Mechanisms of IFN-γ-mediated Resistance against Development of Toxoplasmic Encephalitis

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(Abstract)

Toxoplasma gondii, an obligate intracellular protozoan parasite, establishes a latent, chronic infection by forming cysts preferentially in the brain after replication of tachyzoites in various organs during the acute stage of infection. Chronic infection with T. gondii is one of the most common parasitic diseases in humans. The immune system is required for maintaining the latency of chronic infection. Reactivation of infection can occur in immunocompromised individuals, such as AIDS patients, which results in the development of life-threatening toxoplasmic encephalitis (TE). IFN-γ-dependent, cell mediated immune responses play an essential role in preventing the reactivation of chronic infection of T. gondii in the brain. In my dissertation study, we examined the mechanisms of IFN-γ-mediated prevention of TE by using models of reactivation of chronic infection in BALB/c mice. This strain of mouse is genetically resistant to T. gondii infection and establishes a latent chronic infection as do immunocompetent humans, and therefore provides an excellent model for this purpose.

Our laboratory previously demonstrated that both T cells and IFN-γ-producing non-T cells are required for genetic resistance of BALB/c mice against development of TE. However, the function of T cells required for the resistance is still unclear. Therefore, in the present study, we examined whether IFN-γ production or perforin-mediated cytotoxicity of T cells play an important role in their protective activity against TE. Immune T cells were obtained from infected IFN-γ-knockout (IFN-γ−/−), perforin-knockout (PO), and wild-type (WT) BALB/c mice, and transferred into infected, sulfadiazine-treated athymic nude mice which lack T cells but have IFN-γ-producing non-T cells. Control nude mice that had not received any T cells developed severe TE due to reactivation of infection and died after discontinuation of sulfadiazine treatment. Animals that had received immune T cells from either PO or WT mice did not develop TE and survived. In contrast, nude mice that had received immune T cells from IFN-γ−/− mice developed severe TE and died as early as control nude mice. T cells obtained from
spleens of the animals that had received either PO or WT T cells both produced large amounts of IFN-γ following stimulation with *T. gondii* antigens *in vitro*. In addition, the amounts of IFN-γ mRNA expressed in the brains of PO T-cell recipients did not differ from those of WT T-cell recipients. These results indicate that IFN-γ production, but not perforin-mediated cytotoxic activity, by T cells is required for prevention of TE in genetically resistant BALB/c mice.

In our attempt to identify a T cell population(s) that produces IFN-γ in the brain and plays an important role for prevention of TE, we analyzed T cell receptor (TCR) Vβ chain usage in T cells expressing IFN-γ in the brains of infected BALB/c mice. We found T cells bearing TCR Vβ8 chain to be the most frequent IFN-γ-producing population in the brains of infected animals. To examine the role of IFN-γ production by this T cell population for prevention of TE, Vβ8+ immune T cells purified from spleens of infected BALB/c and IFN-γ−/− mice were transferred into infected, sulfadiazine-treated athymic nude mice. After discontinuation of sulfadiazine treatment, control nude mice that had not received any T cells and animals that had received Vβ8+ T cells from IFN-γ−/− mice all died due to reactivation of infection (TE). In contrast, animals that had received the cells from WT mice survived. These results indicate that IFN-γ production by Vβ8+ T cells in the absence of any other T cell population can prevent reactivation of infection. Thus, Vβ8+ T cells play a crucial role in genetic resistance of BALB/c mice to TE through their production of IFN-γ. When Vβ8+ immune T cells were divided into CD4+ and CD8+ subsets, a potent protective activity was observed only in the CD8+ subset whereas a combination of both subsets provided greater protection than did the CD8−Vβ8+ population alone. These results indicate that CD8+ subset of Vβ8+ T cells is a major afferent limb of IFN-γ-mediated resistance of BALB/c mice against TE, although the CD4+ subset of the T cell population works additively or synergistically with the CD8−Vβ8+ population.

T cells need to enter into the brains of infected mice to demonstrate their protective activity against TE. This migration is mediated, in part, by endothelial adhesion molecules. Since IFN-γ is essential for preventing reactivation of chronic infection with this parasite in the brain, we examined whether this cytokine plays an
important role in expression of lymphocyte and endothelial adhesion molecules and recruitment of T cells into the brain during chronic infection with *T. gondii* using IFN-γ−/− and WT BALB/c mice. Although the number of cerebral vessels expressing intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) increased in both WT and IFN-γ−/− mice following infection, there were more VCAM-1+ vessels in brains of infected WT than infected IFN-γ−/− mice; in contrast, numbers of ICAM-1+ vessels did not differ between strains. We did not detect endothelial E-selectin, P-selectin, MAdCAM-1 or PNAd in any of the brains. Significantly fewer CD8+ T cells were recruited into brains of infected IFN-γ−/− than WT mice. Treatment of infected IFN-γ−/− mice with recombinant IFN-γ restored the expression of VCAM-1 on their cerebral vessels and recruitment of CD8+ T cells into their brains, confirming an importance of this cytokine for up-regulation of VCAM-1 expression and CD8+ T cell trafficking. In infected WT and IFN-γ−/− animals, almost all cerebral CD8+ T cells had an effector/memory phenotype (LFA-1high, CD44high and CD62Lneg) and approximately 38% were positive for α4β1 integrin (the ligand for VCAM-1). In adoptive transfer of immune spleen cells, pre-treatment of the cells with a monoclonal antibody against α4 integrin markedly inhibited recruitment of CD8+ T cells into the brain of chronically infected wild-type mice. These results indicate that IFN-γ-induced expression of endothelial VCAM-1 and its binding to α4β1 integrin on CD8+ T cells is important for recruitment of the T cells into the brain during the chronic stage of *T. gondii* infection. Since we found strong expression of ICAM-1 on endothelia and LFA-1 on T cells in the brains of infected mice, LFA-1/ICAM-1 interaction, in addition to α4β1 integrin/VCAM-1 interaction, may also be involved in this process. As mentioned earlier, CD8+ T cells are crucial for prevention of TE in BALB/c mice. Therefore, IFN-γ-mediated expression of VCAM-1 and its binding to α4β1 integrin for recruitment of CD8+ T cells may play a critical role in genetic resistance of BALB/c mice to development of TE.
DEDICATION

I would like to dedicate this work to my mother, my brothers and my wife in thanks for all of their love and support.
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ATTRIBUTION

I sincerely appreciate the contribution and assistance from each co-author to my studies published. The role of co-authors in each publication is described below.

Chapter 1:
Dr. Hoil Kang: He contributed to some of histological studies.
Dr. Takane Kikuchi: She contributed to semi-quantification of IFN-γ mRNA expressed in the brains of mice.

Chapter 2:
Ms. Jennifer Claflin: She contributed to intracellular staining for IFN-γ on mononuclear cells isolated from the brains of mice.
Dr. Hoil Kang: He contributed to a histological study.

Chapter 3:
Dr. Sara A. Michie: She contributed to quantification of cerebral vessels expressing endothelial adhesion molecules.
Dr. Baohui Xu: He also contributed to quantification of cerebral vessels expressing endothelial adhesion molecules.
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Chapter 1

General introduction

Infection with *Toxoplasma gondii* is ubiquitously observed in humans and animals worldwide. During the acute stage of the infection, tachyzoites quickly proliferate within a variety of nucleated cells in various tissues. In immunocompetent individuals, this stage of infection is usually unnoticed or a benign, self-limiting illness. Following the acute stage, *T. gondii* forms cysts (dormant stage) preferentially in the brain, and establishes a chronic infection. Up to $5 \times 10^8$ people worldwide are considered to be chronically infected with this parasite (1). In immunocompromised individuals, such as AIDS patients, the latent infection can be reactivated, which is initiated by disruption of cysts, then followed by proliferation of tachyzoites. Such reactivation of *T. gondii* infection results in development of life-threatening toxoplasmic encephalitis (TE). Therefore, immune system is required to control chronic infection in the brain and to prevent the development of TE. However, the mechanisms how the immune system functions to prevent reactivation of infection still need to be elucidated.

Murine models have mainly been used to analyze the mechanisms of host resistance to development of TE (2-18). In mice, resistance to TE is under genetic control (5, 19, 20). Strains susceptible to TE (e.g., C57BL/6 and CBA/Ca) develop progressive, and ultimately fatal encephalitis without immunosuppressive treatment (5, 19, 20). In contrast, strains resistant to TE (e.g., BALB/c) are able to control *T. gondii* infection in their brains and establish a latent chronic infection (5, 19, 20), as do immunocompetent humans. Therefore, genetically resistant mouse strains appear to provide an excellent model to analyze the mechanisms how the immune system maintains the latency of chronic infection and prevents development of TE.

By using the TE-resistant BALB/c mouse model, our laboratory previously reported a requirement of IFN-$\gamma$ for their resistance to TE (4, 21). Both T cells and IFN-$\gamma$-producing non-T cells are required for their resistance to the disease (4). In this dissertation, we further examined 1) the function of T cells required for their protective activity, 2) T cell subpopulations important for the IFN-$\gamma$ production and prevention of TE and 3) role of IFN-$\gamma$ in T cell recruitment into the brain following infection.
REFERENCES


Chapter 2
Literature Review

Life cycle of *Toxoplasma gondii*

*Toxoplasma gondii*, an obligate intracellular protozoan parasite, is a globally distributed pathogen which can infect most warm-blooded vertebrates and cause diseases in agricultural animals and humans (1, 2). This parasite is among the most prevalent chronic parasitic infection in humans, infecting from 10% to 25% of the world’s population (2). Although *T. gondii* was first observed in rodents by Nicolle and Manceaux in 1908, the life cycle of this parasite had not been defined until the family Felidae, including domestic cats, was discovered as the definitive host in 1970. *T. gondii* has three stages in its life cycle: the tachyzoites, the bradyzoites (in cysts), and the sporozoites (in oocysts). Cats usually become infected with *T. gondii* by ingestion of cysts present in the organs/tissues of a chronically infected intermediate host. The cyst wall is digested by the proteolytic enzymes in the stomach and intestine, which results in releasing bradyzoites. Bradyzoites penetrate the epithelial cells of the small intestine in the cat. Sexual stages of reproduction of *T. gondii* occur within intestinal epithelial cells and oocysts are formed and excreted in the feces. Oocysts undergo sporulation outside of the body to become infective to other hosts. Sporulated oocysts are very resistant to harsh environment and can survive in soil for several months. Intermediate hosts can be infected by ingestion of food and water contaminated with oocysts or undercooked meat of infected food animals containing tissue cysts. After penetration of sporozoites or bradyzoites into the epithelial cells in the small intestine, the parasite transforms into tachyzoites, tachyzoites then quickly proliferate and disseminate to various tissues. Tachyzoites are able to enter any type of nucleated host cells and multiply within parasitophorous vacuoles. When the infected host cells rupture due to proliferation of the parasite, the released tachyzoites enter new host cells nearby and multiply. Tachyzoites cause pathologic changes if not controlled by the immune system. The IFN-γ-dependent, cell-mediated immune responses play an essential role to control the tachyzoite growth although humoral immunity also contributes to the resistance. Following this acute phase of infection, the parasite is driven to enter the “dormant stage” as bradyzoites
which multiply slowly within tissue cysts. Tissue cysts are formed preferentially in the brain and skeletal muscles, and considered to persist for the life of the host without causing host reaction. This stage of parasite is harbored in different intermediate hosts as a reservoir of *T. gondii* in environment and as potential transmission sources. (The life cycle of *T. gondii* is reviewed from references (3, 4))

**Toxoplastic encephalitis (TE)**

Although tissue cysts stay in the host as a dormant stage, the chronic infection of *T. gondii* could be reactivated when the immune system of the host is compromised. This reactivation is initiated by disruption of cysts, followed by proliferation of tachyzoites and most often results in life-threatening toxoplastic encephalitis (TE). For example, in AIDS patients, TE occurs almost always in individuals with pre-existing antibodies to *T. gondii* (5-7), indicating the development of encephalitis is due to recrudescence of a latent infection. TE has emerged as a major opportunistic infectious disease in the central nervous system of AIDS patients (8). This disease also occurs in non-AIDS immunocompromised patients, such as organ transplant recipients with immunosuppressive therapy (9, 10). On the other hand, immunocompetent individuals are able to maintain latency of chronic infection of *T. gondii* in the brain. Therefore, the immune response is able to prevent reactivation of chronic infection (TE). However, the mechanisms of immune system to control this parasite in the brain during the chronic stage of infection are yet well understood.

**Animal models**

Murine models have been widely used to analyze the mechanisms of host resistance against the development of TE during the late stage of infection (11-27). Resistance to development of TE in mice is under genetic control (14, 28, 29) and strains with the H-2^b^ or H-2^k^ haplotypes (e.g., C57BL/6 and CBA/Ca respectively) are susceptible to TE whereas strains with H-2^d^ haplotype (e.g., BALB/c) are resistant to the disease. Susceptible strains of mice develop progressive, and ultimately fatal encephalitis following infection without any immunosuppressive treatment (14, 28, 29). In contrast, resistant strains are able to control *T. gondii* infection in their brains and establish a latent
chronic infection (14, 28, 29), as do immunocompetent humans. Therefore, genetically resistant strains of mice apparently are a more suitable model for analyzing the mechanisms of host resistance that maintain the latency of a chronic infection and prevent the development of TE.

**Central role of IFN-γ**

It is well documented that IFN-γ-dependent, cell-mediated immune responses are essential for prevention of TE (13, 15, 25, 26, 30), although humoral immunity is also involved (12, 31-33). Neutralization of the activity of IFN-γ by treatment with anti-IFN-γ monoclonal antibody in chronically infected mice results in development of severe encephalitis, which is associated with proliferation of tachyzoites in the brain (15), indicating the absolute requirement of IFN-γ for prevention of TE during the late stage of infection (15, 30). Multiple cell types, such as T cells, NK cells, microglia and blood-derived macrophages have been identified as the source of IFN-γ in the brains of chronically infected mice. Multiple effector mechanisms are also induced by IFN-γ to control *T. gondii* in the brain.

**Producers of IFN-γ**

**T cells** T cells are recruited into the brains of mice following infection with *T. gondii*. Simultaneously depletion of CD4+ and CD8+ T cells is required to induce severe TE in chronically infected mice (30). Brown and Mcleod reported that CD8+ T cells are involved in the resistance by regulating the numbers of *T. gondii* cysts in the brains of mice (34). In an adoptive cell transfer study, both CD4+ T cells and CD8+ T cells displayed abilities to prevent the reactivation of infection (TE) in the recipient mice (13). We determined that IFN-γ production is the function of T cells required for this protective activity against TE in genetically resistant BALB/c mice ((25); this is part of my studies in this dissertation). When T cell receptor (TCR) Vβ chain usage was examined in the T cells that produce IFN-γ in the brains of infected BALB/c mice, the cells bearing TCR Vβ8 chain were found to be the most frequent IFN-γ-producing population ((26); this is part of my studies). In addition, splenic Vβ8+ T cells produced
significantly greater amounts of IFN-γ than did Vβ8+ T cell population following stimulation with tachyzoite antigen *in vitro* (11). Adoptive transfer of Vβ8+ immune T cells, obtained from infected wild-type BALB/c mice prevented development of TE in infected athymic nude mice, which lack T cells (11), indicating that Vβ8+ T cells play a crucial role in the genetic resistance of BALB/c mice against development of TE. Vβ8+ immune T cells from infected IFN-γ−/− mice did not confer any protective activity to recipient nude mice, therefore the protective activity of Vβ8+ T cells is mediated by IFN-γ (26). When Vβ8+ immune T cells were divided into CD4+ and CD8+ subsets, CD8+Vβ8+ T cells conferred much greater resistance to development of TE than did the CD4+Vβ8+ T cells, but the protective activity of CD8+Vβ8+ T cells was less than that of the total Vβ8+ T cell (26). Therefore, the CD8+ subset of Vβ8+ T cells plays a major role in resistance against the development of TE although CD4+ subset works additively or synergistically with the CD8+ subset.

