APPENDIX

A protective role for IL-4 during the early intraperitoneal infection in BALB/c mice.

ABSTRACT

The role of IL-4 during the course of *Toxoplasma gondii* infection was investigated in *Toxoplasma*-resistant BALB/c mice, using IL-4-deficient (IL-4–/–), IL-4 receptor α-deficient (IL-4Rα–/–) mice and their wild type (WT) counterparts. All groups of BALB/c mice (both immunocompetent and gene-deficient animals) were resistant to acute oral infection as demonstrated by comparatively low levels of mortality. Although more WT mice died late in infection following oral challenge than IL-4–/– and IL-4Rα–/– mice, there was no significant difference in mortality rates among the groups of mice. However, following intraperitoneal infection with *T. gondii*, IL-4–/– and IL-4Rα–/– mice displayed increased susceptibility to acute infection as demonstrated by significantly ($P < 0.01$) higher mortality rates compared with WT mice. Increased mortality in intraperitoneally infected IL-4–/– and IL-4Rα–/– mice was associated with more severe pathological changes in their lungs than those of WT mice at 12 days postinfection. However, no significant differences were found in either mortality or pathological changes between IL-4–/– and IL-4Rα–/– mice. At this time, the supernatant of spleen cell cultures derived from IL-4–/– and IL-4Rα–/– contained significantly ($P < 0.05$) higher levels of IL-12, IFN-γ, IL-10 and NO (measured as nitrite) than those derived from WT mice upon stimulation with TLA. However, there was no significant difference in cytokine or nitrite production by splenocytes cultured from IL-4–/– and IL-4Rα–/– mice.

At day 35 post-infection, *in vitro* parasite-specific immunological responses revealed no significant differences in cytokine or nitrite production by spleen cells derived from IL-4–/–, IL-4Rα–/– and WT mice. Similarly, no significant differences were observed in the severity of meningitis, prevascular cuffing, and encephalitis or numbers of cysts in the brains of mice. Together, these results suggest a protective role for IL-4 during the early intraperitoneal infection with *T. gondii* in BALB/C mice. This protective role may be due to the downregulatory effects of IL-4 on proinflammatory cytokines such as IFN-γ, IL-12, and NO, limiting the extent of harmful cytokine-induced inflammation. In addition, the results also indicate no significant role for IL-13 during the acute or chronic phase of disease, as IL-4–/– and IL-4Rα–/– displayed similar disease and immunological phenotypes.

INTRODUCTION

Studies on murine models of *T. gondii* infection indicate that although the size of the challenge dose and the parasite strain can influence the outcome of experimental infection, the progress of a *T. gondii* infection is controlled by a number of host genetic factors operating through the immune system. These genetic controls can influence mortality and long term brain cyst burden following either peroral or parentral infection (Blackwell *et al.*, 1993; McLeod *et al.*, 1989b & 1995). Resistance to *T. gondii* infection, measured as survival, brain cyst formation and toxoplasmic encephalitis is mainly under the control of MHC (H-2) complex. The ability of inbred mice to survive infection with *T. gondii* can also be influenced dramatically by the route of infection (Johnson, 1984). While C57BL/6 (H-2^b haplotype) mice have been found to be highly susceptible to oral but resistant to intraperitoneal infection, in contrast BALB/c mice (H-2^d haplotype) have been found to be comparatively resistant to both oral and intraperitoneal infection. Furthermore, BALB/c mice do not develop pathology in their intestines following peroral infection (Liesenfeld *et al.*, 1996). As BALB/c mice like humans belong to the resistant group of hosts, hence, BALB/c mice can provide a desirable model for studying the human toxoplasmosis.

To further determine the role of the Th2 response in resistance to *T. gondii* infection, we also studied infection in BALB/c IL-4 receptor α-deficient mice in parallel infections with BALB/c IL-4 –/– and WT animals. The IL-4 receptor is a heterodimer complex comprised of the IL-4Rα chain in association with the common γ (γc) chain (Brombacher, 2000; Finkelman *et al.*, 1999). IL-13, a cytokine with similar properties to IL-4, also uses IL-4Rα chain for signalling, along with the ligand-specific chain IL-13Rα1 (Finkelman *et al.*, 1999). Therefore, IL-4Rα–/– mice should be defective in both IL-4 and IL-13 signalling, while IL-4–/– mice would retain IL-13 function. By comparing parallel infections in these mice, we could indirectly assess the contribution of IL-13 in immunity to *T. gondii* infection.

IL-13 is a cytokine structurally similar to IL-4 that is produced by the Th2 subset of $CD4^+$ T cells, mast cells, and by some epithelial cells. Although the amino acid sequence homology between IL-4 and IL-13 is low (~30%), there is a high level of conservation of the tertiary structure between IL-4 and IL-13 (Zurawski & de Vries, 1994). IL-13 shares many but not all of the biological functions of IL-4. IL-13 upregulates a number of surface markers (for example, MHC class II on monocytes and macrophages) while downregulating their production of inflammatory cytokines

(Bancroft *et al.*, 1998) and has been shown to protect mice from LPS-induced lethal endotoxemia by downregulation of IFN-γ activity (Muchamel *et al.*, 1997). Unlike IL-4, IL-13 does not promote T cell growth. However, it has a regulatory role in the development of the Th2 cell lineage (McKenzie *et al.*, 1998b; Zurawski & de Vries, 1994).

