IL-4 Protects Adult C57BL/6 Mice from Prolonged Cryptosporidium parvum Infection: Analysis of CD4+αβ+IFN-γ+ and CD4+αβ+IL-4+ Lymphocytes in Gut-Associated Lymphoid Tissue During Resolution of Infection

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IL-4 Protects Adult C57BL/6 Mice from Prolonged Cryptosporidium parvum Infection: Analysis of CD4⁺αβ⁺IFN-γ⁺ and CD4⁺αβ⁺IL-4⁺ Lymphocytes in Gut-Associated Lymphoid Tissue During Resolution of Infection

Shirley A. Aguirre, Lance E. Perryman, William C. Davis, and Travis C. McGuire

Resistance of adult C57BL/6 mice to severe Cryptosporidium parvum infection is dependent on CD4⁺αβ⁺ TCR lymphocytes. In this study, we demonstrated that treatment with anti-IFN-γ mAb extended oocyst excretion 18 days longer, and anti-IL-4 mAb extended oocyst excretion at least 11 days longer than isotype control mAb treatment. Analysis of the specific activity of anti-IFN-γ mAb present in treated mouse sera suggested that IFN-γ may have a limited role in the resolution phase of infection. Changes were also documented in numbers of CD4⁺αβ⁺IFN-γ⁺ and CD4⁺αβ⁺IL-4⁺ lymphocytes in Peyer’s patches and intraepithelium of adult C57BL/6 mice during resolution of C. parvum infection. Resistance to initial severe infection was associated with CD4⁺αβ⁺IFN-γ⁺ lymphocytes, and eventual resolution of infection was associated with CD4⁺αβ⁺IL-4⁺ lymphocytes. Analysis of cytokine expression following in vitro stimulation with C. parvum Ags during resolution of infection demonstrated consistent increases in CD4⁺αβ⁺IL-4⁺ lymphocytes, but not CD4⁺αβ⁺IFN-γ⁺ lymphocytes. The relevance of CD4⁺αβ⁺IL-4⁺ lymphocytes in protection against C. parvum was then evaluated in C57BL/6 IL-4 gene knockout mice (IL-4⁻⁻). Adult IL-4⁻⁻ mice excreted oocysts in feces approximately 23 days longer than IL-4⁺⁺ mice. Further, anti-IFN-γ mAb treatment increased the severity and the duration of infection in IL-4⁻⁻ mice compared with those in IL-4⁺⁺ mice. Together, the data demonstrated that IFN-γ was important in the control of severity of infection, and either IFN-γ or IL-4 accelerated termination of infection. However, neither IL-4 nor IFN-γ was required for the final clearance of infection from the intestinal tract of adult mice. The Journal of Immunology, 1998, 161: 1891–1900.

Cryptosporidium parvum infects intestinal epithelial cells and causes a diarrheal disease in humans and other mammals (1). The health problems caused by the presence of C. parvum in water supplies have received worldwide recognition as an emerging public health issue because infection can cause a life-threatening disease in young and immunocompetent individuals (2, 3). Individuals with congenital T or B lymphocyte deficiencies, SCID, chemotherapy induced immunodeficiency, or AIDS are at risk of increased morbidity and potentially fatal infections following exposure to C. parvum (1, 3). C. parvum infection also causes diarrheal disease in neonatal ruminants, and these infections result in reservoirs of infectious oocysts that have environmental and zoonotic implications (3). Methods of immunologic control or intervention are needed because of the resistance of the organism to approved drug therapies or conventional water treatments and the lack of effective preventative and interventive therapies (2).

Resolution of C. parvum infection in immunologically intact individuals results in long-term protection from reinfection. Mechanisms of immunity have been dissected using several mouse models to demonstrate that CD4⁺ T lymphocytes or αβ TCR⁺ lymphocytes are required for resolution of C. parvum infection (4–7). The major functional subset of CD4⁺ lymphocytes involved in the protective immune response to C. parvum is not known. Murine CD4⁺ T lymphocytes are heterogeneous and are divided into two major functional subsets, Th1 and Th2 (8). Th1 lymphocytes are defined by the production of IFN-γ, IL-2, and TNF-β and can activate macrophages, mediate delayed-type hypersensitivity, and aid in the production of CTL and IgG2a Abs by B lymphocytes. Th2 lymphocytes express IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 and aid in B lymphocyte production of IgE, IgA, IgG1, and IgG4 Abs. Results from treatment of adult immunocompetent BALB/c mice with anti-IFN-γ mAb indicated that recovery from severe C. parvum infection is independent of IFN-γ (4). In contrast, adoptive lymphocyte transfer experiments in SCID mice suggest that both CD4⁺ T lymphocytes and IFN-γ mediate resolution of established C. parvum infection (9). Recent work in C57BL/6 IFN-γ gene knockout (GKO) mice further indicates that IFN-γ is required to prevent fatal C. parvum infection (10).

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3 Abbreviations used in this paper: GKO, gene knockout; GALT, gut-associated lymphoid tissue; PP, Peyer’s patches; IE, intraepithelium; LP, lamina propria; RPE, R-phycoerythrin.
Host intestinal epithelial cell invasion is required for *C. parvum* infection. Infection of the gut-associated lymphoid tissue (GALT) could be an important first line of host defense for control of infection. When *C. parvum* oocysts are ingested, sporozoites are released into the gut lumen and then infect host intestinal epithelial cells. Within the epithelial mucosa, sporozoites develop into type I or type II meronts. Type II meronts initiate the sexual phase of the life cycle, which, upon completion, yields infectious oocysts (1). GALT that can respond to *C. parvum* infection is compartmentalized into Peyer’s patches (PP), intraepithelium (IE), and lamina propria (LP) (11). PP in the mouse are organized secondary lymphoid structures with clearly defined T and B lymphocyte-dependent areas and consist of B lymphocytes (80%), with the remaining cells composed of CD4+ lymphocytes with fewer CD8+ lymphocytes. In contrast, lymphocytes in the LP and IE are not organized, but are scattered in the mucosa. IE T lymphocytes are predominantly CD3+CD8+ (≥80%) and CD3+CD4+ (≥15%), and the CD3+ lymphocytes express αβ TCR (45%) or γδ TCR (55%). LP lymphocytes are predominantly CD4+αβ TCR+. PP probably act as inductive sites of the host immune response to intestinal pathogens, while the LP and IE act as effector sites of the local immune response.