In regard to cytotoxic activity of T cells, murine CD8+ T cells are able to kill *T. gondii*-infected target cells *in vitro* (35-39) in a major histocompatibility complex (MHC)-restricted manner (37). However, in vivo studies have showed that cytotoxic activity of T cells is not critical for resistance against *T. gondii* infection in mice (25, 40). By using C57BL/6 (genetically susceptible to TE)-background mice, Denker *et al* (40) reported that approximately half of the perforin-deficient animals survived until 150 days after infection whereas CD8+ T cell-deficient mice all died by 50 days after infection, suggesting that perforin-mediated cytolysis by T cells appears to play a limited role in resistance against *T. gondii*. More recently, we ((25); this is part of my dissertation) demonstrated, by using TE-resistant BALB/c mice, that adoptive transfer of immune T cells from perforin-deficient mice into infected nude mice prevented reactivation of infection and development of TE in the recipient mice and all the recipients survived. Therefore, perforin-mediated cytotoxic activity of T cells is not required for genetic resistance of BALB/c mice to development of the disease.

**NK cells** Although NK cells are known primarily for their ability to mediate non-MHC-restricted cytotoxicity against tumor cells, this cell population has also been
recognized to play a role in host resistance to infection through their production of IFN-γ. Sher et al reported that live tachyzoites as well as parasite extracts of *T. gondii* are potent stimulators of IFN-γ production by NK cells (41). IL-12 is required to initiate IFN-γ synthesis by NK cells, and other cytokines, such as tumor necrosis factor (TNF)-α, IL-1β, and IL-15 are also involved in collaboration with IL-12 to potentiate their IFN-γ production (42, 43). *In vivo* studies have shown that IFN-γ production by NK cells is important for resistance against *T. gondii* infection in mice during the early stage of infection (44, 45). However, in the later stage of infection, NK cells do not appear to be critical for the prevention of TE. Kang *et al* reported that depletion of NK cells did not abolish resistance to development of TE in SCID mice that had received adoptive transfer of immune T cells (13). More recently, our study demonstrated that perforin knock out mice did not develop TE and all survived, as did wild-type animals, for 6 months after infection with *T. gondii*, indicating that the perforin-mediated cytotoxic activity of not only T, but also NK, cells is not required for prevention of TE (25).

**Microglia and macrophages as innate producers of IFN-γ**  
T cells play an essential role to control the chronic infection in the brain and prevent the development of TE (13, 30, 34). As mentioned earlier, the protective activity of T cells is mediated by their IFN-γ production (25). However, in addition to T cells, IFN-γ production by cells other than T cells is also required for resistance against this disease in mice (13). The IFN-γ-producing non-T cells do not appear to be NK cells (13). In relation to this, Suzuki *et al* (27) recently demonstrated the identity of non-T, non-NK cells that produce IFN-γ in the brains of nude and SCID mice chronically infected with *T. gondii*. Flow cytometry analysis with immunostaining for IFN-γ revealed that approximately 45-60% of the cells expressing IFN-γ in their brains were positive for CD11b or F4/80 (surface marker for microglia/macrophages). Also large amounts of mRNA for IFN-γ were detected in CD11b+ cells purified from brains of infected mice, but not in the cells obtained from uninfected animals. In the brains of infected SCID mice depleted of NK cells, cells expressing IFN-γ were all positive for CD11b, and the IFN-γ-producing cells were detected in both CD45low and CD45high populations. Taken together, these results
suggest that CD11b<sup>+</sup>CD45<sup>low</sup> microglia and CD11b<sup>+</sup>CD45<sup>high</sup> blood-derived macrophages are the major non-T, non-NK cells expressing IFN-γ in the brains of mice infected with *T. gondii*. It is possible that IFN-γ production by microglia and/or macrophages plays an important role in prevention of TE in collaboration with T cells.

**IFN-γ-mediated recruitment of T cells into the brain**

During primary infection with *T. gondii*, T cells are efficiently recruited into the brains of mice. With the establishment of chronic stage of infection, the numbers of intracerebral T cells are slowly decreased (46). Because T cells are required for prevention of reactivation of chronic infection in the brain, it is clear that T cells need to be present in the brain during the later stage of infection. However, it is unclear how the size of intracerebral T cell pool is regulated. Our recent study ((47); this is part of my dissertation) demonstrated that the continuous recruitment of small numbers of T cells into the brains of mice during chronic stage of infection, and that IFN-γ plays an important role for CD8<sup>+</sup> T cell recruitment into the brain. We also found that up-regulation of expression of vascular cell adhesion molecule-1 (VCAM-1), but not of intercellular cell adhesion molecule-1 (ICAM-1), on cerebral vessels is largely dependent on IFN-γ, and that IFN-γ-mediated expression of endothelial VCAM-1 in its interaction with α4β1 integrin plays an important role in recruitment of CD8<sup>+</sup> T cells into the brains of chronically infected mice (47).

**IFN-γ-mediated effector mechanisms**

**NO production** IFN-γ induces expression of inducible nitric oxide synthase (iNOS) which produces NO (48). Murine peritoneal macrophages become activated after treatment with combination of IFN-γ and TNF-α in *vitro* and the activated cells inhibit tachyzoite growth through generation of NO by iNOS (49). However, *in vivo* studies have shown that mice lacking TNF receptor type 1 (R1) and type 2 (R2) and mice lacking iNOS is able to control the growth of the parasite in the peritoneal cavity following intraperitoneal infection, indicating that TNF-α and iNOS are not essential to control acute infection (18, 19, 21). In contrast to acute stage of infection, mice deficient in TNF R1/R2 or iNOS succumbed to necrotizing TE during the late stage of infection (18, 19,
Treatment of chronic infected wild-type mice with anti-TNF-α mAb or aminoguanidine (an inhibitor of iNOS activity) resulted in development of TE (17, 20). These studies imply that TNF-α and iNOS are critical for maintaining the latency of chronic infection with *T. gondii* in the brain. Gazzinelli *et al* reported that neutralization of IFN-γ or TNF-α in chronic infected mice resulted in decreased iNOS expression and development of severe TE (17), suggesting that activation of iNOS is mediated by IFN-γ and TNF-α for prevention of TE.

Microglia are the resident macrophage population in the central nervous system and appear to be the major effector cells in the brain to control the proliferation of *T. gondii* tachyzoites. With treatment of IFN-γ plus lipopolysaccharide (LPS) *in vitro*, murine microglia become activated and inhibit intracellular proliferation of tachyzoites (50, 51). Blocking the generation of NO with N\(^\text{G}\)-monomethyl-L-arginine ablates their inhibitory activity, indicating that the inhibitory effects of activated microglia are mediated by NO. Freund *et al* (52) reported that an IFN-γ-dependent, NO-independent mechanism exists for inhibition of *T. gondii* replication *in vitro* in activated murine microglia. Human microglia also can be activated *in vitro* following treatment with IFN-γ plus LPS to inhibit intracellular tachyzoite growth (53). However, in contrast to murine cells, NO is not involved in the inhibitory effect of human microglia (53).

IFN-γ activated astrocytes can also inhibit the growth of *T. gondii* *in vitro*. Human astrocytes stimulated with IFN-γ and IL-1β inhibit tachyzoite replication through a NO mediated mechanism (54). However, the inhibitory activity of murine astrocytes activated by IFN-γ is not mediated by NO (55). The role of NO in microglia and astrocytes in resistance to *T. gondii* appears to be different between humans and mice.

**IGTP** IGTP is a member of the 47-kDa family of IFN-γ-inducible GTP-binding proteins (56, 57). Other family members include IRG-47, LRG-47, TGTP, IIGP and GTPI. Expression of these proteins is strongly upregulated by IFN-γ in both hematopoietic and nonhematopoietic cells (56, 58, 59). Three of these family members, IGTP, LRG-47, and IRG-47 have been shown to be important in resistance to *T. gondii* infection. Mice lacking IGTP or LRG-47 are unable to control *T. gondii* infection,
succumbing within 8 to 10 days after parasite inoculation despite the induction of a robust IL-12 induced IFN-γ response (60, 61). Therefore, IGTP and LRG-47 appear to play nonredundant roles in host defense against T. gondii and function downstream of IFN-γ in the signaling pathway of this cytokine. Additionally, IGTP and LRG-47 are able to regulate host resistance to acute T. gondii infections through their ability to inhibit parasite growth within the macrophage (62). However, IRG-47 deficient mice displayed only partially decreased resistance to acute infection with T. gondii, which was not manifested until the chronic stage (60). Thus, these three IFN-γ-inducible GTP-binding proteins appear to have vital, but distinct role in immune response against T. gondii infection. The function of IGTP in host resistance to acute infection with T. gondii is STAT1 dependent and requires the expression of this protein in both hematopoietic and nonhematopoietic cellular compartments (63). This finding implies a direct role for IGTP in the IFN-γ-triggered mechanism that leads to intracellular control of the parasite in multiply cell types and tissues. Halonen et al reported that astrocytes from IGTP knock out mice, failed to limit tachyzoite growth in vitro after stimulation with IFN-γ, indicating IGTP plays a central role in the IFN-γ-induced inhibition of the parasite replication in murine astrocytes (64). However, in vivo studies demonstrated that IGTP knock out mice showed a slightly increased mortality during the late stage of infection when they received microbicidal treatment to establish a chronic infection (63).

**Tryptophan degradation by indoleamine-2, 3-dioxygenase (IDO)** IDO is an IFN-γ-induced enzyme, which converts tryptophan to formylkynurenine and kynurenine in the initial rate-limiting step of tryptophan catabolism. Importance of IDO in IFN-γ-mediated inhibition of tachyzoite growth was described in various human cells, including fibroblast, endothelial cell, and epithelial cells (65-67). Regarding the role of IDO-mediated tryptophan starvation in resistance of the brain against T. gondii, Daubener et al reported that TNF-α and IFN-γ function synergistically in activating IDO in human glioblastoma cell lines (68) and human brain microvascular endothelia cell (69), and the activated cells display a strong toxoplasmostatic effect. This antiparasitic effect was completely blocked with addition of tryptophan to the culture medium (68, 69), suggesting that IFN-γ-mediated induction of IDO is critical for prevention of the parasite
growth in these human brain cells.

IFN-γ-dependent expression of IDO was also observed in the brains and lungs of mice during acute stage of infection (17, 70), suggesting an involvement of IDO and tryptophan degradation in resistance to the parasite. However, the role of IDO in resistance of murine astrocytes against *T. gondii* is unclear since addition of tryptophan had no effect on the inhibition of *T. gondii* growth in murine astrocytes stimulated with IFN-γ in combination with IL-1, IL-6, or TNF-α (55).

**Other cytokines involved in the resistance**

**IL-12**

Dendritic cells and macrophages are important producers of IL-12 in response to *T. gondii* (71). IL-12 is required for induction of IFN-γ-mediated resistance to this parasite during acute stage of infection. Treatment with neutralizing mAbs against IL-12 resulted in 100% mortality in mice after an inoculation of an avirulent strain of *T. gondii*, and their mortality is associated with a marked decrease in IFN-γ production (72). In regard to the role of IL-12 in resistance against chronic stage, Gazzinelli *et al* demonstrated that neutralization of endogenously produced IL-12 did not increase mortality in chronically infected mice (72). More recently, Yap *et al* reported an importance of IL-12 for maintenance of IFN-γ production in T cells for resistance to the chronic infection (73). In this study, IL-12 p40-deficient mice were treated with recombinant to IL-12 during the first 2 weeks of infection with *T. gondii* to establish a chronic infection. Four to 6 weeks after IL-12 withdrawal, the mice exhibited increased brain cyst burdens and succumbed to TE in association with a loss of T cell-dependent IFN-γ production. Therefore, IL-12 appears to be required for the long-term maintenance of IFN-γ-mediated resistance against *T. gondii*.

**IL-4**

CD4+ T cells differentiate to effector Th cells that secrete cytokines coordinating appropriate immune response against various infectious pathogens. The Th cells exhibit characteristic cytokine secretion patterns, which divide them into at least two distinct
subsets (74-77), Th1 and Th2. Th1 cells preferentially produce IL-2 and IFN-$\gamma$ whereas Th2 cells preferentially secrete IL-4, IL-5, IL-6, and IL-10.

IFN-$\gamma$ plays a central role in resistance against *T. gondii* as mentioned earlier. However, IL-4 appears to be involved in controlling the parasite. IL-4 deficient (IL-4$^{-/-}$) mice showed increased mortality when compared to control animals (22, 78, 79). Suzuki *et al* reported that IL-4$^{-/-}$ mice all died during late stage of infection and the mortality was associated with greater numbers of cysts and areas of acute focal inflammation associated with tachyzoites (22). These results indicate that IL-4 is protective against development of TE by preventing formation of cysts and proliferation of tachyzoites in the brain. They also observed that spleen cells from IL-4$^{-/-}$ mice at chronic stage of infection produced significantly less amounts of IFN-$\gamma$ than those of control mice following *in vitro* stimulation with soluble *T. gondii* antigen. Thus, IL-4 appears to play a role to enhance IFN-$\gamma$ production during the late stage of infection, and the impaired ability of IL-4$^{-/-}$ mice to produce IFN-$\gamma$ likely contributes to their susceptibility to development of severe TE (22). Different from these findings, Roberts *et al* reported a significantly higher mortality in IL-4$^{-/-}$ mice than that in wild-type animals during acute stage of infection. *In vitro* culture of spleen cells obtained from infected mice in this stage demonstrated increased IFN-$\gamma$ production in IL-4$^{-/-}$ mice when compared to wild-type animals (78). Therefore, they suggest that IL-4 plays a protective role in preventing mortality by down-regulating proinflammatory cytokine production during the acute stage of infection (78). The differences in the observations in these two articles may be due to the use of different genetic backgrounds of mice and/or the strains of *T. gondii* used in the experiments. In relation to this, genetic background of mice was shown to affect the outcome of IL-4$^{-/-}$ mice following infection with *T. gondii* (79).

**IL-5**

IL-5 is produced predominantly by activated CD4$^+$ T cells (80). This cytokine has several effects on B lymphocytes, such as enhancing IL-2 receptor expression and promoting proliferation and differentiation (81, 82). Zhang *et al* reported that IL-5 deficient (IL-5$^{-/-}$) mice show increased mortality during the late stage of infection and that their mortality is associated with greater numbers of cysts and tachyzoites in their brains.
when compared to infected control mice (83). They also found that in vitro production of IL-12 by spleen cells in response to T. gondii antigen was approximately one third in the IL-5<sup>−/−</sup> mice compared to wild-type controls, and that this decrease correlated with a selective loss of B lymphocytes (83). Therefore, IL-5 seems to play a protective role against T. gondii infection by enhancing the production of IL-12.

**IL-6**

IL-6 has been reported to play a protective role against development of TE by preventing formation of cysts and proliferation of tachyzoites in the brains of infected mice (23, 84). The protective activity of IL-6 appears to be through its ability to stimulate IFN-γ production and induce infiltration and accumulation of different T-cell subsets in brains of infected mice(23, 84). In vitro studies also demonstrated that human fetal microglia treated with IL-6 inhibited intracellular replication of tachyzoites (53) and that IL-6 acts synergistically with IFN-γ to inhibit proliferation of tachyzoites in murine astrocytes (55).

**IL-10**

IL-10 is an important negative regulator of inflammatory responses (85). IL-10 deficient (IL-10<sup>−/−</sup>) mice succumbed to acute stage of infection with T. gondii and their mortality was associated with development of severe immunopathology in the liver (86) and intestine (87), which was mediated by uncontrolled Th1 immune responses. Thus, IL-10 plays an important role to down-regulate IFN-γ-mediated immune response and prevent host immunopathology. Recently, Wilson et al demonstrated an important role of IL-10 in controlling CD4<sup>+</sup> T cell-mediated inflammation in the brains of mice during chronic stage of infection (88). Therefore, IL-10 is a critical down-regulatory cytokine to prevent development of immunopathology in both acute and chronic stage of infection with T. gondii in mice.

