The CD28/B7 Interaction Is Not Required for Resistance to *Toxoplasma gondii* in the Brain but Contributes to the Development of Immunopathology

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Infection of C57BL/6 mice with Toxoplasma gondii leads to chronic encephalitis characterized by infiltration into the brain of T cells that produce IFN-γ and mediate resistance to the parasite. Our studies revealed that expression of B7.1 and B7.2 was up-regulated in brains of mice with toxoplastic encephalitis (TE). Because CD28/B7-mediated costimulation is important for T cell activation, we assessed the contribution of this interaction to the production of IFN-γ by T cells from brains and spleens of mice with TE. Stimulation of splenocytes with Toxoplasma Ag or anti-CD3 mAb resulted in production of IFN-γ, which was inhibited by 90% in the presence of CTLA4-Ig, an antagonist of B7 stimulation. However, production of IFN-γ by T cells from the brains of these mice was only slightly reduced (20%) by the addition of CTLA4-Ig. To address the role of the CD28/B7 interaction during TE, we compared the development of disease in C57BL/6 wild-type (wt) and CD28−/− mice. Although the parasite burden was similar in wt and CD28−/− mice, CD28−/− mice developed less severe encephalitis and survived longer than wt mice. Ex vivo recall responses revealed that mononuclear cells isolated from the brains of chronically infected CD28−/− mice produced less IFN-γ than wt cells, and this correlated with reduced numbers of intracerebral CD4+ T cells in CD28−/− mice compared with wt mice. Taken together, our data show that resistance to T. gondii in the brain is independent of CD28 and suggest a role for CD28 in development of immune-mediated pathology during TE. The Journal of Immunology, 1999, 163: 3354–3362.

I nfection with the protozoan parasite Toxoplasma gondii is usually asymptomatic in immunocompetent individuals, while it can cause life-threatening disease in the immunocompromised host. Patients with AIDS, neoplastic diseases, or transplant recipients are at risk to develop toxoplastic encephalitis (TE) as a consequence of reactivation of a latent T. gondii infection (1–3). In these patients, the development of TE correlates with defects in T cell function, and this highlights the importance of T cells in resistance to T. gondii and prevention of TE (1, 3). Experimental models have confirmed the importance of T cells in resistance to T. gondii and have shown that T cell production of IFN-γ is critical for resistance to TE. Thus, in mice chronically infected with T. gondii, depletion of both CD4+ and CD8+ T cells results in increased severity of TE and death of mice within 2 wk (4). Moreover, administration of anti-IFN-γ to mice with TE resulted in increased parasite numbers and death of mice within 2 wk (4). Interestingly, although CD4+ T cells in combination with CD8+ T cells are involved in resistance to TE, there are contradictory reports on the effects of CD4+ T cells alone. Vollmer and colleagues (5) reported that depletion of CD4+ T cells in C3H/HeN mice resulted in an increase in severity of TE. However, Israelksi et al. (6) suggested that depletion of CD4+ T cells ameliorated the severity of TE. Thus, it appears that CD4+ T cells play a dual role during TE; they mediate resistance to T. gondii in the brain, but may also contribute to the development of immunopathology.

In the last decade, we have gained an understanding of the role of cytokines, in particular IL-12, in the events that lead to the generation of parasite-specific T cells that produce high levels of IFN-γ and restrict resistance to T. gondii (7). However, the role of costimulation in this process is less clear. Activation of T cell responses normally requires two signals. Signal one is provided by the MHC/TCR interaction, which alone fails to activate T cells, while an additional costimulatory signal allows the activation of T cells to progress (8). The interaction of CD28 on T cells with B7 molecules expressed on APC is one of the most important second signals required for T cell activation. The CD28/B7 interaction lowers the threshold of T cell activation and enhances proliferative and effector cell responses of T cells (8). In addition, costimulation via CD28 leads to increased production of growth factors like IL-2 and up-regulates levels of anti-apoptotic factors like Bcl-2 and thereby enhances survival of T cells (9). The importance of the CD28/B7 interaction in the regulation of T cell responses is illustrated by studies in which T cells from mice deficient in CD28 were shown to have severe defects in proliferation and cytokine production when stimulated with alloantigens, mitogen, or anti-CD3 mAb (10, 11). Moreover, blockade of the CD28/B7 interaction has also been shown to inhibit T cell-
mediated responses during graft rejection (12, 13), experimental autoimmune encephalomyelitis (EAE) (14, 15), diabetes (16, 17), and during infection (18, 19).

