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Involvement of Host Defense Mechanisms against *Toxoplasma gondii* Infection in Anhedonic and Despair-Like Behaviors in Mice

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ABSTRACT *Toxoplasma gondii* is a pathogen relevant to psychiatric disorders. We recently showed that reactivation of chronic *T. gondii* infection induced depression-like behaviors in mice. Furthermore, it has been hypothesized that depression-like behaviors are mediated via a host defense mechanism against invading pathogens; proximate mechanisms of this behavioral hypothesis remain unclear. In the present study, we investigate the contribution of indoleamine 2,3-dioxygenase (IDO), inflammation, and interferon gamma (IFN-γ) to anhedonic and despair-related behaviors in *T. gondii*-infected mice by using sucrose preference and forced-swim tests, respectively. First, we confirmed that BALB/c mice exhibited both sickness and depression-like behaviors during acute infection. Treatment of infected wild-type mice with minocycline (anti-inflammatory drug) abated sickness and anhedonic and despair-like behaviors, whereas in *T. gondii*-infected mice, treatment normalized kynurenine/tryptophan (Kyn/Trp) ratios in both plasma and brain tissue. Additionally, *T. gondii* infection failed to induce anhedonic and despair-like behaviors or increase the Kyn/Trp ratio in immunocompromised (IFN-$\gamma^{-/-}$) mice, whereas sickness behavior was observed in both immunocompetent and IFN-$\gamma^{-/-}$ mice following infection. Furthermore, treatment with 1-methyl tryptophan (an IDO inhibitor) did not affect locomotor activity, attenuated clinical scores and anhedonic and despair-like behaviors, and resulted in normal Kyn/Trp ratios in *T. gondii*-infected wild-type mice. Although low levels of serotonin and dopamine were observed in the brain during acute and chronic infections, anhedonic and despair-like behaviors were not detected in the chronic stage of infection. Collectively, our results demonstrated that immune enhancement in response to infection with *T. gondii* resulted in IFN-γ production, IDO activation, and inflammation associated with anhedonic and despair-like behaviors.

KEYWORDS *Toxoplasma gondii*, 1-methyl tryptophan, minocycline, interferon gamma, sickness behavior, anhedonic behavior, despair-like behavior

*T*oxoplasma gondii infection is linked to some mood and psychiatric disorders (1, 2). Furthermore, recent evidence indicated that reactivation of chronic *T. gondii* infection induces depression-related behaviors in mice (3). As the pathogenesis of infection relies largely upon host immunity, after *T. gondii* infection, peripheral macrophages and lymphocytes quickly become activated to kill intracellular tachyzoites (4). Sickness and depression-related behaviors are mediated by proinflammatory cytokines such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) (5–7). However, the distinction between sickness and depressive symptoms in *T. gondii* infection remains unclear. Mice given an experimental immune challenge with bacterial lipopolysaccharides (LPSs) or *Mycobacterium bovis* BCG vaccines exhibited

behaviors specific for sickness and depression-like behaviors. For example, mice exhibited sickness symptoms in the form of reduced body weight and locomotor activity and depressive symptoms such as a reduced preference for sucrose and lower motility in forced-swim and tail suspension tests (8, 9).

Genes shown to promote major depressive disorder in humans were hypothesized to be associated with successful immune responses, protection from microbes, and enhanced survival in the ancestral environment. Therefore, specific depressive symptoms have been suggested to play roles in pathogen host defense (7). IFN-γ has been linked to depressive symptoms by inducing indoleamine 2,3-dioxygenase (IDO) activation and depleting tryptophan (Trp), the only known precursor of serotonin. Increased serum IFN-γ levels in response to peripheral immune stimulation enhanced cerebral IDO activity, which may reduce Trp levels in the brains of mice (10, 11). In addition, Trp depletion is caused by the stress-induced activation of tryptophan 2,3-dioxygenase (TDO), a hepatic enzyme, and/or the ubiquitous enzyme IDO (1, 2, 4, 5). IDO catabolizes Trp into the neurotoxic metabolites kynurenine (Kyn) and kynurenic acid (12, 13). Although the catabolism of Trp is stimulated by the induction of TDO and IDO, it is still argued that a reduction in Trp blood levels under conditions of stress and inflammation decreases the formation of cerebral serotonin (13). Furthermore, circulating Kyn crosses the blood-brain barrier (BBB), whereby it elevates cerebral Kyn levels (14). Hence, circulating Trp can cross the BBB by competing with other amino acids (13). Minocycline (Mino) (expanded-spectrum tetracycline) is widely used to block the expression of proinflammatory cytokines in peripheral and central organs (15–17) and prevent ischemic neuronal death (18, 19). 1-Methyl-DL-tryptophan (1-DL-MT) has become a reference drug for blocking IDO by competing with Trp. However, only 1-L-MT inhibits the enzyme activity of IDO, while 1-D-MT does not (20). Unlike Mino, 1-DL-MT blocks IDO-mediated immune events in rheumatoid arthritis (21–23) and inhibits *T. gondii* multiplication *in vivo* (20, 24). IDO activation in the brain has been proposed to induce Kyn, which may contribute to the depressive symptoms of epilepsy, Alzheimer's disease, and cerebral malaria (25–27).

We selected BALB/c mice to examine immune enhancement, as C57BL/6 mice have an insufficient intracerebral immune response (28, 29). BALB/c mice are considered genetically resistant to *T. gondii* infection and, instead of developing acute fatal toxoplasmic encephalitis, establish a chronic latent infection (30–32). Therefore, we predict that BALB/c mice will exhibit more depression-like behaviors than sickness symptoms. We hypothesized that *T. gondii*-induced depressive behaviors were based on immune enhancement in terms of IFN-γ production, the activation of IDO, and the disruption of serotonergic neurotransmission. To test this hypothesis, we used different approaches to investigate sickness (clinical score and locomotor activity) and depressive (anhedonic and despair-like) behaviors during the acute stage of *T. gondii* infection in wild-type and IFN-γ-deficient mice (BALB/c background) in the context of treatment with Mino or 1-DL-MT to inhibit inflammation or block IDO functions, respectively. Our findings provide insight into immune enhancement associated with the development of anhedonic and despair-like behaviors during the acute stage of *T. gondii* infection.

RESULTS

Attenuation of anhedonic behavior in *T. gondii*-infected mice with Mino or 1-DL-MT treatment or IFN-γ deficiency. Reduced sucrose preference as a putative indicator of anhedonic behavior was measured by using a two-bottle preference test (Fig. 1). Initially, reduced sucrose consumption appeared 7 days after infection with *T. gondii* before reaching a peak level at 10 days postinfection (dpi) and returning to control levels after 2 weeks (Fig. 1A). This result indicates that anhedonic behavior was induced by acute infection with *T. gondii*. To test the role of the inflammatory response in anhedonic behavior, treatment of wild-type mice with an anti-inflammatory agent, Mino, was performed (Fig. 1B). Compared with the *T. gondii*–phosphate-buffered saline (PBS)-treated group, reduced sucrose consumption during acute infection was prevented by treatment with Mino. This indicates that the inflammatory response resulting
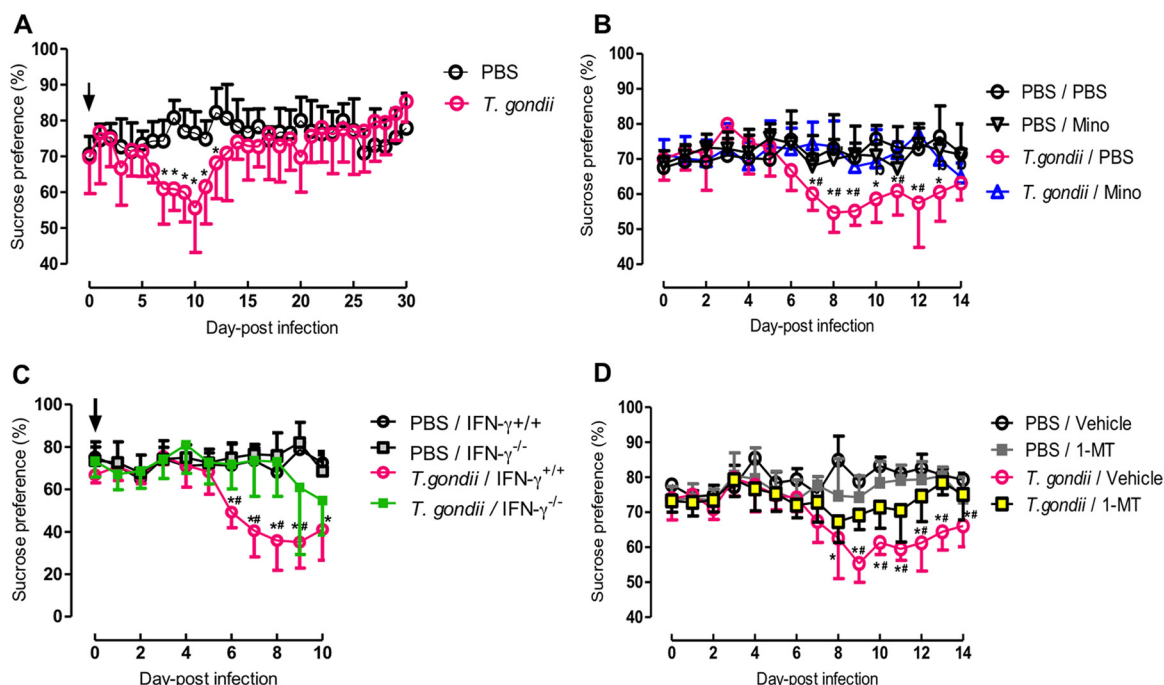
FIG 1 Analysis of anhedonic behavior by sucrose consumption following *T. gondii* infection. (A) Sucrose consumption by wild-type BALB/c mice after injection with PBS or infection with *T. gondii*. Data from two independent experiments are summarized and presented as means ± standard deviations [PBS, $n = 4$ plus 6; *T. gondii*, $n = 2$ plus 6 (two mice died due to infection during trial 1); $F_{(30, 496)} = 4.15$; $P < 0.0001$]. * indicates a significant difference between the PBS-injected and *T. gondii*-infected groups by two-way ANOVA plus Bonferroni *post hoc* analysis. (B) Sucrose consumption of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* under treatment with Mino (10 mg/kg i.p.) or injection with PBS from days 4 to 7 postinfection. Data are representative of data from two independent experiments with similar results and are presented as means ± standard deviations [$n = 6$; $F_{(42, 300)} = 3.27$; $P < 0.0001$]. * indicates a significant difference between the PBS-PBS and *T. gondii*-PBS groups, and # indicates a significant difference between the *T. gondii*-PBS and *T. gondii*-Mino groups by two-way ANOVA plus Bonferroni *post hoc* analysis. (C) Sucrose consumption of wild-type BALB/c mice (IFN-$\gamma^{+/+}$) and IFN-$\gamma^{-/-}$ mice after injection with PBS or infection with *T. gondii*. Data are representative of data from four independent experiments with similar results and are presented as means ± standard deviations [$n = 5$; $F_{(30, 176)} = 3.77$; $P < 0.0001$]. * indicates a significant difference between the PBS-IFN-$\gamma^{+/+}$ and *T. gondii*-IFN-$\gamma^{+/+}$ groups, and # indicates a significant difference between the *T. gondii*-IFN-$\gamma^{+/+}$ and *T. gondii*-IFN-$\gamma^{-/-}$ groups by two-way ANOVA plus Bonferroni *post hoc* analysis. (D) Sucrose consumption of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* under treatment with 1-MT (50 mg/kg subcutaneously) or injection with the vehicle from days 4 to 7 postinfection. Data are representative of data from two independent experiments with similar results and are presented as means ± standard deviations [$n = 6$; $F_{(42, 300)} = 4.24$; $P < 0.0001$]. * indicates a significant difference between the PBS-vehicle and *T. gondii*-vehicle groups, and # indicates a significant difference between the *T. gondii*-vehicle and *T. gondii*-1-MT groups by two-way ANOVA plus Bonferroni *post hoc* analysis.