**Involvement of humoral immunity**

Although cell-mediated immunity plays the major role in resistance against T. gondii, humoral immunity is also involved in controlling the parasite (12, 31-33). Couper
et al demonstrated an importance of parasite-specific IgM in prevention of systemic dissemination of tachyzoites in early acute stage of infection with *T. gondii* (31). Sayles *et al* reported that B cells are required as antibody producers for vaccination-induced resistance to virulent tachyzoites (32). In the chronic stage of infection, B cells play an important role to prevent the development of TE through their production of anti-*T. gondii* antibodies (12). In relation to this, Johnson and Sayles reported that the survival of CD4-deficient mice chronically infected with *T. gondii* was significantly prolonged by administration of immune serum (33).

**Conclusions**

IFN-γ-dependent, cell-mediated immune responses play a central role in resistance against *T. gondii* in the brain. IFN-γ production by both T cells and non-T cells is required for the resistance. Microglia could be one of IFN-γ-producing non-T cells that are essential in the prevention of TE. Three effector mechanisms mediated by IFN-γ have been identified including NO production by iNOS, tryptophan starvation by IDO, and unknown mechanisms mediated by IGTP. IL-12 plays an important role to induce and maintain the IFN-γ production by T cells for prevention of TE. IL-10 also plays an important role in down-regulating immune responses to prevent development of immunopathology in both acute and chronic stage of infection. Other factors, such as genetic background of host and the strains of *T. gondii*, are also important to determine the susceptibility to the development of TE. More studies are needed to obtain better understanding of molecular basis of IFN-γ-mediated prevention of TE and immunopathogenesis of this disease.
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Chapter 3

IFN-$\gamma$ Production, but Not Perforin-Mediated Cytolytic Activity, of T Cells Is Required for Prevention of Toxoplasmic Encephalitis in BALB/c Mice Genetically Resistant to the Disease

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ABSTRACT

We previously showed the requirement of both T cells and IFN-γ-producing non-T cells for genetic resistance of BALB/c mice to development of toxoplasmic encephalitis (TE). In order to define the role of IFN-γ production and perforin-mediated cytotoxicity of T cells in the resistance, immune T cells were obtained from spleens of infected IFN-γ-knockout (IFN-γ⁻⁻), perforin-knockout (PO), and wild-type BALB/c mice, and transferred into infected and sulfadiazine-treated athymic nude mice which lack T cells but have IFN-γ-producing non-T cells. Control nude mice that had not received any T cells developed severe TE and died after discontinuation of sulfadiazine treatment, due to reactivation of infection. Animals that had received immune T cells from either wild-type or PO mice did not develop TE and survived. In contrast, nude mice that had received immune T cells from IFN-γ⁻⁻ mice developed severe TE and died as early as control nude mice. T cells obtained from spleens of the animals that had received either PO or wild-type T cells both produced large amounts of IFN-γ following stimulation with T. gondii antigens in vitro. In addition, the amounts of IFN-γ mRNA expressed in the brains of PO T-cell recipients did not differ from those of wild-type T-cell recipients. Furthermore, PO mice did not develop TE following infection, and their IFN-γ production was equivalent to or higher than that of wild-type animals. These results indicate that IFN-γ production, but not perforin-mediated cytotoxic activity, by T cells is required for prevention of TE in genetically resistant BALB/c mice.
INTRODUCTION

Toxoplasma gondii, an obligate intracellular protozoan parasite, forms cysts and establishes a latent, chronic infection preferentially in the brain after replication of tachyzoites in various organs during the acute stage of infection. Chronic infection with this parasite is likely the most common infection in humans. The requirement for the immune system to maintain the latency of persistent infection is clearly evident from the reactivation of the infection in immunocompromised individuals, that results in development of life-threatening toxoplasmic encephalitis (TE) (18, 44). Murine models have been used to analyze the mechanisms of host resistance to TE. C57BL/6 (H-2^b^) and CBA/Ca (H-2^k^) mice have often been used for these studies (9, 13, 16, 32, 46). However, these strains of mice are genetically susceptible and spontaneously develop progressive and ultimately fatal TE (4, 33, 34). In contrast to these susceptible strains, genetically resistant strains (e.g. BALB/c [H-2^d^]) are able to control T. gondii infection in their brains and develop a latent chronic infection as do immunocompetent humans (4, 33, 34). Therefore, these strains of mice appear to be a suitable model to analyze the mechanisms of host resistance to maintain a chronic infection and to prevent TE.

We have developed a murine model of reactivation of T. gondii infection in the brain using infected, sulfadiazine-treated BALB/c-background IFN-γ-knockout (IFN-γ^-/-^) and athymic nude mice (21, 35). Our recent studies using these animal models demonstrated the requirement of both T cells and IFN-γ-producing non-T cells in the brain for prevention of the reactivation of infection and development of TE (21). However, the function(s) of T cells important for the resistance still needs to be defined. Under collaboration with IFN-γ-producing non-T cells, the function(s) of T cells other than IFN-γ production may play a critical role in the resistance. Cytotoxic activity of T cells (15, 22, 31) may be a function crucial for prevention of TE. In relation to this, Denkers et al (10) reported that cytotoxic activity mediated by perforin plays a limited role in their resistance to T. gondii infection in C57BL/6 mice genetically susceptible to TE. However, it is possible that the cytotoxic activity of T cells plays a more critical role in prevention of TE in genetically resistant BALB/c than in susceptible C57BL/6 mice, hence BALB/c mice are resistant to TE. It is also possible that IFN-γ production by T
cells, in addition to IFN-γ production by the non-T cells, is required for prevention of TE since IFN-γ has been shown to play a central role in controlling *T. gondii* in the brain (12, 32, 35). Unrecognized function(s) of T cells may be critical for prevention of TE.

In the present study, we investigated the role of IFN-γ production and perforin-mediated cytotoxic activity of T cells in prevention of reactivation of chronic infection with *T. gondii* in the brain in genetically resistant BALB/c mice. We found that IFN-γ production by T cells is essential for the resistance to control the parasite in the brain and the cytotoxic activity is dispensable for their protective activity.

**MATERIALS AND METHODS**

**Mice.** Female BALB/c-background IFN-γ knockout, athymic nude and wild-type BALB/c mice were obtained from The Jackson Laboratories (Bar Harbor, Main). Female Swiss-Webster mice were from Taconic (Germantown, N.Y). BALB/c-background perforin knockout (PO) mice (43) were kindly provided by John T. Harty (University of Iowa, Iowa City, Iowa) and bred in our animal facility. Females of the animals were used for studies. All mice were housed in specific pathogen-free conditions, and were 8 to 12 weeks old when used. There were four to six mice for each experimental group.

**Infection with *T. gondii.*** Cysts of the ME49 strain were obtained from brains of Swiss-Webster mice that had been infected intraperitoneally with 10 cysts for 2 to 3 months. Mice were euthanized by asphyxiation with CO2, and their brains were removed and triturated in phosphate-buffered saline (pH 7.2) (37). An aliquot of the brain suspension was examined for numbers of cysts, and after appropriate dilution in phosphate-buffered saline, all animals were infected with 10 cysts perorally by gavage. Mice were treated with sulfadiazine in drinking water (400 mg/l) beginning 4 days (for IFN-γ-/- mice) or 7 days (for nude, PO and wild-type BALB/c mice) after infection for 3-4 weeks. In some experiments, PO and wild-type mice did not receive sulfadiazine after infection.

**Purification and transfer of immune T cells.** At 4 or 5 weeks after infection, spleen cells were obtained from 3 or 4 of IFN-γ-/-, PO and wild-type mice, suspended in
Hanks’ balanced salt solution (Irvine Scientific, Santa Ana, Calif.) with 2% of fetal bovine serum (Sigma Chemical Co, St. Louis, Mo.) and pooled within the same group of the animals. The total T cell population was purified by treating the spleen cells with magnetic beads-conjugated anti-mouse CD4 (GK1.5) plus anti-mouse CD8 (53-6.7) mAbs (Miltenyi, Sunnyvale, Calif.) (21). The purity of the T cells in each of the purified preparations was >95%. A total of $1 \times 10^7$ T cells were injected intravenously from a tail vein to recipient nude mice at 9 and 2 days before discontinuation of treatment with sulfadiazine.

**Culture of T and spleen cells, and detection of IFN-γ in the culture supernatants.** T cells were purified (as described above) from the spleens of nude mice that survived for 90 days after discontinuation of sulfadiazine. T cells were also purified from the spleens of PO and wild-type mice at 6 months after infection. Culture of the purified T and spleen cells with *T. gondii* Ags were performed as previously described (5). Briefly, the cells suspended in RPMI 1640 (Sigma Chemical Co.) with 10% fetal bovine serum (HyClone, Logan, Utah) and penicillin (100 U/ml), streptomycin (100 μg/ml) were placed into flat-bottom wells of 96-well plates (Corstar, Cambridge, Mass.) at a cell density of $4 \times 10^5$ per well and incubated with and without soluble *T. gondii* lysate antigens (20 μg/ml) in a final volume of 200 μl per well for 72 hours. Thereafter, the culture plates were centrifuged and the culture supernatants were collected. The concentration of IFN-γ in the culture supernatants was measured by ELISA using mAbs against IFN-γ (R4-6A2 as capture, XMG1.2 as secondary) obtained from PharMingen (San Diego, Calif.) as described previously (39).

**Histopathology.** When nude mice in the experimental groups developed clinical signs of illness after discontinuation of sulfadiazine treatment, the animals were euthanized at that time (5 or 6 days after discontinuation of the treatment). Their brains were removed and immediately fixed in a solution containing 10% Formalin, 70% ethanol, and 5% acetic acid. When the nude mice in experimental groups did not develop clinical signs of illness after discontinuation of treatment with sulfadiazine, the animals were euthanized for histopathology study on the brains at 90 days after discontinuation.
the treatment. Two to four 5 μm-thick sagittal sections (50 or 100 μm distance between sections) of the brain from each mouse were stained with hematoxylin and eosin. Immunoperoxidase staining using rabbit anti-Toxoplasma IgG was used for the detection of tachyzoites (8). Sections stained with hematoxylin and eosin were evaluated for inflammatory changes. Sections stained by the immunoperoxidase method were evaluated for the numbers of inflammatory areas associated with tachyzoites. The sections stained with the immunoperoxidase method were also used to evaluate numbers of *T. gondii* cysts in the brain when no acute inflammatory changes were observed. The mean value from these sections for each mouse was calculated as the number per section. These values were used for statistical analysis to compare differences between groups.

Semi-quantitative RT-PCR for detection of mRNA for IFN-γ. At 90 days after discontinuation of sulfadiazine treatment, RNA was isolated from brains of infected nude mice by using RNA STAT-60 (TEL-TEST “B”, Inc., Friendswood, Tex.) by following the manufacturer’s instructions. cDNA was synthesized using the RNA as described previously (33, 39). PCR for β-actin and IFN-γ was performed with 5 μl the original cDNA reaction mixture with a Mastercycler 5333 (Eppendorf AG, Hamburg, Germany), using 30 cycles to produce an amount of DNA within a linear range as described previously (33, 39). This number of cycles was determined in preliminary studies using different amounts of cDNA of the sample. Specific primers for β-actin and IFN-γ designed to span at least one intron allowed differentiation of amplified target DNA derived from either cDNA or genomic DNA in the PCR.

The homology of PCR products to the predicted transcript sequence was examined by Southern blot analysis (33, 39). Ten-microliter aliquots of the final PCR mixtures were electrophoresed at 100 V for 1 hr on a 1.5 % agarose gel and denatured. The DNA was then transferred to a Duralon-UV membrane (Stratagene, La Jolla, Calif.) by standard blotting procedure (29) and UV cross-linked. Oligonucleotide probes for β-actin and IFN-γ which hybridize to the PCR products wholly within the region amplified by the primers were end labeled as described for the 3’-end labeling and signal amplification system, and hybridization was detected by scanning of the membranes with a Image Station 440 CF (Eastman Kodak Company, Rochester, N.Y.) as described
previously (38). The quantification of mRNA was performed by densitometry analysis with the Image Station and normalized to the β-actin level.

**Statistical Analysis.** Levels of significance for numbers of areas associated with tachyzoites and cyst numbers in the brain, and the amount of IFN-γ in the culture of supernatants were determined by Student’s t, Alternate Welch t, or Wilcoxon rank sum test. Alternative Welch t test was applied when standard deviations were significantly different between groups tested. The Wilcoxon rank sum test was applied when the standard deviation was zero. Levels of significance for mortality in mice were determined using Fisher’s exact test. Differences which provided P < 0.05 were considered significant.

**RESULTS**

**Effect of adoptive transfer of T cells from IFN-γ−/− mice on mortality and development of TE in infected athymic nude mice.** We have previously reported that non-T cells which produce IFN-γ and T cells are both required for genetic resistance of BALB/c mice to prevent development of TE (21). To analyze whether IFN-γ production by T cells, in addition to that by the non-T cells, is required for the resistance, we purified immune T cells from spleens of infected IFN-γ−/− and wild-type mice, and the purified cells (1 5 10⁷) were injected intravenously into infected, sulfadiazine-treated athymic nude mice. As shown previously (21), the infected nude mice have the IFN-γ−producing non-T cells in their brains. Adoptive transfer of the immune T cells was performed at 9 and 2 days before discontinuation of sulfadiazine treatment. Control nude mice that had not received the cell transfer all died within 10 days after discontinuation of sulfadiazine treatment (Fig. 1A). The animals that had received the T cells from wild-type donor all survived (Fig. 1A). In contrast, nude mice that had received the T cells from IFN-γ−/− mice all died as early as did control animals that had not received the cell transfer (P<0.01 when compared to wild-type T cell recipients, Fig. 1A). Histological studies performed on 5 or 6 days after discontinuation of sulfadiazine treatment revealed large numbers of focal areas associated with tachyzoites in the brains of both control nude mice without cell transfer and those that had received IFN-γ−/− T cells (Fig. 1B and 2A). In
contrast, no such areas were observed in the brains of nude mice that had received wild-type T cells when examined in the same manner as the animals in the other groups (P<0.05 when compared to either IFN-γ−/− T cell recipients or controls without cell transfer, Fig. 1B).

Effect of adoptive transfer of T cells from PO mice on mortality and development of TE in infected athymic nude mice. Next, we examined whether perforin-mediated cytotoxic activity of T cells also plays an important role in prevention of TE. For this purpose, purified immune T cells (1.5 × 10⁷) from infected PO and wild-type mice were transferred to infected, sulfadiazine-treated nude mice. Most animals that had received the T cells from wild-type mice survived until the end of the observation period (90 days after discontinuation sulfadiazine treatment) (Fig. 3A). Nude mice that had received the T cells from PO animals also all survived after discontinuation of sulfadiazine treatment, whereas control nude mice that had not received any T cells all died in 10 days after discontinuation of sulfadiazine (P<0.01, Fig. 3A). Histological studies revealed no inflammatory changes or only mild infiltration of inflammatory cells in limited areas in the brains of nude mice that had received immune T cells from either PO or wild-type animals at the end of the observation period (Fig. 2B and 3B). No inflammatory areas associated with tachyzoites were observed in brains of these groups of mice. In contrast, large numbers of areas associated with tachyzoites were observed in brains of control nude mice without the cell transfer when they developed clinical signs of illness (P<0.05 when compared to the animals that had received either PO T cells or wild-type T cells).

Comparison of IFN-γ production between splenic T cells obtained from nude mice that received immune T cells from PO and wild-type donors. At 90 days after discontinuation of sulfadiazine treatment, T cells were purified from spleens of nude mice that had received immune T cells from PO and wild-type animals. These were the groups of animals that prevented TE and survived until the time that the study was performed (Fig. 3A). The T cells were then stimulated with tachyzoite antigens in vitro to evaluate their IFN-γ production. Because nude mice originally lack T cells before the
cell transfer, the T cells purified from the recipient animals are all donor origin. T cells obtained from the animals that had received PO T cells produced large amounts of IFN-γ when stimulated with the antigens (Fig. 4). The amounts of IFN-γ produced by these T cells did not differ from those produced by T cells obtained from nude mice that had received immune T cells from wild-type animals (Fig. 4).