To further define the involvement of CD4+ T lymphocyte subsets in the resolution of *C. parvum* infection in adult C57BL/6 mice, we investigated the hypothesis that Th2 lymphocyte responses mediate control of *C. parvum* infection. The initial experiments demonstrated that the recovery phase of *C. parvum* infection proceeded despite continued anti-IFN-γ mAb treatment and that anti-IL-4 mAb treatment prevented early termination of *C. parvum* infection. Analysis of the intracellular IFN-γ and IL-4 expression in PP and IE CD4+αβ T lymphocytes in adult immunocompetent C57BL/6 mice inoculated with *C. parvum* oocysts and treated with anti-IFN-γ mAb, anti-IL-4 mAb, or isotype control mAb indicated that 1) CD4+αβ IFN-γ+ lymphocytes were associated with control of severity of early infection; and 2) CD4+αβ IL-4+ lymphocytes were associated with the resolution phase of *C. parvum* infection. Further analysis of the involvement of IL-4 in resolution of infection was investigated in adult C57BL/6 IL-4 GKO (IL-4−/−) mice. IL-4−/− mice developed a prolonged *C. parvum* infection; however, these mice eventually resolved *C. parvum* infection with or without anti-IFN-γ mAb treatment. Therefore, we obtained clear evidence that IL-4 prevented prolonged *C. parvum* infection. In addition, adult C57BL/6 mice eventually recovered from infection in the absence of both IFN-γ and IL-4.

**Materials and Methods**

**Mice**

C57BL/6 mice were purchased from B&K Universal (Kent, WA) and The Jackson Laboratory (Bar Harbor, ME). IL-4 knockout (IL-4−/−) mice were obtained from The Jackson Laboratory. The IL-4 status of the IL-4 knockout mice inoculated with *C. parvum* oocysts was determined by reverse primers used to detect the disrupted first exon of the IL-4 gene were obtained from The Jackson Laboratory. The IL-4 status of the IL-4 knockout mice was determined by radial immunodiffusion (The Binding Site, Birmingham, U.K.) and adjusted to 1 mg/ml. Fivefold serial dilutions of this adjusted mouse serum (1 mg/ml rat IgG1) in a 100-μl volume were incubated with 100 μl containing 4 ng/ml rIFN-γ (PharMingen) at room temperature for 30 min. Ninety-six-well Corning easy wash plates were previously coated with 0.5 μg/ml of purified anti-IFN-γ mAb (XMG1.2) diluted in coating buffer (0.015 M NaHCO3 and 0.03 M Na2HPO4, pH 9.6) and incubated overnight at 4°C. The plates were washed with PBS/1% Tween-20 and blocked for 2 h at room temperature with PBS/0.1% FCS. One hundred microliters of the mouse serum/anti-IFN-γ mixture was added to the 96-well plate in duplicate and incubated overnight at 4°C. After washing, biotinylated anti-IFN-γ mAb (RA426) was added to the plate and incubated for 45 min at room temperature. The plate was washed, streptavidin-horseradish peroxidase was added, and incubation proceeded for 30 min at room temperature. Fresh o-phenylene diamine dihydrochloride substrate was added (100 μl) to the plate and incubated for 1 h. The reaction was stopped with 0.1 N HCl (100 μl), and the plate was read on a Titer-Tek Multiscan MCC/340 ELISA reader (Flow Laboratories, McLean, VA) at 492 nm. The reagents used in this sandwich ELISA were from a kit for measuring murine IFN-γ (PharMingen).

**Three-color flow cytometry for intracellular cytokines**

Lymphocytes (5 × 10^6/ml) were incubated in 2 ml of RPMI medium with 10% FCS and 2 μM monensin (Sigma) for 4 h at 37°C to inhibit cytokine secretion and allow cytokine accumulation within cells (17, 18). The lymphocytes were washed, and Fc receptors were blocked with 2% rabbit serum in PBS for 10 min at room temperature. Lymphocytes were then washed with staining buffer (PBS containing 10% FCS and 0.1% azide) and resuspended to 1 × 10^6 cells/ml in staining buffer; 50 μl of lymphocytes were added to the wells of a 96-well V-bottom plate, followed by 50 μl of biotinylated anti-CD4 mAb and 50 μl of anti-αβ TCR-FITC or anti-αβ TCR-RPE mAbs. After 15 min at room temperature, cells were washed twice with 200 μl staining buffer and then incubated with streptavidin conjugated to Cy-Chrome for 15 min at room temperature. After two wash steps, the lymphocytes were fixed and permeabilized in one step for 10 min
at room temperature in HBSS containing 4% paraformaldehyde, 0.1% saponin, and 10 mM HEPEs (17, 18). Cells were then washed and resuspended in 50 μl of permeabilization buffer (PBS with 10% FCS, 0.1% azide, and 0.1% saponin). Anti-IFN-γ-FTTC or anti-IL-4-PE mAb in 50 μl was added to the cells for a final reaction volume of 100 μl and were incubated at room temperature for 30 min. Cells were washed twice in permeabilization buffer and resuspended in staining buffer for flow cytometry.