Given the critical role of T cells in the control of T. gondii in the brain and the importance of costimulation for T cell activation, we analyzed the role of the CD28/B7 interaction in the regulation of T cell responses during TE. Our studies demonstrate a marked up-regulation of the costimulatory molecules B7.1 and B7.2 in the brain during TE. However, production of IFN-γ by T cells present in the brain during TE and control of the parasite in the brain is largely independent of the CD28/B7 interaction. In addition, we found that mice lacking CD28 have reduced numbers of CD4+ T cells in the brain during TE, and this is associated with a less severe encephalitis and delayed time to death. These findings suggest that although CD28 plays little, if any, role in priming T cells for resistance against T. gondii, it may contribute to the development of T cell-mediated pathology during TE.

**Materials and Methods**

**Mice and infections**

Female CBA/CaJ and C57BL/6 mice were obtained from The Jackson Laboratories (Bar Harbor, ME). C57BL/6 CD28-deficient (CD28−/−) mice (10) originally obtained from Jackson Laboratories were bred within the University Laboratory Animal Resources facilities of the University of Pennsylvania. For experiments, 6- to 10-wk-old mice were inoculated i.p. with 15 cysts of the ME49 strain of T. gondii, which had been prepared from the brains of chronically infected CBA/CaJ mice. At the time of sacrifice, mice to be used were chosen randomly.

**Reagents**

Soluble Toxoplasma Ag (STAg) was prepared from in vitro cultured tachyzoites of T. gondii RH strain RH. Purified parasites were suspended in distilled water and repeatedly freeze-thawed. Following centrifugation (20 min, 800 × g; 15 min, 10,000 × g), the Ag-containing supernatant was stored at −70°C. STAg was titrated to determine the optimal concentration for induction of cytokines and was used at 30 μg/ml. Anti-CD3ε mAb (145-2C11) and anti-IL-2 mAb (IES 6-1A2) were prepared from hybridoma supernatant. CTLA4-Ig, a fusion protein comprising the extracellular domain of human CTLA-4 plus the Fc portion of human IgG, and the CD4 (RM4-5, IgG2a), anti-CD8 (53-5-5, IgG1), biotinylated anti-CD62 ligand (CD62L) (Mel14, IgG2a), anti-CD44 (IM7, IgG2b), anti-CD25 (7D4, rat IgM), PE-labeled anti-B7.2 (RMP-1, IgG2a), and PE-conjugated hamster anti-mouse B7.1 (16-10A1) were provided by Dr. Giorgio Trinchieri (Wistar Institute, Philadelphia, PA). Rat IgG was obtained from Sigma (St. Louis, MO). In titration experiments, the use of 3, 10, or 30 μg/ml of CTLA4-Ig and 5, 10, 20, or 40 μg/ml of anti-IL-2 mAb had similar effects on the production of IFN-γ by brain-associated mono- nuclear cells (BMNC). Thus, CTLA4-Ig was routinely used at a concentration of 30 μg/ml and anti-IL-2 mAb at 20 μg/ml. In previous studies (C.A.H., unpublished observations), we have found that 5 μg/ml of anti-IL-12 will completely abolish the effects of 10 ng/ml of IL-12 on NK cells, and this mAb was routinely used at a concentration of 20 μg/ml.

