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A Role for Inducible Costimulator Protein in the CD28-Independent Mechanism of Resistance to *Toxoplasma gondii*[1]

Eric N. Villegas,[2]* Linda A. Lieberman,* Nicola Mason,* Sarah L. Blass,* Valerie P. Zediak,* Robert Peach,† Tom Horan,‡ Steve Yoshinaga,‡ and Christopher A. Hunter[3]*

Long-term resistance to *Toxoplasma gondii* is dependent on the development of parasite-specific T cells that produce IFN-γ. CD28 is a costimulatory molecule important for optimal activation of T cells, but CD28$^{-/-}$ mice are resistant to *T. gondii*, demonstrating that CD28-independent mechanisms regulate T cell responses during toxoplasmosis. The identification of the B7-related protein 1/inducible costimulator protein (ICOS) pathway and its ability to regulate the production of IFN-γ suggested that this pathway may be involved in the CD28-independent activation of T cells required for resistance to *T. gondii*. In support of this hypothesis, infection of wild-type or CD28$^{-/-}$ mice with *T. gondii* resulted in the increased expression of ICOS by activated CD4$^+$ and CD8$^+$ T cells. In addition, both costimulatory pathways contributed to the in vitro production of IFN-γ by parasite-specific T cells and when both pathways were blocked, there was an additive effect that resulted in almost complete inhibition of IFN-γ production. Although in vivo blockade of the ICOS costimulatory pathway did not result in the early mortality of wild-type mice infected with *T. gondii*, it did lead to increased susceptibility of CD28$^{-/-}$ mice to *T. gondi* associated with reduced serum levels of IFN-γ, increased parasite burden, and increased mortality compared with the control group. Together, these results identify a critical role for ICOS in the protective Th1-type response required for resistance to *T. gondii* and suggest that ICOS and CD28 are parallel costimulatory pathways, either of which is sufficient to mediate resistance to this intracellular pathogen. *The Journal of Immunology*, 2002, 169: 937–943.

Infection with *Toxoplasma gondii* results in the activation of a strong cell-mediated Th1-type immune response characterized by the production of IFN-γ, the major mediator of resistance to infection (1). Given the critical role of T cells in the initiation and maintenance of immunity to *T. gondii* (2–4), it is important to understand the events that regulate their responses. Since the description of the two signal hypothesis of lymphocyte activation, it has been recognized that costimulation is an important process involved in the development and regulation of T cell responses. The B7/CD28 pathway is an important costimulatory interaction involved in T cell activation (5). However, previous studies from this laboratory have shown that CD28$^{-/-}$ mice infected with *T. gondii* have a reduced CD4$^+$ T cell response, but do mount a T cell response that is sufficient to provide resistance to this infection (6, 7). These results suggest that other costimulatory pathways are likely involved in the regulation of T cell responses during toxoplasmosis, or may reflect the ability of *T. gondii* infection to provide a TCR signal that is sufficient to overcome the requirement for CD28-mediated costimulation (8, 9).

Costimulatory functions have been ascribed to molecules such as ICAM-1, CD2, CD30, CD40 ligand, CD44, CD137, and 4-1BB (10–13), but our studies have suggested that neither ICAM-1, CD40 ligand, or CD44 are critical for the development of T cell responses required for resistance *to T. gondii* (7, 14). Recently, inducible costimulator protein (ICOS),[4] a molecule that is homologous to CD28 has been shown to be a potent costimulatory molecule in T cell activation (15, 16). Unlike CD28, which is constitutively expressed on T cells, ICOS is expressed only on activated CD4$^+$ and CD8$^+$ T cells and a subpopulation of memory T cells (15, 16). The ligand for ICOS is B7-related protein 1 (B7RP-1; B7 h, GL50, ICOSL), which is structurally related to members of the B7 family, and is expressed on macrophages, B cells, TNF-α-activated fibroblast cells, as well as on other nonlymphoid tissues (15, 17–19). Early studies demonstrated that ICOS was involved in the production of IL-4, IL-10, and IFN-γ, but not IL-2 by T cells, and subsequent studies reported that ICOS is expressed preferentially on Th2-type T cells and is important during Th2-type immune responses (20–25). Additional studies have shown that ICOS is involved in the regulation of Th1 and Th2 responses to the parasites *Nippostrongylus brasiliensis* and *Leishmania mexicana* as well as the viral pathogens lymphocytic choriomeningitis virus and vesicular stomatitis virus but the importance of ICOS in the outcome of these infections is unclear (26, 27). Nevertheless, since ICOS is capable of providing a costimulatory signal to activate T cells, this was a candidate costimulatory molecule for the CD28-independent regulation of Th1-type T cell responses during toxoplasmosis. The studies presented here reveal that ICOS expression is up-regulated on activated T cells following infection with *T. gondii* and that this is independent of CD28-mediated signaling.

*Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104; †Bristol-Myers Squibb Pharmacology Research Institute, Princeton, NJ 08543; and ‡Amgen Inc., Thousand Oaks, CA 91320

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[2] Current address: Cancer Research Division, Molecular and Cellular Biology Department, Immunology Division, University of California, 441 LSA, Berkeley, CA 94720-3200.

