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Potential Role of the Chemokine Macrophage Inflammatory Protein 1α in Human and Experimental Schistosomiasis

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In human schistosomiasis, the concentrations of the chemokine macrophage inflammatory protein 1α (MIP-1α/CCL3) is greater in the plasma of patients with clinical hepatosplenic disease. The objective of the present study was to confirm the ability of CCL3 to detect severe disease in patients classified by ultrasonography (US) and to evaluate the potential role of CCL3 in *Schistosoma mansoni*-infected mice. CCL3 was measured by enzyme-linked immunosorbent assay in the plasma of *S. mansoni*-infected patients. CCL3-deficient mice were infected with 25 cercariae, and various inflammatory and infectious indices were evaluated. The concentration of CCL3 was higher in the plasma of *S. mansoni*-infected than noninfected patients. Moreover, CCL3 was greater in those with US-defined hepatosplenic than with the intestinal form of the disease. In CCL3-deficient mice, the size of the granuloma and the liver eosinophil peroxidase activity and collagen content were diminished compared to wild-type mice. In CCL3-deficient mice, the worm burden after 14 weeks of infection, but not after 9 weeks, was consistently smaller. The in vitro response of mesenteric lymph node cells to antigen stimulation was characterized by lower levels of interleukin-4 (IL-4) and IL-10. CCL3 is a marker of disease severity in infected humans, and experimental studies in mice suggest that CCL3 may be a causative factor in the development of severe schistosomiasis.

Schistosomiasis is one of the most prevalent helminth diseases in the world and is caused by blood flukes of the *Schistosoma* genus (36). In infected individuals, the granulomatous inflammation in response to egg deposition in the liver in the case of *Schistosoma mansoni* is the major pathological finding and accounts for most of the clinical symptoms. Worm oviposition at 5 to 6 weeks poses a strong Th2 bias in the immune response against infection (6) while also inhibiting the Th1 component (12, 42). Nonetheless, the granulomatous response that is maximal during the first few weeks after initial oviposition is also subjected to immunomodulation. Apart from interleukin-10 (IL-10) (29), other factors may also be involved in this process. For example, recent literature suggests that, in animal models, the granulomatous response that occurs at chronic time points is dependent on the soluble form of the IL-13 receptor α2 (27). In patients, failure in modulating the response might lead to the development severe schistosomiasis later in life (15). Indeed, a direct consequence of the persistence of an exacerbated immune response appears to be the development of large granulomatous reactions associated with intense collagen deposition (20) and the development of hepatosplenic schistosomiasis. Therefore, there is much scientific interest in the understanding of the mechanisms and inflammatory mediators underlying egg-induced granulomatous response, with the ultimate goal of proposing strategies to modulate fibrosis. It is known, however, that prevention of granuloma formation may be dangerous, since lethality has been reported in mice that fail to form granulomas (21).

A few studies have evaluated the role of chemokines in *S. mansoni* granuloma formation. Most of them have been carried out in mice by intravenous administration of egg antigen-conjugated beads (9). Overall, there is good evidence to suggest that chemokines play an important role in defining the leukocyte infiltrate and subsequent immune response that occurs around beads (9). For instance, the chemokines (32), macrophage inflammatory protein 1α (MIP-1α/CCL3) and regulated on activation and normal T-cell expressed and secreted (RANTES/CCL5), are released during the granulomatous response and blocking of their action alters the size and other granuloma characteristics in a model of pulmonary embolization (10, 25, 26, 38). Studies with chemokine receptor-deficient mice also suggested a role for chemokines in the development of granulomas (9, 19, 41, 52).

Fewer studies have evaluated the role of chemokines for human schistosomiasis. A recent study from our group has shown that concentrations of CCL5, eotaxin/CCL11, and CCL3 are elevated in the plasma in patients with chronic schistosomiasis. Importantly, patients with high concentrations of CCL3 were more likely to develop hepatosplenomegaly

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(18). However, this assumption may have been biased, since organomegaly was not confirmed by ultrasonography (US). Moreover, blockade of receptors for CCL3 was associated with a decrease in size of in vitro granuloma around antigen-conjugated beads (18). In the present study, we wanted to confirm and extend our previous data, but now using patients classified by US from a different area of schistosomiasis endemicity. We also investigated likely roles of CCL3 in a murine model of infection that could account for differences in disease morbidity in patients. To this end, the course of *S. mansoni* infection was monitored in CCL3-deficient and wild-type mice.

MATERIALS AND METHODS

Blood collecting and outpatient clinical and US examination. Plasma samples were obtained from patients living in Boa União and Nova União, two adjacent localities, in Minas Gerais, Brazil. Study groups were composed of 32 intestinal disease patients, 13 hepatosplenic patients, and 12 controls. The diameter of the portal and mesenteric veins, the sizes of the liver and spleen, and wall thickening of central and peripheral branches of the portal vein were evaluated according to World Health Organization guidelines (1, 14, 22, 27). The criteria for inclusion in the hepatosplenic group was that patients had to present thickening of the portal branches of >0.3 cm and hepatic and splenic organomegaly as shown by US performed with a portable Hitachi EUB-200 apparatus. This is an important aspect of the present study since sole clinical organomegaly is a bad predictor of disease outcome (1). In our patient population, only grade I fibrosis (periportal thickening of >0.3 and <0.5) was found, confirming previous observations that more severe presentations of the disease seem to be less prevalent probably due to the treatment of infection (2, 3). Patients were treated for infection regardless of their adherence to the study. Those younger than 5 years and those exhibiting liver damage suggestive of other diseases, such as steatosis, in the absence of pipe stem fibrosis, were excluded. The control group was composed of noninfected individuals living in the area of endemicity and healthy volunteers from an area where the disease is not endemic. Negative results for schistosome infection were attested by three consecutive parasitological examinations by the Kato-Katz method (24). All patients signed an informed consent for the use of blood samples.

