IL-10 Is Required for Prevention of Necrosis in the Small Intestine and Mortality in Both Genetically Resistant BALB/c and Susceptible C57BL/6 Mice Following Peroral Infection with *Toxoplasma gondii*

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IL-10 Is Required for Prevention of Necrosis in the Small Intestine and Mortality in Both Genetically Resistant BALB/c and Susceptible C57BL/6 Mice Following Peroral Infection with Toxoplasma gondii

Yasuhiro Suzuki, 2* Alan Sher, † George Yap, ‡ Daniel Park, * Lauri Ellis Neyer, 3*† Oliver Liesenfeld, 4*† Madeline Fort, ‧ Hoil Kang, ‡* and Edgar Gufwoli*

The role for IL-10 in the immunopathogenesis of acute toxoplasmosis following peroral infection was examined in both genetically susceptible C57BL/6 and resistant BALB/c mice. C57BL/6-background IL-10-targeted mutant (IL-10−/−) mice all died in 2 wk after infection with 20 cysts of the ME49 strain, whereas only 20% of control mice succumbed. Histological studies revealed necrosis in the small and large intestines and livers of infected IL-10−/− mice. The necrosis in the small intestine was the most severe pathologic response and was not observed in control mice. Treatment of infected IL-10−/− mice with either anti-CD4 or anti-IFN-γ mAb prevented intestinal pathology and significantly prolonged time to death. Treatment of these animals with anti-IL-12 mAb also prevented the pathology. Significantly greater amounts of IFN-γ mRNA were detected in the lamina propria lymphocytes obtained from the small intestine of infected IL-10−/− mice than those from infected control mice. In common with C57BL/6-background IL-10−/− mice, BALB/c-background IL-10−/− mice all died developing intestinal pathology after infection. Control BALB/c mice all survived even after infection with 100 cysts and did not develop the intestinal lesions. Treatment with anti-IFN-γ mAb prevented the pathology and prolonged time to death of the infected IL-10−/− mice. These results strongly suggest that IL-10 plays a critical role in down-regulating IFN-γ production in the small intestine following sublethal peroral infection with Toxoplasma gondii and that this down-regulatory effect of IL-10 is required for prevention of development of IFN-γ-mediated intestinal pathology and mortality in both genetically resistant BALB/c and susceptible C57BL/6 mice. The Journal of Immunology, 2000, 164: 5375–5382.

Immunity is crucial for controlling acute infection with Toxoplasma gondii. Murine models have been most frequently used for analyzing the protective immune response against T. gondii. The majority of these studies were performed using the i.p. route of infection (1–5). Johnson (6) and Blackwell et al. (7) reported that mortality after acute infection in inbred strains of mice differs depending on the route of infection, suggesting that the mechanism(s) of resistance against acute toxoplasmosis in mice also are influenced by this parameter. Since the peroral route is the natural route of infection with this parasite, it is important to define the mechanism of resistance against this parasite using hosts infected at this mucosal site. Because during natural infection T. gondii first invade the small intestine, identifying the immunoregulatory factors that control the intestinal immune response to the parasite should provide a more accurate understanding of acute infection and disease.

Resistance of mice to mortality following acute peroral infection with T. gondii is under genetic control (7, 8–10). Multiple genes including one linked to the MHC complex are involved in this control (7, 10). Since the MHC encodes major recognition and immunoregulatory molecules, it is likely that the genes responsible for control of resistance against acute toxoplasmosis, or at least some of them, do so by regulating the immune response to the parasite. Thus, to understand the immunopathogenesis of acute toxoplasmosis, it is important to study the factors underlying the genetic regulation of resistance.

With regard to genetic regulation of resistance against peroral infection with T. gondii, IFN-γ has been shown to be required for survival of resistant strains of mice following infection (11, 12). In contrast, we recently found that IFN-γ contributes to the early mortality of infected, genetically susceptible C57BL/6 mice (13). Severe necrosis of the small intestine was observed in association with mortality in infected C57BL/6 mice, and the pathology was shown to be mediated by IFN-γ (13). In this case, local IFN-γ production in the small intestine appears to become overly stimulated and the excess IFN-γ induces the immunopathology and contributes to early mortality (13). These results strongly suggest that regulation of regional IFN-γ production in the small intestine is critical for determining whether this cytokine is protective or

*Department of Immunology and Infectious Diseases, Research Institute, Palo Alto, CA 94301; †Department of Medicine, Division of Infectious Diseases and Geographic Medicine, Stanford University School of Medicine, Stanford, CA 94305; ‡Laboratory of Parasitic Diseases, Immunobiology Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and 4Department of Immunobiology, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA 94304

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Address correspondence and reprint requests to Dr. Yasuhiro Suzuki, Department of Immunology and Infectious Diseases, Research Institute, Palo Alto Medical Foundation, 860 Bryant Street, Palo Alto, CA 94301. E-mail address: ysuzuki@lelandStanford.edu

Current address: Bayer Corporation, 800 Dwight Way, Berkeley, CA 94710.

