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Protective and Memory Immunity to *Histoplasma capsulatum* in the Absence of IL-10

George S. Deepe, Jr. and Reta S. Gibbons

We determined whether the absence of IL-10 in mice influenced protective and memory immunity to *Histoplasma capsulatum*. IL-10−/− mice cleared primary and secondary infection more rapidly than wild-type controls. Administration of mAb to TNF-α or IFN-γ, but not GM-CSF, abrogated protection in naïve IL-10−/− mice; mAb to TNF-α, but not IFN-γ or GM-CSF, subverted protective immunity in secondary histoplasmosis. The inflammatory cell composition in IL-10−/− mice was altered in those given mAb to IFN-γ or TNF-α. More Gr-1+ and Mac-3+ cells were present in lungs of IL-10−/− mice given mAb to IFN-γ, and treatment with mAb to TNF-α sharply reduced the number of CD8+ cells in lungs of IL-10−/− mice. We ascertained whether the lack of IL-10 modulated memory T cell generation or the protective function of cells. The percentage of CD3+, CD44high, CD62low, and IFN-γ+ cells in IL-10−/− mice was higher than that of wild-type at day 7 but not day 21 or 49 after immunization. Fewer splenocytes from immunized IL-10−/− mice were required to mediate protection upon adoptive transfer into infected TCR αβ−/− mice. Hence, deficiency of IL-10 confers a salutary effect on the course of histoplasmosis, and the beneficial effects of IL-10 deficiency require endogenous TNF-α and/or IFN-γ. Memory cell generation was transiently increased in IL-10−/− mice, but the protective function conferred by cells from these mice following immunization is strikingly more vigorous than that of wild-type. The Journal of Immunology, 2003, 171: 5353–5362.

*Histoplasma capsulatum* (Hc) is the single most common etiology of fungal pneumonia. In the United States alone, it is estimated to cause between 200,000 and 500,000 new infections annually (1). Infection with this pathogen is acquired by inhalation of airborne mycelial fragments and conidia that settle within the terminal bronchioles and alveoli. Upon exposure to 37°C, the mycelia and conidia convert to the pathogenic yeast phase.

In naïve mice, the organism initiates a robust Th1 cytokine cascade that is a critically important facet in control of the infectious process. IL-12 is up-regulated within 3 days followed by an increase in IFN-γ (2–4). Endogenous TNF-α and GM-CSF also are necessary for expression of the protective immune response (5–9). In secondary histoplasmosis, TNF-α is required for survival whereas IL-12 and IFN-γ appear to be dispensable (8). Neutralization of endogenous GM-CSF impairs elimination of the fungus but does not lead to a fatal infection (9).

IL-10 is a pleiotropic cytokine that expresses immunosuppressive and anti-inflammatory properties (9). It inhibits the antimicrobial effect of phagocytes and dampens T cell activation (10–14). One of its principal actions is presumed to be regulation of the inflammatory response evoked by Th1 cytokines. Increased production of IL-10 in mice exposed to Hc has been observed when endogenous GM-CSF or TNF-α is neutralized in primary and secondary infection, respectively. Moreover, IL-10 contributes to the progressive infection found in GM-CSF or TNF-α-deficient mice (7, 9). Thus, up-regulation of IL-10 is associated with an inability to restrict fungal growth in specific immunodeficiency states.

In this study, we examined the response of IL-10−/− mice to Hc infection. We sought to determine whether the congenital absence of this cytokine impacted the course of infection. Our results demonstrated that in both primary and secondary infection, Hc is cleared more rapidly than in wild-type mice. The ability of IL-10−/− mice to clear primary infection required endogenous IFN-γ or TNF-α, whereas only the latter was necessary for protective immunity in secondary infection. Memory cell generation was transiently different in immunized IL-10−/− mice, but protective T cells from these immunized IL-10−/− mice were far more potent in mediating resistance.

**Materials and Methods**

**Mice**

C57BL/6 mice, IL-10−/−, and TCR αβ−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Athymic nude mice, which were used to generate ascites, were purchased from the National Cancer Institute (Fredricksburg, MD). Animals were housed in isolator cages and were maintained by the Department of Laboratory Animal Medicine, University of Cincinnati, which is accredited by the American Association for Accreditation of Laboratory Animal Medicine. All animal experiments were done in accordance with the Animal Welfare Act guidelines of the National Institutes of Health.

**Preparation of Hc and infection of mice**

Hc yeast (strain G217B) was prepared as described previously (5). This strain is a prototypical virulent strain of this fungus. To produce infection in naïve mice, animals were infected intranasally (i.n.) with 2 × 10⁹ Hc yeast in a 30-µl vol of HBSS. For secondary histoplasmosis, mice were initially inoculated with 10⁴ yeast i.n. in a volume of 30 µl. Six to 8 wk later, previously exposed animals were rechallenged i.n. with 2 × 10⁹ yeast.