Animals and Infection. Male and female C57BL/6 and CCL3-deficient mice (13) were obtained from the animal facility of the Centro de Pesquisas Ren, Rachou. The animals were 8 weeks old at the beginning of the experiment and kept at the animal facility of the Grupo Interdisciplinar de Estudos em Esquistossomose. Rodent chow and water were given ad libitum. LE strain *S. mansoni* cercariae (50) released from *Biomphalaria glabrata* snails were suspended in saline 0.9% and subcutaneously injected (25 cercariae per animal) by an experienced technician. Within the same experiment, all groups of mice, wild type or knockout, were infected with the same cercaria suspension. To assess parasite burden, mice had to be subjected to mesenteric perfusion for collecting of adult worms. The latter procedure induces a degree of tissue damage and thus prevented the use of the tissues and blood from these animals for the measurement of other parameters. Thus, separate groups of animals were used to assess either parasitological or immunological parameters. Female mice were used for the parasitological studies (egg and worm burdens), and male mice were used for all other parameters described here. Infection-induced morbidity and mortality were observed in CCL3-deficient and wild-type mice throughout the experiment, which lasted 9 or 14 weeks, and so represented the acute and chronic phases of the infection. All experiments were repeated and, since the means of a given variable were similar in these experiments, the results were pooled for presentation.

Determination of egg content in the feces. Animals were kept in individual gridded-floor cages the night prior to sacrifice. In each cage a piece of wet absorbent paper kept feces humid. On the following morning, feces were collected, weighed, homogenized in 10% buffered formaldehyde, and stored. Two aliquots of 100 μl each were counted by light microscopy, and the results are expressed as number of eggs per gram of feces.

Recovering of worms from the portal system. Animals were sacrificed by cervical displacement, and viscera were exposed. A cut was made at the portal vein, and a needle conjugated to a perfusion pump was introduced into the abdominal aorta (45). Saline (0.9%) was perfused and collected in a Becker apparatus. Decantation was allowed for 30 min, and the supernatant was discarded. Washing steps were performed until the supernatant was clear. Male and female worms were counted in a laboratory magnifier.

Determination of egg content in the tissues. The livers, lungs, intestines, and spleens of infected mice were weighted and solubilized in 5% KOH solution until no sign of intact tissue was present. Tissue suspensions were centrifuged at 200 × g for 1 min at room temperature, washed five times, and stored at 4°C. Eggs in aliquots of 100 μl were counted under a light microscope, and values were extrapolated to the total organ content. The tissue egg retention index, a measure of worm fecundity, was calculated as the sum of the absolute number of eggs present in tissues divided by the number or female worms, regardless of whether they were found mated or not (17).

Differential blood cell count and tissue histology. Animals were sacrificed under ether anesthesia, followed by cervical displacement, and 20 μl of blood was collected from each brachial plexus. The blood was diluted in Turk's solution for total cell count. A blood smear was prepared and stained with Giemsa and May-Grunwald stains. Differential blood count of mononuclear cells, neutrophils, and eosinophils was performed from a total of 300 cells. Fragments of liver were removed, fixed in 10% buffered formaldehyde, and stained with either hematoxylin and eosin or Picrus Sirius (30). About 500 μl of blood were collected for the quantification of cytokines.

Cell culture. After sacrifice, animals not subjected to hepatic perfusion had the abdominal wall opened in sterile conditions. Mesenteric lymph nodes (MLN) were removed, and cells were isolated. The resultant cell suspension was diluted to 10^7 cells/ml in HEPES-modified RPMI supplemented with potassium-penicillin at 10 IU/ml and 1 μg of gentamicin-containing 5% fetal calf serum/ml. Cells were plated in the presence of concanavalin A (1 μg/ml), a nonspecific T-cell stimulator (11), and schistosome egg antigen (SEA) (5) or soluble adult worm antigenic preparation (SWAP), both at the concentration of 50 μg/ml. The supernatant was collected 48 h later for cytokine quantification.

Cytokine determination by ELISA. The concentration of cytokines in plasma and culture supernatant was performed by sandwich enzyme-linked immunosorbent assay (ELISA) as described elsewhere (31). The ELISA kits for mouse IL-4, IL-10, IL-13, gamma interferon (IFN-γ), and human CCL3 were obtained from R&D Systems. The antibody pairs and standards used in the assay for mouse IL-1 were kindly provided by Steve Poole. The specific recommendations of the manufacturers were followed. Human plasma samples were subjected to acid-salt precipitation to avoid cross-reactivity in ELISA (18).

Determination of the activity of eosinophil peroxidase (EPO). The assay was performed as described previously (47). Briefly, 100 mg of the liver and intestine of the animals were weighted and homogenized in 1.9 ml of phosphate-buffered saline and centrifuged at 12,000 × g for 10 min. The supernatant was discarded, and the erythrocytes were lysed. The samples were then centrifuged, the supernatant was discarded, and the pellet was suspended in 1.9 ml of 0.5% hexadecyltrimethyl ammonium bromide in phosphate-buffered saline, frozen three times in liquid nitrogen, and centrifuged at 4°C at 12,000 × g for 10 min. The supernatant was used in the enzymatic assay by the addition of an equal amount substrate (1.5 mM o-phenylenediamine–6.6 mM H_2O_2 in 0.075 mM Tris-HCl [pH 8]). The reaction was stopped with 50 μl of 1 M H_2SO_4, and the absorbance was read at 492 nm.

Hydroxyproline determination. Fragments (100 mg) of liver and intestine were removed for hydroxyproline determination as an indirect measure of collagen content (39). Briefly, tissues were homogenized in 0.9% saline, frozen, and lyophilized. The assay was performed with 20 mg of the lyophilized material subjected to alkaline hydrolysis in 300 μl of H_2O plus 75 μl of 10 M NaOH at 120°C for 20 min. An aliquot of 50 μl of the hydrolyzed tissue was added to 450 μl of Chloramine T oxidizing reagent (0.056 M Chloramine T–n-propanol 10% in acetate-citrate buffer [pH 6.5]) and allowed to react for 20 min. A hydroxyproline standard curve was prepared likewise. Color was developed by the addition of 500 μl of 1 M p-dimethylaminebenzaldehyde diluted in n-propanol–perchloric acid (2:1 [vol/vol]). The absorbance was read at 550 nm.

Morphometric analysis. Slides with sections of the liver were analyzed under a light microscope for the characterization of the inflammatory infiltrate and the composition of granulomas. For the quantitative analysis of granuloma area, images were captured with a digital camera (Optronics DEI-470) connected to a microscope (Olympus IX70) and analyzed with Image ProPlus. For each infected animal, the first nine granulomas found in either of three liver sections meeting the criteria of being isolated and of presenting a central viable egg were photographed and analyzed. Granulomas in which more than one or no egg was visible were excluded from the analysis. The area of each of the nine granulomas was measured three times and averaged.