The CD4^+ lymphocyte population was analyzed for αβ^+IL-4^+ and αβ^+IFN-γ^+ lymphocytes by dual parameter FL1-FL2 dot plot. Specificity of the intracellular staining procedure was controlled by preincubation of a twofold excess (micrograms per milliliter) of rIFN-γ with anti-IFN-γ mAb or rIL-4 with anti-IL-4 mAb for 1 h at room temperature to block for 1 h at room temperature in HBSS containing 4% paraformaldehyde, 0.1% saponin. Anti-cytokine mAb preincubation with recombinant cytokine resulted in >99.7% inhibition of intracellular staining, produced <0.3% fluorescent cells, and was used to set the quadrant to determine statistics. Cell fluorescence was measured by FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Two-parameter dot plots demonstrating cytokine staining were created by CellQuest software (San Jose, CA).

To determine intracellular cytokine expression in CD4^+αβ^+ lymphocytes after stimulation with C. parvum Ags or PMA/ionomycin, PE and IE lymphocytes were pooled from five or six mice in each treatment group. Triplicate cultures (3 × 10^5 cells/ml) were incubated for 4 h at 37°C with 5% CO2 in RPMI medium containing 10% FCS, 20 mM sodium bicarbonate, 0.1 mM nonessential amino acids, 2 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPEs, 100 U/ml penicillin, 200 μg/ml streptomycin, 50 μM 2-ME, 2 mM monensin, and C. parvum protein Ags (100 μg/ml), PMA (10 ng/ml) with ionomycin (500 ng/ml), or no stimulation. Following incubation, cells were prepared for flow cytometric staining. The stimulation index for C. parvum Ags was calculated as: % of fluorescent CD4^+αβ^+IFN-γ^+ or CD4^+αβ^+IL-4^+ lymphocytes + C. parvum Ag + % fluorescent CD4^+αβ^+IFN-γ^+ or CD4^+αβ^+IL-4^+ lymphocytes ex vivo.

PP and IE lymphocyte isolation

Small intestinal PP and IE were removed from mice of various treatment groups (five mice per group), and single cell suspensions of lymphocytes were prepared using a described protocol with modifications (19). The number of PP lymphocytes recovered from each mouse was determined, and 10 mg/ml collagenase VIII (Sigma, St. Louis, MO) was used instead of Sephadex. Approximately 6.7 × 10^6 to 1.3 × 10^7 PP lymphocytes were recovered per mouse with >95% viability. The number of IE lymphocytes recovered from each mouse was approximately 3.9 × 10^6 to 1.1 × 10^7 IE lymphocytes with >95% viability.

C. parvum Ags

Soluble C. parvum Ags were prepared from oocyst lysates. Approximately 10^7 oocysts were treated with sodium hypochlorite (1.75%) to sterilize the preparation and to facilitate excystation. Oocysts were pelleted by centrifugation at 1000 g for 10 min at 4°C, resuspended in a 50-ml conical tube of ice-cold 1.75% sodium hypochlorite, and incubated on ice for 8 min, inverting the tube once every minute. Hypochlorite was removed by washing the oocyst preparation four to six times with ice-cold sterile PBS. Washed oocysts were then resuspended in 5 ml of sterile PBS, frozen in liquid nitrogen for 1 min, and thawed in a 37°C water bath. The freeze-thaw process was repeated 14 times to fracture oocysts, which were then left in a 37°C water bath for 3 or 4 days. Soluble C. parvum Ags were separated from insoluble oocyst shells by centrifugation, and the soluble protein concentration was determined by bichinchoninic acid assay (Pierce, Rockford, IL). Soluble C. parvum Ag stocks were determined to be free of endotoxin by a lymphoproliferation assay using noninfected mouse spleen cells and by a Limulus amebocyte lysate assay (Sigma).

Results

Anti-IFN-γ mAb treatment of adult C57BL/6 mice did not block recovery from C. parvum infection

Neutralizing mAb specific for IFN-γ was used to evaluate the role of IFN-γ in the recovery of adult C57BL/6 mice inoculated with C. parvum oocysts. Infected mice treated with anti-IFN-γ mAb for two or three times weekly shed significantly higher numbers of oocysts in feces 4 to 26 days post-oocyst inoculation and reached a peak on day 9 that was 1000-fold greater than that in the control mAb-treated mice (Fig. 1). However, despite continued treatment of mice with anti-IFN-γ mAb, the infection resolved, so that oocysts were no longer detectable in feces on day 30 (Fig. 1). Mice treated with isotype control mAb resolved their mild infection by day 12, so blocking IFN-γ extended oocyst excretion by 18 days.

Serum from each mouse treated with anti-IFN-γ mAb was collected on days 9 and 23 postinoculation to verify that the amount and the sp. act. of anti-IFN-γ mAb in sera of mice on these days were similar, although the pattern of C. parvum oocyst excretion had changed. The concentration of rat anti-mouse IFN-γ IgG1 mAb in the sera of mice was determined by radial immunodiffusion, and the ability of this rat mAb in serum to block recombinant mouse IFN-γ was determined by sandwich ELISA. The mean concentration of anti-IFN-γ mAb in mouse sera on day 23 was 2.2 times that on day 9 (Table I). The increase on day 23 was considered to be a consequence of four additional treatments between days 9 and 23 post-oocyst inoculation. However, the sp. act. of anti-IFN-γ mAb in mouse sera was the same on days 9 and 23 post-oocyst inoculation (Table I). These results substantiated that