**Organ culture for Hc**

Recovery of Hc was performed as described elsewhere (3). Fungal burden was expressed as mean CFU per whole organ ± SEM. The limit of detection is 10² CFU.

**Monoclonal Abs**

Rat anti-mouse IFN-γ (hybridoma XMG 1.6) was prepared at the National Cell Culture Center (Minneapolis, MN). Rat anti-mouse TNF-α (hybridoma XT-22.1) and rat anti-mouse GM-CSF (hybridoma 22-E9) were purified from...
ascites. The cell lines were obtained from Dr. J. Abrams (DNAX, Palo Alto, CA). The concentration of rat IgG in ascites was assessed by ELISA after protein G purification and the amount calculated by linear regression from a rat IgG (Organon Teknika, Durham, NC) standard curve. All Abs contained <5 pg/ml endotoxin as determined by Limulus amebocyte lysate test (BioWhittaker, Walkersville, MD).

**Treatment of mice with neutralizing mAb to TNF-α, IFN-γ, or GM-CSF**

Mice were injected i.p. with 1 mg of mAb to TNF-α, IFN-γ, or GM-CSF at the time of challenge with Hc and each week thereafter. Control animals received an equal amount of rat IgG concomitantly.

**Cytokine measurement**

Lungs from infected mice (n = 5–6) were removed and homogenized in 10 ml of HBSS, centrifuged at 1500 × g, filter sterilized, and stored at −70°C until assayed. The protein concentration of homogenates ranged from 2.3 to 4.8 mg/ml. There were no significant differences (p > 0.05) in protein content among groups of mice. Commercially available ELISA kits were used to measure IFN-γ, IL-4, GM-CSF, and TNF-α (Pierce, Rockford, IL).

**Histology**

Lungs were inflated and excised and tissues were fixed in 10% Formalin and embedded in paraffin blocks. Sections (5 μm) were stained with H&E. Analysis of the sections was performed in a blinded fashion.

**Single-cell suspension from lungs and spleens**

To isolate leukocytes from lungs, mice were sacrificed and lungs were flushed with 5 ml of HBSS by inserting a catheter in the right heart. The lungs were excised and teased apart with forceps and homogenized by sequential passage through 16-, 18-, and 20-gauge needles. Leukocytes were isolated by separation on a 40–70% Percoll (Pharmacia, Piscataway, NJ) gradient (4).

**FIGURE 1.** Burden of infection in Hc-infected wild-type and IL-10−/− mice infected with Hc. In primary infection, mice were infected with 2 × 10^6 yeast i.n., and fungal recovery was assessed in lungs and spleens at days 7, 14, and 21 of infection. In secondary infection, mice were infected with 10^4 yeast i.n. and 49 days later challenged with 2 × 10^6 Hc yeast i.n. Fungal recovery was assessed at days 7 and 14 of infection. Data represent mean ± SEM of six animals per group. One of two experiments is shown. **, p < 0.01.
Spleens were teased apart between the frosted ends of two ground glass slides. Cells were washed three times with HBSS and enumerated.

**FACS analysis**

Lung leukocytes or splenocytes were adjusted to $5 \times 10^3/200 \mu l$ in PBS containing 2% FBS and 0.02% sodium azide and stained with 0.5 µg of one of the following FITC-labeled mAbs (BD PharMingen, San Diego, CA): CD4 (clone RM4-5), CD8 (clone 53-6.7), Ly-6G (Gr-1) (clone RB6-8C5, which recognizes polymorphonuclear cells, and Mac-3 (clone M3/84, detects tissue macrophages) or isotype-matched rat IgG mAb. The samples were washed and fixed in 2% paraformaldehyde until analyzed on a flow cytometer.

To analyze memory phenotype, cells from lungs and spleens were adjusted to $5 \times 10^3/200 \mu l$ in PBS containing 2% FBS and 0.02% sodium azide and stained with 0.5 µg of one of the following mAbs: allophycocyanin-labeled CD62L (clone MEL-14), FITC-conjugated IFN-γ, PE-conjugated CD44, and PerCP protein-labeled CD3. Cells were gated on the CD44high CD62 low population. At least 30,000 events were analyzed.

**Statistics**

Student’s t test was used to compare groups if the data achieved normality, otherwise the Wilcoxon rank sum test was used. Survival data was analyzed using the log rank test.