Comparison of IFN-γ expression in brains between nude mice that received immune T cells from PO and wild-type donors. Amounts of mRNA for IFN-γ were measured by RT-PCR in the total RNA fractions obtained from brains of nude mice that had received immune T cells from PO and those that had received wild-type animals at 90 days after discontinuation of sulfadiazine. Large amounts of IFN-γ mRNA were detected in brains of these two groups of animals (Fig. 5), and the amounts of the mRNA did not differ between these animals (IFN-γ/β-actin ratio, 0.271 ± 0.077 in PO T-cell recipients and 0.183 ± 0.041 in wild-type T-cell recipients; P=0.091). Without cell transfer, only low amounts of mRNA for this cytokine were detectable in the brains of infected nude mice when they were treated with sulfadiazine (Fig. 5; IFN-γ/β-actin ratio, 0.040 ± 0.012, P<0.001 when compared to PO T-cell recipients, P<0.005 when compared to wild-type T-cell recipients).

Mortality and histological changes in the brain in PO mice following infection without treatment with sulfadiazine. PO and wild-type mice were infected with 10 cysts and their mortality was followed for 6 months. All animals in both groups survived until the end of the observation period (Fig. 6A). Histological studies performed at the end of the observation period revealed no inflammatory changes or only mild infiltration of inflammatory cells in limited areas in the brains, mainly in the meninges, in both PO and wild-type animals. In addition, there were no inflammatory areas associated with tachyzoites in brains of both groups of animals. Numbers of cysts observed in their brains were small in both groups of animals, and there were no differences in the cyst numbers (numbers of cysts/sagittal section; 0.33 ± 0.47 in wild-type controls [n=4], 0.58 ± 0.56 in PO mice [n=4]).
Comparison of IFN-γ production by T and spleen cells between infected PO and wild-type mice. T cells and spleen cells were obtained from PO and wild-type mice at 6 months after infection and then stimulated with tachyzoite antigens in vitro to evaluate their IFN-γ production. Large amounts of IFN-γ were detected in the culture supernatants of T cells from both groups of animals, and there were no differences in the amounts of the cytokine detected between these groups (Fig. 6B). In contrast, the amounts of IFN-γ in the culture supernatants of spleen cells were twice larger in the cells obtained from PO mice than in those from wild-type animals (P<0.02; Fig. 6B). The relative percentages of T cells in total spleen cells, calculated by the numbers of T cells purified, did not differ between these mice (data not shown).

DISCUSSION
We investigated the functions of T cells required for genetic resistance of BALB/c mice against reactivation of chronic infection with T. gondii in the brain. Our previous studies demonstrated the requirement of both T cells and IFN-γ-producing non-T cells for this resistance (21). The present study reveals that IFN-γ production by T cells, in addition to non-T cells, is essential for maintaining the latency of chronic infection and for preventing TE. Adoptive transfer of immune T cells from infected IFN-γ−/− mice failed to prevent development of TE and mortality in infected, sulfadiazine-treated athymic nude mice which originally had IFN-γ-producing non-T cells. Their development of the disease and mortality occurred as early as 1 week after discontinuation of sulfadiazine treatment. In contrast to the lack of the protective activity of IFN-γ−/− immune T cells, adoptive transfer of immune T cells from infected PO mice prevented TE and mortality in recipient nude mice, as did the transfer of immune T cells from wild-type BALB/c mice. It has been reported that T. gondii infection induces cytotoxic T cells that lyse infected host cells (15, 22, 31). However, the results of the present study clearly demonstrated the perforin-mediated cytotoxic activity of T cells is not required for preventing reactivation of a chronic infection with this parasite in the brain.

T cells obtained from spleens of nude mice that had received PO immune T cells produced large amounts of IFN-γ, which were equivalent to those produced by T cells
obtained from animals that had received wild-type immune T cells. These results indicate that lack of perforin-mediated cytotoxicity in T cells did not affect their IFN-γ production in response to *T. gondii*. This is further supported by the evidence that IFN-γ mRNA expression did not differ in brains of these two groups of mice that had received either PO or wild-type immune T cells. This unimpaired ability of PO T cells to produce IFN-γ is likely the reason for their potent protective activity to prevent TE. Our findings are consistent with the results by others showing unimpaired production of IFN-γ by PO lymphocytes when stimulated by allogeneic spleen cells (23). We previously reported that adoptive transfer of either CD4⁺ or CD8⁺ T cells prevented development of TE in infected, sulfadiazine-treated nude mice (21). Therefore, IFN-γ production by both CD4⁺ and CD8⁺ T cells likely contributes to this resistance. Contribution of both CD4⁺ and CD8⁺ T cells in resistance against *T. gondii* in the brain was also suggested in T cell depletion study in C57BL/6 mice genetically susceptible to TE (12).

Nude mice that had received T cells from PO mice lacked perforin-mediated cytotoxic activity of T cells, but not of NK cells. In the present study, in addition to these nude mice with the cell transfer, PO mice did not develop TE and all survived as did wild-type animals for 6 months after infection. PO mice lack perforin-mediated cytotoxic activity in both T and NK cells. These results indicate that perforin-mediated cytotoxic activity of not only T but also NK cells is not required for genetic resistance of BALB/c mice against development of TE. This is a contrast to the results in C57BL/6 (susceptible to TE)-background PO mice previously reported by Denkers et al (10), in which accelerated mortality was observed during the chronic stage of infection. In addition, the number of *T. gondii* cysts in the brain did not differ between BALB/c-background PO and wild-type mice in the present study whereas a three-fourfold increase in the cyst numbers was reported in C57BL/6-background PO mice (10). Therefore, it appears that the role of perforin in resistance against *T. gondii* differs between these two strains of mice. Furthermore, because differences in the mortality between these two strains of mice are notable in both PO and wild-type animals, it is likely that perforin-mediated cytotoxic activity does not play a major role in regulating genetic resistance of mice in resistance to chronic infection with *T. gondii* and development of TE.
In regard to the increased number of brain cysts in C57BL/6-background PO mice (10), it would be possible that perforin-mediated cytotoxicity has played an important protective role during the acute stage of infection although no mortality in mice was noted during that period of time. The lack of perforin-mediated cytotoxicity during acute stage may have resulted in accelerated proliferation of tachyzoites in various organs and formation of greater numbers of cysts in the brain. Such increased parasite loading in the brain could have contributed to the accelerated mortality in the later stage of infection, since C57BL/6 mice are genetically susceptible to development of TE during the later stage. In relation to a possible role of cytotoxic activity of T cells during the acute stage of infection, it has been shown that perforin-mediated lysis of tachyzoite-infected cells in vitro does not kill the intracellular parasite and results in release of viable tachyzoites (45). It is possible that these released tachyzoites are effectively killed, at least in part, by antibody-dependent complement-mediated cytolysis (36) and antibody-mediated phagocytosis by macrophages (2).

In the present study, IFN-γ production by T cells did not differ between infected BALB/c-background PO and wild-type mice during the late stage of infection. These results are consistent with our findings on T cells obtained from nude mice that received T cells from infected PO and wild-type animals. In contrast, IFN-γ production by spleen cells was significantly greater in infected PO than wild-type mice although the relative percentages of T cells in total spleen cells did not differ between these mice. These results strongly suggest that the lack of perforin-mediated cytotoxic activity resulted in upregulation of IFN-γ production by non-T cells in the PO mice whereas the ability of T cells to produce IFN-γ is not altered by the deficiency. Since NK cells have been shown to produce this cytokine in response to T. gondii infection (14, 17), it would be possible that NK cells contribute to the upregulated production of IFN-γ by total spleen cells in PO mice. This upregulated IFN-γ production by non-T cells might have played an important role in compensating for the lack of perforin-mediated cytotoxic activity in controlling T. gondii in these animals. Of interest, it was reported that in C57BL/6-background PO mice, IFN-γ production by their spleen cells does not differ from that of wild-types (10). As mentioned earlier, accelerated mortality and increased numbers of T. gondii cysts were observed in these PO mice (10). Therefore, the presence or absence of
upregulation of IFN-γ production by non-T cells may have contributed to the differences in the outcome of infection in PO mice with these two different genetic backgrounds.

In regard to the effector mechanisms of IFN-γ-mediated resistance to control *T. gondii* during the chronic stage of infection, an involvement of TNF receptor p55, inducible nitric oxide synthase, and IFN-γ-induced GTPase has been demonstrated in C57BL/6-background mice (6, 30). It is still unknown whether the effector mechanisms of IFN-γ-mediated resistance in genetically resistant BALB/c mice differ from those in susceptible C57BL/6 mice.

Perforin-mediated cytotoxic activity of T cells is known to play important roles in resistance to many intracellular pathogens (7, 20, 24, 28, 41, 43). On the other hand, it has been shown that the protective activity of T cells to clear out viruses from the brain is noncytolytic and IFN-γ-dependent (25). This strategy for clearance of virus infections without lysing infected cells is probably due to little capability for tissue renewal in the brain (26, 27). The present study demonstrated that the protective activity of T cells to control *T. gondii* in the brain in genetically resistant BALB/c mice is noncytolytic and IFN-γ-dependent. IFN-γ has been shown to play an important role in resistance of the brain against infections with numbers of intracellular microorganisms such as bacteria (19, 42), a fungus (1), and a protozoan parasite (11) other than *T. gondii*, in addition to viruses (3, 25, 40). Our results in *T. gondii*-infected mice may suggest that IFN-γ-mediated, noncytolytic mechanism is a strategy of T cells widely applied for controlling both viral and non-viral intracellular pathogens in the brain without developing tissue damages.
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FIG. 1. Mortality (A) and development of TE (B) in *T. gondii*-infected, sulfadiazine-treated athymic nude mice with adoptive transfer of immune T cells from infected IFN-γ−/−, or wild-type BALB/c mice. Athymic nude mice were infected with 10 cysts of the ME49 strain perorally and treated with sulfadiazine for 3 weeks beginning 7 days after infection. Nine and 2 days before discontinuation of sulfadiazine treatment, mice received an intravenous injection of 1 x 10⁷ immune T cells from infected, wild-type or IFN-γ−/− mice (see Materials and Methods). Histological studies were performed 5 or 6 days after discontinuation of treatment with sulfadiazine. Two to four sagittal sections (distance between sections of 50 μm) were stained with immunoperoxidase stain by using rabbit anti-*T. gondii* IgG and evaluated for the numbers of areas of inflammation associated with tachyzoites. The mean value from these sections for each mouse was calculated as the number per section. These values are shown in the figure and were used for statistical analysis to compare differences between groups. The data shown are the representative of two separate experiments. There were four to five mice in each experimental group in each experiment.
FIG. 2. Histological changes in brains of *T. gondii*-infected, sulfadiazine-treated athymic nude mice with adoptive transfer of immune T cells from infected IFN-γ−/− (A), or PO (B) mice. Athymic nude mice were infected with 10 cysts of the ME49 strain perorally and treated with sulfadiazine for 3 weeks beginning 7 days after infection. Nine and 2 days before discontinuation of sulfadiazine treatment, mice received an intravenous injection of 1 x 10⁷ immune T cells from the donor animals (see Materials and Methods). Histological studies were performed 6 days (the recipients of IFN-γ−/− T cells) or 90 days (the recipients of PO T cells) after discontinuation of treatment with sulfadiazine. Sections were stained with hematoxylin and eosin. The experiments were performed twice, and there were four to six mice in each group in each experiment.
FIG. 3. Mortality (A) and development of TE (B) in *T. gondii*-infected, sulfadiazine-treated athymic nude mice with adoptive transfer of immune T cells from infected PO or wild-type BALB/c mice. Athymic nude mice were infected with 10 cysts of the ME49 strain perorally and treated with sulfadiazine for 3 weeks beginning 7 days after infection. Nine and 2 days before discontinuation of sulfadiazine treatment, mice received an intravenous injection of $1.5 \times 10^7$ immune T cells from infected, wild-type or PO mice (see Materials and Methods). Histological studies were performed at 90 days after discontinuation of treatment with sulfadiazine. In the control animals without the cell transfer, the histological studies were at 6-7 days after discontinuation of the treatment. Two to four sagittal sections (distance between sections of 50 μm) were stained with immunoperoxidase stain by using rabbit anti-*Toxoplasma* IgG and evaluated for the numbers of areas of inflammation associated with tachyzoites. The mean value from these sections for each mouse was calculated as the number per section. These values are
shown in the figure and were used for statistical analysis to compare differences between groups. The data shown are the representative of two separate experiments. There were four to six mice in each experimental group in each experiment.
FIG. 4. Production of IFN-γ by T cells purified from spleens of nude mice that had received immune T cells from PO or wild-type BALB/c mice. Athymic nude mice were infected with 10 cysts of the ME49 strain perorally and treated with sulfadiazine for 3 weeks beginning 7 days after infection. Nine and 2 days before discontinuation of sulfadiazine treatment, mice received an intravenous injection of $1.5 \times 10^7$ immune T cells from infected, wild-type or PO mice (see Materials and Methods). At 90 days after discontinuation of sulfadiazine, T cells were purified from spleens of the recipient animals, and then stimulated with tachyzoite lysate antigens (20 μl/ml) in the presence of antigen-presenting cells (plastic-adherent cells from spleens of normal BALB/c mice) (see Materials and Methods). The experiment shows mean $\pm$ SD values of triplicate cultures. The data shown are the representative of two separate experiments.
FIG. 5. Expression of IFN-γ mRNA in brains of *T. gondii*-infected, sulfadiazine-treated nude mice after adoptive transfer of immune T cells from infected PO or wild-type BALB/c mice. Athymic nude mice were infected with 10 cysts of the ME49 strain perorally and treated with sulfadiazine for 3 weeks beginning 7 days after infection. Nine and 2 days before discontinuation of sulfadiazine treatment, mice received an intravenous injection of $1 \times 10^7$ immune T cells from donor animals (see Materials and Methods). At 90 day after discontinuation of sulfadiazine, their brains were analyzed for amounts of mRNA for β-actin and IFN-γ (see Materials and Methods). NC, negative control; PC, positive control. There were three or four mice in each experimental group.
FIG. 6. Mortality (A) and production of IFN-γ by T and spleen cells (B) in infected PO and wild-type BALB/c mice. Mice were infected with 10 cysts of the ME49 strain perorally. T cells were purified from spleens of the animals at 6 months after infection, and then stimulated with tachyzoite lysate antigens (20 μl/ml) in the presence of antigen-presenting cells (plastic-adherent cells from spleens of normal BALB/c mice) (see Materials and Methods). Total spleen cells were also stimulated with the lysate antigens. The experiment shows mean ± SD values of triplicated cultures. The data shown are the representative of two separate experiments.
Chapter 4

Importance of CD8\(^{+}\)V\(\beta\)8\(^{+}\) T Cells in IFN-\(\gamma\)-Mediated Prevention of Toxoplasmic Encephalitis in Genetically Resistant BALB/c mice

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ABSTRACT

In our attempt to identify a major T cell population(s) that recognizes protective *T. gondii* antigens and produces IFN-γ for prevention of toxoplasmic encephalitis (TE), we found T cell receptor Vβ8⁺ cells to be the most frequent IFN-γ-producing population infiltrated into the brain of *T. gondii*-infected BALB/c mice genetically resistant to the disease. To examine the role of IFN-γ production by this T cell population for the resistance, Vβ8⁺ immune T cells purified from spleens of infected BALB/c and IFN-γ⁻/⁻ mice were transferred into infected, sulfadiazine-treated athymic nude mice. After discontinuation of sulfadiazine treatment, control nude mice that had not received any T cells and animals that had received Vβ8⁺ T cells from IFN-γ⁻/⁻ mice all died due to reactivation of infection (TE). In contrast, animals that had received the cells from BALB/c mice survived. Thus, IFN-γ production by Vβ8⁺ T cells plays an important role in prevention of TE in these animals. When Vβ8⁺ immune T cells were divided into CD4⁺ and CD8⁺ subsets, a potent protective activity was observed only in the CD8⁺ subset whereas a combination of both subsets provided greater protection than did the CD8⁺Vβ8⁺ population alone. These results indicate that CD8⁺ subset of Vβ8⁺ T cells is a major afferent limb of IFN-γ-mediated resistance of BALB/c mice against TE, although CD4⁺ subset of the T cell population works additively or synergistically with the CD8⁺Vβ8⁺ population.
INTRODUCTION

Toxoplasma gondii, an intracellular protozoan parasite, forms cysts and establishes a latent, chronic infection preferentially in the brain after replication of tachyzoites in various organs during the acute stage of infection. It is estimated that up to $5 \times 10^8$ people worldwide are chronically infected with this parasite.\(^{(1)}\) The requirement for the immune system to maintain the latency of chronic infection is clearly evident from the reactivation of the infection in immunocompromised individuals, that results in development of life-threatening toxoplastic encephalitis (TE).\(^{(2, 3)}\) Thus, T. gondii provides an excellent model for analyzing the mechanism of host resistance of the brain against infection.\(^{(4-9)}\) Resistance to development of TE during chronic infection is under genetic control in mice.\(^{(10-12)}\) Strains with the H-2\(^b\) or H-2\(^k\) haplotypes develop progressive and ultimately fatal encephalitis whereas strains with the H-2\(^a\) or H-2\(^d\) haplotypes do not.\(^{(10-12)}\) Since the H-2 complex encodes major recognition and immunoregulatory molecules, it is likely that the genes responsible for control of resistance against TE, or at least some of them, do so by regulating the immune response to the parasite. BALB/c (H-2\(^d\)) is one of strains that are able to control T. gondii infection in their brains and establish a latent chronic infection as do immunocompetent humans.\(^{(10-12)}\) Therefore, these mice appear to be a suitable model to analyze the mechanisms of host resistance to maintain the latency of chronic infection and to prevent TE.