<table>
<thead>
<tr>
<th>Table I. Concentration and specific activity of anti-IFN-γ (XMG.6) mAb in mice sera on days 9 and 23 after C. parvum oocyst inoculation</th>
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<tbody>
<tr>
<td>Anti-IFN-γγ^a (ng/ml), Mean ± SD of 10 Mice</td>
</tr>
<tr>
<td>9 dpi^c</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>1.9 ± 0.6</td>
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</tbody>
</table>

^a Serum was collected from two independent experiments, and the concentration of rat IgG1 was measured on days 9 and 23 for each mouse by radial immunodiffusion.
^b Sp. act. Days postinoculation.
^c Statistical analysis performed by unpaired Student’s t test and p < 0.05 was considered significant.
^d Anti-IFN-γ mAb functional specific activity was defined as ng/ml to inhibit 50% of the maximum OD in an IFN-γ sandwich ELISA.
treated mice had anti-IFN-γ mAb in the serum with the ability to block extracellular IFN-γ. Thus, blocking IFN-γ allowed establishment of a severe Cryptosporidium parvum infection between days 4 to 9 post-oocyst inoculation and extended the infection by 18 days, but did not prevent resolution of infection by day 30 post-oocyst inoculation.

Anti-IL-4 mAb treatment of adult C57BL/6 mice caused prolonged excretion of C. parvum oocysts

Adult C57BL/6 mice were treated with anti-IL-4 mAb before challenge with C. parvum oocysts, and then were treated two or three times weekly to investigate the role of IL-4 in recovery from infection. Anti-IL-4 mAb and isotype control mAb-treated mice shed similar numbers of C. parvum oocysts in feces on days 4 to 7 postinoculation (Fig. 2). However, anti-IL-4 mAb treatment caused significantly higher excretion of oocysts in feces from days 8 to 12 post-oocyst inoculation than that after isotype control mAb treatment (Fig. 2). Infected mice treated with isotype control mAb had no detectable oocysts in feces on day 12. After day 16 post-oocyst inoculation, the numbers of C. parvum oocysts in anti-IL-4 mAb-treated mice declined, but were still detectable on day 23. This result demonstrated that blocking IL-4 did not cause the early establishment of a more severe C. parvum infection as was noted when IFN-γ was blocked in adult C57BL/6 mice (Fig. 1). Nevertheless, blocking IL-4 increased the numbers of oocysts excreted in feces between days 8 and 23 post-oocyst inoculation and prolonged oocyst excretion by at least 11 days.

Resolution of C. parvum infection in adult C57BL/6 mice was associated with increased numbers of CD4+αβ+IL-4+ lymphocytes in GALT

The next experiments analyzed intracellular IL-4 and IFN-γ expression in the CD4+αβ+ lymphocyte populations from GALT during resolution of infection. The CD4+αβ+ lymphocytes were evaluated because CD4+ T lymphocytes or αβ+ TCR lymphocytes are required to prevent persistent C. parvum infection in adult C57BL/6 mice (6, 7). The percentage and number of GALT CD4+αβ+ lymphocytes with intracellular IL-4 or IFN-γ on days 9 and 23 post-oocyst inoculation were determined by flow cytometry (Fig. 3, A–C). Statistical comparisons were made for changes in CD4+αβ+IL-4+ and CD4+αβ+IFN-γ+ lymphocytes on days 0, 9, and 23 post-oocyst inoculation using the Kruskal-Wallis and Mann-Whitney rank sum tests, and p < 0.05 was considered significant.

After C. parvum inoculation of isotype control mAb-treated mice, CD4+αβ+IFN-γ+ lymphocytes increased significantly between days 0 and 9 in both PP and IEL; however, there was a significant decrease in these lymphocytes in both compartments between days 9 and 23 (Fig. 3A). In contrast, CD4+αβ+IL-4+ lymphocytes increased significantly in both compartments between days 0 to 9 and days 9 to 23 (Fig. 3A). These observations demonstrated that compared with day 0, 1) control of the initial severity of infection on day 9 was associated with an increase in numbers of both CD4+αβ+IFN-γ+ and CD4+αβ+IL-4+ lymphocytes; 2) the resolution phase of C. parvum infection on day 23 was inconsistently associated with increased numbers of CD4+αβ+IFN-γ+ lymphocytes; and 3) the resolution phase of C. parvum infection on day 23 was consistently associated with increased numbers of CD4+αβ+IL-4+ lymphocytes in PP and IEL compartments.

After C. parvum inoculation of anti-IFN-γ mAb-treated mice, CD4+αβ+IFN-γ+ lymphocytes were not significantly increased...
in PP but were significantly increased in IE between days 0 and 9 (Fig. 3B). From days 9 to 23, CD4^+αβ^-IFN-γ^-lymphocyte numbers increased significantly in PP, but decreased significantly in IE (Fig. 3B). CD4^+αβ^-IL-4^-lymphocytes increased significantly in both PP and IE compartments on day 9; however, numbers declined significantly in both compartments from days 9 to 23 (Fig. 3B). Even though the CD4^+αβ^-IL-4^-lymphocytes were decreased between days 9 and 23 postinoculation, the numbers on day 23 were still significantly elevated from day 0 and were at levels similar to those in the isotype control mAb-treated mice on day 23 (Fig. 3A). It was concluded that resolution of C. parvum infection in anti-IFN-γ mAb-treated mice was also associated with increased numbers of CD4^+αβ^-IL-4^-lymphocytes. The changes seen in CD4^+αβ^-IFN-γ^-lymphocytes were unexpected because anti-IFN-γ mAb was present extracellularly to block any secreted IFN-γ.

