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Progression of Visceral Leishmaniasis Due to *Leishmania infantum* in BALB/c Mice Is Markedly Slowed by Prior Infection with *Trichinella spiralis*

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We investigated in BALB/c mice the influence of the immunological environment created by the nematode *Trichinella spiralis* on the course of visceral leishmaniasis due to *Leishmania infantum*. On the day of *Leishmania* inoculation (day 0), mice, *T. spiralis* infected 7 days earlier, presented increased gamma interferon (IFN-γ), interleukin-4 (IL-4), and IL-5 mRNA levels locally and systemically and increased the potential of spleen cells to synthesize IFN-γ and IL-4 after activation in vitro. Eighteen days after *Leishmania* inoculation (day 18), corresponding to the acute phase of leishmaniasis, the hepatic amastigote burden in mice coinfected with *L. infantum* and *T. spiralis* (LT mice) was significantly lower ($P < 0.001$) than that in mice infected with *L. infantum* only (L mice). IFN-γ and IL-4 mRNAs were overexpressed in livers of LT and L mice. On day 70, corresponding to the chronic phase, the splenic amastigote load was significantly lower ($P = 0.004$) in LT mice than it was in L mice. Splenic IFN-γ transcripts were overexpressed in both L and LT mice. After *Leishmania*-specific in vitro stimulation, cytokine production was enhanced in both groups, but spleen cells from L mice produced significantly more IFN-γ than did spleen cells from LT mice. Our data (i) generalize previous results indicating the lack of a clear-cut correlation between the outcome of murine visceral leishmaniasis and the type of cytokine pattern and (ii) demonstrate that in LT mice, leishmaniasis takes a markedly milder course than it does in L mice, providing information on the potential consequences of coinfection in a mammalian host.

Murine visceral leishmaniasis due to *Leishmania donovani* or *L. infantum*, the species responsible for human visceral disease, has been much less studied than infection with *L. major* (15, 29, 30), which causes human cutaneous lesions, has been. Infection of mice with *L. major* is characterized by an extreme polarization of Th1 or Th2 responses; the expansion of gamma interferon (IFN-γ)-producing Th1 cells in resistant mice results in control of the disease, whereas susceptibility is correlated with expansion of the interleukin-4 (IL-4)-producing Th2 subset (3, 9–11, 31, 33). However, the correlation between type 1 or 2 cytokines and resistance or susceptibility is not perfect even in this exemplary model (17, 18, 27, 32, 34, 40).

The immune responses to viscerotropic *Leishmania* species have previously been examined in mouse livers (16, 20–26, 38, 41, 45) and spleens (13, 23, 26, 36, 45), the major sites of parasite proliferation in natural disease. The effects of endogenous IL-2 (24, 26), IFN-γ (26), tumor necrosis factor alpha (TNF-α) (41), and granulocyte-macrophage colony-stimulating factor (20) on the hepatic parasite load were studied by treating infected mice with anticytokine antibodies. Early in the disease, all these cytokines contributed to reducing hepatic parasite proliferation, but only TNF-α controlled further progression of infection. The treatment of mice with exogenous IL-2 (24, 26), IFN-γ (26), IL-12 (21), and granulocyte-macrophage colony-stimulating factor (20), but not with TNF-α (41), showed a leishmanicidal effect in the liver. In *L. donovani*-infected mice, the genetically controlled or vaccine-induced rate of cure was not determined by differential production of Th1- or Th2-type cytokines (13).

Helminthic infections, characterized by increased immunoglobulin E (IgE) production, eosinophilia, and mastocytosis, are considered one of the strongest stimuli for a dominance of Th2-type responses (7, 35, 42). They were reported to approach 100% in some developing countries (reference 2 and references therein), and a study of human visceral leishmaniasis in an area where *L. chagasi* is endemic (46) disclosed that over 50% of the population was also infected by different helminthiases. The prevalence of *Leishmania*-helminth coinfection in humans is unknown, but information is needed on the potential effects of coinfection in a mammalian host. In this work, we examined the influence of prior infection with *Trichinella spiralis* on parasitological and immunological aspects of visceral murine leishmaniasis.