IFN-γ is an absolute requirement for maintaining latency in chronic infection with T. gondii and for prevention of TE in BALB/c mice.\(^{(13)}\) We previously reported that IFN-γ production by both T cells\(^{(14)}\) and non-T cells\(^{(15)}\) is essential for their resistance to the disease. In the present study, while attempting to identify a major T cell population(s) that recognizes protective T. gondii antigens and produces IFN-γ in the brain, we examined T cell receptor (TCR) Vβ chain usage in T cells expressing IFN-γ in brains of these animals following infection. We found that Vβ8\(^+\) T cells are the most abundant IFN-γ-producing cells in their brains. By using cell transfer models, we also found that IFN-γ production by Vβ8\(^+\) T cells plays an important role in prevention of TE in these mice and that CD8\(^+\) subset of the Vβ8\(^+\) T cells has the major protective activity.
MATERIALS AND METHODS

Mice

Female BALB/c-background IFN-γ knockout, athymic nude and wild-type BALB/c mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Female Swiss-Webster mice were from Taconic (Germantown, NY). All mice were housed in specific pathogen-free conditions, and were 8 to 12 weeks old when used. There were three to five mice for each experimental group.

Infection with T. gondii

Cysts of the ME49 strain were obtained from brains of Swiss-Webster mice that had been infected intraperitoneally with 10 cysts for 2 to 3 months. Mice were euthanized by asphyxiation with CO2, and their brains were removed and triturated in phosphate-buffered saline (pH 7.2). An aliquot of the brain suspension was examined for numbers of cysts, and after appropriate dilution in phosphate-buffered saline, all animals were infected with 10 cysts perorally by gavage. IFN-γ−/− mice were treated with sulfadiazine in drinking water (400 mg/l) beginning 4 days after infection for 5-6 weeks for controlling proliferation of tachyzoites and establishing a chronic infection. Nude mice were treated with sulfadiazine beginning at 7 days after infection for 3 weeks.

Flow cytometry

At 2, 4 and 8 weeks after infection, mononuclear cells infiltrated into brains and spleen cells were obtained individually from each of 5 BALB/c mice as described previously. The cells were also obtained from uninfected mice as a control. The cells from each mouse were pooled and suspended at a density of 1 x 10^6 cells/ml for spleen cells and 2 x 10^5 cells/ml for mononuclear cells isolated from brains in a volume of 10ml of RPMI (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine, 100u/ml penicillin- streptomycin and 50 μM 2-mercaptoethanol. The cells were stimulated with 5 ng/ml phorbol myristate acetate (PMA) and 500ng/ml ionomycin (Sigma) for four hours in an incubator with 5% CO₂ at 37 C°. GolgiPlug (BD PharMingen) was added for the last two hours of the culture.
After stimulation, the cells were pretreated on ice for 10 min with 10 μl of predetermined optimal concentration of anti-Fc II/III receptors mAb (2.4G2) to block non-antigen-specific binding of antibodies to the Fc II/III receptors. Thereafter, the cells were incubated on ice for 30 min with 10 μl of optimal concentrations of FITC-conjugated mAbs to TCR V chains.\(^{(17)}\) After washing, the cells were fixed and permeabilized with Cytofix/Cytoperm Plus kit (BD PharMingen, San Diego, CA), and then stained with PE-conjugated anti-IFN-γ (XMG1.2) or isotype-control (R3-34) mAbs by following the manufacturer’s instructions.\(^{(18)}\) The mAbs were obtained from BD PharMingen. Analysis of stained cells was performed with an EPICS-XL Flow Cytometer (Beckman-Coulter, Miami, FL). The number of cells analyzed was 10,000 for most samples except for some that had less numbers. The experiment was repeated three times for each time point after infection.

**Purification and transfer of Vβ8\(^+\) T cells**

At 5 or 6 weeks after infection, spleen cells were obtained from 5 each of IFN-γ\(-/-\) and wild-type BALB/c mice, suspended in Hanks’ balanced salt solution (Irvine Scientific, Santa Ana, CA) with 2% of fetal bovine serum (Sigma Chemical Co, St. Louis, MO) and pooled within the same group of the animals. Vβ8\(^+\) T cells were purified by treating the immune spleen cells with FITC-labeled anti-Vβ8 mAbs (BD PharMingen), then with magnetic bead-conjugated anti-FITC mAb (Miltenyi Biotech, Sunnyvale, CA) for MACS.\(^{(17)}\) A total of 2 x 10\(^6\) Vβ8\(^+\) immune T cells were injected intravenously from a tail vein to recipient BALB/c-background nude mice at 9 and 2 days before discontinuation of treatment with sulfadiazine. CD4\(^+\) and CD8\(^+\) subsets of Vβ8\(^+\) immune T cells were also purified by treating immune spleen cells with FITC-labeled anti-Vβ8, biotin-labeled anti-CD4, and PE-labeled anti-CD8 mAbs, then with Texan red-labeled avidin (ICN/Cappel, Costa Mesa, CA) for sorting with FACSVantage (BD Biosciences, Mountain View, CA). After purification, the purity of the targeted T cell population in the area gated for lymphocytes were >95%.
Histopathology

At 7 days after discontinuation of treatment with sulfadiazine, mice were euthanized by asphyxiation with CO₂. Their brains were removed and immediately fixed in a solution containing 10% Formalin, 70% ethanol, and 5% acetic acid. Two to four 5 μm-thick sagittal sections (50- or 100-μm distance between sections) of the brain from each mouse were stained with immunoperoxidase stain by using rabbit IgG antibody against tachyzoite-specific SAG2 and evaluated for the numbers of areas of inflammation associated with tachyzoites. Hematoxylin and eosin stained sections were evaluated for inflammatory changes.

Statistical Analysis

Levels of significance for relative ratios of IFN-γ-producing cells in spleen cells and mononuclear cells isolated from brains, numbers of areas associated with tachyzoites and cyst numbers in the brain were determined by Student’s t or Alternate Welch t tests. Alternative Welch t test was applied when standard deviations were significantly different between groups tested. Levels of significance for mortality in mice were determined using Fisher’s exact test. Differences which provided P < 0.05 were considered significant.

RESULTS

High frequencies of IFN-γ-producing T cells in the brain of BALB/c mice infected with T. gondii

Because of the importance of IFN-γ for genetic resistance of BALB/c mice against development of TE, we compared the frequencies of IFN-γ-producing cells in T cells between brains and spleens of the animals following infection with T. gondii. Mice were infected perorally with 10 cysts of the ME49 strain, and at 2, 4 and 8 weeks later, spleen cells and mononuclear cells infiltrated into their brains were isolated. As a control, spleen cells from uninfected mice were also tested for the presence of IFN-γ-producing cells. In the normal spleen cells, the frequencies of IFN-γ+ cells in T cells were 0.85% (shown as a horizontal line in Fig. 1A). In the spleen cells of infected mice, the frequencies of IFNγ-producing cells in T cells were approximately 3-times higher.
(2.5-2.7%) that than of normal spleen cells at each time point tested (Fig. 1A). Furthermore, IFN-γ-positive cells were detected in much greater frequencies (7-16%) in T cells infiltrated into the brain than those in the spleens during the course of infection (Figs. 1A and 1B; P< 0.005 and P=0.01 at 2 and 8 weeks after infection, respectively). These results indicate that IFN-γ-producing T cells are recruited efficiently into the brain following infection.

**TCR Vβ chain usage in IFN-γ-producing T cells infiltrated into the brains of BALB/c mice following infection with T. gondii**

In order to address a major T cell population(s) that recognize the protective *T. gondii* antigens and produce IFN-γ in the brain following infection, we examined TCR Vβ chain usage of IFN-γ-producing T cells that infiltrated into the brain at 2, 4 and 8 weeks after infection. Eight weeks after infection is the time that BALB/c mice have already developed a latent, chronic infection. The analysis was not performed in uninfected mice since collectable numbers of T cells from brains of uninfected mice were very limited due to effective prevention of T cell entry into normal brain by the blood-brain barrier. At each time point after infection, Vβ8+ cells were the most abundant and distinct population among IFN-γ-expressing cells (Fig. 2). The relative percentages of IFN-γ+ Vβ8+ population in a total number of IFN-γ+ cells were 17.8%, 21.6%, and 15.5% at 2, 4 and 8 weeks after infection, respectively. Vβ6+ and Vβ14+ cells were the second largest populations in IFN-γ-producing cells at each time point (Fig. 2). The frequencies of Vβ8+ cells in IFN-γ-producing cells were significantly greater than those of either Vβ6+ or Vβ14+ cells at 2 and 8 weeks after infection (P<0.05 at 2 weeks, and P<0.01 at 8 weeks). The differences did not reach statistical significance at 4 weeks after infection because of larger variations in the data.

**Requirement of IFN-γ production by Vβ8+ immune T cells for their protective activity against development of TE**

Since Vβ8-bearing cells were found to be the largest IFN-γ-producing T cell population in the brain of *T. gondii*-infected BALB/c mice, we examined the role of IFN-
γ production by Vβ8+ T cells in prevention of TE in these animals. For this purpose, Vβ8+ immune T cells were purified from spleens of infected IFN-γ−/− and wild-type BALB/c mice, and 2 x 10^6 of the purified cells were injected intravenously into infected, sulfadiazine-treated athymic nude mice at 9 and 2 days before discontinuation of sulfadiazine treatment. Control nude mice that had not received any T cells all died between 7 and 10 days after discontinuation of sulfadiazine treatment due to reactivation of infection (TE) (Fig. 3) as we previously reported.(15) Animals that had received Vβ8+ immune T cells from wild-type BALB/c mice all survived (P<0.05) (Fig. 3). In contrast, animals that had received immune Vβ8+ T cells from IFN-γ−/− mice died as early as the control nude mice (Fig. 3). These results indicate that IFN-γ production by Vβ8-bearing T cells is essential for their protective activity to prevent reactivation of chronic T. gondii infection. These results also indicate that IFN-γ production by this T cell population without other T cell population is sufficient for preventing mortality in the recipients.

Role of CD4+ and CD8+ subsets of Vβ8+ T cells for prevention of TE

In order to examine whether CD4+ or CD8+ subsets of Vβ8-bearing T cells play an important role in prevention of TE, we purified each of these subsets of Vβ8-bearing immune T cells from spleens of infected BALB/c mice and transferred the purified cells into infected, sulfadiazine-treated nude mice. In this study, numbers of transferred were less (6 or 7 x 10^5) than that (2 x 10^6) used in the study with a total Vβ8+ T cell population described above. Seven days after discontinuation of sulfadiazine treatment, histological studies were performed on their brains. Many areas of inflammation associated with tachyzoites were observed in brains of control nude mice that had not received any T cells. Numbers of such inflammatory areas tended to be less in brains of animals that had received CD4+ subset of Vβ8+ immune T cells when compared to those in the control nude mice, but the differences did not reach statistical significance (P=0.164: Fig. 4). In contrast, numbers of the inflammatory areas associated with tachyzoites were markedly and significantly less in brains of animals that had received CD8+ subset of Vβ8+ immune T cells than those in the control nude mice (P<0.01; Fig. 4). In animals that had received a total population of Vβ8-bearing immune T cells, the inflammatory areas associated
with tachyzoites were hardly detectable in their brains (Fig. 4). Although numbers of such inflammatory areas were very small in animals with a transfer of either CD8\(^+\) subset of Vβ8\(^+\) or a total Vβ8\(^+\) T cell populations, these numbers were significantly less in the latter than former (P<0.05). In both of these two groups, inflammatory changes in general and numbers of parasite detected in their brains were very limited, indicating that CD8\(^+\) subset of Vβ8\(^+\) and a total Vβ8\(^+\) T cell populations were both efficient to prevent reactivation of the infection although the latter has greater protective activity.

**DISCUSSION**

Since IFN-γ production by T cells is essential for genetic resistance of BALB/c mice to TE,\(^{(13)}\) in the present study we analyzed IFN-γ-producing T cell populations that infiltrated into the brain of these animals following infection with *T. gondii*. The frequencies of IFN-γ-producing cells were found to be 3-5 times greater in T cells isolated from their brains than those from spleens at both acute and chronic stages of infection. These results indicate that IFN-γ-producing T cells are efficiently recruited to the brain in these animals following infection with *T. gondii*. Similar results were recently reported by Kwok et al\(^{(20)}\) using infection with recombinant *T. gondii* expressing β-galactosidase. They demonstrated approximately 4 times greater frequencies of β-galactosidase-specific IFN-γ-producing T cells in the brains than in the spleens of mice at 25 days after infection with the recombinant parasite.

In regard to IFN-γ-producing T cells recruited into the brain following *T. gondii* infection, TCR Vβ8-bearing cells were revealed to be the most abundant population throughout the course of infection. Therefore, Vβ8-bearing cells appear to be a major T cell population that recognizes protective *T. gondii* antigens to produce IFN-γ and effectively infiltrate into the brain of BALB/c mice for prevention of TE following infection. The importance of IFN-γ production by Vβ8\(^+\) T cells for resistance is supported by the results of cell transfer experiments using infected, sulfadiazine-treated nude mice as recipients, which develop severe TE (reactivation of infection) after discontinuation of sulfadiazine if they do not receive protective T cells. Adoptive transfer of Vβ8\(^+\) immune T cells from infected wild-type BALB/c mice prevented
mortality in the recipient nude mice. In contrast, a transfer of Vβ8+ immune T cells from infected IFN-γ−/− mice failed to confer the resistance. In regard to T cell functions in general of IFN-γ−/− mice, it has been reported that these animals have unimpaired ability to develop cytotoxic T cells following various stimulations\textsuperscript{(21-23)}. In addition, we previously reported that serum levels of total anti-Toxoplasma IgG antibodies did not differ between infected IFN-γ−/− and wild-type mice\textsuperscript{(13)}, indicating helper T cells function required for Ig class switching is not impaired in IFN-γ−/− mice. Therefore, their T cell functions do not appear to be generally impaired. Thus, the failure of immune Vβ8+ T cells from infected IFN-γ−/− mice to prevent TE is most likely due to lack of ability to produce IFN-γ in this T cell population, but not due to failure of development of effector cells in the IFN-γ−/− mice. These results strongly suggest that the potent protective activity of Vβ8-bearing T cells against TE is mediated by their production of IFN-γ. Since a transfer of IFN-γ-producing Vβ8+ T cells in the absence of other T cell populations prevented mortality in the recipient nude mice, the cytokine production by this T cell population appears to play a crucial role in genetic resistance of BALB/c mice to TE.