from infection with *T. gondii* played a role in the anhedonic behavior observed in mice. Next, the effects of IFN-γ on anhedonic behavior were examined by using IFN-$\gamma^{-/-}$ mice (Fig. 1C), as IFN-γ is an important cytokine for inflammatory responses. Although reduced sucrose consumption was observed in *T. gondii*-infected wild-type mice from days 6 to 10 postinfection, infected IFN-$\gamma^{-/-}$ mice showed reduced consumption at levels similar to those of infected wild-type animals only at 10 dpi, suggesting a contribution of IFN-γ to anhedonic behavior. Since IFN-γ can activate IDO activity, we used an IDO inhibitor, 1-DL-MT, to treat anhedonic behavior in infected wild-type mice (Fig. 1D). Treatment with 1-DL-MT attenuated the reduction in the preference for sucrose from days 9 to 14 postinfection in *T. gondii*-infected mice. Collectively, these data suggest that anti-inflammatory treatment, deficiency of IFN-γ, or inactivation of IDO may prevent the onset of anhedonic behavior.

Attenuation of despair-like behavior in *T. gondii*-infected mice with Mino or 1-DL-MT treatment or IFN-γ deficiency. To test the effect of *T. gondii* infection on despair-like behavior, we measured the total time spent floating (immobility duration) in a forced-swim test (FST) at 10 and 30 dpi (Fig. 2A). At 10 dpi, but not at 30 dpi, an increased duration of immobility was observed, indicating despair-like behavior during acute infection. To determine whether *T. gondii*-induced despair-like behavior is dependent on inflammatory responses, wild-type mice were treated with Mino (Fig. 2B).
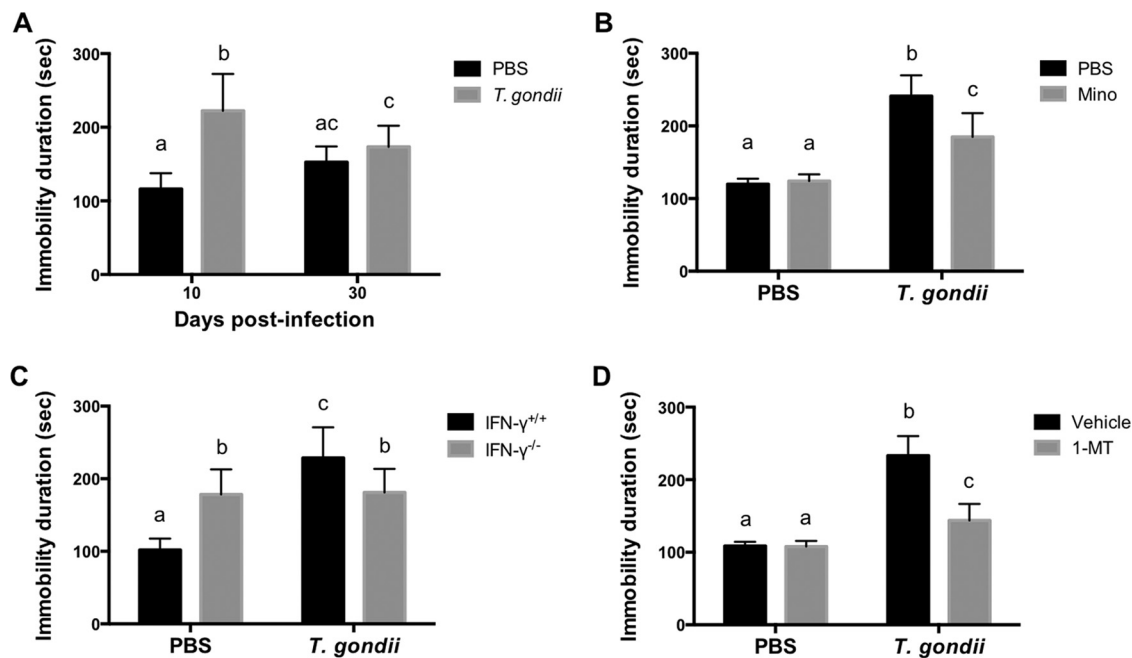
FIG 2 Analysis of despair behavior by duration of immobility following *T. gondii* infection. The total time spent floating (immobility duration) in the FST was measured as a putative indicator of despair behavior. (A) Duration of immobility of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 and 30 dpi. Data from two independent experiments are summarized and presented as means ± standard deviations [PBS, $n = 4$ plus 6; *T. gondii*, $n = 4$ plus 6; $F_{(1, 36)} = 4.24$; $P = 0.0002$]. (B) Duration of immobility of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 dpi under treatment with Mino (10 mg/kg i.p.) or injection with PBS from days 4 to 7 postinfection. Data are representative of data from two independent experiments with similar results and are presented as means ± standard deviations [$n = 5$ or 6 (one mouse died due to infection in the *T. gondii*-infected and PBS-injected group); $F_{(1, 19)} = 10.54$; $P = 0.0042$]. (C) Duration of immobility of wild-type BALB/c mice (IFN-$\gamma^{+/+}$) and IFN-$\gamma^{-/-}$ mice after injection with PBS or infection with *T. gondii* at 10 dpi. Data from three independent experiments are summarized and presented as means ± standard deviations [$n = 3$ plus 5 plus 5; $F_{(1, 48)} = 46.50$; $P < 0.0001$]. (D) Duration of immobility of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 dpi under treatment with 1-MT (50 mg/kg subcutaneously) or injection with the vehicle from days 4 to 7 postinfection. Data are representative of data from two independent experiments with similar results and are presented as means ± standard deviations [$n = 5$ or 6 (one mouse died due to infection in the *T. gondii*-infected and vehicle-injected group); $F_{(1, 19)} = 35.81$; $P < 0.0001$]. Different letters above bars in the graphs indicate statistically significant differences among the groups by two-way ANOVA plus Tukey-Kramer *post hoc* analysis.

Treatment of infected mice with Mino reduced the duration of immobility compared with that of untreated mice, indicating that despair-like behavior is mediated by inflammatory responses. In the case of IFN-$\gamma^{-/-}$ mice, there was no significant difference in durations of immobility between infected and noninfected mice, whereas the duration of immobility of *T. gondii*-infected IFN-$\gamma^{-/-}$ mice was shorter than that of infected wild-type mice (Fig. 2C), indicating IFN-γ-dependent despair-like behavior. However, the duration of immobility of IFN-$\gamma^{-/-}$ mice was longer than that of uninfected wild-type mice (Fig. 2C), suggesting a higher sensitivity of IFN-$\gamma^{-/-}$ mice to despair-like behavior. To evaluate the effects of IDO activity on despair-like behavior, wild-type mice were treated with 1-DL-MT (Fig. 2D). Similar to the results from Mino treatment, a reduced duration of immobility was observed for infected mice treated with 1-DL-MT compared with that for untreated infected animals. Taken together, despair-like behavior observed during acute infection may be induced by IDO activity via inflammatory responses.

