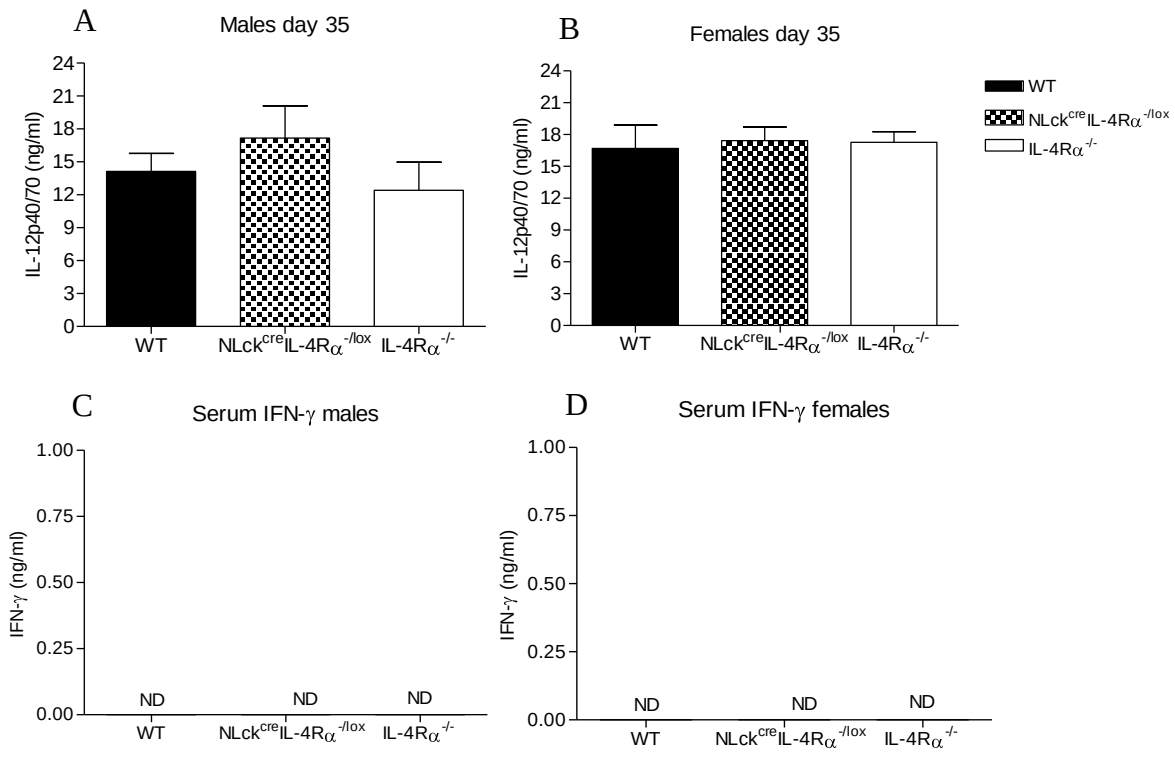


Fig. 5.22 IL-12p40/70 level in the sera of male (A) and female (B) and IFN-γ level in male (C) and female (D) mice at day 35 post-infection with *T. gondii*.

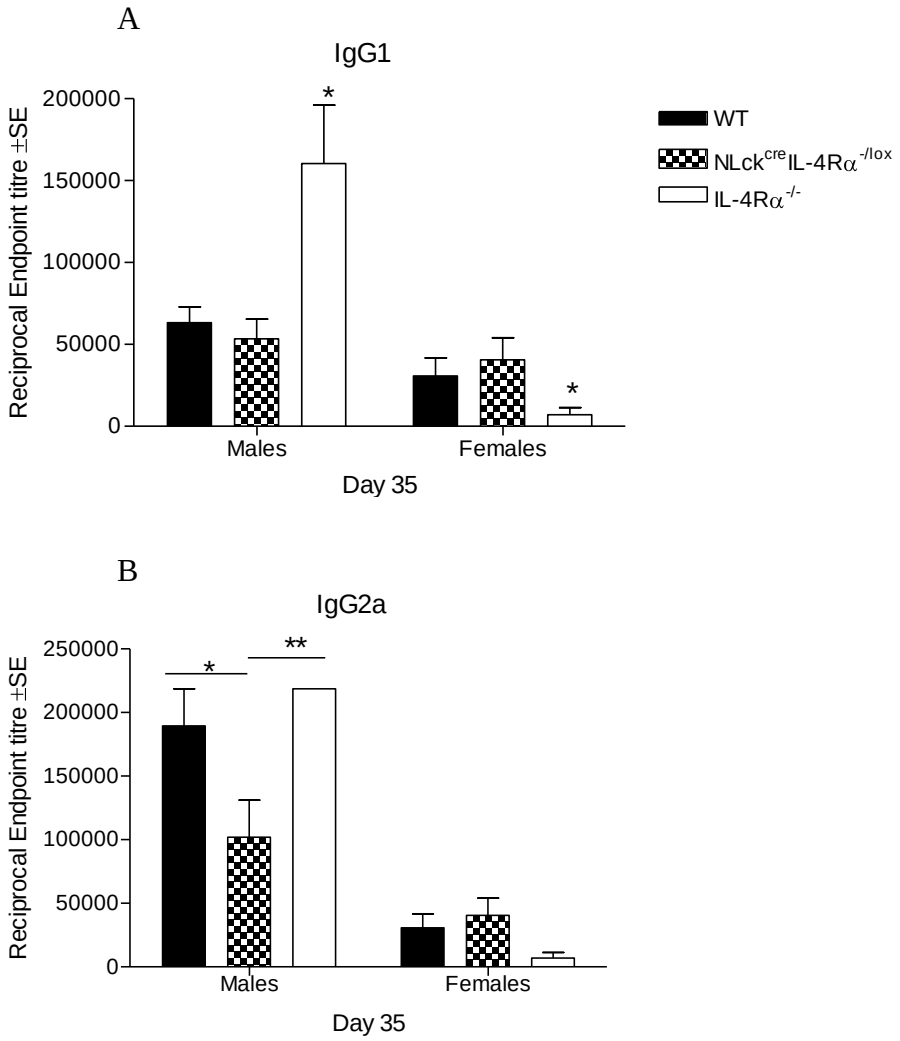


Fig.5.23 Antibody responses in the serum of male and female mice as measured by sandwich ELISA IgG1 (A) and IgG2a (B) levels were quantified in the serum of *T. gondii* infected male and female mice 35 days post-infection. *p<0.05, **p<0.01 compared with WT or NLck^{cre}IL-4R$\alpha^{-/lox}$ of the same sex.

5.1.9 Discussion

In this chapter we used NLck^{cre}IL-4R$\alpha^{-/lox}$ mice, which are deficient in IL-4Rα on CD4^{+} and CD8^{+} T-cells, to further investigate the lymphocyte populations in IL-4/IL-13 mediated protective responses during *T. gondii* infection. We show that male NLck^{cre}IL-4R$\alpha^{-/lox}$ mice are more resistant to infection compared with their WT counterparts, as well as female NLck^{cre}IL-4R$\alpha^{-/lox}$ mice which appeared to be partially susceptible and succumbed to infection during the chronic phase of infection. Male NLck^{cre}IL-4R$\alpha^{-/lox}$ mice were generally more resistant to infection than female mice with a 100% survival rate for the 35 day duration of the study, whereas female mice died with a 20% mortality rate. Although male IL-4R$\alpha^{-/-}$ mice were susceptible and died in this experiment, we have consistently shown over several experiments that male IL-4R$\alpha^{-/-}$ are generally more resistant to infection than their female counterparts. The deaths of male mice in this study may be related to the age of the mice, the severity of the disease and possibly the increased systemic type-1 (IL-12) cytokine response which was not seen in the previous experiments where the mice survived. Similarly, the mortality of female NLck^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/-}$ mice may be associated with elevated levels of IL-12 in the serum coupled with failure to initiate parasite-specific type-1 responses in the early stages of infection (day 9) unlike their male counterparts. IL-12 plays a critical role in initiating the IFN-γ response from NK cells and other T-cell populations which is required for resistance to intracellular infections (Gazzinelli, Hieny et al. 1993), however an unregulated type-1 response can be detrimental for the host and cause lethal pathology. Indeed previous studies in our group have shown that overproduction of type-

1 cytokines in IL-4Rα$^{-/-}$ mice causes death which is associated with excessive pathology in the lungs during the acute phase of *T. gondii* infection (Nickdel et al., appendix). In addition we have also previously shown that male mice are able to survive *T. gondii* infection better than female mice as they have an early IL-12 and IFN-γ response which was delayed in female mice (Walker, Roberts et al. 1997). In agreement, our current study further demonstrates that the sex-dependent difference in resistance to *T. gondii* infection is associated with the ability of male mice to mount a rapid and effective parasite-specific type-1 response in the early stages of infection whereas female mice have a strong non-specific response but a subdued as well as delayed parasite-specific response, and this is independent of IL-4Rα deficiency.

