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Role of CD28 in the Generation of Effector and Memory Responses Required for Resistance to *Toxoplasma gondii*

Eric N. Villegas,* M. Merle Elloso,* Gaby Reichmann,† Robert Peach,‡ and Christopher A. Hunter*§

CD28 deficient (CD28−/−) mice were used to study the role of costimulation in the T cell-mediated, IFN-γ-dependent mechanism of resistance to *Toxoplasma gondii*. These mice were resistant to infection with the ME49 strain of *T. gondii*. Analysis of the immune response of acutely infected CD28−/− mice revealed that IL-12 was required for T cell production of IFN-γ and this was independent of the CD40/CD40 ligand interaction. A similar mechanism of IL-12-dependent, CD28/B7 independent production of IFN-γ by T cells was also observed in wild-type mice. Interestingly, although chronically infected wild-type mice were resistant to rechallenge with the virulent RH strain of *T. gondii*, chronically infected CD28−/− mice were susceptible to rechallenge with the RH strain. This deficiency in the protective memory response by CD28−/− mice correlated with a lack of IL-2 and IFN-γ in recall responses and reduced numbers of CD4+ T cells expressing a memory phenotype. Together, our findings demonstrate that CD28 is not required for the development of a protective T cell response to *T. gondii*, but CD28 is required for an optimal secondary immune response. *The Journal of Immunology*, 1999, 163: 3344–3353.

It is generally accepted that T cell activation requires two distinct signals provided by ligation of the TCR/CD3 complex in the context of either CD4 or CD8 and a second signal that provides costimulation (1). Several molecules on the surface of T cells have been shown to be involved in costimulation but the interaction of CD28 on T cells with B7-1 (CD80) or B7-2 (CD86), on accessory cells, is one of the most important second signals that regulates T cell activation. The CD28/B7 interaction functions to lower the threshold of T cell activation (2) and is important in enhancing cytokine production, preventing development of anergy, and protecting against apoptosis (1, 3, 4). The significance of the CD28/B7 interaction in the regulation of T cell activity is illustrated by studies in which blockade of B7 inhibits T cell-mediated responses during experimental allergic encephalomyelitis (5), graft rejection (6), diabetes (7, 8), and Th2-mediated allergic airway responses to inhaled Ags (9). Moreover, CD28 deficient (CD28−/−) mice are deficient in proliferative responses to polyclonal activators such as alloantigen, Con A, as well as anti-CD3 Ab (10). Nevertheless, there are several reports that T cells can be activated independently of CD28 (8, 9, 11, 12). Most studies on CD28 have focused on the role of this molecule in the initial activation of T cells, but its role in memory responses is less clear. Studies with T cells from TCR transgenic mice suggested that the ability of memory cells to respond to Ag is less dependent on costimulation than that of naive T cells (13) and that the role of B7-mediated signals is to provide costimulation for production of IL-2 rather than IFN-γ (14).

The role of the CD28/B7 interaction in regulating the development of protective T cell-mediated immune responses to infection is still not well understood. Initial studies in mice infected with *Leishmania major* reported that treatment with CTLA4-Ig inhibited the development of a Th2-type response and led to the development of a protective Th1-type response (15). Subsequent studies with CD28−/− mice showed that CD28 had a limited role in the development and differentiation of either Th1 or Th2 subsets during leishmaniasis (16). In addition, Gause and colleagues reported that in mice infected with the nematode *Heligmosomoides polygyrus* the development of a Th2-type response was comparable in CD28−/− and wild-type (WT) mice (17). Similarly, infection of CD28−/− mice with lymphocytic choriomeningitis virus (LCMV) resulted in the generation of a functional T cell response (18). In those studies, the role of CD28 in the development of T cell responses to viral infection was shown to vary with the “strength” of the antigenic stimulus because CD28−/− mice developed anergic CD8+ T cells when stimulated with LCMV peptide. In contrast to the studies with *H. polygyrus*, King and colleagues demonstrated that CD28 was required for the development of a Th2-type response in mice infected with *Schistosoma mansoni* (19). These previous studies examined the role of CD28 in the activation of T cell responses during infection. Few studies have analyzed the role of CD28 in the generation of memory cells following infection. However, studies with influenza virus and *H. polygyrus* have led to the idea that the induction of T cell memory is not dependent on CD28 (20, 21).

