IL-10 Is Critical for Host Resistance and Survival During Gastrointestinal Helminth Infection

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Resistance to many intestinal nematodes is dependent on the induction of polarized type 2 cytokine responses, whereas type 1 responses can exacerbate these infections. The contributions of IL-4 and IL-13 to the development of resistance have been well described for a variety of intestinal parasites; however, the role of IL-10 has not been previously investigated. In this study we infected IL-10-, IL-10/IL-4-, IL-10/IL-12-, IL-4-, and IL-12-deficient mice with *Trichuris muris* to determine whether IL-10 contributes to the development of immunity. Interestingly, *T. muris*-infected IL-10-, IL-4-, and IL-10/IL-4-deficient mice failed to expel the parasite, and animals deficient in IL-10 displayed marked morbidity and mortality. In contrast, double IL-10/IL-12-deficient mice were completely resistant and mounted a highly polarized type 2 cytokine response, demonstrating that the increased susceptibility of IL-10-deficient mice was dependent on IL-12. Further study suggested that the susceptibility of IL-10- and IL-10/IL-4-deficient mice was probably attributable to a marked increase in type 1 cytokine production in those animals. The mortality observed in *T. muris*-infected IL-10- and IL-10/IL-4-deficient mice correlated with increased inflammation, loss of Paneth cells, and absence of mucus in the cecum. Interestingly, survival was enhanced in *T. muris*-infected IL-10/IL-4-deficient mice if a broad spectrum antibiotic was administered, suggesting that an outgrowth of opportunistic bacteria was contributing to the high degree of morbidity and mortality. Overall, these studies reveal a critical role for IL-10 in the polarization of Th2 responses, development of resistance during *T. muris* infection, and maintenance of barrier function in the colon. ***The Journal of Immunology,* 2002, 168: 2383–2392.**

Gastrointestinal nematode infections are among the most common infections worldwide, yet relatively little is known regarding the precise effector mechanisms responsible for generating immunity (1–3). It is clear that T cells and the cytokines they produce have a profound effect on the outcome of infection. Factors that polarize the immune system to a type 1 immune response, as characterized by the induction of IFN-γ and IL-12, inhibit protective immunity (3–5), whereas a polarized type 2 response (IL-4, IL-5, IL-9, IL-13) can limit the severity of infection or ultimately lead to the elimination of the nematode parasite (6–10).

The whipworm *Trichuris muris* is a nematode that causes chronic infections in some mouse strains and is expelled in others (11). This spectrum of infection has also been observed in humans with the closely related parasite *Trichuris trichiura* (12, 13). It has been shown that both IL-4 and IL-13 play a protective role during *T. muris* infection, and IFN-γ induction leads to a permissive host (7, 9, 14–16). Recently, we have shown that IL-13 can mediate expulsion in the absence of both B7 interactions and IL-4 function, but only in the absence of IFN-γ (17). This work supported the idea that both IL-4 and IL-13 are required to mediate protection in the presence of IFN-γ (15). However, the contribution of IL-10, a cytokine often associated with polarized Th2 responses, had not been examined.

In previous work with the helminth parasite *Schistosoma mansoni* we found that in the absence of IL-10 a mixed cytokine response develops following infection. The response was characterized by the simultaneous production of high levels of IFN-γ as well as IL-4, IL-5, and IL-13, and this was in contrast to wild-type (WT)[3] mice that develop a relatively polarized type 2 cytokine response (18–20). These observations suggested that IL-10 plays an important role in polarizing helminth-induced immune responses. Given these observations, in the present study we examined whether IL-10 is a critical determinant in resistance or susceptibility to a gastrointestinal nematode infection. Here, mice deficient in IL-10, IL-10 and IL-4, or IL-10 and IL-12 were used to examine the contribution of IL-10 in situations where either mixed or highly polarized cytokine responses develop. Strikingly, these data demonstrate that IL-10 is not only important for the development of resistance, but this cytokine is also critical for the survival of *T. muris*-infected mice.

Materials and Methods

Mice

The double cytokine-deficient mice (all C57BL/6 background) used in this study were previously described (18). All mice were between 6 and 8 wk of age at the start of each study. All animals were bred at Taconic Farms

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[3] Abbreviations used in this paper: WT, wild type; ES, excretory-secretory; HPRT, hypoxanthine-guanine phosphoribosyl transferase; KO, knockout.

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(Lexington, KY) in barrier facilities and were shipped to the U.S. Department of Agriculture (Beltsville, MD) small animal facility before infection. Animals were maintained in sterile caging, which included filter tops. Importantly, no signs of intestinal inflammation were observed in any animals before infection.

Parasites

T. muris, originally provided by Dr. R. Grencis (University of Manchester, Manchester, U.K.) was maintained in AKR mice. Infections were established by gavage with 350–600 embryonated eggs, and adult worms were gently removed from the cecum and proximal colon using flexible insect forceps. Infective eggs develop into adults 32 days after inoculation in susceptible mouse strains such as AKR. After extensive washing, viable worms were placed in 24-well culture plates containing RPMI 1640 supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml gentamicin at 37°C in an atmosphere of 5% CO_2 in air. Eggs released from female worms over 2 days were collected, washed, placed in a small petri dish in sterile PBS, and maintained at room temperature for 35–40 days to embryonate. The number of eggs that developed into well-formed larvae was adjusted to 5000/ml, and larvae were stored at 4°C. The embryonation procedure is highly variable, and larval morphology within the egg is not a reliable predictor of infectivity. Egg batch infectivity was tested in SCID mice, because they are unable to expel *T. muris* and thus provide a biologically meaningful measure of infectivity. SCID mice were inoculated with a test dose of 400 eggs, and the number of larvae that develop after 14 days was determined. Larvae develop in the cecum and proximal colon of infected mice and can be isolated and counted between 7 and 28 days postinoculation. Briefly, infected tissue was washed free of fecal material and placed in 10 ml of 10 mM EDTA/HBSS without Ca^{2+}/Mg^{2+} for 4 h at 37°C. The suspension was then vortexed for 45 s, and the residual epithelial cell and mucus material was examined for larvae using a dissecting microscope. Egg infectivity was calculated as a percentage of the average number of viable larvae recovered from infected SCID mice over the estimated number of infective eggs inoculated. The inoculation dose changed when mice from different experiments received a different batch of eggs, but the dose was consistently established to achieve a yield of ~100–200 larvae.

Parasite Ag preparations

Adult worms were cultured in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin, and supernatant fluids containing excretory-secretory (ES) Ag were collected every 24 h for 7 days. Parasite eggs and adult worms were removed by centrifugation, supernatant fluids were concentrated and dialyzed against PBS with a concentrator (Amicon, Danvers, MA), and the protein concentration was determined by OD_{280} readings.

