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IL-10 and the Dangers of Immune Polarization: Excessive Type 1 and Type 2 Cytokine Responses Induce Distinct Forms of Lethal Immunopathology in Murine Schistosomiasis

Karl F. Hoffmann,* Allen W. Cheever, † and Thomas A. Wynn2*

To dissect the controversial roles of type 1 and type 2 cytokines to the pathogenesis of schistosomiasis, we generated IL-10/IL-4- and IL-10/IL-12-deficient mice that develop highly polarized type 1 and type 2 cytokine responses, respectively. Interestingly, the Th1-polarized IL-10/IL-4-deficient mice rapidly lost weight at the onset of egg-laying and displayed 100% mortality by wk 9 postinfection. This acute mortality was linked to overexpression of the proinflammatory mediators IFN-γ, TNF-α, and inducible NO and the formation of nonfibrotic granulomas. Elevated serum aspartate transaminase levels confirmed that mortality was in part attributable to acute hepatotoxicity. In contrast, the Th2-polarized IL-10/IL-12-deficient mice developed a progressive wasting disease that correlated with increased hepatic fibrosis, formation of large eosinophil-rich granulomas, a 10-fold increase in IL-4 and IL-13, and significant mortality during the chronic stages of infection. Surprisingly, IL-10-deficient mice displayed pathological features that were characteristic of both extremes, while wild-type mice developed relatively successful long term chronic infections. These data demonstrate that IL-10 significantly suppresses type 1 and type 2 cytokine development in IL-4- and IL-12-deficient mice, respectively, thereby impeding the development of severe egg-induced pathology in the single cytokine-deficient animals. Together, these findings reveal the central regulatory role of IL-10 in the pathogenesis of schistosomiasis, and illustrate that excessive type 1 and type 2 cytokine responses trigger distinct, but equally detrimental, forms of pathology following infection. The Journal of Immunology, 2000, 164: 6406–6416.

Surviving a chronic parasitic disease requires the generation of a controlled immune response that recognizes the invading pathogen and limits a potentially destructive host response. Several studies, using parasites as model systems, have shown that deviating from the host’s natural immune response during infection can lead to severe consequences, including exacerbated tissue pathology and even death (1–3). In schistosomiasis, murine studies have shown that chronic disease is characterized by the establishment of a Th2-associated immune response against eggs trapped in organs such as the liver and intestines. Hallmarks of this Th2-associated immune response include up-regulation of the collagen-inducing cytokines IL-4 (4, 5) and IL-13 (6), down-regulation of the collagen-suppressing cytokine IFN-γ (7), sequestration of parasite eggs by eosinophil-rich granulomas, and development of tissue fibrosis (8). The chronic granulomatous response and resultant fibrosis can eventually lead to portal hypertension, establishment of portal-systemic shunts, intestinal bleeding, and ultimately death. Thus, morbidity and mortality in chronic murine schistosomiasis were hypothesized to develop as a direct consequence of the egg-induced Th2-type response (9).

However, recent studies have provided compelling evidence that challenges the link between Th2-associated immune responses and disease development during schistosomiasis (10, 11). For example, Brunet et al. (10) reported that schistosome-infected IL-4-deficient mice were incapable of developing effective Th2 responses and consequently displayed enhanced morbidity and mortality. Pathology in these mice was attributed to increased production of proinflammatory mediators. Fallon and Dunne (11) also documented enhanced mortality in infected mice that had been previously tolerized to egg Ags. Similar to the IL-4-deficient mice, the egg-tolerized animals displayed decreased Th2 and increased Th1-associated responses following infection. These observations are thus comparable to those found in infected SCID mice, which also fail to develop Th2 responses. Here, as observed in the egg-tolerized or IL-4-deficient mice, morbidity and mortality correlated with the expression of IFN-γ and TNF-α (12). Therefore, these reports suggest that Th2 immune responses in schistosomiasis are, in fact, host protective. Interestingly, this hypothesis is supported by recent studies of patients exhibiting the hepatosplenic form of schistosomiasis. Here, high levels of IFN-γ, TNF-α, soluble TNF-α receptors, and ICAM-1 and low levels of IL-5 were detected in infected individuals exhibiting severe hepatosplenomegaly (13). Together, mouse and human studies question whether severe morbidity and mortality in schistosomiasis are attributable to the development of polarized Th1- or Th2-type immune responses (14).

Recently, we demonstrated that IL-10 is critical for establishing polarized egg-specific Th cell responses in vivo. IL-10-deficient mice challenged i.v. with schistosome eggs (15) or infected with Schistosoma mansoni (16) developed a completely nonpolarized, codominant Th1/Th2-type immune response. Interestingly, the mixed response resulted in a marked increase in the size of egg-induced hepatic granulomas, particularly at the acute stage of infection (16). Nevertheless, it was unclear whether the exacerbated inflammatory response in IL-10-deficient mice was attributable to changes in Th1- or Th2-type cytokine expression. Recently, we crossed IL-10-deficient mice with IL-4- and IL-12-deficient animals to generate double cytokine-deficient mice that develop...
highly polarized Th1- and Th2-type responses, respectively (17). Thus, the double cytokine-deficient animals provided unique tools to directly compare the contributions of polarized Th1- and Th2-type cytokine responses to the pathogenesis of schistosomiasis in mice that are otherwise genetically matched. Moreover, they provided an excellent system to formally define the role of IL-10, which clearly exhibits significant immunoregulatory activity in both murine and human schistosomiasis (18–23).