The overlapping biological functions of IL-4 and IL-13 are due to at least one common component of otherwise distinct receptors. With the recent cloning and characterization of the IL-4Rα, it has been demonstrated that IL-4Rα chain is paired either with the common γ chain (γc) to form an IL-4 receptor (IL-4R type I), or with the IL-13Rα1 chain, which has low but IL-13-specific binding activity, to form the IL-13 receptor (IL-4R type II). The IL-13 receptor is found mainly on nonlymphoid cells, such as macrophages, and can be activated by either IL-13 or IL-4 (Brombacher, 2000; Finkelman *et al.*, 1999).

MATERIAL AND METHODS

Mice

IL-4 gene deficient (IL-4–/–) and IL-4 receptor α gene deficient (IL-4Rα–/–) mice BALB/c (H-$2^{d)}$ background were obtained from The Max Planck Institute for Immunobiology, Freiburg, Germany, and bred under conventional condition at the Department of Immunology, University of Strathclyde. Age-matched wild-type mice (WT) of the same strain combination obtained from the same source were used as controls in all the experiments.

Toxoplasma gondii and Infections

Mice were infected with 10 cysts of the avirulent RRA (Beverley) strain of *T. gondii* intraperitoneally as described in chapter 2.

Monitoring infections and histopathological analysis and collection of peripheral blood

Mice were monitored daily for mortality, and finally they were euthenized under terminal anaesthesia on days 12 or 35 postinfection. Lungs, livers and kidneys from 12 days, and brains from 35 days intraperitoneally infected mice with *T. gondii* were removed for acute and chronic histopathological studies respectively. Tissue samples were immediately fixed in phosphate buffered formalin and appropriate sections were prepared as described previously in chapter 2. The spleens from groups of infected mice were also removed aseptically for *in vitro* cell proliferation and cytokine production assays. Blood sample was collected from mice preinfection and on days 6, 9, and 12 post infection via their tail vein into heparinized capillary tube. Plasma was separated by centrifugation of blood at 14000 rpm for 5 minutes and stored at –70˚C until used for determination of cytokine levels.

Cell proliferation assay

Cell proliferation assays were carried out by using spleen cells derived from intraperitoneally infected mice with *T. gondii* at 12 and 35 days postinfection. Details are available in chapter 2.

Analysis of Supernatants and measurement of Plasma cytokine levels

Cytokine (IFN-γ, IL-10, and IL-12) levels were measured in the supernatants of TLA stimulated (with 20 and 1 μg/ml) spleen cell culture by sandwich (capture) ELISA as described in chapter 2.

Nitrite assay

Cell culture supernatants were analyzed for the production of NO using the Griess reaction assay, which measures the concentration of nitrite, a stable product of the reaction of NO with O_2 as described in chapter 2.

Administration of Nω -Nitro-L-Arginine Methyl (L-NAME) to mice.

Five days after oral infection with *T.gondii,* groups of 5-7 mice were treated with L-NAME (Sigma), the competitive inhibitor of NOS enzyme, at the dose of 200mg/Kg/day, in their drinking water (1g/Litre) for one week (23). Non-infected mice were also L-NAME treated to serve as controls.

Statistical analyses and experimental design

Statistical analysis were performed using the Mann-Whitney U test for the comparison of survival data, stimulation indices, cytokine production, and pathology data. All experiments were performed at least twice with similar findings.

RESULTS

Mortality

All the BALB/c mice infected orally, survived during the early acute phase of infection with *T. gondii* regardless of IL-4–/– or IL-4Rα–/– deficiencies. There were, however, some late mortalities (in Week 3 and thereafter) in WT and IL-4Rα–/– but not IL-4–/– mice infected by this route of infection. Although orally infected WT mice appeared to be slightly more susceptible than IL-4Rα–/– and IL-4–/– mice, there were no significant differences in mortality rates among the three groups of mice over four experiments. In general, male mice had lower mortality rates than females, but the same pattern of mortality was observed in males and females throughout the experiments. In a representative experiment (Figure 5.1A & B), oral infection resulted in 80% survival rate in male WT mice compared with 100% survival rates in both IL-4–/– and IL-4Rα–/– male mice (Figure 5.1A). In female mice, oral infection led to 60% survival rate in WT mice compared with 100 and 71.4% survival rates in IL-4–/– and IL-4Rα–/–mice respectively (Figure 5.1B).