[3] Address correspondence and reprint requests to Dr. Christopher A. Hunter, Department of Pathobiology, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104-6008. E-mail address: chunter@phl.vet.upenn.edu

[4] Abbreviations used in this paper: ICOS, inducible costimulator protein; B7RP-1, B7-related protein 1; BMNC, brain-associated mononuclear cell; Chi-L6, chimeric protein L6; STAg, soluble *Toxoplasma* Ag; TE, toxoplasmic encephalitis; WT, wild type.

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Moreover, ICOS and CD28 are both required for optimal production of IFN-γ by parasite-specific T cells and the in vivo blockade of the B7RP-1-ICOS interaction results in increased susceptibility of $CD28^{-/-}$ mice to infection. Together, these results identify a critical role for ICOS in the protective Th1-type response to *T. gondii* and suggest that ICOS and CD28 are parallel costimulatory pathways, either of which is sufficient to mediate resistance to *T. gondii*.

Materials and Methods

Animals

C57BL/6, Swiss Webster, and CBA/CaJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 CD28-deficient animals (28) were bred and housed in the animal facilities at the University of Pennsylvania (Philadelphia, PA). All mice used were maintained within microisolator caging units at the University Laboratory Animal Resource facilities at the University of Pennsylvania. Six- to 12-wk-old C57BL/6 or $CD28^{-/-}$ mice were used for all experiments with three to six mice per group.

Parasites and infection

Swiss Webster and CBA/CaJ mice were used to maintain the ME49 strain of *T. gondii* and used as sources of tissue cysts for these experiments. For infection with ME49 tissue cysts, brains of chronically infected CBA/CaJ mice were harvested and prepared as previously described (29, 30). Mice were inoculated i.p. with 20 ME49 cysts in a volume of 0.2 ml. Serum samples were collected at different time points and survival was monitored. The RH strain of *T. gondii* was maintained in human foreskin fibroblast as previously described (7) and used to prepare soluble *Toxoplasma* Ag (STAg) as previously described (31). STAg activity was titrated to determine the optimal concentration for splenocyte proliferation and cytokine production (25 μg/ml).

Cytological analysis

At the time of sacrifice, peritoneal exudate cells were harvested with 5 ml of ice-cold 1× PBS without Ca^{2+}/Mg^{2+} (BioWhittaker, Walkersville, MD) and 5×10^5 cells/100 μl were used to prepare cytospins to quantitate parasite burden. Cells were fixed and stained using Protocol Hema3 (Biochemical Sciences, Swedesboro, NJ) as described in the manufacturer's manual, then mounted and sealed using Cytoseal (Stephens Scientific, Kalamazoo, MI).

Reagents

Complete RPMI 1640 (Life Technologies, Gaithersburg, MD) medium containing 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), sodium pyruvate, nonessential amino acids, penicillin (100 U/ml), streptomycin (1 mg/ml), and amphotericin B (25 ng/ml) (BioWhittaker). The 145-2C11 hybridoma for anti-CD3ϵ was provided by Dr. J. Bluestone (University of Chicago, Chicago, IL) and used to prepare purified anti-CD3. HuCTLA-4Ig, a fusion protein comprised of the human CTLA-4 extracellular domain and Fc portion of human IgG, and the chimeric control fusion protein L6 (Chi-L6) was supplied by Bristol-Myers Squibb Research Institute (Princeton, NJ) and used at a concentration of 20 μg/ml. B7RP-1Fc, a fusion protein consisting of the extracellular portion of the mouse B7RP-1 and the Fc portion of human IgG, was used to detect expression of ICOS and was supplied by Amgen (Thousand Oaks, CA). mAb specific to B7RP-1 (1B7; Amgen) was used at a concentration of 20 μg/ml. Infected mice were treated with 1 mg of anti-B7RP-1 or rat Ig (Sigma-Aldrich, St. Louis, MO) at days 0 and 5 postinfection.

Isolation of brain mononuclear cells

Isolation of brain mononuclear cells (BMNCs) were performed as previously described (6). Briefly, mice were anesthetized with 200 mg/kg Kitaset (Fort Dodge Animal Health, Fort Dodge, IA) and 10 mg/kg Xyla-Ject (Phoenix Pharmaceuticals, St. Joseph, MO). Mice were perfused through the left ventricle with 50–60 ml of ice-cold PBS to remove peripheral blood. Brains were then collected, minced, resuspended in complete RPMI 1640, and digested with 100 μg of collagenase/dispase (Roche Diagnostics, Indianapolis, IN) and 300 μg of DNase (Roche Diagnostics) for 45 min at 37°C. Brain digests were then pelleted at $2000 \times g$ for 10 min at 4°C and resuspended in 60% isotonic Percoll solution and overlaid with 30% Percoll solution. The Percoll gradient was centrifuged at $1000 \times g$ for 25 min at 25°C and the top myelin layer was carefully removed before harvesting BMNCs at the 60 and 30% interphase layer. Cells were washed with complete RPMI 1640 before further analysis. Due to the low number of BMNCs obtained per animal, cells from at least three mice were pooled in each experiment.

Analysis of T cell responses

Individual spleens from animals were harvested, dissociated into single-cell suspensions, and depleted of erythrocytes using 0.83% w/v ammonium chloride (Sigma-Aldrich). Cells were washed and resuspended in complete RPMI 1640 before further analysis. Splenocytes or BMNCs were plated at a cell density of 2×10^5 cells/well in a final volume of 200 μl in 96-well plates (Costar, Costar, NY). Cells were stimulated with soluble anti-CD3 (145-2C11) at a final concentration of 1 μg/ml or STAg for 48 h at 37°C/5% CO_2 in the presence of CTLA-4Ig and/or anti-B7RP-1. Levels of IFN-γ from splenocyte cultures were measured using ELISA as previously described (32).

