Published in final edited form as:

Expert Rev Mol Med. ; 13: e31. doi:10.1017/S1462399411002018.

Interferon-gamma- and perforin-mediated immune responses for resistance in the brain against *Toxoplasma gondii*

Yasuhiro Suzuki*, Qila Sa, Marie Gehman, and Eri Ochiai

Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky College of Medicine, 800 Rose Street, Lexington, KY 40536, USA

Abstract

Toxoplasma gondii is an obligate intracellular protozoan parasite, which causes various diseases including lymphadenitis, congenital infection of fetuses and life-threatening toxoplasmic encephalitis in immunocompromised individuals. Interferon-gamma (IFN- γ)-mediated immune responses are essential for controlling tachyzoite proliferation during both acute acquired infection and reactivation of infection in the brain. Both CD4⁺ and CD8⁺ T cells produce this cytokine in response to infection. Murine models demonstrated that both CD4⁺ and CD8⁺ T cells are protective against reactivation of infection, although the latter have more potent protective activity. Various signaling molecules including MyD88, protein kinase C- θ , and nuclear factor- κ B family transcription factors are important for T cells in inducing and/or maintaining their protective function. IL-12, IL-4, IL-6, IL-10, IL-17, IL-27, and IL-33 are involved in regulating resistance to *T. gondii* in the brain. IFN- γ can activate microglia, astrocytes, and macrophages and these activated cells control proliferation of tachyzoites using different molecules depending on the cell types and species of the host. IFN- γ also plays a critical role in recruitment of T cells into the brain after infection by inducing expression of adhesion molecule, VCAM-1, on cerebrovascular endothelial cells and of chemokines such as CXCL9, CXCL10 and CCL5. A recent study revealed that CD8⁺ T cells are able to remove *T. gondii* cysts, the stage of the parasite in chronic infection, from the brain through their perforin-mediated activity. Thus, the resistance to cerebral infection with *T. gondii* requires a coordinated network utilizing both IFN- γ - and perforin-mediated immune responses. Elucidating how these two protective mechanisms function and collaborate in the brain against *T. gondii* will be crucial in developing a new method to prevent and eradicate this parasitic infection.

Keywords

Toxoplasma; IFN- γ ; perforin; T cell; brain; microglia; astrocytes; macrophage; adhesion molecule; chemokine

Introduction

Toxoplasma gondii is capable of infecting many warm-blooded mammals including humans. Acquired infection occurs through ingestion of food and water contaminated with the cyst or oocyst stages of the parasite. Acute infection is characterized by proliferation of tachyzoites and is known to cause various diseases including lymphadenitis and congenital infection of fetuses (1). IFN- γ -mediated immune responses limit proliferation of tachyzoites (2), but the parasite establishes a chronic infection by forming cysts, which can contain hundreds to

*Corresponding author: Yasuhiro Suzuki, Ph.D., Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky College of Medicine, 800 Rose Street, Lexington, KY 40536, USA. yasu.suzuki@uky.edu.

thousands of bradyzoites, primarily in the brain. Chronic infection with *T. gondii* is one of the most common parasitic infections in humans. It is estimated that 500 million to 2 billion people worldwide are chronically infected with this parasite (3, 4). The tissue cysts remain largely quiescent for the life of the host, but can reactivate and cause life-threatening toxoplasmic encephalitis in immunocompromised patients, such as those with AIDS, neoplastic diseases and organ transplants (5, 6). In immunocompetent individuals, recent studies suggested that *T. gondii* is an important cause of cryptogenic epilepsy (7, 8) and likely involved in the etiology of schizophrenia (9–11). Although the cyst stage of the parasite is not affected by any of the current drug treatments and has been generally regarded as untouchable, we recently revealed perforin-mediated activity of CD8⁺ T cells can induce removal of this stage of the parasite from the brain (12). In this review, we discuss the mechanisms of IFN- γ - and perforin-mediated immunity against *T. gondii* in the brain.

Entry of *T. gondii* into the intestine and dissemination into the brain

Infection with *T. gondii* occurs through oral uptake of cysts or oocysts as mentioned in the “Introduction” section. In the small intestine, bradyzoites or sporozoites released from the cysts or oocysts invade epithelium, convert into tachyzoites, and multiply. Using murine models, it was recently demonstrated that tachyzoites are preferentially detected in CD11c⁺ cells in the lamina propria on day 2 and in the mesenteric lymph nodes from day 3 to 7 of infection (13). Therefore, infected CD11c⁺CD11b^{+/−} dendritic cells (DC) appear to be a carrier of parasite, disseminating the infection from the lamina propria to mesenteric lymph nodes. In contrast to the gut-associated lymphoid tissues, CD11c[−]CD11b⁺ monocytes are the major cell population that contains tachyzoites in the blood (13). Therefore, this cell population likely plays an important role in disseminating the infection to various organs, including the brain. In support of this possibility, at 1 day after an intravenous injection of CD11b⁺ blood cells from infected mice into uninfected animals, the parasite is detectable in mononuclear cells obtained from the brains of the recipient animals (13). This is in contrast to an intravenous injection of a small number of free tachyzoite, in which the parasite was not detected 1 day later. Treatment of infected mice with anti-CD11b mAb at 6 and 7 days after infection resulted in 50% reduction in the number of the parasite in their brains detected at 8 days after infection, suggesting an involvement of CD11b integrin in parasite dissemination to the brain. Since macrophages also express CD11b on their surfaces and contribute to dissemination of the parasite to the lymph nodes (14), this cell population may also be involved in carrying tachyzoites into the brain after infection.

DC also play a role in disseminating the parasite into the brain. As mentioned, DC are the main cell population in the gut-associated lymphoid tissue during the early stage of infection. Tachyzoite-infected dendritic cells exhibit hypermotility in vitro (15). When infected DC are injected intravenously into uninfected mice, the parasite can be detected in the brains of the recipients (13, 15).