**Immunohistochemistry**

Brains from C57BL/6 mice, either uninfected or infected for 10 wk with T. gondii, were mounted in OCT compound (Miles Scientific, Naperville, IL), snap-frozen in cooled 2-methylbutane, and stored at −70°C. Cryostat sections (5 μm) were mounted on poly-L-lysine-coated microscope slides and fixed in ice-cold acetone for 10 min. Sections were incubated for 30 min with 0.3% H2O2/0.2 M NaNO3 to quench endogenous peroxidase activity, followed by blocking with 10% goat serum (Vector Laboratories, Burlingame, CA) in HBSS. Sections were then stained for 1 h with primary mAb against B7.1 (16-10A1, PharMingen, San Diego, CA), B7.2 (GL1, PharMingen) or isotype control mAb (PharMingen). After washing in HBSS, sections were incubated with biotinylated anti-hamster IgG Ab or anti-rat IgG Ab (Vector Laboratories). Subsequent incubation of the slides with peroxidase-conjugated avidin-biotin complex (Vectorstain Elite ABC kit, Vector) was performed according to the manufacturer’s instructions. Sections were developed with 3,3′-diaminobenzidine (Vector Laboratories), counterstained with hematoxylin, dehydrated, and mounted.

**Cell preparations**

Spleens were harvested and dissociated into single-cell suspension in complete RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 50 μM 2-ME, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml fungizone (Life Technologies). Erythrocytes were depleted using 0.83% ammonium chloride (Sigma), and cells were washed twice in complete RPMI 1640 before further analysis. For the preparation of mono-nuclear cells from the brain, mice were anesthetized using methoxyflurane and perfused through the left cardiac ventricle with 40 ml of ice-cold PBS to remove peripheral blood. Brains were removed, minced with scissors, and then digested for 1 h at 37°C with 300 μg/ml collagenase/dispace (Boehringer Mannheim, Indianapolis, IN) and 600 μg/ml DNase I (Boehringer Mannheim) in complete RPMI. The dissociated brain tissue was pelleted at 200 × g for 10 min, resuspended in a 60% isotonic Percoll solution (Sigma), and overlaid with a 30% Percoll solution. Discontinuous gradients were centrifuged for 25 min at 1000 × g. After removal of the myelin layer on top of the gradient, BMNC were harvested from the 30% (1.05 g/ml) to 60% (1.07 g/ml) interface and washed twice in complete RPMI before further analysis. Due to the low number of BMNC obtained per animal, cells from at least three mice were pooled per experiment unless otherwise stated.

**Flow cytometric analysis**

Cells were stained directly after their isolation using the following primary mAb (PharMingen or Caltag, San Francisco, CA): rat anti-mouse mAb, IFN-γ (anti-IgG2a, clone XMG1.2); anti-CD28 (RA3-6B2, IgG2b, clone GL1); anti-CD4 (RM4-5, IgG2a), anti-CD8 (53-5-5, IgG1), biotinylated anti-CD62 ligand (CD62L) (Mel14, IgG2a), anti-CD44 (IM7, IgG2b), anti-CD25 (7D4, rat IgM), PE-labeled anti-B7.2 (RMP-1, IgG2a), and PE-conjugated hamster anti-mouse B7.1 (16-10A1). Appropriate isotype control mAb were obtained from PharMingen or Caltag and included in each experiment. To block nonspecific binding via Fc receptors, cells were incubated for 15 min on ice with 50 μg/ml of rat IgG (Sigma) plus 50 μg/ml of Fc block (PharMingen) in FACS buffer (PBS, 0.2% BSA, 4 mM Na2Pi). Cells were then stained for 30 min on ice with primary mAb. After one wash, appropriate samples were incubated for 30 min with PE-conjugated streptavidin (PharMingen). After a further wash, propidium iodide (5 μg/sample) was added. Cells were immediately measured on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA), and analysis was performed using CELLQuest software (Becton Dickinson). Sample data were analyzed by guest on April 16, 2017 http://www.jimmunol.org/ Downloaded from

**Evaluation of histopathology and parasite burden**

At the time of sacrifice, samples of lung, liver, and brain were removed from each mouse and fixed in 10% neutral buffered formalin (Sigma), and embedded in paraffin. Organs were sectioned (5 μm) and stained with hematoxylin and eosin for visualization of pathological changes. To score pathological changes, a blinded analysis was performed using a score of 0 for no pathological changes; 1 for mild disease characterized by few lymphohytic infiltrates in perivascular cuffs and no menin- gitis; 2 for widespread lymphohytic infiltration with localized perivas- cular cuffs and menigitis, widespread areas of necrosis, and neutrophils; and 4 for inflammation throughout the brain with prominent perivascular cuffs and menigitis, widespread areas of necrosis, large numbers of neutrophils, and a prominent glisos. Slides were graded blindly by a single individual. To determine the cyst number, half of each brain was homogenized in 2 ml PBS. Repeated passage through a 21-gauge needle for each brain, three aliquots of 30 μl of the homogenate were scanned microscopically, and the number of cysts were counted and used to esti- mate the total number of cysts per brain.

