Coinfection with *Heligmosomoides polygyrus* Fails To Establish CD8⁺ T-Cell Immunity against *Toxoplasma gondii*[∇]

Imtiaz A. Khan,^{1*} Rubeena Hakak,² Karen Eberle,² Peter Sayles,³
Louis M. Weiss,⁴ and Joseph F. Urban, Jr.⁵

Department of Microbiology and Tropical Medicine and Immunology, George Washington University, Washington, DC 20037¹;
Department of Microbiology, Immunology, and Parasitology, Louisiana State University Health Sciences Center,
New Orleans, Louisiana 70112²; Trudeau Institute, Saranac Lake, New York 12983³; Department of
Medicine and Pathology, Albert Einstein College of Medicine, Bronx, New York 10461⁴; and
USDA/ARS/Beltsville Human Nutrition Research Center, Diet, Genomics,
and Immunology Laboratory, Beltsville, Maryland 20705⁵

Received 7 September 2007/Returned for modification 4 October 2007/Accepted 7 January 2008

CD8⁺ T-cell immunity is important for long-term protection against *Toxoplasma gondii* infection. However, a Th1 cytokine environment, especially the presence of gamma interferon (IFN- γ), is essential for the development of primary CD8⁺ T-cell immunity against this obligate intracellular pathogen. Earlier studies from our laboratory have demonstrated that mice lacking optimal IFN- γ levels fail to develop robust CD8⁺ T-cell immunity against *T. gondii*. In the present study, induction of primary CD8⁺ T-cell immune response against *T. gondii* infection was evaluated in mice infected earlier with *Heligmosomoides polygyrus*, a gastrointestinal worm known to evoke a polarized Th2 response in the host. In the early stage of *T. gondii* infection, both CD4 and CD8⁺ T-cell responses against the parasite were suppressed in the dually infected mice. At the later stages, however, *T. gondii*-specific CD4⁺ T-cell immunity recovered, while CD8⁺ T-cell responses remained low. Unlike in mice infected with *T. gondii* alone, depletion of CD4⁺ T cells in the dually infected mice led to reactivation of chronic infection, leading to *Toxoplasma*-related encephalitis. Our observations strongly suggest that prior infection with a Th2 cytokine-polarizing pathogen can inhibit the development of CD8⁺ T-cell immune response against *T. gondii*, thus compromising long-term protection against a protozoan parasite. This is the first study to examine the generation of CD8⁺ T-cell immune response in a parasitic nematode and protozoan coinfection model that has important implications for infections where a CD8⁺ T-cell response is critical for host protection and reduced infection pathology.

CD8⁺ T-cell immunity plays a critical role in the host protection against *Toxoplasma gondii* infection (2, 17, 33). Cytokines like interleukin-7 (IL-7) and IL-15 are important in the induction and maintenance of CD8⁺ T-cell immune response against several pathogens (15, 16, 39, 42), but our studies have demonstrated that gamma interferon (IFN- γ) is essential for the initiation of robust CD8⁺ T-cell immunity against *T. gondii* (9). In these studies, it was observed that mice unable to produce optimal IFN- γ , due to the absence of IL-12 gene expression, developed severely compromised CD8⁺ T-cell immunity against *T. gondii*, which suggests that a Th1 cytokine environment is critical for optimal development of this protective response.

Heligmosomoides polygyrus is a mouse intestinal nematode that establishes a chronic infection in the duodenum. After two larval molts in the submucosa, the male and female adult worms emerge into the lumen of the intestine and mate, and eggs are excreted in the feces (44). Infection with *H. polygyrus*, although completely enteric, results in systemic dissemination and induction of Th2 cytokine responses (35, 38, 43, 44). Such Th2 cytokine responses have been shown to block type 1 cy-

tokine responses (1, 24) and to induce regulatory T cells (46). Moreover, it has been reported that worm infection can reverse the pathological outcome of allergic and autoimmune reaction (7, 25, 45). Recent studies have demonstrated that *H. polygyrus* infection in mice leads to expansion of regulatory T cells, which may lead to down regulation of immunity against infections dependent on Th1 cytokine response (10). However, the effect of worm infection on the development of CD8⁺ T-cell immunity, important in protecting against many viral, bacterial, and parasitic infections has not been extensively studied (14, 29, 32). We evaluated the effect of prior *H. polygyrus* infection on the generation of CD8⁺ T-cell immune response against *T. gondii* since interference with effective induction of CD8⁺ T-cell responses by worm infection, prevalent in developing countries, could confound development of vaccination and therapeutic protocols for residents, visitors, and immigrants in these areas (36).

MATERIALS AND METHODS

Mice. Female C57BL/6J mice obtained from Jackson Laboratory, Bar Harbor, ME, or the Animal Breeding Facility of the Trudeau Institute were used at ages between 8 and 10 weeks. Mice were raised under barrier-sustained conditions and were free of the common viral pathogens of mice as judged by periodic serological screening of sentinel mice by the University of Missouri Research Animal Diagnostic and Investigative Laboratory, Columbia, MO.

Parasites and infections. ME49 cysts were obtained from brains of chronically infected C57BL/6 mice, and infections were initiated by peroral administration of 10 cysts in 0.1 ml of diluted brain suspension using a 19-gauge gavage needle.

* Corresponding author. Mailing address: Department of Microbiology and Tropical Medicine and Immunology, George Washington University, Washington, DC 20037. Phone: (202) 994-2863. Fax: (202) 994-2913. E-mail: mtmikk@gwumc.edu.

[∇] Published ahead of print on 14 January 2008.