Current address: Institut für Infektionsmedizin, Abteilung für Medizinische Mikrobiologie, Universitätssklinikum Benjamin Franklin der Freien Universität Berlin, 12203 Berlin, Germany.
detrimental in the immunopathogenesis of acute toxoplasmosis and for determining genetic resistance/susceptibility of the host to infection.

IL-10 has been shown to play an important role in down-regulating IFN-γ production in C57BL/6 mice following i.p. infection with T. gondii (14, 15). In these studies, infected, C57BL/6-background IL-10-targeted mutant (IL-10−/−) mice developed enhanced liver pathology characterized by increased cellular infiltration and intense necrosis in association with increased serum levels of IFN-γ as compared with control mice (14). However, it is still unknown whether IL-10 plays an important role in resistance to this parasite through the natural route of infection and whether this cytokine is involved in genetic regulation of resistance to the infection. Therefore, in the present study, we examined the role of IL-10 in the immunopathogenesis of acute toxoplasmosis following peroral infection with T. gondii in both genetically susceptible C57BL/6 and resistant BALB/c mice.

Materials and Methods

Mice

C57BL/6- and BALB/c-background IL-10−/− mice were bred at specific pathogen-free conditions in our animal facilities, and these animals do not develop colitis until 4–6 mo of age under these conditions. These animals had been fully backcrossed to either strains (16). Age- and sex-matched C57BL/6 and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Female and male mice were 8–10 wk old when used. Females were used in most studies. There were three to five mice in each experimental group to study histological changes and five to seven mice in each experimental group to study mortality; four mice were used in each experimental group for detection of mRNA for IFN-γ. Each experiment was performed at least twice.

Infection with T. gondii

Cysts of the ME49 strain were obtained from brains of Swiss-Webster mice (Bantin and Kingman, Fremont, CA) that had been injected i.p. with 10 cysts for 2–3 mo as described previously (17). For peroral infection, C57BL/6-background IL-10−/− and control mice were infected with 20 cysts by gavage. BALB/c-background IL-10−/− and control mice were infected with either 20 or 100 cysts in the same manner.

Histopathology

Groups of three to five mice were euthanized by asphyxiation with CO2 at 9 days after peroral infection with the ME49 strain. Their brains, lungs, hearts, livers, spleens, and small and large intestines were removed and immediately fixed in a solution containing 10% Formalin, 70% ethanol, and 5% acetic acid. Two to four 5-μm-thick sections (50 or 100 μm distance between sections) of each organ from each mouse were stained with hematoxylin and eosin and by the immunoperoxidase method with rabbit anti-T. gondii IgG Ab (18). Sections stained with hematoxylin and eosin were evaluated for inflammatory changes and sections stained by the immunoperoxidase method were evaluated for the number of parasitophorous vacuoles containing T. gondii tachyzoites. Histological changes were consistent between individual mice in the same group and between sections from the same organ of each mouse. Each slide was evaluated by two investigators. The results of each investigator were essentially the same.

Detection of mRNA for IFN-γ in IEL and LPL

RNA was isolated from IEL and LPL using the RNA-STAT RNA extraction kit (Tel-Test B, Friendswood, TX) following the manufacturer’s protocol. The OD at 260 nm was used to estimate the concentration of total RNA. cDNA was synthesized using the RNA as described previously (26, 27). PCR for IFN-γ and β-actin was performed with 2.5 μl of the original cDNA reaction mixture with a GeneAmp 9600 thermocycler (PerkinElmer, Emeryville, CA) as described previously (26, 27). Specific primers for IFN-γ and β-actin (Clontech, Palo Alto, CA) designed to span at least one intron allowed differentiation of amplified target DNA derived either from cDNA or genomic DNA in the PCR.