Sickness behavior in IFN-$\gamma^{-/-}$ mice and wild-type mice treated with Mino and 1-MT. Acute *T. gondii* infection induces sickness behavior in terms of a considerably elevated clinical score (prepared ethogram) and reduced locomotor activity (stride length in a footprint test). Treatment of infected mice with Mino reduced clinical signs and improved the stride length of *T. gondii*-infected mice in the footprint test (Fig. 3A), suggesting that clinical symptoms and locomotor activity may be mediated by inflammatory responses. Conversely, IFN-$\gamma^{-/-}$ mice exhibited clinical symptoms similar to those of wild-type animals, including a marked reduction in locomotor activity, as
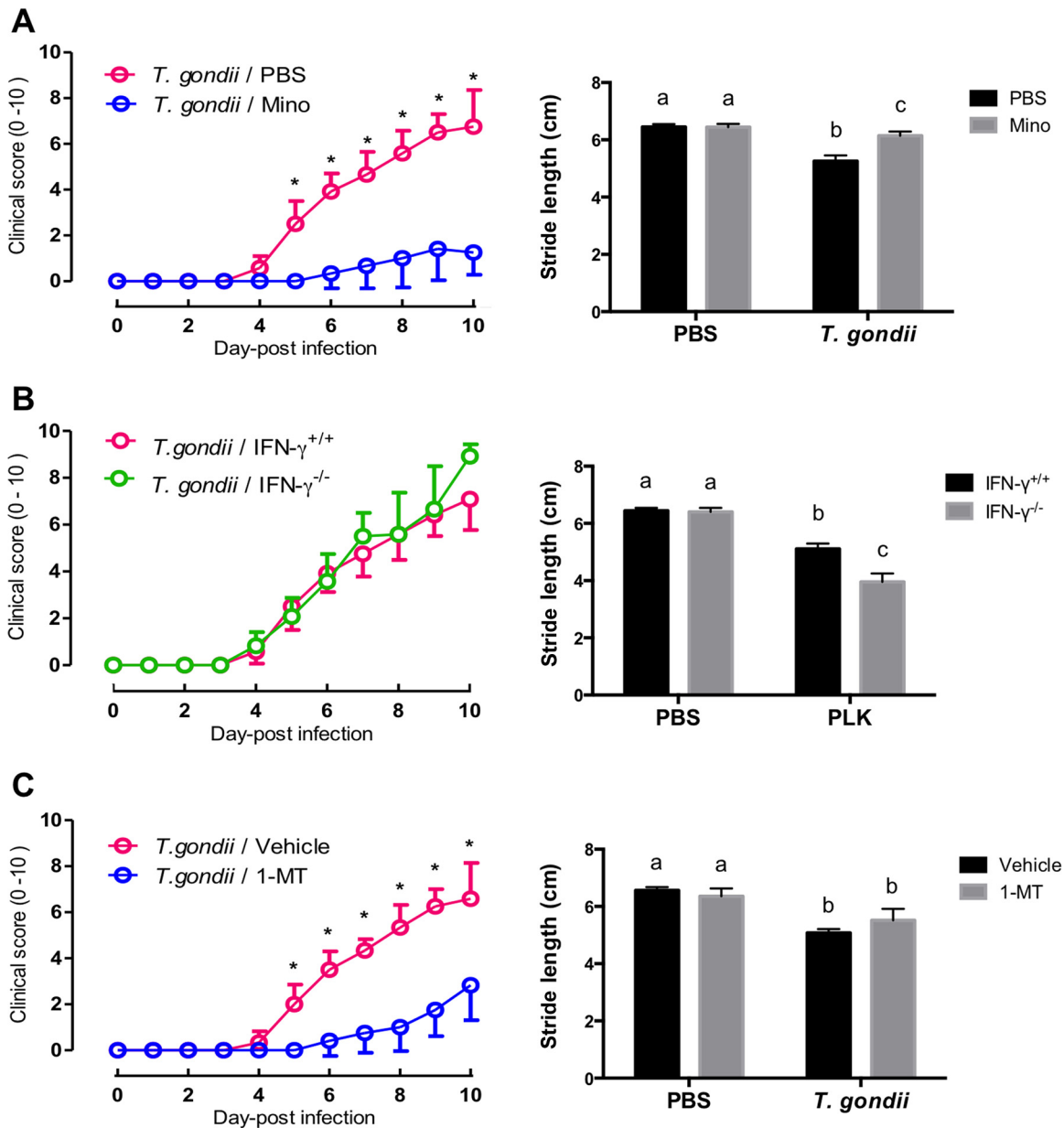
FIG 3 Clinical scores and locomotor activities of mice following *T. gondii* infection. (A) Clinical scores (left) and locomotor activities in terms of stride length in a footprint test (right) of wild-type BALB/c mice after infection with *T. gondii* and treatment with Mino (10 mg/kg i.p.) or injection with PBS from days 4 to 7 postinfection. Clinical scores from two independent experiments are summarized and presented as means $\pm$ standard deviations [$n = 6$ plus 6; $F_{(10, 242)} = 52.97$; $P < 0.0001$]. Locomotor activity data are presented as means $\pm$ standard deviations [$n = 5$; $F_{(1, 16)} = 47.15$; $P < 0.0001$]. (B) Clinical scores (left) and locomotor activities (right) of wild-type BALB/c mice (IFN-$\gamma^{+/+}$) and IFN-$\gamma^{-/-}$ mice after infection with *T. gondii*. Data from two independent experiments are summarized and presented as means $\pm$ standard deviations [clinical score, $n = 6$ plus 6, $F_{(10, 242)} = 3.05$, and $P = 0.0012$; locomotor activity, $n = 5$ plus 5, $F_{(1, 36)} = 81.69$, and $P < 0.0001$]. (C) Clinical scores (left) and locomotor activities (right) of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* under treatment with 1-MT (50 mg/kg subcutaneously) or injection with the vehicle from days 4 to 7 postinfection. Clinical score data from two independent experiments are summarized and presented as means $\pm$ standard deviations [$n = 6$ plus 6; $F_{(10, 242)} = 41.79$; $P < 0.0001$]. Data for locomotor activity are presented as means $\pm$ standard deviations [$n = 5$; $F_{(1, 16)} = 7.877$; $P = 0.0127$]. * indicates significant differences in clinical scores (left) between two groups by two-way ANOVA plus Bonferroni *post hoc* analysis. Different letters above bars in the graphs indicate statistically significant differences among the groups by two-way ANOVA plus Tukey-Kramer *post hoc* analysis (right).

indicated by a shortened stride in the footprint test in infected compared with uninfected wild-type mice (Fig. 3B), suggesting that IFN-γ may not be directly linked to sickness behavior. Treatment with 1-DL-MT attenuated *T. gondii*-induced sickness symptoms, as indicated by a reduction in the clinical score from 5 to 10 dpi, whereas 1-DL-MT
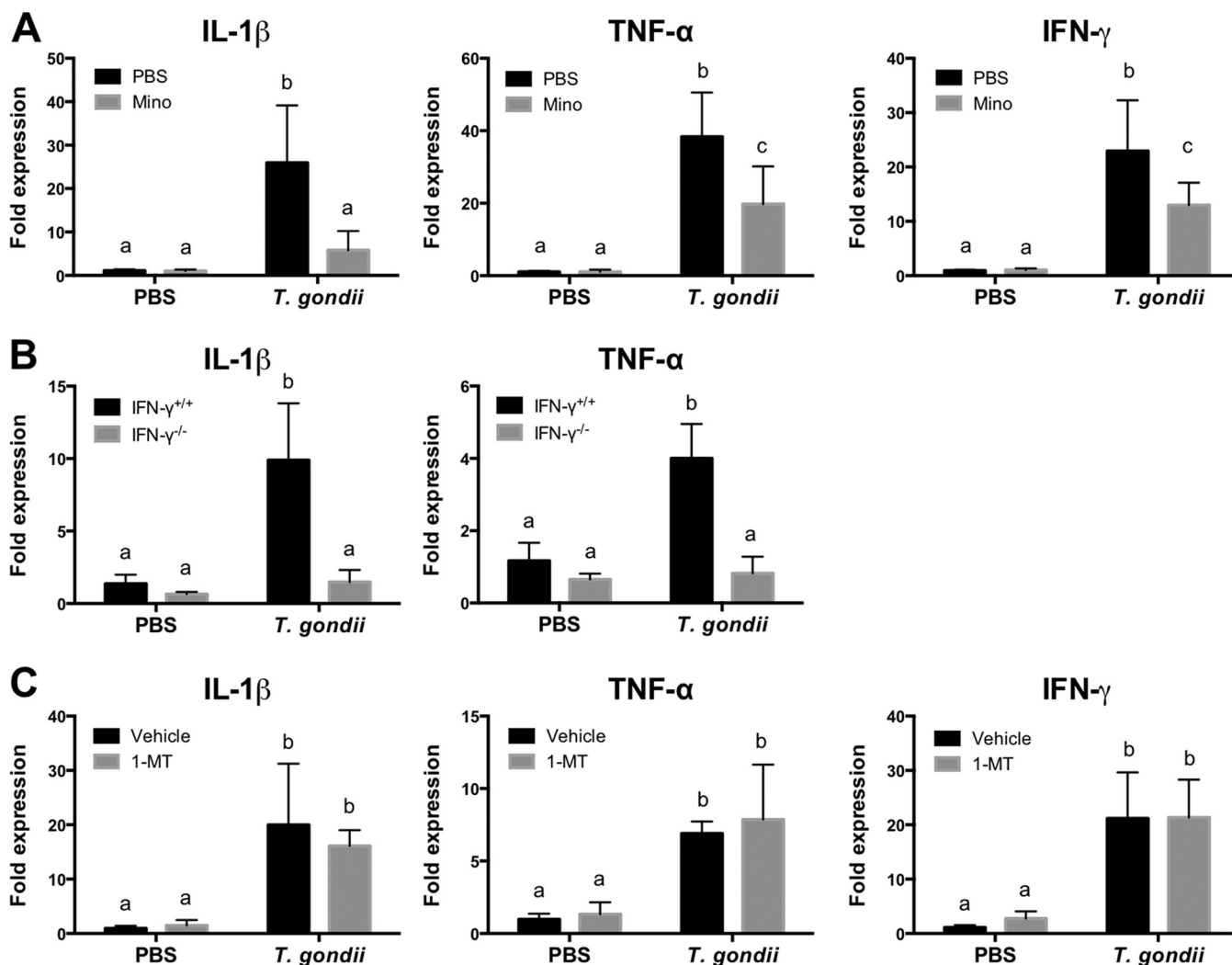
FIG 4 Expression of cytokines in brains of mice following *T. gondii* infection. (A) Expression of IL-1β, TNF-α, and IFN-γ in brains of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 dpi under treatment with Mino (10 mg/kg i.p.) or injection with PBS from days 4 to 7 postinfection. Data are representative of data from two independent experiments with similar results and are presented as means $\pm$ standard deviations [n = 5 or 6 (one mouse died due to infection in the *T. gondii*-infected and PBS-injected group); for IL-1β, $F_{(1, 19)}$ = 13.66 and P = 0.0015; for TNF-α, $F_{(1, 19)}$ = 8.235 and P = 0.0098; for IFN-γ, $F_{(1, 19)}$ = 6.325 and P = 0.0211]. (B) Expression of IL-1β and TNF-α in brains of wild-type BALB/c mice (IFN-$\gamma^{+/+}$) and IFN-$\gamma^{-/-}$ mice after injection with PBS or infection with *T. gondii* at 10 dpi. Data from two independent experiments are summarized and presented as means $\pm$ standard deviations [n = 5 plus 5; for IL-1β, $F_{(1, 36)}$ = 35.98 and P < 0.0001; for TNF-α, $F_{(1, 36)}$ = 50.97 and P < 0.0001]. (C) Expression of IL-1β, TNF-α, and IFN-γ in brains of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 dpi under treatment with 1-MT (50 mg/kg subcutaneously) or injection with the vehicle from days 4 to 7 postinfection. Data are representative of data from two independent experiments with similar results and are presented as means $\pm$ standard deviations [n = 5 or 6 (one mouse died in the *T. gondii*-infected and vehicle-injected group); for IL-1β, $F_{(1, 19)}$ = 0.937 and P = 0.3452; for TNF-α, $F_{(1, 19)}$ = 0.1289 and P = 0.7235; for IFN-γ, $F_{(1, 19)}$ = 0.1108 and P = 0.7429]. Different letters above bars in the graphs indicate statistically significant differences among the groups by two-way ANOVA plus Tukey-Kramer *post hoc* analysis.