Although Vβ8+ cells were the most abundant IFN-γ-producing T cells in the brain of infected BALB/c mice, T cells bearing TCR Vβ chains other than Vβ8 were also found to produce IFN-γ in their brains; for example, Vβ6+ cells were one of the second largest IFN-γ+ populations. In relation to this, we previously demonstrated that Vβ6+ immune T cells did not show an ability to prevent TE as did Vβ8+ T cells\textsuperscript{(17)}. It is unclear why the protective activity of Vβ8+ and Vβ6+ T cells markedly differs even though IFN-γ-producing cells were found in both of these T cell populations in the brains of infected animals. One possibility would be that Vβ8+ immune T cells can be recruited to the brain more efficiently than Vβ6+ immune T cells in the infected mice and therefore the former cell population is more effective for preventing the disease than the latter population. Another possibility is that a function(s) other than IFN-γ production in T cell populations is involved in determining the activity of T cell populations to prevent TE although IFN-γ production is a primary requirement for their protective activity.
When TCR Vβ8-bearing immune T cells from infected BALB/c mice were divided into CD4+ and CD8+ subsets and transferred into infected, sulfadiazine-treated nude mice, we observed a clear difference between these two T cell populations in their activity to prevent TE. Only CD8+ subset of Vβ8+ T cell population demonstrated a potent protective activity at the dose (6 or 7 x 10⁵ cells) used. Although CD4+ subset of Vβ8+ T cells did not show significant protective effects in these studies, the recipients of this T cell population tended to have fewer inflammatory areas associated with tachyzoites in their brains when compared to control mice that had not received any T cells. Therefore, if greater numbers of cells are used for the cell transfer, it is possible that CD4+Vβ8+ T cell population would confer a partial protection against TE. However it is clear that the CD8+ subset of Vβ8-bearing T cells has much greater protective activity to prevent TE than does CD4+ subset of the T cell population. Although the CD4+Vβ8+ T cell population did not have potent activity by themselves, which prevents TE, nude mice that received a transfer of both CD4+ and CD8+ subsets of Vβ8+ T cell population together had significantly fewer inflammatory areas associated with tachyzoites than those that received a transfer of the CD8+ subset alone. Therefore, it appears that CD4+Vβ8+T cells function synergistically or additively with CD8+Vβ8+ T cells to prevent TE, although CD8+Vβ8+ population plays a major role. Since the protective activity of a total immune Vβ8+ T cells is mediated by their production of IFN-γ, and since a transfer of CD8+Vβ8+ T cells alone, but not of CD4+Vβ8+ T cells, conferred a potent protection against TE, CD8+Vβ8+ T cells are most likely a major producer of this cytokine in the total immune Vβ8+ T cells. Although CD4+Vβ8+ T cells would also produce IFN-γ, the cytokine production by this T cell population alone would not be sufficient for protection.

A collaborative effect of total CD4+ and CD8+ T cell subsets in resistance to TE was previously reported by using depletion of these T cell populations in TE-susceptible C57BL/6 mice.(24) It would be possible that an activity of CD4+ and CD8+ subsets of a major protective T cell population(s), such as Vβ8+ cells, contributes to determining the protective activity of total CD4+ and CD8+ T cell subsets to the disease. In relation to our finding of the importance of CD8+ Vβ8+ T cells in prevention of reactivation of T. gondii
infection in the brain, CD8+ T cells have been shown to play an important role in resistance against formation of tissue cysts in the brain after infection.\textsuperscript{(25, 26)} CD8+ Vβ8+ T cells might prevent formation of cysts in addition to preventing reactivation of infection (due to rupture of cysts) in the brain following infection.

McLeod and her colleagues\textsuperscript{(11)} and we\textsuperscript{(12)} previously reported that the $L^d$ gene, an MHC class I gene, is required for resistance against TE in BALB/c mice. Recently, Johnson et al\textsuperscript{(27)} and Kwok et al\textsuperscript{(20)} reported an importance of $L^d$ molecules expressed on the surface of infected cells for their recognition by CD8+ T cells. In the present study, we demonstrated that IFN-γ production by Vβ8+ T cells is important for prevention of TE in these mice, and that CD8+ subset of the Vβ8+ T cells possessed the major protective activity. It would be possible that CD8+Vβ8+ T cells are a major T cell population that recognizes the protective \textit{T. gondii} antigen(s) presented by $L^d$ molecules and thereby this T cell population plays a critical role in IFN-γ-mediated genetic resistance of BALB/c mice to development of TE following infection with \textit{T. gondii}.
ACKNOWLEDGMENTS

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Fig. 1. Detection of intracellular IFN-γ in T cells isolated from brains and spleens of BALB/c mice infected with *T. gondii*. Mice were infected with 10 cysts of the ME49 strain, and 2, 4 and 8 weeks later, lymphocytes infiltrated into the brain of mice were isolated, stimulated with PMA and ionomycin, and stained with PE-labeled anti-IFN-γ or isotype control mAbs in combination with FITC-labeled antiCD3 mAb, followed by flow cytometry (see the Materials and Methods). The panel A shows mean values and standard deviations from three experiments performed. Spleen cells from uninfected mice were stimulated and stained for IFN-γ as a control. A horizontal line in panel A indicates the mean value (0.85%) of frequencies of IFN-γ+ cells in the normal spleen cells. Panel B shows representative staining of lymphocytes isolated from brains (4 weeks after infection): left panel, stained with PE-labeled isotype control mAb; right panel, stained with PE-labeled anti-IFN-γ mAb.
Fig. 2. TCR Vβ chain usage in IFN-γ-expressing lymphocytes infiltrated into brains of BALB/c mice following infection with *T. gondii*. At 2, 4, and 8 weeks after infection with 10 cysts of the ME49 strain, lymphocytes infiltrated into the brain of mice were isolated, stimulated with PMA and ionomycin, and TCR Vβ usage in T cells expressing IFN-γ was analyzed by flow cytometry (see Materials and Methods). Three experiments were performed at each time point and the mean value from three experiments was calculated for each TCR Vβ chain. The panel shows these values at each time point after infection.
Fig. 3. Mortality in *T. gondii*-infected, sulfadiazine-treated nude mice that received Vβ8+ immune T cells from either infected IFN-γ−/− or wild-type BALB/c mice. Nude mice were infected with 10 cysts of the ME49 strain perorally and treated with sulfadiazine for 3 weeks beginning 7 days after infection. Vβ8-bearing T cells were obtained from IFN-γ−/− and wild-type BALB/c mice at 5 and 6 weeks after infection (see Materials and Methods). IFN-γ−/− mice were treated with sulfadiazine beginning at 4 days after infection. Vβ8+ immune T cells (2 x 10⁶) were injected intravenously into infected nude mice at 9 and 2 days before discontinuation of sulfadiazine.
Fig. 4. Development of TE in *T. gondii*-infected, sulfadiazine-treated nude mice that received either CD4$^+$ or CD8$^+$ subsets of V$\beta$8$^+$ immune T cells from infected BALB/c mice. Nude mice were infected with 10 cysts of the ME49 strain perorally and treated with sulfadiazine for 3 weeks beginning 7 days after infection. Nine and 2 days before discontinuation of sulfadiazine treatment, mice received an intravenous injection of either CD4$^+$ or CD8$^+$ subsets of V$\beta$8$^+$ immune T cells purified from spleens of infected BALB/c mice (see Materials and Methods). Histological studies were performed 7 days after discontinuation of treatment with sulfadiazine. Two to four saggital sections (distance between sections of 50 $\mu$m) were stained immunoperoxidase stain by using tachyzoite-specific SAG2 antibody and evaluated for counting.
Chapter 5

Importance of IFN-γ-Mediated Expression of Endothelial VCAM-1 on Recruitment of CD8⁺ T Cells into the Brain during Chronic Infection with *Toxoplasma gondii*

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ABSTRACT

IFN-γ is essential for preventing reactivation of chronic infection with *Toxoplasma gondii* in the brain. We examined the role of IFN-γ on lymphocyte and endothelial adhesion molecule expression and T cell recruitment into the brain during chronic infection with *T. gondii* in IFN-γ knockout (IFN-γ−/−) and wild-type mice. Although the number of cerebral vessels expressing intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) increased in both wild-type and IFN-γ−/− mice following infection, there were more VCAM-1+ vessels in brains of infected wild-type than infected IFN-γ−/− mice; in contrast, numbers of ICAM-1+ vessels did not differ between strains. We did not detect endothelial E-selectin, P-selectin, MAdCAM-1 or PNAd in any of the brains. Significantly fewer CD8+ T cells were recruited into brains of infected IFN-γ−/− than wild-type mice. Treatment of infected IFN-γ−/− mice with recombinant IFN-γ restored the expression of VCAM-1 on their cerebral vessels and recruitment of CD8+ T cells into their brains, confirming an importance of this cytokine for up-regulation of VCAM-1 expression and CD8+ T cell trafficking. In infected wild-type and IFN-γ−/− animals, almost all cerebral CD8+ T cells were LFA-1high, CD44high and CD62Lneg and approximately 38% were α4β1 integrin+. In adoptive transfer of immune spleen cells, pre-treatment of the cells with a monoclonal antibody against α4 integrin markedly inhibited recruitment of CD8+ T cells into the brain of chronically infected wild-type mice. These results indicate that IFN-γ-induced expression of endothelial VCAM-1 and its binding to α4β1 integrin on CD8+ T cells is important for recruitment of the T cells into the brain during the chronic stage of *T. gondii* infection, although LFA-1/ICAM-1 interaction may also be involved in this process.
INTRODUCTION

Following infection with *Toxoplasma gondii*, an obligate intracellular protozoan parasite, tachyzoites invade and proliferate in a variety of cells throughout the body during the acute stage of infection. The proliferation of tachyzoites is suppressed by IFN-\(\gamma\)-dependent, cell-mediated immune responses \(^1\)-\(^3\) and, to a lesser degree, humoral immunity \(^4\)-\(^6\), leading to the development of chronic infection characterized by *T. gondii* cysts primarily in the brain. Chronic infection with *T. gondii* is one of the most common parasitic infections in humans \(^7\),\(^8\). People with immunodeficiencies such as AIDS are at risk for reactivation of infection and development of life-threatening toxoplasmic encephalitis (TE) \(^9\),\(^10\). Murine models of the disease have demonstrated that IFN-\(\gamma\) production by T cells is essential for prevention of the reactivation of infection \(^11\),\(^12\). IFN-\(\gamma\) production can activate microglia \(^13\),\(^14\) and astrocytes \(^15\),\(^16\) to inhibit proliferation of tachyzoites.

The blood-brain barrier prevents most intravascular leukocytes from entering the parenchyma of the normal brain \(^17\). However, large numbers of leukocytes are able to migrate from blood vessels into a brain that is injured by infection, ischemia or an autoimmune disease such as multiple sclerosis. This migration is mediated, in part, by endothelial adhesion and activation molecules that are found in injured brain but not in normal brain \(^17\). In mice with TE, endothelial cells of cerebral vessels up-regulate their expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) \(^18\),\(^19\). Mice with a deficiency in VCAM-1 expression have unimpaired infiltration of T cells into their brains during TE \(^20\). In contrast to the animals that develop TE, strains of mice genetically resistant to chronic infection with *T. gondii* establish a latent chronic infection as do immunocompetent humans \(^21\)-\(^23\), and these animals have little inflammation in their brains during the chronic infection \(^21\)-\(^23\). Therefore, the mechanisms of T cell recruitment into the brain in the hosts with the latent chronic infection could be distinct from those in the hosts that have severe inflammatory responses caused by TE. In order to understand how the immune system prevents reactivation of *T. gondii* infection, we must first determine which lymphocyte and endothelial adhesion molecules control the recruitment of T cells from blood vessels into the brain parenchyma during latent chronic infection with *T. gondii*. 

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IFN-γ is required for maintaining the latency of chronic infection with *T. gondii* [11,24-26]. IFN-γ treatment of endothelial cells up-regulates the expression of several adhesion molecules, including ICAM-1 and VCAM-1, that bind to counterreceptors on circulating lymphocytes [27,28]. Thus, IFN-γ may play an important role in inducing expression of endothelial adhesion molecules and promoting infiltration of T cells into the brain during the chronic stage of *T. gondii* infection. We have previously developed murine models of chronic infection using genetically resistant BALB/c mice and utilized this model to analyze the mechanisms of host immunity to prevent TE [11,24,25]. In the present study, we used *in vivo* migration studies in the BALB/c model to directly evaluate the ability of T cells to migrate from blood vessels into the brains of IFN-γ−/− and wild-type mice with chronic infection with *T. gondii*. We also examined the effects of IFN-γ on the expression of adhesion molecules by endothelial and T cells in the brains of infected mice. We found that up-regulation of expression of VCAM-1, but not of ICAM-1, on cerebral vessels is largely dependent on IFN-γ. Moreover, endothelial VCAM-1 and lymphocyte α4β1 integrin are important for recruitment of CD8+ T cells into the brains of chronically infected mice.

**MATERIALS AND METHODS**

*Mice*

Female BALB/c-background IFN-γ−/− and wild-type BALB/c mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Female Swiss-Webster mice were obtained from Taconics (Germantown, NY). All mice were housed under specific-pathogen-free conditions and were 8-12 weeks old when used. There were 3-5 mice in each experimental group.

*Infection with T. gondii*

Cysts of the ME49 strain were obtained from brains of Swiss-Webster mice that were intraperitoneally infected with 10 cysts 2-3 months before sacrifice. Each experimental mouse was given 10 cysts perorally by gavage [25]. To control the proliferation of tachyzoites in IFN-γ−/− mice so that a chronic infection could be established, sulfadiazine supplemented drinking water (400 mg/l) was given to these
animals for 3 weeks beginning 4 days after infection. In one experiment, wild-type BALB/c mice received sulfadiazine for 11 days beginning 14 days after infection.

**Immunohistochemistry for endothelial adhesion molecules**

Brains from IFN-γ−/− and wild-type mice were removed 25 days after infection, bisected along the median sagittal plane and snap-frozen in OCT compound (Sakura Finetek, Torrance, CA). Acetone-fixed frozen sections were stained as previously described with monoclonal antibodies (mAbs) against ICAM-1 (clone YN1/1.7; ATCC, Manassas, VA), VCAM-1 (MK-2.7; ATCC), E-selectin (10E9.6, BD PharMingen, San Diego, CA), P-selectin (RB40, ATCC), MAdCAM-1 (MECA-367, provided by Dr. E. Butcher, Stanford, CA), PNAd (MECA-79, provided by Dr. E. Butcher), and irrelevant negative control antigens. Briefly, the sections were sequentially incubated with rat primary antibody, biotin-conjugated anti-rat IgG or anti-rat IgM, peroxidase-streptavidin, diaminobenzidine/hydrogen peroxide, and methylene blue counterstain. There were two washes with phosphate-buffered saline (PBS) between each step. Three or more sections, at least 200 μm apart, were stained for each adhesion molecule in each brain. Slides were examined by light microscopy. The numbers of vessels expressing VCAM-1 or ICAM-1 per mm² of brain area were determined by image analysis as described.