Flow cytometric analysis

To assess surface expression of ICOS on T cells, purified splenocytes were resuspended in FACS buffer (1× PBS, 0.2% BSA fraction V, and 4 mM sodium azide) at a concentration of 1×10^7 cells/ml. A total of 10^6 cells was preincubated with saturating concentrations of Fc Block for 20 min on ice and stained with PE or PerCP-conjugated anti-CD4, FITC-conjugated anti-CD8 (BD PharMingen, San Diego, CA), and B7RP-1Fc (1 mg/ml) for 30 min on ice. Cells were washed with FACS buffer and then incubated with biotinylated goat anti-human IgG (Vector Laboratories, Burlingame, CA) for 30 min on ice. Cells were washed with FACS buffer and incubated with allophycocyanin-conjugated streptavidin (BD PharMingen) for an additional 20 min on ice. Cells were then washed, resuspended in FACS buffer, and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). For intracellular detection of IFN-γ, purified splenocytes were plated in a 96-well plate (Costar) at a density of 4×10^5 cells/well in a final volume of 200 μl. Cells were stimulated with STAg (25 μg/ml) for 72 h, then PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (10 μg/ml; Sigma-Aldrich) were added to cultures during the last 5 h of stimulation. Cells were harvested, washed, resuspended in FACS buffer, and then stained with various fluorochrome-conjugated Abs as described above. Cells were then washed with FACS buffer, fixed with 4% w/v paraformaldehyde, washed again, and permeabilized with 0.1% w/v saponin in FACS buffer. After permeabilization, cells were stained with allophycocyanin-conjugated anti-IFN-γ (BD PharMingen) for 30 min on ice. Cells were washed once with 0.1% saponin buffer and then with FACS buffer. Analysis of the cells was performed using a FACSCalibur flow cytometer (BD Biosciences). Results were analyzed using CellQuest software (BD Biosciences). Ab concentrations were empirically determined to give optimal staining for flow cytometric analyses.

Statistics

INSTAT software (GraphPad, San Diego, CA) was used for unpaired or paired two-tailed Student's *t* test. A value of $p < 0.05$ was considered to be significant.

Results

Infection with T. gondii *results in increased expression of ICOS on activated T cells*

The recent identification of the B7RP-1-ICOS interaction and its role in providing a costimulatory signal for T cell activation suggested a possible alternative costimulatory signal involved in the CD28-independent mechanism of T cell-mediated resistance to *T. gondii* (6, 7). To determine whether ICOS was involved in the immune response to *T. gondii*, experiments were performed to compare the expression of ICOS in uninfected and infected mice. C57BL/6 mice were infected i.p. with 20 cysts of the ME49 strain of *T. gondii* and at 7 days after infection, splenocytes were harvested, then analyzed for expression of ICOS. In uninfected C57BL/6 animals, only a small percentage of T cells expressed ICOS and all of these T cells were $CD44^{high}$ (Fig. 1*A*). By day 7 after infection, there was small increase in the percentage of $CD8^+$ T cells which expressed ICOS and a marked expansion in the percentage of $CD4^+$ T cells expressing ICOS (Fig. 1*B*). Although a slight variation in the magnitude of ICOS up-regulation on

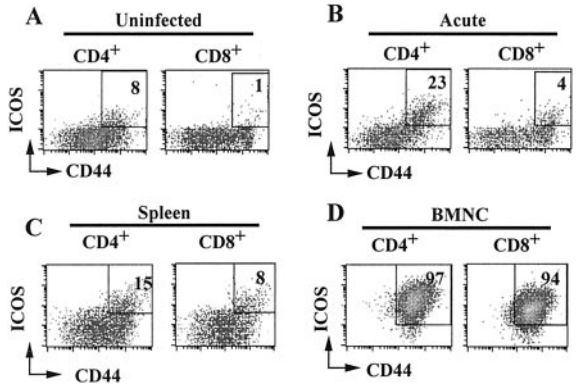

FIGURE 1. Infection-induced expression of ICOS by T cells. ICOS expression on $CD4^+$ and $CD8^+$ T cells from spleen cells of uninfected (*A*) or 7-day infected (*B*) C58BL/6 mice. ICOS expression on $CD4^+$ and $CD8^+$ splenic T (*C*) cells or on BMNCs (*D*) from 8-wk infected C57BL/6 mice. Numbers indicated within each boxed region represent the percentage of $ICOS^+$, $CD44^{high}$-expressing $CD4^+$ or $CD8^+$ T cells. Data are representative of two to four independent experiments.