Following intraperitoneal infection of mice with *T. gondii*, IL-4–/– and IL-4Rα–/– mice displayed increased susceptibility to acute infection as demonstrated by significantly ($P < 0.01$) higher mortality rates compared with WT mice. However, there were no significant differences in mortality rates between IL-4–/– and IL-4Rα–/– mice. Again this pattern was observed in both male and female mice, though male mice had consistently lower mortality rates than females. In the representative experiment (Figure 5.1C & D), while all the WT mice (both male and female) infected intraperitoneally with *T. gondii*, survived during the 35 days course of study, infection resulted in 27.3 and 15.4% mortality rates in male IL-4–/– and IL-4Rα–/– mice respectively (Figure 5.1C). Similarly in females, intraperitoneal infection caused 58.3 and 46.2% mortality rates in IL-4–/– and L-4Rα–/– mice respectively (Figure 3.1D).

Histopathology

Sections of Lung, liver and kidney from WT, IL-4–/– and IL-4Rα–/– mice infected intraperitoneally with *T. gondii* were examined for histopathological changes at day 12 postinfection, the day immediately followed the time of death in previous experiments. At this time point, livers and lungs from infected IL-4–/–, IL-4–/– and WT displayed different degrees of pathological changes, whereas no pathological changes were observed in the kidneys of these animals. The pathological changes in

livers consisted of inflammation of the portal tracts and acini, presence of fibrin thrombi in the blood vessels, and various size (small and large) necrosis of hepatic tissue (Table 5. 1). No significant differences in the severity of liver pathology were noted among IL-4–/–, L-4Rα–/– and WT mice. The inflammatory changes in the lungs of infected animals included interstitial pneumonitis and pulmonary oedema. A few lungs from IL-4–/– mice also had a number of thromboembolism in the blood vessels (Table 5. 2). At day 12 postinfection, lungs from IL-4–/– and IL-4Rα–/– mice showed significantly ($P < 0.05$) greater degrees of interstitial pneumonia than those of WT mice, whereas, no significant difference was observed in severity of pulmonary oedema among three groups of mice. There were also no significant differences in any of these inflammatory changes between IL-4–/– and IL-4Rα–/– mice (Fig. 5.2).

At day 35 postinfection, brains of WT, IL-4–/– and IL-4Rα–/– mice infected intraperitoneally with *T. gondii* examined for histopathologic changes and cyst burden, The brains of all infected mice irrespective of mouse substrain, showed different degrees of histopathological changes from mild to moderate meningitis, prevascular cuffing, and mild to moderate inflammation of the brain tissue. The brains of infected mice also contained various numbers of cysts (Table 5.3). However, no significant differences in any of the pathological changes and cyst burden in the brains of mice were observed among the groups of mice.

Comparison of *T. gondii* -specific splenocyte responses from IL-4–/–, IL-4Rα–/– and WT mice infected intraperitoneally with *T. gondii* .at day 12 post infection.
The ability of spleen cells derived from WT, IL-4–/– and IL-4Rα–/– mice infected intraperitoneally with *T. gondii* to proliferate in response to Toxoplasma Lysate Antigen (TLA) stimulation was assessed on day 12 post infection (Figure 5.4). A marked proliferation was observed in TLA-stimulated splenocyte cultures derived from infected WT, IL-4–/– and IL-4Rα–/–mice compared with those derived from noninfected mice. There were, however, no significant differences among the stimulation indices of splenocytes derived from infected WT, IL-4–/– and IL-4Rα–/– mice.

Supernatant of spleen cell cultures derived from WT, IL-4–/– and IL-4Rα–/– mice infected intraperitoneally with *T. gondii* were examined for cytokine production in response to TLA stimulation at day 12 post infection (figure 5.5). Cell culture supernatants from IL-4–/– and IL-4Rα–/– mice were found to contain significantly

(P<0.05) higher quantities of IL-12 (1.863 ± 0.296 and 1.441 ± 0.126 versus 0.798 ± 0.178), IFN-γ (65.67 ± 8.532 and 65.125 ± 0.592 versus 40.382 ± 3.257), and IL-10 (1.546 ± 0.362 and 1.076 ± 0.182 versus 0.438 ± 0.024) than those from WT mice when stimulated with 20 μg of TLA per ml. Similarly, spleen cells derived from IL-4–/– and IL-4Rα–/– mice also produced significantly (P < 0.05) more NO, measured as nitrite, (18.129 ± 0.941 and 18.034 ± 1.499 versus 11.52 ± 1.349) than splenocytes derived from WT mice when stimulated with 20 μg of TLA per ml.

Comparison of *T. gondii* -specific splenocyte responses from IL-4–/–, IL-4Rα–/– and WT mice infected intraperitoneally with *T. gondii* at day 35 post infection.

T. gondii -specific splenocyte proliferative responses from WT, IL-4–/– and IL-4Rα–/– mice infected intraperitoneally with *T. gondii* were assessed on day 35 post infection (Figure 2.7). Splenocytes derived from infected WT, IL-4–/– and IL-4Rα–/– mice displayed a significant proliferation in response to TLA stimulation compared with those from noninfected mice. However, there were no significant differences among the stimulation indices of lymphocytes from infected WT, IL-4–/– and IL-4Rα–/– mice.