MATERIALS AND METHODS

Mice and experimental protocol. Five-week-old female BALB/c mice (Iffa Credo, L'Arbresle, France), maintained in a positive pressure chamber (Flufrance), were used for experimentation at 7 to 8 weeks of age. Mice were split into following groups of 10 animals: (i) mice infected with *L. infantum* only (L mice), (ii) mice infected with *T. spiralis* only (T mice), (iii) mice coinfected with *L. infantum* and *T. spiralis* (LT mice), and (iv) noninfected littermates (used as age-matched controls). First, two groups of mice (T and LT mice) were infected with *T. spiralis* larvae; next, 7 days later, two groups of mice (L and LT mice) were infected with *L. infantum*. Hereafter, the day of infection with *L. infantum* is termed day 0. Eighteen and seventy days after *L. infantum* inoculation, five mice from each group were anesthetized (sodium thiopental [50 μg/g of body weight]) and sacrificed. Livers, spleens, and diaphragms were taken under sterile conditions and weighed. Blood samples for serum separation were obtained from the orbital chamber and pooled within each group. Blood samples for the preparation of peripheral blood mononuclear cells (PBMC) were drawn from the vena cava and also pooled.

Parasites and infection. *L. infantum* MON1 (MHOM/FR/94/LPN101) was maintained by serial passages in Syrian hamsters. The promastigote form was cultured under standard conditions (39) and used for infection after three in vitro

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passages as stationary-phase cells from 7-day-old cultures (2.5×10^7 promastigotes/ml). After two washings (15-min sedimentation at 2,500 × *g* and 4°C), promastigotes were resuspended in 0.9% NaCl at 2×10^8 cells/ml and injected into the caudal vein at 10^8 promastigotes/mouse. The evaluation of *Leishmania* infection was carried out by blinded microscopic enumeration with Giemsa-stained liver and spleen touch prints. The parasite burden was expressed in Leishman Donovan units (LDU) (37) as follows: LDU = number of amastigotes per 1,000 nucleated cells × organ weight (in grams) × 2×10^5. Results below are presented in millions.

T. spiralis was maintained by passages in Wistar rats. For infection and *T. spiralis* antigen preparation, infective muscle larvae (ML) were isolated from rats by pepsin-chlorhydric acid digestion. Briefly, minced muscles were incubated for 2 h at 37°C in 1% pepsin (1:10,000; Sigma), dissolved in 60 mM HCl, under constant stirring. After muscle digestion, ML were allowed to sediment for 30 min at room temperature, washed six times in 0.9% NaCl, and counted in a Nageotte cell. On average, 10^3 larvae/g of infected muscle were collected. Mice received orally through a soft feeding tube (Marquat; Genie Biomedical) 600 ML in 0.5 ml of 0.9% NaCl. For evaluation of the *T. spiralis* load on days 18 and 70 (corresponding to 25 and 77 days after *T. spiralis* infection, respectively), ML were isolated from pooled diaphragms by the same pepsin digestion procedure and blindly counted.

Isolation of PBMC. From 1.5 to 2 ml of blood/group of five mice was collected. PBMC were isolated by centrifugation of blood over lymphocyte separation medium (Eurobio, Les Ulis, France). Interface cells (2×10^6 to 6×10^6 PBMC) were washed four times in 0.9% NaCl at 4°C, and then 1 ml of RNAzol solution (RNA-B; Bioprobe) was immediately added on ice. Tubes were vigorously vortexed and stored at −80°C prior to RNA preparation.