Flow cytometry

One × 106 IEL and LPL were pretreated on ice for 10 min with 10 μl of a predetermined optimal concentration of anti-FcyRII/III receptors (2.4G2) to block non-Ag-specific binding of Abs to the FcγRII/III receptors. Thereafter, the cells were incubated on ice for 30 min with 10 μl of optimal concentrations of PE-conjugated anti-CD4 mAb (RM4-5) and FITC-conjugated anti-CD8 mAb (53–6.7). The mAbs were obtained from Pharmingen (San Diego, CA). Analysis of stained cells was performed with a FACScan (Becton Dickinson, Mountain View, CA). Dead cells were gated out on the basis of propidium iodide staining.

Statistical analysis

Levels of significance for amounts of mRNA for IFN-γ in LPL and IEL, length of ileum with necrosis, numbers of parasitophorous vacuoles containing tachyzoites in the ileum, and time to death of mice were determined using Student’s t, alternate Welch t, or Wilcoxon rank sum test. Alternative
Strain of mice. Necrosis was also observed in the large intestine in which large numbers of tachyzoites were detected in either control mice or normal IL-10−/− mice. These consisted of necrosis of hepatocytes as well as pulmonary infiltration of mononuclear cells. Nevertheless, the same changes were also observed although less frequently in infected control mice (data not shown).

Results

Mortality in C57BL/6-background IL-10−/− and control mice following a low-dose infection

IL-10−/− and control mice were infected perorally with 20 cysts of the ME49 strain. IL-10−/− mice all (12/12) died from 8 to 13 days after infection, whereas only 20% (2/10) of control mice died (p = 0.0001, Fig. 1). IL-10−/− mice appeared to be healthy until 6 days after infection; thereafter, they quickly developed piloerection, huddled, and lost mobility.

Comparison of histological changes in the organs of infected C57BL/6-background IL-10−/− and control mice

Because of the remarkable difference in mortality between IL-10−/− and control mice, we examined their brains, hearts, lungs, spleens, livers, small intestines, and large intestines at 9 days after infection to determine whether histological changes differ between infected IL-10−/− and control mice. The most pronounced histological differences between these strains of mice were observed in the small intestine. Severe necrosis of the villi and mucosal cells was observed in large areas of the small intestine, mostly in the ileum, in IL-10−/− mice (Figs. 2 and 3D). In contrast, such histological changes were not observed in either normal or infected control mice or normal IL-10−/− mice (Figs. 2 and 3, A–C). There was a tendency for IL-10−/− mice to show greater numbers of tachyzoites than did control mice; however, the difference did not reach statistical significance (the numbers of parasitophorous vacuoles/cm of ileum = 96.3 ± 104 for IL-10−/− vs 34.7 ± 26.8 for control animals; p = 0.058). The small intestine was the only organ in which large numbers of tachyzoites were detected in either strain of mice. Necrosis was also observed in the large intestine of infected IL-10−/− mice, although only a few tachyzoites were detected in this organ. Inflammatory changes were also observed in livers and lungs of IL-10−/− mice. These consisted of necrosis of hepatocytes as well as pulmonary infiltration of mono...
anti-IFN-γ mAb to neutralize activity of endogenous IFN-γ daily beginning at 6 days and the animals were necropsied for histopathologic examination at 9 days after infection. Severe necrosis was observed in large areas of the ileum of mice treated with control IgG but not in mice treated with anti-IFN-γ mAb ($p = 0.014$, Fig. 6). To examine whether IL-12 plays an important role in induction of the IFN-γ-mediated immunopathology, IL-10−/− mice were treated with anti-IL-12 mAb at 2 h before and 7 days after infection. Mice treated with anti-IL-12 did not develop intestinal pathology ($p = 0.014$, Fig. 6). In contrast, mice treated

FIGURE 3. Histological changes in the ileum of C57BL/6-background IL-10−/− and control mice following peroral infection with *T. gondii*. Mice were infected with 20 cysts of the ME49 strain and histological studies were performed on their small intestine at 9 days after infection. *A*, normal control mouse; *B*, infected control mouse; *C*, normal IL-10−/− mouse; *D*, infected IL-10−/− mouse. Hematoxylin and eosin stain.