**Flow cytometry**

Twenty-five days after infection, anesthetized mice were perfused intracardially with PBS to remove intravascular leukocytes. Mononuclear cells were isolated from brains and spleens from 5 mice for each group as described previously. The cells were incubated on ice for 10 min with predetermined optimal amount of anti-FcγII/III receptors mAb (2.4G2). The cells were then incubated for 30 min with a FITC-conjugated mAb to either CD4 (RM4-5) or CD8 (53-6.7) combined with a PE-conjugated mAb to LFA-1 (2D7), CD44 (IM7), CD62L (MEL-14), or α4 integrin (9C10). A biotin-conjugated mAb to β7 integrin (M293) was also added to the staining with anti-α4 integrin mAb. After washing, the cells stained with the anti-α4 and anti-β7 mAbs were incubated with avidin-PerCP for 30 min. All mAbs and avidin-PerCP were from BD PharMingen. Cells were analyzed on an EPICS-XL flow cytometer (Beckman-Coulter,
Miami, FL). 10,000 cells in the gated area for lymphocytes were analyzed. The number of T cells expressing α4β1 integrin (VLA-4) was calculated by subtracting the numbers of α4β7 T cells from total numbers of α4 integrin⁺ T cells, since the integrin α4 chain is expressed as a heterodimer with either β1 or β7.³²

_Treatment of IFN-γ⁻/⁻ mice with recombinant IFN-γ (rIFN-γ)_

In one experiment, IFN-γ⁻/⁻ mice were injected intravenously beginning 20 days after infection with 1μg (0.2-1.0 x 10⁵ Units) of murine rIFN-γ (BD PharMingen) in 0.2 ml of saline every other day for three times. As a control, IFN-γ⁻/⁻ mice were injected in the same manner with 0.2 ml of saline. One day after the final injection (25 days after infection), the mice were euthanized and their brains were frozen for immunohistochemical studies as described above.

_Adoptive transfer of 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled immune spleen cells_

Spleen cells from chronically infected wild-type BALB/c mice were suspended in labeling media composed of 50% RPMI1640 (Sigma Chemical Co, St. Louis, MO), 48.5% Hanks’ balanced salt solution (GIBCO, Carlsbad, CA) and 1.5% fetal bovine serum (Sigma). The cells were then incubated with 5μM CFSE mixed isomers (Molecular Probes, Eugene, OR) in labeling media at a concentration of 5 x 10⁷ cells/ml for 15 min at 37°C. Labeled cells were washed with equal volumes of fetal bovine serum and labeling media. Three or 5 x 10⁷ CFSE-labeled cells were injected intravenously into infected IFN-γ⁻/⁻ and/or wild-type mice. Two or 18 hrs after the transfer, mononuclear cells were isolated from the brains of recipient mice, stained with PE-conjugated mAbs to CD4 or CD8 and analyzed by flow cytometry. 2 x 10⁴–2 x 10⁵ cells in the gated area for lymphocytes were analyzed. In one experiment, infected IFN-γ⁻/⁻ mice were treated with rIFN-γ as described above and received CFSE-labeled cells one day after the last injection of rIFN-γ.
In vivo blocking of T cell homing by a monoclonal antibody (mAb) against $\alpha_4$ integrin

CFSE-labeled immune spleen cells were treated with 10 $\mu$g/ml of anti-$\alpha_4$ mAb (PS/2) or a control mAb (9B5; anti-human CD44) on ice for 10 min. Thereafter, the cells ($5 \times 10^7$) were transferred into BALB/c mice infected with *T. gondii* for 3 months. Two hours after the cell transfer, mononuclear cells were obtained from their brains and stained for flow cytometry as described above.

**Statistical analysis**

Numerical data are presented as mean ± standard deviation. Student’s *t*-test was used to evaluate differences between treatment groups. *P* < 0.05 is considered to be significant.

**RESULTS**

Expression of endothelial adhesion molecules on cerebral vessels of IFN-$\gamma^{-/-}$ and wild-type BALB/c mice infected with *T. gondii*

To determine if IFN-$\gamma$ affects expression of adhesion molecules by cerebrovascular endothelial cells during the later stage of *T. gondii* infection, we stained frozen sections of brains from BALB/c-background IFN-$\gamma^{-/-}$ and wild-type mice with antibodies against ICAM-1, VCAM-1, E-selectin, P-selectin, MAdCAM-1 and PNAd at 25 days after infection. IFN-$\gamma^{-/-}$ mice were treated with sulfadiazine for 3 weeks from day 4 after infection to establish a chronic infection; unless treated, these animals die within 2 weeks after infection with uncontrolled proliferation of tachyzoites. A few vessels in brains of uninfected IFN-$\gamma^{-/-}$ and wild-type mice expressed VCAM-1 (4.34 ± 1.13 and 4.51 ± 2.55 vessels/mm$^2$ of tissue, respectively) (Figure 1A). Infection with *T. gondii* caused an increase in vascular VCAM-1 expression in both strains (*P*<0.05; Fig. 1A). However, the mean number of VCAM-1-expressing vessels in brains of infected wild-type mice was 2.5-times greater than in infected IFN-$\gamma^{-/-}$ mice (*P*<0.01) (Fig. 1A). Infected IFN-$\gamma^{-/-}$ mice treated with rIFN-$\gamma$ (1 $\mu$g intravenously on days 20, 22 and 24 after infection) had significantly more intracerebral VCAM-1$^+$ vessels than did infected IFN-$\gamma^{-/-}$ mice (*P*<0.01) (Fig. 1A). Although there were fewer VCAM-1$^+$ vessels in the brains of
infected rIFN-γ-treated IFN-γ−/− mice than in brains of infected wild-type mice, the difference was not statistically significant (P=0.08) (Fig. 1A).

Very few vessels in brains of uninfected IFN-γ−/− and wild-type mice had detectable levels of ICAM-1. After *T. gondii* infection, both strains had an 18-30-fold increase in the number of ICAM-1+ vessels (P<0.05; Fig. 1B). More importantly, the number of ICAM-1-expressing cerebral vessels in infected wild type mice was equal to that in infected IFN-γ−/− mice and infected IFN-γ−/− mice treated with rIFN-γ (Fig. 1A).

We did not detect expression of E-selectin, P-selectin, MAdCAM-1 or PNAd on cerebral vessels in infected or uninfected mice of either strain. Treatment with sulfadiazine did not alter the expression of vascular adhesion molecules in infected wild-type mice (data not shown). These results indicate that ICAM-1 and VCAM-1 are the cerebrovascular endothelial adhesion molecules whose expression is upregulated during chronic infection with *T. gondii*. The induction of VCAM-1 is largely dependent on IFN-γ whereas the induction of ICAM-1 is independent of IFN-γ.

Numbers of CD8+ and CD4+ T cells in the brains of IFN-γ−/− and wild-type BALB/c mice infected with *T. gondii*

VCAM-1 has been shown to be important for T cell migration into various tissues during chronic inflammation, including experimental autoimmune encephalomyelitis. Since IFN-γ was found to be important for up-regulation of endothelial VCAM-1 expression in the brains of infected mice, it is possible that IFN-γ plays an important role for inducing T cell migration into the brain during infection. Our first step in addressing this possibility was to determine how many T cells are in the brains of infected IFN-γ−/− mice, as compared to infected wild-type mice. We found fewer CD8+ cells in brains of infected IFN-γ−/− mice than in brains of infected wild-type mice at 25 days after infection (P<0.05) (Fig. 2A). In contrast, there was no significant difference between strains in the number of intracerebral CD4+ T cells (Fig. 2A). These results suggest that IFN-γ plays a major role, either directly or indirectly, in the development of CD8+ T cell infiltration in brains of mice with chronic *T. gondii* infection.
Numbers of CD8+ and CD4+ T cells in the spleens of IFN-γ−/− and wild-type BALB/c mice infected with T. gondii

Since there are fewer CD8+ T cells in the brains of infected IFN-γ−/− mice than in infected wild-type mice, we asked whether there are fewer CD8+ T cells in lymphoid tissues of infected IFN-γ−/− mice than in infected wild-type mice. In contrast to the brain, there were significantly more CD4+ T cells and CD8+ T cells in spleens of infected IFN-γ−/− than in spleens of infected wild-type mice (P<0.005 and P< 0.01, respectively) (Fig. 2B). In uninfected animals, there were no differences between strains in the absolute numbers of splenic CD4+ and CD8+ T cells (2.2 ± 1.2 x 10^7 [n=2; IFN-γ−/−] vs. 2.0 ± 0.3 x 10^7 [n=2; wild-type] in CD4+ T cells; 0.98 ± 0.21 x 10^7 [n=2; IFN-γ−/−] vs. 0.64 ± 0.39 x 10^7 [n=2; wild-type] in CD8+ T cells). These results indicate that the paucity of CD8+ T cells in the brains of IFN-γ−/− mice is not due to a systemic lack of CD8+ T cells.

Recruitment of CD8+ and CD4+ T cells from the bloodstream into brains of IFN-γ−/− and wild-type BALB/c mice infected with T. gondii

We used in vivo lymphocyte migration assays to directly evaluate the migration of T cells from blood vessels into brains of infected IFN-γ−/− and infected wild-type mice. Mononuclear cells from spleens of chronically infected wild-type mice were labeled with a fluorescent marker (CFSE) and injected intravenously into IFN-γ−/− and wild-type mice (3 x 10^7 cells/mouse) at 25 days after infection. Eighteen hours after the transfer, mononuclear cells were isolated from brains of the recipients, and numbers of CFSE-labeled CD4+ and CD8+ T cells were determined by flow cytometric analysis. The absolute number of donor CD8+ T cells (CFSE+CD8+) (Fig. 3 left panel) and the relative number of donor CD8+ T cells (CFSE+CD8+/total T cells) (Fig. 3 right panel) in the brains of IFN-γ−/− recipients were significantly less than in the brains of wild-type recipients (P<0.01 and P< 0.005, respectively). Although fewer CFSE+ CD4+ T cells were recovered from brains of IFN-γ−/− than wild-type recipients, the difference was statistically significant in one of two experiments (absolute numbers of cell/brain: 0.84 ± 0.18 x 10^3 vs. 4.1 ± 0.2 x 10^3 [P<0.01], and 0.60 ± 0.17 x 10^3 vs.1.08 ± 0.27 x 10^3 [P=0.06]). In every experiment, the intracerebral donor T cells had one discrete peak of
CFSE fluorescence, indicating that the cells had not divided after transfer. Thus, the difference in number of intracerebral donor T cells between wild-type and IFN-γ−/− mice is due to differences in recruitment of the cells into the brain, rather than in proliferation after entering the brain. Therefore, these results indicate that IFN-γ is important in the recruitment of CD8+ T cells and, to a lesser extent, CD4+ T cells, into the brains of mice chronically infected with *T. gondii*.

Effects of rIFN-γ treatment on recruitment of CD8+ T cells into brains of *T. gondii*-infected IFN-γ−/− mice

To further address the role of IFN-γ in recruitment of CD8+ T cells into the brain, infected IFN-γ−/− mice were injected with rIFN-γ (1 μg intravenously on days 20, 22 and 24 after infection) or saline. One day after the last injection, the mice were given CFSE-labeled lymphocytes from spleens of chronically infected wild-type mice. As a control, infected wild-type mice also received the CFSE-labeled lymphocytes. Eighteen hours after the cell transfer, mononuclear cells were isolated from brains of the recipients for flow cytometry. As in the previous experiment (Fig. 3), the absolute number of CFSE+ CD8+ T cells in brains of untreated IFN-γ−/− mice was significantly less than in brains of wild-type mice (P<0.05; Fig. 4 left panel). In contrast, the absolute number of CFSE+ CD8+ T cells in brains of IFN-γ−/− mice treated with rIFN-γ was significantly greater than in untreated IFN-γ−/− mice (P<0.001; Fig. 4 left panel) and equal to that in wild-type controls (Fig. 4 left panel). Moreover, the relative number of CFSE+ CD8+ T cells in total T cells in brains of IFN-γ−/− mice treated with rIFN-γ was significantly higher than in untreated IFN-γ−/− mice (P<0.05; Fig. 4 right panel) and equal to that in wild-type controls (Fig. 4 right panel). These results confirm the importance of IFN-γ in recruitment of CD8+ T cells into the brains of mice during chronic infection with *T. gondii*.

Adhesion molecules on CD4+ and CD8+ T cells in the brains of IFN-γ−/− and wild-type BALB/c mice infected with *T. gondii*

Since IFN-γ is important for expression of VCAM-1 on cerebral vessels and in recruitment of CD8+ T cells into brains of *T. gondii*-infected mice, we asked if there are
differences in expression of adhesion molecules on T cells in brains of infected IFN-γ−/− and wild-type mice. In both strains, more than 90% of brain CD8+ and CD4+ T cells had an effector/memory (TEM) phenotype (CD44high, LFA-1high and CD62Lneg) (Fig. 5). In contrast, 23-50% of spleen CD8+ and CD4+ T cells displayed LFA-1high or CD62Lneg (data not shown). Among intracerebral CD8+ T cells, 11-34% expressed α4β7 integrin (the major ligand for MAdCAM-1) and about 38% expressed α4β1 integrin (the major ligand for VCAM-1) (Figs. 5B and D).

**Effects of anti-α4 mAb on the migration of T cells into brains of mice infected with T. gondii**

Although there were many VCAM-1+ vessels in the brains of infected wild-type mice, a minority of the CD8+ T cells in brains expressed α4β1 integrin. It is possible, however, that the T cells downregulated their expression of α4β1 integrin after infiltrating into the brain. Alternatively, some of T cells might be from local proliferation of cells that had migrated into brain. To directly examine the role of α4 integrin in recruitment of CD8+ T cells into the brain during chronic infection with *T. gondii*, we looked at the ability of an inhibitory mAb to α4 integrin to block migration of adoptively-transferred CD8+ T cells from blood vessels into brains of infected wild-type mice. The mAb would block α4β1/VCAM-1 and α4β7/MAdCAM-1 binding. However, because there was no MAdCAM-1 detected on intracerebral vessels in the infected mice, we would be looking at an inhibitory effect of the mAb on α4β1/VCAM-1 binding. Lymphocytes from spleens of chronically infected wild-type mice were labeled with CFSE, incubated with anti-α4 or negative control mAb, and transferred intravenously into chronically infected wild-type mice (3 months after infection). Mice were sacrificed two hours after the transfer. The numbers of CFSE-labeled CD8+ T cells in their brains were determined using immunofluorescent staining and flow cytometry. The anti-α4 mAb blocked about 90% of the migration of donor CD8+ T cells and 80% of the migration of donor CD4+ T cells into brains, as compared to the negative control mAb (P<0.001 and P<0.005 for CD8+ and CD4+ cells, respectively; Fig. 6). These results indicate that the binding of lymphocyte α4β1 integrin to endothelial VCAM-1 plays a
major role in recruitment of CD8$^+$ and CD4$^+$ T cells into the brains of mice chronically infected with *T. gondii*.

**DISCUSSION**

Although T cells are essential for maintaining the latency of chronic *T. gondii* infection in the brains of BALB/c mice$^{25,34}$, the adhesion mechanisms that control the migration of T cells from blood vessels into the brains of these mice have not been defined. Before migrating into tissue, T cells must bind to the luminal surface of blood vessel endothelial cells at the site of exit from the bloodstream; this is a multistep process, with sequential interactions between molecules on the endothelial surface and their counter-receptors on the T cells. In the present study, we found that IFN-γ is crucial for induction of endothelial VCAM-1 expression in the cerebral vessels of BALB/c mice following infection with *T. gondii*. Moreover, we show that interaction of endothelial VCAM-1 with α4β1 integrin on the surface of CD8$^+$ T cells plays an important role in recruitment of this T cell population into the brain during the chronic stage of infection.