RNA preparation. Four fragments of each spleen (10 to 15 mg/fragment) and liver (20 to 25 mg/fragment) were separated from the rest of the organ and immediately immersed in liquid nitrogen. For each organ, four lots were composed and stored in 2-ml congelation tubes (Costar) at −170°C; each lot consisted of five equal-weight fragments (one from each mouse) pooled within each experimental group. To prepare RNA samples, ice-cold RNAzol solution (RNA-B; Bioprobe) was added directly to a frozen lot of spleen or liver fragments (1 ml/50 mg of tissue) and tissues were ground. RNAs were prepared according to the manufacturer's instructions, analyzed on a 1% agarose gel, quantitated by spectrometry, and stored at −80°C until used.

PCR analysis. For each cDNA preparation, reaction mix [1× reverse transcriptase (RT) buffer (Boehringer), 1 mM deoxynucleotides (Promega), 3 μg of $oligo(dT)_{10}$ (Boehringer) per ml, 0.6 mM spermidine (Sigma), 0.8 U of RNasin (Promega) per μl] was prepared with (RT^+ solution) or without (RT^- solution) 0.5 U of avian myeloblastosis virus RT (Boehringer) per μl. For each sample, 2 μg of denatured RNA was added to RT^+ and RT^- solutions, and reverse transcription was carried out in a final volume of 25 μl at 42°C for 90 min. cDNAs were used immediately or stored at −20°C. The PCR oligonucleotide primers chosen for IFN-γ, IL-4, IL-5, and β-actin have previously been described (5). The PCR conditions (except for β-actin) were as follows. Each cDNA (or mock cDNA from control RT^- solution) (5 μl) in 25 μl of reaction mix (1× *Taq* polymerase buffer [Appligene], 0.2 mM deoxynucleotides [Promega], 1 μM primers each [R&D Systems], 0.014 U of *Taq* DNA polymerase [Appligene] per μl) was amplified after denaturation (2 min at 90°C) by 30, 35, and 40 cycles (denaturation for 1 min at 90°C, annealing for 1 min at 55°C, and polymerization for 1 min at 70°C), with termination by elongation at 70°C for 7 min. For β-actin, denaturation was carried out at 94°C. Lymphokine transcript levels were determined after normalization to the β-actin level to compensate for intersample differences in RNA integrity, cDNA synthesis, or PCR efficiency. In some series of experiments (see Fig. 2), liver and spleen cDNA samples were normalized independently, whereas in other series (see Fig. 2), cDNA samples from different organs were normalized together. RT-PCR products were subjected to electrophoresis on 1% agarose gels, photographs were scanned with an Ultro-Scan laser densitometer, and the signal intensities (SI) at a nonsaturating number of cycles (see the legend to Fig. 2) were analyzed by using NIH Image 1.54. The results were expressed as follows: (SI for infected mice − SI for control mice)/SI for control mice.

Spleen cell preparation and culture. Suspensions of spleen cells were obtained by squeezing pooled spleens (from a group of five mice) with a syringe plunger in 10 ml of RPMI 1640, allowing the debris to settle out for 2 min, and lysing erythrocytes contained in the supernatant in ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 10^{-4} M EDTA [pH 7.25]) for 3 min at room temperature. After three washes in RPMI 1640, cells were resuspended in complete culture medium (39) at 2.5×10^6 cells/ml, seeded at 500 μl/well on 24- or 48-well plates, and cultured at 37°C in a 5% CO_2, humidified atmosphere. Cell activation was initiated 16 to 24 h later. Specific cell stimulation was performed by the addition of live parasites (1×10^6, 2×10^6, and 4×10^6 promastigotes/ml) or leishmanial antigens (10, 20, and 80 μg/ml). Polyclonal activation was achieved by incubation with concanavalin A and phorbol 12-myristate 13-acetate at the concentrations indicated in figure legends. After an additional 48 h of incubation, plates were stored at −20°C until cytokine production was measured.

Lymphokine assays. The IFN-γ and IL-4 levels in lysates of spleen cells were determined by indirect sandwich enzyme-linked immunosorbent assays (ELISAs) (Duoset Genzyme), which detect 10 pg of cytokines per ml, according to the manufacturer's instructions.