**Results**

**IL-10−/− mice manifest accelerated clearance of Hc**

IL-10−/− and wild-type naive mice were infected with $2 \times 10^6$ Hc yeast i.n. and sacrificed at days 7, 14, and 21 of infection. Lungs and spleens were removed, and fungal burden was assessed. On day 7, CFU in lungs and spleens of IL-10−/− mice did not differ ($p > 0.05$) from those of wild-type (Fig. 1). On days 14 and 21, the organs of IL-10−/− mice contained less CFU ($p < 0.01$) compared with wild-type.

**FIGURE 2.** Effect of cytokine neutralization on fungal recovery and survival of IL-10−/− mice infected with Hc. IL-10−/− mice were infected with $2 \times 10^6$ Hc yeast i.n. and administered either mAb to TNF-α (A and B), IFN-γ (C and D), or GM-CSF (E and F), and fungal burden was assessed at day 7 of infection. Controls received an equal amount of rat IgG. The data represent mean ± SEM of six animals per group. ***, p < 0.01. Survival of mice ($n = 7$) given mAb or rat IgG is depicted in G. One of two experiments is shown.
We next examined the ability of IL-10−/− mice to control secondary infection with Hc yeast. Mice were immunized with 10⁴ yeast i.n. and 49 days later infected with 2 × 10⁶ yeast i.n. At days 7 and 14, the lungs and spleens of both groups were assessed for the number of CFU in organs. By day 7, the lungs and spleens of IL-10−/− mice contained exceedingly fewer CFU (p < 0.01) than wild type (Fig. 1). In spleens, the CFU were below the limits of detection (10² CFU). By day 14, Hc CFU were not detected in either lungs or spleens of IL-10−/− mice, whereas Hc was present in wild-type mice at day 14 (p < 0.01; Fig. 1).

**Regulation of immunity in IL-10−/− mice**

Since IL-10−/− mice eliminated Hc more rapidly in both primary and secondary infection, we sought to determine whether the augmented host response by these mice was dependent on the presence of endogenous cytokines known to mediate protective immunity to this fungus in wild-type mice (2–9). Naive IL-10−/− mice were administered mAb to TNF-α, IFN-γ, or GM-CSF on the day of infection with 2 × 10⁵ yeast i.n. mAb was continued each week thereafter. A group of mice was sacrificed on day 7, and the fungal burden in lungs and spleens was quantified. IL-10−/− mice given mAb to TNF-α, IFN-γ, or GM-CSF contained significantly higher numbers of CFU (p < 0.01) in lungs and spleens than IL-10−/− mice given rat IgG (Fig. 2, A–F). A separate group of mice was followed for survival. All mice administered mAb to TNF-α or IFN-γ succumbed to infection by day 14, whereas all infected controls and 80% of mice receiving mAb to GM-CSF survived for 40 days (Fig. 2G). The differences in survival between the groups of mice given mAb to TNF-α or IFN-γ were significant (p < 0.01) as compared with infected controls or those treated with mAb to GM-CSF. At the end of the observation period, the surviving mice were sacrificed and their lungs and spleens were cultured for Hc. All organs contained <10⁵ CFU.

Control of the protective response also was examined in immunized IL-10−/− mice administered mAb to the cytokines listed above. Mice were immunized with 10⁴ yeast i.n., and 49 days later they were infected with 2 × 10⁵ yeast i.n. and concomitantly given mAb to TNF-α, IFN-γ, or GM-CSF or an equal amount of rat IgG. On day 7 of infection, only mice whose endogenous TNF-α had been neutralized manifested significantly elevated CFU (p < 0.01) in lungs and spleens (Fig. 3). Separate groups of mice were followed for survival and only the mice given mAb to TNF-α succumbed to secondary infection (Fig. 3).

**Neutralization of endogenous cytokines and the inflammatory response in IL-10−/− mice**

The cell subpopulations within the lungs of infected IL-10−/− mice and those given mAb to TNF-α, IFN-γ, or GM-CSF were analyzed on day 7 of infection to determine whether neutralization of endogenous cytokine altered the composition of the inflammatory cells. IL-10−/− mice given mAb to IFN-γ contained significantly (p < 0.01) more total cells, Gr-1+ cells, and Mac-3+ cells than wild-type mice or IL-10−/− mice given rat IgG or TNF-α (Table I). IL-10−/− mice given mAb to TNF-α contained far fewer CD8+ cells than any of the other groups (Table I). Administration of mAb to GM-CSF did not alter the inflammatory cell composition in lungs of IL-10−/− mice.