*Toxoplasma gondii* is an important opportunistic infection in patients with defects in T cell function (22). Infection with *T. gondii* results in an acute phase of disease during which NK and T cells produce IFN-γ, the major mediator of resistance to *T. gondii* (23). Normally, this response leads to the control of the infection;
However, T. gondii has a latent cyst stage that ensures that the host remains chronically infected (22). Resistance to this chronic phase of infection is dependent on CD4+ and CD8+ T cells (24), as is immunity to rechallenge with a virulent strain of T. gondii (25, 26). The role of the CD28/B7 interaction in the regulation of protective T cell responses to infection with T. gondii is not clear. Our previous studies demonstrated that the CD28/B7 interaction was required for maximal NK cell responses to T. gondii in SCID mice (27). More recent studies have shown that in humans serologically negative for T. gondii, infection of their monocytes with T. gondii results in increased expression of B7-1 and B7-2 and that these co-stimulatory ligands were required for the ability of their T cells to proliferate and produce IFN-γ in response to T. gondii in vitro (28).

Given the importance of costimulation for T cell activation and the role of T cell production of IFN-γ for resistance to T. gondii, we decided to analyze the role of the CD28/B7 interaction in the generation of protective T cell responses during toxoplasmosis. We assessed T cell responses in WT and CD28−/− BALB/c mice during the acute and chronic phase of infection with T. gondii. Our studies demonstrate that the activation of T cells following infection is independent of the CD28/B7 interaction. Rather, it appears that IL-12 is a critical factor that allows T cell activation and resistance to this pathogen to develop independently of the CD28/B7 interaction. Moreover, our studies reveal that CD28 is required for the ability of chronically infected mice to mount a protective secondary response against a virulent strain of T. gondii. These latter studies suggest an important role for CD28 in either the generation or maintenance of memory cell populations.

Materials and Methods

Animals

Female Swiss Webster, CBA/CaJ, and male BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). BALB/c CD28−/− (10) were obtained from The Jackson Laboratory and were bred and maintained within Thoren caging units (Thoren Caging Systems, Hazleton, PA) within the animal facilities at the University of Pennsylvania. 129/B6 CD40 ligand (CD40L)-deficient mice (CD40L−/−) (29) were provided by Immunex (Seattle, WA) and were bred and maintained within Thoren caging units at the animal facilities at the University of Pennsylvania. Mice were between 6 and 8 wk of age when used for experiments.

Parasites

Soluble Ags of T. gondii (STAg) were prepared from RH strain tachyzoites as previously described (30). STAg was titrated to determine the optimal concentration for splenocyte proliferation and was used at 25–40 μg/ml for these experiments. Virulent RH or TS-4 tachyzoites were maintained in vitro at 37°C or 34°C, respectively, using human foreskin fibroblasts cultured in DMEM (Life Technologies, Rockville, MD) supplemented with 1% FCS (HyClone, Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) (Bio-Whittaker). Erythrocytes were depleted using 0.83% w/v ammonium chloride (Sigma, St. Louis, MO). Cells were then washed three times and resuspended in complete RPMI 1640 before being plated at a cell density of 4 × 105 cells per well in a final volume of 200 μl in 96-well plates (Costar, Cambridge, MA). Cells were stimulated with soluble anti-CD3 (1–10 μg/ml) or parasite Ag (10–50 μg/ml), and supernatants were harvested after 24 and 48 h, respectively. IFN-γ, IL-2, and IL-4 levels were measured using two site ELISAs as previously described (32, 33). IL-12 (p40) levels were measured using mAb C17.8 and biotinylated C15.6 (grown from hybridomas provided by G. Trinchieri, Wistar Institute). Cytotoxins of 33Cr-labeled YAC-1 cells (American Type Culture Collection, Manassas, VA) was used to measure NK cell cytolytic activity as previously described (34).

Cytofluorometric analysis

After dissociation and lysis of erythrocytes, cells were resuspended at a final concentration of 1 × 106 cells/ml in FACS buffer composed of 1× PBS, 0.2% BSA fraction V (Sigma), and 4 mM sodium azide. Then, 106 cells were stained with mAbs specific for CD4, CD8, CD44, CD45RB, or CD62L for 20 min on ice in the presence of saturating amounts of Fc Block (PharMingen, San Diego, CA). Cells were then washed and analyzed using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). For biotinylated Abs, cells were stained and washed as described above, then incubated with FITC- or PE-conjugated streptavidin (PharMingen) or biotinylated anti-streptavidin (PharMingen) and FACS buffer and analyzed. Each Ab and streptavidin reagents were used at dilutions empirically determined to give optimal staining for flow cyto- metric analyses. Results were analyzed using Cell Quest software (Becton Dickinson).