FIG. 1. Survival of *H. polygyrus*-infected mice after *T. gondii* challenge. (A) Photomicrographs of gut and liver of dually infected mice. Five- to eight-week-old female C57BL/6 mice were infected with *H. polygyrus* L₃, and subsequently 8 days later, the mice were inoculated perorally with *T. gondii* cysts. At day 10 p.i., the mice were sacrificed and tissues were subjected to histopathological analysis: (i) Ileum from a dually infected mouse showing mild enteritis with slight hypercellularity in the lamina propria. (ii) Ileum from a *T. gondii*-infected mouse showing subacute enteritis of moderate severities, consisting of increased numbers of mononuclear cells in the lamina propria, blunting of the intestinal villi, and parasites in the intestinal mucosa (arrows). (iii) Liver from a dually infected mouse showing the presence of small numbers of focal inflammatory infiltrates (arrow). (iv) Liver from a *T. gondii*-infected mouse showed multiple medium foci of inflammatory infiltrates consisting of various numbers of mononuclear cells with occasional neutrophils throughout the parenchymal and portal areas together with the presence of perivascularitis and vasculitis of moderate severities in medium to large blood vessels (arrows) (hematoxylin and eosin; bar = 100 μ m). (B) Infection with *H. polygyrus* results in increased survival rates of mice concomitantly infected with *T. gondii* (Toxo). Age-matched C57BL/6 mice were coinfectd with *H. polygyrus* and *T. gondii* as described above, and survival was monitored. There were six animals per group, and data are representative of one experiment. (C) Survivors from the experiment shown in panel B were sacrificed at day 30 post-*T. gondii* infection, and brains were evaluated for cyst number. Data are presented as means $\pm$ standard deviations.

Sham-infected mice received similarly diluted brain suspensions from uninfected mice. Two hundred infective third-stage larvae (L₃) of *H. polygyrus* were administered via the peroral route. Infection with *T. gondii* cysts was performed on day 8 post-worm challenge.

Intracellular staining. IFN- γ was determined by intracellular staining using a Cytofix/CytoPerm kit (BD PharMingen, San Jose, CA) as previously described (5). Cells were labeled with fluorescein isothiocyanate-conjugated anti-CD8a and CytoChrome-conjugated anti-CD3 (e-Biosciences, San Diego, CA), fixed and permeabilized with paraformaldehyde, and then stained with phycoerythrin (PE)-conjugated anti-IFN- γ (e-Biosciences). Stained cells were analyzed by flow cytometry, and the results were evaluated by using Cell Quest software.

Quantification of parasite burden. Quantification of parasite burden in the spleen, liver, gut, and brain was performed at day 7 postinfection (p.i.) by quantitative PCR (22). DNA was isolated from tissues with the Qiamp tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Parasite

DNA was amplified with primers specific for a 35-fold repetitive sequence of the B1 gene (5'-TCTTTAAAGCGTTCGTGGTC-3' and 5'-GGAAGTGCATCCG TTCATGAG-3'), which is found in all known parasite strains (3). A 134-bp competitive internal standard containing the same primer template sequences as the 194-bp B1 PCR fragment was synthesized and amplified along with parasite DNA. Amplification was performed using a 50- μ l reaction mixture containing 1.24 U Amplitaq DNA polymerase; 1 $\times$ PCR buffer (Promega, Madison, WI); 0.2 mM each of dGTP, dATP, dTTP, and dCTP; and 0.4 mM of each B1 primer. For each reaction, a known amount of DNA from the tissues was amplified with various amounts of the internal standard. The levels of parasite load were estimated by comparison to the internal controls.

IFN- γ ELISA. Serum IFN- γ levels of the infected animals were assayed at day 10 following *T. gondii* infection using an enzyme-linked immunosorbent assay (ELISA) kit from Biolegend (San Diego, CA).

TLA preparation. *Toxoplasma* lysate antigen (TLA) was prepared from tachyzoites of the RH strain of *T. gondii* (18).

Preparation of HpAg. Antigenic extract from *H. polygyrus* (HpAg) was obtained from adult worms, isolated from the duodenum of female BALB/c mice infected 14 days earlier with 200 infective 3rd stage larvae (L_3). The worms settled in 10 changes of 50 ml of RPMI 1640 medium maintained at 37°C, and were then incubated at same temperature for 1 h in RPMI 1640 medium plus a standard antibiotic mixture of penicillin (100 µg), streptomycin (100 U), and amphotericin (10 µg) (culture medium). Subsequently, they were then placed in fresh culture medium and distributed at approximately 400 adult worms per 2 ml in 24-well culture plates. Medium was changed at day 3, and the wells containing worms with >90% viability were pooled at day 6 and extract was prepared using a Dounce homogenizer on ice until all worms were sheared. The suspension was centrifuged at $4,000 \times g$, and the clear supernatant was aseptically passed through a 0.2-µm filter. The solution was stored at -20°C until used further for T-cell stimulation.

Proliferation and cytotoxicity. CD4 or CD8⁺ T cells were purified by magnetic separation (16). Purified cells (>95% pure) (1×10^5 cells/well) were cultured with TLA (15 µg/ml) or HpAg (20 µg/ml) in presence of irradiated feeder cells. After a 5-day incubation, radioactive [³H]thymidine was added to the wells and proliferation was measured by a standard procedure in our laboratory (6).

For the cytotoxic assay, purified CD8⁺ T cells were cultured in presence of TLA and irradiated feeder cells and cytotoxic activity was determined after 5-day incubation (13). Briefly CD8⁺ T cells were harvested and incubated with ⁵¹Cr-labeled *T. gondii*-infected macrophages at various effector/target ratios in 96-well U-bottom plates. After a 4-h incubation, the supernatants were measured for radioactive release and the percentage of cytotoxic response was calculated (19).