did not alleviate the reduced stride length caused by infection (Fig. 3C), suggesting that IDO inhibition may reduce clinical symptoms but may not affect locomotor activity. Considered with observations of anhedonic and despair-like behaviors, inflammatory responses during acute infection (including IDO activation) may induce both sickness and depressive-like behaviors. However, sickness and depressive-like behaviors may result from independent pathways, as in the case of IFN-$\gamma^{-/-}$ mice.

Expression of proinflammatory cytokines in IFN-$\gamma^{-/-}$ mice and wild-type mice treated with Mino and 1-DL-MT. *T. gondii* infection increased the expression levels of IL-1β, TNF-α, and IFN-γ in the brain (Fig. 4). Correlation analysis of levels of proinflammatory cytokine expression and depressive-like behavior indicated anhedonic and despair-like behaviors, and levels of IFN-γ and IL-1β were strongly and moderately correlated, respectively (see Table S2 in the supplemental material). Furthermore,

TABLE 1 Effects of minocycline and 1-methyl-DL-tryptophan treatments on plasma levels of the inflammatory cytokines IFN-γ and IL-1β in mice infected with *T. gondii* at 10 days postinfection[a]

Treatment	Mean level of protein (pg/ml) ± SD	
	IL-1β	IFN-γ
PBS-PBS	ND	ND
PBS-Mino	47.2 ± 4.6 a	82.5 ± 28.4 a
T. gondii-PBS	645.6 ± 34.6 b	410.89 ± 87.7 b
T. gondii-Mino	24.1 ± 4.4 a	136.6 ± 7.6 c
PBS-vehicle	ND	ND
PBS–1-MT	ND	26.5 ± 5.3 a
T. gondii-vehicle	676.0 ± 32.5	459.6 ± 43.2 b
T. gondii–1-MT	619.5 ± 26.7	433.3 ± 40.0 b

[a]Data represent average mean values ± standard deviations {n = 5 or 6; one mouse each died due to infection in the *T. gondii*-PBS group [for IL-1β, $F_{(3, 22)}$ = 231.7 and P = 0.0001; for IFN-γ, $F_{(3, 22)}$ = 329.4 and P = 0.0001] and the *T. gondii*-vehicle group [for IL-1β, $F_{(3, 22)}$ = 156.7 and P = 0.0001; for IFN-γ, $F_{(3, 22)}$ = 326.0 and P = 0.0001]}. Different letters indicate statistically significant differences among the groups by one-way ANOVA plus Tukey-Kramer *post hoc* analysis. ND, not detected.

inhibitory effects of Mino on the expression of IL-1β, TNF-α, and IFN-γ mRNAs were observed in brains at 10 dpi (Fig. 4A). In addition, plasma levels of IL-1β and IFN-γ were reduced by Mino treatment in infected mice (Table 1). These results indicate that the changes in proinflammatory cytokine expression were associated with altered behaviors after *T. gondii* infection. IFN-γ$^{-/-}$ mice exhibited an attenuation of IL-1β and TNF-α mRNAs (Fig. 4B); however, 1-DL-MT treatment did not reduce mRNA expression levels of IL-1β, TNF-α, and IFN-γ in infected mice (Fig. 4C). In addition, compared with untreated and *T. gondii*-infected mice, 1-DL-MT treatment did not alter the production of IL-1β and IFN-γ after *T. gondii* infection (Table 1). Therefore, these results suggest an involvement of IDO activity in the induction of anhedonic and despair-like behaviors as well as clinical symptoms during acute *T. gondii* infection.

Expression of IDO in IFN-γ$^{-/-}$ mice and wild-type mice treated with Mino and 1-DL-MT. The expression of IDO1 and its other form IDO2 has been described in both mice and humans (33). To determine whether the expression of IDO1 and IDO2 was correlated with altered behavior exhibited during *T. gondii* infection, quantitative reverse transcriptase PCR (qRT-PCR) of brains was performed (Fig. 5). As shown in Table S2 in the supplemental material, the expression levels of IDO1 and IDO2 in the brain correlated with the duration of immobility but not with sucrose preference, suggesting that the expression of IDO1 and IDO2 plays a role in the induction of despair-like behaviors. At 10 dpi, decreased expression levels of IDO1 and IDO2 were observed in Mino-treated wild-type mice compared with *T. gondii*-infected mice (Fig. 5A). A decreased expression level of IDO1 was also observed in IFN-γ$^{-/-}$ mice compared with infected wild-type mice (Fig. 5B). However, 1-DL-MT treatment did not affect IDO2 mRNA expression levels but significantly increased IDO1 expression levels in *T. gondii*-infected mice at 10 dpi (Fig. 5C). Thus, the genetic deletion of IFN-γ and the blockage of proinflammatory cytokines by Mino were associated with reduced IDO expression. However, 1-DL-MT treatment was not associated with reduced IDO expression in *T. gondii*-infected mice.

