Unimpaired Down-Modulation of the Hepatic Granulomatous Response in CD8 T-Cell- and Gamma Interferon-Deficient Mice Chronically Infected with *Schistosoma mansoni*

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The granulomatous response to schistosome eggs is a CD4 T-cell-dependent, Th2-cytokine-dominated immunopathologic response. As infection proceeds to chronicity, both granuloma formation and egg-induced cytokine production become downregulated, and previous experiments have implicated CD8 T cells in this process. One mechanism by which CD8 T cells could suppress immunopathology is through the production of the counterregulatory cytokine gamma interferon (IFN-γ), but no in vivo evidence exists to directly support this hypothesis. In this study, we analyzed hepatic granuloma formation and egg-induced cytokine production in *Schistosoma mansoni*-infected gene knockout mice deficient in either CD8 lymphocytes or IFN-γ. Surprisingly, we found that neither immunologic component plays an essential function in the control of granuloma and cytokine responses during either the acute or chronic stage of infection. Thus, other mechanisms may be more important in the regulation of immunopathology in schistosomiasis.

Infection with the parasitic helminth *Schistosoma mansoni* induces the formation of granulomatous lesions around eggs deposited in hepatic and intestinal tissues (32). Studies of schistosome-infected mice have indicated that this tissue response is dependent on the activity of T cells. Upon egg deposition, the population of egg antigen-responsive CD4 T cells rapidly develops a Th2-like phenotype, synthesizing and secretory high levels of interleukin-4 (IL-4), IL-5, IL-13, and IL-10 and low levels of gamma interferon (IFN-γ) (16, 25, 31, 34). This response leads to the induction of blood and tissue eosinophilia and elevated serum immunoglobulin E, two hallmarks of helminth infection.

In experimentally infected mice, granulomatous lesions reach a maximum size at about 8 weeks postinfection, approximately 2 to 3 weeks after the sexual maturation of adult worm pairs. Several weeks thereafter, the lesions forming around newly laid eggs decrease progressively in volume (5, 13), and by 16 to 30 weeks, the volume is approximately half that of acute, florid granulomas. Reduced cellular immunity measured by dermal delayed-type hypersensitivity reactivity or in vitro proliferative or lymphokine responsiveness to schistosome egg antigen (SEA) has also been reported during the chronic phase. This process of spontaneous diminution of immune reactivity was originally called endogenous desensitization and is now generally considered to be a form of immunologic downmodulation. Because of its potential relevance to the establishment of a balance between tissue protection and disease during chronic schistosomiasis in humans, much attention has been focused on the cellular mechanisms underlying this process of downmodulation in murine models.

Adoptive transfer of splenic and lymph node cells from chronically infected mice into acutely infected mice decreases the size of nascent granulomas (10). Depletion of CD8 T cells abolishes this inhibitory effect (7, 8). Furthermore, experiments with cell populations isolated from granulomas indicate a requirement for CD8 T cells for in vitro suppression of SEA-induced proliferation as well as in vivo downmodulation of lung granuloma size in adoptively transferred recipients (8, 28). The aforementioned observations suggest that CD8 T cells are responsible for downregulating CD4 effector function during chronic infection. The mechanism of CD8 T-cell-suppressive activity, however, remains poorly understood.

CD8 T cells may exert regulatory effects by secreting counterregulatory cytokines (2). In particular, IFN-γ is known to be a potent inhibitor of Th2 proliferation and in some cases has been shown to explain the suppressive activity of CD8 clones on Th2 responses (17). Several lines of circumstantial evidence suggest a potential role for IFN-γ in immune downmodulation of the granuloma response. For example, upon adoptive transfer of lymphoid cells from chronically (20-week) infected mice, IFN-γ secretion by explanted lung granulomas remains relatively stable while levels of IL-2 and IL-4 decrease (8). In addition, endogenous IFN-γ has been observed to have a suppressive effect on granuloma formation in both an in vitro system (21) and in a pulmonary egg injection model (33). Moreover, exogenous IFN-γ has been shown to downregulate pulmonary granuloma size (24) and hepatic fibrosis (11), a sequela of schistosome granuloma formation.