**In vitro recall response**

Spleen cells and BMNC prepared at 8–12 wk postinfection were plated in complete RPMI at 1 × 106/ml in a final volume of 200 μl. Cultures were left untreated or were stimulated with 30 μg/ml STAg or 1 μg/ml anti-CD3 mAb. Where indicated, CTLA4-Ig (30 μg/ml), anti-IL-2 mAb (20 μg/ml), or anti-IL-12 mAb (20 μg/ml) was added, while control cultures were incubated with Chi-L6 (30 μg/ml) or rat IgG (20 μg/ml). Supernatants were collected after 48 h of incubation at 37°C. Cytokines were determined by sandwich ELISA as previously described (20) using the following mAb pairs: IFN-γ, RA2-65 and biotinylated AN-18; JES6-112 and biotinylated JES6-5H4; IL-12, C17.8 and biotinylated C15.6. Cytokine concentrations were determined from the appropriate standard curves using recombinant cytokines (Genzyme, Cambridge, MA).
F4/80 revealed that the majority of the cells expressing B7.1 or B7.2 were (MFI 65.75) and B7.2 (MFI = 115). Two-color analysis revealed that the majority of the cells expressing B7.1 or B7.2 were F4/80+ macrophages/microglia (50–60%), while only 2% were B220+ B cells (data not shown). Together, the immunohistochemical staining plus the flow cytometric analysis revealed a marked up-regulation of B7 expression in the brain during TE.

Different costimulatory requirements for splenic and intracerebral T cells during TE

Because the expression of B7 molecules in the brain was up-regulated during TE, we assessed the role of B7-mediated costimulation in the regulation of intracerebral T cell responses during TE. BMNC were isolated from wt mice at 10 wk postinfection and were stimulated with STAg or anti-CD3 mAb in the presence or absence of CTLA4-Ig, a B7 antagonist. Because IFN-γ is the major mediator of resistance against T. gondii (22), we measured the production of IFN-γ by BMNC and compared it to the production of IFN-γ by splenocytes from the same mice. As shown in Fig. 2, stimulation of splenocytes with STAg or anti-CD3 resulted in production of IFN-γ, and this was almost completely inhibited (85–95%) in the presence of CTLA4-Ig (p = 0.001 and <0.0001, respectively). Stimulation of BMNC from these mice with STAg or anti-CD3 also induced the production of high levels of IFN-γ, but the addition of CTLA4-Ig only resulted in a 15–30% reduction in the production of IFN-γ by cells stimulated with STAg (p = 0.0013) and failed to significantly reduce the production of IFN-γ by cells stimulated with anti-CD3 (p = 0.16). These results suggest that, during TE, T cell production of IFN-γ in the brain is largely CD28 independent.

Given the importance of IL-2 for the production of IFN-γ during T. gondii infection (23), it seemed likely that IL-2 was required for the production of IFN-γ by intracerebral T cells during TE. At 10 wk postinfection, splenocytes cultured in medium produced little IL-2, but the production of IL-2 was enhanced upon stimulation with STAg or anti-CD3 mAb (Fig. 3A). Neutralization of IL-2 in these cultures led to a strong reduction (65–70%) in the STAg- or anti-CD3-induced production of IFN-γ (p = 0.037 and 0.0017, respectively; Fig. 3B). In contrast, no IL-2 was detected in the supernatants of BMNC, and the addition of anti-IL-2 mAb to these cultures had no significant effect on the production of IFN-γ by unstimulated cells or cells stimulated with STAg or anti-CD3 (p = 0.7, 0.08, and 0.2, respectively; Fig. 3C).

IL-12 is another cytokine involved in stimulating production of IFN-γ by T cells (24, 25) and is required for the CD28-independent production of IFN-γ by splenic T cells during acute toxoplasmosis (5). Therefore, we tested if IL-12 was required for the CD28-independent production of IFN-γ by intracerebral T cells.