Transepithelial migration of tachyzoites might also be involved in passage of the parasite through the blood-brain barrier. Using in vitro models with monolayers of several different cell lines (Madin Darby Canine Kidney cell, colorectal adenocarcinoma-derived Caco2 cell, and placenta-derived BeWo cell), an interaction of intercellular adhesion molecule 1 (ICAM-1) with the parasite adhesin MIC2 was shown to be important for transmigration of tachyzoites through a monolayer of host cells (16). A contribution of this interesting mechanism in dissemination of tachyzoites into the brain remains to be elucidated.

Requirement of IFN- γ production by T cells to control *T. gondii* tachyzoites in the brain

IFN- γ is the absolute requirement for controlling tachyzoite proliferation during the acute stage of *T. gondii* infection. T cells are also essential for resistance against acute infection since athymic nude and SCID mice which lack T cells succumb to acute infection and their mortality is associated with proliferation of large numbers of tachyzoites in various organs including the brain. In resistance to acute infection in general, CD8⁺ T cells are the major effector limb of the protective cellular immunity although CD4⁺ T cells are also involved. The protective activity of the T cells is predominantly mediated by IFN- γ . Both CD4⁺ and CD8⁺ T cells infiltrate the brain of mice following infection, and the T cells are the main source of IFN- γ (17–19).

T cells and IFN- γ are essential for maintaining the latency of the chronic infection in the brain and preventing reactivation of infection (TE). CD8⁺ T cells are known to be the major mediator of resistance, and this is consistent with evidence that the *H-2L^d*, a MHC Class I gene, confers resistance to TE in mice (20, 21). The protective activity of the T cells is through their production of IFN- γ (22). T cells bearing T cell receptor V β 8 are the most abundant population that produces IFN- γ in the brains of infected BALB/c mice (23). Furthermore, adoptive transfer of V β 8⁺ T cells alone into infected nude mice prevents the development of TE (23, 24). When immune V β 8⁺ T cells were divided into CD4⁺ and CD8⁺ T cell populations, the CD8⁺ population conferred much greater resistance to development of TE than did the CD4⁺ population (23). The protective activity of total V β 8⁺ T cells was greater than that of CD8⁺V β 8⁺ T cells (23). Therefore, the CD8⁺ population plays a major role in the activity of V β 8⁺ immune T cells against reactivation of infection in the brain although the CD4⁺ population works additively or synergistically with the CD8⁺ population. An importance of CD4⁺ T cells for optimum IFN- γ production by cerebral CD8⁺ T cells was also shown in CB6 (BALB/c $\times$ C57BL/6) mice infected with *T. gondii* (25). The mechanisms by which CD4⁺ T cells enhance IFN- γ production and protective activity of CD8⁺ T cells are unclear at this moment. One possible mechanism is that IL-4 produced by CD4⁺ T cells upregulates IL-12 production of dendritic cells. IL-4 alone or together with IFN- γ efficiently enhances the production of bioactive IL-12 in mouse and human DC (26) (see “IL-4” section below for additional information). Since IL-12 is important for the maintenance of IFN- γ production in T cells mediating resistance to chronic infection (27), it is possible that IL-4 produced by CD4⁺ T cells is involved in the activity of this T cell population to enhance IFN- γ production by CD8⁺ T cells during the chronic stage of *T. gondii* infection. This possibility is supported by evidence that STAT6, a molecule involved in IL-4 signaling, is important for activation of CD8⁺ T cells and their IFN- γ production (28).

In regard to the protective activity of CD4⁺ T cells, it was previously shown that adoptive transfer of CD4⁺ immune T cells conferred a partial protection against reactivation of infection in the recipient athymic nude mice whereas the same number of a total population of immune T cells completely prevented the reactivation of infection (29). Therefore, a large number of CD4⁺ immune T cells alone could confer a protection against reactivation of *T. gondii* infection. However, it is unclear how long the protective effect lasts in the absence of CD8⁺ T cells. The presence of both T cell subsets is critical for long-term maintenance of the latency of the chronic infection in the brain.

A number of signaling molecules have been shown to be important for induction and/or maintenance of the protective T cell responses during infection with *T. gondii*. In T cell receptor signaling in response to the parasite, mice deficient in two Tec kinases, Rlk and Itk, had increased mortality associated with increased brain cyst numbers and decreased IFN- γ

production by splenocytes following in vitro stimulation with a low dose of *T. gondii* antigens (30). Protein kinase C- θ (PKC- θ) is another enzyme involved in the signaling in the T cell response. Infection of mice deficient in this enzyme (*Pkc θ ^{-/-}*) resulted in impaired production of IFN- γ in both CD4⁺ and CD8⁺ T cells, and the animals succumbed to necrotizing TE (31). The impaired IFN- γ production by T cells in infected *Pkc θ ^{-/-}* mice is associated with decreased activation of transcription factors including nuclear factor (NF)- κ B, AP-1, and MAPK pathways. T cell expression of Myeloid differentiation factor 88 (MyD88) is also important for antigen-specific IFN- γ production by T cells after infection (32), although the mechanisms by which MyD88 mediates the T cell response need to be determined.

In regard to NF- κ B family transcription factors, RelB, c-Rel, NF- κ B1, and NF- κ B2 are all involved in regulating T cell responses to *T. gondii* infection (33–36). For example, infected NF- κ B1-deficient mice developed TE associated with a local decrease in the number of CD8⁺ T cells and IFN- γ production (36). A transfer of naïve T cells from the deficient animals to SCID mice conferred less protection against infection than the T cells from wild-type animals. c-Rel-deficient mice survived the acute phase of infection but developed severe TE associated with decreased numbers of CD4⁺ T cells and reduced production of IFN- γ in the their brains during the later stage of infection (34).