In this study five distinct single or double cytokine-deficient mouse lines were infected with S. mansoni, and the effects on disease development were compared with those in wild-type (WT) animals. The results from this study clearly show that the development of extreme Th1, Th2, or mixed Th1/Th2 cytokine-producing phenotypes is highly detrimental during schistosome infection. Nevertheless, the pathological consequences associated with each response are distinct and develop at different times following infection. These findings reveal the central regulatory role of IL-10 to the pathogenesis of schistosomiasis and demonstrate that the maintenance of IL-10 expression during acute and chronic schistosome infection is critical for host survival. Moreover, they clarify the controversial roles of Th1/Th2-type cytokine responses to the pathogenesis of schistosomiasis (9, 24) and illustrate that an imbalance in either Th1- or Th2-type cytokine expression can contribute to morbidity during infection.

Materials and Methods

Mice, parasites, and Ag preparations

The double cytokine-deficient mice (C57BL/6) used in this study were previously described (17). All mice were obtained from Taconic Farms (Germantown, PA) and were between 6 and 8 wk of age at the start of each study. All mice were housed in a National Institutes of Health Association for the Accreditation of Laboratory Animal Care-approved animal facility and were fed an enriched diet (NIH-07) fortified with vitamins and minerals as specified by the National Institutes of Health veterinary staff. Cercariae of a Puerto Rican strain of S. mansoni (Naval Medical Research Institute) were obtained from infected Biomphalaria glabrata snails (Biomedical Research Institute, Rockville, MD). Soluble egg Ag preparation (SEA) was derived from homogenized eggs as previously described (25).

Infections and weight measurements

Mice were percutaneously exposed to S. mansoni (Puerto Rican strain) by immersion of their tails in water containing 20–30 parasites for 40 min. Weight measurements were taken every week starting 5 wk postinfection and continued until wk 18 postinfection. The change in weight was expressed as the mean weight at each time point subtracted from the initial weight at 5 wk postinfection and expressed as weight gain or weight loss (grams).

Lymphocyte culture, nitrite measurement, and cytokine assays

For in vitro cytokine measurements, mesenteric lymph nodes (MLN) were removed aseptically 8 wk after infection, and single-cell suspensions were prepared. Mesenteric nodes were assayed individually, and cells were plated in 24-well tissue culture plates at a final concentration of 3 × 10^6 cells/ml in RPMI supplemented with 2 mM glutamine, 25 mM HEPES, 10% FCS, 50 μM 2-ME, penicillin, and streptomycin. Spleens were pooled and cells were plated at a final concentration of 4 × 10^6 cells/ml. Cultures were incubated at 37°C in an atmosphere of 5% CO₂.

Histopathology and fibrosis measurement

The collagen content of the liver, determined as hydroxyproline, was measured as described previously (26). Approximately half the liver was fixed in Bouin-Hollande solution, and histologic sections were processed and stained with Giemsa (Hist-Path of America, Clinton, MD). The diameters and eosinophil contents of granulomas (30/mouse) surrounding single, mature, and viable eggs were measured using an ocular micrometer, and the volume of each granuloma was calculated assuming a spherical shape. Eggs in the liver and intestines were counted separately after digestion in 4% KOH at 37°C (26).

RT-PCR detection of cytokine mRNAs

Two 25-mg portions of each liver were combined and homogenized in 1 ml of RNA STAT-60 using a tissue lytomer (Omni, Waterbury, CT), and total RNA was isolated as recommended by the manufacturer. The RNA was resuspended in diethylpyrocarbonate-treated water and quantitated spectrophotometrically. A RT-PCR procedure was performed as previously described (27) to determine relative quantities of mRNA for IFN-γ, TNF-α, and hypoxanthine phosphoribosyltransferase (HPRT). The primers and probes for all genes were previously published (27, 28). The amplified DNA was analyzed by electrophoresis, Southern blotting, and hybridization with cytokine-specific probes. The chemiluminescent signals were quantified using a 600 ZS scanner (Microtek, Torrance, CA). The amount of PCR product was determined by comparing the ratio of cytokine specific signal density to that of HPRT-specific signal density for individual samples (five mice per group). Arbitrary densitometric units for individual samples were subsequently multiplied by a factor of 100 and compared with those for control mice (uninfected mouse liver). Amplification of HPRT served as an internal control for the amount of RNA and cDNA from each sample.

Measurement of serum TNF-α, IFN-γ, and aspartate transaminase

Serum TNF-α, IFN-γ, and aspartate transaminase levels were measured 8 wk postinfection. Serum TNF-α levels were measured by a capture ELISA kit supplied by R&D Systems, and IFN-γ levels were quantitated by capture ELISA as previously described. Cytokine levels were calculated using standard curves constructed with recombinant murine cytokines. Measurement of the liver-associated enzyme aspartate transaminase (AST) used a colorimetric assay modified from a commercial kit (Sigma, St. Louis, MO) (29).
Statistics

Hepatic fibrosis (adjusted for egg number) decreases with increasing intensity of infection (worm pairs) and was therefore, compared by analysis of covariance, using the log of total liver eggs as the covariate and the log of hydroxyproline per egg. Variables that did not change with infection intensity were compared by one-way ANOVA or Student’s t test. SEA-specific Ab isotype values and in vitro secreted cytokine profiles were graphed according to the StatView program (SAS Institute, Cary, NC). Bars from bottom to top show the 10th, 25th, 50th, 75th, and 90th percentiles, respectively, of the tested samples. Single outliers are indicated as circles. Values for secreted cytokine proteins, semiquantitative RT-PCR, serum Ab data, and serum cytokine levels were compared using Student’s two-tailed t test. p, 0.05 was regarded as significant. A minimum of two separate experiments were performed for all data.