Antigen preparation. Leishmanial antigen preparation for functional studies was carried out as described previously (39). *T. spiralis* ML were suspended at 5,000 ML/ml in water containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 1,000 U of aprotinin per ml) and disrupted first in a Dounce homogenizer, then by five cycles of freeze (liquid nitrogen)-thaw (37°C), and finally by eight sonications (1 min) on ice. The suspensions were centrifuged (15,000 × *g* at 4°C for 15 min), and the supernatants were stored at −80°C. The protein concentrations were determined with a MicroBCA protein assay reagent kit (Pierce).

Detection of anti-*T. spiralis* IgE. Specific IgE in the sera of infected and control mice was detected by a classic ELISA procedure. Briefly, wells of high-binding-capacity microtiter plates (Greiner) were coated overnight with 1 μg of *T. spiralis* soluble extract in 0.1 ml of 0.1 M phosphate buffer (PB) pH 7.2. After being washed with PB, plates were saturated with 1% bovine serum albumin (BSA) in 0.2 M Tris-HCl buffer (Tris-BSA) for 30 min and incubated with duplicate 0.1-ml aliquots of mouse serum samples, previously diluted 1/20 with Tris-BSA, for 1 h. Plates were extensively washed with PB-saline containing 0.1% Tween 20 and further incubated for 1 h with 0.1 ml of horseradish peroxidase-labeled rat monoclonal anti-mouse IgE (Pharmingen), diluted 1/1,000 in Tris-BSA. After being washed as described above, 0.1 ml of substrate solution (0.1 M PB-citrate [pH 5.5], 0.02% H_2O_2, 3 mg of OPD · 2HCl per ml) was added, and plates were incubated for 30 min in the dark. All procedures were carried out at room temperature. The enzymatic reaction was quenched with 0.1 ml of 2 N H_2SO_4, and the absorbances were read at 492 nm in an automatic plate reader (MR 5000; Dynatech).

Statistical analysis. The differences between experimental groups were analyzed by Student's *t* test (StatWorks).

RESULTS

Clinical evaluation of infected and coinfected mice. Wasting, hepatomegaly, and splenomegaly are known aspects of human visceral leishmaniasis. The weights of *L. infantum*-infected BALB/c mice (L mice) followed the standard weight curve. No hepatomegaly was observed in either group (mean liver weight ± standard deviation [SD] was 1.3 ± 0.2 g). In contrast, spleen weights increased from 140 ± 32 mg on day 0 to 198 ± 37 and 148 ± 34 mg on day 18 and to 442 ± 63 and 249 ± 131 mg on day 70 for L and LT mice, respectively. The degree of splenomegaly correlated with the leishmanial burden (see below).

Parasite burdens in infected and coinfected mice. The *L. infantum* loads in the livers and spleens of L and LT mice are shown in Fig. 1. Eighteen days after *L. infantum* inoculation (day 18), the hepatic amastigote load (Fig. 1A) of LT mice (mean ± SD, 33.4 ± 10.0 LDU; range, 23 to 44 LDU) was significantly lower ($P < 0.001$) than that of L mice (116.9 ± 34.0 LDU; range, 73 to 150 LDU). On day 70, the hepatic load of L mice decreased, as expected (references 23 and 45 and our unpublished observations). In L mice, it was 16.1 ± 7.9 LDU (range, 9 to 24 LDU), roughly the same as that in LT mice (26.8 ± 19.2 LDU; range, 15 to 49 LDU). Unlike in the liver, in the spleen *L. infantum* multiplication continued between days 18 and 70 (references 36 and 45 and our unpublished observations). On day 18, the splenic parasite burden was very low and statistically not different between these two groups (L mice, 0.2 ± 0.1 LDU [range, 0.2 to 0.3 LDU]; LT mice, 0.5 ± 0.5 LDU [range, 0.1 to 1.5 LDU]). It increased notably on day 70 in the spleens of L mice (6.6 ± 2.4 LDU; range, 5 to 9 LDU), and at that time it was significantly higher ($P = 0.004$) than was the leishmanial burden in LT mice (0.8 ± 0.9 LDU; range, 0 to 2 LDU).