In order to further dissect the male/female dichotomy in disease development and outcome, we compared the bodyweight regulation over a period of 35 days following intra-peritoneal infection with 10 cysts of *T. gondii*. Similar to our observation in the previous chapter (4), female IL-4Rα$^{-/-}$ mice were able to regain their lost bodyweight following the wasting acute phase, whereas male mice did not fully recover the lost bodyweight and maintained the significantly reduced bodyweight for the course of the experiment. As discussed in the previous chapter, the bodyweight divergence is possibly related to the difference in cytokine expression profiles once stabilization of disease has occurred as described in a few studies by Arsenijevic et al, although they used female mice of a different mouse strain in their study. As far as we are aware, a male/female difference and direct IL-4/IL-13 involvement in weight regulation in response to *T.*

gondii infection has not been reported yet. Sex hormones affect cells of the innate and adaptive immune response directly and indirectly as macrophages, $CD4^+$ and $CD8^+$ T-cells have been shown to have both intracellular and plasma membrane testosterone and estrogen receptors (Benten, Lieberherr et al. 1999; Benten, Lieberherr et al. 1999; Roberts, Walker et al. 2001). Thus there is a possibility that the sex-hormone mediated responses could be influencing the immune response and metabolic pathways in an IL-4 dependent manner during *T. gondii* infection. Nevertheless, this IL-4/IL-13 response does not appear to be mediated via $CD4^+$ or $CD8^+$ T-cells as both Lck^{cre}IL-4R$\alpha^{-/lox}$ and $NLck^{cre}$IL-4R$\alpha^{-/lox}$ male mice were able to recover their lost bodyweights fully and at a similar rate to that of their WT counterparts.

Furthermore, we studied the immune response of both female and male mice by examining the pathological changes in the lung, liver and brains, as well as *ex-vivo* cytokine production and lung cytokine expression to understand the mechanism through which IL-4 mediates protective responses during *T. gondii* infection. Male IL-4R$\alpha^{-/-}$ mice had significant pathology in the lungs compared with WT and $NLck^{cre}$IL-4R$\alpha^{-/lox}$ mice at day 9 post infection, which is consistent with our observation in the previous chapter (4) and generally in previous studies. In the previous chapter male Lck^{cre}IL-4R$\alpha^{-/lox}$ mice displayed minimal degrees of lung peribronchial inflammation at day 9 post-infection, whereas $NLck^{cre}$IL-4R$\alpha^{-/lox}$ mice display no pathology in this area of the lung at this time-point. Nonetheless in a further study Lck^{cre}IL-4R$\alpha^{-/lox}$ mice also showed no pathology in the lungs compared with WT and IL-4R$\alpha^{-/-}$ mice as early as day 9 post-

infection suggesting that IL-4Rα signaling via $CD4^+$ T-cells and $CD8^+$ T-cells may contribute to the development of pathology in the lungs during the early phase and chronic phase ($CD4^+$ T-cells only) of *T. gondii* infection. The significantly reduced degrees of lung pathology in male $NLck^{cre}$IL-4$R\alpha^{-/lox}$ mice can be correlated with the marked reduced parasite-specific splenocyte IFN-γ production following infection, which, interestingly, was not seen in Lck^{cre}IL-4$R\alpha^{-/lox}$ mice. In the acute phase it is critical that there is a robust type-1 response to control tachyzoite replication, however this early cytokine response must be regulated to prevent tissue damage and lethal immunopathology as seen in IL-$4^{-/-}$ mice (Nickdel et al., appendix).

While IL-4Rα signaling is not necessary for the generation of type-2 $CD4^+$ T-cells (Brewer, Conacher et al. 1999), in the absence of IL-4 signaling on T-cell populations a more type-1 polarised phenotype with overproduction of cytokines such as IFN-γ is produced. Conversely, our data shows that there is not only a reduction of splenocyte nitrite production from $NLck^{cre}$IL-4$R\alpha^{-/lox}$ mice, which was also seen in splenocytes deficient of IL-4Rα on $CD4^+$ T-cells only in the previous chapter (4), but a reduced splenocyte IFN-γ response as well, which was not seen in $CD4^+$ T-cell specific and global IL-$4R\alpha^{-/-}$ splenocytes. This clearly demontrates that $CD4^+$ and $CD8^+$ T-cell responses are affected differently by the absence of IL-4 during *T. gondii* infection. The defective IFN-γ response in $NLck^{cre}$IL-4$R\alpha^{-/lox}$ mice is evident in TLA stimulated cultures and not present in ConA stimulated cultures, which suggests that it is a parasite specific event. This observation also raises the possibility that IL-4 plays an important

role in the development of efficient $CD8^+$ T-cell responses during parasite infections. Indeed defective IFN-γ responses have been observed in other infections, where $CD8^+$ T-cells isolated from spleen cells of *Schistosoma mansonii* infected IL-$4^{-/-}$ mice produced less IFN-γ compared with WT $CD8^+$ T-cells (Pedras-Vasconcelos, Brunet et al. 2001), while several other studies have shown that IL-4 is essential for the development of protective $CD8^+$ T-cell responses against tumours (Schuler, Qin et al. 1999) and infections with other protozoan parasites such as *Leishmania* and *Plasmodium* (Stager, Alexander et al. 2003; Morrot, Hafalla et al. 2005). Earlier studies also showed that in $CD4^{-/-}$ mice, systemic *T. gondii* specific $CD8^+$ T-cells produce less IFN-γ and are not effectively maintained (Casciotti, Ely et al. 2002) and also that IL-4Rα signaling is essential for $CD8^+$ T-cell effector mechanisms in absence of $CD4^+$ T-cell help (Marsland, Schmitz et al. 2005). More recently, studies have shown that IFN-γ production by splenocyte $CD8^+$ T-cells but not $CD4^+$ T-cells was significantly reduced in *T. gondii* infected STAT-$6^{-/-}$ mice than in WT mice, and that the cytotoxic activity of these $CD8^+$ T-cells was much lower in STAT-$6^{-/-}$ mice which resulted in increased proliferation of cysts in the brains of these mice whereas WT mice could control parasite numbers (Jin, Takamoto et al. 2009). However, this study was done using a different mouse strain (C57BL/6) and these observations were made at day 28 post-infection, but not at day 14 post-infection where they did not observe any differences between WT and STAT-$6^{-/-}$ mice. In our study, the reduced IL-4 mediated IFN-γ production by spleen cells was evident at a much early time-point, day 9 post-infection, in addition we show that it is not sex-dependent, as female NLck^{cre}IL-4Rα$^{-/lox}$ mice also display defective

splenocyte IFN-γ production compared with the WT and IL-4Rα$^{-/-}$ mice.

Interestingly, although the type-2 response (IL-4 and IL-10) was generally very low in both male and female mice, TLA-stimulated splenocytes from infected female NLck^{cre}IL-4Rα$^{-/lox}$ mice, but not male mice, produced significantly lower levels of IL-4 compared with WT mice. Some studies have demonstrated that IL-4Rα signaling can also play an important role in the induction of Th1 responses, specifically by promoting dendritic cell maturation (Lutz, Schnare et al. 2002) or alternatively inhibiting IL-10 effects (Yao, Li et al. 2005) thus promoting IL-12 production. Nevertheless, a clear correlation cannot be made between the reduced IL-4 response in the absence of IL-4Rα on CD4^{+} and CD8^{+} T-cells and type-1 cytokine response in female mice at this particular time point (day 9) as the IFN-γ levels were too low to be detected. Furthermore there was no significant difference in splenocyte IL-12 production, as well as type-1 mRNA transcripts in the lungs of *T. gondii* WT, NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice at this time-point.

More recently, the role of alternatively activated macrophages in the susceptibility to infections is being investigated with much interest as they have been shown to be essential for survival during some infections (Herbert, Holscher et al. 2004). We have shown in chapter 3 that the highly susceptible IL-4Rα$^{-/-}$ mice display excessive lung pathology and suggested that the significantly reduced expression of the marker FIZZ1 in the lungs of these mice may be a susceptibility factor during *T. gondii* infection. In

this chapter, we demonstrate that infection with *T. gondii* induces a strong induction of alternative macrophage activation markers in the lungs, which appears to be independent of IL-4Rα signaling in both male and female mice, although the female response is generally lower than that observed in male mice. In agreement with our observation in chapter 3, FIZZ1 transcript expression was significantly reduced in the lungs of female mice deficient of IL-4Rα signaling compared with their WT controls. Nonetheless, at day 12 post-infection female IL-4Rα$^{-/-}$ mice express significantly increased levels of YM1 and Arginase 1 transcripts in the lungs which are all markers for alternatively activated macrophages. Alternatively activated macrophages are thought to be involved in tissue remodeling, wound healing and generation of Th2 responses (Mosser 2003) though the mechanisms through which they function are not clear. Arginase 1 is associated with collagen deposition (Munder, Eichmann et al. 1999). Alternative activation of macrophages are defined as being IL-4/IL-13 mediated; it is therefore surprising to observe upregulation of markers of alternative macrophage activation in the absence of IL-4/IL-13 signaling. It is therefore possible that the parasite itself is inducing alternative macrophage activation. IL-4/IL-13-independent alternative macrophage activation has been shown to be induced during some nematode infections (Loke, MacDonald et al. 2000; Herbert, Holscher et al. 2004; Nair, Gallagher et al. 2005). Recently a few studies have come out demonstrating IL-4/IL-13-independent induction of alternative macrophage activation markers. Macrophage inhibitory factor (MIF) homologues from the filarial nematode *Brugia malayi* were shown to induce the expression of alternative activation markers Arginase1, FIZZ1 and YM1 *in vitro* and *in*