Histological egg densitometry. Images covering 1,500 μm² from liver sections of male wild-type and CCL3$^{-/-}$ mice were captured with a digital camera (Optronics DEI-470) connected to a microscope (Olympus IX70) with a magnification of × 100. The number of images taken was about 30 to 50 for each animal and was equal to the highest number of whole snapshots that could be taken without including serial slices in a given fragment of stained tissue. The schistosome eggs per image were counted, and the average value was entered as
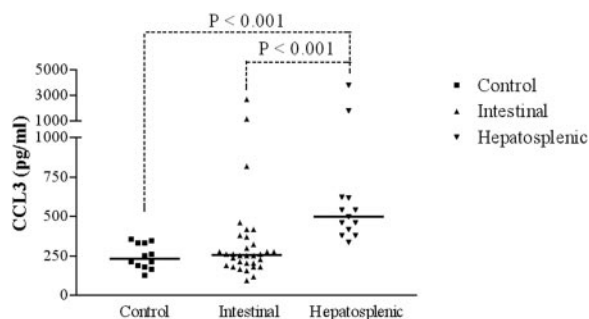
FIG. 1. Chemokine levels in the plasma of schistosome-infected patients and control individuals. Individual concentrations are shown with the median of each group expressed as a horizontal line. Patients were classified as intestinal ($n = 32$) or hepatosplenic ($n = 13$) according to clinical and US examinations. A control group was composed of 12 individuals. Plasma samples were processed for elimination of cross-reactivity and used for CCL3 determination by ELISA. The data were analyzed by the Kruskal-Wallis test, followed by Dunn's comparison test.

an individual value for each animal. The final value is referred to here as the "egg densitometric index" and was used to compare to egg counts obtained from whole-tissue digestions performed in female mice. As hydroxyproline content was only measured in livers and intestines of male mice, the egg densitometric index was useful to calculate a ratio describing the collagen deposition per tissue egg content.

Statistical analysis. Results for mice are expressed as means ± the standard error of the means (SEM) and were analyzed by analysis of variance, followed by the Newman-Keuls multiple comparison test. MLN cultures were analyzed by using an unpaired t test when detectable levels of cytokines were found only in infected groups. Human data were analyzed by Kruskal-Wallis test, followed by Dunn's multicomparison test. A cutoff value of MIP-1α that differentiated patients with or without hepatosplenomegaly was selected empirically. Relative risk was calculated by using the Fisher exact test in a 2 × 2 contingency table.

RESULTS

Concentration of CCL3 in plasma of patients. To confirm and validate our previous finding that CCL3 marked a group of patients with severe disease (18), we evaluated the concentrations of CCL3 by sandwich ELISA in plasma samples of US-assessed schistosome-infected individuals. In agreement with previous findings, infected patients had greater concentration of CCL3 in plasma than noninfected individuals ($P < 0.05$). The concentrations of CCL3 were significantly higher in plasma of patients with organomegaly than in those without organomegaly ($P < 0.001$, Fig. 1). The value of 400 pg/ml was found to be best to separate patients with or without organomegaly. Indeed, infected patients with a concentration of CCL3 of >400 pg/ml had a 14-fold greater chance of having organomegaly than those with lower concentrations of this chemokine ($P < 0.001$). However, there was no tendency for patients with high levels of CCL3 within the intestinal group to exhibit higher levels of fibrosis (not shown).

Infection indices in CCL3-deficient and wild-type mice. Initial experiments were designed to assess whether CCL3 deficiency interfered with worm burden and/or fecundity. No statistically significant difference in the lethality rates of infected mice was found, although percentages tended to be smaller in the CCL-3-deficient (28.5%) than in the wild-type group (37.5%) at 14 weeks postinfection. Of the approximately 25 injected cercariae, ca. 10 adult worms were recovered after
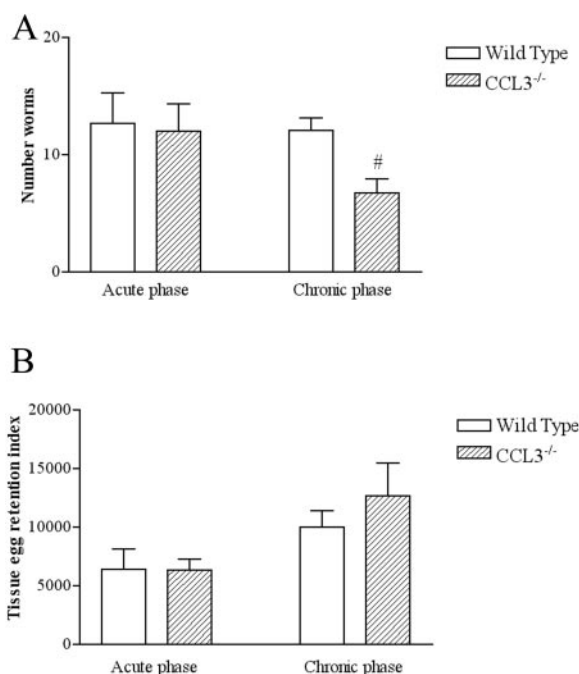

FIG. 2. Total number of worms in the portal system and fecundity of *S. mansoni* harvested from wild-type or CCL3-deficient mice. Values are the means ± the SEM of worms from perfusates of female mice. (A) The male and female adult mated and unmated worms present in the portal system of infected wild-type (□; acute phase, $n = 6$; chronic phase, $n = 12$) or CCL3-deficient (▨; acute phase, $n = 6$; chronic phase, $n = 11$) mice were counted after hepatic perfusion at 9 (acute phase) or 14 (chronic phase) weeks after infection. (B) The ratio between the total number or eggs present in all analyzed tissues, i.e., lungs, liver, intestine, and spleen divided by the number of female worms gives the tissue egg retention index for each group. The numbers of female worms are equal to those used to calculate the averages shown in panel A. #, $P < 0.01$ as determined by the Newman-Keuls test for CCL3-deficient mice compared to wild-type ones at the same phase of infection.

perfusion from either wild-type or CCL3-deficient mice at 9 weeks postinfection (Fig. 2A). At 14 weeks, there was a similar number of worms in the wild-type group, but a we observed a significant ($P < 0.01$) reduction in CCL3-deficient mice (Fig. 2A). Despite changes in worm numbers, the tissue egg retention index, as calculated by the total number of eggs trapped in tissues divided by the total number of female worms, was equal in CCL3-deficient and wild-type mice during both the acute and the chronic phases (Fig. 2B).