Cell cultures and cytokine assays

Single-cell suspensions were prepared from the mesenteric lymph nodes and spleens by routine methods (21). RBC were lysed by osmotic treatment with ACK lysis buffer (Biofluids, Rockville, MD). Cells were placed in RPMI 1640 medium supplemented with 10% FCS that had been heat inactivated for 30 min at 57°C, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES. Cell populations were plated at 3–4 $\times 10^6$ cells/well in 24-well plates and cultured in medium alone, Con A (Sigma-Aldrich, St. Louis, MO) at a final concentration of 5 μg/ml, or parasite ES Ag at a final concentration of 10 μg/ml. Additional cell cultures were incubated in the presence of anti-CD4 mAb (50 μg/ml; clone GK1.5) to block APC/T cell interactions. All cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air. Cell-free supernatant fluids were harvested at 72 h, and cytokine concentrations were determined by cytokine-specific ELISAs. IFN-γ, IL-5, and IL-10 levels were measured by specific two-site ELISA as previously described (22). The capture Ab (clone RMMG-1) for IFN-γ was obtained from BioSource (Rockville, MD), and the secondary Ab was a rabbit anti-mouse IFN-γ produced in-house (National Institutes of Health, Bethesda, MD). The detector Ab, peroxidase donkey anti-rabbit Ig, was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Ab pairs for IL-5 and IL-10 were obtained from BD PharMingen (San Diego, CA), and streptavidin peroxidase was purchased from Kirkegaard & Perry (Gaithersburg, MD). IL-4 levels were determined be proliferation of CT4S cells. IL-13 and serum TNF-α levels were measured using capture ELISA kits supplied by R&D Systems (Minneapolis, MN) following the manufacturer's instructions. Cytokine levels were calculated with standard curves using recombinant murine cytokines.

RT-PCR detection of cytokine mRNAs

Portions of the cecum were snap-frozen and then homogenized in RNAzol B (Tel-Test, Friendswood, TX) using a tissue Polytron (Brinkmann Instruments, Westbury, NY), and total RNA was isolated as recommended by the manufacturer. An RT-PCR procedure was performed as previously described (23) to determine relative quantities of mRNA for hypoxanthine-guanine phosphoribosyl transferase (HPRT), IFN-γ, TNF-α, IL-4, IL-10, and IL-13. The primers and probes for these genes have been previously described (23, 24). The PCR conditions and cycle numbers were strictly defined for each cytokine primer pair, such that a linear relationship between input RNA and final PCR product was obtained. Positive and negative controls were included in each assay to confirm that only cDNA were detected and that none of the reagents was contaminated with cDNA or extraneous PCR products. 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 International, 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). Amplification of HPRT served as an internal control for the amounts of RNA and cDNA from each sample.

Histology

Tissue samples from the cecum were placed in Bouin-Hollande fixative and processed routinely. Sections were stained with H&E, Wright-Giemsa, Warthin-Starry silver stain, or periodic acid-Schiff for histopathologic evaluation. Sections were read without knowledge of the identity of the mice and were scored 1–4+ for degree of inflammation, number of Paneth cells, and submucosal edema. The thickness of the epithelium was measured, and an estimate was made of the percentages of eosinophils, lymphocytes, polymorphonuclear neutrophils, and macrophages present in the inflammatory infiltrate.

Statistics

Statistical significance was determined using one-way ANOVA (nonparametric) and Tukey's multiple comparison test. Significance was assumed for values of $p < 0.05$. Results shown are representative of at least two independent experiments.

Results

Mice deficient in IL-10, IL-4, or IL-10 and IL-4 fail to eliminate T. muris *larvae*

To determine whether mice with varying degrees of polarized Th cell responses exhibit different profiles of resistance to *T. muris* infection, we infected WT mice and mice deficient in a variety of Th1- and Th2-associated genes. Specifically, mice deficient in IL-10, IL-10 and IL-4, IL-10 and IL-12, IL-12, or IL-4 were inoculated with *T. muris*. We previously demonstrated that IL-10/IL-4

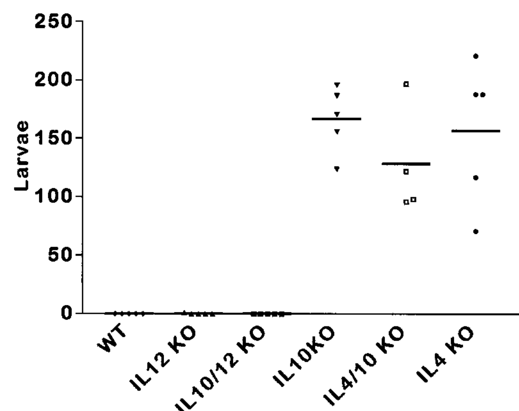

FIGURE 1. Determination of larval burden in *T. muris*-infected WT and cytokine-deficient mice. WT and cytokine-deficient mice (five per group) were inoculated orally with 600 embryonated eggs. Mice were sacrificed 20 days later, and the numbers of parasites per mouse were determined. Results shown are representative of four independent experiments.

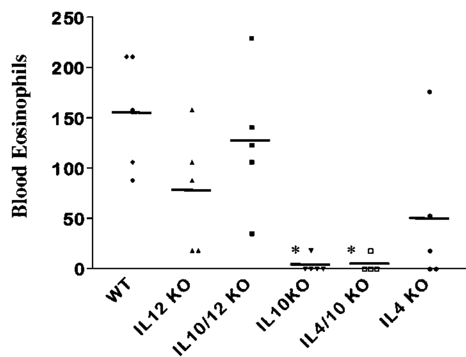

FIGURE 2. Blood eosinophil response in *T. muris*-infected WT and cytokine-deficient mice. WT and cytokine-deficient mice (five per group) were inoculated orally with 600 embryonated eggs. Mice were sacrificed 20 days later, and eosinophils were counted in fresh blood by using the Unopette test (BD Biosciences, Franklin Lakes, NJ). Results shown are representative of four independent experiments. The asterisk indicates that the group is significantly different from both WT and IL-10/12-deficient mice.

knockout (KO) mice develop highly polarized Th1-type responses to *S. mansoni* eggs and/or irradiated parasites, while IL-4- and IL-10-deficient mice generate a diminished type 2 and mixed type 1/type 2 cytokine responses, respectively (18–20). As such, these animals, all on the same genetic background, provided an ideal system to examine the role of IL-10 in immune regulation following infection with *T. muris*. As shown in Fig. 1, on day 20 postinfection IL-10, IL-4, and IL-10/IL-4 double-deficient mice retained *T. muris* larvae within the cecum and proximal colon. In marked contrast, WT, IL-12-deficient, and IL-10/IL-12-deficient mice, which were hypothesized to be skewed toward a type 2 cytokine response, had no larvae on day 20 postinfection. In addition, animals that expelled the *T. muris* larvae developed much higher levels of circulating blood eosinophils, providing indirect evidence that they were indeed generating a Th2-type response. More specifically the circulating blood eosinophils were statistically higher in the infected WT and IL-10/IL-12 KO mice than in the infected IL-10 KO and IL-4/IL-10 KO mice ($p < 0.05$; Fig. 2).