Intracellular cytokine staining

Erythrocyte-depleted splenocytes from chronically infected WT or CD28−/− mice were plated in a 24-well plate (Costar) at a density of 5 × 105 cells per well in a final volume of 1 ml. Cells were then stimulated with STAg (25 μg/ml) for 72 h. PMA (50 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma) and brefeldin A (10 μg/ml; Sigma) were added to the cultures during the last 5 h of stimulation. Cells were then harvested and resuspended in FACS buffer at a final concentration of 1 × 107 cells per ml. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) was performed on all samples following fixation for 30 min on ice. Cells were washed with 0.1% w/v paraformaldehyde. Cells were washed again and permeabilized with 0.1% saponin in FACS buffer. After permeabilization, cells were stained with PE-conjugated anti-IFN-γ (PharMingen) for 20 min on ice. Cells were then washed with FACS buffer and fixed with 1% w/v paraformaldehyde. Cells were then analyzed using a FACScalibur flow cytometer (Becton Dickinson).

Statistics

INSTAT software (GraphPad, San Diego, CA) was used for unpaired two-tailed Student’s t test or paired t test evaluations. Values of p < 0.05 were considered significant.

Histology

At different times postinfection, samples of lung, liver, and brain were removed from each mouse, fixed in 4% formaldehyde/70% ethanol/0.8% acetic acid, and embedded in paraffin. Organs were sectioned and stained with hematoxylin and eosin for visualization of pathological changes. T. gondii parasites and Ags were detected in tissues of infected mice using polyclonal rabbit Abs against T. gondii (31). Cytospins of peritoneal exudate cells (PECs) were prepared as previously described and used to estimate the percentage of cells infected with T. gondii (27).
Results

Resistance to T. gondii is independent of CD28

Infection of WT and CD28−/− male BALB/c mice i.p. with 20 cysts of the ME49 strain of T. gondii did not result in death of these mice for a period of at least 4 mo. When a higher parasite dose (100 cysts) was used to infect BALB/c CD28−/− mice, we saw increased mortality of these mice during the acute phase of the infection in two of three experiments (data not shown). These results are similar to a previous report from Suzuki and colleagues in which BALB/c mice, infected i.p. with increasing numbers of parasites, were more susceptible to the acute phase of infection (i.e., 80 cysts resulted in 75% mortality) (35). Nonetheless, histological analysis and cyst counts of the brains of the chronically infected BALB/c WT and CD28−/− mice revealed that there were no differences in parasite burden or pathology (G. Reichmann, E. N. Villegas, and C. A. Hunter, unpublished observations). Given the important role for CD28 in the activation of T cell responses in many experimental systems, these results were surprising. Therefore, we decided to investigate why CD28 was not required for resistance to T. gondii. As a measure of the initial response to T. gondii, we assayed parasite burden, serum levels of IFN-γ and IL-12, as well as the ability of NK cells to lyse the YAC-1 tumor cell line. At day 5 postinfection, there were no significant differences in the numbers of infected cells in the peritoneum (<1% of cells infected for WT and CD28−/− mice) or of the serum levels of IFN-γ and IL-12 (Fig. 1A). However, there was a 2- to 4-fold decrease in the levels of NK cell cytotoxic activity for the YAC-1 tumor cell target (Fig. 1B). These latter results are consistent with our previous studies, which showed that blockade of the CD28/B7 interaction in SCID mice infected with T. gondii results in decreased NK cell activity (27).