Recent evidence indicates that CD8 T cells from schistosome-infected mice display a type 1 cytokine profile characterized by the selective production of IFN-γ (26). Since the SEA-responsive CD4 T-cell population is predominantly Th2-like and therefore presumably susceptible to the reported antiproliferative effects of IFN-γ (15, 27), CD8 T cells may exert their regulatory effects by secretion of that cytokine. In this study, we tested this hypothesis directly by analyzing the development and downmodulation of the egg granuloma response in mice with mutations resulting in deficiencies in either CD8 T-cell development or IFN-γ production.

**MATERIALS AND METHODS**

**Animals and infection.** Mice with a disrupted IFN-γ gene (GKO mice) (12) were provided by Genentech, Inc., and were bred onto the C57BL/6 background for seven generations before use. β-2 microglobulin knockout (β-2 µKO) ani-
nals (20), originally provided by B. Koller (University of North Carolina), were backcrossed onto the B6 background for 10 generations. TAP knockout (TAP KO) mice (30) were obtained from Luc Van Kaer (St. Jude Childrens Research Hospital) and are on a mixed B6 and 129 background. CD8α gene knockout (CD8 KO) mice (14) backcrossed onto the B6 background for six generations were obtained from T. Mak (Ontario Cancer Institute). Age- and sex-matched C57BL/6 or (B6 × 129)F1, mice were used as the appropriate controls. Mice were bred and maintained at the American Association for Laboratory Animal Care-accredited NIAID Animal Care Facility.

The NMRI strain of *S. mansoni* was provided by the Biomedical Research Institute (Rockville, Md.). Mice (8 to 10 weeks of age) were infected by percutaneous exposure of the tail to 25 to 35 cercariae. Animals were sacrificed at 8 and 16 or 18 weeks postinfection, time points which correspond to the acute and chronic stages of infection, respectively.

**Parasite antigen preparation.** Eggs were isolated from livers of 8-week-infected mice, and a soluble fraction was prepared by previously published methods (4). The resulting antigen preparation (SEA) was aliquoted and frozen at −80°C.

**Determination of granuloma volumes and tissue fibrosis.** Histologic sections of liver tissue were prepared and stained as described by Litt (22). Granulomas around single eggs containing fully embryonated miracidia were measured by using a micrometer eyepiece, and the diameters were used to calculate the mean granuloma volume for each mouse. Fibrosis was measured in samples of liver tissue by chemical quantitation of hydroxyproline as previously described (6).

**In vitro stimulation of spleen cells.** Single-cell suspensions were prepared from individual spleens by passing them through a sterile fine wire mesh. Erythrocytes were removed by hypotonic lysis. Spleen cells (3 × 10^6/ml) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (HyClone), 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, and 500 μg 2-mercaptoethanol in 24-well plates (Costar) and stimulated with SEA (15 μg/ml). Culture supernatants were harvested after 48 h for IL-4 bioassay or after 72 h for IL-5 and IFN-γ determination.

**Quantitation of cytokines.** IL-4 was quantified by means of a bioassay using CT4.S indicator cells. IL-5 and IFN-γ levels were determined by using previously described two-site enzyme-linked immunosorbent assays (34) and recombinant cytokine reference standards.

**Quantitation of CD8 T-cell levels.** The percentages of CD8 T cells were quantitated by staining spleen cell suspensions with fluorescein isothiocyanate-conjugated anti-CD8α (Pharmingen) and performing flow cytometric analysis on 10^6 cells per sample, using an EPICS cytofluorometer. The gate was set to include all viable splenocytes.