After C. parvum inoculation of anti-IL-4 mAb-treated mice, CD4^+αβ^-IFN-γ^-lymphocytes increased significantly in PP and IE between days 0 and 9 (Fig. 3C). From days 9 to 23, CD4^+αβ^-IFN-γ^-lymphocyte numbers decreased significantly in PP and IE (Fig. 3C). CD4^+αβ^-IL-4^-lymphocytes also increased significantly in both PP and IE compartments from days 0 to 9; however, numbers declined significantly from days 9 to 23 in PP and remained the same in IE on days 9 and 23 (Fig. 3C). It was concluded that the increased numbers of CD4^+αβ^-IFN-γ^-lymphocytes in PP and IE on day 9 did not result in early resolution of C. parvum infection. The prolonged excretion of C. parvum oocysts in the anti-IL-4 mAb-treated mice was attributed to blocking of extracellular IL-4 by the mAb treatment.

**In vitro recall response of GALT to C. parvum Ags was consistently associated with increased CD4^+αβ^-IL-4^-lymphocytes**

To determine whether CD4^+αβ^-lymphocytes with intracellular IFN-γ and IL-4 were C. parvum Ag specific, the in vitro recall response to C. parvum Ags was investigated in mice during resolution of C. parvum infection and in uninfected control mice. The percentages of CD4^+αβ^-IFN-γ^- and CD4^+αβ^-IL-4^-lymphocytes in PP and IE after stimulation with C. parvum Ags in isotype control mAb-treated, C. parvum-infected mice and in uninfected control mice are shown in Figure 4. Stimulation of isolated lymphocytes with C. parvum Ags consistently produced stimulation indexes of ≥4 in PP and IE for CD4^+αβ^-IL-4^-lymphocytes and ≥1 for CD4^+αβ^-IFN-γ^-lymphocytes from isotype control mAb-treated mice. Similar results were obtained in mice challenged with C. parvum oocysts and treated with either anti-IFN-γ mAb or anti-IL-4 mAb (data not shown). A stimulation index <0 in uninfected control mice after in vitro stimulation with C. parvum Ags indicates that IL-4 expression in CD4^+αβ^-lymphocytes from infected mice was C. parvum Ag specific (Fig. 4). Stimulation with ionomycin and PMA was included to show nonspecific IFN-γ and IL-4 cytokine production by CD4^+αβ^-lymphocytes (Fig. 4). This nonspecific stimulation resulted in a stimulation index of >2 for IFN-γ and IL-4.

**Adult C57BL/6 IL-4^-/- mice, but not IL-4^-/+ mice, had a C. parvum intestinal epithelial infection 30 days post-oocyst inoculation**

To further investigate the relevance of CD4^+αβ^-IL-4^-lymphocytes in the resolution of C. parvum infection in an adult C57BL/6 mouse model, initial experiments evaluated C. parvum infection in intestines from adult C57BL/6 IL-4^-/- mice at 30 days post-oocyst inoculation. C. parvum intestinal infection scores in IL-4^-/- mice were significantly greater than those in IL-4^-/+ mice (p = 0.004) (Table II). C. parvum organisms were consistently seen in the jejunum and ileum of IL-4^-/- mice, but were inconsistently seen in the pylorus, duodenum, cecum, and colon. No organisms were found in the intestines of IL-4^-/+ mice. Although C. parvum organisms were present in the epithelium of IL-4^-/- mice, intestinal sections lacked significant pathologic changes indicative of the resolution phase of infection. In a separate experiment to determine whether the severity of prolonged C. parvum infection in IL-4^-/- mice on day 30 could be increased by blocking the early protective effect of IFN-γ, IL-4^-/- and IL-4^-/+ mice were treated with anti-IFN-γ mAb. This treatment caused significantly higher C. parvum infection scores on day 30 in IL-4^-/- mice colon compared with those in anti-IFN-γ mAb treated IL-4^-/+ mice (Table II). IL-4^-/- mice had substantial numbers of organisms, and histopathologic changes were seen in the pylorus, duodenum, jejunum, ileum, and colon. The changes included crypt

**Table II. C. parvum intestinal scores in IL-4^-/+ mice 30 days postinoculation**

<table>
<thead>
<tr>
<th>Intestinal Infection Scores*</th>
<th>Mean ± SD (no. of mice)</th>
<th>p**</th>
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</thead>
<tbody>
<tr>
<td>IL4^-/-</td>
<td>IL4^-/+</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3.6 ± 0.5 (5)</td>
<td>0.0 ± 0.0 (5)</td>
</tr>
<tr>
<td>Anti-IFN-γ</td>
<td>4.8 ± 1.1 (5)</td>
<td>0.8 ± 0.4 (5)</td>
</tr>
</tbody>
</table>

*Intestinal infection score was described in Materials and Methods.

**Mean-Whitney Rank Sum test.

Anti-IFN-γ mAb administration was described in Figure 1.
abscessation, epithelial hyperplasia, villus blunting, villus thickening, and infiltration of eosinophils, neutrophils, lymphocytes, and plasma cells in the LP. These observations demonstrated that *C. parvum* infection in adult C57BL/6 IL-4−/− mice could be made more severe on day 30 by blocking the early protective effect of IFN-γ by anti-IFN-γ mAb treatment.

As expected, CD4+αβ+IL-4+ lymphocytes were absent from PP and IE in the *C. parvum*-infected IL-4−/− mice (Fig. 5). However, infection caused a significant increase in CD4+αβ+IL-4+ lymphocytes in PP and IE of IL-4+/+ mice and a significant increase in CD4+αβ+IFN-γ+ lymphocytes in PP and IE of both IL-4+/+ and IL-4−/− mice (Fig. 5). Further, the numbers of CD4+αβ+IFN-γ+ lymphocytes in PP and IE of both IL-4+/+ and IL-4−/− mice were similar (*p < 0.05*), indicating that these Th1 lymphocytes were insufficient to explain resolution of infection in IL-4+/+ mice.