Statistical analyses

Statistical analyses were performed using the INSTAT or PRISM software (GraphPad, San Diego, CA). Unless otherwise stated, unpaired two-tailed Student’s t tests were performed. Survival curves were analyzed using a logrank test, and pathology scores were analyzed using the Mann-Whitney U test as previously described (21). Values of p < 0.05 were considered significant.

Results

Expression of costimulatory molecules during toxoplasmic encephalitis

Because immune-mediated control of T. gondii infection in the brain is T cell dependent (4) and optimal T cell stimulation requires costimulatory signals in addition to Ag recognition through the TCR, we investigated the expression of the costimulatory molecules B7.1 and B7.2 in the brains of mice with TE. Immunohistochemical analysis revealed that these molecules were not expressed in the brains of uninfected C57BL/6 mice. However, in brains from mice infected for 10 wk with T. gondii, expression of B7.1 and B7.2 was up-regulated. Both molecules were expressed in perivascular cuffs and in inflammatory foci within the brain parenchyma and were associated with areas of infiltration (data not shown). We were unable to detect any differences in the patterns of B7 expression from mice that differed in their severity of TE. Flow cytometric analysis of mononuclear cells isolated from the brain confirmed these results (Fig. 1). BMNC from uninfected mice expressed very low levels of B7.1 (mean fluorescence intensity (MFI) = 12.3) and B7.2 (MFI = 10.3). In contrast, BMNC isolated at 10 wk postinfection expressed higher levels of B7.1 (MFI = 65.75) and B7.2 (MFI = 115). Two-color analysis revealed that the majority of the cells expressing B7.1 or B7.2 were F4/80+ macrophages/microglia (50–60%), while only 2% were B220+ B cells (data not shown). Together, the immunohistochemical staining plus the flow cytometric analysis revealed a marked up-regulation of B7 expression in the brain during TE.

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FIGURE 1. Expression of B7.1 and B7.2 on BMNC during TE. Mononuclear cells isolated from the brains of normal or T. gondii-infected mice (10 wk postinfection) were stained with anti-B7.1 mAb or anti-B7.2 mAb (bold lines) or respective isotype control mAb (thin line) and analyzed by FACS as described in Materials and Methods. Data shown are representative of three experiments.

FIGURE 2. Effect of B7 blockade on production of IFN-γ by splenocytes and BMNC. Spleen cells (A) and BMNC (B) were prepared at 10 wk postinfection. Pooled cells from three mice were stimulated with STAg (30 μg/ml) or anti-CD3 mAb (1 μg/ml) in the presence of 30 μg/ml CTLA4-Ig or Chi-L6 for 48 h. The levels of IFN-γ in the culture supernatants were determined by ELISA. The data shown are the means ± SD from triplicate cultures. Similar results were seen in six additional experiments with three to five mice.
during chronic toxoplasmosis. At 10 wk postinfection, stimulation of splenocytes with STAg resulted in a 4-fold increase in the production of IL-12 compared with no stimulation or stimulation with anti-CD3 mAb (Fig. 4A). BMNC isolated from these chronically infected mice produced high levels of IL-12 when cultured in medium alone, and these IL-12 levels were not significantly increased in the presence of STAg or anti-CD3 mAb (Fig. 4B). Neutralization of IL-12 in cultures of splenocytes stimulated with STAg or anti-CD3 did not significantly affect the production of IFN-γ (p = 0.4 and 0.5, respectively; Fig. 4B). Similar results were observed with unstimulated BMNC or cultures stimulated with STAg or anti-CD3 (p = 0.8, 0.2, and 0.6, respectively; Fig. 5C). Together, our results suggest that, in contrast to T cell responses in the periphery, the response of T cells in the brain during TE, as measured by the production of IFN-γ, is largely CD28 independent and that neither IL-2 or IL-12 are required for the production of IFN-γ by these cells.