Increased susceptibility of IL-10- and IL-4-deficient mice correlates with the development of an elevated parasite-specific IFN-γ response

Mice that expelled *T. muris* larvae failed to exhibit an Ag-specific IFN-γ response (Fig. 3*A*), whereas the draining lymph node and spleen cells (data not shown) from susceptible (IL-10-, IL-4-, and IL-10/IL-4-deficient) mice generated a potent IFN-γ response upon restimulation with parasite Ag. In contrast, lymph nodes (Fig. 3*B*) and spleens (data not shown) from resistant (WT, IL-12 KO, IL-10/12 KO) mice produced IL-4 in response to parasite Ag, while in susceptible mice no IL-4 was detected. We also determined the levels of two other type 2-associated cytokines, IL-5 and IL-13, and only the highly polarized IL-10/IL-12 KO mice produced high levels of these cytokines upon restimulation (Fig. 3, *C* and *D*). Moreover, cultures stimulated in the presence of anti-CD4 mAbs suggested that type 2 cytokine production is highly dependent on $CD4^+$ T cells, while a portion of the IFN-γ response is clearly $CD4^+$ T cell independent. Given the dichotomous roles of IL-5 and IFN-γ in eosinophil activation and recruitment (25, 26), these observations provide a logical explanation for the high peripheral eosinophil responses of WT and IL-10/12 KO mice and the low frequency in IL-10- and IL-10/IL-4-deficient animals (Fig. 2). These findings also illustrate how IL-10 and IL-12 interact to dampen Th2 responses following *T. muris* infection (19, 20).

Local production of IFN-γ and TNF-α mRNA increases in infected IL-10- and IL-10/IL-4-deficient mice

RNA was extracted from the cecum of *T. muris*-infected WT, IL-10, IL-4, IL-12, IL-10/IL-4, and IL-10/IL-12 KO mice. Little or no

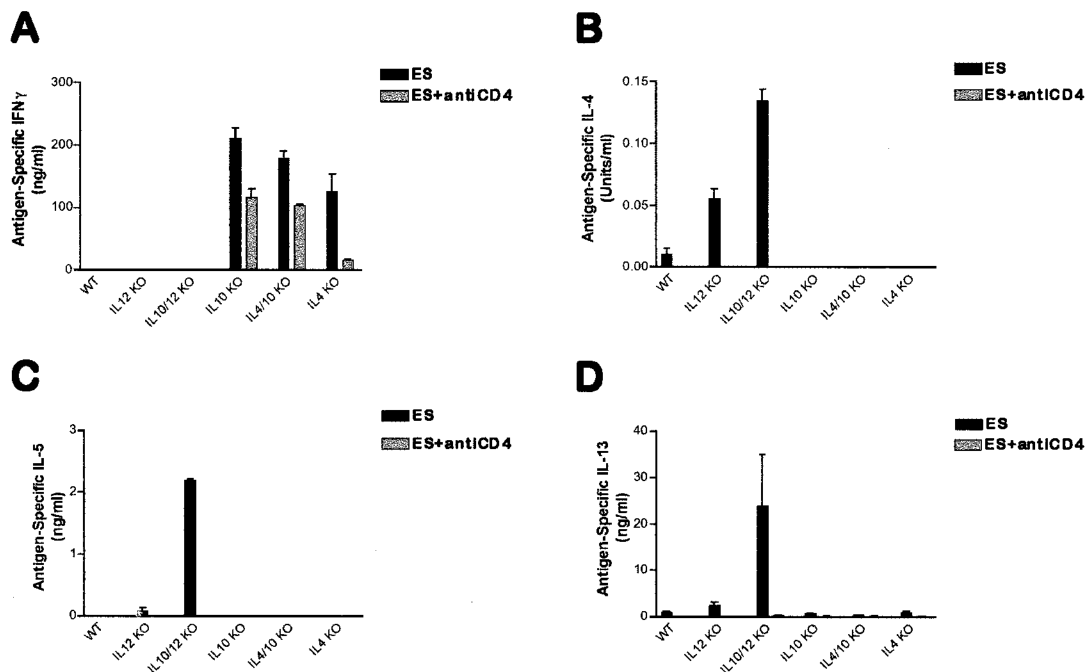

FIGURE 3. Ag-specific cytokine profiles in *T. muris*-infected WT and cytokine-deficient mice. WT and cytokine-deficient mice (five per group) were inoculated orally with 600 embryonated eggs. Mesenteric lymph nodes were harvested 20 days later, and single-cell suspensions were cultured with ES Ag. Cultured cells were also incubated in the presence of anti-CD4 mAb (50 μg/ml; clone GK1.5) to block APC/T cell interactions. Supernatant fluids were harvested at 72 h and used in capture ELISAs as described in *Materials and Methods*. *A*, IFN-γ; *B*, IL-4; *C*, IL-5; *D*, IL-13. Results are shown as the mean ± SD.

cytokine mRNA was detected in the tissues of any group before infection (data not shown). However, susceptible mice (IL-10-, IL-4-, and IL-10/IL-4-deficient) had elevated levels of transcripts for IFN-γ in the gut as shown in Fig. 4*A* following infection, which was in complete agreement with our in vitro lymph node and spleen cell results (Fig. 3*A*). A second cytokine used as a marker for a biased type 1 response, TNF-α, was most evident in the highly polarized IL-10/IL-4 KO mice (Fig. 4*B*). Of the groups capable of producing IL-4, no major differences in IL-4 mRNA were observed in the large intestine (Fig. 4*C*). Interestingly, this was in contrast to lymph node and spleen cell cultures restimulated with parasite Ag, where resistant mice clearly produced more IL-4 than the susceptible IL-10-deficient mice (Fig. 3*B*). Consistent with the IL-4 mRNA findings, a significant IL-13 mRNA response (Fig. 4*D*) was detected in the cecum of all groups of infected mice regardless of genotype; however, decreased levels were consistently observed in the susceptible IL-10- and IL-10/IL-4-deficient mice. These findings suggest that a gut-specific IFN-γ response is a much better indicator of a susceptible phenotype than the presence or absence of a local IL-4/IL-13 response.

IL-10 deficiency leads to weight loss and death following T. muris *infection*

A dramatic weight loss was observed in infected IL-10 and IL-10/IL-4 KO mice between days 18 and 21 (Fig. 5*A*), and this resulted in 100% mortality by day 25 postinfection (Fig. 5*B*). The IL-10/IL-4-deficient mice also displayed a slight, but consistent, shortened time to death compared with the single IL-10-deficient mice. Strikingly, however, no mortality was observed in the IL-4-deficient group, and there was also no weight loss despite the fact that these animals displayed worm burdens nearly identical with or higher than those of the IL-10- and IL-10/IL-4-deficient mice. The IL-10/IL-12 KO mice also displayed no weight loss or death and ultimately completely cleared their infections, suggesting that the mortality observed in the IL-10-deficient mice is likely linked to overproduction of the type 1-associated cytokines IL-12, TNF-α, and IFN-γ.