Results

The egg-induced cytokine response in schistosomiasis is regulated by IL-4, IL-10, and IL-12

Given the controversial roles of type 1 and type 2 cytokines in the pathogenesis of schistosomiasis (24, 30, 31), we examined the regulatory role of IL-10 in mice that manifested extreme Th1- or Th2-producing phenotypes (17). For these studies, IL-10/IL-4-deficient and IL-10/IL-12-deficient mice were infected with 20–30 S. mansoni cercariae, and disease progression was examined in detail during both the acute and chronic stages of infection and compared with that in WT mice as well as animals exhibiting single cytokine deficiencies. Evidence that the double cytokine-deficient mice generated a polarized type 1 (IL-10/IL-4 deficient) or type 2 (IL-10/IL-12 deficient) immune response following infection was determined by examining the cytokine-producing profiles of SEA-stimulated lymphocytes ex vivo (Fig. 1). SEA-specific serum Ab isotype profiles 8 wk postinfection also indicate the extent of immune polarization (Fig. 2). As expected (32), lymphocytes from infected WT mice produced significant amounts of the Th2-type cytokine IL-5 (Fig. 1B) but produced relatively little IFN-γ (Fig. 1A). By contrast, IL-10-deficient mice developed the most nonpolarized immunological phenotype of all the mice examined in the study. MLN (Fig. 1) and spleen cell cultures (data not shown) prepared from the double IL-10/IL-4-deficient mice produced abundant IFN-γ and little or no IL-5 upon restimulation with SEA. Additionally, infected IL-10-deficient mice generated high serum titers of both type 1 (IgG2b; Fig. 2A) and type 2 (IgG1 and IgE; Fig. 2, B and C)-associated Ab isotypes.

Mice with a single deficiency in IL-4 developed a markedly reduced Th2-type response (Fig. 1B), consistent with previous observations (6, 33, 34). The continued production of IL-10 in the absence of IL-4 (15) clearly explains the inability of IL-4-deficient mice to manifest an extreme Th1 phenotype following S. mansoni infection. Indeed, in this study only MLN (Fig. 1) and spleen cell cultures (data not shown) prepared from the double IL-10/IL-4-deficient mice produced abundant IFN-γ and little or no IL-5 upon restimulation with SEA. Additionally, high titers of SEA-specific

![FIGURE 1](http://www.jimmunol.org/)

![FIGURE 2](http://www.jimmunol.org/)
IgG2b Abs were detected in the sera of IL-10/IL-4 deficient mice (Fig. 2A). There were also little SEA-specific IgG1 and no circulating IgE Abs detectable (Fig. 2, B and C), further demonstrating that these mice were developing a strongly polarized type 1 immune response. In fact, compared with the other mice in this study, the highest ratios of SEA-specific IFN-γ/IL-5 and IgG2b/IgG1 Ab were observed in the mice simultaneously deficient in IL-4 and IL-10.

Conversely, mice deficient in IL-12 alone showed relatively little change in their cytokine or Ab responses compared with WT animals (Figs. 1 and 2). This is probably explained by the lack of a significant IL-12 response in infected WT animals (35). In contrast, the double IL-10/IL-12-deficient mice generated a robust SEA-specific IL-5 response 5–10 times greater than the response in WT or IL-12-deficient mice. Moreover, unlike the single IL-10-deficient animals, these mice did not exhibit a marked IFN-γ response, demonstrating that a highly polarized and heightened type 2 response was generated only in the combined absence of IL-10 and IL-12. These mice also displayed the lowest serum titers of the type 1-associated IgG2b Ab (Fig. 2A) and the highest total IgE Ab response (Fig. 2C).

A critical protective role for IL-10 is revealed during chronic S. mansoni-infection

Because the IL-10-deficient (mixed response), IL-10/IL-4-deficient (Th1-dominant) and IL-10/IL-12-deficient (Th2-dominant) mice generated such unique cytokine-producing profiles following infection, the mice provided an excellent opportunity to examine the contribution of IL-10 as well as immune deviation to disease progression during schistosomiasis. Again, as described above, mice were infected with 20–30 S. mansoni cercariae and examined weekly for a total of 18 wk for signs of illness. One set of animals was sacrificed on wk 8 postinfection to determine whether there were significant differences in parasite burdens or fecundity. The remaining animals were monitored for changes in weight and for survival throughout the 18 wk.