Statistical analysis. Statistical analysis, unless otherwise noted, was conducted with unpaired Student's *t* test (31).

RESULTS

Dually infected mice exhibit increased survival and reduced intestinal pathology. C57BL/6 mice infected orally with *H. polygyrus* were subsequently challenged 8 days later with *T. gondii* cysts. At day 10 p.i. (post-*Toxoplasma* infection), some of the dually infected mice and those challenged with *T. gondii* alone were sacrificed and ileum and liver were subjected to histopathological analysis. Both tissues from *T. gondii*-infected mice showed signs of inflammation and necrosis (Fig. 1Ai and iv) as reported earlier (21, 27), while the inflammatory reaction was reduced in dually infected mice (Fig. 1Ai and iii). Two of six mice from the *T. gondii*-infected group succumbed to infection, but no mortality was observed in mice carrying the dual infection (Fig. 1B).

Reduced inflammation in the dually infected mice led to a decreased ability to clear parasites, as shown by the enhanced cyst load in their brains. A significantly higher number of cysts was detected in the brains of dually infected mice than in the controls infected with *T. gondii* alone ($P = 0.005$) (Fig. 1C).

To determine the effect of worm infection on the level of Th1 cytokine response during *T. gondii* infection, sera from these animals were isolated at day 10 p.i. and levels of IFN-γ were determined by ELISA. As expected, sera from mice infected with *T. gondii* alone showed high levels of IFN-γ that were significantly reduced in dually infected mice ($P = 0.003$) (Fig. 2). The decrease in the serum IFN-γ levels was not followed by an increase in Th2 cytokines. Levels of IL-4 in the dually infected mice were similar to those in mice infected with *T. gondii* alone (data not shown).

CD4⁺ and CD8⁺ T-cell response against *T. gondii*. Since Th1 cytokines are important for the induction of a primary CD8⁺ T-cell immune response against *T. gondii* (9), the development of functional CD8⁺ T cells in the dually infected mice was determined. Mice were sacrificed at day 10 p.i., and spleens and mesenteric lymph nodes (MLNs) were collected and pooled. CD4 and CD8⁺ T cells were isolated, and antigen-

FIG. 2. Serum IFN-γ levels of dually infected mice. C57BL/6 mice carrying worm infection were challenged perorally with 10 cysts of *T. gondii* (Toxo). At day 10 p.i., the serum collected from dually infected and control mice were pooled (three mice per group) and analyzed by ELISA for levels of IFN-γ.

specific proliferation was measured. T-cell subsets from both spleens (Fig. 3Ai and iii) and MLNs of *T. gondii*-infected mice (Fig. 3Aii and iv) were able to proliferate in response to TLA stimulation. However, T-cell subsets isolated from dually infected mice exhibited a significant decrease in response to TLA (Fig. 3Ai to iv). There was a significant reduction in the IFN-γ-positive CD4 and CD8⁺ T-cell numbers noted in both spleens and MLNs of dually infected mice (Fig. 3Bi and ii).

No difference in CD4⁺ T-cell proliferative response to HpAg stimulation between the mice carrying dual or worm infection alone was observed (Fig. 3Ci). CD8⁺ T cells isolated from both dually or worm-infected mice showed background proliferation when stimulated with HpAg (Fig. 3Bii). Thus, *T. gondii* infection apparently does not alter the T-cell response to worm infection.

To determine if the failure to develop antigen-specific T-cell response against *T. gondii* in the dually infected mice is transient or long term, the assay was repeated at day 30 post-*T. gondii* infection. Although CD4⁺ T cells from dually infected mice at this time point are able to respond to TLA (Fig. 4Ai and iii), CD8⁺ T cells continue to exhibit nonresponsiveness to TLA stimulation (Fig. 4Aii and iv). Similarly, while increase in the frequency of IFN-γ-producing CD4⁺ T cells in both MLNs and spleens was detected at this time point (Fig. 4Bi and ii), the number of IFN-γ-positive CD8⁺ T cells was reduced in the spleens and MLNs of dually infected mice (Fig. 4Bi and ii).

CD8⁺ T cells from dually infected mice fail to protect or exhibit antigen-specific cytotoxic response. To further analyze the *T. gondii*-specific CD8⁺ T-cell function in the dually infected mice, cytotoxic response against *T. gondii*-infected macrophages was assayed. *T. gondii*-infected mice are known to develop strong CD8⁺ T-cell-mediated cytotoxic activity against infected cells (8, 19, 37). Purified CD8⁺ T cells from *T. gondii*-infected mice were incubated with radiolabeled *T. gondii*-infected target cells, and cytotoxic activity was measured by isotope release. As shown in Fig. 5A, CD8⁺ T cells from the dually infected mice exhibited minimal cytotoxic activity even at high effector/target ratios. Conversely, CD8⁺ T cells from the mice infected with *T. gondii* alone exhibited a strong lysis of *T. gondii*-infected targets (Fig. 5A).