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**Figure 3.** Neutralization of TNF-α alters the course of secondary histoplasmosis. Groups of mice (n = 6) were immunized with 10⁴ Hc yeast and 8 wk later were infected with 2 × 10⁶ yeast i.n. Concomitantly, mice were administered mAb to IFN-γ, TNF-α, or GM-CSF or rat IgG. Mice were sacrificed on day 7 of infection and lungs (A) and spleens (B) were cultured for Hc. The results indicate mean ± SEM. **, p < 0.01. C, A survival curve of Hc-immunized mice (n = infected with 2 × 10⁶ yeast i.n. and treated with rat IgG or mAb to TNF-α (n = 10/group). One of two or three experiments is shown.
In secondary infection, there were no significant differences between the wild-type and IL-10⁻/⁻ mice in the number of CD4⁺, CD8⁺, Gr1⁻, and Mac3⁺ cells at day 7 of infection. In IL-10⁻/⁻ mice given mAb to TNF-α, the number of CD8⁺ cells was significantly lower than the number in wild-type or IL-10⁻/⁻ mice given rat IgG (Table II). The remaining populations were similar between these two groups of mice.

Endogenous GM-CSF is necessary for survival from primary Hc infection and contributes to the generation of an effective protective response in secondary histoplasmosis (9). Since neutralization of this cytokine did not markedly subvert immunity in IL-10⁻/⁻ mice, we determined whether this lot of mAb altered protection in wild-type mice. Treatment with mAb to GM-CSF of naive mice resulted in a high mortality as compared with mice given rat IgG. In secondary infection, administration of mAb to GM-CSF blunted clearance of the infection (data not shown). These results are similar to previous data (6).

**Cytokine response in the lungs of infected mice**

To determine whether the inimical effects of cytokine neutralization with perturbations in cytokine generation, we measured the production of IFN-γ, TNF-α, IL-4, and GM-CSF in lungs of infected mice at day 7 (Fig. 4). These cytokines were selected because they are known to modulate the course of histoplasmosis in mice (2–9). Levels of the cytokine that had been neutralized were excluded from analysis.

In primary infection, the levels of IFN-γ in lungs were significantly elevated \((p < 0.01)\) only in IL-10⁻/⁻ mice given rat IgG compared with wild-type mice (Fig. 4). The levels of TNF-α in lungs of wild-type mice were not different from those in IL-10⁻/⁻ mice given rat IgG, mAb to IFN-γ, or mAb to GM-CSF. IL-4 levels were increased in lungs of IL-10⁻/⁻ mice treated with rat IgG \((p < 0.01)\), mAb to GM-CSF \((p < 0.01)\), or mAb to IFN-γ \((p < 0.05)\) as compared with wild-type mice. Lung GM-CSF was greater in IL-10⁻/⁻ mice given rat IgG, mAb to TNF-α, or mAb to IFN-γ \((p < 0.01)\) than that found in wild type. Moreover, the value in IL-10⁻/⁻ mice treated with mAb to IFN-γ was significantly elevated \((p < 0.01)\) over that of IL-10⁻/⁻ mice.

In secondary Hc, IFN-γ levels in lungs were sharply reduced in rat IgG-treated IL-10⁻/⁻ mice \((p < 0.01)\), and TNF-α was elevated \((p < 0.01)\) in IL-10⁻/⁻ animals (Fig. 5). IL-4 was markedly increased \((p < 0.01)\) in lungs of IL-10⁻/⁻ mice administered rat IgG or mAb to TNF-α. The levels in mAb to TNF-α-treated mice were greater \((p < 0.01)\) than those administered rat IgG. GM-CSF was similar among wild-type, IL-10⁻/⁻ given rat IgG, and mAb to TNF-α.

**Histopathological appearance of lungs of IL-10⁻/⁻ and wild-type mice**

We sought to determine whether alterations in survival and number of inflammatory cells were associated with differences in the pathological appearance of the lungs. Analysis was restricted to wild-type and IL-10⁻/⁻ mice as well as those whose survival was shortened by neutralization with mAb to cytokine. Lung pathology was examined at day 7 of infection. The lungs of wild-type mice exhibited a moderate to severe admixture of neutrophil and mononuclear cell infiltration in the perivascular spaces and lymphocytes were noted in the alveolar spaces (Fig. 5A). The lesions in the lungs of IL-10⁻/⁻ mice contained a mild to moderate mixture of neutrophils and mononuclear cells. Perivascular cuffing with lymphocytes was present, and there was extension of these cells into the alveolar spaces (Fig. 5B). The histological appearance of the inflammatory response in the lungs of IL-10⁻/⁻ mice given mAb to TNF-α was similar to that of rat IgG-treated IL-10⁻/⁻ mice (Fig. 5C). On the other hand, the lungs of infected IL-10⁻/⁻ mice administered mAb to IFN-γ manifested severe to very severe neutrophil and mononuclear cell infiltrates. There was extension of lymphocytes from the perivascular area to the alveoli with marked perturbation of the alveolar architecture (Fig. 5D).