These results indicate that CCL3 deficiency does not alter worm fecundity. However, tissue- and phase-specific analysis reveals important differences in egg burdens calculated in female mice through the digestion of whole tissues with 5% KOH solution. During both the acute and chronic phases, a smaller number of eggs per gram of feces was found in CCL3-deficient mice (Fig. 3A). At the chronic phase other tissue specific differences were found. A reduction in the absolute number of eggs deposited in the liver (Fig. 3B, $P < 0.05$) and spleen (Fig. 3C, $P < 0.05$) was observed in the CCL3-deficient group. There was no difference in the number of eggs in the intestines (Fig. 3D) or lungs (not shown). Confirming data on egg counts, the evaluation of egg number by histology demonstrated a smaller number of
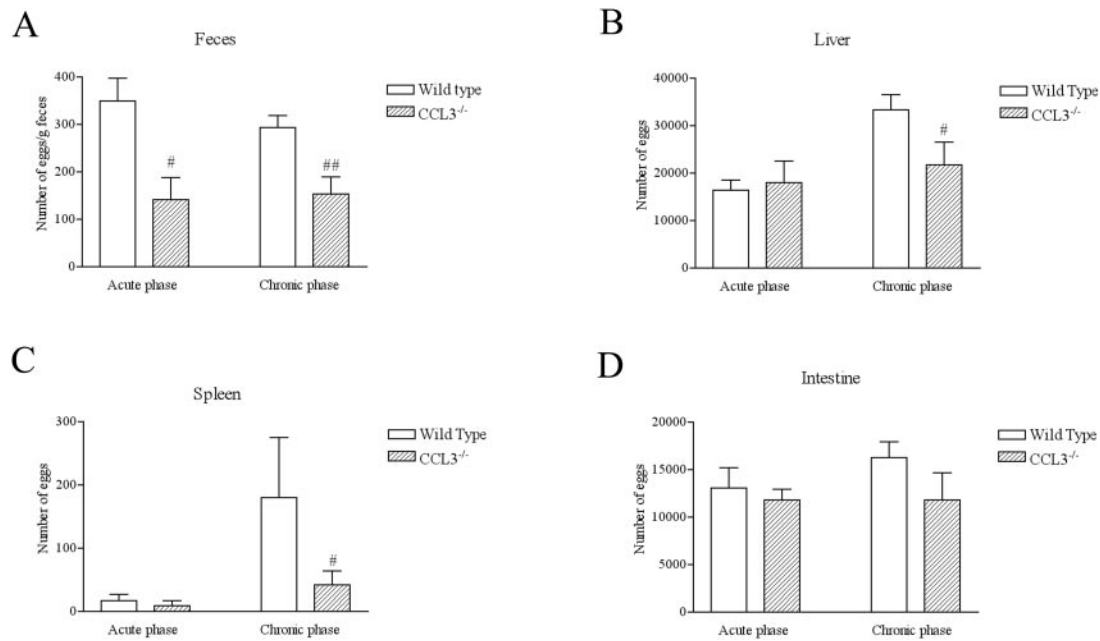
FIG. 3. *S. mansoni* egg burdens in different tissues from wild-type or CCL3-deficient mice. Values are the means ± the SEM of eggs counted from tissues or feces from female mice. At the day prior to sacrifice, animals were kept in special cages for the collection of their feces. Eggs in the feces (A) of wild-type (□; acute phase, $n = 6$; chronic phase, $n = 12$) or CCL3-deficient (▨; acute phase, $n = 6$; chronic phase, $n = 11$) mice are expressed per grams. After sacrifice, mice had their livers (B), spleens (C), and intestines (D) excised, weighed, and dissolved in 5% KOH. After the counting of aliquots in a light microscope, the total number of eggs in the tissues was calculated. # and ##, $P < 0.05$ or $P < 0.01$, respectively, as determined by the Newman-Keuls test for MIP-1a/CCL3-deficient mice compared to wild-type mice at the same phase of infection.

eggs in the liver of CCL3-deficient compared to wild-type mice during the chronic phase of infection (3.7 ± 0.2 versus 2.6 ± 0.3, respectively; $P < 0.05$ [unpaired t test]). The difference in egg density was not accompanied by changes in organ mass between the two groups (results not shown).

Hepatic and intestinal inflammation in CCL3-deficient and wild-type mice. Eosinophils represent the major cellular component of Th2 granulomas (4). The eosinophil relative content in tissues was deduced by EPO activity. There was minimal EPO activity in the liver of noninfected animals both groups (Fig. 4A). *S. mansoni* infection induced an increase in the EPO activity in the liver during the acute phase of infection, and this was persistent at 14 weeks. Comparisons within the same phase of infection demonstrated that the levels of EPO in the liver of CCL3-deficient mice were similar at 9 weeks. There was a significant 15% reduction of liver EPO ($P < 0.05$) at 14 weeks compared to wild-type mice (Fig. 4A).

Just as no statistical difference was found in the egg number in the intestines of wild-type animals compared to CCL3-deficient animals, the EPO activity was also similar between these groups in this organ (Fig. 4B). Blood eosinophilia is found in infected animals, but no difference was observed between the two groups (Fig. 4C).

Next, we evaluated whether CCL3 deficiency interfered with the size of liver granulomas. Comparison between the acute and chronic phases shows an approximate 50% reduction in granuloma size both in wild-type and CCL3-deficient mice (Fig. 5A), indicating extensive immunomodulation. The area of granulomas formed in the liver of CCL3-deficient animals was similar to those of wild-type mice in the acute phase of the

infection but significantly smaller ($P < 0.01$) at 14 weeks with a 28% reduction (Fig. 5A). Despite the clear reduction in granuloma size, there was no apparent tendency of livers of CCL3-deficient mice to present larger stretches of hepatic degeneration or fibrosis. Since no major differences have been observed between the two animal groups at 9 weeks, the following results will focus on the chronic phase of the infection.