As shown in Table I, there were no differences in the establishment of infection in any group of mice. All groups harbored a similar number of worm pairs, and total worm and tissue egg burdens were not significantly different. Egg viability was not assessed. Nevertheless, there were marked differences in how the animals dealt with their infections. There were no signs of cachexia in any mice up to 5 wk postinfection. Interestingly, however, after the onset of egg laying (~5 wk postinfection), several groups of cytokine-deficient mice began losing weight (Fig. 3A). The single IL-10-deficient mice did not show significant weight change up to wk 12 postinfection, at which time they began a gradual decline. Shortly following the drop in weight, a high rate of mortality was observed (Fig. 3B), with <50% of the mice surviving through wk 18. Surprisingly, those mice that survived past wk 16 showed a significant weight gain during the last weeks of the study. Mortality increased, however, when the IL-10 deficiency was coupled with a deficiency in either IL-12 or IL-4. Most dramatic was the rapid weight loss seen in the infected IL-10/IL-4-deficient mice (Fig. 3A). These mice lost an average of >2 g of body weight between 6 and 8 wk postinfection and were all dead by 9 wk (Fig. 3B). Mice deficient for both IL-10 and IL-12 (Th2-polarized) also displayed a higher rate of mortality than the single IL-10-deficient animals, although it was much more gradual than in the IL-10/IL-4-deficient (Th1-polarized) mice. Indeed, the IL-10/12-deficient mice gained weight up to wk 9 postinfection, at which point they then began a very gradual decline. The weight loss again correlated closely with some mortality. Although the IL-4-deficient mice also showed excessive weight loss between wk 7 and 13 postinfection (Fig. 3A), there was no significant decrease in survival compared with infected WT controls (Fig. 3B). Moreover, similar to the single IL-10-deficient mice, the IL-4-deficient animals gradually gained weight as they entered the chronic stage of infection. In marked contrast to the other mice in the study, the IL-12-deficient and WT mice gained weight throughout the study. This correlated well with the near complete absence of mortality among these animals.

Table I. Parasitology

<table>
<thead>
<tr>
<th>Group</th>
<th>Worm Pairs</th>
<th>Total Worms</th>
<th>Eggs/Worm Pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10/12 KO (n = 9)</td>
<td>4.2 ± 0.6</td>
<td>10.6 ± 1.4</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>IL-12 KO (n = 9)</td>
<td>3.0 ± 0.5</td>
<td>8.3 ± 1.3</td>
<td>8.6 ± 0.7</td>
</tr>
<tr>
<td>IL-10 KO (n = 9)</td>
<td>4.9 ± 0.8</td>
<td>11.7 ± 1.4</td>
<td>9.8 ± 1.5</td>
</tr>
<tr>
<td>WT (n = 10)</td>
<td>4.7 ± 0.8</td>
<td>11.4 ± 1.7</td>
<td>9.4 ± 1.2</td>
</tr>
<tr>
<td>IL-4 KO (n = 10)</td>
<td>3.2 ± 0.4</td>
<td>8.4 ± 0.9</td>
<td>9.0 ± 1.1</td>
</tr>
<tr>
<td>IL-10/12 KO (n = 6)</td>
<td>3.0 ± 0.5</td>
<td>8.5 ± 1.2</td>
<td>9.9 ± 2.2</td>
</tr>
</tbody>
</table>

* All data are means for each group (± SE). n, number of mice in each group.
The morbidity and mortality of infected IL-10- and IL-10/IL-4-deficient mice correlate with elevated serum levels of IFN-γ and TNF-α

In an attempt to explain the increased mortality of mice deficient in IL-10, we examined in detail the production of IFN-γ and TNF-α, two candidate markers of cytokine-induced shock (36). Lymphocytes obtained from both IL-10- and IL-10/IL-4-deficient mice produced the most IFN-γ in vitro when restimulated with SEA (Fig. 1A). Therefore, we also examined the cytokine response in vivo by examining the serum levels of IFN-γ and TNF-α (by ELISA) and by analyzing the cytokine mRNA response within the granulomatous tissues (by RT-PCR) at 7.5–8 wk postinfection. Consistent with their lymphocyte responses, mice harboring deficiencies in IL-10 or IL-10/IL-4 had the highest levels of IFN-γ in the serum (Fig. 4B) and IFN-γ mRNA expression in the liver (Fig. 4A). The pattern of TNF-α mRNA expression was similar in these mice, although WT and IL-4-deficient mice also exhibited a marked response (Fig. 4C). In contrast to their tissue mRNA responses, the pattern of TNF-α expression in the serum was more variable among the groups, although it was interesting to note that all the mice that displayed some weight loss during infection (Fig. 3A) also exhibited detectable serum levels of TNF-α (Fig. 4D).

Thus, a combined increased local and systemic IFN-γ and TNF-α response correlated most closely with the high degree of mortality observed in the IL-10- and IL-10/IL-4-deficient mice. Nevertheless, these findings failed to explain the significant mortality observed in the IL-10/IL-12-deficient mice, as these animals clearly exhibited a markedly reduced IFN-γ response, both locally and systemically (Figs. 1A and 4, A and B).

The systemic type 1 response and morbidity of IL-10 and IL-10/IL-4-deficient mice are associated with significant hepatotoxicity and NO expression following infection

Overproduction of NO can induce tissue damage (37) and may contribute to morbidity during schistosome infection (38). Because IFN-γ and TNF-α are both potent activators of NO (39, 40), and both cytokines were markedly up-regulated in IL-10- and IL-10/IL-4-deficient mice, we examined NO production following infection. Splenocytes were obtained from the various cytokine-deficient mice 8 wk postinfection and were placed in culture in the presence (Fig. 5B) or absence (Fig. 5A) of SEA. Interestingly, NO levels (as assessed by nitrite production) closely mirrored the IFN-γ response of SEA-stimulated MLN cells (Fig. 1A) as well as the tissue (Fig. 4A) and serum (Fig. 4B) IFN-γ expression profiles. A slight increase in nitrite levels was detected even in unstimulated cultures from the IL-10-, IL-10/IL-4-, and IL-4-deficient mice (Fig. 5A). A similar, but more marked, expression followed SEA stimulation, although the IL-10- and IL-10/IL-4-deficient mice displayed the strongest response. Little or no induction of NO was detected in the cultures obtained from WT, IL-12-deficient, or IL-10/IL-12-deficient mice.