FIGURE 4. Effect of depletion of CD4+ T cells on development of necrosis in the ileum of C57BL/6-background IL-10−/− mice following peroral infection with *T. gondii*. Mice were treated with anti-CD4 mAb and infected with 20 cysts of the ME49 strain. Control mice were treated with control IgG and infected with the ME49 strain. Histological studies were performed on their small intestine at 9 days after infection. Two sections of the entire length of small intestine from each mouse were examined. Four mice were used for each group. Essentially identical results were obtained in two independent experiments. Results are expressed as mean length of ileum with necrosis in centimeters.

FIGURE 5. Effect of depletion of CD4+ T cells on mortality in C57BL/6-background IL-10−/− mice infected perorally with *T. gondii*. Mice were treated with anti-CD4 mAb and infected with 20 cysts of the ME49 strain. Control mice were treated with control IgG and infected with the ME49 strain. Four mice were used for each group.
with anti-IL-12 mAb at 3 days after infection developed the pathology (data not shown). These results indicate that IL-12 plays a critical role in the induction of IFN-γ-mediated necrosis in the small intestine in IL-10−/− mice during the early stage of infection with T. gondii.

IL-10−/− mice were also treated with anti-IFN-γ mAb to examine its effect on mortality. For this purpose, mice were treated with anti-IFN-γ mAb daily for 5 days beginning 6 days after infection. Mice treated with anti-IFN-γ mAb survived significantly longer than mice treated with control IgG (p = 0.0002, Fig. 7). These results indicate that IFN-γ mediates development of necrosis in the small intestine in IL-10−/− mice following peroral infection with T. gondii and that this cytokine contributes to early death of the same animals.

Expression of IFN-γ in IEL and LPL of the small intestine in infected C57BL/6-background IL-10−/− and control mice

Since necrosis in the small intestine occurred in IL-10−/− mice but not in control C57BL/6 animals following peroral infection with 20 cysts of the ME49 strain and since the necrosis was found to be mediated by IFN-γ, we examined whether IFN-γ expression is accelerated in the small intestine of infected IL-10−/− mice. For this purpose, IEL and LPL were obtained from the small intestines of IL-10−/− and control mice at 7 days after infection. Significantly greater IFN-γ/β-actin mRNA ratios were detected in LPL obtained from infected IL-10−/− mice than that from infected control animals (p = 0.003, Fig. 8), whereas IFN-γ/β-actin mRNA ratios did not differ in IEL from these mice (p = 0.122, Fig. 8).

Large amounts of IL-10 mRNA were detected in both IEL and LPL of the control animals (data not shown). Both IEL and LPL obtained from the IL-10−/− animals had significantly higher rel-
C57BL/6 mice following a sublethal low-dose infection, we examined whether IL-10 is required for genetic resistance of BALB/c mice against infection. BALB/c-background IL-10<sup>−/−</sup> and control mice were infected perorally with 100 cysts of the ME49 strain. This is a dose in which genetically resistant BALB/c mice survive but susceptible C57BL/6 mice die during the acute stage (9, 13). BALB/c-background IL-10<sup>−/−</sup> animals all died from 9 to 11 days after infection whereas control animals all survived (Fig. 9A). To examine whether the IL-10<sup>−/−</sup> mice are able to survive when infected with fewer cysts, they were infected perorally with 20 cysts. The IL-10<sup>−/−</sup> animals again all died from 9 to 11 days after infection (Fig. 9B). Control mice all survived (Fig. 9B). Of interest, the BALB/c-background IL-10<sup>−/−</sup> mice died at the same time as did C57BL/6-background IL-10<sup>−/−</sup> mice infected in parallel with the BALB/c-background mice (Fig. 9B). These results indicate that genetic resistance of BALB/c mice against mortality following peroral infection with <i>T. gondii</i> requires IL-10.

Histological studies were performed on brains, lungs, hearts, livers, spleens, and small and large intestines of BALB/c-background IL-10<sup>−/−</sup> and control mice at 9 days after infection with 100 cysts. The most remarkable differences in histological changes in these animals were observed in the small intestine. Severe focal necrosis of tissues was detected in the small intestine of only IL-10<sup>−/−</sup> mice. Necrotic areas were also detectable in some areas of the large intestine of these animals, and the intestines were the only organs in which such pathology was observed.