Regarding epitopes recognized by CD8⁺ T cells, H-2^d-restricted epitopes of GRA6, GRA4, ROP7, SAG1 and SAG3 of *T. gondii* are identified in mice (37–40). The reactivity of CD8⁺ T cells to GRA4, GRA6, and ROP7 peaked 2, 4, and 6–8 weeks after infection (37, 39), and these changes would probably reflect changes in antigens available in association with conversion of *T. gondii* from the tachyzoite to the cyst stage during the course of infection. The GRA6 epitope, HF10, has a potent activity to stimulate IFN- γ production by CD8⁺ T cells obtained from infected mice with H-2^d haplotype, and an immunization with this epitope peptide prevented mortality of B10.D2 (H-2^d), but not C57BL/6 (H-2^b) mice after challenge infection (37). Recently, an H-2^b-restricted epitope of tgd057 was also identified (40, 41). Tgd057-specific CD8⁺ T cells obtained from ES-cloned mice following somatic cell nuclear transfer of individual nuclei from tgd057-tetramer⁺ CD8⁺ T cells into ES cells also mediated a significant protection to lethal challenge infection when transferred into recipient C57BL/6 mice (40). In humans, HLA-A02 supertype-restricted epitopes of SAG2C, SAG2D, SAG2X, SAG3, GRA6, GRA7, MIC1, MICA2P, and SPA, and HLA-A03 supertype-restricted epitopes of SAG1, SAG2C, GRA6, GRA7 and SPA were recently identified (42–44). In addition, an immunization of transgenic mice expressing these human HLA Class I molecules (HLA-A0201 or HLA-A1101 [an HLA-A03 supertype allele]) with those identified epitope peptides conferred a protection associated with reduced parasite burden against challenge infection (42, 45). Therefore, these epitopes appear to be promising candidates for human vaccine to induce the protective immune responses against *T. gondii* infection.

With the use of *T. gondii* transfected to express ovalbumin (OVA) and OT-1 CD8⁺ T cells specific to OVA peptide in conjunction with two-photon microscopy of living brain tissue, antigen-specific cerebral CD8⁺ T cells are shown to make transient contacts with granuloma-like structures containing parasites and with individual CD11b⁺ antigen-presenting cells (46). Another study showed that movement of brain infiltrating OT-1 T cells is closely associated with an infection-induced reticular system of fibers (47). In the study, 7 to 14 days after a transfer of OT-1 cells into infected mice, a reduction in parasite burden in the brains of the recipients occurred (47). However, the parasite burden gradually increased thereafter in association with an increase in PD-1 expression in the transferred OT-1 cells, suggesting that T cells recruited to the brain during *T. gondii* infection downregulate their ability to act as effector cells over time.

Importance of IFN- γ production by innate immune cells for controlling tachyzoites in the brain

In addition to T cells, IFN- γ production by cells other than T cells was suggested to be important for prevention of reactivation of *T. gondii* infection in the brain (TE) in chronically infected mice (29). In the study, athymic nude, SCID and IFN- γ -deficient (*Ifng*^{-/-}) mice were infected with *T. gondii* and treated with sulfadiazine to establish chronic infection and received adoptive transfer of immune spleen or T cells from infected wild-type mice. After discontinuation of sulfadiazine to initiate reactivation of infection, infected athymic nude and SCID mice did not develop TE, whereas the infected IFN- γ -deficient mice developed the disease (29). Before cell transfer, IFN- γ mRNA was detected in brains of the nude and SCID mice but not in brains of the *Ifng*^{-/-} mice. IFN- γ mRNA was also detected in brains of infected SCID mice depleted of NK cells, and such animals did not develop TE after receiving the immune cells (29). These results suggest that IFN- γ production by non-T, non-NK cells, in addition to T cells, is important for prevention of reactivation of *T. gondii* infection in the brain. CD11b⁺ CD45^{low} microglia and CD11b⁺ CD45^{high} blood-derived macrophages are identified as the major non-T, non-NK cells which express IFN- γ in the brains of mice infected with *T. gondii* (48, 49). Therefore, 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. Since microglia reside within the brain parenchyma, IFN- γ production by these brain-residing cells may play an important role as an innate defense system that senses the early stage of tachyzoite proliferation and upregulates chemokine production to facilitate T cell accumulation into the site of the parasite growth (see “Regulatory role of IFN- γ in recruitment of T cells into the brain” for the role of IFN- γ in chemokine expression). Information regarding the production of IFN- γ by microglia in response to *T. gondii* is still limited at this moment. However, microglial expression/production of this cytokine has been observed in response to various stimuli, including lipopolysaccharide (LPS), beta-amyloid protein structure, and IL-12 (50–52). Microglial expression of IFN- γ was also observed in the brain samples from multiple sclerosis patients (53). Therefore, it appears that microglia could produce this cytokine in various disease settings. Further studies are needed to define how IFN- γ production by microglia contributes to prevention of TE in collaboration with T cells.

IFN- γ -mediated effector mechanisms to control tachyzoites in the brain

1) Microglia

Microglia appear to be major effector cells in prevention of proliferation of *T. gondii* tachyzoites in the brain. Both human (54) and murine (55) microglia become activated in vitro to inhibit intracellular proliferation of tachyzoites following treatment with IFN- γ plus LPS. TNF- α and IL-6 are involved in activation of human microglia (54). Nitric oxide (NO) mediates the inhibitory effect of activated murine microglia on intracellular replication of tachyzoites since treatment of these cells with N^G-monomethyl-L-arginine (which blocks the generation of NO) ablates their inhibitory activity (56). In murine microglia activated by a combination of IFN- γ and TNF- α , both NO-dependent and -independent mechanisms are involved in their resistance (57). In contrast to these observations in murine microglia, it was reported that NO is not involved in the inhibitory effect of activated human microglia against *T. gondii* (54). In vivo, following *T. gondii* infection, microglia become activated to produce TNF- α , and IFN- γ mediates the activation (58). IFN- γ -mediated activation of microglia in collaboration with autocrine TNF- α is likely one of the resistance mechanisms of the brain against *T. gondii*.

Granulocyte-macrophage colony stimulating factor (GM-CSF) and transforming growth factor- β (TGF- β) are other cytokines which appear to be involved in the induction of effector functions of microglia. Expression of mRNA for these cytokines increases in the brain following infection (59, 60). Murine microglia can be activated by treatment with GM-CSF (61) or a combination of IFN- γ and TGF- β (55) in vitro and inhibit intracellular multiplication of tachyzoites. The inhibitory effect of microglia activated by GM-CSF is due to their synthesis of NO (61).