In secondary infection, the lungs of wild-type mice at day 7 of infection manifested a predominantly mononuclear cell infiltrate with a smaller proportion of neutrophils (Fig. 5E). The inflammatory

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**Table I. FACS profile of lung cells from mice infected for 7 days with Hc**

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Wild type</th>
<th>IL-10⁻/⁻ + rat IgG</th>
<th>IL-10⁻/⁻ + mAb to TNF-α</th>
<th>IL-10⁻/⁻ + mAb to IFN-γ</th>
<th>IL-10⁻/⁻ + mAb to GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>16.60 ± 2.46</td>
<td>24.21 ± 9.01</td>
<td>11.20 ± 2.85</td>
<td>122.64 ± 9.22b</td>
<td>15.56 ± 1.89</td>
</tr>
<tr>
<td>CD4⁺</td>
<td>1.92 ± 0.13</td>
<td>2.81 ± 1.33</td>
<td>1.44 ± 0.16</td>
<td>2.93 ± 0.66a</td>
<td>2.23 ± 0.19</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>1.42 ± 0.12</td>
<td>1.07 ± 0.36</td>
<td>0.11 ± 0.07f</td>
<td>2.03 ± 0.30b</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>Gr-1⁻</td>
<td>6.20 ± 0.34</td>
<td>13.27 ± 6.74</td>
<td>9.59 ± 2.11</td>
<td>93.62 ± 11.24b</td>
<td>7.9 ± 0.51</td>
</tr>
<tr>
<td>Mac-3⁺</td>
<td>3.17 ± 0.48</td>
<td>5.12 ± 2.16</td>
<td>4.57 ± 0.97</td>
<td>22.74 ± 4.52b</td>
<td>2.83 ± 0.45</td>
</tr>
</tbody>
</table>

*Mean number of cells ± SEM from five to six animals per group.

b \(p < 0.01\) compared to wild type.

f \(p < 0.01\) compared to wild type.

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**Table II. Inflammatory response in mice on day 7 of secondary infection with Hc**

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Wild type</th>
<th>IL-10⁻/⁻ + rat IgG</th>
<th>IL-10⁻/⁻ + mAb to TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>8.44 ± 2.86</td>
<td>8.54 ± 1.98</td>
<td>5.47 ± 1.99</td>
</tr>
<tr>
<td>CD4⁺</td>
<td>4.00 ± 1.39</td>
<td>3.58 ± 0.95</td>
<td>2.57 ± 0.10</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>0.38 ± 0.04</td>
<td>0.54 ± 0.11</td>
<td>0.08 ± 0.03d</td>
</tr>
<tr>
<td>Gr-1⁻</td>
<td>2.54 ± 1.10</td>
<td>3.01 ± 0.79</td>
<td>1.79 ± 0.60</td>
</tr>
<tr>
<td>Mac-3⁺</td>
<td>0.86 ± 0.27</td>
<td>1.45 ± 0.46</td>
<td>0.88 ± 0.30</td>
</tr>
</tbody>
</table>

*Mean number of cells ± SEM from five to six animals per group.

d \(p < 0.01\) compared to wild-type and IL-10⁻/⁻ mice given rat IgG.
cells were associated with moderate to severe perivascular cuffing and alveolar extension. Alveolar occlusion was noted. The response in the lungs of IL-10/−/− mice was markedly less than that of wild-type mice. The inflammation consisted principally of mild to moderate inflammation with a predominance of mononuclear cells (Fig. 5F).

Memory response by immunized IL-10/−/− mice
Immunized IL-10/−/− mice manifested accelerated clearance of Hc when challenged 49 days following exposure. To determine whether the differences between wild-type and knockout mice could be attributed to altered proportions of memory cells, mice were immunized with 10⁴ yeast, and at days 7, 21, and 49 following exposure the lungs and spleens were analyzed for the presence of memory cells that produced IFN-γ. As a control, uninfected mice were analyzed similarly. Since both CD4⁺ and CD8⁺ cells contribute to host resistance (15), the CD3⁺ population was analyzed. In wild-type mice, there was an increase in the percentage of IFN-γ⁺ cells that peaked on day 21 and declined thereafter. The pattern observed in IL-10/−/− mice differed since the peak was observed on day 7 followed by a decline (Table III). At day 7 after exposure to yeast, the percentage of CD3⁺, CD44high, CD62Llow, and IFN-γ⁺ cells in the lungs and spleens of Hc-immunized wild-type mice was significantly less than that found (p < 0.01) in the organs of immunized IL-10/−/− mice. By day 21, the percentage of these cells was similar in the spleens and lungs in both groups of mice and remained similar on day 49 (Table III). Moreover, since the numbers of cells did not differ between the two groups, the absolute numbers of memory cells followed the same trend as did the percentages (data not shown).