Morbidity and mortality of T. muris*-infected IL-10- and IL-10/IL-4-deficient mice correlates with elevated serum TNF-α levels*

TNF-α has been implicated in cytokine-induced shock and weight loss. Therefore, we examined circulating TNF-α in the serum of naive and *T. muris*-infected cytokine-deficient mice (Fig. 6). There was no statistical difference in serum TNF-α levels between any of the groups before infection. However, infected IL-10 and IL-10/IL-4 KO mice produced significantly higher amounts of TNF-α than WT, IL-12 KO, and IL-10/12 KO mice ($p < 0.001$). Thus, mice retaining *T. muris* larvae on day 20 postinfection (Fig. 1) and displaying high mortality (Fig. 5) had higher systemic levels of TNF-α. These data suggest that the absence of severe morbidity in infected IL-4-deficient mice may have been due to the development of more moderate IFN-γ (Figs. 3*A* and 4*A*) and TNF-α (Fig.

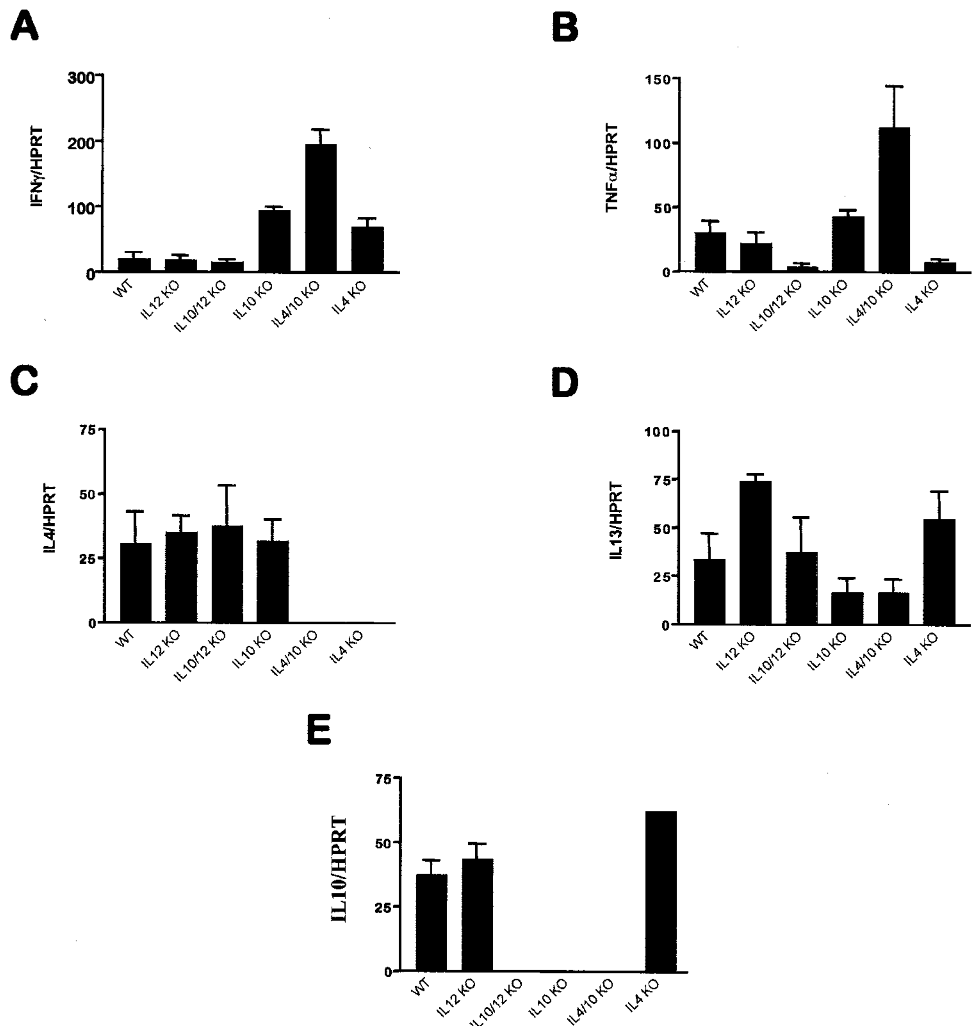

FIGURE 4. Cytokine mRNA response in the cecum of *T. muris*-infected mice. WT and cytokine-deficient mice (five per group) were inoculated orally with embryonated eggs. Mice were sacrificed 20 days later, and RNA was extracted from portions of the cecum. Cytokine gene expression was determined by RT-PCR. The data are expressed as the ratio of cytokine-specific signal density over that of HPRT. The ratios were determined from individual samples (five mice per group) and are shown as the means ± SEM. *A*, IFN-γ; *B*, TNF-α; *C*, IL-4; *D*, IL-13; *E*, IL-10.

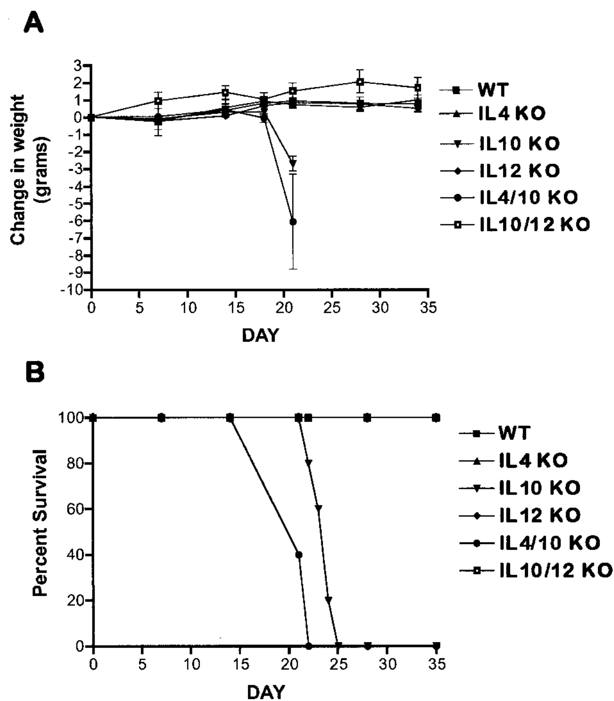

FIGURE 5. Assessment of morbidity and mortality following *T. muris* infection. Five mice per group were inoculated with 350 *T. muris* eggs, and weight change as well as mortality were recorded over a 35-day period. *A*, Change in weight was expressed as the mean weight at each time point subtracted from the initial weight on day 0 and expressed as weight gain or loss in grams. *B*, Mortality curves are shown as the percentage of survival.