vivo (Prieto-Lafuente, Gregory et al. 2009) which may explain the expression of these markers in absence of IL-4Rα signaling in our results, as *T. gondii* also secretes MIF (Sommerville et al., submitted). *Mycobacterium tuberculosis*, as well as *T. gondii* were also shown to induce expression of Arginase1 in a STAT-6 independent manner (El Kasmi, Qualls et al. 2008). Furthermore, this study demonstrates that TLR signaling induces Arginase1 in macrophages; therefore arginase is probably not an ideal marker for alternatively activated macrophages (El Kasmi, Qualls et al. 2008). It is thought that upon host cell entry, *T. gondii* injects rhoptry kinases, specifically ROP16, which is involved in phosphorylation of STAT-6 (Saeij, Boyle et al. 2006; Saeij, Coller et al. 2007) thus activating downstream IL-4 responses. This provides a viable explanation for our observation of induction of alternative macrophage activation in the absence of IL-4/IL-13 signaling in IL-4Rα$^{-/-}$ mice. It also represents a sophisticated way for the parasite to evade the host immune responses. Nevertheless, ROP16 associated STAT-6 phosphorylation was only demonstrated during infection with type I and type III, but not in type II strains of *T. gondii* (Saeij, Boyle et al. 2006; Saeij, Coller et al. 2007) which is the strain type that we used in our study. Additional work is needed to further define the dynamics of IL-4/IL-13 independent alternative macrophage activation during *T. gondii* infection.

Among the several factors mentioned to be important during *T. gondii* infection, p47 GTPases are reported to play a significant role in controlling parasite replication during *T. gondii* infection (Butcher, Greene et al. 2005). We observed a significant increase of

lung LRG47 in NLck^{cre}IL-4Rα$^{-/lox}$ mice and both LRG47 and Igtp GTPases in IL-4Rα$^{-/-}$ mice. However, there was also significantly increased bradyzoite burden in the lungs of IL-4Rα$^{-/-}$ mice compared with WT mice at the same time, which suggests that the expression of these particular GTPases is not limiting parasite replication in these mice. Interestingly, we observed an increased tachyzoite to bradyzoite conversion of the parasite in female NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ compared with WT mice. Around 10-14 days post-infection tachyzoites are reported to differentiate into the slower dividing bradyzoites (Lyons, McLeod et al. 2002) and there is some evidence that this conversion is indirectly facilitated by nitric oxide stress (Bohne, Heesemann et al. 1994). On the other hand, we showed a diminished splenocyte NO response in female NLck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice at this time point. Nevertheless, it is likely that there are several other factors associated with this parasite conversion, but it is clear that lack of IL-4Rα signaling on CD4^{+} T-cells and on CD8^{+} T-cells favours the tachyzoite to bradyzoite conversion as this effect was also pronounced in the previous chapter in Lck^{cre}IL-4Rα$^{-/lox}$ mice.

CHAPTER 6

In vivo studies to determine the role of *T. gondii* in induction of alternatively activated macrophages

Abstract

A type-1 cytokine dependent response inducing classically activated macrophages is crucial for parasite control during *T. gondii* infection in BALB/c mice, but can also contribute to development of lethal immunopathology as demonstrated in our previous studies. Type-2 cytokines such as IL-4 and IL-13 antagonize classical macrophage activation inducing alternatively activated macrophages. In the previous chapters we observed differential expression of markers of alternatively activated macrophages in the lung tissue following *T. gondii* infection. In this study we further investigated the expression of alternative macrophage markers in broncheoalveolar lavage (BAL) macrophages from *T. gondii* infected BALB/c wild-type mice and IL-4R$\alpha^{-/-}$ mice. Our results show that FIZZ1 mRNA levels remain unchanged, whereas YM1 expression is significantly increased in both WT and IL-4R$\alpha^{-/-}$ BAL macrophages following *T. gondii* infection. More significantly, we show that Arginase1 mRNA is significantly increased in IL-4R$\alpha^{-/-}$ BAL macrophages following infection but not in WT macrophages. Moreover, the increased Arginase1 mRNA coincided with higher tachyzoite mRNA burden. Our observations show that *T. gondii* directly induces YM1 and arginase1 but not FIZZ1 expression in macrophage populations independently of IL-4/IL-13 signaling, and these molecules possibly support tachyzoite proliferation in the lung.

6. 1 Introduction

Toxoplasma gondii is an obligate intracellular parasite that infects almost all nucleated cells. Cell invasion by the parasite tachyzoites is an important event that determines parasite survival, replication and manipulation of the host cellular response (Dubremetz 1998; Denkers 2003). *T. gondii* presents some organelles involved in host cell attachment, penetration and in the formation of the parasitophous vacuole (PV) and these include micronemes, rhoptries and dense granules which attach to the parasite membrane, discharging their (protein) contents in sequential, rapid series of events (Dubremetz 1998). Recent studies have implicated some of these proteins in the parasite's success in its ability to subvert host immune response and survive within the host cells despite the vigorous immune response (Laliberte and Carruthers 2008).

The outcome of protozoan infections is dependent on the tightly regulated Th1/Th2 balance; the order, timing and relative strength of the type-1 vs type-2 immune response are critical for host defense. Infection with *T. gondii* elicits both a type-1 and type-2 immune response (Denkers, Kim et al. 2003). In the acute stages of infection, the induction of a strong Th-1 response is critical for parasite control and host survival and IFN-γ is well recognized as the major mediator of protective type-1 mediated host responses (Suzuki, Orellana et al. 1988; Gazzinelli, Hakim et al. 1991; Gazzinelli, Xu et al. 1992). A type-1 cytokine pro-inflammatory response induces classically activated macrophages which is essential for parasite control. Classically activated macrophages display enhanced MHC class II expression and enhanced ability to present antigens and

eliminate intracellular parasites. The anti-toxoplasma action of classically activated macrophages is widely attributed to production of nitric oxide (NO) and reactive nitrogen intermediates (RNI) which represents an essential IFN-γ-mediated macrophage effector mechanism (Adams, Hibbs et al. 1990; Yap and Sher 1999). Moreover, studies using iNOS deficient mice have shown that $iNOS^{-/-}$ mice are highly susceptible to *T. gondii* infection and the increased mortality is related to uncontrolled parasite growth (Scharton-Kersten, Yap et al. 1997). Although a highly polarized type-1 cytokine response is essential to control parasite proliferation, it can also be detrimental to the host if it is unregulated and persists too long. IL-4 is a central mediator in the induction of type-2 mediated responses, and inhibits the production of IFN-γ by various mononuclear cells (Peleman, Wu et al. 1989; Wagner, Fischer et al. 1989). Previous studies by our group and others have demonstrated that mice deficient in IL-4 (Roberts, Ferguson et al. 1996; Suzuki, Yang et al. 1996) and IL-4Rα (Nickdel et al.,, appendix) are highly susceptible to *T. gondii* infection and the mortalities are associated with damage to the lung tissue which is mediated by an unregulated type-1 pro- inflammatory response.

In a type-2 environment, macrophages sequentially change their functional phenotype and undergo a distinctly different (to classically activated macrophages) and more anti-inflammatory activation programme. Alternative activation of macrophages has been limited to activation in the presence of IL-4 and IL-13 through a common receptor chain (IL-4Rα), whereas other anti-inflammatory activation programs involving IL-10,

glucocorticoids and TGF-β are termed regulatory macrophages (Mosser and Edwards 2008). IL-4-dependent macrophages recruited *in vivo* actively suppress the proliferation of lymphocytes (Loke, MacDonald et al. 2000) and macrophages exposed to IL-4 are also unable to produce NO and are less efficient at killing intracellular pathogens. IL-4 down-regulates the production of NO by iNOS and alternatively stimulates arginase activity in macrophages (Corraliza, Soler et al. 1995; Modolell, Corraliza et al. 1995). iNOS and Arginase1 compete for the common substrate L-arginine. iNOS metabolises L-arginine to generate NO which is a critical effector mechanism for restricting *T. gondii* replication (Adams, Hibbs et al. 1990). On the other hand, Arginase1 hydrolyses L-arginine to urea and L-orthinine, which are required for collagen deposition and production of extracellular matrix. Thus arginase production may be involved in the resolution of tissue damage and cell growth during infections. Furthermore, several studies have shown that IL-4-induced alternatively activated macrophages express large amounts of chitinase-like molecules such as YM1 and YM2 (Raes, De Baetselier et al. 2002), novel resistin-like molecule α secreted protein (RELM-α, or found in inflammatory zone-1 FIZZ1) which are also involved in the synthesis of extracellular matrix (Gratchev, Guillot et al. 2001). The function of these molecules and/or alternatively activated macrophages in host defense has however not yet been elucidated. Several studies have shown that some helminth (Rodriguez-Sosa, Satoskar et al. 2002; Herbert, Holscher et al. 2004; Noel, Raes et al. 2004; Anthony, Urban et al. 2006) and protozoan (Iniesta, Carcelen et al. 2005) infections widely induce the expression of these markers of alternative macrophages and that these macrophages may

be involved in the resolution of inflammation or alternatively exarcebate disease progression respectively. Thus generation of alternatively activated macrophages can therefore play a protective role or be detrimental for the host depending on the infectious agent.