When supernatants of spleen cell culture derived from infected WT, IL-4–/– and IL-4Rα–/– mice infected examined for cytokine production on day 35 postinfection, no significant differences were observed in IL-12, IFN-γ and IL-10 production by spleen cells derived from WT, IL-4–/– and IL-4Rα–/– mice when stimulated with either 1 or 20 μg of TLA per ml. (Figure 5.6).

The effect of Nω - Nitro - L - Arginine Methyl (L-NAME) on survival of WT, IL-4-/- and IL-4Rα-/- mice

Since higher mortality and associated lung pathology in gene-deficient mice during *T. gondii* infection were found to be related to NO production, the role of NO was investigated in WT, IL-4–/– and IL-4Rα–/– mice. Inhibition of NO production by daily oral administration of L-NAME to intraperitoneally infected female WT, IL-4–/– and IL-4Rα–/– mice from day 5 to 11 postinfection, resulted in significantly (P< 0.05) higher mortality compared with control mice which received water (Figure 5. 8). L-NAME-treated WT and gene-deficient animals showed 80 and 100% mortality by 12 days post infection respectively. However, control survival following infection was 60% in IL-4–/– and 80% in both WT and IL-4Rα–/– animals. L-NAME treatment had no detrimental effect on noninfected animals as measured by mortality.

DISCUSSION

In this study, we used genetically resistant BALB/c mice to investigate the role of IL-4 during the course of *T. gondii* infection. The results of our study indicate that BALB/c mice are comparatively resistant to acute oral infection, regardless of whether they are immunocompetent or lacking IL-4 activity. Although WT mice appeared to be slightly more susceptible to late oral infection than IL-4–/– and IL-4Rα–/– mice, there were no significant differences in mortality rates among the groups of mice over four experiments. Following intraperitoneal infection, while immunocompetent BALB/c mice remained resistant during the 35 days course of study, IL-4–/– and IL-4Rα–/– mice displayed some degrees of susceptibility to acute infection as demonstrated by significantly ($P < 0.01$) higher mortality rates compared to WT mice. These findings again confirm the genetic resistance of BALB/c mice to both peroral and intraperitoneal infection with *T. gondii*, as previously described by Blackwell *et al.*, (1993). In addition, these observations reveal an important role for IL-4 in resistance to acute intraperitoneal but not oral infection with *T. gondii* in BALB/c mice. While the outcome of peroral infection in BALB/c mice was not affected by impaired IL-4 function, the resistance of these mice during the acute phase of intraperitoneal infection dramatically decreased in the absence IL-4 activity.

The enhanced mortality of gene-deficient mice over WT mice during the early intraperitoneal infection with *T. gondii* was found to be related to increased pathological changes in the lungs of both IL-4–/– and IL-4Rα–/– mice compared with those of WT mice. Furthermore, evaluation of *in vitro* splenocyte immune response from intraperitoneally infected mice revealed that increased inflammatory changes in the lungs of gene-deficient mice were accompanied by the elevated levels of IL-12, IFN-γ, IL-10 as well as nitrite in the supernatants of spleen culture from these animals compared with those of their WT counterparts. Taken together, these results suggest an inhibitory role for IL-4 on Th1-mediated immune response during acute intraperitoneal infection in BALB/c mice, preventing the development of type 1 cytokine-mediated lung pathology in these animals. These findings support the previous observations in our laboratory by Roberts *et al.*, (1997) and Alexander *et al.*, (1998) that the overproduction of proinflammatory cytokines such as IFN-γ and IL-12 by splenocytes from IL-4-deficient mice during early *T. gondii* infection may be deleterious to the host, although their findings were obtained with different mouse strains.

IFN-γ has been shown to have a protective role throughout all phases of *T. gondii* infection (Suzuki *et al.* 1988a and 1989b). Induction of a strong but regulated IFN-γ response serves to suppress acute and uncontrolled tachyzoites replication as well as establish and maintain latency during the chronic phase of infection. IFN-γ synthesis during the initial phase of infection is regulated by two major antagonist cytokines, IL-12 and IL-10. While IL-12 is the central mediator initiating the synthesis of IFN-γ, IL-10 appears to modulate both IL-12 and IFN-γ synthesis *in vivo*, avoiding an excessive immune response that could cause extensive inflammation and host tissue damage (Neyer *et al.*, 1997). Although IFN-γ may play multiple roles in resistance to the parasite, macrophage activation is generally believed to be the critical effector function (Anderson & Remington, 1974 ; Gazzinelli *et al.*, 1996b). Macrophage activation results in synthesis of nitric oxide (NO) which is effective in the control of *T. gondii* infection (James, 1995). However, if uncontrolled these responses can lead to severe immunopathologic changes and even to host death. Interestingly, it has been demonstrated that those mouse strains with the highest mortality rates during acute phase of infection are those producing the most IFN-γ (McLeod *et al.*, 1989a). Similarly, in this study we found the IL-4–/– and IL-4Rα–/– mice produce more splenocytes IFN-γ and NO early in infection *ex vivo*, and are more susceptible, as measured by mortality in acute phase of disease than their immunocompetent counterparts. Therefore, our results imply that impairment of IL-4 activity (either the absence of cytokine or its receptor) accounts for the overproduction of proinflammatory cytokines during the acute phase of intraperitoneal infection with *T. gondii* in BALB/c mice which may in part be responsible for the enhanced inflammatory reactions in the lungs of IL-4–/– and IL-4Rα–/– mice.