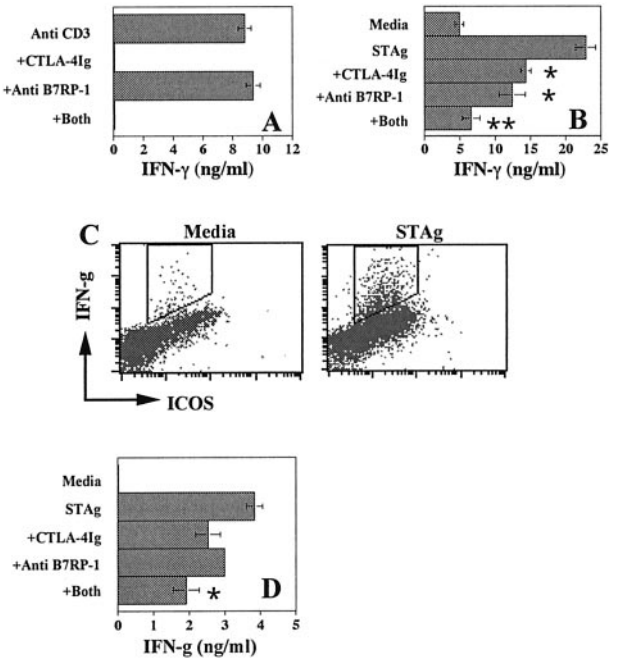

FIGURE 2. Role of ICOS and CD28 in the production of IFN-γ during toxoplasmosis. *A*, Splenocytes from uninfected C57BL/6 mice stimulated with soluble anti-CD3 (1 μg/ml) for 48 h in the presence of CTLA-4Ig (20 μg/ml), anti-B7RP-1 (20 μg/ml), or in combination. IFN-γ levels in supernatants were measured by ELISA. *B*, Splenocytes from C57BL/6 infected for 7 days were stimulated with STAg (25 μg/ml) and the effects of CTLA-4Ig or anti-B7RP-1 alone (*, $p < 0.003$) or in combination (**, $p < 0.0001$) on the production of IFN-γ were assessed. *C*, Splenocytes from 7-day infected C57BL/6 mice were stimulated with STAg for 3 days. Intracellular cytokine detection of IFN-γ and ICOS expression on $CD4^+$ T cells were assessed as described in *Materials and Methods*. The boxed region represents IFN-γ^+ and $ICOS^+$ $CD4^+$ T cells. *D*, BMNCs from chronically infected (>6 wk postinfection) animals were isolated and stimulated with STAg in the presence of CTLA-4Ig, anti-B7RP-1, or in combination, and levels of IFN-γ in supernatants were measured by ELISA. Results presented are the means ± SD of a single experiment with three to six mice per experimental group, *, $p < 0.001$. Data are representative of two to four independent experiments.

$CD4^+$ and $CD8^+$ T cells was observed in four independent experiments, the levels of ICOS expressed on T cells from infected animals was consistently and reproducibly higher than uninfected C57BL/6 mice. In contrast, at 6 wk postinfection, ICOS expression on splenic $CD4^+$ T cells had decreased compared with splenic T cells from 7-day infected animals, albeit ICOS expression on splenic T cells from 6-wk infected animals remained higher than those of uninfected mice (Fig. 1*C*).

Although C57BL/6 mice are able to control the acute phase of infection, they do develop toxoplasmic encephalitis (TE) in which large numbers of $CD4^+$ and $CD8^+$ T cells infiltrate into the brain, a process required to control parasite replication at this site (3, 4). Moreover, the majority of T cells present in the brain during TE display an effector/memory phenotype (i.e., $CD25^{low}$, $CD44^{high}$, and $CD62L^{low}$) (6). Whereas the brains of uninfected mice contain no lymphocytes, analysis of brain mononuclear cells from chronically infected wild-type (WT) mice revealed that >90% of the $CD44^{high}$, $CD4^+$, and $CD8^+$ T cells expressed ICOS (Fig. 1*D*). Together, these data reveal that following infection, the activation of T cells in the spleen is accompanied by increased expression of ICOS during the acute phase of infection. As the infection is controlled in the periphery, the numbers of $ICOS^+$ T cells in the spleen return to near-normal levels. However, T cells isolated from the brains of mice with TE continued to express high levels of ICOS. Together, these results suggest that the infection-induced expression of ICOS is restricted to parasite-specific effector T cells.

CD28 and ICOS are both required for parasite-specific production of IFN-γ

Since the ability of T cells to produce IFN-γ is essential for resistance to *T. gondii* (1), the role of ICOS and CD28 in the ability of splenocytes from naive and acutely infected mice to produce IFN-γ was compared. Stimulation of naive splenocytes with anti-CD3 resulted in the production of IFN-γ and, as previously reported (33), this response was almost completely blocked by CTLA-4Ig ($p < 0.0004$). In contrast, anti-B7RP-1 had no effect on the production of IFN-γ by these resting T cells (Fig. 2*A*, $p = 0.7$). These results indicate that although CD28 is critical in activation and production of IFN-γ by resting T cells, ICOS plays a minimal role in this initial activation process. When splenocytes from acutely infected mice were stimulated with STAg, the production of IFN-γ by parasite-specific T cells was partially inhibited by the addition of CTLA-4Ig (Fig. 2*B*, $p < 0.003$). Similarly, the addition of anti-B7RP-1 also had an inhibitory effect on the production of IFN-γ in these cultures (Fig. 2*B*, $p < 0.001$). Moreover, the addition of CTLA-4Ig plus anti-B7RP-1 resulted in an additive effect and reduced the levels of IFN-γ production to background (Fig. 2*B*, $p < 0.0001$). Similar results were observed when infected splenocytes from C57BL/6 mice were stimulated with anti-CD3 (data not shown). Further analysis of Ag-stimulated splenocytes from acutely infected animals revealed that few $CD8^+$ T cells were IFN-γ^+ (data not shown) but that >75% of the $CD4^+$ T cells that expressed IFN-γ^+ also expressed ICOS (Fig. 2*C*).