CD200/CD200 receptor (CD200R) interaction negatively regulates microglial function during *T. gondii* infection (62). Infection induces an upregulation of CD200R on microglia in the brain. Infected CD200-deficient mice have significantly increased numbers of microglia as well as increased expression of TNF and inducible NO synthase (NOS2) when compared to infected wild-type animals. The increased microglial activation is associated with moderately decreased intracerebral parasite burden and an increased survival rate.

The presence of an IFN- γ -independent CD40-autophagy pathway to control intracellular replication of tachyzoites in microglia was recently reported (63). Microglia from mice lacking the autophagy molecules, Beclin 1 or Atg7, failed to kill intracellular tachyzoites in vitro after stimulation through CD40, whereas these microglia were able to kill the parasite when stimulated with IFN- γ and TNF- α . In addition, these mutant mice are more susceptible to cerebral and ocular toxoplasmosis than wild-type animal despite upregulation of IFN- γ , TNF- α , and NOS2.

2) Astrocytes

Astrocytes are another effector cell population in prevention of *T. gondii* growth in the brain. Human astrocytes become activated following treatment with IFN- γ plus IL-1 β to inhibit intracellular proliferation of tachyzoites (64). This inhibitory effect is mediated by NO. TNF- α induced a significant reduction in intracellular multiplication of the parasite in human astrocytoma-derived cells whereas IL-1 α induced an increase in parasite multiplication (65). TNF- α and IFN- γ were synergistic in activation of indoleamine 2,3-dioxygenase (IDO) in human glioblastoma cell lines and native astrocytes (66). This IDO activity resulted in a strong toxoplasmostatic effect in glioblastoma cells activated by treatment with a combination of these cytokines (66).

Murine astrocytes are also able to inhibit proliferation of tachyzoites in vitro. Pretreatment of murine astrocytes with IFN- γ resulted in 65% inhibition of tachyzoite replication (67). In contrast to human astrocytes, the inhibitory effect of activated murine astrocytes is not mediated by NO or by IDO (67) but by Irgm3 (IGTP), one of p47 GTPases (68). Following infection of IFN- γ -activated astrocytes with tachyzoites, multiple p47 GTPases, including Irga6 (IIGP1) and IGTP, accumulate at vacuoles containing the parasite (69, 70). Vacuolar accumulations of these two p47 GTPases culminate in disruption of the parasitophorous vacuoles (the vacuole containing tachyzoites) and finally of the parasite itself. IGTP as well as IIGP1 in part are required for the IFN- γ -mediated restriction of *T. gondii* growth in murine astrocytes (69, 70). The importance of astrocytes in controlling *T. gondii* in the brain was demonstrated in GFAP-Cre gp130^{fl/fl} mice lacking expression of gp130, the signal-transducing receptor for IL-6 family cytokines, in their astrocytes (71). Infected GFAP-Cre gp130^{fl/fl} mice developed a lethal necrotizing TE associated with loss of GFAP⁺ astrocytes in inflammatory lesions and impaired parasite control. In vitro studies with GFAP-Cre gp130^{fl/fl} astrocytes showed that they inhibited growth of *T. gondii* efficiently after stimulation with IFN- γ , but neighboring uninfected cells became apoptotic. Therefore, astrocytic gp130 expression is crucial for survival of GFAP⁺ astrocytes in the brain of infected mice, and this protective effect of gp130 in astrocytes appears to contribute to controlling cerebral proliferation of tachyzoites.

3) Macrophages

Activation of macrophages by IFN- γ is critical for controlling tachyzoite proliferation during the acute stage of *T. gondii* infection (2). Since macrophages, in addition to T cells, migrate into the brain following infection (48, 72, 73), this cell population is likely involved in resistance to cerebral toxoplasmosis. NO production by NOS2 was first shown to be a mechanism of macrophages activated by IFN- γ to restrict tachyzoite growth in vitro (74). However, recently an importance of IGTP for their protective activity became clear. IGTP is required for killing of tachyzoites by macrophages activated by IFN- γ (75, 76). Studies using peritoneal macrophages from mice primed with live attenuated tachyzoites showed that the IFN- γ -induced, IGTP-mediated killing was preceded by parasitophorous vacuole indentation, vesiculation, disruption, and stripping of the parasite plasma membrane (76). Denuded parasites are then enveloped in autophagosome-like vacuoles, which ultimately fuse with lysosomes (76). In peritoneal macrophages activated in vitro by IFN- γ plus LPS, autophagy protein Atg5 is required for recruitment of IIGP1 to parasitophorous vacuole membrane and parasite clearance (77). However, autophagosomes enveloping tachyzoites were not detected in these cells. Differences in how macrophages become activated might be related to the different observations in these studies.

P2X₇-mediated killing of tachyzoites by human and murine macrophages was recently reported (78). P2X₇ receptor activation by extracellular ATP kills tachyzoites in infected cells, and this killing occurs in parallel with host cell apoptosis. Although this killing mechanism itself does not require IFN- γ , P2X₇ receptor expression can be upregulated by this cytokine. Therefore, this mechanism may also play a role as a part of IFN- γ -mediated resistance to *T. gondii*. An importance of P2X₇ in resistance to the parasite is supported by evidence for association of polymorphisms of the gene *P2RX7*, which encodes P2X₇ receptor, with clinical signs of congenital toxoplasmosis (79).

Regulatory role of IFN- γ in recruitment of T cells into the brain

T cells need to enter the brain to demonstrate their protective activity against TE, although the blood-brain barrier limits access of lymphoid cells from the periphery. IFN- γ plays an important role in induction of vascular cell adhesion molecule-1 (VCAM-1) on cerebral vessels and recruitment of immune T cells into the brain in TE-resistant BALB/c mice during the chronic stage of infection (80). Numbers of VCAM-1 expressing vessels were significantly greater in brains of infected wild-type mice than in brains of the IFN- $\gamma^{-/-}$ mice, and significantly fewer CD8⁺ T cells were recruited into brains of the latter than the former animals. Treatment of infected IFN- $\gamma^{-/-}$ 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 adoptive transfer of immune spleen cells, pre-treatment of the cells with α 4 integrin mAb markedly inhibited recruitment of CD8⁺ T cells into the brain of chronically infected wild-type mice. Therefore, IFN- γ -induced expression of endothelial VCAM-1 and its binding to α 4 β 1 integrin on CD8⁺ T cells is important for recruitment of the T cells into the brain during the chronic stage of *T. gondii* infection. By using a similar cell transfer method, an importance of α 4 β 1 integrin for T cell recruitment into the brain was also observed in TE-susceptible C57BL/6 mice (47). On the other hand, a TE-susceptible strain of mouse with neonatal inactivation of VCAM-1 gene did not show an impaired leukocyte recruitment into the brain after infection (81). These results may suggest that VCAM-1-mediated T cell recruitment into the brain during *T. gondii* infection could be compensated for by other cell adhesion molecules over time, or neonatal inactivation of VCAM-1 may have resulted in expression of a molecule, which is not usually expressed in wild-type mice, to compensate for the absence of VCAM-1.