The cytokine IL-4 is known to promote humoral rather than classical cell-mediated immunity, and has been previously shown to inhibit certain macrophage functions and to potentiate the effect of IL-10 on macrophages (Oswald *et al*., 1992). In addition, IL-4 plays a major role in controlling the development of cell-mediated immunity through its effects on Th0 cells, resulting in STAT 6 induction and T-cell differentiation to the Th2 phenotype (Quelle *et al*., 1995; Swain *et al*., 1990). While some studies suggest a protective role for this cytokine in avoiding pathologic changes (as discussed above), others suggest that absence of this mediator may be beneficial to the host in surviving acute infection (Villard *et al.*, 1995; also previous chapters). Part of this difference may be due to the use of different mouse or parasite strains, however, it may also be related in part to the different routes of infection (previous chapters).

On the other hand, resistance to *T. gondii* infection requires the development of a finely tuned balance between the production of proinflammatory and regulatory cytokines by the regional Th1-mediated immune response, and the outcome of infection is dependent upon this delicate cytokine balance. Therefore, the absence of any of proinflammatory or regulatory cytokines may disturb the delicate balance and lead to a different outcome of disease. Thus, C57BL/6 mice which are resistant to intraperitoneal infection with *T. gondii*, when deficient in IL-10, succumb to early infection with an avirulent parasite strain (ME-49) while producing elevated levels of IFN-γ and IL-12 (Neyer *et al.*, 1997). Similarly, BALB/c mice, which are resistant to peroral infection and development of intestinal pathology, in the absence of cytokine IL-10, succumb during acute oral infection with a severe intestinal pathology as a result of IL-12 and IFN-γ overproduction (Suzuki *et al.*, 2000). Significantly, these investigators had previously reported that IL-12 and IFN-γ were protective against development of intestinal pathology in BALB/c mice (Liesenfeld *et al.*, 1996). Indeed, they argued that these cytokines were the mechanism underlying the genetic resistance of BALB/c (H-2^d) mice to peroral infection with *T. gondii*. Therefore, the absence of a single cytokine may totally undermine the genetic basis of the resistance in a specific mouse strain, whereas the same cytokine may have minor effects in another strain of mice.

An interesting observation in our study was the upregulation of both proinflammatory (IL-12 and IFN-γ) and antiinflammatory (IL-10) cytokines during the acute *T . gondii* infection in the absence of IL-4 activity. These finding may possibly be explained by the fact that during acute *T. gondii* infection, macrophages are the major source of proinflammatory cytokine IL-12, as well as the anti-inflammatory mediator IL-10, although this cell population also appears to be the main target of IL-10 suppressive action (Moore *et al.*, 1993). Interestingly, several reports have indicated that IL-12 itself can induce T cells to produce IL-10, suggesting a model in which IL-12 may limit its own production by induction of its own negative regulator, IL-10 (Daftarian *et al.*, 1996 ; Trinchieri, 1996; Meyaard *et al.*, 1996; Jeannin *et al.*, 1996; Windhagen *et al.*, 1996). IL-10 has been shown to be crucial for protecting mice through the acute phase of *T. gondii* infection by inhibiting the " cytokine shock" and allowing establishment of chronic phase (Neyer et al., 1997).

We also observed an increased splenocyte NO production by IL-4–/– and IL-4Rα–/– compared with WT mice. This finding indicates that IL-4 down-regulates the

induction of iNOS in macrophages, and that the NO plays a detrimental role during the acute intraperitoneal infection with *T. gondii*. Thus, some of the pathological changes associated with acute intraperitoneal infection involve NO-mediated effects. However, oral treatment with the synthetic NOS inhibitor L-NAME of the infected mice resulted in enhanced mortality in both WT and gene-deficient mice relative to untreated controls. This would clearly suggest a protective rather than an exacerbative function for NO during acute intraperitoneal infection in BALB/c mice. Nevertheless, studies by Scharton-Kersten, *et al*., (1997b) shows that the control of early parasite growth in mice following intraperitoneal infection appears to be iNOS-independent.