On day 18 (25 days after *T. spiralis* ingestion), the ML burden in the diaphragms of LT mice was notably lower than that of T mice (4.7 ± 1.1 and 24.9 ± 3.7 ML/mg of diaphragm, respectively) and still slightly lower on day 70 (18.1 ± 2.7 and 28.6 ± 4.3 ML/mg, respectively).

Immunological status of mice at the time of infection with *L. infantum*. The immunological statuses of T mice and uninfected controls were determined on the day of *L. infantum* inoculation. The mRNA expression levels of type 1 and type 2

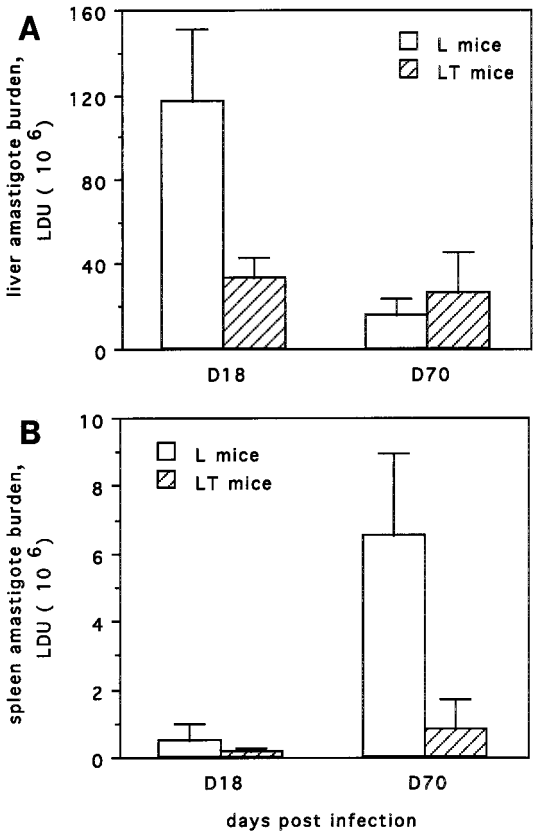

FIG. 1. Effects of prior infection with *T. spiralis* on amastigote burden in the livers (A) and spleens (B) of BALB/c mice, as determined 18 (D18) and 70 (D70) days after *L. infantum* inoculation. Mice were either infected on day 0 with *L. infantum* (L mice) or first infected with *T. spiralis* and then 7 days later infected on day 0 with *L. infantum* (LT mice). Parasitic loads were assessed by blinded microscopic enumeration with Giemsa-stained liver and spleen imprints. The data shown are means ± SDs for five mice in each group and at each time point, from one representative experiment of the two performed. (In the experiment not shown, the hepatic load on day 18 and the splenic load on day 70 for L mice were significantly higher than those for LT mice [$P = 0.03$ and 0.04, respectively].)

cytokines were assessed in blood, liver, and spleen samples by RT-PCR, and the in vitro capacity of spleen cells to produce cytokines was measured by ELISAs. Figure 2 shows that IFN-γ transcripts were overexpressed in blood and liver samples and that IL-4 transcripts were overexpressed in blood, liver, and spleen samples of T mice compared to the levels in controls; IL-5 mRNAs were detected only in infected animals (the SI is indicated in arbitrary units). Figure 3A and C show that under polyclonal in vitro stimulation to gauge the potential of preinfected cells for development into either Th1 or Th2 cells, the production levels of both IFN-γ and IL-4 in T mice were much higher than those in control mice.