Finally, CD8⁺ T cells from the dually infected mice were

FIG. 3. Antigen-specific response (proliferation and IFN- γ production) by T-cell subsets in dually infected mice at day 10 post-*T. gondii* infection. (A and C) Proliferation. Five- to eight-week-old C57 BL/6 mice were infected with *T. gondii* (Toxo) and *H. polygyrus*. At day 10 post-*T. gondii* infection, mice were sacrificed and spleens and MLNs were collected and pooled (three mice per group). CD4 and CD8⁺ T cells from the tissues isolated by affinity purification (>95% pure as determined by fluorescence-activated cell sorter analysis; 1×10^5 cells/well) were cultured with 1×10^5 irradiated feeder cells and stimulated with 15 μ g/ml of TLA or 20 μ g/ml of HpAg. Lymphoproliferation was indicated by [3 H]thymidine incorporation after 72 h of incubation. The experiment was performed twice with similar results, and data are representative of one experiment. (B) IFN- γ -positive cells. Splenocytes and MLNs were harvested at day 10 after *T. gondii* infection, pooled ($n = 3$), and cultured in vitro with phorbol myristate acetate, ionomycin, and monensin for 4 h. The cultured cells were surface stained for CD4 or CD8 before intracellular staining for IFN- γ . Data are expressed as means $\pm$ standard deviations (per lymphoid tissue) and are representative of two independent experiments.

FIG. 4. Antigen-specific response in dually infected mice at day 30 post-*T. gondii* infection. (A) Proliferation. Dually infected mice were sacrificed at day 30 post-*T. gondii* (Toxo) infection, and antigen-specific proliferation of T-cell subsets was determined. The experiment was performed twice with similar results, and the data are representative of one experiment. (B) IFN- γ -positive cells. The absolute number of IFN- γ -positive CD4 and CD8 $^{+}$ T-cell subsets was determined at day 30 post-*T. gondii* infection as previously described. Data are expressed as means $\pm$ standard deviations (per lymphoid tissue) and are representative of two separate experiments.

evaluated for their ability to protect naïve animals against a parasite challenge. CD8 $^{+}$ T cells isolated from *T. gondii*-infected, dually infected, and noninfected controls were isolated and transferred intravenously to naïve syngeneic animals. The mice were subsequently challenged perorally 24 h later with 35 cysts. As shown in Fig. 5B, all of the mice that received CD8 $^{+}$ T cells from the *T. gondii*-infected mice survived until the termination of the experiment. In contrast, mice receiving cells from dually infected mice succumbed to infection similar to control mice treated with CD8 $^{+}$ T cells isolated from uninfected mice (Fig. 5B).

CD4 $^{+}$ T-cell depletion reactivates the infection in dually infected mice. Previous studies have demonstrated that simultaneous depletion of CD4 $^{+}$ and CD8 $^{+}$ T cells is needed

for reactivation of latent *T. gondii* infection in chronically infected mice (11). In these studies, it has been reported that depletion of CD4 $^{+}$ T cells alone had no effect, while treatment with anti-CD8 antibody led to only partial reactivation of chronic infection. As CD8 $^{+}$ T cells in dually infected animals were nonresponsive, we determined the effect of anti-CD4 antibody treatment on these chronically infected animals. Similar to earlier findings (11), depletion of CD4 $^{+}$ T cells had no effect on the mice infected with *T. gondii* alone (Fig. 6A) and all the mice in this group continued to survive similar to control animals treated with rat immunoglobulin G (IgG). Interestingly, depletion of CD4 $^{+}$ T cells in the dually infected mice proved to be lethal as all the animals in this group died by day 15 after the start of

FIG. 5. Effector response of CD8⁺ T cells from dually infected mice. (A) Antigen-specific cytotoxicity. MLNs from the mice carrying dual infection or *T. gondii* (Toxo) or worm infection alone were isolated at day 14 post-*T. gondii* infection. The tissues (three mice per group) were pooled, and CD8⁺ T cells were isolated and cultured in presence of 15 μ g/ml of TLA and irradiated feeder cells. After 5 days of culture, CD8⁺ T cells were collected and incubated with ⁵¹Cr-labeled *T. gondii*-infected macrophages at various effector/target ratios. Four hours later, cytotoxic activities were measured by radioactive release. Data are representative of two separate experiments. (B) Adoptive transfer. Purified CD8⁺ T cells from the MLNs of dually infected mice or mice carrying *T. gondii* or worm infection were isolated at day 14 p.i. Purified cells (5×10^6) from each group were adoptively transferred to naïve mice (six animals per group). Twenty-four hours later, the mice were challenged perorally with 35 *T. gondii* cysts. Each experiment was performed twice with similar results, and the data are representative of one experiment.

antibody treatment. Dually infected controls administered rat IgG did not exhibit any mortality.

To determine if the mortality of anti-CD4-depleted dually infected mice is due to reactivation of latent *T. gondii* infection, the levels of *T. gondii* DNA in the brain, liver, spleen, and lung of dually infected mice were measured. Treatment with anti-CD4 led to a significant increase in *T. gondii* DNA load in all the tissues tested (Fig. 6B). Conversely, depletion of CD4 in the mice infected with *T. gondii* alone or the dually infected mice administered rat IgG did not exhibit an increase in the parasite load in these tissues.

To confirm that the depletion of CD4⁺ T cells led to *Toxoplasma*-related encephalitis in the dually infected mice, the brains from these mice were subjected to histopathological analysis. The assay showed that similar to the data obtained in Fig. 6B, compared to mice infected with *T. gondii* alone, dually infected mice exhibited a marked increase in cyst burden (Fig.