Tryptophan metabolism in IFN-γ$^{-/-}$ mice and wild-type mice treated with Mino or 1-DL-MT. To determine the mechanism by which alterations in Trp degradation contribute to depressive-like behavior following *T. gondii* infection, we measured Trp, Kyn, and 5-hydroxytryptamine (5-HT) levels in brains as well as plasma levels of Trp and Kyn (Fig. 6). The calculated Kyn/Trp ratio in brains at 10 dpi was ~3.6-fold higher than that at 30 dpi, whereas in plasma, it was ~1.8-fold higher (Fig. 6A). It was noted that *T. gondii* infection did not affect Trp turnover to 5-HT at 10 and 30 dpi in wild-type mice, as measured by comparing the ratios of 5-HT/Trp (Fig. 6A). At 10 dpi, Kyn/Trp ratios in plasma and brain exhibited a weak to moderate correlation with anhedonic behaviors and a moderately strong correlation with despair-like behaviors (see Table S2 in the
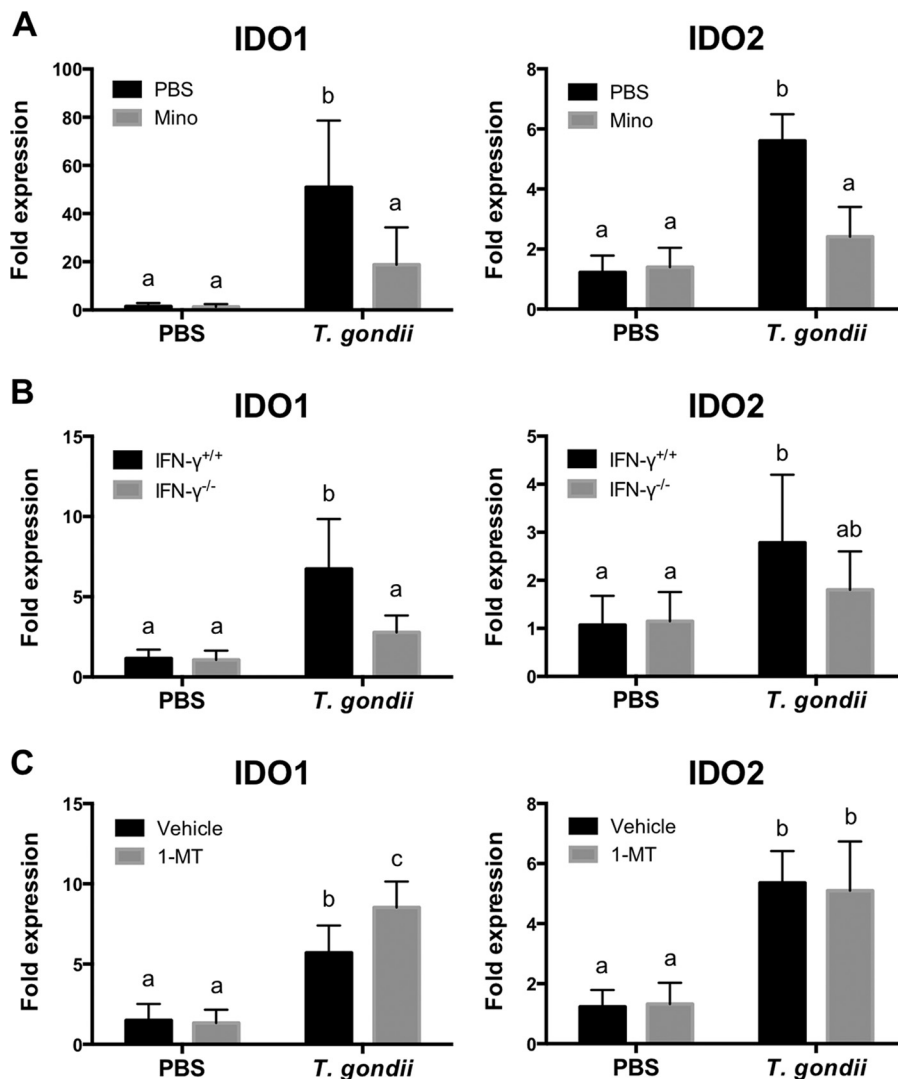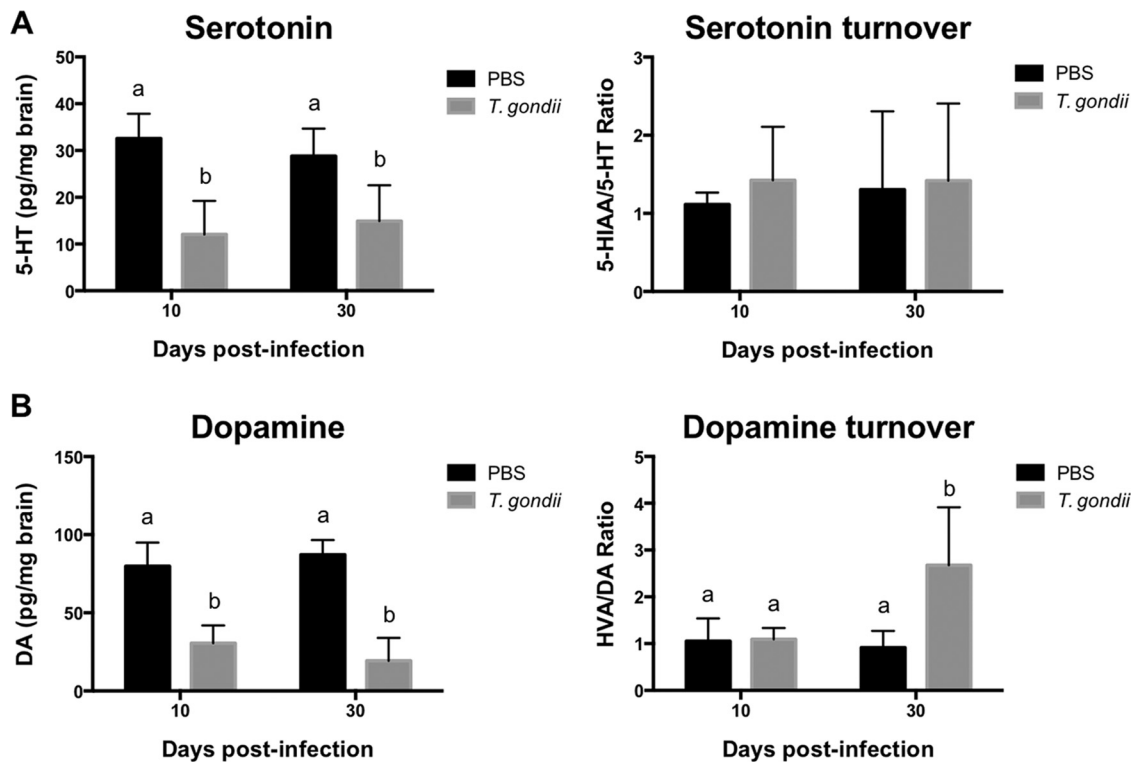
FIG 5 Expression of IDO in brains of mice following *T. gondii* infection. (A) Expression of IDO1 and IDO2 in brains of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 dpi under treatment with Mino (10 mg/kg i.p.) or injection with PBS from days for 4 to 7 postinfection. Data are representative of data from two independent experiments with similar results and are presented as means ± standard deviations [$n = 5$ or 6 (one mouse died due to infection in the *T. gondii*-infected and PBS-injected group); for IDO1, $F_{(1, 19)} = 6.480$ and $P = 0.0197$; for IDO2, $F_{(1, 19)} = 26.09$ and $P < 0.0001$]. (B) Expression of IDO1 and IDO2 in brains of wild-type BALB/c mice (IFN-$\gamma^{+/+}$) and IFN-$\gamma^{-/-}$ mice after injection with PBS or infection with *T. gondii* at 10 dpi. Data from two independent experiments are summarized and presented as means ± standard deviations [$n = 5$ plus 5; for IDO1, $F_{(1, 36)} = 13.06$ and $P = 0.0009$; for IDO2, $F_{(1, 36)} = 3.313$ and $P = 0.077$]. (C) Expression of IDO1 and IDO2 in brains of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 dpi under treatment with 1-MT (50 mg/kg subcutaneously) or injection with the vehicle from days 4 to 7 postinfection. Data are representative of data from two independent experiments with similar results and are presented as means ± standard deviations [$n = 5$ or 6 (one mouse died due to infection in *T. gondii*-infected and vehicle-injected group); for IDO1, $F_{(1, 19)} = 7.313$ and $P = 0.0141$; for IDO2, $F_{(1, 19)} = 0.158$ and $P = 0.6954$]. Different letters above bars in the graphs indicate statistically significant differences among the groups by two-way ANOVA plus Tukey-Kramer *post hoc* analysis.

supplemental material). To further investigate the effects of the inflammatory response and IFN-γ action on Trp metabolism, we measured Kyn/Typ and 5-HT/Trp ratios in Mino-treated wild-type mice (Fig. 6B) and IFN-$\gamma^{-/-}$ mice (Fig. 6C). Reproducibly, *T. gondii* infection markedly increased the Kyn/Trp ratio in both plasma and brain. These altered ratios of Kyn/Trp were significantly reduced in the plasma and brains of Mino-treated wild-type mice and in IFN-$\gamma^{-/-}$ mice, whereas the 5-HT/Trp ratio was not affected (Fig. 6B and C). In parallel, treatment of *T. gondii*-infected wild-type mice with
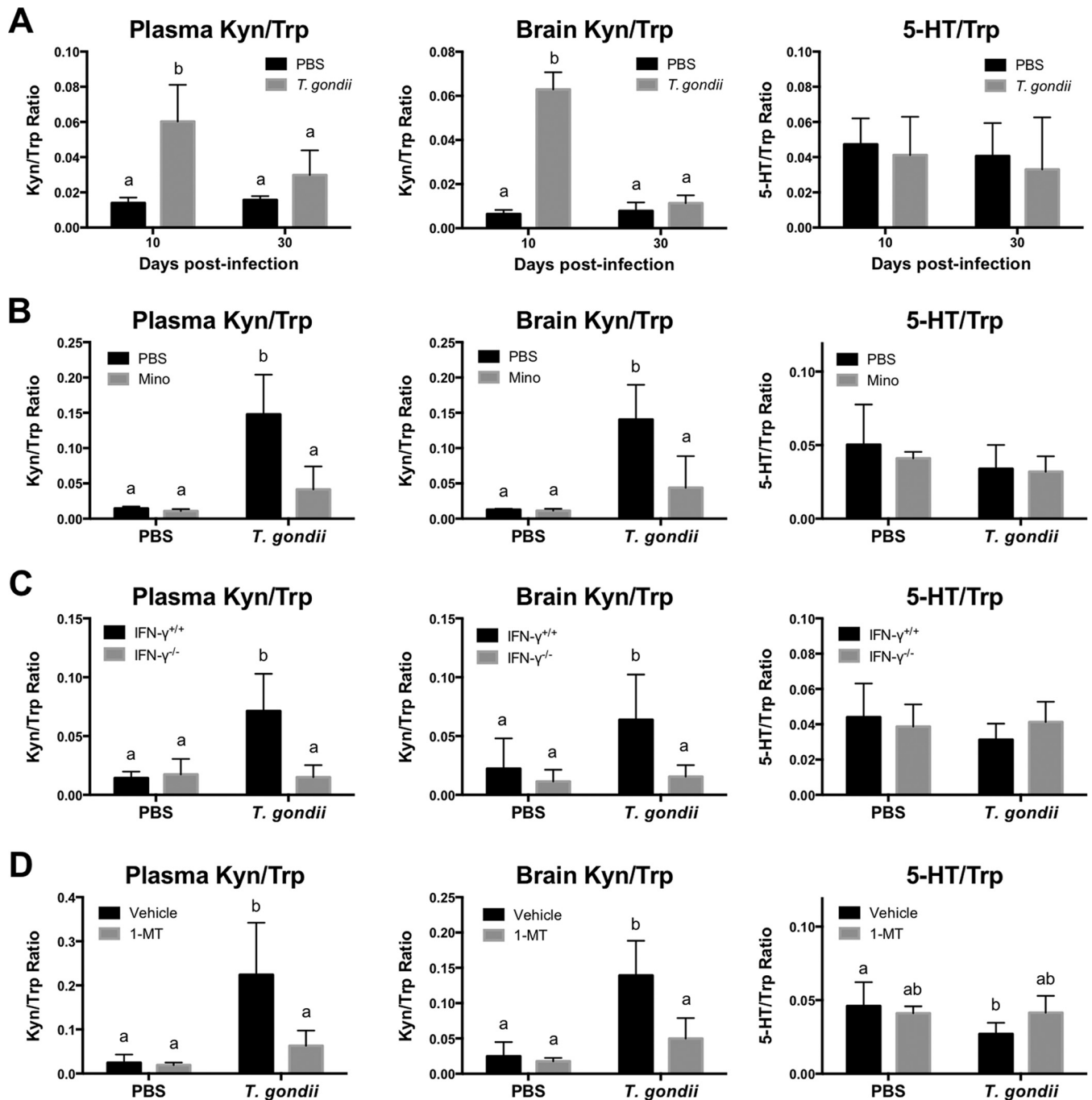
FIG 6 Trp turnover to Kyn or 5-HT in mice following *T. gondii* infection. Trp turnover to Kyn (Kyn/Trp ratio) in plasma and brain and Trp turnover to brain serotonin (5-HT/Trp ratio) were determined. (A) Trp turnover in wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 and 30 dpi. Plasma Kyn/Trp ratios are presented as means ± standard deviations [$n = 5$; $F_{(1, 16)} = 8.039$; $P = 0.0119$]. Data for brain Kyn/Trp and brain 5-HT/Trp ratios from two independent experiments are summarized and presented as means ± standard deviations [$n = 4$ or 5 plus 5; for the brain Kyn/Trp ratio, $F_{(1, 32)} = 274.6$ and $P < 0.0001$; for Trp turnover, $F_{(1, 36)} = 0.01294$ and $P = 0.9101$]. (B) Trp turnover in wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 dpi under treatment with Mino (10 mg/kg i.p.) or injection with PBS from days 4 to 7 postinfection. Data are presented as means ± standard deviations [$n = 5$ or 6 (one mouse died in the *T. gondii*-infected and PBS-injected group); for the plasma Kyn/Trp ratio, $F_{(1, 19)} = 15.91$ and $P = 0.0008$; for the brain Kyn/Trp ratio, $F_{(1, 19)} = 12.44$ and $P = 0.0023$; for Trp turnover, $F_{(1, 19)} = 0.2663$ and $P = 0.6118$]. (C) Trp turnover of wild-type BALB/c mice (IFN-γ$^{+/+}$) and IFN-γ$^{-/-}$ mice after injection with PBS or infection with *T. gondii* at 10 dpi. Data from two independent experiments are summarized and presented as means ± standard deviations [for the plasma Kyn/Trp ratio, $n = 4$ plus 4, $F_{(1, 28)} = 21.44$, and $P < 0.001$; for the brain Kyn/Trp ratio, $n = 5$ plus 5, $F_{(1, 36)} = 6.016$, and $P = 0.0192$; for the brain 5-HT/Trp ratio, $n = 5$ plus 5, $F_{(1, 36)} = 3.19$, and $P = 0.0825$]. (D) Trp turnover of wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 dpi under treatment with 1-MT (50 mg/kg subcutaneously) or injection with the vehicle from days 4 to 7 postinfection. Data are presented as means ± standard deviations [$n = 5$ or 6 (one mouse died due to infection in the *T. gondii*-infected and vehicle-injected group); for the plasma Kyn/Trp ratio, $F_{(1, 16)} = 7.838$ and $P = 0.0129$; for the brain Kyn/Trp ratio, $F_{(1, 19)} = 11.51$ and $P = 0.0031$; for Trp turnover, $F_{(1, 19)} = 4.43$ and $P = 0.0489$]. Different letters above bars in graphs indicate statistically significant differences among the groups by two-way ANOVA plus Tukey-Kramer *post hoc* analysis.