6) responses. Indeed, the high mortality observed in the IL-10/IL-4-deficient mice, in contrast to that in IL-4-deficient animals, reinforces the conclusion that IL-10 plays an important protective role in both WT and IL-4-deficient hosts.

Mortality in T. muris*-infected IL-10- and IL-10/IL-4-deficient mice correlates with increased inflammation in the cecum, loss of Paneth cells, and a marked reduction in mucus*

Importantly, no significant differences in gut pathology were observed in uninfected mice before infection. When uninfected WT, IL-10, and IL-10/IL-4 KO mice were compared, none showed mast cells or Paneth cell metaplasia, and the appearance of eosinophils was rare and identical in all three groups. Their inflammation scores were 0.8 ± 0.7, 1 ± 0, and 0.9 ± 1, respectively. Gut mucus was also similar in these animals, with scores of 3, 3, and 3.33 reported in uninfected WT, IL-10 KO, and IL-10/IL-4 KO animals, respectively. In contrast, the lamina propria and submucosa contained a marked inflammatory infiltrate in all infected groups (Fig. 7) with eosinophils forming 60–70% of the infiltrating cells, except in the IL-10 KO (27%) and IL-10/IL-4 KO mice (10%). Instead, activated macrophages were more prominent in the IL-10 (38%) and IL-10/IL-4 KO mice (58%). All other groups had between 13 and 17% macrophages in the inflammatory infiltrate. Additionally, Paneth cells were most numerous in mice displaying a protective Th2-type phenotype. Mucosal thickness averaged ~180 μm in infected mice, without any obvious variation between the groups. However, there was extensive mucosal ulceration in IL-10/IL-4 KO mice. Interestingly, intestinal epithelial cell mucus was markedly reduced or absent in IL-10 and IL-10/IL-4 KO mice, and these animals also exhibited a more significant inflammatory cell infiltrate in the muscle layer (Fig. 8). Representative photomicrographs from WT, IL-10 KO, IL-10/IL-4 KO, and IL-10/12 KO mice are shown in Fig. 8. Obvious disruption of gut villi was seen in the IL-10 and IL-10/IL-4 KO groups, while the IL-10/12 KO animals displayed pathology similar to that of WT mice. Goblet cells were only visible in the WT and IL-10/12 KO mice and were stained bright blue. Paneth cells are not visible at this magnification. However, the marked submucosal edema was obvious in the IL-10 and IL-4/IL-10 KO mice at this magnification. These representative photomicrographs are provided at this magnification to illustrate the general appearance of the gut, and the more specific histological analysis is provided in Fig. 7.

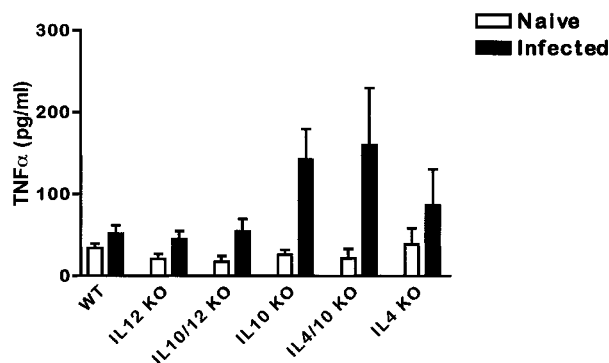

FIGURE 6. Systemic TNF-α levels in naive and *T. muris*-infected mice. WT and cytokine-deficient mice (five per group) were inoculated orally with 600 embryonated eggs. Serum was collected on day 20 and used in a capture ELISA. TNF-α levels were determined for individual samples and expressed as the mean ± SD.

Highly susceptible IL-10/IL-4-deficient mice can be protected from death by the administration of a broad spectrum antibiotic

We suspected that the highly susceptible IL-10/IL-4 KO and IL-10 KO mice might be dying from an outgrowth of opportunistic bacteria triggered by the *T. muris* larva-induced intestinal damage. Therefore, starting 1 day before infection groups of mice received a broad spectrum antibiotic, neomycin sulfate, in their drinking water as well as an every other day gavage for the duration of the study (43 days) to insure adequate hydration of the infected mice. As expected, WT mice expelled their parasites with normal kinetics with or without antibiotic treatment (data not shown). Importantly, however, some of the IL-10/IL-4 KO mice were clearly protected by the antibiotic (Fig. 9). The untreated, but infected, IL-10/IL-4 KO mice had a median survival time of 21 days, while three of five mice treated with the antibiotic survived until the termination of the study on day 43. Surprisingly, little change in mortality was observed in the treated IL-10 KO. The percent survival of the IL-10/IL-4 KO mice receiving antibiotics was statistically significant compared with that in the untreated controls ($p < 0.004$).

T. muris*-infected IL-10/IL-4-deficient mice treated with antibiotic show a reduced inflammatory response in the cecum and regained the ability to produce mucus*

As in previous studies there was extensive mucosal ulceration in IL-10/IL-4 KO mice on day 21 postinfection, which was significantly reduced upon administration of antibiotic (Fig. 10). In addition, inflammation in the lamina propria and edema of the submucosa were reduced in the IL-10/IL-4 KO group receiving antibiotic. These mice were also now producing mucus, compared with the complete lack of mucus in the untreated animals. The IL-10 KO mice receiving antibiotic failed to exhibit these characteristics and, not surprisingly, succumbed to *T. muris* infection (Figs. 9 and 10).