As well as in murine models, lung involvement has been reported to occur during disseminated toxoplasmosis in immunosuppressed patients (Vietzke, Gelderman et al. 1968; Gleason and Hamlin 1974). Lung macrophages are an essential component of the first line of defense for most pulmonary infections. The plasticity of macrophages and high complexity of the *in vivo* environment results in the induction of various types of macrophages during protozoan infections and differentially influences the course of disease. Moreover, many protozoan parasites have evolved and acquired strategies to evade the host immune response. Our aim in this chapter was to investigate the role of *T. gondii* in induction of alternatively activated macrophages as a possible strategy to survive within lung macrophages and evade host elimination.

6.2 Materials and Methods

6.2.1 Experimental animals and Infection

Female BALB/c and IL-4R$\alpha^{-/-}$ (BALB/c background) mice were used for all *in vivo* studies. All experimental mice were age matched, and used between 8-12 weeks of age ($n \geq 5$). The low virulent Berveley strain of *T. gondii* was used to infect the mice as previously described (Materials and Methods, chapter2). Briefly, Cysts were harvested from the brains of infected mice, enumerated and subsequently experimental mice were infected with 10 cysts intraperitoneally. The mice were maintained under specific pathogen free conditions, and used in accordance with UK Home Office guidelines.

6.2.2 Bronchoalveolar lavage

Alveolar macrophages were isolated at day 9 post-infection with *T. gondii* using a bronchoalveolar lavage (BAL) technique adapted from Stokes *et. al* with minor modifications (Stokes, Thorson et al. 1998) 9 days post-infection with *T. gondii*. The lungs were flushed with 1ml of warm PBS containing 0.6mM EDTA using a 21G canula attached to a 1ml syringe. The lavage procedure was repeated 3 times and the recovered fluid pooled. The pooled lavage was centrifuged for 5minutes at 1400rpm at 4°C. The supernatant was removed and the pellet containing alveolar macrophages was retained and resuspended in 1ml of cold supplemented RPMI (RPMI 1640 medium, 10% v/v FCS, 10mM L-glutamine, 10 mM Sodium pyruvate) and subsequently plated in 24-well tissue culture plates. The cells were allowed to adhere overnight by incubating at 37°C with 5% CO_2 supplementation. Following incubation, the non-adherent cells were

removed and discarded and the adherent macrophages scraped off the wells using 1ml supplemented RPMI media and a cell scraper. The recovered alveolar macrophages were then used immediately to extract RNA.

6.2.3 RNA extraction, cDNA synthesis and quantitative RT-PCR

RNA was extracted from the cells as previously described; and cDNA synthesized and quantitave Real Time PCR were performed as described in chapter 2. The genes that were examined were IL-12p40, NOS2, Arginase1, FIZZ1, YM1 and the parasite specific genes SAG1 and *T. gondii* cyst antigen (TgCyst).

6.3 Results

IL-12p40 and NOS2 mRNA transcript expression were significantly increased in *T. gondii* infected WT and IL-4R$\alpha^{-/-}$ -derived BAL macrophages compared with uninfected controls (Fig. 6.1A, B). But there was no difference between infected WT and IL-4R$\alpha^{-/-}$ for both genes. NOS2 transcripts were slightly lower in IL-4R$\alpha^{-/-}$ infected macrophages compared with infected WT control; however the difference was not significant (Fig. 6.1B)

Markers of alternative macrophage activation were differentially expressed in BAL macrophages following *T. gondii* infection. FIZZ1 mRNA transcript expression in BAL macrophages remained unchanged following *T. gondii* infection (Fig. 6.2A), whereas YM1 expression was significantly increased in WT and IL-4R$\alpha^{-/-}$ BAL macrophages following infection with *T. gondii* ($p<0.05$) compared with uninfected controls (Fig. 6.2B). Nonetheless, there was no significant difference between infected WT and IL-4R$\alpha^{-/-}$. On the other hand, Arginase1 mRNA transcript expression was significantly increased in *T. gondii* infected IL-4R$\alpha^{-/-}$ BAL macrophages compared with uninfected controls ($p<0.05$) but not in infected WT macrophages (Fig. 6.2C). Arginase1 expression in *T. gondii* infected IL-4R$\alpha^{-/-}$ BAL macrophages was also significantly higher than in infected WT macrophages ($p<0.05$).

Furthermore, we examined parasite burden in BAL macrophage samples by measuring the expression of tachyzoite specific gene SAG1 and bradyzoite specific gene TgCyst-

antigen. BAL macrophages from IL-4Rα$^{-/-}$ mice displayed increased level of tachyzoite transcripts compared with their WT counterparts, whereas the level of bradyzoite transcripts was similar to that observed in WT derived macrophages (Fig. 6.3). In general, there was higher parasite burden in infected IL-4Rα$^{-/-}$ macrophages compared with WT controls.

Overall, infection with *T. gondii* elicited increased type-1 cytokine and mRNA expression in BAL macrophages irrespective of IL-4Rα deficiency. At this stage, infection with *T. gondii* did not affect FIZZ1 transcript expression in BAL macrophages. However YM1 transcripts were considerably increased in both WT and IL-4Rα$^{-/-}$ mice following *T. gondii* infection and Arginase1 transcripts were highly upregulated in macrophages from IL-4Rα$^{-/-}$ mice but not in WT derived macrophages following infection with *T. gondii*. The increased Arginase1 transcription was concomitant with a higher tachyzoite mRNA burden in absence of IL-4Rα in macrophages.

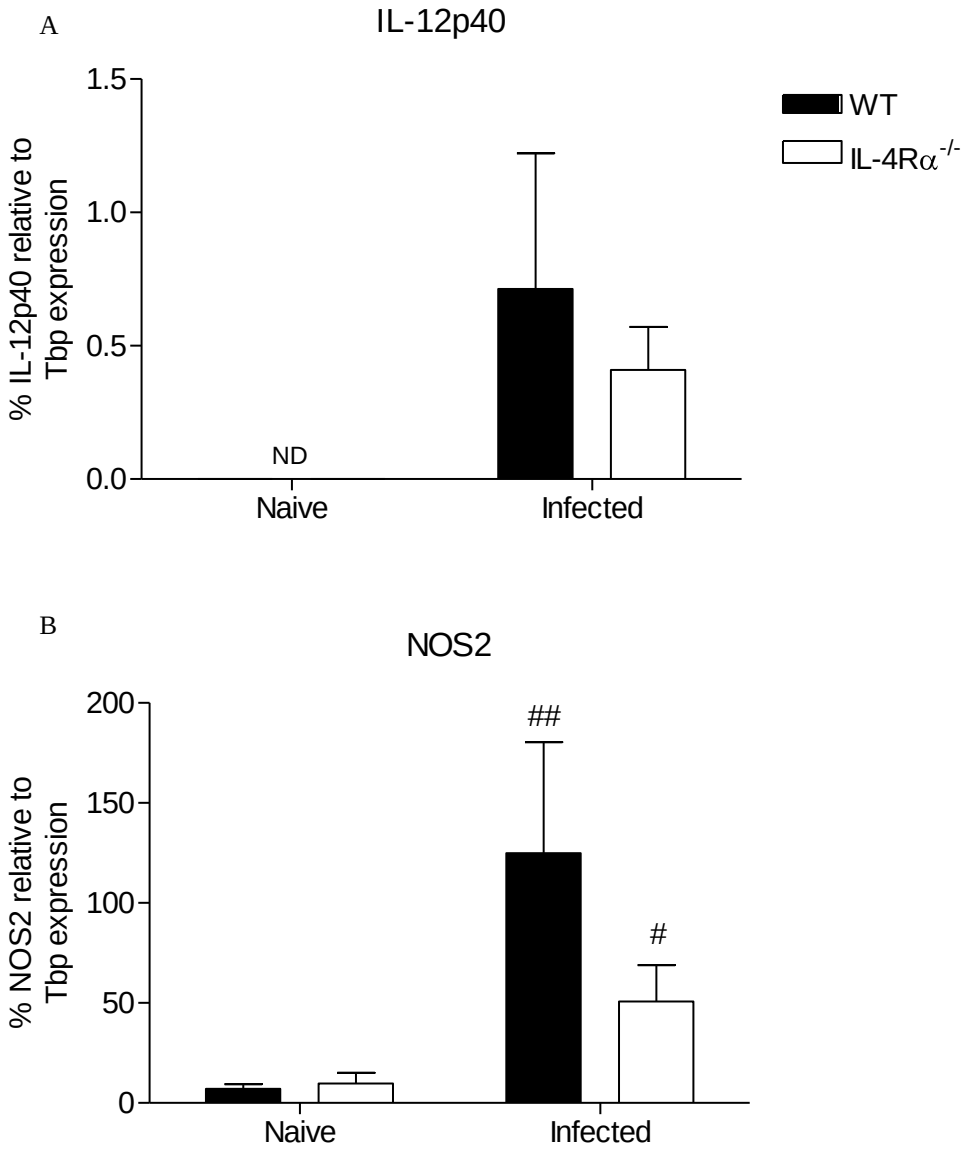


Fig. 6.1 mRNA transcript expression of IL-12p40 (A) and NOS2 (B) in BAL macrophages derived from *T. gondii* infected WT (solid bar) and IL-4R$\alpha^{-/-}$ (open bar) mice 9 days post-infection. # $p<0.05$, ## $p<0.01$ compared with naïve (uninfected) corresponding control. n=5, Representative of 2 independent experiments