To explain the mechanisms by which inhibition of NO production exacerbates inflammation, it must be remembered that NO is produced by three different NOS isoforms, thus it has a broad and widespread of physiological functions. While endothelial and neuronal NOS are constitutively expressed, the macrophage NOS (iNOS) is induced by cytokines and other immunological stimuli. Activation of macrophages by IFN-γ results in iNOS gene induction and synthesis of NO which is an important anti-*T. gondii* controlling factor (Langermans *et al*., 1992; Sibley *et al*., 1991). Although cytostatic/cytocidal activity of NO forms part of the host defence mechanism, the excessive or inappropriate production of NO can lead to tissue damage possibly through the formation of the potent oxidizing and nitrating agent, peroxynitrite, by a coupling of NO with superoxide (O_2-) (reviewed, Bogdan et al., 2000). Under physiological conditions, NO released from endothelial cells regulates vascular tone and maintains blood vessel patency by preventing platelet aggregation and down-regulating adhesion molecule expression (Kubes *et al*., 1991). However, mediators released during the acute phase of infection, including histamine, 5-hydroxytryptamine, bradykinin, and platelet-activating factor evoke the release of endothelial NO, causing vasodilatation and vascular permeability, thus facilitating edema formation and trafficking of inflammatory cells (Paul-Clark *et al*., 2001). Therefore, selective inhibition of eNOS during acute inflammation, is expected to cause vasoconstriction, followed by a reduction in blood delivery to the inflamed site. This sequence may ultimately lead to reduced cellular diapedesis and exudation and thus a reduction in inflammation. However, it has recently been demonstrated by Paul-Clark *et al*., (2001) that NOS inhibitors have different effects on inflammation depending on their route of administration. The results of their study shows that local administration of NOS inhibitors exacerbates inflammation, whereas giving NOS inhibitors systematically ameliorates inflammation.

L-arginine analogues such as L-NAME, are the pharmacological agents most commonly used to inhibit NO production, but they have poor selectivity between NOS isoforms, thereby inhibiting both iNOS and eNOS during inflammatory reaction. Therefore, systemic administration of L-arginine analogues, e.g., by the oral route, inhibits eNOS, while may result in an anti-inflammatory effect by reducing exudate formation and cellular influx (Tracey *et al*., 1995),but also may interfere with immune cell trafficking to the site of infection, allowing parasite replication and parasite-induced pathology.

On the other hand, it has been demonstrated by many studies that NO generation reduces O_2- levels, while its inhibition increases O_2- production both *in vitro* and *in vivo* (Rodenas et al., 1998; Paul-Clark *et al*., 2001). Therefore, inhibiting NO production removes the brakes on O_2- generation by PMNs and macrophages. As O_2- has been associated with tissue damage and loss of function during inflammatory episodes (Vega *et al*., 1998), it is conceivable that one of the contributors to an enhanced inflammatory response, consequent to NOS inhibition, is O_2- production. In addition to selectivity and route of administration, the dose and time of administration of the NOS inhibitors are also important factors in determining the outcome of inflammation.

The results of the present study also demonstrate that the intraperitoneally infected IL-4–/– and IL-4Rα–/– BALB/c mice succumbed to severe lung pathology rather than liver pathology during acute *T. gondii* infection. In broad agreement with these results, the studies of Derouin & Garin, (1991) on the blood and tissue kinetics of the *T. gondii* during acute and chronic infection in mice demonstrated that following intraperitoneal infection with *T. gondii*, the parasites were first detected in the lungs of infected animals on either days 2 or 4 postinfection, according to the inoculum size, and the infected mice died as a result of an early involvement of lungs, with pneumonia as the principal cause of death. In a separate study, Hunter *et al.*, (1993) also reported 100% mortality in SCID mice infected with *T. gondii* due to inflammation of the lung tissue on day 7 postinfection.

In the present study, we found no significant differences in brain cyst burden and associated brain inflammatory reactions among WT, IL-4–/– and IL-4Rα–/– mice over a series of experiments and up to 35 days postinfection. These results confirm the observations by Alexander *et al.*, (1998) that reported no significant difference in brain cyst burden between WT and IL-4–/– BALB/c mice over a period of 120 days

postinfection. Consistent with these results we found no significant difference in IL-12, IFN-γ and IL-10 production by spleen cells of WT, IL-4–/– and IL-4Rα–/– mice at 35 days postinfection.

In summary, the results of the present study strongly suggest a protective role for IL-4 during the acute but not chronic phase of intraperitoneal infection with *T. gondii* in genetically resistant BALB/c mice. Moreover, the results obtained with IL-4Rα–/– mice imply no significant role for IL-13 during either acute or chronic phases of *T. gondii* infection, since the absence of IL-13 effect in addition to IL-4 activity in IL-4Rα–/– mice did not produce any difference in the outcome of *T. gondii* infection compared with that in IL-4–/– mice. However, in addition to IL-13Rα1 which is a common receptor between IL-13 and IL-4 cytokines, another receptor for IL-13 has been recently cloned, IL-13Rα2, which binds IL-13 with a high affinity independently of IL-4Rα (Caput *et al.*, 1996; Donaldson *et al.*, 1998). Thus, the presence of this receptor in IL-4Rα–/– mice may partially permit IL-13 to function in the absence of IL-4Rα.