In addition to the regulatory activity of IFN- γ on expression of VCAM-1, this cytokine plays an important role in induction of expression of various chemokines in the brains of mice during acute systemic (82, 83) and chronic infection (84) with *T. gondii*. During the chronic stage of infection, CXCL9, CXCL10, and CCL5 are abundant in both mRNA and protein levels in the brains of infected BALB/c mice (84). Combined in situ hybridization and immunohistochemistry demonstrated an expression of CXCL10 and CCL2 in astrocytes and of CXCL9 and CCL5 in microglia (85). CXCR3, the receptor for CXCL9 and CXCL10, is expressed predominantly on activated T cells, and this chemokine receptor is considered to play the primary role in recruitment of effector T cells into the site of Th1-type immune response (86, 87). CCL5 is one of three ligands that bind to CCR5, and in addition to CXCR3, CCR5 is preferentially expressed on Th1 cells and plays an important role in infiltration of these T cells in Th1-type reactions (88). CCR5 is also expressed on macrophages, which are important effector cells activated by IFN- γ to prevent proliferation of *T. gondii* tachyzoites (2). Therefore, an expression of CCL5 in combination with CXCL9 and CXCL10 could play an important role in recruiting T cells to maintain a type 1 immune response and facilitating migration of effector macrophages into the brain to control the parasite. During the acute stage of *T. gondii* infection, CXCL10 has been shown to play an important role in inducing massive influx of T cells into the lungs and livers and controlling the parasite in these organs (89). Another chemokine receptor, CCR2, the only known functional receptor for CCL2, is also involved in regulating cell migration into the brain following *T. gondii* infection (90). CCR2-deficient (CCR2^{-/-}) mice presented greater parasitic burden associated with reduced CD4⁺ and Mac1⁺ and increased CD8⁺ cell migration in the peripheral organs during the acute phase and in the brain at the initial phase of chronic infection. Infected CCR2^{-/-} mice also had reduced NOS2⁺ cell numbers in these organs.

In addition to the adhesion molecules and chemokines described above, metalloproteinases (MMPs) and their inhibitor (tissue inhibitor of metalloproteinase-1; TIMP-1) appear to be involved in regulation of T cell infiltration into the brain during *T. gondii* infection. An expression of MMP-8, MMP-10 and TIMP-1 is upregulated in the brains of mice after *T. gondii* infection, and infected TIMP-deficient mice have decreased perivascular accumulation of lymphocyte populations, increased CD4⁺/CD8⁺ ratio, and decreased parasitic burden in the brain (91). The mechanisms by which MMPs and TIMP-1 regulate T cell trafficking into the brain during infection remain to be elucidated.

IL-6 also appears to be involved in cerebral migration of T cells during *T. gondii* infection. Lymphocyte preparations isolated from brains of infected *IL6*^{-/-} mice had significantly lower ratios of $\gamma\delta$ T cells and CD4⁺ $\alpha\beta$ T cells but higher ratios of CD8⁺ $\alpha\beta$ T cells than those of infected control mice (18). Of interest, no differences were detected in the ratios of these T cell subsets in spleens between these animals (18). These results suggest that IL-6 is another player that regulates T cell infiltration into the brain during *T. gondii* infection, although the mechanisms of this regulatory function of IL-6 are unclear.

Participation of cytokines other than IFN- γ in controlling *T. gondii* tachyzoites in the brain

1) IL-12

Interleukin (IL)-12 is critical for induction of IFN- γ production following infection with *T. gondii*. Neutralization of IL-12 with antibodies to this cytokine results in 100% mortality in mice infected with an avirulent strain of *T. gondii*, and the mortality is associated with decreased IFN- γ production (92). Dendritic cells, macrophages and neutrophils produce IL-12 in response to the parasite. In dendritic cells, a binding of a profilin-like protein of the

parasite to Toll-like receptor (TLR) 11 stimulates their IL-12 production (93). Mice deficient in the gene for MyD88, an adaptor molecule essential for most TLR as well as IL-1 and IL-18 signaling, are susceptible to acute infection with an avirulent strain and their susceptibility is associated with impaired IL-12 responses to the parasite (94). The binding of IL-12 to its receptor leads to the activation of signal transducer and activator of transcription (STAT) 4, and this signaling cascade is crucial for IFN- γ -producing cells. In agreement with this, STAT4-deficient mice are susceptible to acute infection with *T. gondii* and their mortality is associated with a defect in the ability to produce IFN- γ in response to infection (95). Tp12 (Tumor progression locus 2) belonging to p38 mitogen-activated protein kinase is also involved in this IL-12-induced IFN- γ production (96). In addition to the importance of IL-12 for induction of IFN- γ production by T cells, IL-12 is also important for the maintenance of IFN- γ production in T cells mediating resistance to chronic infection (27).