Adult C57BL/6 IL-4−/− mice developed a prolonged *C. parvum* infection, but eventually resolved infection

To determine the duration of the prolonged infection in adult IL-4−/− mice, *C. parvum*-infected IL-4+/+ and IL-4−/− mice were monitored over a 60-day period (Fig. 6). Oocyst excretion in IL-4−/− mice was not significantly different from that in IL-4+/+ mice on days 4 to 7 post-oocyst inoculation (Fig. 6A). However, oocyst excretion between days 8 to 30 postinoculation was significantly higher in feces from IL-4−/− mice than in those from IL-4+/+ mice (Fig. 6). Oocyst excretion became undetectable in feces from IL-4+/+ mice on day 12 and in feces from IL-4−/− mice on day 35 (Fig. 6A). These observations demonstrated that IL-4−/− mice remained infected with *C. parvum* for at least 23 days longer than IL-4+/+ mice. Nonetheless, IL-4−/− mice resolved infection by day 35 postinoculation.

IL-4−/− mice were next treated with anti-IFN-γ mAb to determine whether in vivo neutralization of endogenous IFN-γ would result in a more severe and prolonged *C. parvum* infection. Anti-IFN-γ mAb treatment allowed establishment of a severe *C. parvum* infection in IL-4−/− mice that was not significantly different from that in anti-IFN-γ mAb-treated IL-4+/+ mice on days 4 to 23 postinfection (Fig. 6B). After day 23, IFN-γ-depleted IL-4−/− mice shed significantly higher numbers of oocysts in feces compared with IFN-γ-depleted IL-4+/+ mice. IL-4+/+ mice treated with anti-IFN-γ mAb resolved *C. parvum* infection as expected; oocyst excretion in feces of these mice was undetectable on day 35 postinoculation (Fig. 6B). IL-4−/− mice treated with anti-IFN-γ mAb also resolved *C. parvum* infection; oocysts were undetectable in feces of these mice by day 55 postinoculation. In conclusion, blocking of IFN-γ in IL-4−/− mice resulted in the same early increase in severity of infection as that noted when IFN-γ was blocked in IL-4+/+ mice. In summary, *C. parvum* infection was prolonged by at least 23 days in IL-4−/− mice compared with that in IL-4+/+ mice and by at least 20 days in anti-IFN-γ mAb-treated IL-4−/− mice compared with that in anti-IFN-γ mAb-treated IL-4+/+ mice.

Discussion

In this work, adult C57BL/6 mouse models were used to confirm the role of IFN-γ in limiting the severity of early *C. parvum* infection. A new finding was the documentation of an important role for IL-4 in the early resolution of *C. parvum* infection in mice treated with anti-IL-4 mAb and in IL-4−/− mice. Even though an early role for IFN-γ and subsequent roles for IFN-γ and IL-4 were demonstrated in protective immunity, neither was required for CD4+αβ+ lymphocytes to eventually resolve *C. parvum* infection.

Blocking IFN-γ by mAb treatment resulted in a more severe early *C. parvum* infection in adult C57BL/6 mice. The demonstration that IFN-γ was necessary to limit severity of infection in adult C57BL/6 mice corroborated the results of similar experiments using anti-IFN-γ mAb treatment in adult BALB/c mice (4). In addition, we found that significantly lower oocyst excretion in isotype control mAb-treated C57BL/6 mice on day 9 post-oocyst inoculation was associated with increased numbers of CD4+αβ+IFN-γ+ lymphocytes in both IE and PP compartments. Of interest, increased numbers of CD4+αβ+IFN-γ+ lymphocytes also occurred in both IE and PP compartments in anti-IFN-γ mAb-treated mice. It was assumed that this accumulation of intracellular IFN-γ was a compensatory response to blocking extracellular IFN-γ with mAb or possibly that blocking IFN-γ by mAb treatment was ineffective at the gut level. That the anti-IFN-γ mAb
treatment had an in vivo blocking effect on IFN-γ was demonstrated by the increase in severity of the gut infection in these treated mice and by the ability of mAb in sera taken from these treated mice to block IFN-γ in the sandwich ELISA. The early effect of IFN-γ, which limits C. parvum infection, may result from several general mechanisms (20), including 1) activation of macrophages, 2) increased MHC class II expression on APC, 3) induction of nitric oxide synthase and nitric oxide synthesis, 4) activation of NK cells, 5) specific cytotoxic responses, and 6) B cells switching to IgG2a. In vitro studies suggest that IFN-γ may also mediate early protection by inhibition of parasite replication in the epithelium. Specifically, IFN-γ has been shown to inhibit Toxoplasma gondii replication in a dose-dependent manner in a rat intestinal cell line IEC6 (21), and similar inhibition in a human fibroblast cell line (22) was due to induction of indolamine 2,3-dioxygenase and depletion of tryptophan (23, 24). IFN-γ may also alter the intestinal epithelium by decreasing chloride ion secretion and increasing β2 integrin-dependent neutrophil adhesion and MHC class II expression (25), resulting in resistance to penetration by C. parvum sporozoites or merozoites.