Evolution of cytokine mRNA expression in livers and spleens of infected and coinfected mice. The in situ cellular responses to disease in the livers and spleens of L and LT mice were assessed in terms of IFN-γ, IL-4, and IL-5 mRNA expression by RT-PCR. Note that no direct comparison between cytokine mRNA levels in the liver and spleen should be made here; in these series of experiments, the RT-PCR analyses of RNAs from these two organs were performed independently. Figure 2 shows that on days 18 and 70 in the liver, IFN-γ mRNAs were overexpressed for L and LT mice, whereas IL-4 mRNAs were more greatly increased for LT mice. In the spleen, the most striking feature was overexpression of IFN-γ mRNAs in L and LT mice on day 70, compared with moderate increases in IFN-γ mRNAs on day 18 and in type 2 (IL-4 and IL-5) transcripts.

Capacity of in vitro-activated spleen cells to produce type 1 and type 2 cytokines. The capacity of spleen cells to respond to *Leishmania*-specific in vitro stimulation was analyzed by measuring IFN-γ and IL-4 production. Spleen cells were activated by 48-h incubation with live promastigotes (at 1×10^6, 2×10^6, and 4×10^6 parasites/ml) or total leishmanial antigens (at 10, 20, and 80 μg/ml), and the cytokine levels were measured in cell lysates. On day 18, there was no detectable cytokine production induced by cell stimulation in either group, whereas on day 70, spleen cells did respond to specific activation. Figure 3B and D show that on day 70, after stimulation with live parasites cells from L mice produced markedly more IFN-γ than did cells from LT mice. Promastigote-induced IL-4 secretion was slightly higher for L mice than it was for LT mice, and both were higher than that for controls. The same profiles and orders of magnitude for cytokine production were induced by total leishmanial antigens (not shown). Cells from all groups of infected mice showed basal cytokine levels that were greater than those of controls, probably due to the presence of parasites in spleen cell preparations (estimated to be 0.5×10^5 to 1×10^5/well for L mice and 1×10^4 to 1.5×10^4/well for LT mice [corresponding to 0.2 and 0.03 μg of leishmanial proteins, respectively]). We found by cytofluorimetry that T lymphocytes, B lymphocytes, and macrophages represented 55 to 65, 30 to 40, and 10 to 18% of spleen cells, respectively, and that there was no major differences between the results for various experimental groups of mice.

IgE response. Anti-*T. spiralis* IgE and anti-*L. infantum* IgE in pooled serum of five mice from each experimental group were measured by ELISA. Specific anti-*T. spiralis* larva IgE levels, expressed as optical density readings, were as follows (mean ± SD): 0.10 ± 0.01 on day 0 for T mice; 0.19 ± 0.01 and 0.14 ± 0.001 on day 18 for T and LT mice, respectively; 0.28 ± 0.003 and 0.40 ± 0.004 on day 70 for T and LT mice, respectively; 0.070 ± 0.01 for L mice and controls. Specific anti-*L. infantum* IgE was not detectable in any group of mice.

DISCUSSION

We studied the influence of the immunological environment created by preinfection with the nematode *T. spiralis* on the course of murine visceral leishmaniasis. In general, intracellular parasitism is associated mainly with type 1 cytokine responses, whereas extracellular helminths induce predominantly type 2 responses (12, 19, 35, 42). Our working assumption was that the course of leishmaniasis in mice infected first with *T. spiralis* and then 1 week later with *L. infantum* (LT mice) would be more severe than that in mice infected with *Leishmania* only (L mice). Unexpectedly, in LT mice, both pathologies, leishmaniasis and trichinellosis, appeared to take a markedly milder progression than in simply infected L or T mice.