As liver fibrosis is a major alteration associated with morbidity, we evaluated the hydroxyproline content of livers from wild-type and CCL3-deficient mice. As shown in Fig. 5B, the amount of hydroxyproline present in the liver, an indirect measure of collagen content, is increased in infected animals compared to noninfected ones. The hydroxyproline content in the liver of wild-type animals was higher than that of CCL3-deficient ones (Fig. 5B). No statistically significant differences were found for the hydroxyproline concentrations in the intestine in any group of mice (results not shown).

To dissect whether collagen deposition depended directly on the action of CCL3 or was resultant from the decreased egg retention in the liver of CCL3-deficient mice, we calculated in the same animal the ratio between the hydroxyproline content and the number of eggs in tissue, as assessed histologically. Thus, the ratio between a marker of fibrosis and a marker of egg burden was similar in wild-type and CCL3-deficient mice (0.62 ± 0.11 versus 0.67 ± 0.13, respectively).

Figure 6A and B shows the normal architecture of the liver of noninfected wild-type and CCL3-deficient animals which were apparently similar. In Fig. 6C, we noticed a strong egg-induced granulomatous response in the liver of a wild-type mouse at 14 weeks postinfection. There was an intense eosin-
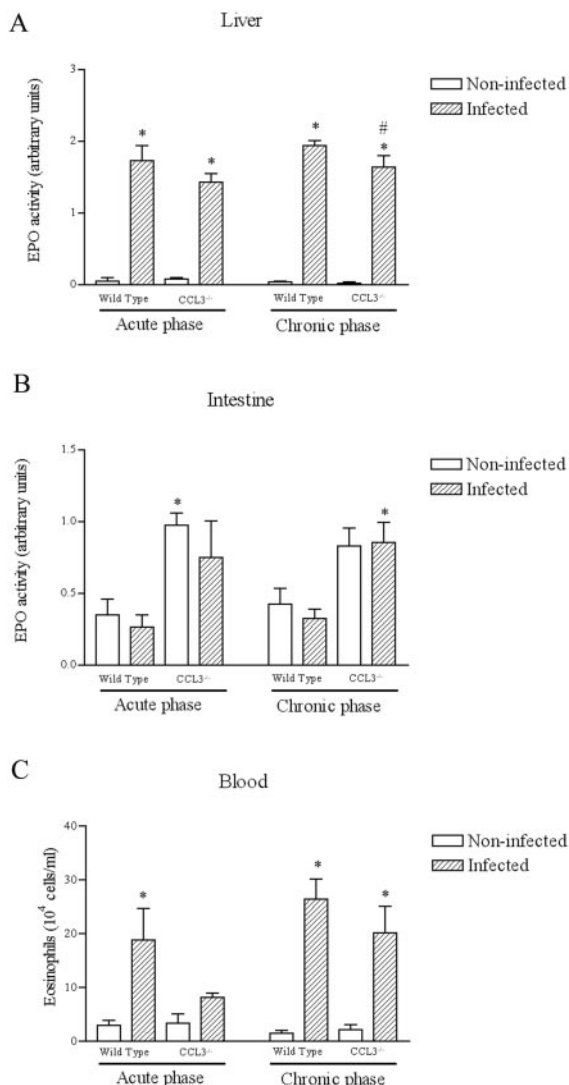
FIG. 4. Blood eosinophils and EPO activity in the livers and intestines of wild-type and CCL3-deficient mice. Values are the means ± the SEM from male mice. At 9 (acute phase) or 14 (chronic phase) weeks after infection, a fragments of the livers (A) and intestines (B) of noninfected (□) or infected (▨) wild-type (acute phase noninfected, n = 4; infected, n = 6; chronic phase noninfected, n = 8; infected, n = 12) and CCL3-deficient (acute phase noninfected, n = 5; infected, n = 4; chronic phase noninfected, n = 8; infected, n = 12) mice were weighed and processed for quantification of EPO activity. Blood smears were also prepared, fixed, stained with hematoxylin and eosin, and then subjected to differential cell counting. The percentages of eosinophils are shown in panel C. ✳, $P < 0.001$ (in panel A), $P < 0.05$ (in panel B), or $P < 0.01$ (in panel C) as determined by the Newman-Keuls test for infected versus noninfected mice of the same phenotype at the same phase of infection. #, $P < 0.05$ for infected CCL3-deficient compared to infected wild-type mice at the same phase of infection.

ophilic infiltrate and extensive collagen deposition, as shown by Picrus Sirius staining. However, Fig. 6D illustrates the result shown in Fig. 5A that granulomas around eggs in the CCL3-deficient group were smaller than that of wild-type mice.

Cytokine production by MLN cells during the chronic phase of infection. The in vitro cytokine production from SEA-stimulated MLN cells from mice is shown in Fig. 7. As expected,
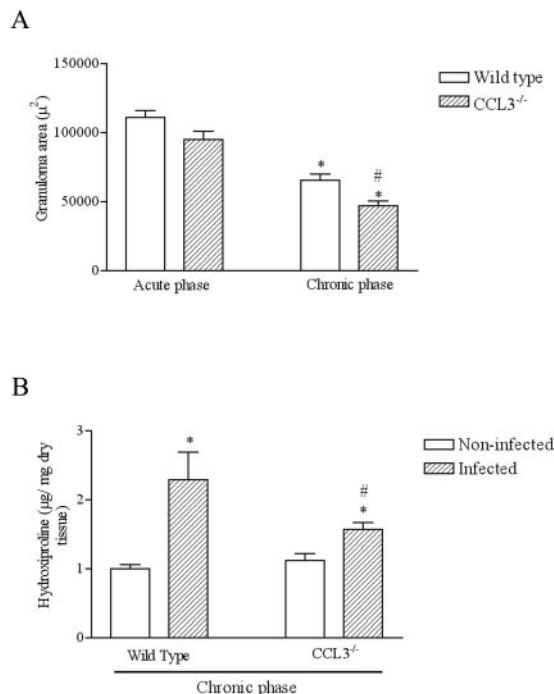