**RESULTS AND DISCUSSION**

**Granuloma formation and egg-specific cytokine responses in mice deficient in CD8 T cells.** To directly assess whether CD8 T cells are required for the processes leading to the formation and downmodulation of granulomatous responses to schistosome eggs, three different CD8 T-cell-deficient gene knockout lines were utilized. β2 microglobulin- and TAP-deficient mice express very few stable major histocompatibility complex class I complexes and therefore do not positively select the normal complement of CD8 T cells (23). We also used mice in which the CD8α gene had been genetically disrupted. Fluorescence-activated cell sorting analysis of splenocytes from infected mice confirmed the absence of CD8-positive cells in the CD8 KO mice and indicated that spleens of infected β2-μKO and TAP KO mice contained less than 1.0% of these cells during both acute and chronic infection. In contrast, 8% (range, 5 to 10%) of splenocytes from infected wild-type (WT) mice were CD8 positive. No consistent differences were observed in the recovery of adult worms from the infected knockout and WT control animals (data not shown), indicating that all of the mouse strains are equally susceptible to infection.

As shown in Fig. 1, acute-stage (8-week) granuloma volumes in β2-μKO, TAP KO, and CD8 KO mice were not significantly different from those observed in simultaneously infected C57BL/6 or (B6 × 129)F1, hybrid WT control animals. More importantly, at the chronic stage (16 weeks postinfection), the newly formed granulomas in all three CD8-deficient strains decreased in size to the same extent as the lesions in WT animals. Infected CD8 KO and WT animals were also assayed for hepatic fibrosis. No differences in liver hydroxyproline levels were evident between the two mouse strains at either the acute- or chronic-stage time points examined (data not shown).

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**FIG. 1.** Granuloma formation in β2-μKO and B6 (A), TAP KO and (B6 × 129)F1 (B), and CD8 KO and B6 (C) mice during acute (8-week) and chronic (16-week) stages of *S. mansoni* infection. Data shown represent mean granuloma diameters ± standard error of five infected mice at each time point. Experiments with each mutant strain were repeated at least once.
The egg-specific cytokine responses of the same infected knockout and WT animals were evaluated following in vitro stimulation of spleen cells with SEA (Table 1). Representative data for CD8 KO mice are shown in Table 1. Acute-stage splenocytes from knockout or WT mice both produced high levels of IL-4 and IL-5 and relatively low levels of IFN-γ. These Th2 and Th1 cytokine responses decreased by 30 to 70% at the chronic stage in both mouse strains. No significant differences in cytokine production were noted between the knockout and WT animals at either time point. Similarly, no significant difference in the percentage of eosinophils in granulomas developing in knockout and WT mice was observed (data not shown).

Taken together, the foregoing experiments indicate that major histocompatibility complex class II-dependent CD4 T cells in the absence of CD8 lymphocytes can orchestrate normal granuloma formation. More importantly, the data indicate that modulation of granulomatous tissue and egg-specific cytokine responses is unaffected by the absence of CD8 cells. It is worth noting that mice of the same CD8 KO strain used in our experiments exhibited marked defects in the regulation of CD4-dependent tissue inflammation in a model of experimental allergic encephalomyelitis (19), thus providing a precedent for the use of these animals in assessing CD8 immunoregulatory function.

Granuloma formation and downmodulation in GKO mice. To examine the role of IFN-γ in the development and downmodulation of granulomas, mice deficient in this cytokine were infected with *S. mansoni* and analyzed as described above. Worm burdens and fecundity were not observed to differ between GKO and WT animals (data not shown). Moreover, at the acute stage of infection, the GKO mice developed granulomas which did not differ significantly from those of WT animals. Furthermore, downmodulation of granuloma size during chronic infection was evident and occurred to the same extent as in the infected controls (Fig. 2). In addition, measurements of hepatic hydroxyproline levels failed to reveal differences between infected GKO and WT mice at either of the two time points examined (data not shown).

Surprisingly, despite the absence of endogenous IFN-γ, Th2 cytokine (IL-4 and IL-5) production in response to SEA stimulation by splenocytes at the acute stage of infection was not significantly altered in GKO compared to control mice (Table 2). Furthermore, the production of these Th2 cytokines was diminished to the same or to an even greater extent (e.g., IL-5) in GKO animals during chronic infection (Table 2). Consistent with these observations, the percentage of eosinophils in granulomas at both time points was equivalent in GKO and WT mice (data not shown).