Protective efficacy of cells from immunized wild-type and IL-10/−/− mice
We next evaluated the protective function of cells from immunized mice using an adoptive transfer system into TCRαβ−/− mice. Splenocytes were harvested from unimmunized wild-type and IL-10/−/− mice or from animals immunized with 10⁴ yeast at day 49 postexposure, and 2 × 10⁸ splenocytes were transferred into TCRαβ−/− mice. These mice were infected with 2 × 10⁶ yeast i.n. 8 h later. As a control, a group of mice received no cells. Mice given immunized splenocytes survived for 70 days whereas those

FIGURE 4. Cytokine profile of IL-10−/− mice given mAb to cytokines. In primary infection (right-hand panel), IL-10−/− mice were infected with 2 × 10⁶ Hc yeast i.n. and given mAb to IFN-γ, TNF-α, or rat IgG, and cytokine was measured by ELISA in the homogenates of lungs. In secondary Hc (left-hand panel), IL-10−/− mice were infected with 10⁶ yeast i.n. and 8 wk later challenged with 2 × 10⁶ Hc yeast i.n. At this time, mice received rat IgG or mAb to TNF-α. Cytokine levels were quantified in the lungs of animals. Data represent mean ± SEM of six animals per group. **, p < 0.01 compared with wild type. #, p < 0.01 compared with IL-10−/− mice given rat IgG.
given naive splenocytes from wild-type or IL-10−/− mice did not manifest enhanced survival (Fig. 6).

Subsequently, we sought to determine whether the ability of splenocytes to transfer protection was dependent on the cell number. TCR−/− mice were injected with 2 × 10^3, 2 × 10^5, or 2 × 10^7 splenocytes from immunized wild-type or IL-10−/− animals, and survival was monitored. Control animals received no cells. Transfer of 2 × 10^3 wild-type immune splenocytes was not different from those that received no cells. Transfer of 2 × 10^5 immune cells from wild-type mice was associated with survival of 40% of the mice, whereas 2 × 10^7 cells conferred survival in 100% of the mice. Transfer of as few as 2 × 10^3 cells from immunized IL-10−/− mice resulted in a significant survival (p < 0.01) of TCR−/− mice infected with Hc (Fig. 7A).

To validate that the protective effect of transferred cells was dependent on the presence of T cells, we eliminated CD4− and CD8− cells from immunized wild-type and IL-10−/− mice and transferred 2 × 10^7 splenocytes into TCR−/− mice 8 h before infection with Hc. Immunized controls received an equal amount of rat IgG. Transfer of splenocytes deficient in CD4− and CD8− T cells abrogated protection (Fig. 7B).

Table III. Memory T cell evolution following immunization with viable Hc

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Organ</th>
<th>Day 0 Percentage (± SEM) CD3+CD44highCD62lowIFN-γ+ Cellsa</th>
<th>Day 7</th>
<th>Day 21</th>
<th>Day 49</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Lung</td>
<td>&lt;0.1</td>
<td>1.02 ± 0.45</td>
<td>3.45 ± 0.22</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>IL-10−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>Spleen</td>
<td>&lt;0.1</td>
<td>8.33 ± 0.78b</td>
<td>2.84 ± 0.33</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>IL-10−/−</td>
<td></td>
<td></td>
<td>0.81 ± 0.24</td>
<td>1.97 ± 0.20</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.64 ± 0.31b</td>
<td>1.66 ± 0.29</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

a Mean ± SEM of n = 5–12 animals.
b p < 0.01 compared to wild type.
In this study, we have demonstrated that a deficiency of endogenous IL-10 in mice results in an accelerated elimination of Hc from visceral and lymphoid tissue in both primary and secondary infection. Enhanced clearance of Hc by IL-10−/− mice was accompanied by increased lung levels of IFN-γ, a cytokine known to be critically important in controlling fungal burden in naive, but not Hc-immune mice (2). The salutary effect of IL-10 deficiency was abolished by neutralization of TNF-α or IFN-γ, but not GM-CSF, in primary infection, whereas in secondary histoplasmosis, only neutralization of TNF-α led to a dramatic loss of protective immunity. The results strongly suggest that endogenous IL-10 modulates host control of Hc infection.