6Ci, ii, vi, and vii). This was associated with an inflammatory response in these mice (Fig. 6Cvi and viii). Cysts were found in the dually infected mice which were in the process of rupture with detectable extracellular bradyzoites (Fig. 6Cvii and vii). Cysts were rarely seen in mice infected with *T. gondii* alone (Fig. 6Ci and ii), and there was minimal inflammation in these mice that was not changed by anti-CD4 treatment (Fig. 6Civ). In contrast, fewer cysts were demonstrated in the dually infected mice treated with anti-CD4 than those receiving control IgG (Fig. 6Cix and x), which is consistent with reactivation of the latent cysts seen in the dually infected mice (Fig. 6Cvi and vii). Compared to mice with *T. gondii* infection alone (Fig. 6Civ) anti-CD4 treatment led to significantly more inflammation in dually infected mice (Fig. 6Cix and x).

DISCUSSION

IFN- γ -producing CD8⁺ T cells are important for long-term protection against *T. gondii* infection (16). While CD8⁺ T cells have been demonstrated to play an essential effector role, CD4⁺ T cells act synergistically to limit chronic infection (12). Earlier reports from our laboratory have shown that Th1 cytokines are important for the generation of robust CD8⁺ T-cell immunity against *T. gondii* (9). In the present study, elicitation of CD8⁺ T-cell immunity against *T. gondii* in coinfection with *H. polygyrus*, a worm which is known to induce a strong Th2 cytokine response (35), was evaluated. Compared to mice infected with *T. gondii* alone, dually infected mice failed to develop antigen-specific CD8⁺ T-cell immunity against the parasite. During the early phase of infection, both CD4 and CD8⁺ T-cell subsets were nonresponsive to in vitro antigenic stimulation. However, *H. polygyrus*-specific CD4⁺ T cells at the same time point continued to remain responsive in dually infected animals, suggesting that *T. gondii* infection does not alter the host immune response against the worm. Subsequently, at day 30 p.i. while CD4⁺ T-cell response to TLA was restored, CD8⁺ T cells continued to be nonresponsive to stimulation by *T. gondii* antigens. Moreover, CD8⁺ T cells from the dually infected mice were unable to lyse *T. gondii*-infected targets and failed to protect naïve animals in adoptive transfer experiments. The nonresponsive state of CD8⁺ T cells in the dually infected mice was further characterized by CD4 depletion studies. Treatment of these mice with anti-CD4 antibody led to reactivation of latent toxoplasmosis. Earlier studies by Gazzinelli et al. (11) showed that depletion of CD4⁺ T cells alone had no effect on chronically infected mice, while treatment with a combination of antibodies to deplete CD4 and CD8⁺ T cells resulted in reactivation of *T. gondii* infection. The same study demonstrated that depletion of CD8⁺ T cells alone led to partial reactivation of latent infection in mice carrying chronic *T. gondii* infection. Our present study demonstrates that mice infected with *H. polygyrus* and *T. gondii* fail to generate effector CD8⁺ T-cell immunity and protection against the parasite is totally dependent on CD4⁺ T cells. Hence, depletion of CD4⁺ T cells leaves the host defenseless, resulting in latent *T. gondii* reactivation and multiplication of parasites in the tissues.

Previous studies related to coinfection of *T. gondii* with a helminth have focused on the outcome of acute infection. In one study conducted recently, it was reported that *T. gondii*

FIG. 6. CD4⁺ T-cell neutralization of mice carrying chronic *T. gondii* infection. C57BL/6 mice infected with *T. gondii* and *H. polygyrus* or *T. gondii* alone were administered 0.5 mg of anti-CD4 starting at day 31 after *T. gondii* infection. The control mice received similar doses of rat IgG. The antibody treatment was continued for 3 successive days and twice a week thereafter. (A) Mortality. The mice were monitored daily for morbidity or mortality until the termination of the experiment. There were six mice per group, and the experiment was performed twice with similar results. (B) Parasite load. Some of the antibody-treated mice were sacrificed at day 17 post-antibody treatment, and brain, liver, spleen, and lung were evaluated for the parasite load by quantitative PCR. (C) Histopathological analysis. Brains of some of the infected mice were analyzed by histopathological studies. Reactivation is shown. (i and ii) Sections of brain from mice infected with *T. gondii* and treated with control antibody ($\times 20$). In panel ii, an arrow points to a tissue cyst. (ii) Cysts were rare and there was minimal inflammation present in these sections. (iii) Sections of *H. polygyrus*-infected mice treated with antibody to CD4 showed no inflammation or abnormalities in tissue sections ($\times 20$). (iv) Tissue sections prepared from *T. gondii*-infected mice treated with antibody to CD4 were similar to those in mice infected with *T. gondii*, with rare foci of inflammatory cells seen ($\times 20$). (v) Sections from mice infected with *H. polygyrus* alone showed no inflammation or abnormalities ($\times 20$). (vi, vii, and viii) Sections from mice infected with *H. polygyrus* and *T. gondii* and treated with control IgG showed a marked increase in the number of cysts in the tissue (vi, $\times 10$; vii and viii, $\times 40$). Arrows in panel vi point to groups of multiple cysts seen at low power ($\times 10$). Panel vii demonstrates a cyst in the process of rupturing, releasing *T. gondii* (arrow) that can invade adjacent cells. Lymphocyte infiltration is present in the vicinity of the rupturing cyst. Panel viii demonstrates focal infiltrates and a developing glial nodule (arrows). Such infiltrates were very common in the brains of dually infected mice. (ix and x) Sections ($\times 20$) from *T. gondii*-infected mice previously infected with *H. polygyrus* and treated with antibody to CD4 showed a decrease in the number of cysts and areas of inflammation (arrow) compared to dually infected mice treated with control antibody (vi, vii, and viii).