Systemic levels of AST, a liver enzyme associated with hepatocyte injury, was evaluated to determine whether morbidity and mortality were possibly attributable to egg-induced tissue damage and liver malfunction (29). Circulating AST levels were examined in all mice 7.5–8 wk postinfection. As shown in Fig. 6, AST levels were significantly elevated in all infected animals. However, the most dramatic increase was observed in IL-10- and IL-10/IL-4-deficient mice. These mice also displayed a robust type 1 response (high IFN-γ, TNF-α, and iNO), demonstrating a strong correlation...
among type 1 cytokines, AST expression, and morbidity following infection. Importantly, the double IL-10/IL-12-deficient animals confirmed the association with a robust type 1 response for this activity, because these animals (Th2-polarized) failed to exhibit a similarly elevated AST response. Nevertheless, the latter animals also exhibited significant mortality, suggesting that other mechanisms contribute to morbidity during infection.

Distinct patterns of egg-induced inflammation, hepatic fibrosis, and tissue eosinophilia were observed in the type 1 and type 2 polarized mice

Differences in the egg-induced granulomatous response could also contribute to the morbidity and mortality observed in the IL-10 and double cytokine-deficient mice. Consistent with many previous observations, WT mice generated hepatic granulomas (Fig. 7A) rich in eosinophils (Fig. 7B) and displayed increased levels of hydroxyproline (Fig. 7C), a quantitative measure of tissue fibrosis, 8 wk postinfection. Given the lack of a significant change in the cytokine response of infected IL-12-deficient mice (Fig. 1), it was not too surprising that hepatic pathology was not significantly affected by the single IL-12 deficiency. In marked contrast to these animals, however, IL-10-deficient mice developed large granulomas (Fig. 7A). Nevertheless, while their granulomas were larger, there was no significant increase in hepatic fibrosis (Fig. 7C), compared with that in WT mice. There was also no change in the cellular composition of the granulomas, which was consistent with previous observations (16). Granuloma size was increased in IL-4-deficient mice, although the magnitude of the increase was less marked than that in IL-10-deficient animals (Fig. 7A). Moreover, unlike the IL-10-deficient mice, tissue fibrosis decreased (Fig. 7C), and significantly fewer eosinophils were observed in the granulomas of IL-4-deficient vs WT mice (Fig. 7B).

FIGURE 5. NO expression by Ag-stimulated splenocytes prepared from infected multicytokine-deficient mice. Spleens were harvested on day 56, and single-cell suspensions were cultured with medium alone (A) or SEA (B). The supernatants were collected 72 h after stimulation and used in the Greiss reaction to assay for nitrite. The bars represent the average nitrite levels for each group of mice (n = 10) ± SD.

FIGURE 6. Assessment of AST levels in infected mice as a quantitative measure of hepatotoxicity. Sera were taken from mice 8 wk postinfection and analyzed for AST, a marker of hepatocellular damage. The horizontal gray line indicates the AST levels in uninfected C57BL/6 mice. All six groups exhibited significantly elevated levels following infection, yet the IL-10- and IL-10/IL-4-deficient mice were the only animals that showed an increased response compared with that in the infected WT group.

FIGURE 7. Granuloma size, tissue eosinophilia, and hepatic fibrosis were assessed 8 wk postinfection. Mice were infected with 20–30 cercariae and sacrificed 8 wk postinfection to measure liver granuloma volumes (A), the percentage of granuloma-associated eosinophils (B), and hepatic fibrosis (C), expressed as micromoles of liver hydroxyproline per 10,000 tissue eggs. Data were obtained from groups of mice containing 5–21 mice/group (*, p < 0.05, by Student’s t test for granuloma volumes and eosinophil measurements, by analysis of covariance for hepatic fibrosis). Due to significant mortality in multiple groups of mice after 8 wk of infection, quantitative histological comparisons could not be evaluated at later times.
Much more striking, however, were the pathological changes observed in the double cytokine-deficient animals. Here, although the double IL-10/IL-4-deficient mice developed a significant egg-induced inflammatory response, lesion size was much smaller than in mice exhibiting the individual (IL-4 or IL-10) cytokine deficiencies (Fig. 7A). Again, it is important to note that these mice, unlike their single cytokine-deficient counterparts, developed the most extreme Th1-polarized response following infection (Fig. 1).

The near complete ablation of the type 2 response in the IL-10/IL-4-deficient mice also resulted in the most dramatic change in the phenotype of the developing granulomas. In fact, their lesions were almost completely devoid of eosinophils (Fig. 7B), and the fibrotic response was significantly reduced compared with that in WT mice (Fig. 7C). In contrast to these observations, the double IL-10/IL-12-deficient animals developed large (Fig. 7A) eosinophil-rich granulomas (Fig. 7B) similar in size to those in IL-10-deficient mice. More importantly, however, in contrast to the WT or single cytokine-deficient animals, the double IL-10/IL-12-deficient mice displayed a significant increase in hepatic fibrosis (Fig. 7C). Indeed, these mice were the only animals in the study that displayed a marked and highly significant increase in this important parameter of disease compared with WT mice.