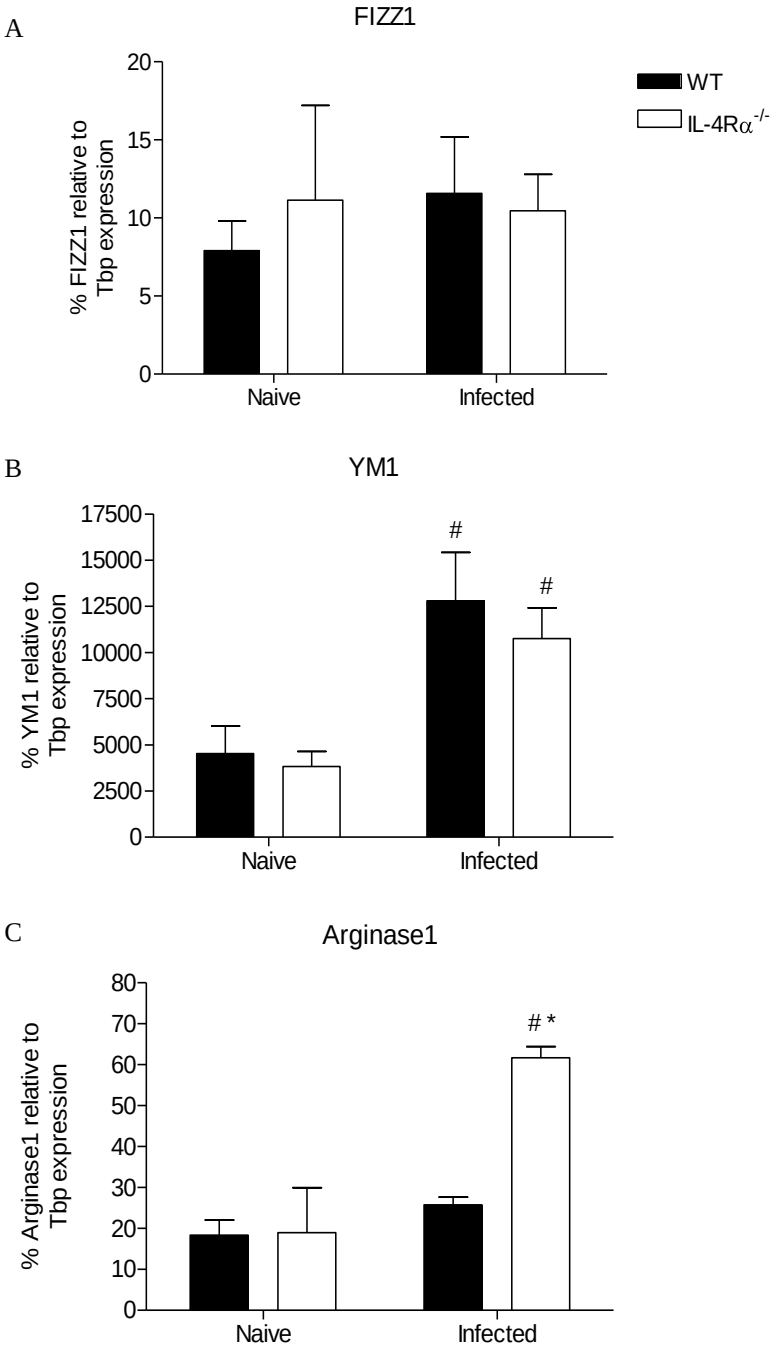


Fig. 6.2 Expression of mRNA transcripts of alternatively activated macrophage markers FIZZ1 (A), YM1 (B) and Arginase1 (C) in BAL macrophages derived from uninfected (naïve) and *T. gondii* infected BALB/c WT (solid bar) and IL-4Rα$^{-/-}$ (open bar) mice. # $p<0.05$ compared with corresponding naïve control, *$p<0.05$ compared with infected WT control

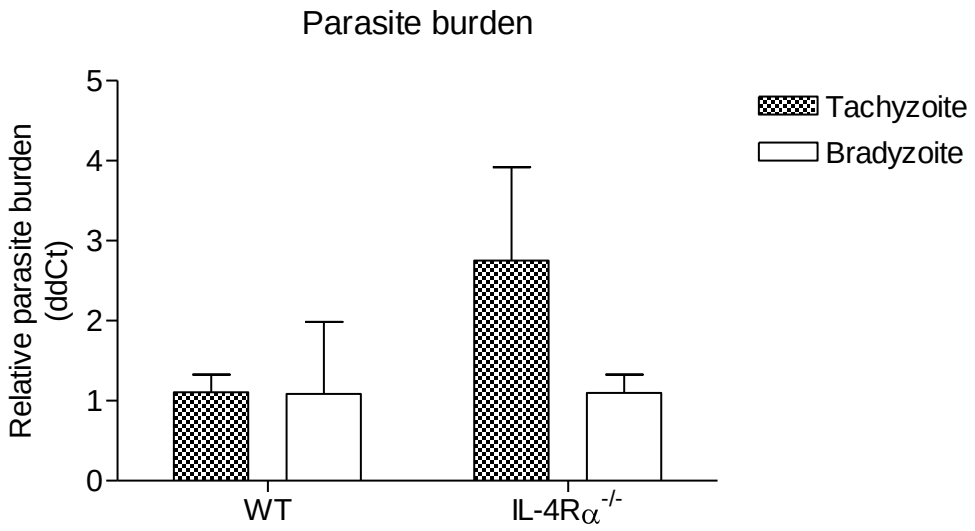


Fig. 6.3 Parasite burden. Tachyzoite (SAG1) and bradyzoite (TgCyst antigen) mRNA transcript expression in BAL macrophages derived from *T. gondii* infected WT and IL-4R$\alpha^{-/-}$ female mice 9 days post-infection as measured by qRT-PCR.

6.4 Discussion

Our previous results have shown that signaling via IL-4Rα is essential for survival of BALB/c mice during *T. gondii* infection, and that mortalities in IL-4Rα$^{-/-}$ mice were associated with excessive lung inflammation which was associated with upregulated type-1 cytokine responses. We also observed an enhanced upregulation of markers of alternative macrophage activation in the lung tissue following *T. gondii* infection in both WT and IL-4Rα-/-mice at different time points. To further dissect the role of IL-4Rα signaling in pulmonary mediated immune responses, alveolar macrophages from IL-4Rα$^{-/-}$ and WT (BALB/c) mice were assessed for their ability to express type-1 cytokines and markers for alternatively activated macrophages during acute *T. gondii* infection. Herein we show that during *in vivo T. gondii* infection markers of alternative macrophage activation YM1 and Arginase1 are significantly upregulated in BAL macrophages from BALB/c mice in the absence of IL-4/IL-13 mediated signaling. Moreover, Arginase1 mRNA transcripts remained at basal levels in WT macrophages whereas in IL-4Rα$^{-/-}$ macrophages Arginase1 mRNA induction increased 3 fold at 9 days post-infection with *T. gondii*. FIZZ1 expression remained unchanged in BAL macrophage populations following *T. gondii* infection. Studies done to characterize cell-specific expression of mouse FIZZ1 have shown that in normal lungs FIZZ1 mRNA is expressed at low levels in the large airways in epithelial cells and in scattered isolated cells in the peribronchiolar stroma. Whereas in OVA-induced allergic inflammation, FIZZ1 mRNA was markedly increased with widespread expression in bronchial epithelial cells and cells associated with the alveolar wall, no signal was observed in

alveolar macrophages (Holcomb, Kabakoff et al. 2000). Therefore, future studies will involve examining the cell-specific expression of alternative macrophage activation markers, including FIZZ1, by immunohistocytochemistry in the lungs of *T. gondii* infected mice.