Resistance of CD28−/− mice to infection with T. gondii

To test our hypothesis that the intracerebral T cell response during TE is independent of the CD28/B7 interaction, we inoculated wt and CD28−/− mice with 15 cysts of the ME49 strain of T. gondii and monitored the survival of these mice. In accordance with our previous results in BALB/c mice,5 both wt and CD28−/− mice were resistant to acute toxoplasmosis and developed a chronic infection. C57BL/6 wt mice, which are susceptible to TE, started to die at ~2 mo postinfection, with 80% mortality by 6 mo postinfection (Fig. 5A). However, CD28−/− mice survived significantly longer, with 60% of the mice surviving at 6 mo postinfection. Similar survival patterns were observed in two additional experiments in both female and male mice. Statistical analysis of the

survival curves from these experiments revealed a $p < 0.0001$ for each of the three survival curves.

To analyze the ability of wt and CD28$^{-/-}$ mice to control parasite growth in the brain, we determined the number of cysts at this site. At 8 and 12 wk postinfection, large numbers of cysts were detected in the brains of wt and CD28$^{-/-}$ mice (Table I). However, at both time points, cyst numbers were not significantly different in wt and CD28$^{-/-}$ mice ($p > 0.5$). These results were confirmed by immunohistochemical staining of T. gondii in brain sections. Between 8 and 12 wk postinfection in both wt and CD28$^{-/-}$ mice, parasites were rarely seen within inflammatory foci, but intact cysts were scattered throughout otherwise normal areas of the brain (data not shown). In addition, lung, liver, and brain were assessed for histopathology between 8 and 12 wk postinfection. In both mouse strains, only mild inflammation was observed in lung and liver. As expected, the brain was the main organ affected during chronic toxoplasmosis, and both wt and CD28$^{-/-}$ mice developed TE. Wt mice developed moderate to severe encephalitis and meningitis characterized by perivascular cuffs, random foci of necrosis and gliosis within the parenchyma, and multifocal infiltrates of inflammatory cells within the meninges (Fig. 5, B and C). The inflammatory infiltrates within the perivascular cuffs and in the meninges consisted primarily of lymphocytes, with fewer plasma cells, macrophages, and neutrophils. The parenchymal foci of necrosis contained a higher proportion of neutrophils and often times nuclear debris. In the brains of infected CD28$^{-/-}$ mice, we also observed perivascular cuffs, random foci of necrosis and gliosis, as well as meningitis (me) between wt and CD28$^{-/-}$ mice (hematoxylin and eosin staining, magnification $\times100$). Similar pathology was observed in two repeat experiments with three to five mice per group.

Table I. Brain cyst number in chronically infected wt and CD28$^{-/-}$ mice$^a$

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Weeks Postinfection</th>
<th>Mice per Group</th>
<th>WT</th>
<th>CD28$^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>4</td>
<td>4009 ± 873.35</td>
<td>6430 ± 5685.72</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>5</td>
<td>1434 ± 947.06</td>
<td>1403 ± 696.31</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>5</td>
<td>450 ± 322.7</td>
<td>556 ± 277.06</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>3</td>
<td>1093 ± 603.32</td>
<td>867 ± 151.44</td>
</tr>
</tbody>
</table>

$^a$ The number of cysts per brain was determined as described in Materials and Methods. Values are the mean cyst number ± SD, $p > 0.5$. 

![Figure 5](http://www.jimmunol.org/content/3358/pdf)
not require CD28. In contrast, these findings suggest that stimulation through CD28 during chronic toxoplasmosis results in increased severity of TE and associated mortality.

Intracerebral response of chronically infected CD28−/− mice
Because CD28−/− mice infected with T. gondii survived significantly longer and showed less severe brain pathology than wt mice, we compared the intracerebral T cell responses of chronically infected wt and CD28−/− mice. In accordance with the reduced brain pathology in CD28−/− mice, fewer mononuclear cells were obtained from the brains of CD28−/− mice than from wt mice between 10 and 12 wk postinfection. In a representative experiment with four mice per group, $4.17 \times 10^6 \pm 0.49 \times 10^6$ mononuclear cells were isolated from CD28−/− brains and $5.66 \times 10^6 \pm 0.62 \times 10^6$ mononuclear cells from wt brains ($p < 0.01$). Similar results were observed in four experiments. Analysis of the recall response of intracerebral T cells showed that stimulation of BMNC from wt mice with STAg resulted in the production of high levels of IFN-γ (Fig. 6). In contrast, stimulation of BMNC from infected CD28−/− mice induced ~40–50% less IFN-γ ($p < 0.05$).