FIG 7 Levels of serotonin and dopamine in brains of mice following *T. gondii* infection. 5-HT and its turnover to 5-hydroxyindoleacetic acid (5-HIAA) (5-HIAA/5-HT ratio) as well as DA and its turnover to homovanillic acid (HVA) (HVA/DA ratio) were measured in brains of mice. (A) 5-HT and its turnover in wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 and 30 dpi. Data from two independent experiments are summarized and presented as means ± standard deviations [n = 5 plus 5; for 5-HT, $F_{(1, 36)}$ = 2.538 and P = 0.1199; for the 5-HIAA/5-HT ratio, $F_{(1, 36)}$ = 0.1521 and P = 0.6989]. (B) DA and its turnover in wild-type BALB/c mice after injection with PBS or infection with *T. gondii* at 10 and 30 dpi. Data from two independent experiments are summarized and presented as means ± standard deviations [n = 5 plus 5; for DA, $F_{(1, 36)}$ = 5.27 and P = 0.0276; for the 5-HIAA/5-HT ratio, $F_{(1, 36)}$ = 0.1521 and P = 0.6989]. Different letters above bars in graphs indicate statistically significant differences among the groups by two-way ANOVA plus Tukey-Kramer *post hoc* analysis.

1-DL-MT normalized the Kyn/Trp ratio in both the plasma and brain (Fig. 6D). Next, we examined the levels of 5-HT and dopamine (DA) in the brain (Fig. 7). Lower levels of 5-HT and dopamine were observed in infected wild-type mice at both 10 and 30 dpi. Dopamine metabolism was upregulated in infected mice at 30 dpi as the result of lower DA levels and higher DA turnover rates than those in uninfected mice (Fig. 7B). Thus, 5-HT and dopamine may not be directly involved in depression-like behavior because the behavioral changes were not seen at 30 dpi. Together, these findings indicate that *T. gondii* enhanced Trp catabolism toward Kyn and not 5-HT.

Effect of Mino and 1-DL-MT on parasite growth *in vivo* and *in vitro*. At 14 dpi, the number of parasites in brain DNA was measured by quantitative PCR (qPCR) (Fig. 8). We found that treatment of infected mice with Mino and 1-MT reduced the brain parasite load (Fig. 8A). This effect was validated *in vitro* in *T. gondii*-infected microglia and astrocyte cell lines, MG6 and OS3, respectively. Pretreatment with Mino (5 to 40 nM) and 1-DL-MT (0.05 to 1 μM) had dose-dependent effects on restricting *T. gondii* replication in MG6 and OS3 cells (Fig. 8B and C). No effect on cell viability at the selected dose ranges was observed (data not shown). These findings indicate that both 1-DL-MT and Mino exhibit significant anti-*Toxoplasma* activities *in vivo* and *in vitro*.

DISCUSSION

Causes of depression do not stem from a single source; rather, depression is likely to be caused by a combination of factors. Perpetuation of the neurotropic parasite *T. gondii* may further complicate this syndrome. In the present study, we observed anhedonic and despair-like behaviors during the acute stage of *T. gondii* infection.
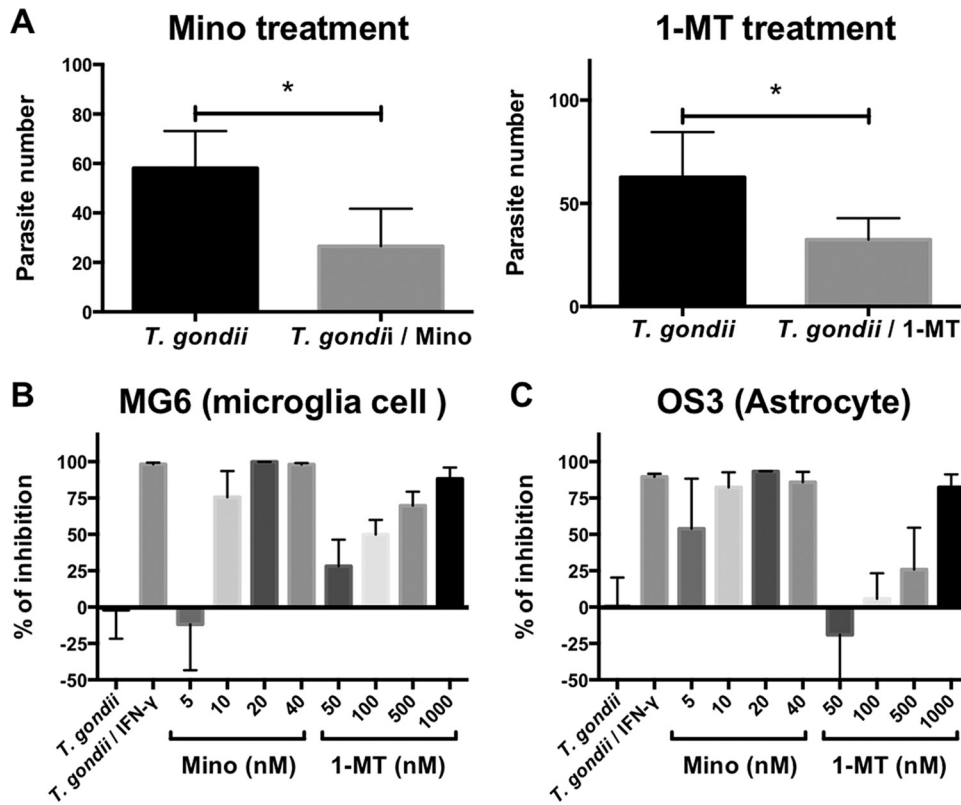
FIG 8 Effects of 1-MT and Mino on *T. gondii* growth *in vivo* and *in vitro*. (A) Numbers of parasites in the brains of wild-type BALB/c mice after infection with *T. gondii* at 14 dpi under treatment with Mino (10 mg/kg i.p.) or injection with PBS from days 4 to 7 postinfection and under treatment with 1-MT (50 mg/kg subcutaneously) or injection with the vehicle from days 4 to 7 postinfection. Data are presented as means ± standard deviations ($n = 5$ or 6; one mouse each died due to infection in the *T. gondii*-infected and PBS-injected groups and in the *T. gondii*-infected and vehicle-injected groups). * indicates significant differences between groups by Student's *t* test. (B and C) Effects of IFN-γ (250 U/ml), Mino (5 to 40 nM), and 1-MT (50 to 1,000 nM) on *T. gondii* growth in murine microglia MG6 (B) and murine astrocyte OS3 (C) cells. Data are presented as means ± standard deviations ($n = 3$).

Therefore, immune enhancement may be a prerequisite for the development of such a depressive phenotype. Here, we hypothesized that the activation of host immunity mediates depression-like behaviors in *T. gondii*-infected mice. Our results support the view that interactions of the immune system and IFN-γ, IDO, and Trp metabolism play crucial roles in developing *T. gondii*-induced depression-like behaviors. In animal models, studies of the Kyn pathway and the Trp catabolic shunt indicate that these mechanisms are conserved not only between humans and rodents but also in lower organisms such as yeast (34). Several lines of evidence illustrate that depression-like behavior is associated with an enhancement of Trp catabolism caused by either inflammation or the release of proinflammatory cytokines (34, 35). Investigation of this pathway following infection with a chronic pathogen, such as *T. gondii*, will likely continue to advance our understanding of mechanisms by which neurotropic parasites induce some psychiatric disorders (36). The induction of depressive-like behaviors in *T. gondii*-infected mice and enhancement of Trp catabolism toward Kyn may be relevant to interpreting studies of psychobehavioral disorders in *T. gondii*-infected humans.