Because our results indicated that the initial activation of the protective immune response to T. gondii was intact in the absence of CD28, we analyzed the ability of T cells from uninfected and infected CD28−/− mice to produce IFN-γ. As shown in Fig. 2A, T cells from uninfected WT mice produced IFN-γ in response to stimulation with soluble anti-CD3. This effect was dependent on B7 molecules because the addition of CTLA4-Ig to the cultures inhibited the production of IFN-γ (Fig. 2B). When the responses of WT mice were assessed, we observed that infection for 5 days resulted in enhanced production of IFN-γ in response to anti-CD3 (Fig. 2A). However, in contrast to splenocytes from uninfected WT mice, a significant proportion of the IFN-γ produced was not inhibited by CTLA4-Ig (Fig. 2C). Thus, in WT mice infected with T. gondii a significant portion of the IFN-γ produced is independent of the CD28/B7 interaction. In contrast to WT mice, T cells from uninfected CD28−/− mice failed to produce appreciable levels of IFN-γ in response to anti-CD3 and CTLA4-Ig did not alter this response (Fig. 2, A and B). However, by day 5 of infection, stimulation of splenocytes from CD28−/− mice with anti-CD3 resulted in the production of elevated levels of IFN-γ in comparison to uninfected CD28−/− mice (Fig. 2A). Moreover, these levels of IFN-γ were not affected by the addition of CTLA4-Ig to the cultures (Fig. 2C). Thus, infection of CD28−/− mice with T. gondii results in enhanced production of IFN-γ by T cells stimulated with anti-CD3.

To characterize the generation of Ag-specific T cells following infection with T. gondii, we assessed the ability of splenocytes from infected mice to produce IFN-γ in response to STAg. Splenocytes from infected WT mice produced significant levels of IFN-γ in response to STAg, and this was reduced by ~60% in the presence of CTLA4-Ig (Fig. 3A). Splenocytes from infected CD28−/− mice produced reduced levels of Ag-specific IFN-γ in comparison to infected WT mice (Fig. 3A). Interestingly, the recall responses of cells isolated from the peritoneum of WT mice that had been infected i.p. revealed that the levels of IFN-γ produced in response to STAg were only inhibited by 25–30% in the presence of CD28−/− mice (Fig. 3B). Moreover, the levels of IFN-γ produced by peritoneal cells isolated from infected CD28−/− mice were similar to the levels produced by WT mice. The source of these responses in the peritoneum of CD28−/− mice were shown to involve both CD4+ and CD8+ T cells as in vivo depletion of either of these subsets resulted in reduced production of IFN-γ (Fig. 3C). Similar results were observed with WT mice (data not shown). Thus, at the local site of infection the T cell response to infection was comparable in WT and CD28−/− mice, and it is likely that the serum levels of cytokines that were detected (see Fig. 1) are a function of the immune response at the local site of infection rather than in the spleen. Together, these observations demonstrate a requirement for CD28-mediated costimulation for the ability of splenocytes to produce optimal levels of parasite specific IFN-γ in vitro. However, our results identify a CD28-independent component to these T cell responses, which is observed in WT and CD28−/− mice and which is sufficient to protect against infection with T. gondii.

FIGURE 1. Analysis of early immune responses to T. gondii in WT and CD28−/− (KO) mice. Mice were infected i.p. with T. gondii, sacrificed on day 5 postinfection, serum was collected, and splenocytes were used to measure NK cell cytolytic activity. A, Serum levels of IFN-γ and IL-12 were measured by ELISA, and the data presented are the means ± SEM from eight experiments each containing three to five mice per experiment. There was no statistical difference between the experimental groups. For uninfected mice sacrificed at the same time, serum levels of these cytokines were typically <19 pg/ml for IFN-γ and <2000 pg/ml for IL-12 p40. B, The NK cell cytolytic activity for 51Cr-labeled YAC-1 cells presented is the pooled data from a single experiment containing three mice per experimental group. Similar results were seen in a repeat experiment.

FIGURE 2. IFN-γ and IL-12 production by splenocytes from infected CD28−/− mice. A, Splenocytes from infected WT and CD28−/− mice were isolated, restimulated with anti-CD3, and IL-12 p40 and IFN-γ levels were measured by ELISA, and the data presented are the means ± SEM. Splenocytes from infected CD28−/− mice produced is independent of the CD28/B7 interaction. Moreover, these levels of IFN-γ were not inhibited by CTLA4-Ig. The results of three experiments are shown, with each experiment containing four mice per group. Similar results were seen in a repeat experiment.