IFN-γ was not necessary for resolution of an established C. parvum infection in adult C57BL/6 mice treated with mAb to IFN-γ. Previous work using anti-IFN-γ mAb (1 mg/mouse/wk) treatment of adult BALB/c mice indicated that recovery from a severe self-limited C. parvum infection was independent of IFN-γ (4). We determined that adult C57BL/6 mice treated two or three times weekly with a higher dose (2 mg/mouse) of mAb to IFN-γ also resolved C. parvum infection. Our conclusion that resolution of infection was independent of IFN-γ was further confirmed by demonstrating that the amount and the sp. act. of the anti-IFN-γ mAb in sera from treated mice during high oocyst excretion on day 9 post-oocyst inoculation and in those from treated mice during low oocyst excretion on day 23 after inoculation were similar. Since blocking IFN-γ activity early allowed more severe infection on day 9, the same or a higher quantity of Ab with the same sp. act. should have blocked IFN-γ on day 23. This result indicated that some other mechanism was required for resolution of C. parvum infection in anti-IFN-γ mAb-treated C57BL/6 mice. These results are in contrast to those obtained in adult C57BL/6 IFN-γ GKO mice, which have a severe acute C. parvum infection with mucosal destruction and die as early as 2 wk post-oocyst inoculation (10). It is difficult to assess whether mechanisms other than IFN-γ could resolve C. parvum infection in adult C57BL/6 GKO mice because of the early death in these mice. The reason for the early death of C. parvum-infected adult GKO mice is not known, but may be due to the severity of immunologic defects present in these mice, including reduced MHC class II expression on macrophages, impaired ability of macrophages to make nitrogen intermediates, uncontrolled proliferative responses in splenocytes, enhanced T cell CTL activity, and decreased resting splenic NK cell activity (20, 26–28). Cumulative immune defects in adult C57BL/6 GKO mice deficient in IFN-γ since conception may be more severe than the immune defects caused by 4 wk of anti-IFN-γ mAb treatment in adult immunocompetent C57BL/6

FIGURE 6. C. parvum oocyst excretion in five adult C57BL/6 mice (IL-4+/+) and five adult C57BL/6 IL-4 GKO (IL-4−/−) mice receiving no treatment (A) or anti-IFN-γ mAb treatment (B). Oocyst counts and inoculum are described in Figure 1. The symbols (○) indicates IL-4+/+ group and □ indicates IL-4−/− group) represent the mean number of excreted oocysts per gram of feces on a given day, and the vertical bars on each symbol are the SD. Note the change in scale on the y-axis of A and B.
mice. Even though rigorous anti-IFN-γ mAb treatment indicated that a depletion of IFN-γ alone was insufficient to block eventual recovery, some residual IFN-γ-mediated effector mechanisms may have remained and contributed to resolution of *C. parvum* infection. However, the presence of similar numbers of Th1 lymphocytes in IL-4−/− mice (which were unable to resolve *C. parvum* infection on day 30) and in IL-4+/+ mice (which resolved *C. parvum* infection on day 30) suggests that Th1 have no role or a limited role in resolution. In addition, IL-12 treatment, which has an IFN-γ-dependent protective effect, failed to ameliorate established *C. parvum* infections in neonatal immunocompetent and neonatal immunodeficient mice, supporting the lack of IFN-γ and IFN-γ-dependent effector mechanisms in this resolution (29). Further, inability of recombinant IFN-γ treatment to significantly reduce *C. parvum* infection in the large intestines and biliary tract of immunosuppressed rats also indicates that some mechanism other than IFN-γ resolves *C. parvum* infection (30).

In adult immunodeficient mouse models, a role for IFN-γ and IFN-γ-mediated effects such as nitric oxide in the control of *C. parvum* infection was demonstrated in a number of experiments, but none of these experiments provided conclusive evidence for a role for IFN-γ in resolution of *C. parvum* infection. *C. parvum* infection in congenitally athymic nude adult mice was enhanced after anti-IFN-γ mAb treatment and remained severe after stopping the treatment (4). Administration of the nitric oxide inhibitor, N-nitro-l-arginine methyl ester, enhanced oocyst excretion in congenitally athymic nude adult mice (31). Treatment of SCID mice with anti-IFN-γ mAb caused enhanced *C. parvum* infection at 3 wk compared with that in control SCID mice (32). However, it is difficult to assess the role of IFN-γ in the resolution of *C. parvum* infection in immunodeficient mouse models that lack functional CD4+ lymphocytes, such as SCID mice and congenitally athymic nude mice, because it is known that CD4+ T lymphocytes are required for recovery (4, 6). In other experiments, *C. parvum* infection was not resolved in 17 days in SCID mice reconstituted with spleen cells depleted of 1) CD4+ T cells, 2) IFN-γ, and 3) both CD4+ T cells and IFN-γ (9). The conclusion made that IFN-γ was required to resolve *C. parvum* infection in these reconstituted SCID mice may have been different if the mice had been observed for longer periods, as in this study.

A prolonged, but self-limited, *C. parvum* infection in IL-4-deficient adult C57BL/6 mice was associated with normal Th1 responses in gut lymphocytes. Depletion of IL-4 by mAb treatment did not cause early enhanced oocyst excretion in adult C57BL/6 mice, as was seen with anti-IFN-γ mAb treatment, but did allow oocyst excretion to persist in feces at least 11 days longer than in isotype control mAb-treated mice. Similarly, *C. parvum* oocyst excretion in IL-4−/− mice persisted 23 days longer than that in IL-4+/+ mice. Further, IL-4−/− mice and IL-4+/+ mice had the same number of CD4+αβ+IFN-γ− lymphocytes in IE and PP on day 30 postinoculation, yet *C. parvum* organisms were present in the intestinal epithelium of IL-4−/− mice and not in IL-4+/+ mice. These observations demonstrated the importance of IL-4 in preventing a prolonged *C. parvum* infection. Unlike other infections in IL-4−/− mice, a deficient Th1 response seen with *T. gondii* (33) or *Leishmania major* (34) or an exacerbated Th1 response seen in *Mycobacterium bovis* granulomas (35) was not seen in PP and IE lymphocytes of IL-4−/− mice on day 30 post-C. parvum oocyst inoculation. Therefore, extension of the time needed for the IL-4−/− mice to resolve *C. parvum* infection was attributed to a deficiency of IL-4 production.