Table 1. Cytokine responses of CD8 KO and WT mice to in vitro stimulation with SEA during acute and chronic infection

<table>
<thead>
<tr>
<th>Cytokine measured</th>
<th>Wk postinfection</th>
<th>Cytokine level (mean ± SE of 5 mice/time point)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B6 mice</td>
<td>CD8 KO mice</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>8</td>
<td>0.37 ± 0.06</td>
<td>0.77 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.27 ± 0.06 (28)</td>
<td>0.29 ± 0.07 (62)</td>
</tr>
<tr>
<td>IL-4</td>
<td>8</td>
<td>1.48 ± 0.61</td>
<td>1.72 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.62 ± 0.07 (58)</td>
<td>0.79 ± 0.07 (54)</td>
</tr>
<tr>
<td>IL-5</td>
<td>8</td>
<td>2.99 ± 0.40</td>
<td>3.55 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>1.96 ± 0.39 (35)</td>
<td>1.76 ± 0.29 (50.4)</td>
</tr>
</tbody>
</table>

* Similar results were obtained in a subsequent experiment and in experiments with TAP and β-2 μKO mice. IFN-γ levels are expressed in nanograms/milliliter; IL-4 and IL-5 levels are expressed in units/milliliter.

** Percentage decrease in cytokine secretion compared to the 8-week value.

*** Student t test comparison of WT versus knockout mice.

IFN-γ is detected in the early stages of the host response to eggs and has been proposed to play a role in the regulation of granuloma formation. Thus, in both anti-IFN-γ-treated mice and GKO mice, primary granulomas induced in the lung by egg injection are larger than those in control animals (35). In contrast, neutralization of IFN-γ in infected mice during the acute stage of hepatic granuloma response failed to result in a change in lesion size (29), a conclusion supported by a preliminary study of granuloma formation in GKO animals (1). The observations reported here both confirm these earlier findings on acute-stage granuloma formation and extend the analysis to chronic egg pathology. Our results indicate that while IFN-γ clearly plays a role in the regulation of lesion size in the pulmonary granuloma model, the cytokine is not required for either the formation or downmodulation of hepatic granulomas arising from natural infection. Nevertheless, recent experiments using mice vaccinated with IL-12 plus egg antigens reveal a correlation between increased IFN-γ production and decreased granuloma formation and tissue fibrosis (33). Thus, while IFN-γ at the low levels produced during natural infection does not appear to be a major homeostatic regulator of the granuloma response, exogenously induced IFN-γ may exert a modulating influence on egg-induced pathology.

The experiments reported herein were undertaken to test the hypothesis that CD8 T cells are the primary effectors of immune downmodulation of the T-cell-dependent hepatic granuloma response to schistosome eggs and that the regulatory function of these cells is mediated by IFN-γ secretion (3). Until the advent of gene knockout technology, complete depletion of CD8 T cells or IFN-γ by antibody depletion with xenogeneic antibodies over a course of more than 15 weeks was virtually impossible. The results that we have obtained unequivocally show that normal development and downmodulation of hepatic granulomatous responses proceed in the complete or nearly complete absence of either of these two key putative immunoregulatory elements. A potential caveat, which could explain the discrepancies with previous findings (7, 8), is that compensatory mechanisms which substitute for the functions normally provided by CD8 cells or IFN-γ may arise in the knockout mice. While such compensation has been observed in the β-2 μKO mice (23), we know of no previous reports documenting these mechanisms in the CD8 KO and GKO animals used in this study. Thus, the

![FIG. 2. Granuloma formation in GKO and B6 mice during acute (8-week) and chronic (18-week) stages of *S. mansoni* infection. Data shown represent mean granuloma diameters ± standard error of five infected mice at each time point and are representative of three independent experiments performed.](http://iai.asm.org)
present set of observations, at the very least, argue that CD8 T lymphocytes or the IFN-γ-mediated suppression in regulating the granulomatous response to schistosomes eggs during natural infection.

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REFERENCES


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