FIGURE 3. IFN-γ production by splenocytes from infected CD28−/− mice. A, Splenocytes from infected WT and CD28−/− mice were isolated, restimulated with anti-CD3, and IFN-γ levels were measured by ELISA, and the data presented are the means ± SEM. Similar results were seen in a repeat experiment. B, The NK cell cytolytic activity for 51Cr-labeled YAC-1 cells presented is the pooled data from a single experiment containing three mice per experimental group. Similar results were seen in a repeat experiment.
IL-12 is required for the CD28-independent activation of T cells after infection

Because of the important role of IL-12 in the generation of protective T cell responses to T. gondii (36, 37) and the identification of a role for the CD40/CD40L interaction in costimulation of T cell responses (38-40) and resistance to the intracellular parasite Leishmania (29, 41, 42), we chose to study the role of these factors in the CD28-independent activation of T cells to produce IFN-γ following infection. The addition of anti-IL-12 to splenocytes from CD28−/− mice infected for 5 days reduced the production of IFN-γ in response to anti-CD3, whereas anti-CD40L had no significant effect (Fig. 4A). Similarly, treatment of CD28−/− mice with a neutralizing mAb specific for IL-12, but not an Ab specific for CD40L, before infection inhibited the infection-induced activation of T cells, as measured by production of IFN-γ (Fig. 4B). Moreover, splenocytes from CD28−/− mice that were infected and treated with anti-IL-12 in vivo and were stimulated in vitro with anti-CD3 or STAg in the presence of anti-IL-12 produced minimal levels of IFN-γ (data not shown). The significance of these studies was illustrated by the fact that administration of anti-IL-12 resulted in the death of the CD28−/− mice within 10 days of infection. In contrast, mice treated with anti-CD40L or deficient in CD40L survived for at least 4 wk after infection (Fig. 4C).

Because our in vitro and in vivo studies demonstrated that IL-12 was important for the CD28-independent activation of T cells to produce IFN-γ after infection with T. gondii, we then tested whether IL-12 was capable of enhancing the responses of splenocytes from uninfected CD28−/− mice stimulated with anti-CD3. The addition of IL-12 to WT splenocytes stimulated with anti-CD3 resulted in a 5-fold increase in the levels of IFN-γ produced (Fig. 4D), while the addition of IL-12 to splenocytes from uninfected CD28−/− mice resulted in a 10- to 20-fold increase in the levels of IFN-γ. Similar results were observed with splenocytes from infected WT and CD28−/− mice stimulated with anti-CD3 alone or in combination with IL-12 (data not shown). Although the levels of IFN-γ produced by splenocytes from CD28−/− mice are reduced compared with those obtained with cells from WT mice, these data support our in vivo results that, following infection, IL-12 can stimulate T cell production of IFN-γ independently of CD28.

CD28 is required for the generation of protective recall responses to T. gondii

To determine the requirements for CD28-mediated costimulation during memory T cell-mediated immune responses, we assessed the ability of chronically infected mice to resist a challenge with...
the virulent RH strain of *T. gondii*. Although the RH strain of *T. gondii* stimulates high systemic levels of IL-12 and IFN-γ, it is still a lethal infection, and protective immunity in immunized mice is dependent on CD4 and CD8 T cell responses (25, 26). Whereas naive WT and CD28−/− mice succumbed to challenge with RH between days 8–10 postinfection (Fig. 5A), WT mice chronically infected with ME49 for 12 wk were resistant to rechallenge with the RH strain and remained alive for 30 days postchallenge (Fig. 5B). In contrast, chronically infected CD28−/− mice rechallenged with RH died between 12–14 days after rechallenge (Fig. 5B).

Similar results were obtained when a lower dose of RH (2000 tachyzoites i.p.) were used to challenge these mice or if WT and CD28−/− mice were immunized with the temperature-sensitive mutant of *T. gondii*, Ts-4, and then challenged with 10,000 RH strain of *T. gondii* (data not shown).