In the present study, *T. gondii* infection induced depression-like behavior in the forms of anhedonic and despair-like behaviors in mice during the acute stage. Additionally, sickness behavior was observed in mice following acute infection. As behavioral symptoms of sickness and depression are triggered by proinflammatory cytokines (17), it may be difficult to determine whether *T. gondii* directly induced depression-like behavior or whether generalized inflammatory responses induced by infection triggered behavioral changes. Although both sickness and depression-like behaviors are

induced by the same proinflammatory cytokines, IDO induction has been proposed to lie at the interface between chronic inflammatory disease and depression (17). Although *T. gondii*-infected IFN-$\gamma^{-/-}$ mice showed clinical scores similar to those of infected wild-type mice, infected IFN-$\gamma^{-/-}$ mice did not exhibit anhedonic behavior (reduced sucrose preference) at 6 to 9 dpi, except for 10 dpi, compared with uninfected IFN-$\gamma^{-/-}$ mice. At 10 dpi, both wild-type BALB/c and IFN-$\gamma^{-/-}$ mice showed comparably severe clinical symptoms following infection. These symptoms at 10 dpi were categorized as sickness-related symptoms. Additionally, the locomotor activity of IFN-$\gamma^{-/-}$ mice infected with *T. gondii* was reduced compared with that of infected wild-type BALB/c mice. In fact, IFN-$\gamma^{-/-}$ mice showed a reduced intake of both water and sucrose (total fluid intake), which ranges from 0 to 2 ml of each bottle during 24 h. Actually, these IFN-$\gamma^{-/-}$ mice mostly succumbed after 10 days of infection. Thus, the severe reduction of locomotor activity may result in a reduced sucrose preference (or total fluid intake) in IFN-$\gamma^{-/-}$ mice infected with *T. gondii* at 10 dpi. It should be noted that uninfected IFN-$\gamma^{-/-}$ mice showed a longer duration of immobility in the FST than did uninfected wild-type mice. Furthermore, *T. gondii* infection did not increase the duration of immobility in IFN-$\gamma^{-/-}$ mice at 10 dpi compared with uninfected IFN-$\gamma^{-/-}$ mice (Fig. 2C). This result suggests that sickness and anhedonic behaviors may occur in *T. gondii*-infected mice by independent mechanisms. However, at present, it is unclear why the genetic deletion of IFN-γ induced despair behavior (increased duration of immobility in the FST) in uninfected IFN-$\gamma^{-/-}$ mice compared with uninfected wild-type mice. Perhaps, the relationship between IFN-γ and Trp metabolism may be linked to the immunogenetics of major depression, as reported previously in some correlation studies (11). Similarly, a deficiency of Toll-like receptor 2 was found to be associated with the induction of schizophrenia-like symptoms in mice (37). Collectively, these results suggest that the deficiency of an immunity-related gene (IRG), such as IFN-γ, was associated with despair-like and anhedonic behaviors in uninfected mice.

IFN-γ is essential for host defense mechanisms and the survival of mice during *T. gondii* infection. IFN-γ-deficient mice succumb to acute infection (4). Furthermore, mice lacking IL-12 or T cells that regulate or produce IFN-γ, respectively, do not survive the chronic stage of *T. gondii* infection (38–40). IFN-γ can suppress symptoms in *T. gondii* infection and, in fact, can inhibit *T. gondii* proliferation through various mechanisms, including (i) the depletion of arginine and the production of free radical nitric oxide via enzymatic activation (41); (ii) the disruption of parasitophorous vacuoles via IFN-γ-inducible genes, such as IRGs and guanylate-binding proteins (GBPs), in murine macrophages (42–44); or (iii) the starvation of an essential amino acid, Trp, mediated through IDO activity, as shown in human fibroblasts (45), but a direct effect of IDO on parasites in mice remains unclear. Thus, the activation of IFN-γ and IDO was associated with the depletion of Trp, suggesting a correlation between host defense mechanisms and the display of anhedonic and despair-like behaviors. Therefore, it is plausible that anhedonic and despair-like behaviors were dependent on IFN-γ or IDO activation rather than simply being linked to sickness behaviors.

Aside from the association of sickness symptoms with depression-like behavior during the acute stage of *T. gondii* infection, our findings support a role for the Trp-to-Kyn shunt in depression pathophysiology induced by *T. gondii* infection. Anhedonic and despair-like behaviors during the acute stage were present with low 5-HT levels (associated with a high Kyn/Trp ratio in plasma and brain) and normal 5-HT turnover. However, such behaviors were not displayed at 30 dpi in the context of low brain 5-HT and DA levels. In support of these results, we found that Kyn/Trp ratios in the brain of infected mice at 60 dpi did not change from those in control uninfected mice, indicating lower IDO activity in the chronic stage (3). Thus, the development of anhedonic and despair-like behaviors may not be simply dependent on lower levels of serotonergic and dopaminergic neurotransmission during the acute stage of *T. gondii* infection in mice. It has been reported that IDO induction, Trp degradation, and Kyn formation are completely absent in *T. gondii*-infected IFN-γ-deficient mice (46, 47). Our data also confirmed the importance of IFN-γ for IDO activity induction and the resultant

depression-like behaviors. Since IFN-γ-mediated IDO activation may induce such behaviors in mice following infection with *T. gondii* during the acute stage, *T. gondii* infection can induce changes in Trp metabolism via proinflammatory cytokines, especially IFN-γ.

However, at present, it is unclear why the sustained decreases in DA and 5-HT levels were not reflected in the precipitation of anhedonic and despair-like symptoms in *T. gondii*-infected mice during chronic infection. On the other hand, low levels of 5-HT and DA coexisted with anhedonic and despair-like behaviors during acute infection. Thus, the contribution of DA and 5-HT to depression-like behaviors could not be concluded. Compared with uninfected mice, the unchanged 5-HT/Trp ratio and serotonin turnover and the higher Kyn/Trp ratio indicated enhanced Trp catabolism toward Kyn in infected mice during acute infection. Together, these findings suggest that enhanced Trp catabolism toward Kyn resulting from the inflammatory response, IFN-γ action, and IDO activity-dependent mechanisms of *T. gondii* infection may be related to the appearance of anhedonic and despair-like behaviors during acute infection.

In the present study, Mino abrogated *T. gondii*-induced anhedonic and despair-like behaviors; it also normalized Kyn/Trp ratios in the brain and plasma in *T. gondii*-infected mice. It is well known that proinflammatory cytokines are involved in depression-like behaviors through the generation of a neuroreactive Trp metabolite, Kyn. A Kyn metabolite, quinolinic acid, acts as an *N*-methyl-D-aspartate (NMDA) receptor agonist (48) and is implicated in the development of core symptoms of depression-like anhedonia and behavioral despair following the peripheral administration of cytokine-inducing bacterial LPS (8). Our data showed that treatment with Mino inhibited IDO expression in the brains of *T. gondii*-infected mice, resulting in the normalization of Kyn/Trp ratios in both the plasma and brains of infected mice. Mino, a prototype anti-inflammatory drug, was used to prevent ischemic neuronal death. In addition, Mino protects neurons from glutamate toxicity and blocks the expression of proinflammatory cytokines in both peripheral and central organs (17–19). Mino is also a selective inhibitor of microglia activation, and it inhibits apoptosis by decreasing the levels of IL-1β, TNF-α, and their converting enzymes caspase 1 and caspase 3 (49, 50). Additionally, Mino reduced the permeability of the BBB by inhibiting IL-1β, TNF-α, matrix metalloproteinase 2 (MMP-2), MMP-9, and vascular cell adhesion molecule (VCAM) expression as well as reducing the transmigration of T cells across the fibronectin matrix barrier (51, 52). Therefore, Mino may be a potential therapeutic for psychiatric diseases, including early schizophrenia, multiple sclerosis, Huntington disease, and Parkinson's disease (49, 50, 52–54, 72). Activated microglia contribute to neuronal apoptosis; thus, the inhibition of microglia activation by Mino may represent a novel therapeutic strategy for treating neuronal apoptosis in cases of toxoplasmic encephalitis (55). Mino also prevented anhedonia and sickness behavior in mice challenged with LPS (56). Although LPS induces sickness behaviors followed by depression-like behaviors, this appeared within a relatively short time course compared with immune challenge with the BCG vaccine (9, 57) or *T. gondii* infection. In the present study, we treated *T. gondii*-infected mice with Mino for 4 days at doses of 10 mg/kg of body weight intraperitoneally (i.p.). Our treatment regimen decreased clinical symptoms induced by infection without inducing side effects. Furthermore, Mino decreased the brain parasite burden (Fig. 5A), thus conferring the strongest protective effect against *T. gondii* in murine microglia and astrocyte cells (Fig. 4B and C). *T. gondii* tachyzoites invade murine microglia, astrocytes, and neurons; thereafter, the parasite forms cysts within these cells (58). Our data for parasite growth indicated that microglia have 17 times the ability of astrocytes to control *T. gondii*. Within the brain, microglia are reported to be the major effector cells for the prevention of *T. gondii* tachyzoite proliferation (58). At the neuronal level, Mino salvaged glutamate toxicity in cultured primary neurons (16). Thus, Mino may have the potential to control acute toxoplasmosis, while the anti-*Toxoplasma* mechanism of Mino is still unknown.

1-DL-MT was previously used as a reference drug for blocking IDO activity in human and murine cells (21, 23, 59). In clinical trials, 1-DL-MT has been used as a vaccine

adjuvant and an add-on immunotherapeutic agent for cancer patients (60). As IDO triggers Trp depletion in the cellular microenvironment, it can also inhibit the proliferation of T cells and permit tumor cells to escape the immune system (61). Therefore, IDO inhibition by 1-DL-MT reverses the immunosuppressive effects of IDO (62). Interestingly, chronic treatment with 1-L-MT or 1-DL-MT results in controversial antiparasitic activity, but subchronic treatment exerts some anti-*Toxoplasma* activity (20, 24). Furthermore, IDO$^{-/-}$ mice and mice with IDO inhibited by 1-DL-MT exhibit reduced *T. gondii* mRNA expression in the lungs (24). In our study, 1-DL-MT treatment attenuated anhedonic and despair-like behaviors in *T. gondii*-infected mice without an obvious effect on proinflammatory cytokine expression, suggesting that the effect of 1-DL-MT is independent of proinflammatory cytokine expression. Furthermore, IDO activity may be involved in anhedonic and despair-like behaviors, and such behaviors are unlikely to be linked to cytokine production in *T. gondii*-infected mice. Our results indicate that 1-DL-MT, like Mino, significantly reduced the parasite load *in vivo* and *in vitro* (Fig. 8). It would be of interest in future studies to examine mechanisms underlying the ability of Mino and 1-DL-MT to control the growth of *T. gondii*.