Lipoxin A₄ (LXA₄), an eicosanoid product generated from arachidonic acid, is an important downregulator of IL-12 production to prevent pathogenic inflammatory responses in the brain during the chronic stage of *T. gondii* infection. Wild-type mice produced high levels of serum LXA₄ beginning at the onset of chronic infection. 5-lipoxygenase (5-LO) is an enzyme critical in the generation of LXA₄, and mice deficient in 5-LO (*Alox5*^{-/-}) succumbed to infection during the chronic stage displaying a marked inflammation in their brains (97). The increased mortality in the (*Alox5*^{-/-}) animals is not due to defective control of the parasite but due to enhanced inflammatory responses associated with elevations of IL-12 and IFN- γ , and their mortality is completely prevented by the administration of a stable LXA₄ analogue. Therefore, LXA₄ is important for down-regulation of proinflammatory responses during the chronic stage of *T. gondii* infection. Recent studies showed that lipoxins activate two receptors (AhR and LXAR) in dendritic cells, and that this activation triggers expression of suppressor of cytokine signaling (SOCS)-2. SOCS-2-deficient mice succumb to chronic infection with *T. gondii* in association with elevated IL-12 and IFN- γ responses and reduced brain cysts (98), as observed in *Alox5*^{-/-} animals (98).

2) IL-4

Although the IFN- γ -mediated immune responses play the essential role in resistance to *T. gondii* infection, Th2-type immune responses are also involved in the protective immunity. IL-4-deficient (*Il4*^{-/-}) mice all died during the late stage of infection whereas control mice all survived (99). Histological study revealed significantly greater numbers of cysts and areas of acute focal inflammation associated with tachyzoites in brains of *Il4*^{-/-} than control mice during the later stage of infection (99). These results indicate that IL-4 is protective against development of TE by preventing formation of cysts and proliferation of tachyzoites in the brain. Spleen cells of control mice at 8 weeks after infection were found to produce significantly greater amounts of IFN- γ following stimulation in vitro with *T. gondii* antigens than those of *Il4*^{-/-} mice (99). Therefore, IL-4 plays a role to enhance IFN- γ production during the late stage of infection. The impaired ability of *Il4*^{-/-} mice to produce IFN- γ likely contributes to their susceptibility for development of severe TE.

STAT6 is a molecule involved in IL-4 and IL-13 signaling. In agreement with the observations in *Il4*^{-/-} mice, significantly greater numbers of *T. gondii* cysts were recovered from the brains of STAT6-deficient (*Stat6*^{-/-}) than wild-type mice. CD8⁺ T cells obtained from cerebrospinal fluids and spleens of infected wild-type mice produced greater amounts of IFN- γ than the T cells from infected *Stat6*^{-/-} animals (28). In addition, a transfer of CD8⁺ T cells from wild-type to *Stat6*^{-/-} mice reduced cyst numbers in the brains of the recipients. Transfer of splenic adherent cells from wild-type to *Stat6*^{-/-} mice induced activation of CD8⁺ T cells and decreased brain cyst numbers. Therefore, STAT6 signaling is

important in CD8⁺ T cell activation following *T. gondii* infection, possibly through regulation of the activity of antigen-presenting cells. In this regard, it has been shown that IL-4 alone or together with IFN- γ efficiently enhances the production of bioactive IL-12 in mouse and human DC (26). A lack of this positive regulatory effect of IL-4 on IL-12 production by DC most likely contributes to the reduced resistance of *Il4*^{-/-} and *Stat6*^{-/-} mice to *T. gondii* infection described above.

Another study reported that greater numbers of cysts and more severe histological changes were observed in brains of control than *Il4*^{-/-} mice in the late stage of infection although the former mice were more resistant against death during the acute stage than the latter mice (100). Since the strains of *T. gondii* differ between the studies mentioned, the strains and virulence of the parasite may have contributed to the different outcomes in the studies.

3) IL-10

IL-10 is an important negative regulator of inflammatory responses (101). IL-10-deficient (*Il10*^{-/-}) mice all die during the acute stage of infection with *T. gondii* and their mortality is associated with development of severe immunopathology mediated by Th1 immune responses in the liver (102) and intestine (103). Therefore, IL-10 is crucial for down-regulating IFN- γ -mediated immune responses and preventing development of pathology caused by the immune responses. When IL-10^{-/-} mice were treated with sulfadiazine to control proliferation of *T. gondii* in the early stage of infection, the animals survived the acute stage but developed lethal inflammatory responses in their brains in the later stage of infection (73). The importance of IL-10-dependent signaling for survival of mice during the acute and chronic stages of infection is confirmed by treating infected wild-type animals with anti-IL-10 receptor antibody (104). Therefore, IL-10 plays an important down-regulatory role to prevent immunopathology and mortality during the course of infection with *T. gondii*.

Various populations of T cells, such as Th2 and T_{reg}, are able to produce IL-10. However, conventional IFN- γ -secreting T-bet⁺Foxp3⁻ Th1 cells were shown to be the major producers of IL-10 in *T. gondii*-infected mice. These IL-10⁺IFN- γ ⁺ CD4⁺ T cells possess potent activity to prevent intracellular multiplication of tachyzoites within macrophages, while profoundly suppressing IL-12 production by antigen-presenting cells (104). In addition, these IL-10-producing T cells are generated from IL-10⁻ IFN- γ ⁺ CD4⁺ T cells following re-stimulation with tachyzoite antigens. These results indicate that IL-10 production by CD4⁺ T cells after infection with *T. gondii* does not require a distinct regulatory Th subset but can be generated in Th1 cells as a part of the effector response to the parasite, which provides a crucial negative feedback loop for prevention of pathogenic over-stimulation of the Th1 response.

There is another interesting evidence on the negative feedback loop of Th1 response in *T. gondii* infection. Tyk2, a member of Jak family of nonreceptor tyrosine kinases, mediates the biological effects of IL-12 and IFN- α β and promotes IFN- γ production by Th1 cells, and Tyk2-null mice are susceptible to infection with the parasite. However, Tyk2 is also required for production of IL-10 by immune CD4⁺ T cells after challenge infection of vaccinated mice (105). The Tyk2-dependent IL-10 production is mediated by IFN- γ , indicating a negative autoregulation of Th1 effector response in infection. Therefore, Tyk2 plays a dual-function mediating induction and suppression of Th1 effector response, most likely for maximizing pathogen clearance while minimizing immunopathology.