To analyze the reason for the reduced production of IFN-γ by BMNC isolated from CD28−/− mice, we compared the expression of CD62L, CD44, and CD25 by intracerebral and splenic T cell populations in C57BL/6 wt and CD28−/− mice. Naïve T cells express CD62Lhigh, CD44low; however, upon stimulation, expression of CD62L is down-regulated while expression of CD44 is increased (26). At 10 wk postinfection, splenocytes from chronically infected CD28−/− mice had fewer CD4+ T cells with a CD62Llow, CD44high phenotype than splenocytes from chronically infected wt mice (Fig. 7A). In contrast, splenocytes from infected CD28−/− and wt mice had similar percentages of CD8+ T cells with a CD62Llow, CD44high phenotype. We have also shown similar results for splenic T cells from BALB/c CD28−/− mice with chronic toxoplasmosis. Further analysis showed that, at 10 wk postinfection, none of the splenic CD8+ T cells and only 5% of the splenic CD4+ T cells expressed the early activation marker CD25 (Fig. 7A), suggesting that the predominant T cell populations in the spleens of mice with chronic toxoplasmosis are resting cells. Phenotypic analysis of T cells isolated from brains of wt mice with TE revealed that almost all CD4+ and CD8+ T cells expressed this phenotype. In contrast to splenic T cells, ~20% of the CD4+ and 10% of the CD8+ T cells isolated from the brains expressed CD25, indicating that intracerebral T cells are effector cells rather than resting memory cells. Although intracerebral T cells from chronically infected wt and CD28−/− mice were phenotypically similar, there was an ~50% reduction in the percentage of CD4+ T cells in the brains of CD28−/− mice ($p < 0.01$, paired t test) compared with wt mice (Fig. 8), while the percentage of intracerebral CD8+ T cells was similar in CD28−/− and wt mice. This decreased percentage of CD4+ T cells was accompanied by an increased percentage of macrophages. For example, in one experiment the percentage of F4/80+ cells in wt BMNC was 19.5%, whereas the BMNC from CD28−/− mice contained 29.6% F4/80+ cells ($p = 0.001$). Interestingly, FACS analysis of spleens from chronically infected wt and CD28−/− mice did not reveal any significant difference in the percentages of CD4+ and CD8+ T cells ($p = 0.77$ and 0.73, respectively). Thus, the decreased susceptibility of CD28−/− mice to TE is accompanied by decreased numbers of CD4+ T cells in the brain and is associated with reduced levels of IFN-γ at this site.

Discussion

The CD28/B7 interaction provides an important costimulatory signal required for the activation of T cell responses (8). In this study, we have demonstrated that, in mice chronically infected with T.
gondii, the CD28/B7 pathway is a major contributor to the production of IFN-γ in recall responses by parasite-specific T cells in the periphery, but has a less significant contribution to the production of IFN-γ by parasite specific T cells from the brain. These results indicate that T cell responses to T. gondii in the brain are largely independent of the CD28/B7 interaction, and this is supported by our studies, which showed that, in the absence of CD28, mice were able to control parasite growth in the brain and did not become more susceptible to the infection.

Given the important role of the CD28/B7 interaction in the regulation of T cell responses and the importance of T cells for resistance to TE, it was surprising to us that the CD28/B7 interaction does not contribute significantly to the protective T cell response in the brains of mice with chronic toxoplasmosis. Phenotypic characterization of T cells isolated from the brains of mice with TE revealed that they exhibited an effector phenotype characterized by the expression of CD62Llow, CD44high and low levels of the activation marker CD25. Because effector T cells are less dependent on costimulation through CD28 than naive T cells (27, 28), it is possible that the minimal role of CD28/B7 in activation of intracerebral T cells during TE simply reflects the activation status of these T cells. Whether other costimulatory molecules play a role in the CD28-independent activation of these effector T cells during TE is not known, but we have ruled out a role for endogenous IL-12 or IL-2 as second signals involved in T cell activation at this site. This contrasts with the role of these cytokines in promoting T cell responses in the periphery during toxoplasmosis (29–31).