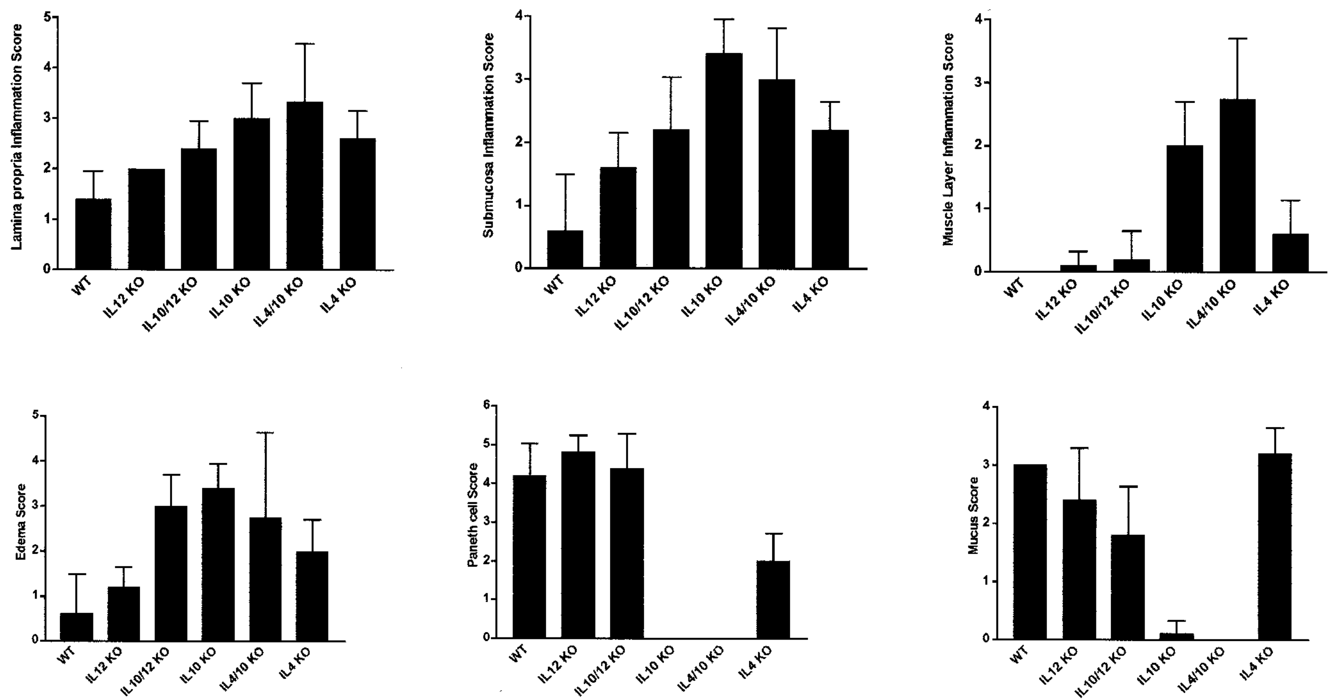

FIGURE 7. Assessment of tissue pathology in the cecum following *T. muris* infection. WT and cytokine-deficient mice (five per group) were inoculated orally with 350 embryonated eggs. Mice were sacrificed 20 days later. Tissue samples were taken from the cecum, placed in Bouin-Hollande fixative, and processed routinely. Sections were stained for histopathologic evaluation with Wright-Giemsa for general inflammation scoring or with periodic acid-Schiff for mucin assessment. Sections were read without knowledge of the identity of the mice, and each variable was scored 1–4+.

Discussion

There has been significant work characterizing the immune response to gastrointestinal parasites, yet in many cases the precise mechanisms responsible for protection are still unknown. It is clear for *T. muris* infection, which is an excellent model of *Trichuris* infection in humans and pigs, that eosinophils, mast cells, and Ab-mediated cellular cytotoxicity are not critical for mediating resistance (27). A majority of mouse strains are resistant to infection and expel *T. muris* larvae between 14 and 21 days postinfection. This expulsion has been correlated with development of a polarized Th2-type immune response (11, 28). In contrast, susceptible mouse strains develop polarized Th1-type immune responses and harbor larvae that mature into adults (7, 29). Therefore, the type and magnitude of the cytokine response have a major influence on the development of a resistant vs susceptible phenotype following *T. muris* infection. It has been suggested that one target of cytokine action may be on gut smooth muscle contractility and intestinal epithelial cell fluid secretion, which regulate the resistant or permissive state of the host (3, 30). Importantly, this study for the first time identifies IL-10 as a critical component of the resistance mechanism.

Our study demonstrates that a deficiency in IL-10 can lead to increased susceptibility to *Trichuris* infection and even death. Interestingly, this lethal outcome is not unique to *T. muris* infections, because mortality of IL-10-deficient mice has also been observed during *Toxoplasma gondii*, *Trypansoma cruzi*, *Plasmodium chabaudi chabaudi*, and *S. mansoni* infection (20, 31–35). The IL-10-deficient animals are also much more susceptible to endotoxic shock (36–38). These studies demonstrated directly or through correlation that overproduction of IFN-γ and TNF-α contributes to the increased morbidity and mortality. Indeed, we now show that mortality in *T. muris*-infected IL-10- and IL-10/IL-4-deficient mice correlates with a marked increase in both local and systemic production of IFN-γ and TNF-α. IL-12 was also implicated as a key mediator of the mortality observed during acute toxoplasmosis (32, 33). Our studies with *T. muris* infection now confirm that the mortality, weight loss, and increase in IFN-γ and TNF-α observed in IL-10-deficient mice is highly dependent on IL-12, because infected IL-10/12 KO mice failed to develop these traits. It is notable that secondary bacterial invasion and enhanced mucosal pathology in the colon of pigs infected with *T. suis* are associated with increased IL-12 gene expression in the mesenteric lymph nodes draining the site of infection (39).

Our hypothesis that TNF-α contributes to the morbidity and mortality observed in the IL-10- and IL-10/IL-4-deficient mice is in contrast to a recent report that suggests a protective role for TNF-α during *T. muris* infections (40). These authors clearly demonstrate that blockade of TNF-α, by the use of a neutralizing Ab or TNF receptor KO mice, prevents worm expulsion, which suggests that TNF-α is important to the resistance mechanism. Nevertheless, in our study we observed markedly increased levels of TNF-α in the IL-10- and IL-10/IL-4-deficient mice, yet these animals were incapable of expelling their parasites. These observations suggest that the effects of TNF-α may be context dependent. Indeed, we and others have suggested that TNF-α may exhibit different functional activities depending on whether it is produced in a Th1- or Th2-dominant milieu. Thus, as Artis et al. (40) proposed, TNF-α appears to be a critical component of the protective Th cell type 2 response, but during a type 1- dominant response the activities of TNF-α may be lethal, as observed in our experiments. Artis et al. (40) also reported a decrease in IL-9 production in their TNF-α-defective mice and, because IL-9 has been linked with resistance during both *T. muris* and *Trichinella spiralis* infection, the susceptibility they observed may actually be due to a decrease in IL-9 activity (41, 42). Alternatively, the timing, site of expression, and/or duration of exposure to TNF-α may lead to different outcomes (20, 43, 44). Thus, it is possible that endogenous production or exogenous administration of TNF-α early during an infection