When BMNCs from chronically infected mice were isolated and stimulated with STAg, there was increased production of IFN-γ compared with unstimulated cells. The addition of CTLA-4Ig or anti-B7RP-1 only resulted in a small, but significant, reduction in the levels of IFN-γ produced in response to Ag (Fig. 2*D*, $p < 0.005$ and 0.003, respectively). However, when both costimulatory pathways were blocked, there was a 50% reduction in levels of IFN-γ produced (Fig. 2*D*, $p < 0.001$). Together, these results indicate that following infection with *T. gondii*, costimulation through ICOS, in combination with CD28, is required for optimal

production of IFN-γ. Thus, in WT mice there are distinct CD28- and ICOS-dependent components required for optimal production of IFN-γ.

Infection-induced expression of ICOS is independent of CD28

The data presented in the previous section support the hypothesis that ICOS has a role in the regulation of IFN-γ responses during toxoplasmosis and that its effects may be independent of CD28. However, previous in vitro studies have reported that the ability of activated T cells to express ICOS was largely dependent on costimulation through CD28 (20, 34). Therefore, to help determine whether ICOS was involved in CD28-independent pathways of resistance to *T. gondii*, studies were performed to assess whether the infection-induced increase in expression of ICOS occurred in $CD28^{-/-}$ mice. Analysis of ICOS expression on T cells revealed that a small percentage of $CD4^+$ (3%) and $CD8^+$ T cells (1%) express ICOS and expression was restricted to $CD44^{high}$ T cells (Fig. 3*A*). However, as described previously, infection of $CD28^{-/-}$ mice results in normal numbers of activated $CD8^+$ T cells, but there are decreased numbers of activated $CD4^+$ T cells (6, 7). This defect, as well as the infection-induced increase in the number of splenocytes, makes it difficult to directly compare the percentage of activated $CD4^+$ T cells that express ICOS in $CD28^{-/-}$ and WT mice. Therefore, the data presented in Fig. 3*B* show the total numbers of $CD4^+$ and $CD8^+$ T cells which express ICOS in the spleens of uninfected and infected mice. At day 7 postinfection, analysis of ICOS expression revealed that there was no significant difference ($p = 0.26$) in the total numbers of $ICOS^+CD8^+$ T cells in WT and $CD28^{-/-}$ mice, and although uninfected and infected $CD28^{-/-}$ mice had fewer $ICOS^+CD4^+$ T cells than WT controls (uninfected, $p < 0.02$; infected, $p < 0.01$), there was a 2-fold increase in the numbers of $ICOS^+CD4^+$ T cells in the infected $CD28^{-/-}$ mice (Fig. 3*B*). In contrast to the spleen, analysis of $CD4^+$ and $CD8^+$ T cells isolated from the brains of chronically infected $CD28^{-/-}$ mice revealed that the majority of these T cells were $CD44^{high}$ and expressed high levels of ICOS (Fig. 3*C*). These results demonstrate that although there are fewer $ICOS^+$ T cells in the spleens of acutely infected $CD28^{-/-}$ mice compared with WT mice, there is still a significant increase in the number of $CD28^{-/-}$ T cells which have up-regulated ICOS during the acute phase of infection. Moreover, by the chronic phase of the infection, expression of ICOS by T cells from the brains of infected $CD28^{-/-}$ mice was comparable to that observed in chronically infected WT mice. Thus, the ability of T cells to increase expression of ICOS in response to infection can occur independently of CD28.

CD28-independent, ICOS-dependent effector T cell responses during toxoplasmosis

To determine the possible role of ICOS as an alternative costimulatory molecule in the CD28-independent resistance to *T. gondii* (6, 7), splenocytes from infected $CD28^{-/-}$ mice were stimulated with STAg and production of IFN-γ by T cells in the presence of CTLA-4Ig, anti-B7RP-1, or in combination were measured by ELISA. Splenocytes from $CD28^{-/-}$ mice infected for 7 days and stimulated with STAg produced low levels of IFN-γ and the addition of CTLA-4Ig did not alter IFN-γ levels (7) (data not shown). However, the addition of anti-B7RP-1 resulted in a marked decrease ($p < 0.002$) in the production of IFN-γ (Fig. 4*A*). To further explore the in vitro role of ICOS in regulating effector T cell function in the absence of CD28, BMNCs from the brains of chronically infected $CD28^{-/-}$ animals were stimulated with toxoplasma Ag, and IFN-γ production in the presence or absence of anti-B7RP-1 was measured. BMNCs from chronically infected