Nevertheless, there are many molecules, including heat-stable Ag (32), 4-1BB ligand (33, 34), ICAM-1 (35), or VCAM-1 (36), that have costimulatory activity for T cells and that may provide a second signal for effector T cell responses in the brain. The CD40 ligand interaction is also an important costimulatory pathway (37, 38), and in our preliminary studies the absence of CD40 ligand did not affect the activation of T cells during TE. Nevertheless, the CD28/B7 interaction would occur at these sites. Other studies have shown that the B7/CTLA-4 interaction has a limited role in the regulation of T cell responses during TE. Nevertheless, our immunohistochemical studies localized the increased expression of B7 during TE to inflammatory foci that contain T cells. This colocalization of T cells and B7-expressing accessory cells suggests that the CD28/B7 interaction would occur at these sites. Other studies that have examined the expression of B7 in the brain during inflammation have reported increased expression of B7.1 and B7.2 in multiple sclerosis lesions (42) and in murine EAE (15, 43).

Although systemic administration of CTLA4-Ig or anti-B7.1 plus anti-B7.2 did not affect the severity of established EAE (44, 45), the intracerebral administration of CTLA4-Ig following the onset of clinical EAE ameliorated disease (46). Thus, the CD28/B7 pathway has a role in the local regulation of this autoimmune inflammatory disease of the brain. Our findings that in the absence of CD28 (and presumably B7-mediated activation of T cells) there is a reduction in the severity of TE, but no change in parasite burden, suggests that CD28/B7 interactions may be involved in the inflammatory process within the brain during TE.

The most interesting aspect of these studies was the finding that, in the absence of CD28, mice developed less severe TE and survived for a longer period of time. This finding correlated with a reduced production of IFN-γ and a reduced number of CD4+ T cells in the brains of CD28−/− mice. The events that lead to the development of pathology in the brains of mice susceptible to TE are complex. There is a requirement for both CD4+ and CD8+ T cells to mediate resistance to T. gondii at this site (4, 47), but the presence of a chronic inflammatory reaction within the brain would also result in immunopathology and contribute to the development of disease. A role for CD4+ T cells in mediating immunopathology during toxoplasmosis has been described during the acute phase of disease (48–51), and, relevant to our studies, depletion of CD4+ T cells was shown to ameliorate the severity of TE in C3H/HeN mice chronically infected with T. gondii (6).

While our data suggest that CD28/B7 costimulation may contribute to the development of immunopathology during TE, our studies do not distinguish whether CD28-mediated costimulation is required in the periphery or at the local site of inflammation, the brain. Costimulation through CD28 may directly lead to increased production of IFN-γ by pathogenic T cells because CD28 signaling increases the stability of cytokine mRNA, including IFN-γ (52–54). Alternatively, CD28 signaling induces the expression of survival factors like Bcl-xL, which protects T cells against Fas-mediated cell death (9, 55). Thus, during TE, CD28 costimulation may prolong the survival of activated T cells, which, in the end, may lead to increased numbers of pathogenic T cells. Indeed, our data show that CD28−/− mice with TE do have reduced numbers of CD4+ T cells with a CD62Llow, CD44high phenotype in the spleen and, concurrently, reduced numbers of effector CD4+ T cells in the brain.

The CD28/B7 interaction is generally regarded as one of the most important costimulatory pathways involved in the activation of T cell responses (8). However, the role of the CD28/B7 interaction in the immune response to infection remains unclear. CD28−/− mice infected with lymphocytic choriomeningitis virus could still generate cytotoxic T cells and be induced to show delayed-type hypersensitivity after infection (10). In addition, the absence of CD28 does not alter the T cell-dependent outcome of infection with Leishmania major (56) or Heligmosomoides polygyrus (57). The studies presented here add to our knowledge of the
role of CD28 in resistance to an important opportunistic pathogen and suggest that during TE, this interaction has a more important contribution to the development of immunopathology than to protective responses. Strategies designed to antagonize costimulation through CD28 may prove useful in the management of the pathological consequences of TE without affecting anti-parasite effector mechanisms.

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References
response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-γ, and TNF-α. J. Immunol. 157:798.