Eventual resolution of infection in IL-4−/− mice, with or without anti-IFN-γ mAb treatment, demonstrated that neither IL-4 nor IFN-γ was required for recovery. One explanation for resolution of the infection in these mice may still involve a Th2 response, because IL-4 is often only one of multiple signals that can induce redundant Th2 protective effects. IL-9 (36, 37) and IL-13 (38) can mimic or enhance some of the effects of IL-4. IL-4 may decrease time to recovery from *C. parvum* infection through its ability to drive Th2 differentiation (39, 40), cause multiple effects on the immune system, and influence gut physiology (41). Information regarding surrogate Th2 cytokine expression, such as IL-9 or IL-13, in IL-4−/− mice is still incomplete (35, 42, 43). Even though Th2 mechanisms in the absence of IL-4 are plausible explanations for eventual resolution of *C. parvum* infection in IL-4−/− mice, our findings do not discriminate between this explanation and the possibility of other Th1 mechanisms in the absence of IFN-γ.

Another possible explanation for the ability of IL-4−/− mice to eventually resolve *C. parvum* infection is a mucosal Ab response. The gut contains high levels of IgA-producing plasma cells, and IgA can be transported across the epithelium into the gut lumen to prevent invasion of micro-organisms (8, 44, 45). IL-4−/− mice have been shown to have normal serum levels of IgA, indicating that IL-4 has no obligatory role in differentiation of B lymphocytes to IgA-producing plasma cells (42). Other evidence for a role for Ab includes in vitro studies with human mAb specific to *C. parvum*, which inhibited *C. parvum* infection of human enterocyte lines (46), and passive administration of Ab in the form of hyperimmune bovine serum or *C. parvum*-neutralizing mAb, which had beneficial effects against *C. parvum* challenge in mice (13, 47–49) and caused clinical improvement in *C. parvum*-infected AIDS patients (50, 51). However, an argument against a singular role for Ab is provided by the recovery of B cell-depleted BALB/c mice from *C. parvum* infection (52).

Our study provided other intriguing findings that should be addressed. As might be predicted, removal of extracellular IFN-γ increased the numbers of CD4+αβ−IL-4+ lymphocytes almost 10-fold in IE on day 9 compared with that in isotype control mice, consistent with Th1 and Th2 cross-regulation (8). Similarly, removal of extracellular IL-4 increased the number of CD4+αβ−IFN-γ− lymphocytes almost 3-fold in IE on day 9 compared with that in isotype control mice. However, removal of extracellular IFN-γ or IL-4 unexpectedly caused 2- and 3-fold increases in CD4+αβ−IFN-γ− and CD4+αβ−IL-4− lymphocytes, respectively, in the IE on day 9. In contrast, removal of extracellular IFN-γ did not affect CD4+αβ+IFN-γ− lymphocytes in PP on day 9, and removal of IL-4 caused an almost 4-fold increase in CD4+αβ+IL-4− lymphocytes in PP on day 9. The above observations suggest that IFN-γ and IL-4 expression are regulated differently in these gut compartments.

The protective immune functions we described for both IFN-γ and IL-4 in *C. parvum* infection occur in a similar manner for some other intracellular protozoal infections as well. Neutralization of endogenous IFN-γ by mAb treatment impairs, but does not completely abrogate, protective immunity in *Plasmodium chabaudi* infection (53). Also, IL-4 deficiency did not block control of primary infection in *P. chabaudi*, but did enhance later recrudescent parasitemia (54). Neutralization of IFN-γ by mAb treatment blocked acute resistance to *T. gondii* infection (55), while deficiency of IL-4 caused fatal *T. gondii*-induced encephalitis in IL-4−/− C57BL/6 mice (33). Production of IL-4 is associated with control of *L. major* infections in resistant mouse strains (56), while defective Th1 (34) or IL-4 deficiency prevented clearance of *L. major* organisms in adult IL-4−/− BALB/c mice (57). Therefore,
IL-4 may play a role in host protective immunity to certain intra-cellular protozoa in which IFN-γ also has a documented role. Elicitation of redundant immune mechanisms induced by C. parvum at the site of infection and a better understanding of cytokine signals involved in the Th1 and Th2 responses will help define the mechanism(s) that CD4+ αβ⁺ lymphocytes use to finally resolve C. parvum infection.

In summary, new evidence for the role of IL-4 in protective immunity in adult C57BL/6 mice against C. parvum was based on the following: 1) CD4⁺ αβ⁺ IL-4⁺ lymphocyte numbers increased in IE and PP of mice during the resolution phase of infection; 2) blocking IL-4 by mAb treatment in adult mice prolonged oocyst excretion in feces at least 11 days longer than that in isotype control mAb treated mice; and 3) genetic deficiency of IL-4 in C57BL/6 mice prolonged oocyst excretion approximately 23 days longer than that in IL-4-intact (IL-4⁺) mice. Even though our results demonstrated an important role for both IFN-γ and IL-4 in the early termination of C. parvum infection in immunocompetent and IL-4⁻/⁻ C57BL/6 mouse models, recovery of these mice from C. parvum infection occurred independent of both IFN-γ and IL-4. Since it is known that CD4⁺ αβ⁺ lymphocytes are required to terminate C. parvum infection and that Th1 and Th2 immune responses can be stimulated by more than one cytokine pathway, studies aimed at dissection of surrogate Th1 and Th2 cytokine mechanisms are warranted.

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References

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