We first examined endothelial adhesion molecule expression on cerebral vessels of BALB/c mice following *T. gondii* infection. We observed a marked increase in the number of intracerebral vessels that expressed ICAM-1 and VCAM-1 in the infected animals. Our results agree with those of Deckert-Schluter$^{18,19}$, who found upregulation of ICAM-1 and VCAM-1 on cerebral vessels of genetically susceptible mice with TE. Thus, there is prominent expression of ICAM-1 and VCAM-1 on cerebrovascular endothelial cells during the later stage of *T. gondii* infection, regardless of the presence or absence of severe inflammatory changes. We did not detect E-selectin, P-selectin, MAdCAM-1 or PNAd on endothelia in the brains of the infected BALB/c mice. There was no published information on the expression of these adhesion molecules on cerebral vessels following *T. gondii* infection. Thus, ICAM-1 and VCAM-1 are the vascular adhesion molecules selectively up-regulated in the brains of mice after *T. gondii* infection.

We found significantly more VCAM-1 expressing vessels in brains of infected wild-type mice than in brains of infected IFN-γ$^{-/-}$ mice. In contrast, there were no differences between strains in the numbers of ICAM-1-expressing intracerebral vessels.
In addition, treatment of infected IFN-γ−/− mice with rIFN-γ restored the expression of VCAM-1 on their cerebral vessels but did not affect their ICAM-1 expression. Thus, IFN-γ is crucial for expression of VCAM-1 on the cerebrovascular endothelial cells in BALB/c mice following infection with *T. gondii*. Although our study does not address the molecular mechanisms of VCAM-1 induction, IFN-γ is known to upregulate the expression of VCAM-1 on primary mouse brain capillary endothelial cells in an *in vitro* model of the blood-brain barrier, consisting of a co-culture of the endothelial and glial cells. Thus, IFN-γ is likely to play a direct role in inducing VCAM-1 expression on vascular endothelial cells in the brain of infected mice. Secondary involvement of other cytokines, however, cannot be ruled out.

IFN-γ receptor-deficient (IFN-γR−/−) 129/Sv mice were previously shown to fail to up-regulate expression of ICAM-1 on cerebral vessels following acute infection with *T. gondii*. This is in contrast to our finding that ICAM-1 expression in infected IFN-γ−/− BALB/c mice was equivalent to that of infected wild-type animals. One possible reason for the difference in these observations is the genetic background of mice. The present study was performed in BALB/c-background mice, which are genetically resistant to development of TE, while the previous study was performed in 129/Sv-background mice, which are genetically susceptible to TE. Another factor that may have contributed to the differences in ICAM-1 expression is the timing after infection for examining the expression of the endothelial adhesion molecule. The IFN-γR−/− mice were examined 10 days after infection, when their brains contained many proliferating tachyzoites and a severe, acute inflammatory infiltrate. In contrast, in our studies, IFN-γ−/− mice established a chronic infection by receiving treatment with sulfadiazine and vascular adhesion molecule expression was examined during the chronic stage of infection (25 days after infection). Therefore, it is possible that upregulation of ICAM-1 expression on cerebral vessels occurs through an IFN-γ-mediated mechanism in the early stage of infection and through an IFN-γ-independent mechanism in the later stage of infection.

We observed significantly fewer CD8+ T cells in brains of infected IFN-γ−/− mice than in brains of infected wild-type mice; in contrast, there was no significant difference between strains in the number of intracerebral CD4+ T cells. In both strains, essentially
all of the intracerebral T cells were CD44\textsuperscript{high}, LFA-1\textsuperscript{high} and CD62L\textsuperscript{neg} TEM cells. After adoptive transfer of CFSE-labeled immune spleen cells into infected mice, there were fewer donor CD8\textsuperscript{+} T cells in the brains of IFN-\(\gamma\)-/- mice than wild-type mice. Thus, IFN-\(\gamma\) plays a crucial role for the recruitment of CD8\textsuperscript{+} T cells into the brains of mice during the chronic stage of infection with \textit{T. gondii}. We\textsuperscript{35} and others\textsuperscript{47,48} reported that CD8\textsuperscript{+} T cells are important for resistance against the parasite in the brain. In BALB/c mice, IFN-\(\gamma\) production by T cells is essential for their genetic resistance against TE\textsuperscript{11}, and 8-15% of the T cells in brains of infected BALB/c mice produce this cytokine\textsuperscript{35}. Therefore, 76% reduction in recruitment of CD8\textsuperscript{+} T cells into the brain, which was observed in the IFN-\(\gamma\)-/- mice, could make a crucial impact on numbers of IFN-\(\gamma\)-producing effector T cells in their brains. In this regard, we previously reported that adoptive transfer of immune T cells into infected nude mice (which express IFN-\(\gamma\) in their brains through non-T cells) resulted in a marked increase in the amounts of this cytokine expressed in their brains, whereas such an increase did not occur in infected IFN-\(\gamma\)-/- animals after receiving the immune T cells\textsuperscript{11,25}. Thus, IFN-\(\gamma\)-mediated recruitment of CD8\textsuperscript{+} T cells may play a crucial role in resistance of BALB/c mice against chronic infection with \textit{T. gondii} by facilitating an infiltration of IFN-\(\gamma\)-producing effector cells into their brains.

The binding of endothelia VCAM-1 to lymphocyte \(\alpha 4\beta 1\) integrin is important in the migration of T cells into many sites of chronic inflammation\textsuperscript{38-40}. In our adoptive transfer experiments, anti-\(\alpha 4\) integrin mAb markedly inhibited the migration of CD8\textsuperscript{+} T cells from infected donor mice into the brains of chronically infected host mice. Although the anti-\(\alpha 4\) integrin mAb blocks binding of \(\alpha 4\beta 1\) to VCAM-1 and \(\alpha 4\beta 7\) to MAdCAM-1, the absence of endothelial MAdCAM-1 in the brains of our host mice indicates that VCAM-1 and \(\alpha 4\beta 1\) integrin are important for the recruitment of CD8\textsuperscript{+} T cells into the brains of \textit{T. gondii}-infected mice. As discussed earlier, induction of VCAM-1 on cerebral vessels in the infected mice is mostly dependent on IFN-\(\gamma\). Thus, IFN-\(\gamma\)-dependent expression of VCAM-1 is probably important in IFN-\(\gamma\)-mediated recruitment of CD8\textsuperscript{+} T cells into the brains of infected animals.

In addition to CD8\textsuperscript{+} T cells, the migration of CD4\textsuperscript{+} T cells to the brains of infected mice was inhibited by anti-\(\alpha 4\) integrin mAb. In the studies comparing recruitment of the
T cell populations into the brains of infected IFN-γ⁻/⁻ and wild-type mice, the number of CD4⁺ T cells recruited into the IFN-γ⁻/⁻ mice were 3.6-fold less than that recruited into the wild-type animals, but the difference was not as large as the 9.2-fold difference observed in CD8⁺ T cells. This might be due to the difference in the time between transfer of T cells and detection of the cells recruited into the brain in these two experiments. Such time period was 2 hours in the former with anti-α4 integrin mAb, whereas it was 18 hours in the latter with IFN-γ⁻/⁻ mice. CD4⁺ T cells may be able to migrate into the brains of infected mice through a mechanism(s) other than the α4β1 integrin/VCAM-1 system over a longer time period.

Our functional studies indicate that α4β1 integrin is important for the recruitment of most T cells into brains of mice with chronic T. gondii infection. However, only 38% of CD8⁺ cells and 71% of CD4⁺ T cells in brains of infected wild-type mice expressed α4β1 integrin (Fig. 5). The adhesion molecules that are expressed on a lymphocyte depend on a number of factors, including the maturation and activation state of the cell. Thus, the T cells may have down-regulated their expression of α4β1 after entering the brain. Moreover, this down-regulation might have occurred more efficiently in CD8⁺ T cells than CD4⁺ T cells.

It was previously reported that neonatal inactivation of the VCAM-1 gene did not impair the recruitment of CD4⁺ and CD8⁺ T cells into the brains of T. gondii-infected Sv129xC57BL/6 mice. Sv129xC57BL/6 mice are susceptible to TE while BALB/c mice are resistant to the disease. Therefore, the role of VCAM-1 on T cell migration into the brain following infection might differ between these strains, and this difference may play a role in determining their resistance/susceptibility to TE through affecting T cell populations to infiltrate into the brain.

Lymphocyte LFA-1 and endothelia ICAM-1 are involved in the activation-dependent firm adhesion of lymphocytes to endothelia in a wide range of secondary lymphoid tissues and chronically inflamed tissues. We found strong expression of ICAM-1 on endothelia and LFA-1 on T cells in the brains of T. gondii-infected mice. Thus, the LFA-1/ICAM-1 system, in addition to α4β1 integrin/VCAM-1 system, could be involved in T cell trafficking into the brains of these mice. It was previously reported
that α4β1 integrin/VCAM-1 and LFA-1/ICAM-1 mediate the recruitment of T cells into the brains of rodents with experimental autoimmune encephalomyelitis.\textsuperscript{41-44}

IFN-γ has been shown to activate microglia\textsuperscript{13,14} and astrocytes\textsuperscript{15,16} to prevent intracellular proliferation of \textit{T. gondii}. The present study reveals that IFN-γ is important for expression of VCAM-1 and recruitment of CD8\textsuperscript{+} T cells into the brains of infected animals through this adhesion molecule. Thus, IFN-γ functions not only as an essential effector molecule to control the parasite but also as a regulatory molecule to facilitate effector T cell infiltration into the brain to maintain the protective immunity in this organ during chronic infection with \textit{T. gondii}.
ACKNOWLEDGEMENTS

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Fig. 1. Expression of VCAM-1 (A) and ICAM-1 (B) on cerebral vessels of IFN-γ−/− and wild-type mice infected with *T. gondii*. IFN-γ−/− and wild-type (WT) mice were infected with 10 cysts of the ME49 strain perorally. The IFN-γ−/− mice were treated with sulfadiazine for 3 weeks beginning on day 4 after infection. Some infected IFN-γ−/− mice were injected intravenously with 1 μg (0.2-1 x 10⁵ units) of murine rIFN-γ on days 20, 22 and 24 after infection. All mice were sacrificed 25 days after infection. The number of cerebral vessels that expressed VCAM-1 or ICAM-1 was determined by frozen section immunohistochemistry staining and quantitative image analysis (see Materials and Methods). The data shown are the mean values ± standard deviations from 3-4 mice in each group. *P<0.01 when compared with infected WT mice. **P=0.01 when compared with untreated, infected IFN-γ−/− animals. P<0.005 when compared VCAM-1 expression between uninfected and infected WT mice. P< 0.05 when compared VCAM-1 expression between uninfected and infected IFN-γ−/− mice. P<0.05 when compared ICAM-1 expression between uninfected and infected WT mice and between uninfected and infected IFN-γ−/− mice.
Fig. 2. Numbers of CD4$^+$ and CD8$^+$ T cells in brains (A) and spleens (B) of IFN-γ$^{-/-}$ and wild-type mice infected with *T. gondii*. IFN-γ$^{-/-}$ and wild-type (WT) mice were infected as described in Fig. 1. The absolute numbers of CD4$^+$ and CD8$^+$ T cells were determined by 2 color immunofluorescent staining and flow cytometric analysis of mononuclear cells from brains and spleens of mice at 25 days after infection (see Materials and Methods). The data shown are the mean values ± standard deviations from data obtained from three separate experiments. There were 3-5 mice in each group in each experiment. *P<0.05, **P<0.01, and ***P<0.005 compared to WT mice.
Fig. 3. Recruitment of CD8$^+$ T cells into brains of IFN-γ$^{-/-}$ and wild-type mice infected with *T. gondii*. IFN-γ$^{-/-}$ and wild-type (WT) mice were infected as described in Fig. 1. CFSE-labeled lymphocytes (3 x 10$^7$) from spleens of chronically infected WT mice were injected intravenously into IFN-γ$^{-/-}$ and WT mice at 25 days after infection. Eighteen hours after the transfer, mononuclear cells were isolated from brains of the recipients. The absolute (left panel) and relative (% CFSE$^+$ CD8$^+$ /total T cells) numbers (right panel) of donor CD8$^+$ T cells in each brain was determined using flow cytometry (see Materials and Methods). There were three mice in each group, and the data shown are the mean values ± standard deviations from triplicate staining in each group. *P<0.01 compared to WT mice. **P<0.005 compared to WT mice.
Fig. 4. Effects of treatment with rIFN-γ on recruitment of CFSE-labeled CD8^+ T cells into brains in IFN-γ^-/- mice infected with T. gondii. IFN-γ^-/- and wild-type (WT) mice were infected as described in Fig. 1. IFN-γ^-/- mice were injected intravenously with 1 μg (0.2-1 x 10^5 units) of murine rIFN-γ on days 20, 22 and 24 after infection. Lymphocytes from spleens of infected WT mice were labeled with CFSE, and the labeled cells (3 x 10^7) were injected intravenously into IFN-γ^-/- and BALB/c mice at 25 days after infection. Eighteen hours after the cell transfer, mononuclear cells were isolated from brains of the recipients. The absolute (left panel) and relative (% CFSE^+ CD8^+ /total T cells) numbers (right panel) of donor CD8^+ T cells in each brain was determined using flow cytometry (see Materials and Methods). There were three mice in each group, and the data shown are the mean values ± standard deviations from triplicate staining in each group. *P<0.05 when compared with WT mice and P<0.001 when compared with IFN-γ^-/- animals treated with rIFN-γ. ** P<0.05 when compared with either WT or IFN-γ^-/- mice treated with IFN-γ.
Fig. 5. Phenotype of T cells in the brains of IFN-γ⁻/⁻ and wild-type mice infected with *T. gondii*. IFN-γ⁻/⁻ and wild-type (WT) mice were infected as described in Fig. 1. Mononuclear cells pooled from brains of five mice of each strain at 25 days after infection. The expression of adhesion molecules and subset markers on CD4⁺ (A, C) and CD8⁺ (B, D) T cells was determined by immunofluorescent staining and flow cytometry (see Materials and Methods). The data shown in panels A and B are representative of
three separate experiments. The data shown in panels C and D are the mean values ± standard deviations from three separate experiments.
Fig. 6. Effects of anti-α4 integrin mAb on the migration of T cells into the brains of wild-type mice chronically infected with *T. gondii*. Lymphocytes from spleens of chronically infected BALB/c mice were labeled with CFSE, incubated with anti-α4 integrin or control mAb, and injected intravenously (5 x 10^7 cells/mouse) into WT mice that had been infected for 3 months. Two hours after the cell transfer, mononuclear cells were isolated from brains of the recipients, and numbers of CFSE-labeled CD4^+ and CD8^+ T cells in the cell preparations were determined using flow cytometry (see Materials and Methods). There were three mice in each group, and the data shown are the mean values ± standard deviations from triplicate staining in each group. *P<0.005 and **P<0.001 when compared to the cells treated with control mAb.
Chapter 6

General conclusions

We examined the mechanisms of IFN-\(\gamma\)-mediated resistance against development of TE using a mouse strain (BALB/c) genetically resistant to the disease. Our studies revealed that T cells prevent TE through their IFN-\(\gamma\) production, and their cytotoxic activity mediated by perforin is dispensable for the disease prevention. T cells bearing T cell receptor V\(\beta\)8 chain were identified as the most frequent IFN-\(\gamma\)-producing T cell population in the brains of infected mice, and IFN-\(\gamma\) production by V\(\beta\)8\(^+\) T cells alone confers resistance against TE in athymic nude mice that lack T cells. CD8\(^+\) subset of V\(\beta\)8\(^+\) T cells is a major afferent limb of the IFN-\(\gamma\)-mediated resistance against TE, although CD4\(^+\) subset of the T cell population works additively or synergistically with the CD8\(^-\)V\(\beta\)8\(^+\) population. We also found that IFN-\(\gamma\) plays an important role in expression of endothelial adhesion molecules on cerebral vessels and recruitment of T cells into the brain during chronic infection with *T. gondii*. Our studies showed that up-regulation of expression of VCAM-1, but not of ICAM-1, on cerebral vessels is largely dependent on IFN-\(\gamma\), and that IFN-\(\gamma\)-mediated expression of endothelial VCAM-1 in its interaction with \(\alpha 4\beta 1\) integrin on lymphocytes plays an important role in recruitment of CD8\(^+\) T cells into the brains of chronically infected mice.