Analysis of the serum levels of IFN-γ and IL-12 7 days after challenge of naive WT mice with the RH strain revealed high levels of IFN-γ and IL-12 (Fig. 6, A and B) associated with a high parasite burden (Fig. 6E). In contrast, chronically infected WT mice rechallenged with RH produced low levels of IL-12 and IFN-γ (Fig. 6, A and B), had a low parasite burden (Fig. 6E), no detectable parasites in the liver, and low numbers of parasites.
We interpret these data as showing that chronically infected WT mice mount a prompt secondary response that controls early parasite replication and as a consequence fails to induce high levels of systemic IL-12 and IFN-γ. In contrast, chronically infected CD28−/− mice rechallenged with RH displayed a different pattern of responses compared with WT mice. Analysis of serum levels of IFN-γ and IL-12 7 days after challenge of CD28−/− mice with the RH strain revealed that naive mice produced high levels of IFN-γ and IL-12 (Fig. 6, C and D) associated with a high parasite burden (Fig. 6E). Similarly, chronically infected CD28−/− mice rechallenged with RH produced comparable levels of IL-12 and IFN-γ (Fig. 6, C and D) on day 7 postinfection. At this time point, parasites were readily detected in peritoneal cells (Figs. 6E and 7B), and large numbers of parasites were detected using immunohistochemistry in the livers of these mice (data not shown). By 14 days after infection, the numbers of parasites in the peritoneum of these mice had been reduced (Fig. 6E), and a similar reduction was also observed in the liver (data not shown). However, large numbers of parasites were still present in the lungs (Fig. 7D). We interpret these data as showing that chronically infected CD28−/− mice do not fully control the initial challenge with RH and as a consequence mount a response that is similar in strength to the primary response seen in naive CD28−/− mice. However, although chronically infected CD28−/− mice are susceptible to rechallenge with RH, they do have elements of a protective secondary response that is illustrated by the decreased parasite burden compared with naive CD28−/− mice and the ability to clear parasites from the peritoneum and liver.

To determine why CD28−/− mice chronically infected with ME49 have a defect in their ability to resist rechallenge with the RH strain of T. gondii, we decided to characterize their memory cell responses. Analysis of parasite Ag-specific recall responses from WT mice infected for 12 wk revealed the presence of a memory response characterized by the production of IFN-γ and IL-2 after stimulation with STAg (Fig. 8, A and C). At this time point, the addition of CTLA4-Ig to the cultures almost completely ablated this recall response. In chronically infected CD28−/− mice, we could not detect the production of IFN-γ or IL-2 in response to STAg (Fig. 8, B and D). Furthermore, the addition of CTLA4-Ig to these cultures did not alter the production of these cytokines. Thus, unlike the acute phase of infection (see Fig. 3A), the ability of
memory cells to produce IFN-γ is dependent on CD28. The addition of IL-2 to splenocytes from chronically infected WT or CD28−/− mice did not stimulate the production of IFN-γ. However, IL-2 did enhance the production of IFN-γ by these splenocytes from chronically infected mice stimulated with STAg (Fig. 8, E and F).

Spleen cells from chronically infected WT and CD28−/− mice were analyzed to determine whether the T cell populations present during infection expressed markers characteristic of a memory phenotype (CD62Llow, CD45RBlow, CD44high). Our results revealed that CD4+ T cells from chronically infected CD28−/− mice showed a 2- to 4-fold reduction in the percentage of memory-type T cells as compared with chronically infected WT mice (Fig. 9, A, B, and E). There were comparable numbers of “memory” CD8+ T cells present in the spleens of chronically infected CD28−/− and WT mice (Fig. 9, C–E). Analysis of age-matched, uninfected controls for memory cell populations revealed that in the absence of CD28 there was a reduction in the percentage of CD4+ T cells expressing a memory phenotype (WT, 29%; CD62Llow; CD28−/−, 15%; CD62Lhigh). Thus, the defect in protective recall responses that we observed in CD28−/− mice correlates with decreased numbers of memory T cells, specifically the CD4+ T cell response.

To further dissect the correlation between the reduction in “memory” CD4+ T cells and defects in IFN-γ production in chronically infected CD28−/− mice, intracellular staining for IFN-γ was performed. Results showed a high percentage of CD8+ T cells that were positive for IFN-γ in unstimulated cultures from WT mice. However, this percentage was increased when these cultures were stimulated with STAg (Table I). In these cultures, only a small percentage of CD4+ T cells were positive for IFN-γ, and even following stimulation with STAg alone or STAg plus IL-2 this percentage remained small (7–8%). When splenocytes from CD28−/− mice were examined, the percentage of CD8+ T cells that were positive for IFN-γ in unstimulated cultures was lower.