4) IL-17 and IL-27

A unique subset of CD4⁺ T cells, “Th-17”, produces IL-17 and this T cell population has been suggested to mediate inflammation in models of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. In infection with *T. gondii*, IL-17 has been shown to play both protective and pathogenic roles. IL-17 receptor-deficient mice are more susceptible to acute infection with the parasite (106). Their mortality was associated with increased parasite burden in the organs including the brain and with a defect in the migration of neutrophils to infected sites during the early stage of infection. Therefore, IL-17-mediated induction of neutrophil migration to the infected sites during the initial stage of infection appears to play an important role in resistance against acute infection with *T. gondii*. However, the activity of Th-17 cells needs to be appropriately down-regulated by IL-27 to prevent the development of severe inflammatory changes in the brain during the later stage of infection. Chronically infected IL-27 receptor-deficient mice developed severe inflammation in their brain mediated by CD4⁺ T cells and the pathology was associated with a prominent IL-17 response (107). In addition, treatment of naïve primary T cells with IL-27 *in vitro* suppressed the development of Th-17 cells induced by IL-6 and TGF- β , and the suppressive effect was dependent on the intracellular signaling molecule STAT1 (107). Therefore, IL-27 plays a crucial role in prevention of Th-17-mediated inflammatory responses in the brain during the chronic stage of *T. gondii* infection.

5) IL-33

IL-33 is a member of the IL-1 family with the ability to downregulate IFN- γ production by Th1 cells and upregulate Th2 response. Mice deficient in T1/ST2, a component of the IL-33 receptor, demonstrated increased susceptibility to *T. gondii* infection that correlated with increased pathology and greater parasite burden in the brain (108). Real-time PCR analysis of cerebral cytokine levels revealed increased mRNA levels of IFN- γ , TNF- α , and NOS2 in infected T1/ST2-deficient animals. The mechanisms mediated by IL-33 receptor to control *T. gondii* in the brain remain to be analyzed.

Immune responses to the cyst stage of *T. gondii* in the brain and requirement of perforin-mediated activity in anti-cyst immune responses by CD8⁺T cells

The basis of persistence of chronic infection with *T. gondii* is the tissue cyst, which remains largely quiescent for the life of host, but can reactivate and cause disease. This stage of the parasite is not affected by any of the current drug treatments and has been generally regarded as untouchable. However, we recently revealed that the immune system can eliminate *T. gondii* cysts from the brains of infected hosts when immune T cells are transferred into infected immunodeficient animals that have already developed large numbers of the cysts (12). This T cell-mediated immune process was associated with accumulation of microglia and macrophages around tissue cysts. Since the accumulated phagocytes penetrate within the cyst, these cells appear to be the main effector cells that destroy the cysts and eliminate them from the brain after initiation of this process by immune T cells. CD8⁺ immune T cells possess a potent activity to remove the cysts. Of interest, the initiation of this process by CD8⁺ T cells does not require their production of IFN- γ , the major mediator to prevent proliferation of tachyzoites during acute infection, but does require perforin. Perforin is the major molecule that mediates cytolysis of target cells by CD8⁺ cytotoxic T cells. Therefore, our results suggest that CD8⁺ T cells induce elimination of *T. gondii* cysts through their perforin-mediated cytotoxic activity. In relation to our observation, a study using two photon microscopy in C57BL/6 mice with ovalbumin-expressing *T. gondii* showed that ovalbumin-specific CD8⁺ T cells accumulated in regions

containing isolated parasites (tachyzoites) but not around cysts in the brain. C57BL/6 mice are genetically susceptible to chronic infection with the parasite and continuous tachyzoite proliferation occurs in their brain. Therefore, it is possible that the activity of cerebral CD8⁺ T cells of these animals is downregulated in this environment and does not have a potent anti-cyst activity.

Perforin-mediated cytolytic activity by T cells in resistance against *T. gondii* infection was less appreciated before, when compared to the absolute requirement of IFN- γ to control tachyzoite proliferation during the acute stage of infection. Perforin-knockout mice survive acute infection (22, 109). *In vitro* studies demonstrated that the lysis of tachyzoite-infected cells by cytotoxic CD8⁺ T cells results in release of viable parasites (110). However, the situation of the parasite in the cyst stage seems different. Whereas the cyst resides within an infected cell (111, 112) as do tachyzoites, bradyzoites within the cyst are surrounded by a thick cyst wall, which is unique to the cyst. Therefore, cytolysis of cyst-containing cells by perforin-mediated activity of CD8⁺ T cells followed by quick accumulation of large numbers of microglia and macrophages would probably provide the parasite little time to escape from the coordinated attack by the T cells and phagocytes. Denkers *et al* (109) reported a three- to fourfold increase in brain cyst numbers in perforin-knockout mice in the later stage of infection. The absence of perforin-dependent anti-cyst activity of CD8⁺ T cells in these animals may have contributed to their observation.

Outstanding research questions

IFN- γ -mediated immune responses are essential for controlling proliferation of tachyzoites during the acute stage of infection and for prevention of reactivation (TE) of infection with *T. gondii*. For maintaining the latency of chronic infection in the brain and prevention of TE, IFN- γ production by T cells is essential. CD8⁺ T cells are the major mediator of the protective immunity, and CD4⁺ T cells are important for optimum IFN- γ production by cerebral CD8⁺ T cells. However, the detailed role of each of CD4⁺ and CD8⁺ T cell populations and their interactions in the protective immunity in the brain during the course of infection still remains to be elucidated. Recent discovery of H-2^d- and H-2^b-restricted CD8⁺ T cell epitopes provides a powerful tool for this analysis. However, additional information regarding *T. gondii* epitopes for both CD4⁺ and CD8⁺ will facilitate to gain better understanding of the development and maintenance of the protective T cell responses in the brain during the course of infection in association with conversion of *T. gondii* from the tachyzoite to cyst stage. It is also important to depict how T cell responses differ between TE-resistant and -susceptible hosts. We also need better understanding of the mechanisms of recruitment of T cells into the brain through the blood brain barrier and into the site of parasite growth within the brain.