**Production of the profibrogenic cytokines, IL-4 and IL-13, is increased in infected IL-10- and IL-10/IL-12-deficient mice**

Previous studies demonstrated that IL-4 directly stimulates collagen production in fibroblasts (4). More recently, IL-13 was shown to exhibit a similar functional activity in vitro, and in vivo studies demonstrated that IL-13 plays the dominant role in the development of hepatic fibrosis during infection with *S. mansoni* (6). The pattern of IL-4 and IL-13 production was therefore investigated in the various cytokine-deficient mice 8 wk postinfection. Consistent with previous observations, WT mice showed significant SEA-specific IL-13 (Fig. 8A) and IL-4 (Fig. 8B) responses following infection (6), and there was no dramatic change in this response in IL-12-deficient mice. In marked contrast, however, almost a 10-fold increase in both IL-4 and IL-13 was observed in the MLN cell cultures prepared from IL-10- and IL-10/IL-12-deficient mice.

Thus, there was a strong correlation between maximal hepatic fibrosis (Fig. 7C) and IL-4 and IL-13 levels, particularly in the double IL-10/IL-12-deficient mice, in which the levels of anti-fibrotic IFN-γ (7) were clearly diminished (Fig. 1A). Perhaps not surprisingly, IL-13 production was significantly decreased in IL-4-deficient mice and was almost undetectable in the majority of IL-10/IL-4-deficient mice, which, again, correlated well with the modest fibrotic response observed in these animals. IL-4 and IL-13 mRNA expression in the granulomatous livers showed a similar pattern (data not shown).

Liver sections were also stained with picrosirius red, which stains collagen, to demonstrate the marked difference in hepatic fibrosis in the infected IL-10/IL-12- and IL-10/IL-4-deficient mice. Again, as shown in Fig. 9, A and D, both groups exhibited a significant egg-induced inflammatory response 8 wk postinfection. Nevertheless, collagen, which forms an impressive ring around lesions in IL-10/IL-12-deficient mice (Fig. 9B), was almost absent from the granulomas in the IL-10/IL-4-deficient group (Fig. 9E). The polarized light image shown in Fig. 9C illustrates the dense pattern of collagen deposition that formed around the granulomas in IL-10/IL-12-deficient mice.

**Discussion**

The results from this study clearly show that type 1 and type 2 cytokines can both contribute to morbidity during murine schisto-

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** IL-4 and IL-13-producing profiles in infected cytokine-deficient mice. Mice were infected with 20–30 cercaria, MLN were harvested 56 days postchallenge, and single-cell suspensions were placed in culture with SEA. Supernatants were collected 72 h after stimulation and used in capture ELISAs as described in Materials and Methods. IL-13 (A) and IL-4 (B) levels were assayed, and data were plotted using box plots. Data were obtained from groups of mice containing 6–11 mice/group. ND, none detected.

some infection. IL-10 emerged as the most critical immunoregulatory factor, because only the IL-10-deficient and double cytokine-deficient mice developed the most extreme infection-related tissue pathology. In one extreme, the IL-10/IL-4-deficient Th1-polarized mice rapidly succumbed to infection and developed much less fibrotic hepatic granulomas compared with WT mice. In contrast, the IL-10/IL-12-deficient Th2-polarized mice developed a slow and prolonged wasting disease that resulted in significant mortality during the chronic stages of infection. These latter mice also formed much larger granulomas compared with the IL-10/IL-4-deficient animals and exhibited a significant increase in hepatic fibrosis. Together, these data demonstrate that highly polarized Th1- and Th2-type cytokine responses trigger distinct, but equally detrimental, forms of egg-induced pathology in schistosomiasis, particularly when combined with a deficiency in IL-10.

Although pathological changes were noted in all knockout animals, the most dramatic outcome of infection was observed in the double IL-10/IL-4-deficient mice. These mice uniformly lost weight beginning at the onset of egg-laying, and all had succumbed to infection within 9 wk postinfection. Given the rapid onset of cachexia in these mice, overproduction of type 1 cytokines was suspected as a possible explanation (1, 2, 10). Indeed, cells from these mice, unlike the other animals in the study, produced abundant IFN-γ and little of the Th2 cytokines IL-4, IL-5, and IL-13. There was also detectable systemic IFN-γ and TNF-α, and splenocytes restimulated with SEA produced abundant iNO, suggesting a heightened state of macrophage activation in IL-10/IL-4-deficient mice. The marked elevation in AST levels suggested that significant hepatocyte damage probably contributed to the
acute mortality. Moreover, although the mice developed egg-induced granulomas, the phenotype of the lesions was completely different from that in the other mice in the study. In fact, their granulomas were almost devoid of eosinophils and were much less fibrotic. Thus, this atypical granulomatous response could also contribute to morbidity by failing to efficiently contain or neutralize hepatotoxins produced by the developing miracidia within the egg (41).

Brunet et al. reported similar infection-related mortality in mice deficient for IL-4 (10). In their studies, IL-4-deficient mice began to lose weight at the onset of egg deposition, and almost 100% mortality was reported by 8–10 wk postinfection. Similar to our observations in infected IL-10/IL-4-deficient mice, they observed elevated expression of type 1 cytokines. They suggested that IL-4 and possibly other type 2 cytokines were required to inhibit the deleterious effects of the proinflammatory mediators TNF-α and iNO. Surprisingly, in our studies we observed little mortality in the infected IL-4-deficient group, despite excessive weight loss during the acute stage of infection. In fact, similar survival was noted for IL-4-deficient and WT mice through wk 18 postinfection. The IL-4-deficient mice used in both studies were on the same genetic background (C57BL/6), and the parasites used (Puerto Rican strain) were from the same source, so we have no specific explanation for the discrepancy. However, differences in the numbers of parasites used to infect our mice, diet (42), and housing conditions could affect disease progression during infection. Regardless, the data collected from the infected IL-10/IL-4-deficient mice support the deleterious role for highly polarized type 1 cytokine responses during infection with *S. mansoni*.