**Effect of treatment with anti-IFN-γ mAb on development of necrosis in the small intestine and time to death in infected BALB/c-background IL-10<sup>−/−</sup> mice**

To examine whether IFN-γ contributes to the intestinal pathology and mortality in BALB/c-background IL-10<sup>−/−</sup> mice following infection, the IL-10<sup>−/−</sup> animals were injected with 2 mg of anti-IFN-γ mAb daily beginning 5 days after infection with 100 cysts. Animals treated with anti-IFN-γ mAb survived significantly longer than those treated with control IgG (<i>p</i> < 0.001, Fig. 10). Histological studies were performed on their small intestines at 9 days after infection. Numbers of areas of focal necrotic regions were significantly less in the animals treated with anti-IFN-γ mAb than those treated with control IgG (<i>p</i> = 0.021, Fig. 11). These results indicate that IFN-γ plays a critical role in development of the intestinal pathology and mortality in BALB/c-background IL-10<sup>−/−</sup> mice following peroral infection with <i>T. gondii</i>.

**Discussion**

The present study reveals a requirement for IL-10 in the prevention of intestinal pathology and mortality in both genetically resistant BALB/c and susceptible C57BL/6 mice following peroral infection with <i>T. gondii</i>. Infection with a low dose of <i>T. gondii</i> cysts resulted in 100% mortality in either strain of animals deficient in IL-10 but not in control animals. In each strain of the deficient animals, the intestine was the organ that had the most severe histological changes. This is the first evidence for the importance of IL-10 in resistance against infection with <i>T. gondii</i> through the natural route of infection and for the function of this cytokine in the protection against infection.
The genetic regulation of this process. Our results imply that IL-10 may also be a critical determinant of host resistance to toxoplasmosis in humans.

In contrast to remarkable differences in mortality and intestinal pathology between infected IL-10 /– and control mice, there were no differences in the numbers of tachyzoites detected in the small intestines in these animals. This evidence argues that IL-10 is not directly controlling parasite growth but regulating the immune response induced. This possibility is supported by the evidence that neutralization of IFN-γ by treatment with anti-IFN-γ mAb inhibited intestinal pathology and prolonged time to death in both BALB/c- and C57BL/6-background IL-10 /– mice following infection. Thus, IFN-γ mediates the pathology and contributes to mortality in both strains of IL-10 /– mice. Since the intestinal lesions were the most severe histological changes in these animals, the IFN-γ-mediated pathology appears to contribute to their mortality. Studies using RT-PCR revealed significantly greater IFN-γ/β-actin mRNA ratios in LPL obtained from the small intestine of infected C57BL/6-background IL-10 /– than control mice, strongly suggesting that IL-10 plays a crucial role in down-regulation of IFN-γ production by the LPL and in prevention of IFN-γ-mediated pathology in the intestine following peroral infection with T. gondii. This is further supported by our finding of enhanced expression of IL-10 mRNA by LPL of infected control mice. Of interest, the IFN-γ/β-actin mRNA ratios did not differ in IEL from the small intestine of infected IL-10 /– and control mice. Thus, the role of IL-10 in down-regulation of IFN-γ production may differ between IEL and LPL following infection or it may act at different time points in the LPL. Since the numbers of LPL obtained from the small intestines of infected IL-10 /– mice were similar to those of IEL (6–8 × 10⁶ cells/mouse on average), and since IFN-γ/β-actin mRNA ratios were significantly up-regulated in LPL as mentioned above, overproduction of IFN-γ by LPL appears to contribute to their intestinal pathology although the cytokine produced by IEL may also partially participate in the pathology.

IFN-γ has been shown to be critical for prevention of tachyzoite proliferation and mortality in animals infected with T. gondii (11, 12, 21). Our observation of the importance of down-regulation of IFN-γ production by IL-10 indicates that both the induction of IFN-γ and its regulation by IL-10 are key elements in host resistance to this parasite.

Depletion of CD4⁺ T cells by treatment with anti-CD4 mAb was shown to prevent intestinal necrosis and early mortality in infected IL-10 /– mice. Since IFN-γ, most likely produced by LPL, was demonstrated to mediate the pathology and mortality in the infected IL-10 /– animals in the present study and since the relative percentages of CD4⁺ T cells increased in their intestinal lymphocyte populations, IFN-γ production by CD4⁺ T cells in LPL appears to be an important factor which contributes to both pathologic sequelae. CD4⁺ T cells may also play an indirect role by stimulating CD8⁺ T cells to produce IFN-γ. However, CD8⁺ T cells do not seem to play a major role in the intestinal pathology since depletion of CD8⁺ T cells did not prevent the pathology in our previous study using lethally infected C57BL/6 mice (13). Furthermore, the present study demonstrates that the development of intestinal pathology in infected IL-10 /– mice is IL-12 dependent. Since IL-12 is a potent inducer of Th1-type cytokine production by CD4⁺ T cells (29–31), IL-12-mediated IFN-γ production by intestinal CD4⁺ T cells is likely to be a critical determinant of the marked susceptibility of IL-10 /– mice to peroral infection with T. gondii. NK cells may also contribute partially to their pathology and mortality since it has been reported that T. gondii infection induces IL-12-dependent IFN-γ production by NK cells (32, 33).