FIG. 5. Area of the granulomas formed around eggs and hydroxyproline content in the liver of wild-type and CCL3-deficient mice at the acute and chronic phases of infection. Hydroxyproline content was used as a measure of collagen deposition. Values are the means ± the SEM of liver samples from male mice at the chronic phase of the infection. (A) At 9 (acute phase) or 14 (chronic phase) weeks after infection, paraffin-fixed liver slices of wild-type (□ acute phase, n = 8; chronic phase, n = 12) or CCL3-deficient (▨; acute phase, n = 8; chronic phase, n = 12) mice were stained with eosin and hematoxylin. Isolated granuloma images were captured and analyzed in the software KS300 2.0 for the measurement of the area. ✳, $P < 0.01$ as determined by the Newman-Keuls test for infected mice at the acute phase compared to infected mice of the same phenotype at the chronic phase; #, $P < 0.01$ for CCL3-deficient mice compared to wild-type mice at the same phase of infection. (B) At 14 weeks (chronic phase) after infection, a fragment of the liver of noninfected (□) or infected (▨) wild-type (noninfected, n = 4; infected, n = 6) and CCL3-deficient (noninfected, n = 4; infected, n = 7) animals was used for the quantification of hydroxyproline. ✳, $P < 0.05$ for infected mice compared to noninfected ones of the same phenotype; #, $P < 0.05$ for infected MIP-1α/ CCL3-deficient mice compared to wild-type mice.

noninfected mice of both groups produced little or no cytokine against SEA stimulation. In contrast, MLN cells from infected animals produced cytokines of both the Th1 (IFN-γ) and the Th2 (IL-4, IL-10, and IL-13) type. As observed in Fig. 7A and B, the cytokines IL-4 ($P < 0.01$) and IL-10 ($P < 0.001$) were produced at lower levels by cells from CCL3-deficient mice. However, the production of TNF-α (Fig. 7C), IFN-γ (Fig. 7D), and IL-13 (Fig. 7E) were similar in the two mouse strains.

Table 1 shows the negative and positive control groups for the data given in Fig. 7 and also shows the responses to SWAP stimulation. Unstimulated MLN cells of both wild-type and CCL3-deficient mice produced very little or no IL-4, IL-10, IL-13, IFN-γ, and TNF-α, regardless of the infection status. Nonetheless, concanavalin A-stimulated MLN cells from infected animals of both groups produced higher amounts of IL-4 ($P < 0.001$ and < 0.01 for wild-type and CCL3-deficient mice, respectively) and IL-10 ($P < 0.05$ and < 0.01 for wild-
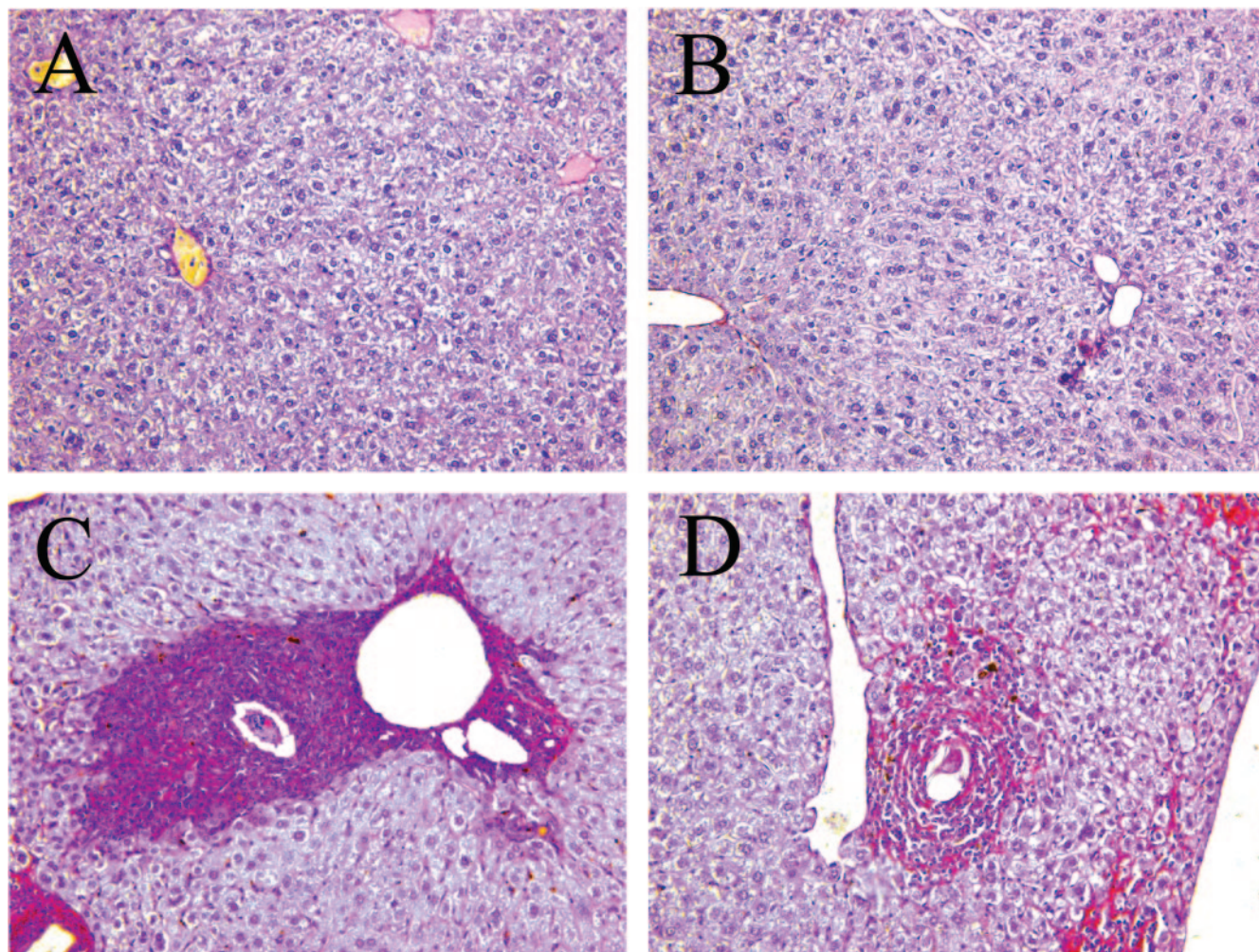
FIG. 6. Histological sections of the liver of noninfected or infected male wild-type and CCL3-deficient animals at the chronic phase of infection. Images are from Picrus Sirius-stained histological sections representative of the group. (A) Normal architecture of the liver of a noninfected wild-type animal. (B) Normal architecture of the liver of a noninfected CCL3-deficient animal. (C) Appearance of the liver of a wild-type infected mouse at the chronic phase of the infection. Notice the strong granulomatous response formed around the eggs. (D) Appearance of the liver of a CCL3-deficient infected mouse at the chronic phase of the infection. Many of the alterations described in panel C are also visible.