### Table 1. Production of IFN-γ by CD4+ or CD8+ T cells from chronically infected WT and CD28−/− mice

<table>
<thead>
<tr>
<th>Conditions</th>
<th>CD4+ (%)</th>
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<tbody>
<tr>
<td>Unstimulated</td>
<td></td>
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<tr>
<td>STAg</td>
<td>4.30</td>
<td>5.72</td>
</tr>
<tr>
<td>IL-2</td>
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</tr>
<tr>
<td>STAg + IL-2</td>
<td>8.24</td>
<td>8.84</td>
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a Percentage of IFN-γ-positive cells were determined by intracellular cytokine staining. Erythrocyte-depleted spleen cells from chronically infected mice were stimulated with STAg, IL-2, or both for 72 h. Results are representative of two separate experiments containing three to four mice per group.

b Percentage of CD4+ T cells (WT, 24%; CD28−/−, 25%) and CD8+ T cells (WT, 10%; CD28−/−, 7%) in culture.
than that observed in WT mice (9% vs 18%), and this percentage was not increased when cultures were stimulated with STAg. However, the addition of IL-2 alone, or in combination with STAg, resulted in an increase in the percentage of CD8<sup>+</sup> T cells that were positive for IFN-γ (17–20%). When the response of CD4<sup>+</sup> T cells from CD28<sup>−/−</sup> mice were compared with WT mice, there were fewer cells positive for IFN-γ, and this percentage remained low even after stimulation with STAg plus IL-2 (3%). These data have to be interpreted with care because the percentage of IFN-γ-positive cells does not always correlate with levels of IFN-γ protein produced in these cultures (see Fig. 8). Nevertheless, in combination with the IFN-γ protein data shown in Fig. 8, we interpret the intracellular staining as showing that CD8<sup>+</sup> T cells from chronically infected CD28<sup>−/−</sup> mice are defective in their ability to produce IFN-γ unless provided with a source of exogenous IL-2.

Discussion

Our results demonstrate that the development of effector T cell responses required for resistance to T. gondii is independent of CD28. However, CD28 is required for the generation of maximal responses of T cells ex vivo during the primary immune response against ME49 infection and during a secondary immune response that can mediate protection against rechallenge with a virulent strain of T. gondii. Although the CD28/B7 interaction is one of the most important costimulatory pathways for T cells there are several studies that show that activation of T cells following infection is independent of CD28. CD28<sup>−/−</sup> mice infected with LCMV 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 (16) or H. polygyrus (17). Thus, CD28 is not required for all T cell responses in vivo, suggesting that alternative costimulatory pathways exist. Indeed, other molecules such as CD2, CD5, CD9, CD11a, CD44H, 4-1BB, and heat stable Ag have been implicated in the initial activation of naive T cells independently of CD28 (39, 43–45). Nevertheless, despite the presence of these alternative costimulatory pathways, our results demonstrate a requirement for CD28 in the development of a memory cell response which is required for survival following rechallenge with the virulent RH strain of T. gondii.

Our findings that IL-12 can act, independently of CD28, as a signal for T cell production of IFN-γ confirms the central role of IL-12 in resistance to T. gondii (36, 37, 46). The activation of lymphocytes to produce IFN-γ can occur in several ways: the classical MHC/TCR interaction in the presence of costimulation for either naive or educated Th1 cells in the presence of IL-12 is probably the best characterized pathway (47). There is also the “innate” ability of activated T cells to produce IFN-γ in response to IL-12 and costimulation, independently of stimulation through the TCR (48). Our studies show that the induction of high levels of IL-12, following infection with ME49, is sufficient to generate protective effector T cells. These findings are in agreement with recent studies that demonstrated that stimulation of human PBMCs with maximal concentrations of PHA to produce IFN-γ was IL-12 dependent, whereas at lower levels of T cell activation a CD28-dependent pathway was observed (49). Together with our in vivo studies, it appears that, in the presence of high levels of IL-12, CD28 is not required for activation of T cells to produce IFN-γ or to protect mice during the acute phase of toxoplasmosis. Moreover, because CD28<sup>−/−</sup> mice survive for >4 mo, resistance to the chronic phase of infection appears to be CD28 independent. This is supported by our analysis of the immune response of C57BL/6 CD28<sup>−/−</sup> mice during toxoplasmic encephalitis, which revealed that there are sufficient numbers of Ag-specific effector T cells present in the brain that are able to inhibit parasite replication.5

Recent studies have reported that naive T cells express low levels of the IL-12R, and that maximal responsiveness to IL-12 is dependent on stimulation through CD28, which results in up-regulation of the IL-12R (50, 51). Our results show that the ability of T cells to produce IFN-γ in response to IL-12 is independent of CD28, but is reduced in comparison to cells that can receive costimulation through CD28. However, it should be noted that stimulation through CD28 does not simply up-regulate IL-12R expression but also results in increased stability of IFN-γ mRNA, which enhances production of IFN-γ protein (52). Thus, the reduced levels of IFN-γ produced by CD28<sup>−/−</sup> T cells in our experiments may not be a result of reduced responsiveness to IL-12 but rather may be a consequence of decreased stability of IFN-γ mRNA.