infection reversed an established Th2 response to *Nippostrongylus brasiliensis* infection (26). Conversely, coinfection did not affect the *T. gondii*-induced intestinal necrosis or the number of parasites in the dually infected mice. An earlier study using *Schistosoma mansoni* demonstrated that Th1 cytokine production

and *T. gondii*-mediated intestinal pathology in the dually infected mice were substantially reduced (28). In the present study, we observed that the characteristically strong Th2 response to *H. polygyrus* was not seen in the dually infected mice (data not shown), but a decrease in IFN- γ levels and severe

intestinal pathology mediated by *T. gondii* was observed. The focus of our study was to evaluate the affect of coinfection with *H. polygyrus* on the development of CD8⁺ T-cell immune response against *T. gondii*. Interestingly, we observed that the *T. gondii*-specific CD4⁺ T-cell response, which in the dually infected mice is down regulated during acute infection, recovers at later stages, while CD8⁺ T-cell immunity is not restored. These observations raise an important question about the possibility of separate antigens involved in the induction of CD4 and CD8⁺ T-cell response. It is possible that due to conversion from the acute to chronic stage, parasite antigens involved in the induction of CD8⁺ T-cell immunity are not available or the processing of antigens by dendritic cells is altered. In fact, Chen et al. demonstrated that mice infected with *H. polygyrus* and subsequently exposed to *Citrobacter rodentium* failed to clear the bacterial infection and the mice expressed enhanced pathology related to a skewing of dendritic cell activity in the intestine (4). Others have demonstrated altered dendritic cell responses to nonparasite antigen induced by excretory/secretory products released by *H. polygyrus* (34). Alternatively, Metwali et al. showed that *H. polygyrus* induces a population of regulatory CD8⁺ T cells that control inflammation at mucosal surfaces that could contribute to more generalized suppression of *T. gondii*-induced inflammation in coinfecting mice (30).

Our studies demonstrate that prior infection to *H. polygyrus* leads to development of suboptimal adaptive immunity against *T. gondii*, especially CD8⁺ T-cell response, which has been reported to be as essential for protection against long-term protection against *T. gondii* (20). While earlier reports used a coinfection model to study the outcome of immune response during acute *T. gondii* infection (26, 28), this is the first study to focus on generation of the adaptive immune response against the parasite in mice. These findings emphasize the importance of Th1 cytokine milieu for induction of antigen-specific adaptive immunity against *T. gondii*. Earlier studies from our laboratory reported that p40^{-/-} mice, which are unable to produce optimal IFN- γ levels, exhibit a diminished CD8⁺ T-cell response against *T. gondii* infection (9). Exogenous IFN- γ treatment in these mice helps to restore normal CD8⁺ T-cell immunity. Although overwhelming of the Th2 cytokine environment created by *H. polygyrus* infection alone is not observed in the dually infected mice (data not shown), the worm infection inhibits the development of a *T. gondii*-specific CD4⁺ T-cell response in the short term and induction of CD8⁺ T-cell immunity against the parasite in both the short and long terms. Moreover, recent studies have shown that *H. polygyrus* induces distinct subsets of cells with suppressive properties and phenotypic characteristics similar to those of regulatory T cells (10). Suppression of CD8⁺ T-cell immunity against *T. gondii* in *H. polygyrus*-infected mice due to regulatory T-cell response remains to be studied. Nevertheless, our observations have far-reaching implications in other infections such as viruses (41) or diseases like cancer (23), where CD8⁺ T-cell immunity is critical for host protection. Immunotherapeutic interventions to control diseases may not be effective, especially in underdeveloped areas where both nematode and other infections are present. Based on our data, this factor should be seriously considered while developing vaccination strategies against these agents. In fact, a recent review has suggested that vaccine efficacy using both crude and defined antigens can be

severely impaired in animals coinfecting with helminths and protozoan and bacterial pathogens (40). These observations along with effects of coinfection on inflammation indicate a need for more definitive studies to explore parasite-related products as immune modulators and the mechanisms related to control of infection and tissue reactivity.

ACKNOWLEDGMENT

This work was supported by NIH grant AI33325 awarded to I.A.K.