Our results show that some of the markers commonly associated with IL-4-induced alternatively activated macrophages are highly expressed in absence of IL-4/IL-13 signaling, and more significantly Arginase1 is preferentially induced in absence of IL-4/IL-13 signaling during *in vivo T. gondii* infection. In support of our observations, *in vitro* studies by our group have also demonstrated that *T. gondii* can induce arginase transcription and activity in bone marrow derived macrophages (Menzies, Henriquez et al.). Previously it was reported that IL-4/IL-13 signaling through the common IL-4Rα receptor was required for Arginase1 induction (Rutschman, Lang et al. 2001). Nevertheless, accumulating evidence has now shown that Arginase-1 expressing macrophages are induced directly by some intracellular pathogens and they play different roles in those infections. Macrophage-specific Arginase1 contributes to the resolution of *Schistosomaisis* by inhibiting $CD4^+$ T-cell effector functions thereby limiting tissue damage induced by excessive type-2 inflammation (Pesce, Ramalingam et al. 2009). On the other hand, Arginase1 induction in *Leishmania* infection was shown to be detrimental and was associated with development of disease and favoured the growth of *Leishmania* parasites inside the macrophage (Iniesta, Carcelen et al. 2005). Furthermore, Arginase1 expression was shown to be induced in murine macrophages by

infection with *Mycobaterium tuberculosis* and *T. gondii* in a TLR-dependent manner (El Kasmi, Qualls et al. 2008). The TLR-mediated Arginase1 expression was independent of the STAT-6 pathway. In addition, *T. gondii* was also demonstrated to directly trigger STAT-6 activation thus obstructing the innate type-1 immunity elicited by the infection (Ahn, Kim et al. 2009) and a polymorphic parasite encoded kinase ROP16, from the typeI and typeIII *T. gondii* strains was shown to be responsible for the indirect phosphorylation of STAT-6 (Saeij, Coller et al. 2007). Macrophage migration inhibitory factor (MIF) homologues from filarial nematodes have recently been shown to synergize with IL-4 to induce Arginase1, YM1 and FIZZ1-expressing macrophages (Prieto-Lafuente, Gregory et al. 2009). As *T. gondii* also expresses MIF (Sommerville et al., submitted), this may provide an explanation for the induction of alternatively activated macrophages in the absence of IL-4/IL-13 signaling during *T. gondii* infection. These observations also suggest that Arginase1 may not be an ideal marker for alternative macrophage activation as it is induced in the absence of IL-4. The increased expression of Arginase1 may be beneficial for the parasite and represents a mechanism for the parasite to evade NO and RNI-mediated killing by depleting L-arginine which is a common substrate for iNOS thus limiting NO production. In addition because *T. gondii* is an auxotroph for arginine and polyamines, re-directing L-arginine to the generation of polyamines which are involved in cell growth could be a strategy by the parasite to support its growth and survival. Additionaly, studies by the Bzik laboratory have shown that arginine depletion results in tachyzoite to bradyzoite switch (Fox, Gigley et al. 2004).

YM1 is a chitinase-like secretory lectin that forms crystals in the lungs of mice exhibiting hyperactivity of alveolar macrophages (Raes, Noel et al. 2002). Unlike Arginase1, YM1 mRNA expression was induced in WT and IL-4R$\alpha^{-/-}$ macrophages following infection with *T. gondii* suggesting that its expression is not entirely IL-4-mediated. YM1 is also thought to be involved in cell remodeling, however it is possible that *T. gondii* is inducing YM1 expression to promote its own survival and persistence within the macrophage. Nevertheless, more work needs to be undertaken to establish the role of the induction of alternatively activated macrophages. The results in this chapter are significant as the induction of various types of macrophages during *T. gondii* infection affect the course of disease. Thus it is important to understand the modulation of macrophage activation by the parasite as the results provide a basis for possible drug targets and therapeutic strategies for controlling this infection.

CHAPTER 7

GENERAL DISCUSSION

7.1 General discussion

IL-4 and IL-13 are related cytokines which signal through the common IL-4Rα to induce anti-inflammatory type-2 response. Although a type-1 cytokine response, mediated by IFN-γ, plays a critical role in the prevention of intracellular replication of *T. gondii* and toxoplasmic encephalitis (TE) during acute and chronic toxoplasmosis in murine models (Suzuki, Orellana et al. 1988) a number of cytokines including IL-4 are also important in mediating interactions between immune response cells and regulating activation of effector functions that prevent parasite proliferation (Gazzinelli, Wysocka et al. 1994; Kasper, Matsuura et al. 1995; Khan, Matsuura et al. 1995). More significantly, IL-4 has been shown to be essential for survival during murine *T. gondii* infection (Roberts, Ferguson et al. 1996; Suzuki, Yang et al. 1996) possibly by inhibiting the strong type-1 cytokine mediated responses during acute infection but also by facilitating type-1 responses during chronic disease. There have been limited studies showing the immune cell populations involved in mediating the protective IL-4/IL-13 signaling pathway effects during *T. gondii* infection. The purpose of the studies undertaken herein was therefore to examine the functional target of IL-4, specifically investigating the role of IL-4/IL-13 signaling through macrophages, CD4+ and CD8+ T-cells during *T. gondii* infection in male and female mice. Consistent with previous studies from our laboratory, we demonstrated that BALB/c mice deficient in IL4/IL-13 mice were more susceptible to *T. gondii* infection compared with their WT counterparts (Nickdel et al., appendix). Moreover female mice were more susceptible to infection compared with male mice which was consistent with previous studies (Roberts,

Cruickshank et al. 1995; Roberts, Ferguson et al. 1996).

Macrophages are essential mediators of inflammation and wound healing. Depending on the cytokine environment and subsequent activation state, they play a role in restricting parasite replication in various infections and modulate inflammatory responses to prevent immunopathology. We have shown that mice deficient in IL-4/IL-13 signaling on macrophages/neutrophil populations ($LysM^{cre}IL\text{-}4R\alpha^{-/lox}$) were highly susceptible to *T. gondii* infection and succumbed to infection during the acute phase of infection with similar kinetics to global $IL\text{-}4R\alpha^{-/-}$ mice. The increased mortality was attributed to excessive lung pathology which was caused by an upregulated type-1 immune response. Indeed following *T. gondii* infection, macrophages and neutrophils produce IL-12 which interacts with other cytokines to stimulate IFN-γ production by NK cells (Gazzinelli, Amichay et al. 1996). In the absence of IL-4/IL-13 signaling via macrophages there is evidently an overproduction of IFN-γ, which is detrimental and lethal when not regulated. On the other hand, mice deficient in IL-4 signaling on $CD4^+$ T-cells were resistant to *T. gondii* infection, whilst mice lacking IL-4Rα signaling on both $CD4^+$ and $CD8^+$ were partially susceptible to *T. gondii* infection and started dying in the late stages of infection suggesting that IL-4 signaling on $CD4^+$ T-cells is not critical for survival during early stages of *T. gondii* infection whereas IL-4 signals on $CD8^+$ T-cells may play some protective role in the late stages of infection.

In contrast to macrophage-specific $IL\text{-}4R\alpha^{-/-}$ mice, there was no lung pathology

observed in $CD4^+$T-cell specific IL-4R$\alpha^{-/-}$ mice in the late stages (day 35) and in some experiments in early stage (day 9) post-infection with *T. gondii* compared with the degrees of pathology observed in the lungs of infected IL-4Rα intact (WT) and global IL-4R$\alpha^{-/-}$ mice. Likewise $CD4^+$and $CD8^+$T-cell specific IL-4R$\alpha^{-/-}$ mice displayed no lung pathology in the early phase (day 9) of *T. gondii* infection which was significantly lower than the degrees observed in lung tissues from WT and global IL-4R$\alpha^{-/-}$ mice. This suggests that IL-4Rα signaling via $CD4^+$ and $CD8^+$ T-cells mediates pathology in the lungs following *T. gondii* infection. T-cells are essential for resistance against *T. gondii* infection as athymic nude mice are highly susceptible to *T. gondii* infection (Gazzinelli, Xu et al. 1992). Nonetheless, our observations show that IL-4 signaling via macrophages/neutrophils and not $CD4^+$/$CD8^+$ T-cells is essential to prevent lung immunopathology and for survival during acute stage *T. gondii* infection. IL-4Rα signaling through $CD4^+$ and $CD8^+$ T-cells conferred partial susceptibility in the late stages of infection, but more importantly appeared to contribute to lung pathology during acute and chronic *T. gondii* infection. Macrophages are typically the first cells to receive the danger signals and early immunity to *T. gondii* has been shown to be dependent on macrophages and neutrophils among other innate immune cells (reviewed Alexander and Hunter 1998). Hence it is befitting that IL-4 signaling through macrophages plays a more prominent role in determining the outcome of infection in the initial phase compared with signals on the cells of the adaptive response which become more important in the chronic phase of infection.