type and CCL3-deficient mice, respectively),but no difference was found in IFN-γ, IL-13, or TNF-α production. Similar results were observed for SWAP-stimulated cells, where noninfected animals did not respond stimulation, producing no detectable levels of cytokines. There was always cytokine production in infected animals.

DISCUSSION

We have recently demonstrated a correlation between elevated concentrations of CCL3 and the likelihood of presentation of severe schistosomiasis in humans (18). The present study had two main purposes: (i) to confirm the latter findings by using US to identify patients with severe disease and (ii) to investigate the existence of a causal relationship between CCL3 and morbidity in an animal model of S. mansoni infection. Using plasma of patients categorized according to US findings, we were able to confirm previous findings (18) that levels of CCL3 in plasma were a good marker of hepatosplenic

schistosomiasis. Indeed, patients with hepatosplenic disease had greater concentrations of this chemokine in plasma than those with intestinal disease or controls. Moreover, infected patients with CCL3 concentrations in plasma of >400 pg/ml had a 14-fold-greater chance of presenting with hepatosplenomegaly than those with lower concentrations. Thus, the two studies, which comprise two distinct populations, strongly suggest that CCL3 concentrations could be good markers of severity in S. mansoni infection.

Important differences were found in S. mansoni infection in mice in the absence of CCL3. (i) The numbers of eggs in the liver and adult worms were decreased in CCL3-deficient mice at the chronic phase of the infection. (ii) Chronic-phase granulomas were smaller in CCL3-deficient mice than in wild-type mice. (iii) The livers of infected CCL3-deficient mice had a lower hydroxyproline content. Of note and in contrast to the findings at the chronic phase, there were no dramatic changes in infection or inflammatory indices at 9 weeks.

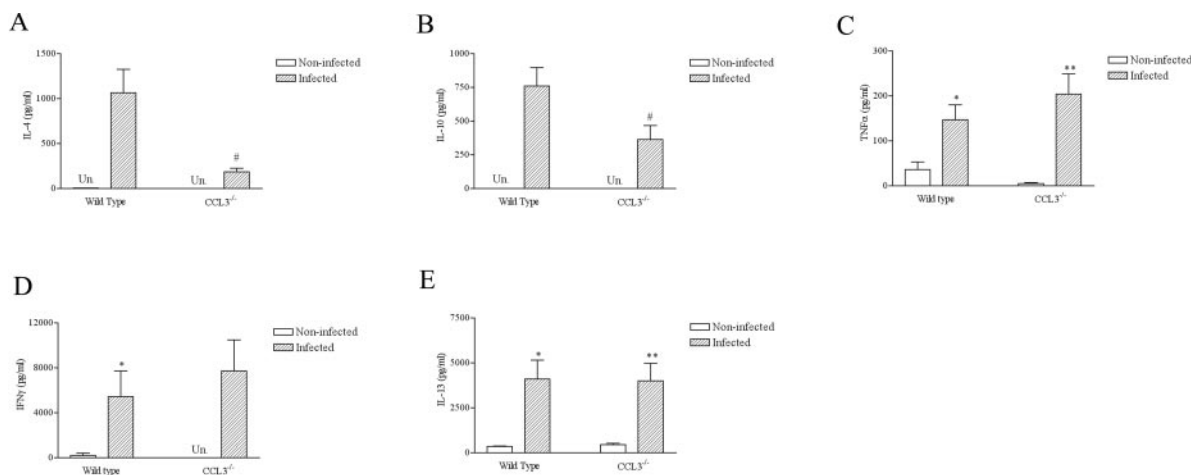We have shown here that the parasitic load of chronically

FIG. 7. Cytokine production by MLN cells from male infected and noninfected wild-type and CCL3-deficient mice in response to SEA stimulation at the chronic phase of the infection. Values are the means ± the SEM of supernatants from four mice. At 14 weeks postinfection, lymph node cells were obtained and stimulated with SEA. The supernatant was collected 48 h later for the quantification of IL-4 (A), IL-10 (B), TNF-α (C), and IFN-γ (D). $*$ and $**$, $P < 0.05$ or $P < 0.01$, respectively, for infected mice compared to noninfected ones of the same phenotype as determined by the Newman-Keuls test; #, $P < 0.05$ for infected CCL3-deficient compared to infected wild-type mice as determined by unpaired t test. Un., undetectable.

infected CCL3-deficient animals is reduced compared to wild-type animals. There are two possible explanations for this. First, CCL3 deficiency may interfere with the immune response to adult worms, making it more efficient and so capable of causing their clearance. Second, the absence of CCL3 may directly or indirectly alter worm survival. The testing of these hypotheses is beyond the scope of the present study and is currently under way in our laboratory. In any case, further studies are needed to address this question.

It is worth emphasizing that all groups of animals were infected with the same cercaria suspension by the same person in a random order and with no delay between groups, so that differences in cercarial virulence or viability could not account for the latter findings. Besides, worm burdens were investigated in groups of female wild-type and CCL3-deficient mice, excluding the possibility that sex-specific susceptibilities to infection could have biased the results (16, 33).