In summary, the present study suggests that depression-like behaviors are likely mediated by host defense mechanisms against *T. gondii* infection. As a host immune response against acute *T. gondii* infection, IFN-γ produced by T cells or natural killer cells stimulates IDO activity. Metabolites of IDO activity, Trp to Kyn, resulted in depression-like behaviors. Treatment with Mino and 1-DL-MT ameliorated *T. gondii*-induced anhedonic and despair-like behaviors, suggesting the potential of this drug to treat core depression symptoms. As these drugs are highly lipid soluble and capable of penetrating the BBB (17), they may be of potential interest for future clinical studies. Since these drugs inhibit *Toxoplasma* growth, their use for *T. gondii* infection may be beneficial. Moreover, their effect was associated with symptomatic relief of anhedonia and despair as well as an improvement of clinical symptoms or locomotor activity. Our results demonstrate that immune enhancement in response to infection of immunocompetent mice with *T. gondii* resulted in IFN-γ production, IDO activation, and inflammation associated with sickness and anhedonic and despair-like behaviors. In contrast, after inhibition of IFN-γ production, sickness symptoms were displayed without the other behaviors. The inactivation of IDO improved clinical symptoms and depression-like behaviors. In conclusion, host defense mechanisms against *T. gondii* infection may be involved in anhedonic and despair-like behaviors in mice.

MATERIALS AND METHODS

Ethics statement. This study was performed in strict accordance with recommendations of the *Guidelines for Proper Conduct of Animal Experiments* (73). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (permit numbers 24-13, 24-15, 25-59, and 25-61). All injections were performed under isoflurane anesthesia, and every effort was made to minimize animal suffering.

Animals. According to our established experimental model (3, 63), experiments were performed by using wild-type female BALB/c mice (7 weeks old). Female IFN-γ$^{-/-}$ mice (BALB/c background) were obtained from Clea Japan (Tokyo, Japan) and maintained at the National Research Center for Protozoan Diseases (Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan). Animals were examined after 1 week of accommodation under specific-pathogen-free conditions and under stable conditions (12-h light/dark cycles; light on from 07:00 h to 19:00 h). Food and water were administered *ad libitum*, and all behavioral experiments commenced at 09:00 h.

***Toxoplasma gondii* culture and infection.** The type II PLK strain of *T. gondii* was utilized; the parasites were maintained as tachyzoites in Vero cell culture. After syringe lysis purification, wild-type and IFN-γ$^{-/-}$ mice were either i.p. infected with 10^3 tachyzoites or injected with sterile PBS, as previously described (42, 63). At designated time points, mice were decapitated without anesthesia, blood was collected into heparinized tubes to obtain plasma, and organs were instantly frozen in liquid nitrogen for storage at −80°C until further analyses.

Treatments and experimental groups. At 4 dpi, groups of *T. gondii*-infected and PBS-injected mice were treated with either Mino (Sigma-Aldrich, St. Louis, MO) (10 mg/kg i.p.) or PBS once a day for 4 days (16). Similarly, groups of *T. gondii*-infected and PBS-injected mice were subcutaneously treated with 50 mg/kg 1-DL-MT) (Sigma) or its vehicle once a day for 4 days starting at 4 dpi. Injections of 1-DL-MT were prepared in 0.1 N HCl, neutralized with an equal volume of 0.1 M NaOH, buffered with 2× PBS, and filtered through a 0.2-μm syringe filter.

Cell culture. A murine microglia cell line (MG6) was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% heat-inactivated fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), 10 μg/ml bovine insulin (Sigma), and 100 μM 2-mercaptoethanol (Sigma) (64, 65). An astrocyte cell line (OS3) was cultured in modified Eagle's medium (MEM; Sigma) containing 10% heat-inactivated FBS and 5 μg/ml bovine insulin (66). Both the MG6 and OS3 cell lines were provided by Riken BRC through the National Bio-Resource Project of MEXT, Japan.

Sucrose preference test. Anhedonic behavior is putatively considered a reduction in sucrose preference (67). First, mice were habituated with two bottles of water for 1 week, followed by one bottle of 1% sucrose and one bottle of water 2 days before treatment. The bottle position was switched every day according to a reward test protocol (3, 67). The total consumption of each fluid was measured daily, and sucrose preference was calculated by using the following formula: [sucrose intake/(water intake + sucrose intake)] $\times$ 100.

FST. FST evaluations were performed as described previously (68) and in our previous study (3). The FST was conducted under normal light for 6 min, and the time of immobility was then analyzed. Mice were individually placed into the water-filled FST cylinder (12-cm diameter, filled to a 25-cm water depth; Coulbourn Instruments, White Hall, PA). The water temperature was adjusted to within a thermoneutral range (31°C $\pm$ 1°C) for rodents. Immobility was defined as mice remaining motionless, except for necessary movements to maintain floating. The first 2 min of the test allowed accommodation. The duration of immobility within a 6-min session was recorded as an immobility score. Analysis was performed offline by an experienced observer blind to experimental groups. After the testing period, mice were towel dried and returned to their housing conditions.

Clinical score. Ethograms were customized according to the appearance of clinical symptoms during *T. gondii* infection within home and naive cages, as previously reported (3). In brief, scores varied from 0 (no signs) to 10 (all signs). Recorded signs included hunching, piloerection, warmth-seeking behavior (hiding in the corner of the cage and beneath bedding), ptosis (drooping or falling of the upper eyelids), sunken eyes, ataxia, reluctant movement, deficient evacuation and touch reflexes, and lying on the belly. Each symptom was equivalent and worth 1 point, and there were exactly 10 measures.

Footprint test. Each mouse was exposed to the footprint test (69) once a day, with some modifications. In brief, the feet of mice were brushed with black and red (right and left forefeet, respectively) and green and yellow (right and left hind feet, respectively) nontoxic paints. Animals were then allowed to walk along a 50-cm-long and 10-cm-wide enclosed runway with 10-cm-high walls. All mice performed three training runs before being subsequently tested with one run per day. Stride length was measured as the average distance of forward movement between each stride. For each step, three values from one run were measured, excluding footprints made at the beginning and the end of the run, where the animal was initiating and finishing movement, respectively. The mean value for each set of three values was used in subsequent analyses.

Quantitative reverse transcriptase PCR. Total RNA was extracted from the left halves of brains by using Tri reagent (Sigma). Reverse transcription was performed by using Superscript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. Amplification was performed by using a standard protocol recommended by the manufacturer (2 min at 50°C, 10 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min). Samples were run in duplicate. Amplification, data acquisition, and data analysis were carried out in an ABI Prism 7900HT sequence detection system (Thermo Fisher), and calculated cycle threshold (C_T) values were exported to Microsoft Excel for analysis. Expression levels of each gene relative to the value for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were calculated by using the $2^{\Delta CT}$ method (user bulletin no. 2; Perkin-Elmer Applied Biosystems, Waltham, MA). The optimal reference gene was selected based on the Cotton EST database. Specific primers for each gene were designed by using PRIMER EXPRESS software (Perkin-Elmer Applied Biosystems). A list of primer sequences is shown in Table S1 in the supplemental material.

DNA isolation and qPCR detection of *T. gondii*. qPCR was performed on purified DNA from brain homogenates, cultured cells, and egressed parasites in the culture medium by using Tri reagent (Sigma), as previously described (42). Briefly, after DNA purification, 50 ng of DNA extracted from brain homogenates or total cellular DNA was used for the amplification of parasite DNA with primers specific for the *T. gondii* B1 gene (70). Parasite numbers were calculated by interpolation on a standard curve, with C_T values plotted against a known parasite concentration. MG6 and OS3 cells were pretreated with 1-DL-MT (0.05 to 1.0 μM), Mino (5.0 to 40.0 nM), or IFN-γ (250 U/ml) for 24 h. After the medium was changed, cells were treated again with 1-DL-MT or Mino and infected with *T. gondii* (PLK strain; multiplicity of infection [MOI] of 0.25) for an additional 48 h. Data are presented as percentages of control inhibition in *T. gondii*-infected cells according to a previously described formula (42). The percentage of inhibition was calculated as {[(mean value of the control) − (value of the test sample)]/(mean value of the control)} $\times$ 100.

High-performance liquid chromatography (HPLC). Major monoamines, L-Trp, and their metabolites were examined in supernatants obtained from the right brain hemispheres by using an SC-5ODS column (Eicompak) and an electrochemical detector according to the monoamine analysis application manual (Eicom, Kyoto, Japan) and as previously described (3). Wet samples (100 mg) were homogenized in 0.5 ml of 0.2 M perchloric acid (including 100 μM EDTA-2Na and 1 μg/ml isoproterenol as an internal standard). External calibration was done by using freshly prepared standards. Final standard concentrations (100 pg), prepared daily from a stock in 20 mM acetic acid and stored at 4°C until use, were injected into the system. The mobile phase (pH 3.5) consisted of 0.075 M NaH$_2$PO$_4$ and 25 mM EDTA in 0.1 M citrate acetate buffer (83%) and methanol (17%). The flow rate was maintained at 0.5 ml/min throughout

chromatographic runs. Chromatographs were quantified by using PowerChrom software version 2.5 (eDAQ Pty. Ltd., Densitone East, Australia).

ELISA. Levels of plasma cytokines (IFN-γ, IL-1β, IL-6, and TNF-α) were measured by using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences Pharmingen, Piscataway, NJ) according to the manufacturer's recommendations. The amount of cytokine produced was calculated by using a standard cytokine curve performed on each immunoplate. Plasma L-Trp was assayed with a Bridge-It L-Trp fluorescence assay (Mediomics, St. Louis, MO) by using a fluorescence microplate reader (the Trp detection limit was 4.114 μmol at an optical density [OD] value of 0.036, using 485-nm excitation and 665-nm emission). Plasma L-Kyn (Sigma) was assayed by using a competitive ELISA with mouse L-Kyn monoclonal conjugated antibody (Abcam, Tokyo, Japan) (the Kyn detection limit was 35.5 nmol at an OD value of 0.042); readings were plotted against an L-Kyn standard curve.

Kynurenine production assay. IDO activity is directly correlated with the concentration of Kyn, its stable conversion product from Trp, as described previously (3, 42, 71).

Statistical analysis. Statistical analyses were performed by using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Results are presented as means ± standard deviations. The significance of differences was evaluated by two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons procedure or Student's t test. Data for sucrose preference were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. A P value of <0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00007-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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