In regard to *T. gondii* epitopes recognized by human CD8⁺ T cells, HLA-A0201- and HLA-A03-restricted epitopes were recently identified. In addition, an immunization of transgenic mice expressing these human HLA Class I molecules (HLA-A0201 or HLA-A1101 [an HLA-A03 supertype allele]) with those identified epitope peptides conferred a protection associated with reduced parasite burden against challenge infection. These epitopes appear to be promising candidates for human vaccine to induce the protective immune responses. Utilizing the information from murine models and searching for additional or more dominant epitopes could improve the efficacy of the vaccination to confer protection to *T. gondii* infection.

In regard to effector mechanisms to control *T. gondii* in the brain, multiple cell types, such as microglia, astrocytes, and macrophages, become activated by IFN- γ to prevent intracellular proliferation of tachyzoites through NO, IDO, p47 GTPases, and/or unidentified

molecules. IFN- γ -independent mechanisms that require CD40 or P2X₇ receptor were recently revealed to participate in controlling tachyzoites in microglia and macrophages, respectively. However, the information currently available is mostly derived from in vitro studies using these cell types. The role of these effector mechanisms to control tachyzoite growth in each of microglia, astrocyte, neuron, and macrophage in vivo are needed to understand the mechanisms to control *T. gondii* in the brain.

In addition to the IFN- γ -mediated protective immunity against tachyzoites, it was recently revealed that the immune system uses perforin-mediated activity of CD8⁺ T cells to remove tissue cysts from the brain. Therefore, the resistance to *T. gondii* in the brain requires a coordinated collaboration of IFN- γ - and perforin-mediated immune responses. It is crucial to elucidate how perforin-mediated activity of CD8⁺ T cells eliminates *T. gondii* cysts from the brain, and how the IFN- γ - and perforin-mediated protective mechanisms are coordinated to compose effective resistance to the parasite in the brain. Elucidating these mechanisms will be crucial to develop a new method to prevent and eradicate *T. gondii* infection.

Acknowledgments

We thank the peer reviewers for their critical reading of this manuscript and helpful comments. This work is supported by grants from National Institutes of Health (AI073576, AI078756, and AI077887) and from The Stanley Medical Research Institute (08R-2047). We appreciate Sara Perkins and James Hester for their assistance in preparing the manuscript.

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Further reading and resources

Yarovinsky, F. 2008. Toll-like receptors and their role in host resistance to *Toxoplasma gondii*. Immunol. Lett. 119: 17–21. This article provides an overview on the role of Toll-like receptors in resistance to *T. gondii* infection.

Munoz, M., O. Liesenfeld, M.M. Heimesaat. Immunology of *Toxoplasma gondii*. Immunol. Rev. 2011. 240: 269–285. This article provides an overview of the immune responses to *T. gondii* in general, including the immune responses in the intestine and those in congenital and ocular toxoplasmosis.

Toxoplasma gondii database is a genome and functional genomic database in which sequence information is integrated with various genomic-scale data including community annotation, ESTs, gene expression and proteomics data.

<http://toxodb.org/toxo/>

A

B

Fig. 1.

Schematic figures of effector mechanisms mediated by IFN- γ and perforin in resistance against *T. gondii* in the brain.

Panel A. IFN- γ -mediated effector mechanisms against tachyzoites in the brain

Microglia: Microglia activated by IFN- γ plus lipopolysaccharide inhibit proliferation of tachyzoites through nitric oxide (NO) production by inducible nitric oxide synthase (NOS2). An involvement of both NO-dependent and -independent mechanisms is suggested in the resistance of murine microglia activated by a combination of IFN- γ and TNF- α . The mechanisms of the NO-independent inhibitory effect is unknown.

Astrocytes: Murine astrocytes activated by IFN- γ inhibit proliferation of tachyzoites by Irgm3 (IGTP), one of p47 GTPases. TNF- α and IFN- γ synergistically activate indoleamine 2,3-dioxygenase in human glioblastoma cell lines and native astrocytes, and this IDO activity results in a strong toxoplasmostatic effect in these activated cells.

Macrophages: NO production by NOS2 was first shown to be a mechanism of macrophages activated by IFN- γ to restrict tachyzoite growth in vitro. However, recent studies indicated that p47 GTPases such as IGTP and IIGP1 are critical for the activity of activated macrophages to restrict tachyzoite growth. Studies using peritoneal macrophages from mice primed with live attenuated tachyzoites showed that the IFN- γ -induced, IGTP-mediated killing was preceded by parasitophorous vacuole (the vacuole containing tachyzoites) indentation, vesiculation, disruption, and stripping of the parasite plasma membrane. Denuded parasites are then enveloped in autophagosome-like vacuoles, which ultimately fuse with lysosomes. In peritoneal macrophages activated in vitro by IFN- γ plus LPS, autophagy protein Atg5 is required for recruitment of IIGP1 to the parasitophorous vacuole membrane and parasite clearance.

Panel B. IFN- γ -independent effector mechanisms against tachyzoites in the brain

Microglia: CD40-CD40L interaction induces autophagy-mediated control of tachyzoites within microglia. Microglia from mice lacking the autophagy molecules, Beclin 1 or Atg7, failed to kill intracellular tachyzoites in vitro after stimulation through CD40.

Macrophages: P2X₇ receptor activation by extracellular ATP kills tachyzoites in infected macrophages, and this killing occurs in parallel with host cell apoptosis. Although this killing mechanism itself does not require IFN- γ , P2X₇ receptor expression can be upregulated by this cytokine.

Panel C. Perforin-mediated effector mechanisms against tachyzoites in the brain

Tissue cysts of *T. gondii* are formed mostly within neurons and astrocytes in the brain.

Immune T cells can eliminate the cysts from the brain when the T cells are transferred into infected immunodeficient animals that have already developed large numbers of the cysts.

The T cell-mediated immune process was associated with accumulation of microglia and macrophages around tissue cysts. Since the accumulated phagocytes penetrate within the cyst, these cells appear to be the main effector cells that destroy the cysts and eliminate them from the brain after initiation of this process by immune T cells. CD8⁺ immune T cells have a potent activity to remove the cysts, and their anti-cyst activity requires perforin. Perforin is the major molecule that mediates cytolysis of target cells by CD8⁺ cytotoxic T cells.

Therefore, CD8⁺ T cells appear to induce elimination of *T. gondii* cysts through their perforin-mediated cytotoxic activity.