These results are congruent with those found in experimental mycobacterial disease, murine infection with Leishmania donovani, Leishmania major, Coccidioides immitis, Candida albicans, or Listeria monocytogenes. In contrast, the lack of IL-10 promotes susceptibility to experimental infection with Trichuris muris, Plasmodium chabaudi chabaudi, and candidiasis in IL-12-deficient mice (16–27). Although the absence of endogenous IL-10 was associated with a decrement in the parasite burden in mice infected with Toxoplasma gondii or Trypanosoma cruzi, these animals succumb to an aggressive Th1 inflammatory response (28, 29). Collectively, the data indicate that divergent actions of IL-10 on host immunity cannot be explained simply by the fact that a microbe is intracellular or extracellular. Both intra- and extracellular organisms can be found among the group in which the absence of IL-10 is advantageous and detrimental to the protective immune response.

IL-10 markedly inhibits production of numerous cytokines including IFN-γ and TNF-α (10). In primary infection, we detected a pronounced enhancement of IFN-γ production in the lungs of IL-10−/− mice as compared with wild-type mice, but levels of TNF-α were similar between the two groups. The interplay between IL-10 and TNF-α was not apparent in Hc infection, and the elevated IFN-γ probably contributed to the enhanced control of fungal burden. In secondary histoplasmosis, neither IFN-γ nor TNF-α was increased when compared with wild type. In fact, levels of the former were significantly less, yet the mice exhibited enhanced elimination of Hc. The beneficial effect of a deficiency of IL-10 was independent of significant increases in the generation of protective cytokines.
IL-10 exerts a number of immunological effects including inhibition of inflammation as well as suppression of microbicidal action of phagocytes (10–14). In IL-10−/− mice, the inflammatory response to Hc was similar to that of wild-type animals as assessed either by histopathology or by FACS quantification of inflammatory cell infiltrates in the lungs. Thus, it appears that endogenous IL-10 is not a major mediator of inflammation induced by Hc. This observation is similar to that found in IL-10−/− mice infected with L. donovani in which there was not a massive infiltration with Th1 inflammatory cells (19). The failure to mount an aggressive inflammatory response in L. donovani-infected IL-10−/− mice was attributed to the fact that the parasite resides principally within spleens and livers and is not as disseminated as T. gondii or T. cruzi (19). This explanation is unlikely to be true for the model of Hc, since this organism is widely disseminated in mice (30). Therefore, limitation of the Th1 immunopathology may be mediated by another cytokine such as IL-13 (31, 32).

In primary infection, the fungal burden in IL-10−/− mice was similar to that of wild-type mice at the early stage of infection (≤day 7). This span of time corresponds to the phase when innate immunity is responsive; acquired immunity is not triggered until day 14 (30). The beneficial effect in mice devoid of IL-10 was principally observed during the phase when acquired immunity is activated. This contention is further supported by the accelerated clearance of the fungus in secondary infection. Generation of innate immune effectors such as toxic oxygen radicals and nitrogen intermediates may be dampened by IL-10 (10), and the expectation would be that the absence of IL-10 would lead to a more vigorous production of these microbicidal molecules. Consequently, it might be anticipated that yeast would be eliminated more rapidly during the first 7 days of infection. The failure to observe a difference in fungal burden between wild-type and IL-10−/− mice implies that the putative modulatory effect of IL-10 on effector molecules from phagocytes was not crucial to early elimination of Hc. One explanation for this result is that Hc yeast thrive intra-cellularly despite robust generation of toxic oxygen radicals by phagocytes (33). NO also is important in host control of Hc, especially within the first 7 days of infection (2). If the absence of IL-10 augmented the release of this intermediate, it did not translate into increased resistance to infection.

Despite the propitious effects of a deficiency in IL-10, treatment with mAb to TNF-α or IFN-γ produced elevated CFU and abolished protective immunity. Neutralization of IFN-γ exacerbates L. donovani in IL-10−/− mice, but the severity of infection is not comparable to that of wild-type mice nor does it lead to a high rate of death among IL-10-deficient animals (19). In contrast, the protective effect associated with a lack of IL-10 in Hc-infected mice was not sufficiently powerful to prohibit the imetical effects of neutralizing the biological activity of endogenous TNF-α or IFN-γ, and the absence of IL-10 did not counterbalance the lack of TNF-α or IFN-γ. Thus, the deficiency of IL-10 is not an absolute protective mechanism, and the presence of endogenous TNF-α or IFN-γ is essential for host control even when that IL-10 is absent.

The findings with neutralization of TNF-α or IFN-γ in naive IL-10−/− mice parallel those in wild-type animals. Neutralization of endogenous TNF-α or IFN-γ results in the death of wild-type C57BL/6 mice in primary Hc infection (2–9). One disparate result is that IL-10−/− recipients given mAb to GM-CSF survived although their fungal burden was modestly but significantly increased. On the other hand, wild-type recipients of mAb to GM-CSF succumb to infection (9). We validated the biological effect of GM-CSF neutralization by treating wild-type mice with mAb to this cytokine and found that it caused mice to succumb to infection. There is a clear difference between IL-10−/− and wild-type mice on their dependence for endogenous GM-CSF in limiting the progression of infection. In this case, the absence of IL-10 protects against the lack of GM-CSF.