It is widely known that there is a clear dichotomy in the manifestation of disease phenotype between male and female mice following *T. gondii* infection (Kittas and Henry 1980). Previous studies in our laboratory have also demonstrated that male mice are more resistant to *T. gondii* infection compared with their female counterparts as they produced IL-12 and IFN-γ earlier and in greater quantities than their female counterparts (Roberts, Cruickshank et al. 1995; Walker, Roberts et al. 1997). In agreement, our results show that splenocytes from *T. gondii* infected female mice were unable to produce IFN-γ in the early stages of infection (day 9), and only showed the capacity to respond to antigen and produce IFN-γ and NO at day 12 post-infection whereas male mice mounted earlier and more robust type-1 cytokine responses. Moreover, male mice also mounted a higher type-2 response than female mice, including an antigen specific IL-10 induction, which regulates the effects of IFN-γ and maintains a healthy Th1/Th2 balance. Sex-associated hormones can influence the function of cells of the innate immune response such as macrophages, thus ultimately affecting the adaptive immune response involving T-cells. Mature $CD8^+$ T-cells, which are critical mediators of protective IFN-γ responses, have also been shown to express estrogen receptors (Cohen, Danel et al. 1983). Estrogen has been shown to modulate NK cell activity and increase macrophage phagocytic capacity *in vitro* and has the capacity to upregulate the expression of IFN-γ (Grasso and Muscettola 1990), while testosterone can inhibit IFN-γ production (Araneo, Dowell et al. 1991). Thus female IL-4R$\alpha^{-/-}$ mice may be more susceptible to infection due to increased non-specific systemic IFN-γ production, which can prove lethal in the absence of counter-regulatory IL-4 action on macrophages and T-

cells.

In addition, we also demonstrated that whilst the surviving female IL-4R$\alpha^{-/-}$ mice were able to regain their lost bodyweight after the wasting 12 days of acute disease, the more resistant male IL-4R$\alpha^{-/-}$ counterparts failed to fully recover their lost bodyweights. Generally the regulation of bodyweight and studies on metabolism during *T. gondii* infection are very limited. Arsenijevic *et al* have linked the divergence in weight gain following *T. gondii* infection to cytokine expression profiles in mice that regain the bodyweights and those that remain at the reduced bodyweight indefinitely. They showed that the gainers had higher serum IFN-γ whilst non-gainers had a more prominent type-2 cytokine expression once stabilization of weight had occurred. Nonetheless, we did not observe any difference in systemic IFN-γ between male (non-gainers) and female (gainers) mice during the chronic phase of *T. gondii* infection (Arsenijevic, Girardier et al. 1997; Arsenijevic, Girardier et al. 1998). Some studies have associated the inhibition of NO production with increased weight loss and mortality following bone marrow transplantation procedures in mice (Drobyski, Keever et al. 1994). Therefore the impaired NO responses by splenic cells observed in our studies may also be implicated in the lack of ability of IL-4R$\alpha^{-/-}$ male mice to recover their bodyweights following acute *T. gondii* infection. From our observations, we can postulate that IL-4 plays an important role in mediating an effect/s on cells including $CD4^+$ and $CD8^+$ T-cells which facilitates bodyweight recovery after cachexia in female mice but not in male mice following infection with *T. gondii*.

The protective activity of $CD8^+$ T-cells is largely mediated by IFN-γ, and $CD8^+$ T-cells are major sources of IFN-γ during the acute stage of *T. gondii* infection (Suzuki, Orellana et al. 1988; Gazzinelli, Hakim et al. 1991). Previous studies have demonstrated that both CD4+ and CD8+ T-cells obtained from spleens of *T. gondii* infected mice are able to produce this cytokine following stimulation with *T. gondii* tachyzoite antigen (Gazzinelli, Hakim et al. 1991). The interaction between $CD4^+$ and $CD8^+$ T-cells in the protective responses against *T. gondii* infection are less well defined. In our studies we demonstrated that splenocytes from infected IL-4Rα intact (WT) mice are able to produce IFN-γ. We also show that IFN-γ production is significantly increased in splenocytes derived from macrophage specific IL-4R$\alpha^{-/-}$ and in global IL-4R$\alpha^{-/-}$ mice compared with WT mice, whereas splenocytes from $CD4^+CD8^+$T-cells specific IL-4R$\alpha^{-/-}$ ($NLck^{cre}$IL-4R$\alpha^{-/lox}$) but not $CD4^+$ T-cell specific IL-4R$\alpha^{-/-}$ (Lck^{cre}IL-4R$\alpha^{-/lox}$) mice produced significantly less IFN-γ compared with WT and IL-4R$\alpha^{-/-}$ mice. Importantly, this reduction was antigen specific as ConA-stimulated $CD4^+CD8^+$ T-cell specific IL-4R$\alpha^{-/-}$ derived splenocytes did not display this reduced IFN-γ response. IL-4 is generally recognized to polarize naïve $CD4^+$ T-cells into type-2 cytokine secreting cells and this is achieved through the IL-4Rα in a STAT-6-dependent manner (Cunningham, Serre et al. 2004). Nonetheless, consistent with our observations, some studies using *Leishmania* and *Plasmodium yoelii* infection models have demonstrated that IL-4Rα deficient $CD8^+$ T-cells developed severely impaired memory responses and reduced IFN-γ responses, thus showing that IL-4 signaling is essential for the development of efficient protective $CD8^+$ T-cell responses (Stager, Alexander et al. 2003; Morrot,

Hafalla et al. 2005). In addition studies using *T. gondii* have also shown that IL-4Rα signaling is essential for $CD8^+$ T-cell effector mechanisms in the absence of $CD4^+$ T-cell help (Marsland, Schmitz et al. 2005) and more recently $CD8^+$ T-cells but not $CD4^+$ T-cells isolated from *T. gondii* infected STAT-$6^{-/-}$ mice were shown to have significantly reduced IFN-γ production compared with $CD8^+$ T-cells from WT mice although this was done using a different mouse strain (C57BL/6) (Jin, Takamoto et al. 2009). Our findings, taken together with the recently published studies, show that IL-4Rα signaling via $CD8^+$ T-cells but not $CD4^+$ T-cells is essential to increase efficient IFN-γ responses by splenic T-cells following *T. gondii* infection.

Furthermore we also showed that splenocytes derived from *T. gondii* infected global IL-4Rα-deficient, $CD4^+$ T-cell specific IL-4R$\alpha^{-/-}$ as well as $CD4^+$ T-cell and $CD8^+$ T-cell specific IL-4Rα-deficient mice displayed significantly reduced NO production compared with splenocytes from infected IL-4Rα intact (WT) mice following stimulation with soluble *Toxoplasma* antigen. Moreover, splenocytes from $CD4^+$ and $CD8^+$ T-cell specific IL-4R$\alpha^{-/-}$ ($NLck^{cre}$IL-4R$\alpha^{-/lox}$) mice produced significantly lower NO compared with splenocytes from $CD4^+$ T-cell specific IL-4R$\alpha^{-/-}$ mice. These observations raise a possibility that IL-4Rα signaling on $CD4^+$ and $CD8^+$ T-cells augments NO responses during *T. gondii* infection. NO production is an important effector mechanism by classically activated macrophages which controls intracellular *T. gondii* proliferation and $iNOS^{-/-}$ C57BL/6 mice were shown to be highly susceptible to *T. gondii* infection (Khan, Schwartzman et al. 1997; Scharton-Kersten, Yap et al. 1997).

On the other hand, high NO levels have been shown to be counter-protective and can be detrimental to the host (Niedbala, Wei et al. 1999). Our observations seem to contradict the established notion that IL-4 is a counter-inflammatory mediator that could inhibit inflammatory responses mediated by T-cells thus we suggest that in the absence of IL-4Rα signaling via $CD4^+$ and $CD8^+$ T-cells NO production is reduced during *T. gondii* infection to limit type-1 cytokine mediated immunopathology. More studies would need to be undertaken to uncover the mechanisms through which NO responses are regulated and the role of IL-4Rα signaling during *T. gondii* infection.

In addition to NO production novel IFN-γ dependent macrophage effector functions including tryptophan degradation by IDO and upregulation of p47 GTPases have been implicated in restricting intracellular parasite growth (Pfefferkorn 1984; Butcher, Greene et al. 2005). We could not draw any conclusive evidence of the involvement of IDO and p47 GTPases in *T. gondii* infection as we observed different patterns of mRNA transcripts expression depending on the time point we investigated in the lung following *T. gondii* infection. The differential expression may be due to constantly changing type-1/type-2 cytokine environment, as we investigated the acute phase of infection where the immune response is very dynamic. Additionally, the stage-specific surface antigen expression by *T. gondii* (SAG1, TgCyst) influence the immune response and subsequently the IFN-γ/IL-4-dependent macrophage effector mechanisms hence the different pattern of expression of these markers. Nevertheless, IDO expression was generally reduced in IL-4R$\alpha^{-/-}$ mice and in some cases in $CD4^+$ T-cell IL-4R$\alpha^{-/-}$ mice.