It is not clear in the literature whether mice can mount an effective immune response against adult *S. mansoni* worms. Our results, however, do indicate that the difference in worm burdens appeared in CCL3-deficient mice only after 9 weeks of infection and was responsible for the slight tendency, although not statistically significant, of wild-type mice to have higher mortality rates than CCL3-deficient mice. If a change in immune response was the reason for adult worm killing, then a difference in MLN responses to SWAP stimulation between wild-type and CCL3-deficient mice could have been noticeable. However, among cytokines that have been shown to have a role in worm killing in other models of parasitic infection, we have shown that CCL3-deficient mice responded to SWAP stimulation, producing less IL-4, IFN-γ, and TNF-α than wild-type animals. This is more suggestive of a less intense response to egg antigens, secondary to decreased parasitic load, rather than an enhanced immune response to adult worms in this group. On the other hand, CCL3 may be an important factor for the production of Th2 cytokines in vitro. Moreover, other studies investigated the role of CCL3 in models of Th2 inflam-

mation (18, 34, 35). These have usually shown an exacerbation of the response against SEA in the presence of high CCL3 concentrations or an inhibition when CCL3 was blocked with antibodies. These results also corroborate the data shown herein, so that a contribution of CCL3 in some Th2 cytokine amplification loops should not be excluded.

CCL3 binds to the chemokine receptors CCR1 and CCR5 (29) and is chemotactic for monocytes (51, 54), lymphocytes (48), and eosinophils (40), among other cell types, which are important for egg-induced granuloma formation (4). Indeed, similar to the inhibition observed in our model of infection, one study based on the pulmonary embolization model demonstrated a 20 to 40% inhibition in granuloma size in anti-CCL3-treated animals (26). It is noteworthy that the inhibition observed (28%) may be underestimated as the egg itself accounts for some of the granuloma area that, if excluded, would revert in a larger percent inhibition. The slight fall in EPO activity found in the present study is in agreement with previous data in that eosinophils should be less recruited in the absence of CCL3 (49). A reduction in the number of eosinophils may have contributed to the smaller size of granulomas present in the liver of CCL3-deficient mice. Reduction of other cell types is also a possibility, considering the lack of selectivity of the chemotactic activity of CCL3 toward any specific cell type present in the schistosome egg granuloma. Regardless of the cellular composition, the smaller granuloma area found in CCL3-deficient mice is in agreement with the lower granuloma index previously reported for peripheral blood mononuclear cells treated with met-RANTES, a CCR1 and CCR5 antagonist (37), in a model of in vitro granuloma (18). Thus, a reduced egg content in tissues associated with smaller granulomas could reflect diminished morbidity in CCL3-deficient mice.

We have shown that the liver collagen content of infected CCL3-deficient mice was smaller than that of wild-type mice. There is indeed evidence in the literature to suggest that CCL3 is involved in fibrosis both in humans and in mice (44). CCL3 is detectable in the bronchoalveolar lavage fluid of patients

TABLE 1. In vitro cytokine production from MLN cells (chronic phase)

Cytokine	Infection status[a]	Mean production[b] ± SEM (pg/ml) in:		
		Cell medium	Concanavalin A	SWAP
IL-4				
Wild type	NI	ND	6 ± 2	ND
	I	ND	97 ± 22‡	150 ± 58
CCL3$^{-/-}$	NI	ND	4 ± 3	ND
	I	ND	98 ± 9†	29 ± 15
IL-10				
Wild type	NI	ND	61 ± 7	ND
	I	ND	231 ± 37*	244 ± 81
CCL3$^{-/-}$	NI	ND	14 ± 2	ND
	I	ND	306 ± 85†	121 ± 63
IL-13				
Wild type	NI	300 ± 7	353 ± 37	411 ± 31
	I	30 ± 8	2,886 ± 558	1,208 ± 301
CCL3$^{-/-}$	NI	390 ± 51	401 ± 59	514 ± 75
	I	179 ± 121	4,044 ± 1,286*	1,291 ± 524
IFN-γ				
Wild type	NI	123 ± 72	7,049 ± 5,799	ND
	I	168 ± 13	6,989 ± 1,800	1,458 ± 430
CCL3$^{-/-}$	NI	11 ± 8	1,277 ± 586	ND
	I	226 ± 49	4,885 ± 1,969	253 ± 75
TNF-α				
Wild type	NI	9 ± 4	109 ± 36	7 ± 2
	I	7 ± 3	111 ± 9	30 ± 8*
CCL3$^{-/-}$	NI	4 ± 1	62 ± 8	3 ± 1
	I	12 ± 10	76 ± 12	14 ± 5

[a] NI, not infected; I, infected.
[b] ND, not detectable. *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$. P values were determined by the Newman-Keuls test for male infected mice compared to noninfected mice of the same phenotype.

with sarcoidosis and idiopathic pulmonary fibrosis (43). Moreover, anti-CCL3 antibody treatment in mice inhibits bleomycin-induced inflammatory infiltrate and fibrosis (41). Besides, several reports have shown the role of IL-4 in granulomatous inflammation and its interference on various aspects of the response, including collagen synthesis (7, 8, 28). So, it is possible that the inhibition of IL-4 production observed in CCL3-deficient mice could account for at least some of the observed inhibition of collagen deposition. From these evidences, it could have been possible that CCL3 also affected the amount of collagen that is produced in the liver. However, unlike the pipe stem fibrosis found in humans, the granulomas contain most of the disease-induced collagen deposition in mice. Accordingly, by calculating the ratio between the hydroxyproline content and the liver egg content, we could observe that the difference in fibrosis deposition was eliminated if the effect of egg burdens was excluded. These data indicate that the reduced collagen content of liver of CCL3-deficient mice was resulted mainly from the decreased number of eggs present in these animals. A direct effect of CCL3 on fibrogenesis in our model could not be demonstrated but remains a possibility that deserves further investigation.

In summary, we have shown that CCL3 was elevated in the plasma of patients with US-defined hepatosplenomegaly and marked a group under greater risk of developing severe disease.

We also showed that CCL3 deficiency is associated with decreased morbidity in a murine model of infection. This may derive from distinct actions of this chemokine, i.e., one that results in reduced worm burdens and their associated consequences and whose mechanism is unknown. One such associated consequence, whose importance is undisputed in schistosomiasis, is a reduction in collagen content that was demonstrated in CCL3-deficient mice. Second, CCL3 deficiency was associated with decreased granulomas, possibly reflecting a direct action of the chemokine on the various cell types that compose the granulomatous inflammation (46). Altogether, these results indicate that schistosomiasis is milder in the absence of CCL3 and suggest that CCL3 may be a causative factor in the development of severe schistosomiasis in humans.

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