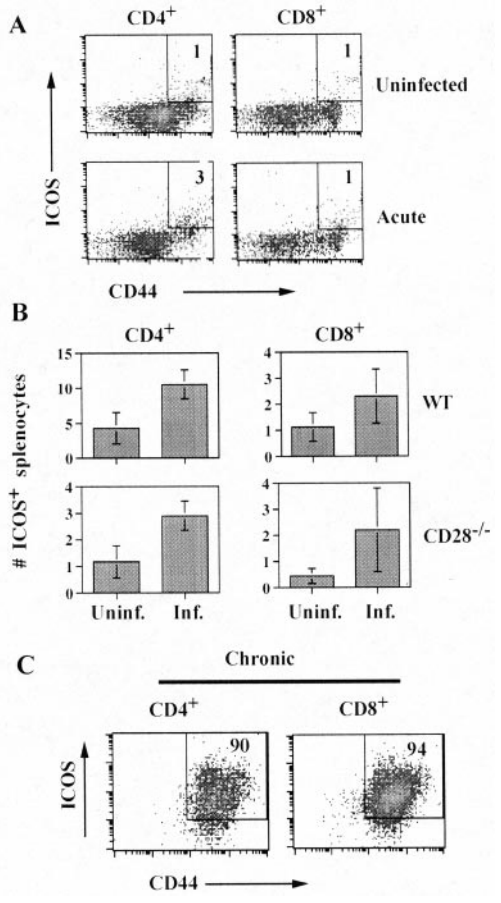

FIGURE 3. CD28-independent expression of ICOS following infection with *T. gondii*. *A*, Expression of ICOS on uninfected (Uninf.) and infected (Inf.) $CD4^+$ and $CD8^+$ splenic T cells from $CD28^{-/-}$ mice infected with *T. gondii* at 7 days postinfection. *B*, Total $CD4^+$ or $CD8^+$ splenic T cells expressing ICOS were quantitated and compared between uninfected and 7-day infected WT and $CD28^{-/-}$ mice. Results presented are the means of three independent experiments containing three to six mice per experimental group. Data presented in *A* and *B* are representative of four independent experiments. *C*, Expression of ICOS on $CD44^{high}CD4^+$ or $CD8^+$ T cells from BMNCs of chronically infected $CD28^{-/-}$ mice. Numbers indicated within each boxed region represent percentage of $ICOS^+$-, $CD44^{high}$- expressing $CD4^+$ or $CD8^+$ T cells. Similar results were seen in a repeat experiment.

$CD28^{-/-}$ mice stimulated with STAg produced significantly higher levels of IFN-γ compared with unstimulated cells ($p < 0.001$), and anti-B7RP-1 significantly inhibited ($p < 0.001$) the response to STAg (Fig. 4*B*). These results strengthen the hypothesis that the B7RP-1/ICOS pathway is required for the CD28-independent mechanisms involved in the production of IFN-γ by parasite-specific T cells during the acute and chronic phases of infection.

Blockade of the ICOS-B7RP-1 interaction leads to increased susceptibility of $CD28^{-/-}$ mice to infection with T. gondii

To determine the in vivo role of ICOS, WT mice were infected with *T. gondii* and treated with anti-B7RP-1 at days 0 and 5 after infection. Initial studies revealed no significant difference in mortality, serum levels of IFN-γ, or parasite burden in treated WT mice compared with control treated mice (data not shown). However, $CD28^{-/-}$ mice treated with anti-B7RP-1 on days 0, 3, and 5 of infection showed increased susceptibility to infection compared with infected $CD28^{-/-}$ mice that received the sham treatment

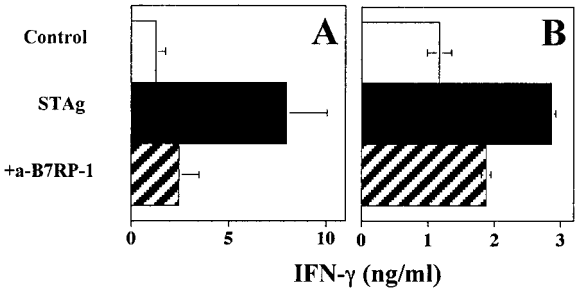

FIGURE 4. CD28-independent, ICOS-dependent effector T cell function following infection with *T. gondii*. *A*, Splenocytes from infected $CD28^{-/-}$ mice ($n = 4$) stimulated with STAg for 48 h in the presence of Chi-L6 or anti-B7RP-1 and levels of IFN-γ in supernatants were measured by ELISA. Data are representative of four independent experiments. *B*, BMNCs from chronically infected $CD28^{-/-}$ mice were harvested as described in *Materials and Methods* and stimulated with STAg for 48 h in the presence of control Chi-L6 or anti-B7RP-1. Presence of IFN-γ in supernatants was measured by ELISA. Results presented are the means ± SD of a single experiment with three to five mice per experimental group with similar results obtained in a repeat experiment.