REFERENCES

1. Bashir, M. E., P. Andersen, I. J. Fuss, H. N. Shi, and C. Nagler-Anderson. 2002. An enteric helminth infection protects against an allergic response to dietary antigen. *J. Immunol.* **169**:3284–3292.
2. Brown, C. R., and R. McLeod. 1990. Class I MHC genes and CD8⁺ T cells determine cyst number in *Toxoplasma gondii* infection. *J. Immunol.* **145**:3438–3441.
3. Burg, J. L., C. M. Grover, P. Pouletty, and J. C. Boothroyd. 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J. Clin. Microbiol.* **27**:1787–1792.
4. Chen, C. C., S. Louie, B. A. McCormick, W. A. Walker, and H. N. Shi. 2006. Helminth-primed dendritic cells alter the host response to enteric bacterial infection. *J. Immunol.* **176**:472–483.
5. Combe, C. L., T. J. Curiel, M. M. Moretto, and I. A. Khan. 2005. NK cells help to induce CD8⁺ T-cell immunity against *Toxoplasma gondii* in the absence of CD4⁺ T cells. *Infect. Immun.* **73**:4913–4921.
6. Combe, C. L., M. M. Moretto, J. D. Schwartzman, J. P. Giggley, D. J. Bzik, and I. A. Khan. 2006. Lack of IL-15 results in the suboptimal priming of CD4⁺ T cell response against an intracellular parasite. *Proc. Natl. Acad. Sci. USA* **103**:6635–6640.
7. Cooke, A., P. Tonks, F. M. Jones, H. O'Shea, P. Hutchings, A. J. Fulford, and D. W. Dunne. 1999. Infection with *Schistosoma mansoni* prevents insulin dependent diabetes mellitus in non-obese diabetic mice. *Parasite Immunol.* **21**:169–176.
8. Denkers, E. Y., A. Sher, and R. T. Gazzinelli. 1993. CD8⁺ T-cell interactions with *Toxoplasma gondii*: implications for processing of antigen for class-I-restricted recognition. *Res. Immunol.* **144**:51–57.
9. Ely, K. H., L. H. Kasper, and I. A. Khan. 1999. Augmentation of the CD8⁺ T cell response by IFN- γ in IL-12-deficient mice during *Toxoplasma gondii* infection. *J. Immunol.* **162**:5449–5454.
10. Finney, C. A., M. D. Taylor, M. S. Wilson, and R. M. Maizels. 2007. Expansion and activation of CD4⁺CD25⁺ regulatory T cells in *Heligmosomoides polygyrus* infection. *Eur. J. Immunol.* **37**:1874–1886.
11. Gazzinelli, R., Y. Xu, S. Hieny, A. Cheever, and A. Sher. 1992. Simultaneous depletion of CD4⁺ and CD8⁺ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. *J. Immunol.* **149**:175–180.
12. Gazzinelli, R. T., F. T. Hakim, S. Hieny, G. M. Shearer, and A. Sher. 1991. Synergistic role of CD4⁺ and CD8⁺ T lymphocytes in IFN- γ production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. *J. Immunol.* **146**:286–292.
13. Guan, H., M. Moretto, D. J. Bzik, J. Giggley, and I. A. Khan. 2007. NK cells enhance dendritic cell response against parasite antigens via NKG2D pathway. *J. Immunol.* **179**:590–596.
14. Iizuka, K., C. Nakajima, Y. M. Iizuka, M. Takase, T. Kato, S. Noda, K. Tanaka, and O. Kanagawa. 2007. Protection from lethal infection by adoptive transfer of CD8 T cells genetically engineered to express virus-specific innate immune receptor. *J. Immunol.* **179**:1122–1128.
15. Kasper, L. H., T. Matsuura, and I. A. Khan. 1995. IL-7 stimulates protective immunity in mice against the intracellular pathogen, *Toxoplasma gondii*. *J. Immunol.* **155**:4798–4804.
16. Khan, I. A., and L. Casciotti. 1999. IL-15 prolongs the duration of CD8⁺ T cell-mediated immunity in mice infected with a vaccine strain of *Toxoplasma gondii*. *J. Immunol.* **163**:4503–4509.
17. Khan, I. A., W. R. Green, L. H. Kasper, K. A. Green, and J. D. Schwartzman. 1999. Immune CD8⁺ T cells prevent reactivation of *Toxoplasma gondii* infection in the immunocompromised host. *Infect. Immun.* **67**:5869–5876.
18. Khan, I. A., and L. H. Kasper. 1996. IL-15 augments CD8⁺ T cell-mediated immunity against *Toxoplasma gondii* infection in mice. *J. Immunol.* **157**:2103–2108.
19. Khan, I. A., J. A. MacLean, F. S. Lee, L. Casciotti, E. DeHaan, J. D. Schwartzman, and A. D. Luster. 2000. IP-10 is critical for effector T cell trafficking and host survival in *Toxoplasma gondii* infection. *Immunity* **12**:483–494.
20. Khan, I. A., M. Moretto, X. Q. Wei, M. Williams, J. D. Schwartzman, and F. Y. Liew. 2002. Treatment with soluble interleukin-15/Ralpha exacerbates intracellular parasitic infection by blocking the development of memory CD8⁺ T cell response. *J. Exp. Med.* **195**:1463–1470.
21. Khan, I. A., J. D. Schwartzman, T. Matsuura, and L. H. Kasper. 1997. A