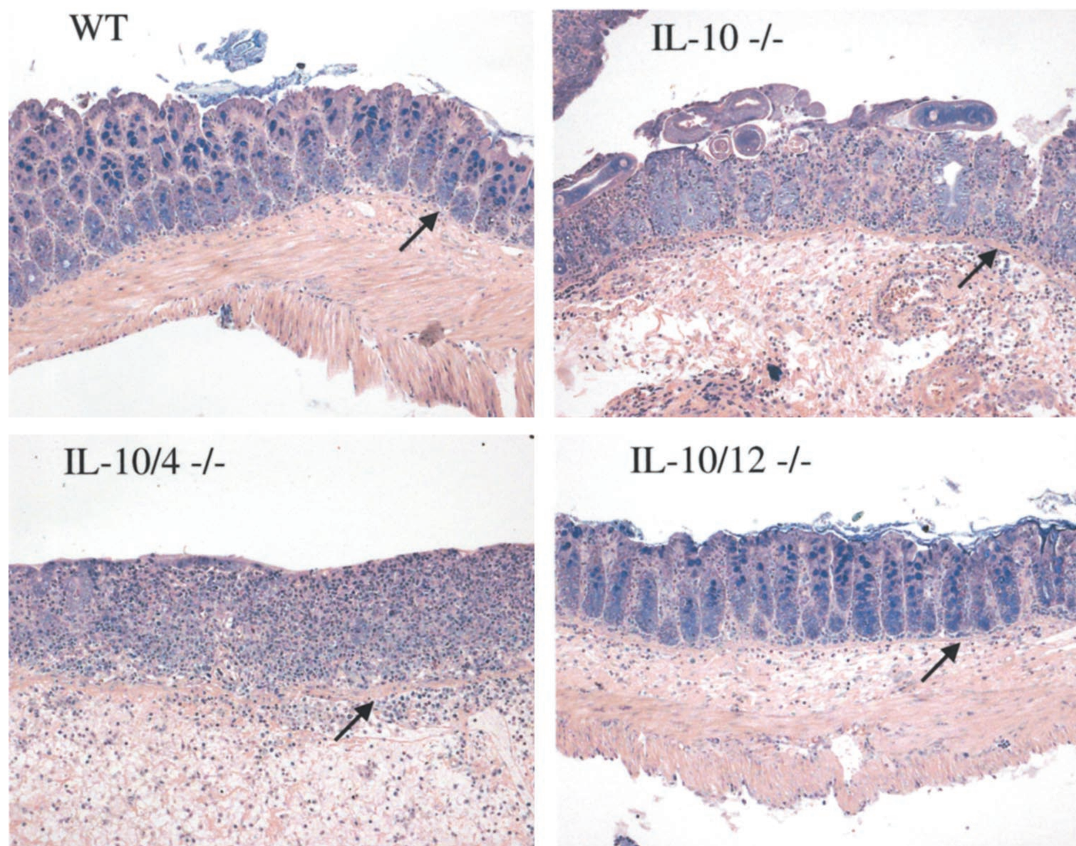

FIGURE 8. Representative photomicrographs of cecal pathology following *T. muris* infection. WT and cytokine-deficient mice were inoculated orally with 350 embryonated eggs. Mice were sacrificed 21 days later. Tissue samples were taken from the cecum, placed in Bouin-Hollande fixative, and processed routinely. Sections were stained for histopathologic evaluation with Wright-Giemsa stain. Representative photomicrographs are shown. Note the location of parasites near the villi in the IL-$10^{-/-}$ mice. Arrows indicate the muscularis mucosae. Goblet cells are stained bright blue, while Paneth cells are not easily visible at this magnification. Note the marked submucosal edema in the IL-10 and IL-10/IL-4 KO mice and the absence of goblet cells. Also note the absence of severe pathology in the IL-10/IL-$12^{-/-}$ animals. Magnification, ×110.

may help direct resistance (40), while the severe pathology observed in our study may be due to overproduction of the cytokine late in the infection.

One of the more interesting findings from our histological evaluation was that intestinal epithelial cell mucus was markedly reduced or absent in IL-10 and IL-10/IL-4 KO mice. These groups also exhibited a more extensive inflammatory cell infiltrate in the muscle layer. Similar morphological changes in the intestinal tract have been observed during *Salmonella typhimurium* infections, which include generalized tissue inflammation, edema, and changes in mucin production (45). It has been proposed that TNF-α mediates tissue damage, cachexia, loss of mucus production, and death caused by bacterial septicemia (46). Thus, it is possible that the increase in TNF-α in IL-10- and IL-10/IL-4-deficient mice contributes to the tissue pathology observed during *T. muris* infection.

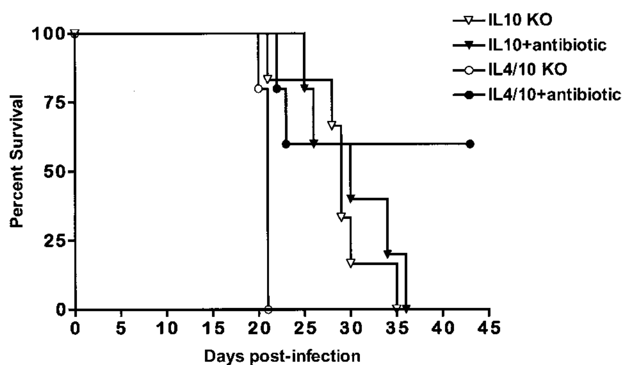

FIGURE 9. Mortality following *T. muris* infection with and without antibiotic treatment. Five mice per group were inoculated with 400 *T. muris* eggs, and mortality was monitored over a 43-day period. Mice were given a broad spectrum antibiotic, neomycin sulfate, in the drinking water at a concentration of 2.4 mg/ml and a 0.5-ml oral gavage of the same solution every other day starting on day −1. Fresh antibiotic solutions were prepared daily. Control mice received a gavage of water. Representative results from two independent experiments are shown. Mortality curves are shown as the percentage of survival.

We have previously shown that *T. suis* infection in young pigs can lead to secondary bacterial infections in the proximal and distal colon (47). The growth of opportunistic bacteria appears to contribute to the development of severe intestinal pathology and death. In support of this conclusion, when pigs were treated with a broad spectrum antibiotic, intestinal pathology was dramatically reduced (47). We now report similar findings with the hyper-Th1-polarized IL-10/IL-4 KO mice, which were the only animals that developed severe ulcerating lesions. When a broad spectrum antibiotic was given the majority of the treated mice survived, while 100% mortality was observed among the untreated IL-10/IL-4 KO mice. There was also a marked reduction in pathology at 3 wk postinfection in the antibiotic-treated animals, and they regained some of the mucus response. These observations may be related to a changing pattern of Paneth cell expression, because the secretion of protective factors by these cells and mucus-producing goblet