The impact of cytokine neutralization on production of other regulatory cytokines was explored in IL-10−/− mice as a potential explanation for the altered protective immune response in conjunction with administration of mAb to TNF-α, IFN-γ, or GM-CSF. In primary infection, there were no marked alterations in cytokine levels in lungs that could account for the lack of host control. Although IL-4, which is known to exacerbate experimental Hc infection, was elevated in lungs of IL-10−/− mice given mAb to GM-CSF or to IFN-γ, these levels did not differ from those of IL-10−/− mice. Thus, marked deficiencies in noncognate protective cytokines (TNF-α, IFN-γ, or GM-CSF) were not detected in recipients of mAb to cytokines. Furthermore, there was no up-regulation in the disease-exacerbating cytokine, IL-4, in IL-10−/− mice given mAb to cytokines. A likely explanation for the impaired protective response following neutralization of TNF-α, IFN-γ, or GM-CSF is impaired generation of NO. In histoplasmosis, the three cytokines alluded to above diminish NO production that leads to a loss of host control in infection (2, 7, 9). In addition, in IL-10−/− mice administered mAb to TNF-α, there was a sharp decrease in the number of CD8+ cells. Since this subpopulation contributes to host resistance to Hc (15, 34), another reason why TNF-α neutralization is associated with a poor outcome is the decrement in CD8+ cells in the lungs.

In secondary infection, the only perturbation was an elevation of IL-4 in recipients of mAb to TNF-α. It is unlikely that the increased levels of IL-4 impaired clearance of Hc although it is known to directly alter host clearance of this fungus in wild-type mice and to suppress killing of intracellular pathogens (31, 35). Previously, we reported that neutralization of TNF-α in immunized C57BL/6 mice rechallenged with Hc failed to limit replication of the fungus and succumbed to overwhelming histoplasmosis. The lungs of these mice contain high levels of IL-4 and IL-10, but restoration of protective immunity requires neutralization of both cytokines not just one (7).

The accelerated clearance of Hc in immunized IL-10−/− mice prompted an examination of the memory response in these animals. T cells were the focus because they are the pivotal lymphocyte population that regulates immunity to this fungus (15). The percentage of memory cells that were IFN-γ+ following immunization with 103 yeast was higher at day 7 for IL-10−/− mice than for wild-type mice, whereas at day 21, the reverse was true. By day 49, the percentage in the two groups was exceedingly low and did not differ. Memory cells from both groups experienced an expansion and contraction phase (36), but the kinetics of this modulation differed.

Adaptive transfer studies suggested that cells from IL-10−/− mice were more potent in inducing protection on a per cell basis. Transfer of 2 × 105 or 2 × 106 splenocytes from immunized IL-10−/− mice were able to prolong survival in TCR αβ−/− mice. On the other hand, cells from immunized wild-type only transferred protection when 2 × 107 cells were used. We confirmed that the results we observed with adoptive transfer were caused by T cells since elimination of CD4+ and CD8+ cells abrogated adoptive immunity.

Little is known regarding the influence of IL-10 on memory T cell generation. Most of the attention on regulation of memory cell generation and maintenance by cytokines has focused on IL-2, -7, and -15 (37–40). An analysis of memory cell generation in IL-10−/− and wild-type mice has been conducted following vaccination with keyhole limpet hemocyanin. In those mice, elevated numbers of memory CD4+ cells and reduced numbers of CD8+...
cells were detected (41). The accelerated clearance in immunized IL-10−/− mice was not a consequence of increased memory cell number since these values did not differ from those of wild-type mice at the time of rechallenge (day 49 postexposure). The results suggest that the absence of IL-10 does not result in a sustained increase in the memory cell pool. Rather, the accelerated clearance observed in immunized IL-10−/− mice is most likely a result of enhanced biological activity expressed by cells from these mice.

In summary, we have explored the contribution of IL-10 to the course of histoplasmosis in both primary and secondary infection. IL-10−/− mice manifested accelerated elimination of the fungus, but only during the period when cell-mediated immunity is activated. The absence of IL-10 did not alter the innate immune response. The protective effect associated with the lack of IL-10 was breached by neutralization of TNF-α or IFN-γ in naive mice and by TNF-α in immune mice. Thus, the absence of IL-10 is not an absolute protective mechanism. T cells from IL-10−/− mice manifest a more potent capacity to transfer protection than those of wild type. Thus, the absence of IL-10 enhances on a per cell basis the protective activity of T cells.

References
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