D

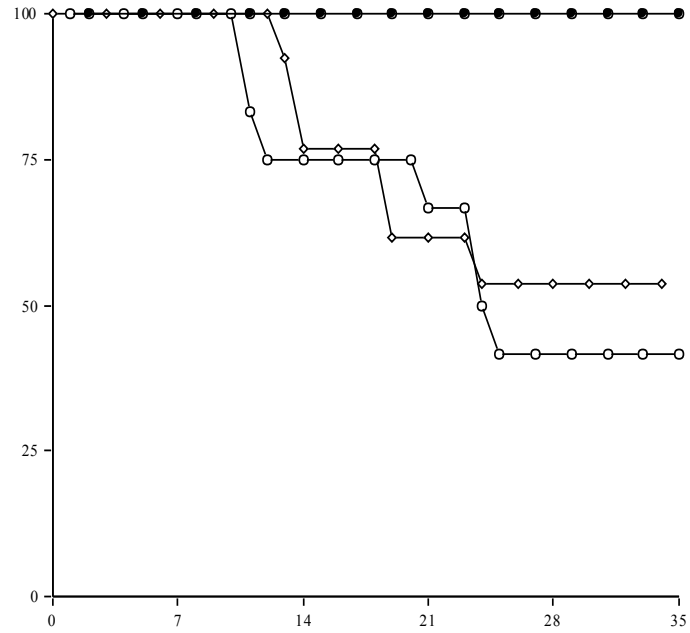

Figure 1.

Percent survival of male and female WT (closed circles), IL-4–/– (open circles), and IL-4Rα–/– (open squares) mice infected with *T. gondii*. Mice were infected with 10 *T. gondii* cysts by either oral (A & B, not shown) or intraperitoneal (C & D) route (n=5-7/group). There were no singificant differences in survival rates among either male (A) or female (B) WT, IL-4–/– and IL-4Rα–/– mice infected by the oral route. However, WT mice infected by the intraperitoneal route had significantly greater survival than IL-4–/– and IL-4Rα–/– mice infected by the same route irrespective of gender, although females (D) had greater mortality rates than males (C, not shown). No significant difference was observed in mortality between intraperitoneally infected IL-4–/– and IL-4Rα–/– mice. Results are representative of 4 replicate experiments.

Table 5.2 Lung pathological analysis following *T. gondii* infection

Mouse strain	Histological score for:		Pulmonary Thromboembolism *(n)*
	Interstitial Pneumonitis	Pulmonary Oedema	
Wild Type	2	0	0
	2	1	0
	2	1	0
	2	0	0
IL-4–/–	3	0	0
	3	0	0
	3	0	0
	2	1	0
IL-4Rα–/–	3	0	1
	3	0	0
	3	0	1
	2	1	0

Table showing histological scores for inflammation in the lung of T. gondii infected mice. The increased mortality was associated with increased lung pathology in IL-4-/- and IL-4Rα-/- compared with WT mice.

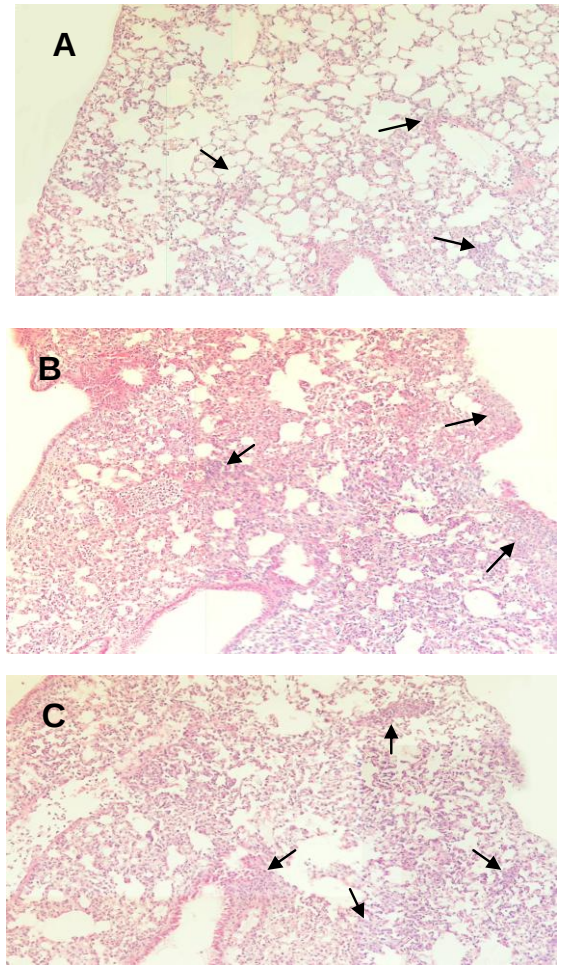

Fig. 5.2 Lung tissue sections showing higher degrees of interstitial inflammation (arrows) in the lungs of IL-4-/- (B) and IL-4Rα-/- (C) than in WT (A) mice following intraperitoneal *T. gondii* infection with 10 cysts.