Interestingly, the maintenance of a significant IL-10 response in the absence of IL-4 (6) is a likely explanation for the minimal morbidity and mortality observed in our infected IL-4-deficient mice. Metwali et al. reported little default toward a more dominant Th1-type response in infected IL-4-deficient animals, which is consistent with our observations (34). Although a modest increase in IFN-γ was observed in IL-4-deficient mice, a much more dramatic increase in IFN-γ was found in mice deficient for both IL-4 and IL-10. In fact, the major immunological difference measured between the IL-4-deficient and the IL-10/IL-4-deficient mice is in IFN-γ levels (Ag specific (Fig. 1A), hepatic message (Fig. 4A), and systemic (Fig. 4B)). Elevated IFN-γ levels probably contribute to the high mortality observed in the IL-10/4− vs IL-4-deficient animals. These data formally demonstrate that IL-10 reduces the Th1 default and provides a significant degree of protection to IL-4-deficient mice. Results from a previous study suggested that this IL-10 is probably derived from a source other than Th2 lymphocytes, because little IL-10 is produced by Ag-stimulated cells from IL-4-deficient mice (6). In this previous study IL-10 mRNA expression was increased in the granulomatous tissues, whereas expression of the prototypical Th2 cytokines IL-5 and IL-13 was diminished (6), further suggesting that the majority of IL-10 is derived from cells other than Th2 lymphocytes.
Surprisingly, the single IL-10-deficient mice showed higher mortality than animals exhibiting a deficiency in IL-4 (Fig. 3B). This was surprising because, unlike the IL-4-deficient mice, expression of Th2-type cytokines was clearly unimpaired in the IL-10-deficient group. In fact, production of IL-13 and IL-4 was elevated compared with that in WT mice (Fig. 8). It was previously hypothesized that Th2-type cytokines might protect mice from the lethal effects of proinflammatory mediators (10, 24). Interestingly, however, IL-10-deficient mice also displayed a marked type 1 response, which was of a similar magnitude as the double IL-10/IL-4-deficient animals. Elevated serum levels of IFN-γ, TNF-α, and the liver enzyme AST suggested that these mice were also suffering from the hepatotoxic effects of sustained type 1 cytokine production despite the presence of a significant type 2 response. Thus, while some protection was apparently conferred by the type 2 response (IL-10-deficient animals lived longer than the Th1-polarized IL-10/IL-4-deficient mice), there was no evidence that proinflammatory mediator production was impaired. This suggests that the maintenance of IL-10, rather than a classical Th2-type response, is the more critical factor contributing to host survival during infection.

In previous studies we showed that development of egg-induced hepatic fibrosis in schistosomiasis is significantly ameliorated by sensitizing mice to egg Ags in the presence of IL-12 before infection with *S. mansoni* (25). These animals developed smaller and less fibrotic granulomas than the control infected mice and showed markedly increased Th1 and decreased Th2-type cytokine expression. Importantly, however, there was no mortality in these mice, even through the chronic stages of infection, despite the development of a sustained and polarized type 1 cytokine response. Nevertheless, unlike the IL-10- and IL-10/IL-4-deficient mice that were incapable of producing IL-10, the egg/IL-12-sensitized Th1-polarized mice developed a significantly elevated IL-10 response in the granulomatous tissues, which was sustained at least through wk 12 postinfection (25). In fact, the production of IL-10 in IL-12-treated mice is commonly observed in vivo (43, 44). Thus, it appears that IL-12-vaccinated animals benefit from an increased IFN-γ (antifibrotic) and reduced IL-4/IL-13 (profibrogenic) response, but, perhaps even more importantly, an unimpaired IL-10 response spares them from the potentially harmful and toxic effects of the polarized type 1 response. Similar IL-12-based vaccination studies are being performed in IL-10-deficient mice to confirm this hypothesis. Regardless, these findings suggest that a highly polarized type 1 response, while beneficial in terms of preventing tissue fibrosis, is potentially harmful, particularly when established in an IL-10-deficient setting.

Given the pathogenic role of the type 1 response in infected IL-10- and IL-10/IL-4-deficient mice, an improved course of infection was probable in the double IL-10/IL-4-deficient animals, because these mice were relatively deficient in type 1 cytokines due to the absence of an endogenous IL-12 response. Surprisingly, however, these mice exhibited a high rate of mortality following infection, which exceeded even the rate observed in the single IL-10-deficient group. In fact, <50% of the IL-10/IL-12-deficient mice survived through wk 13 postinfection. Initially, the animals appeared to improve when compared with the IL-10/IL-4-deficient mice, because they clearly gained weight through wk 9 postinfection and demonstrated no mortality. Nevertheless, they slowly began to lose weight as they entered the more chronic stages of the infection, which was quite dramatic compared with that in the WT and IL-12-deficient groups (Fig. 3A). The modest iNO response of SEA-stimulated splenocytes confirmed the absence of a significant type 1 response (Fig. 5), and the relatively low serum AST level indicated that there was little hepatotoxicity following infection (Fig. 6). These observations suggested that IL-10/IL-12-deficient animals were probably dying from a mechanism distinct from that operating in the IL-10/IL-4-deficient mice.