- dichotomous role for nitric oxide during acute *Toxoplasma gondii* infection in mice. *Proc. Natl. Acad. Sci. USA* **94**:13955–13960.
22. Kirists, M. J., E. Mui, and R. Mcleod. 2000. Measurement of the efficacy of vaccines and antimicrobial therapy against infection with *Toxoplasma gondii*. *Int. J. Parasitol.* **30**:149–155.
 23. Klebanoff, C. A., L. Gattinoni, and N. P. Restifo. 2006. CD8+ T-cell memory in tumor immunology and immunotherapy. *Immunol. Rev.* **211**:214–224.
 24. Kullberg, M. C., E. J. Pearce, S. E. Hieny, A. Sher, and J. A. Berzofsky. 1992. Infection with *Schistosoma mansoni* alters Th1/Th2 cytokine responses to a non-parasite antigen. *J. Immunol.* **148**:3264–3270.
 25. La Flamme, A. C., K. Ruddenklau, and B. T. Bäckström. 2003. Schistosomiasis decreases central nervous system inflammation and alters the progression of experimental autoimmune encephalomyelitis. *Infect. Immun.* **71**:4996–5004.
 26. Liesenfeld, O., I. R. Dunay, and K. J. Erb. 2004. Infection with *Toxoplasma gondii* reduces established and developing Th2 responses induced by *Nippostrongylus brasiliensis* infection. *Infect. Immun.* **72**:3812–3822.
 27. Liesenfeld, O., J. Kosek, J. Remington, and Y. Suzuki. 1996. Association of CD4+ T cell-dependent, interferon-gamma-mediated necrosis of the small intestine with genetic susceptibility of mice to peroral infection with *Toxoplasma gondii*. *J. Exp. Med.* **184**:597–607.
 28. Marshall, A. J., L. R. Brunet, Y. van Gessel, A. Alcaraz, S. K. Bliss, E. J. Pearce, and E. Y. Denkers. 1999. *Toxoplasma gondii* and *Schistosoma mansoni* synergize to promote hepatocyte dysfunction associated with high levels of plasma TNF-alpha and early death in C57BL/6 mice. *J. Immunol.* **163**:2089–2097.
 29. Martin, D. L., D. B. Weatherly, S. A. Laucella, M. A. Cabinian, M. T. Crim, S. Sullivan, M. Heiges, S. H. Craven, C. S. Rosenberg, M. H. Collins, A. Sette, M. Postan, and R. L. Tarleton. 2006. CD8+ T-cell responses to *Trypanosoma cruzi* are highly focused on strain-variant trans-sialidase epitopes. *PLoS Pathog.* **2**:e77.
 30. Metwali, A., T. Setiawan, A. M. Blum, J. Urban, D. E. Elliott, L. Hang, and J. V. Weinstock. 2006. Induction of CD8+ regulatory T cells in the intestine by *Heligmosomoides polygyrus* infection. *Am. J. Physiol. Gastrointest. Liver Physiol.* **291**:G253–G259.
 31. Neter, J., W. Wasserman, and M. Kuter. 1985. Applied linear statistical models. Irwin, Homewood, IL.
 32. Orr, M. T., N. N. Orgun, C. B. Wilson, and S. S. Way. 2007. Cutting edge: recombinant *Listeria monocytogenes* expressing a single immune-dominant peptide confers protective immunity to herpes simplex virus-1 infection. *J. Immunol.* **178**:4731–4735.
 33. Parker, S. J., C. W. Roberts, and J. Alexander. 1991. CD8+ T cells are the major lymphocyte subpopulation involved in the protective immune response to *Toxoplasma gondii* in mice. *Clin. Exp. Immunol.* **84**:207–212.
 34. Segura, M., Z. Su, C. Piccirillo, and M. M. Stevenson. 2007. Impairment of dendritic cell function by excretory-secretory products: a potential mechanism for nematode-induced immunosuppression. *Eur. J. Immunol.* **37**:1887–1904.
 35. Setiawan, T., A. Metwali, A. M. Blum, M. N. Ince, J. F. Urban, D. E. Elliott, Jr., and J. V. Weinstock. 2007. *Heligmosomoides polygyrus* promotes regulatory T-cell cytokine production in the murine normal distal intestine. *Infect. Immun.* **75**:4655–4663.
 36. Stepek, G., D. J. Buttle, I. R. Duce, and J. M. Behnke. 2006. Human gastrointestinal nematode infections: are new control methods required? *Int. J. Exp. Pathol.* **87**:325–341.
 37. Subauste, C. S., A. H. Koniaris, and J. S. Remington. 1991. Murine CD8+ cytotoxic T lymphocytes lyse *Toxoplasma gondii*-infected cells. *J. Immunol.* **147**:3955–3959.
 38. Svetic, A., K. B. Madden, X. D. Zhou, P. Lu, I. M. Katona, F. D. Finkelman, J. F. Urban, Jr., and W. C. Gause. 1993. A primary intestinal helminthic infection rapidly induces a gut-associated elevation of Th2-associated cytokines and IL-3. *J. Immunol.* **150**:3434–3441.
 39. Tan, J. T., B. Ernst, W. C. Kieper, E. LeRoy, J. Sprent, and C. D. Surh. 2002. Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *J. Exp. Med.* **195**:1523–1532.
 40. Urban, J. F., Jr., N. R. Steenhard, G. I. Solano-Aguilar, H. D. Dawson, O. I. Iweala, C. R. Nagler, G. S. Noland, N. Kumar, R. M. Anthony, T. Shear-Donohue, J. Weinstock, and W. C. Gause. 2007. Infection with parasitic nematodes confounds vaccination efficacy. *Vet. Parasitol.* **148**:14–20.
 41. van Leeuwen, E. M., I. J. ten Berge, and R. A. van Lier. 2007. Induction and maintenance of CD8+ T cells specific for persistent viruses. *Adv. Exp. Med. Biol.* **590**:121–137.
 42. Villinger, F., R. Miller, K. Mori, A. E. Mayne, P. Bostik, J. B. Sundstrom, C. Sugimoto, and A. A. Ansari. 2004. IL-15 is superior to IL-2 in the generation of long-lived antigen specific memory CD4 and CD8 T cells in rhesus macaques. *Vaccine* **22**:3510–3521.
 43. Wahid, F. N., and J. M. Behnke. 1992. Stimuli for acquired resistance to *Heligmosomoides polygyrus* from intestinal tissue resident L3 and L4 larvae. *Int. J. Parasitol.* **22**:699–710.
 44. Wahid, F. N., J. M. Behnke, R. K. Grecis, K. J. Else, and A. W. Ben-Smith. 1994. Immunological relationships during primary infection with *Heligmosomoides polygyrus*: Th2 cytokines and primary response phenotype. *Parasitology* **108**:461–471.
 45. Wang, C. C., T. J. Nolan, G. A. Schad, and D. Abraham. 2001. Infection of mice with the helminth *Strongyloides stercoralis* suppresses pulmonary allergic responses to ovalbumin. *Clin. Exp. Allergy* **31**:495–503.
 46. Wilson, M. S., M. D. Taylor, A. Balic, C. A. Finney, J. R. Lamb, and R. M. Maizels. 2005. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J. Exp. Med.* **202**:1199–1212.

Editor: W. A. Petri, Jr.