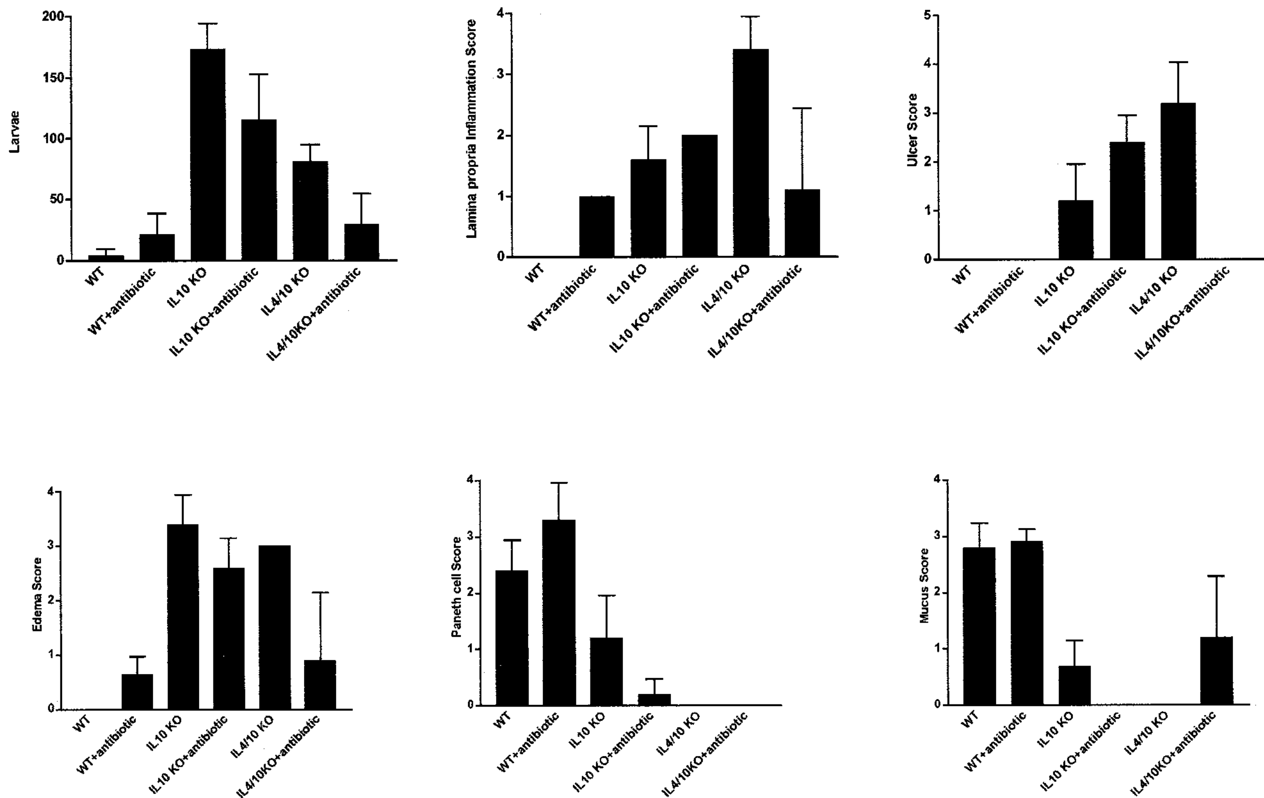

FIGURE 10. Larval burdens and tissue pathology in the cecum following *T. muris* infection with and without antibiotic treatment. WT and cytokine-deficient mice (five per group) were inoculated orally with 400 embryonated eggs. Mice were sacrificed 21 days later. Tissue samples were taken from the cecum, placed in Bouin-Hollande fixative, and processed routinely. Sections were stained for histopathologic evaluation with Wright-Giemsa for general inflammation scoring or with periodic acid-Schiff for mucin assessment. Sections were read without knowledge of the identity of the mice and were scored 1–4+.

cells stimulates epithelial cell continuity and mucosal barrier function and provides α-defensins, β-defensins, and intestinal trefoil factor that inhibit mucosal invasion by enteric bacteria in both small and large intestines (48–51). Paneth cells are typically absent from the normal colon but may appear in chronic inflammatory diseases of the colon such as ulcerative colitis and Crohn's disease (52, 53). Our data demonstrate that they also appear following infection with *T. muris* and suggest that IL-4 and IL-10 may contribute to Paneth cell development. Baseline levels of Paneth cells as well as goblet cells are increased in the small intestine following infection with *T. spiralis* (54), although these cells are not required for worm expulsion from the small intestine (55). The induction of these cells in the cecum and colon, however, may act as a barrier to prevent parasites and bacteria from maintaining their attachment to the mucosal surface. Therefore, the absence of Paneth cells and mucus, as observed in untreated IL-10 and IL-10/IL-4 KO mice (Fig. 10), could provide the parasite a distinct advantage as well as facilitate bacterial attachment and invasion (50, 54–56). Nevertheless, to date it remains unknown whether Paneth cell products have a direct effect on the nematode (55). There was, however, a consistent decrease in larval burdens in the antibiotic-treated animals, which suggests that this may be an additional mechanism controlling susceptibility.

Unexpectedly, the antibiotic effect was much less dramatic in IL-10-deficient mice. In this study, the effect on survival was significant in only one of two experiments. Interestingly, both IL-10 and IL-10/IL-4 KO groups treated with antibiotic showed a significant decrease in larval burden ($p < 0.5$). Nevertheless, the IL-10 KO groups consistently developed greater larval burdens; therefore, the partial reduction in parasite burden observed in the antibiotic-treated group may have been inadequate to significantly alter the outcome of infection. Furthermore, larval burdens alone are unlikely to be the only factor contributing to the high rate of mortality. Indeed, we consistently observed large larval burdens in IL-4 KO mice, yet these mice developed more modest intestinal pathology and never died from their infections. In contrast, IL-10/IL-4 KO mice often had lower larval burdens than IL-4- or IL-10-deficient mice but consistently displayed a high degree of morbidity and mortality. Together these observations, in addition to those discussed above, suggest that several factors contribute to morbidity following infection with *T. muris*, including persistent parasite load, outgrowth of opportunistic bacteria, overproduction of type 1-associated cytokines, and degree of intestinal damage. In all cases, IL-10 appears to be a critical immunoregulatory factor.

The relative roles of IL-4 and IL-13 in host protection have been studied in mice infected with a variety of gastrointestinal nematodes, including *T. muris* (8, 9, 15, 17, 57). In this work, *T. muris* expulsion was inhibited in mice deficient in either IL-4 or IL-13. From these studies it was concluded that both cytokines are required for the development of protective immunity. Nevertheless, a requirement for both cytokines was not observed in IFN-γ-deficient mice, which suggests other cytokines, including IFN-γ, may also play important regulatory roles during *Trichuris* infection (17). The present studies contribute important new information by demonstrating that IL-10 is critical to the resistance mechanism. Indeed, this is the first study to show a role for IL-10 in protective immunity during a gastrointestinal nematode infection. Our study suggests that, among the Th2-associated cytokines studied to date,

IL-10 is particularly important because the cytokine not only promotes resistance but also protects infected animals from developing lethal immunopathology. Similar findings were not reported in studies examining *T. muris* infection in IL-4- or IL-13-deficient mice. The importance of IL-10 to protection is also emphasized by the finding that relatively normal IL-4/IL-13 mRNA responses develop in the gut of IL-10-deficient mice (Fig. 4), yet these animals remain susceptible. The development of a significant IFN-γ response appears to be the primary explanation for the increased susceptibility of the IL-10-deficient mice, because IFN-γ-defective IL-10/12-deficient mice were resistant to *T. muris* infection. Importantly, these later findings clearly demonstrate that IL-10 is not playing a direct role in resistance but, rather, operates by suppressing the counter-regulatory type 1 cytokine response. Additional studies will be needed, however, to determine whether the important findings observed for IL-10 in *T. muris* infection will extend to other gastrointestinal parasites.

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