IL-4 and IL-13 trigger a characteristic 'alternative' state of activation in macrophages which is distinct from the IFN-γ 'classical' activation or the deactivation phenotype associated with IL-10 and TGF-β (Mosser 2003). Our studies demonstrated that *T. gondii* infection induced a marked increase of markers of alternative macrophage activation in the lungs of both male and female mice, irrespective of IL-4Rα deficiency. This suggested that *T. gondii* infection was inducing alternative macrophage activation independently of IL-4/IL-13 mediated signals. In general, female mice expressed lower levels of FIZZ1, YM1 and arginase1 mRNA transcripts in the lungs compared with male mice at day 9 post-infection with *T. gondii*. FIZZ1 expression was significantly reduced in the lungs of female IL-4Rα$^{-/-}$ mice in most cases in our studies. Alternatively activated macrophages are widely reported to be generated in and contribute to helminth and nematode infections control, which are largely type-2 dependent (Anthony, Urban et al. 2006; Zhao, Urban et al. 2008). Nevertheless, the exact role of alternatively activated macrophages in host defence and adaptive responses to protozoan parasites remains a largely unknown area. There is evidence that macrophages are recruited to various sites of inflammation where they might partake in the down-regulation of inflammatory responses and subsequent tissue repair (Wynn 2004). It is also thought that they are involved in providing negative regulatory signals to protect the host from excessive inflammatory responses (Goerdt, Politz et al. 1999). We therefore postulate that alternatively activated macrophages may be involved in limiting lethal immunopathology as we observed excessive damage to the lungs of IL-4Rα$^{-/-}$ mice and

in macrophage/neutrophil specific IL-4Rα deficient mice following infection compared with WT mice, and these mice also displayed a significant reduced expression of lung FIZZ1 mRNA.

Although there was a significant level of alternatively activated macrophage induction following *T. gondii* infection in lung tissues, studies on BAL macrophages from infected mice revealed that YM1 and arginase1 but not FIZZ1 mRNA transcripts were significantly increased in BAL macrophages in the absence of IL-4/IL-13 signaling. Various types of cells are recruited into the inflammatory site, in our model the lung, following infection, and these observations demonstrate that *T. gondii* differentially induces the markers of alternative macrophage activation YM1 and Arginase 1 in macrophages independently of IL-4/IL-13 signaling whilst FIZZ1 remains at basal expression levels. In fact, studies done to determine the cell-specific expression of mouse FIZZ1 during inflammatory responses in the lung have shown that FIZZ1 mRNA is predominantly expressed in bronchial epithelial cells, and not in alveolar macrophages (Holcomb, Kabakoff et al. 2000). Therefore, this may also explain the unaltered expression of FIZZ1 mRNA in BAL macrophages. In line with our findings, previous *in vitro* studies in our laboratory demonstrated that *T. gondii* (Beverly and RH strain) were able to induce Arginase 1 expression and activity in bone-marrow derived macrophages in the absence of IL-4 stimulation (Menzies, Henriquez et al.). In addition, recent evidence has shown that Arginase-1, YM1 and FIZZ1 expression in macrophages can be directly induced by parasites such as *S. mansoni* (Pesce, Ramalingam et al. 2009; Pesce,

Ramalingam et al. 2009), bacterial infections such as *M. tuberculosis* (El Kasmi, Qualls et al. 2008) and protein homologues such as MIF from the helminth *Brugia malayi* (Prieto-Lafuente, Gregory et al. 2009). Studies using macrophage-specific STAT-$6^{-/-}$ mice have also demonstrated that *M. tuberculosis* and *T. gondii* are able to induce arginase expression in a TLR-dependent and STAT-6-independent manner (El Kasmi, Qualls et al. 2008). More recently, Nair et al (2009) and Pesce et al (2009) have proposed that alternatively activated macrophage derived FIZZ1 and Arginase 1 respectively, limited lethal immunopathology in the lungs and liver respectively during *S. mansoni* infection, in part through down-regulating $CD4^{+}$ T-cell type-2 cytokine responses. In the *T. gondii* infection model, it is very likely that the induction of Arginase 1 is a mechanism utilized by the parasite to subvert the classically activated anti-parasitic activity by utilizing the common substrate (L-Arginine) required for NO production. Arginase expressing macrophages promote the production of polyamines and L-proline which are involved in cell proliferation and collagen synthesis (Goerdt and Orfanos 1999), and hence they could also be supporting parasite replication and competitively inhibiting NO-mediated killing. Induction of Arginase 1 is particularly significant as it is emerging as a pertinent strategy for parasites to suppress protective T-cell functions (Modolell, Choi et al. 2009; Nair, Du et al. 2009; Pesce, Ramalingam et al. 2009) and also to support parasite growth inside macrophages (Iniesta, Carcelen et al. 2005). Indeed, in most of our studies we observed a higher parasite burden in the lungs of IL-4R$\alpha^{-/-}$ mice compared with WT mice. Moreover, there was a significant increase in conversion from the active tachyzoite stage to the latent bradyzoite form of the

parasite in the lungs in the absence of IL-4Rα signaling. The mechanisms that regulate parasite conversion are not well defined, but it is thought that NO stress induces tachyzoite to bradyzoite conversion (Bohne et al., 1993, Lyons et al., 2002). Studies by Fox et al. have also shown that arginine depletion results in tachyzoite to bradyzoite stage switch (Fox, Gigley et al. 2004). We generally observe a defective NO response by splenocytes deficient in IL-4 signaling, and a significant increase of Arginase 1 expression in IL-4Rα$^{-/-}$ lung macrophages competitively inhibiting NO production and potential NO stress. Therefore it is possible that arginine depletion by Arginase 1, together with other macrophage effector mechanisms, including IDO expression may be compromising parasite metabolic processes by tryptophan starvation and in turn contributing to the conversion of the parasite stage from the tachyzoite to the more dormant (less energy consuming) bradyzoite form. Nevertheless, more studies need to be undertaken to further test this hypothesis.

In conclusion, we have demonstrated in tissue specific IL-4Rα$^{-/-}$ mice that IL-4Rα signaling via macrophages/neutrophils promotes survival and limits lethal type-1 mediated immunopathology in female BALB/c mice during acute *T. gondii* infection. On the other hand, IL-4Rα signaling via CD4^{+} T-cells is not critical for survival whilst signaling through both CD4^{+} and CD8^{+} T-cells offers partial protection during the chronic stage of infection. In fact, IL-4Rα signaling on CD4^{+} and CD8^{+} T-cells mediated pathology in early and chronic infection respectively. We have also shown the gender-dependent differences in adaptive immunity to *T. gondii*, whereby female mice

were more susceptible to infection due to their delayed and less vigorous type-1 response whereas males mounted early and efficient antigen specific responses. Moreover, we have shown that IL-4 signaling on $CD8^+$ T-cells but not $CD4^+$ T-cells plays an important role in the development of efficient IFN-γ responses, and that IL-4Rα signaling on T-cells is required for robust NO production by splenocytes in response to infection. More significantly, our findings revealed that *T. gondii* infection promoted alternative macrophage activation in an IL-4/IL-13 independent manner *in vivo*. We suggest that *T. gondii* is directly inducing Arginase 1 and YM1 expression as a strategy to evade NO-mediated anti-parasitic mechanisms and instead hydrolyze L-arginine to produce components which supports its own proliferation within macrophages. Furthermore, in absence of IL-4/IL-13 signaling there is increased tachyzoite to bradyzoite parasite form conversion which may contribute to parasite persistence as bradyzoite are less antigenic. These results together with supporting evidence from other protozoan infection models provide a basis for drugs targeting arginine metabolism. Finally, our understanding of how IL-4 modulates the innate and adaptive responses including macrophage activation and how *T. gondii* manipulates the immune responses should provide therapeutic strategies and targets for drugs to combat *T. gondii* and other infections.

Table 5. Summary of IL-4 knock-out mouse models and *T. gondii* infection

Gene KO	Mouse strain	*T. gondii* strain/ route of infection	Sex	Mortality rate	Pathology	aaMØ markers	Parasite burden	Other
IL-4-/- (Roberts et al., 1996)	B6/129	RRA Beverley 20 cysts Oral	M	17% mortality, acute	Less TE compared with WT	N/A	Few brain cysts	↑ IFN-γ, ↓ IL-10
			F	76.9% mortality, acute	Less TE compared with WT	N/A	Few brain cysts	↑ IFN-γ, ↓ IL-10
IL-4Rα-/-	BALB/c	RRA Beverley cysts 10 cysts IP	F	50%-60% mortality, Acute phase	Severe lung inflammation	-Markedly increased but ↓FIZZ in whole lung -YM1 and Arginase 1 increased in BAL MØs	Increased tachyzoite to bradyzoite conversion in lung	↑ IFN-γ, ↓ IDO
Macrophage/neutrophil IL-4Rα-/-	BALB/c	RRA Beverley cysts 10 cysts IP	F	50-60% mortality, Acute phase	Severe lung inflammation	Slightly reduced in whole lung tissue compared to WT	Increased tachyzoite to bradyzoite conversion in lung	
CD4+ T-cell IL-4Rα-/-	BALB/c	RRA Beverley cysts 10 cysts IP	M	100% survival	Lack of lung inflammation in chronic phase	Increased lung YM1		Robust type-1 response, but less than WT
			F	14% mortality		Increased, but reduced lung YM1	Increased tachyzoite to bradyzoite conversion in lung	Delayed parasite specific type-1 response↓ IDO,
CD4+CD8+ T-cell IL-4Rα-/-	BALB/c	RRA Beverley cysts 10 cysts	M	100% survival	Lack of lung inflammation in acute phase	Increased		Reduced splenic IFN-γ production

		IP	F	20% mortality, chronic phase	Lack of liver inflammation in chronic phase	Increased	Increased tachyzoite to bradyzoite conversion	Delayed type-1 cytokine response

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