On the day of *Leishmania* inoculation, mice infected with *T. spiralis* 7 days earlier presented a hyperstimulated immunological state, compared to that of controls. It involved both type 1 (IFN-γ) and type 2 (IL-4 and IL-5) cytokines. It was manifest both locally (in the liver and spleen) and systemically (in the blood). It was characterized at the transcriptional level by overexpression of IFN-γ, IL-4, and IL-5 mRNAs and at the protein level by the potential of spleen cells to synthesize IFN-γ and IL-4 after in vitro polyclonal stimulation. This apparent lack of cross-regulation between type 1 and type 2 activities in early infection with *T. spiralis* (8, 14, 28) suggests

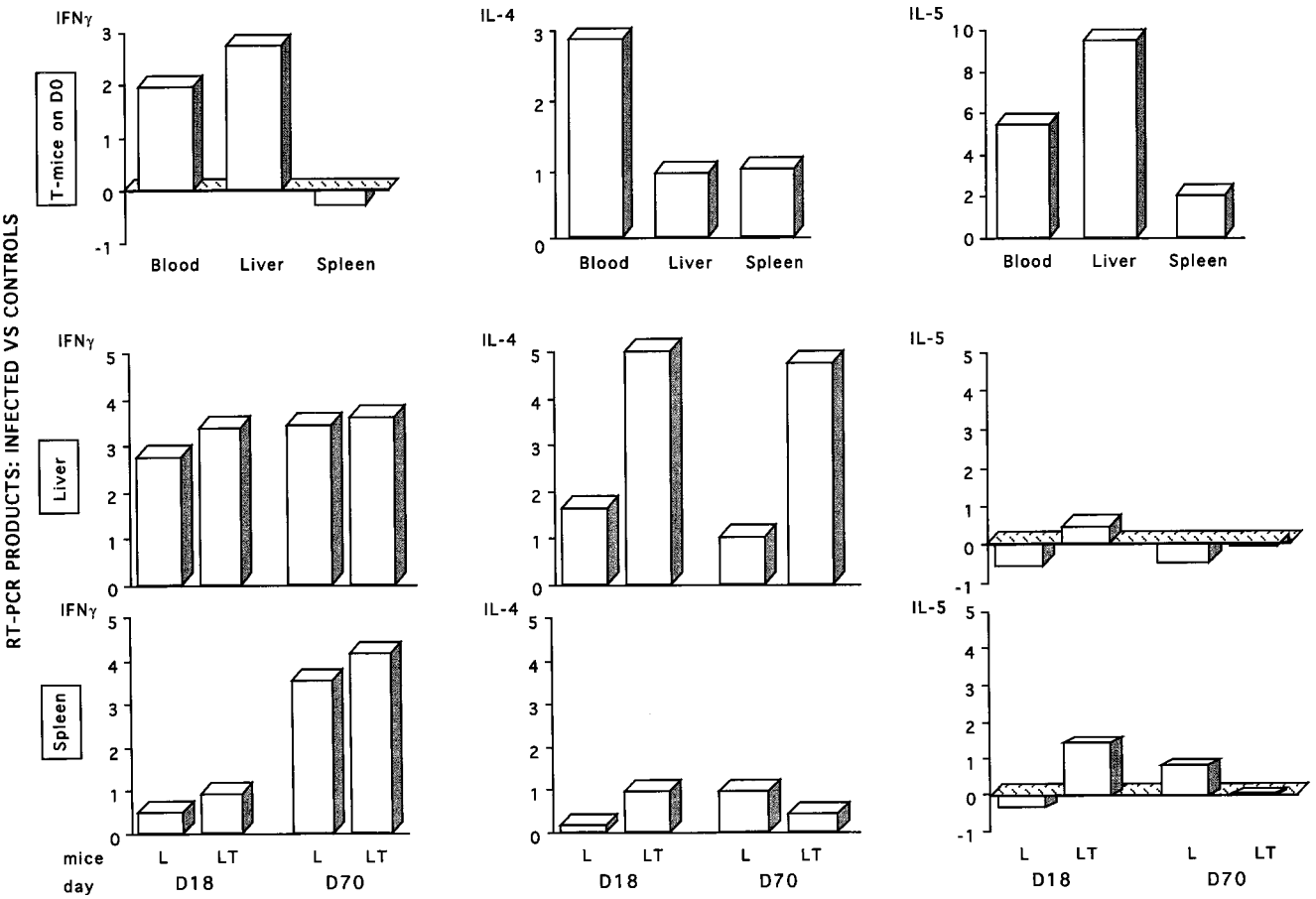

FIG. 2. RT-PCR analysis of cytokine mRNAs. (Top) cDNAs synthesized from transcripts expressed on day 0 (D0) in mice infected 7 days earlier with *T. spiralis* (T mice) and in age-matched controls in blood, liver, and spleen samples and normalized to β-actin cDNA levels. (Middle and bottom) cDNAs synthesized from transcripts and normalized to β-actin cDNA levels in pooled samples of livers and spleens, respectively, of L, LT, and age-matched control mice 18 (D18) and 70 (D70) days after *L. infantum* inoculation. Photographs of electrophoresed RT-PCR products were analyzed by densitometry. Each histogram represents the following ratio: (SI for infected mice − SI for control mice)/SI for control mice (except for IL-5 on day 0, as indicated in the text). The results correspond to SI at a nonsaturating number of cycles of DNA polymerization as follows: for top histograms, IFN-γ, 35 cycles; IL-4, 35 (blood) and 40 (liver and spleen) cycles; IL-5, 40 cycles. For middle histograms, all cDNAs were amplified for 35 cycles. For bottom histograms, IFN-γ and IL-4, 30 cycles; IL-5, 35 cycles.

that cells other than those which belong to Th1 and Th2 subpopulations contribute to cytokine production (12, 19).

The next assessment was carried out 18 days after *Leishmania* inoculation. Day 18 of leishmaniasis in BALB/c mice (*Lsh*^{s} *H-2*^{d}) infected with viscerotropic *Leishmania* species corresponds to the acute phase of disease, as defined by hepatic parasite proliferation (4). We found that although on day 18 the amastigote burden in the livers of L mice was indeed high, in the livers of LT mice it was significantly lower ($P < 0.001$). Our analysis of the immune state in the liver at the same stage of the disease (day 18) showed that both IFN-γ and IL-4 mRNAs were overexpressed by L and LT mice.