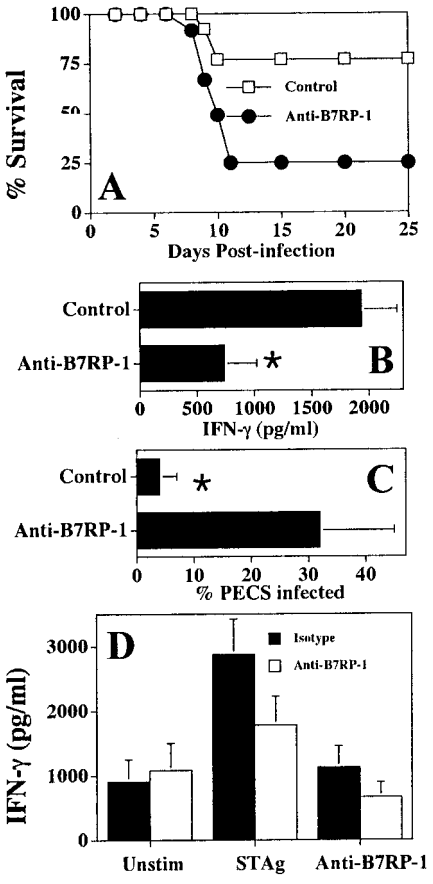

FIGURE 5. In vivo blockade of the B7RP-1/ICOS pathway results in susceptibility of $CD28^{-/-}$ mice to infection with *T. gondii*. *A*, Survival of $CD28^{-/-}$ mice infected with 20 cysts of ME49 i.p. and treated with 1 mg of anti-B7RP-1 at days 0 and 5. Results were pooled from two independent experiments (control treated, $n = 13$; anti-B7RP-1 treated, $n = 12$). *B*, Serum levels of IFN-γ at day 5 postinfection from control-treated or anti-B7RP-1-treated $CD28^{-/-}$ mice (∗, $p < 0.03$). Results presented are means ± SD of a single experiment containing four mice per experimental group and are representative of four independent experiments. *C*, Effects of in vivo anti-B7RP-1 treatment on parasite burden in the peritoneum of $CD28^{-/-}$ mice (∗, $p < 0.005$). Results presented are means ± SD of a single experiment containing four to five mice per experimental group. Similar results were obtained in four independent experiments. *D*, Production of IFN-γ by splenocytes from $CD28^{-/-}$ mice treated in vivo with an isotype control (■) or anti-B7RP-1 (□) and infected with *T. gondii* for 7 days. Cells were stimulated with STAg alone or in combination with anti-B7RP-1 for 48 h and the production of IFN-γ was measured by ELISA. Results presented are from a single experiment with four mice per group and similar results were seen in a repeat experiment.

(Fig. 5*A*). The ability of anti-B7RP-1 treatment to increase susceptibility of $CD28^{-/-}$ mice to infection correlated with a significant reduction in serum levels of IFN-γ (Fig. 5*B*, $p < 0.03$) and an increase in the parasite burden compared with sham-treated $CD28^{-/-}$ mice (Fig. 5*C*, $p < 0.005$). Analysis of splenocytes from infected $CD28^{-/-}$ mice treated with anti-B7RP-1 revealed that they produced reduced levels of IFN-γ when stimulated with STAg compared with control $CD28^{-/-}$ mice. Although these results failed to reach statistical significance ($p = 0.17$) a similar trend (7.9 vs 4.1 ng; $n = 3$ mice/group) was also observed in a repeat experiment. However, for both experimental groups, the addition of anti-B7RP-1 in vitro resulted in a significant reduction in the levels of IFN-γ (Fig. 5*D*, $p < 0.03$ for both experimental groups). Taken together, these results demonstrate that in the absence of CD28, ICOS has an important role in the production of IFN-γ and resistance to *T. gondii*.

Discussion

Studies from this laboratory have shown that although CD28 was required for optimal $CD4^+$ T cell responses during toxoplasmosis, there was a significant CD28-independent component which was sufficient for resistance to this infection (6, 7). The discovery of ICOS and its ability to provide costimulation to T cells suggested that this may be an alternative pathway to mediate CD28-indpendent activation of T cells required for resistance to *T. gondii*. Indeed, studies presented here indicate that the B7RP-1-ICOS interaction largely accounts for the CD28-independent component of T cell production of IFN-γ during the acute phase of toxoplasmosis. In addition, although blockade of the B7RP-1-ICOS interaction did not affect the overall susceptibility of WT mice to *T. gondii*, $CD28^{-/-}$ mice treated with anti-B7RP-1 became susceptible to infection. In agreement with these findings, previous studies have shown that ICOS is involved in the regulation of CD28-dependent and CD28-independent Th1 and Th2 responses to the nematode *N. brasiliensis* and the viral pathogens lymphocytic choriomeningitis virus and vesicular stomatitis virus (26). However, the importance of ICOS in the outcome of these infections was unclear and blockade of ICOS in $CD28^{-/-}$ mice did not alter the time of expulsion of *N. brasiliensis*. Similarly, $ICOS^{-/-}$ mice are slightly more resistant to infection with *L. mexicana* than WT mice, although the Th1 and Th2 responses specific for *L. mexicana* in these mice are compromised (27). Thus, the data presented here provide the first demonstration that ICOS has a critical role in resistance to a pathogen.

Following the identification of ICOS, a consensus quickly emerged that the B7RP-1-ICOS interaction was more important in Th2- than Th1-type responses. Thus, blockade of the B7RP-1-ICOS interaction inhibited Th2- but not Th1-mediated lung inflammation (21), and several studies have shown that Th2 cells express higher levels of ICOS than do Th1 cells (20, 26). Moreover, ICOS-deficient mice have severe defects in Th2 responses, CD40-mediated Ab class switching, and germinal center formation but produced normal levels of IFN-γ (22–24). However, this paradigm has been challenged by studies which demonstrated that

blockade of the B7RP-1-ICOS interaction ameliorated experimental allergic encephalomyelitis and graft rejection, which are both mediated by Th1 cells (25, 35). The studies presented here extend those findings and identify an important role for ICOS in Th1-type responses required for resistance to an intracellular pathogen.