Whether IL-12 alone is sufficient to provide a second signal required for T cell production of IFN-γ or if other costimulatory molecules are involved in this process is not known. We have partially addressed this issue by examining the possible role of the CD40/CD40L interaction in this process. This costimulatory pathway can regulate the ability of accessory cells to provide costimulation through B7 (53, 54), as well as direct activation of T cells (55–57). Our in vitro and in vivo studies with anti-CD40L as well as CD40L-deficient mice demonstrate that this interaction is not required for initial resistance to T. gondii. These observations are similar to recent studies with Histoplasma capsulatum and Mycobacterium tuberculosis (58, 59) but differ from studies with Leishmania species, Pneumocystis carinii, and Cryptosporidium parvum, in which CD40L is required for the generation of protective T cell responses (29, 41, 42, 60, 61). Interestingly, mice deficient in CD40L are more susceptible than WT mice to the chronic phase of infection with T. gondii (A. Sher, unpublished observations, and our own unpublished data).

There are few reports on the role of CD28 in the generation of memory T cell responses. Based on studies that examined T cell responses to influenza virus and H. polygyrus in CD28<sup>−/−</sup> mice, it has been proposed that induction of T cell memory does not require CD28 (20, 21) but may be important for the generation of effector T cells from either naive or memory T cells (20). However, our studies with CD28<sup>−/−</sup> mice chronically infected with T. gondii reveal a defective memory response. Thus, CD28 is required for optimal memory responses in this experimental system, and other costimulatory molecules do not substitute for this interaction. Nevertheless, it is important to note that although chronically infected CD28<sup>−/−</sup> mice were susceptible to rechallenge with the RH strain of T. gondii, and had a reduced number of T cells expressing a memory phenotype as well as defective production of IFN-γ, they did display partial resistance to the RH strain compared with naive mice. This was illustrated by the delay in time to death of chronically infected mice (14 days) vs naive mice (8 days). In addition, the parasite burden seen in rechallenged mice at day 7 postinfection (10–15% infected PECs) was less than that observed in naive mice (>90% infected PECs) at the same time point. Moreover, although the chronically infected CD28<sup>−/−</sup> mice died with prominent inflammatory lesions in the lungs associated with large numbers of parasites, there had been a reduction in the numbers of parasites found in the peritoneum and liver between day 7 and 14 postchallenge. Thus, even in the absence of CD28, there are signs of a parasite-specific memory T cell response in vivo, and this can be revealed in vitro by addition of IL-2 to the

These cultures show that efficiency of infection is lower when the strain of T. gondii was due to the ability of CD4+ T cells to produce IL-2 and thereby provide help for CD8+ T cell production of IFN-γ (25). Indeed, studies with LCMV have also shown that there is a requirement for CD4+ T cell production of IL-2 for sustained production of IFN-γ by CD8+ T cells following infection (62).

The studies presented here do not clearly distinguish whether the defect in recall responses observed in chronically infected mice is due to failure to generate a complete memory cell response and/or if costimulation is required for the ability of memory cells to provide effecter functions. The ability of CTLA4-Ig to block the in vitro recall responses observed with chronically infected WT mice suggests that CD28 is needed for the ability of these memory cells to produce IFN-γ. In contrast, our studies that identified a reduction in the numbers of CD4+ “memory” phenotype cells in chronically infected mice support the idea that CD28 is required for the generation or maintenance of memory T cell responses. There are several possible mechanisms whereby CD28 may affect memory cell responses. In particular, stimulation through CD28 leads to the production of cytokines (such as IL-2) that may be required for proliferation and subsequent differentiation of memory cells from either Ag-specific naive or effecter cell populations. Alternatively, the anti-apoptotic effects induced via stimulation through CD28 (63) may be important in the maintenance and expansion of long-lived memory cell populations. Gaining an understanding of the role of CD28 in the generation/maintenance of memory cells and their effector functions may help in the design of vaccines to protect against infection as well as the design of intervention strategies to manage chronic immune-mediated diseases.

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