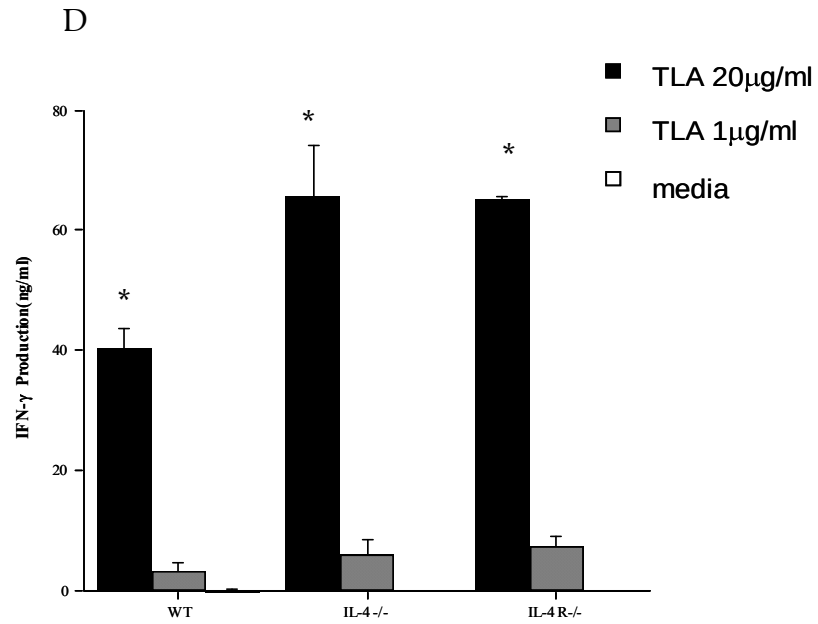

Figure 2.

Comparison of TLA-induced splenocyte responses of female WT, IL-4–/– and IL-4Rα–/– mice infected with 10 *T. gondii* cysts by the intraperitoneal route. Splenocytes were isolated from mice 12 days post infection and stimulated with 20 μg/ml of TLA (closed bars) and 1 μg/ml of TLA (hatched bars) or unstimulated (open bars) for 96 hours. Proliferation was assessed over the final 18 hours by incorporation of tritiated thymidine (A, not shown). Supernatants were collected from parallel cultures after 72 hours and analyzed for IFN-γ (D). Statistically significant differences (by Mann-Whitney U test) between the responses of WT, IL-4–/– and IL-4Rα–/– mice are marked (*). Results are representative of 2 replicate experiments.

FIGURE LEGENDS

Figure 2. (graphs not shown)
Comparison of TLA-induced splenocyte responses of female WT, IL-4–/– and IL-4Rα–/– mice infected with 10 *T. gondii* cysts by the intraperitoneal route. Splenocytes were isolated from mice 12 days post infection and stimulated with 20 μg/ml of TLA (closed bars) and 1 μg/ml of TLA (hatched bars) or unstimulated (open bars) for 96 hours. Proliferation was assessed over the final 18 hours by incorporation of tritiated thymidine (A). Supernatants were collected from parallel cultures after 72 hours and analyzed for nitrite (B), IL-12 (C), and IL-10 (E). Statistically significant differences (by Mann-Whitney U test) between the responses of WT, IL-4–/– and IL-4Rα–/– mice are marked (*). Results are representative of 2 replicate experiments.

Figure 3. (graphs not shown)
Comparison of TLA-induced splenocyte responses of male WT, IL-4–/– and IL-4Rα–/– mice infected with 10 *T. gondii* cysts by the intraperitoneal route. Splenocytes were isolated from mice 35 days post infection and stimulated with 20 μg/ml of TLA (closed bars) and 1 μg/ml of TLA (dashed bar) or unstimulated (open bars) for 96 hours. Proliferation was assessed over the final 18 hours by incorporation of tritiated thymidine (A). Supernatants were collected from parallel cultures after 72 hours and analyzed for IL-10 (B), IL-12 (C), IFN-γ (D). No statistically significant differences were (by Mann-Whitney U test) found between the responses of WT, IL-4–/– and IL-4Rα–/– mice. Results are representative of 3 replicate experiments.

Figure 4. (graphs not shown)
Percent survival of control and L-NAME treated WT (A), IL-4–/– (B) and IL-4Rα–/– (C) female mice infected with *T. gondii*. Mice were infected with 10 *T. gondii* cysts by the intraperitoneal route. Treated groups (closed circles) received approximately 2 mg/kg of L-NAME per day (1g/L in their drinking water) from day 5 post infection. Untreated mice received normal drinking water (open circles). Both WT, IL-4–/– and IL-4Rα–/– mice treated with L-NAME had significantly reduced survival compared with their untreated controls.