Unlike the IL-10/IL-4-deficient mice, which developed smaller lesions, the IL-10/IL-12-deficient mice formed granulomas that were as large as those in the IL-10-deficient group (Fig. 7A). This suggests that the increase in granulomatous inflammation observed in IL-10-deficient mice is probably controlled by changes in type 2 rather than type 1 cytokine expression. Perhaps more importantly, however, the IL-10/IL-12-deficient mice, in contrast to the IL-10- or IL-10/IL-4-deficient animals, also showed a marked and highly significant increase in hepatic fibrosis (Fig. 7C). Although the IL-10-deficient mice also displayed a similarly elevated type 2 response (Fig. 8), we hypothesize that the simultaneous expression of IFN-γ (1A) prevents the development of an overexuberant fibrotic response in these animals. Thus, the severe hepatic fibrosis observed in the IL-10/IL-12-deficient mice is probably attributable to both the increased expression of the profibrogenic cytokines IL-4 and IL-13 (6, 45, 46) and the decreased production of the collagen-suppressing cytokine, IFN-γ (7). Thus, morbidity in the double IL-10/IL-12-deficient mice may be attributable to the marked exacerbation in both egg-induced inflammation and hepatic fibrosis. Such a hypothesis is consistent with the delayed morbidity and mortality in the IL-10/IL-12-deficient compared with the IL-10/IL-4-deficient mice. Moreover, collagen deposition slowly accumulates throughout infection and would probably contribute to morbidity only during the more chronic stages of the infection.

TNF-α could also be contributing to the morbidity of the infected IL-10/IL-12-deficient mice. Serum levels of TNF-α were markedly increased in all mice exhibiting a deficiency in IL-10 (Fig. 4D). Indeed, this attribute appeared to be somewhat unrelated to the dominance of type 1/type 2 cytokines, although IL-4-deficient mice also displayed a response. It was particularly interesting that all the animals that exhibited some degree of weight loss during infection also developed detectable serum levels of TNF-α. Only WT and IL-12-deficient mice continued to gain weight throughout the 18 wk of the study (Fig. 3A), which correlated perfectly with the absence of a detectable serum TNF-α response in these mice (Fig. 4D). Given the well-known antagonistic nature of type 2-associated cytokines to the production of TNF-α, it was somewhat unexpected to see such a response in the IL-10/IL-12-deficient group. Nevertheless, previous studies have suggested that IL-10 may be the most critical type 2 cytokine responsible for suppressing TNF-α production (47). A role for TNF-α in the development of acute morbidity in infected type 1-polarized mice was recently described in schistosomiasis (10). Interestingly, TNF-α was also characterized as a critical cofactor for a Th2-dependent response (48). Therefore, as proposed previously in an experimental tuberculosis model, TNF-α may exhibit unique functional activities in type 1 vs type 2 cytokine-dominant responses (49).

We are currently investigating the possible dichotomous role of TNF-α in schistosomiasis by neutralizing the cytokine in our Th1-polarizing (IL-10/IL-4) and Th2-polarizing (IL-10/IL-12) mice.

The question was recently raised whether schistosome pathology in humans results from type 1 vs type 2 dominant responses. Fallon (24) and others have suggested from both murine and human studies that Th2 cytokine responses do not produce serious pathology and that their primary function in schistosomiasis is protective, because they serve as anti-inflammatory mediators. However, the results from the current study clearly show that highly polarized type 2 as well as type 1 responses exhibit proinflammatory activity. Therefore, in the setting of schistosomiasis it seems inappropriate to strictly label type 1 responses as proinflammatory...
and type 2 responses as anti-inflammatory. In fact, although egg-
induced inflammation can be either type 1- or type 2-mediated,
unique pathological features are associated with each response.
Although we would agree that one function of the type 2 response is to regulate potentially pathogenic type 1 responses (24, 30), we believe that the results from this study show that type 2 cytokines are not just regulatory factors (50), but also induce important pathological changes and contribute to morbidity in murine schistosomiasis.

Recently, we described the distinct and nonredundant contribu-
tions of the type 2 cytokines, IL-4 and IL-13, to the development of egg-induced hepatic pathology (6). Although IL-4 played the dominant role in the generation of the egg-specific type 2 response, both IL-4 and IL-13 were required for the development of eosinophil-rich granulomas. More importantly, however, IL-13 was shown to be the dominant mediator of hepatic fibrosis, and this activity was believed to be a direct effect of the cytokine because it stimulated collagen deposition by fibroblasts. Previous studies documented the collagen-suppressing activity of the type 1 cytokines IFN-γ and IL-12 (7, 25). Together, these findings suggest that type 2 rather than type 1 cytokines are the key mediators of hepatic fibrosis and that type 2 responses as anti-inflammatory are important for dampening pathogenic type 1 immune responses.

We thank Drs. Alan Sher, Matthias Hesse, Monica Chiairamonte, David Sacks, and Rhian Hayward for critically reviewing this manuscript. We also thank Dr. Fred Lewis, Claudia Gryzwaicz, and Chris Rowe at the Biomedical Research Institute for providing the parasites used in this study. Sandy Cooper, Marti Cain, and Sherry Copeland at the Animal Care Branch also provided invaluable assistance with weighing/bleeding the mice described in this study.

Acknowledgments

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