Analysis of ICOS expression following infection with *T. gondii* revealed distinct patterns associated with different phases of infection. During the acute phase of infection, high levels of ICOS were expressed on activated $CD4^+$ T cells from the spleen, consistent with the model that $CD4^+$ T cells are the major source of T cell-derived IFN-γ during acute infection (36). In contrast, during toxoplasmic encephalitis, when the brain is the site most affected, expression of ICOS by splenic T cells had decreased, while $CD4^+$ and $CD8^+$ T cells isolated from the brains of infected mice expressed high levels of ICOS. Since ICOS can provide costimulation for both $CD4^+$ (15, 23, 25, 35, 37) and $CD8^+$ (38) T cells to make IFN-γ, this finding is consistent with their role in producing IFN-γ required for resistance to toxoplasmic encephalitis (3, 39). However, in the studies with T cells from the brains of mice with TE, blockade of ICOS alone had a small but reproducible inhibitory effect on their ability to produce IFN-γ. When the blockade was combined with CTLA4-Ig, there was an additive effect, although significant levels of IFN-γ were still produced. Nonetheless, the simultaneous blockade of CD28 and ICOS led to a 50% reduction in levels of IFN-γ produced by these T cells and suggest a role for these costimulatory pathways in the pathogenesis of toxoplasmic encephalitis. Similarly, although the administration of anti-B7RP-1 to infected $CD28^{-/-}$ mice resulted in reduced serum levels of IFN-γ and reduced production of IFN-γ in recall responses, there were still significant levels of IFN-γ and some of these mice did survive the acute phase of infection. There are several possible explanations for these findings and perhaps the blockade of the B7RP-1-ICOS interaction in these studies was incomplete and studies with $ICOS^{-/-}$ mice would be needed to address this issue. Alternatively, the studies presented here have focused on the role of ICOS in the regulation of T cell responses but have not addressed whether ICOS has a role in the regulation of NK cell production of IFN-γ which provides an innate mechanism of resistance to *T. gondii* (40, 41). Our previous studies have shown that infection of severe combined immunodeficient mice with *T. gondii* stimulates NK cells to express CD28 and that the CD28-B7 interaction is involved in the regulation of NK cell production of IFN-γ (42). Related to the studies presented here, although the NK cell responses to *T. gondii* are blunted in $CD28^{-/-}$ mice (7), these NK cells may still contribute to the low systemic levels of IFN-γ seen in infected $CD28^{-/-}$ mice treated with anti-B7RP-1. Alternatively, it is also possible that the administration of anti-B7RP-1 may inhibit NK cell responses since preliminary studies indicate that although resting NK cell do not express ICOS, IL-2-activated NK cells can express low levels of ICOS (our unpublished observations). Whether this has any functional significance for NK cell responses during toxoplasmosis is unclear and is the subject of ongoing studies.

Initial in vitro studies demonstrated that the stimulation of T cells with anti-CD3 resulted in ICOS expression which was largely dependent on the B7-CD28 interaction (20) and suggested that some of the effects attributed to CD28 could be due to signaling through ICOS. Since CD28 is expressed constitutively, whereas ICOS expression is inducible, a model in which stimulation through CD28 was required for the activation of T cells and increased expression of ICOS was proposed. However, when human $CD4^+$ T cells are stimulated with suboptimal levels of anti-CD3, ICOS can provide costimulation (34) and has also been shown to have a role in the costimulation of $CD28^{-/-}$ T cells (26). The in vivo data presented here show that in the absence of CD28 there is a marked reduction in the total number of $CD4^+$ T cells that express ICOS. Although these data confirm the important role of CD28 for optimal $CD4^+$ T cell responses during toxoplasmosis, we interpret the reduced levels of ICOS as a result of reduced numbers of activated $CD4^+$ T cells in these mice. However, the high levels of ICOS observed on $CD4^+$ and $CD8^+$ T cells isolated from brains of $CD28^{-/-}$ mice with TE demonstrate that stimulation through CD28 is not required for expression of ICOS during toxoplasmosis.

The close structural and functional relationship between ICOS and CD28 raises the question of whether these molecules are functionally redundant or whether they have distinct roles in the regulation of T cell responses required for resistance to infection. For example, in mice chronically infected with *T. gondii*, the CD28-mediated production of IL-2 is important for the ability of $CD8^+$ T cells to make IFN-γ in recall responses and more recently studies have indicated an important role for IL-2 during ICOS-mediated costimulation (34). Thus, although these may be parallel pathways, both may be required for optimal T cell responses in this particular experimental system. Nevertheless, during toxoplasmosis, WT mice treated with anti-B7RP-1 are resistant to *T. gondii*, but in the absence of CD28, ICOS is critical for resistance. These findings correlate with the presence of CD28-dependent and ICOS-dependent pathways for the production of IFN-γ during toxoplasmosis and suggest that ICOS and CD28 are parallel costimulatory pathways, either of which is sufficient to mediate the production of IFN-γ and resistance to *T. gondii*. Several questions about the role of ICOS in the regulation of immunity to *T. gondii* remain unresolved. Our studies have not addressed whether the B7RP-1-ICOS interaction is restricted to the regulation of IFN-γ production or whether these molecules may also regulate other T cell functions necessary for resistance to *T. gondii*. Thus, further studies are required to delineate the functional relationship between CD28 and ICOS and how each pathway integrates to provide an optimal T cell response necessary for resistance to *T. gondii*.

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