Buzoni-Gatel et al. (34) have recently reported that CD8⁺ IEL produce IFN-γ and play a protective role against peroral infection with T. gondii. Our findings suggest that in the case of the small intestine, CD4⁺ T cells in LPL are likely the major source of the IFN-γ production responsible for disease induction in T. gondii-infected IL-10 /– mice. Whether the same CD4⁺ LPL population plays a role in IFN-γ-dependent control of parasite growth in the intestine in the presence of IL-10 remains to be established.

IL-10 /– mice have been shown to spontaneously develop chronic enterocolitis (35). This colitis is mediated by IFN-γ produced by CD4⁺ Th1-type T cells of LPL. (16, 36, 37). Normal intestinal microflora is suggested to play a causal role in induction of the detrimental Th1-type immune response in the colon of the animals (35). The present study suggests that IFN-γ production by CD4⁺ T cells of LPL induces necrosis in the small intestine in T. gondii-infected IL-10 /– mice. Thus, CD4⁺ T cells appear to be the major source of this cytokine in LPL during the pathogenic response to two highly distinct microorganisms.

Control C57BL/6 mice die during the acute stage of infection when infected with a high dose (100) of T. gondii cysts because of their genetic susceptibility (9, 13). We previously reported that CD4⁺ T cell-dependent IFN-γ-mediated necrosis in the small intestine occurs in these mice following lethal infection (13). This is in contrast to the consequences of sublethal infection described in the present study in which control C57BL/6 mice were able to prevent pathology through a down-regulatory effect of IL-10 on IFN-γ production. Thus, it appears that the role of IL-10 in the pathogenesis of acute toxoplasmosis differs depending on the dose of infection in genetically susceptible C57BL/6 mice. It may be that lethal infection causes a suppression of IL-10 production in the susceptible animals, resulting in the same pathologic consequences observed in IL-10 /– mice. Alternatively, the lethal (high) dose infection may induce a particularly strong and early IFN-γ response that cannot be down-regulated by the ensuing IL-10 response.

**FIGURE 11.** Effect of treatment with anti-IFN-γ mAb on development of necrosis in the ileum of BALB/c-background IL-10 /– mice following peroral infection with T. gondii. Mice were injected i.p. with 2 mg of anti-IFN-γ mAb 5 days after infection. Control mice were injected with 2 mg of control IgG in the same manner. Histological studies were performed on their small intestine at 9 days after infection. Two sections of the entire length of small intestine from each mouse were examined. Four mice were used for each group. Essentially identical results were obtained in two independent experiments for each study.

![Image](http://www.jimmunol.org/)
When necrosis of the small intestine occurred in infected IL-10−/− mice, large numbers of tachyzoites were detected only in that organ. In previous studies using i.p. infection, necrosis in the liver was noted as a major pathologic correlate of mortality in these mice (14). In recent work, we have observed large numbers of the parasite in the liver but not in the intestine following i.p. infection (our unpublished data). This evidence strongly argues that the regulation of IFN-γ-mediated regional immune responses in the target organ(s) where large numbers of tachyzoites proliferate is critical for determining susceptibility to infection. The organs involved appear to differ depending on the route and the stage of infection.

The data from the present study strongly suggest a crucial role of IL-10 in preventing the IFN-γ-mediated protective immune response from becoming pathogenic during the acute stage of T. gondii infection initiated by the peroral route. Thus, resistance against T. gondii requires the development of a finely tuned balance between the host protective and host detrimental effects of the regional Th1-immune response. It is of interest that there were no differences in time to death between BALB/c and C57BL/6-background IL-10−/− mice following infection, although control BALB/c and C57BL/6 mice markedly differ in their susceptibility to development of intestinal pathology (13) and mortality (9, 13) after infection. Thus, IL-10 is an essential determinant of host resistance to T. gondii, and in its absence the genetic regulation of this process is overridden.

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