We then showed that preinfection of mice with *T. spiralis* continued to exert its protective effect on the course of leishmaniasis beyond the acute phase. At the end of the eighth or ninth week after infection, the hepatic parasite counts in BALB/c mice drop to very low levels (23, 45). This stage of disease is referred to as the chronic or recovery phase (4). During this phase, however, *Leishmania* organisms do proliferate in the spleen and the splenic burden steadily increases (references 36 and 45 and our unpublished observations). We showed that in contrast to L mice, which allowed notable parasite multiplication between days 18 and 70, LT mice delayed, in fact almost prevented, amastigote proliferation; the splenic load of LT mice, as measured 10 weeks after *Leishmania* infection, was significantly lower ($P = 0.004$) than that of L mice. A concurrent analysis of the cellular immune response in the spleen on day 70 showed that IFN-γ mRNA was overexpressed in both L and LT mice. In vitro cytokine production after *Leishmania*-specific stimulation was also enhanced in both groups, but spleen cells from L mice showed a much higher capacity to produce IFN-γ than did cells from LT mice.

An attempt at establishing a direct correlation between parasitological and immunological states at a given time would probably be unreliable. For instance, on day 70 the splenic amastigote load of L mice was high in spite of high IFN-γ levels and that of LT mice was low in spite of high IL-4 levels. However, the parasite burden, as determined at a given time, is more the result of all previous immune responses rather than a direct reflection of the concurrent immunological environment. The protection from *L. infantum* manifested by LT mice on days 18 and 70 may have been due partly to high levels of IFN-γ present locally and systemically on day 0, but it has previously been shown that in murine visceral leishmaniasis IFN-γ is not a unique protective cytokine (41). A recent study (45) of cytokine responses at 6 weeks after *L. infantum* infection of BALB/c mice showed that in vitro-stimulated spleen cells produced IFN-γ, whereas T cells isolated from liver gran-

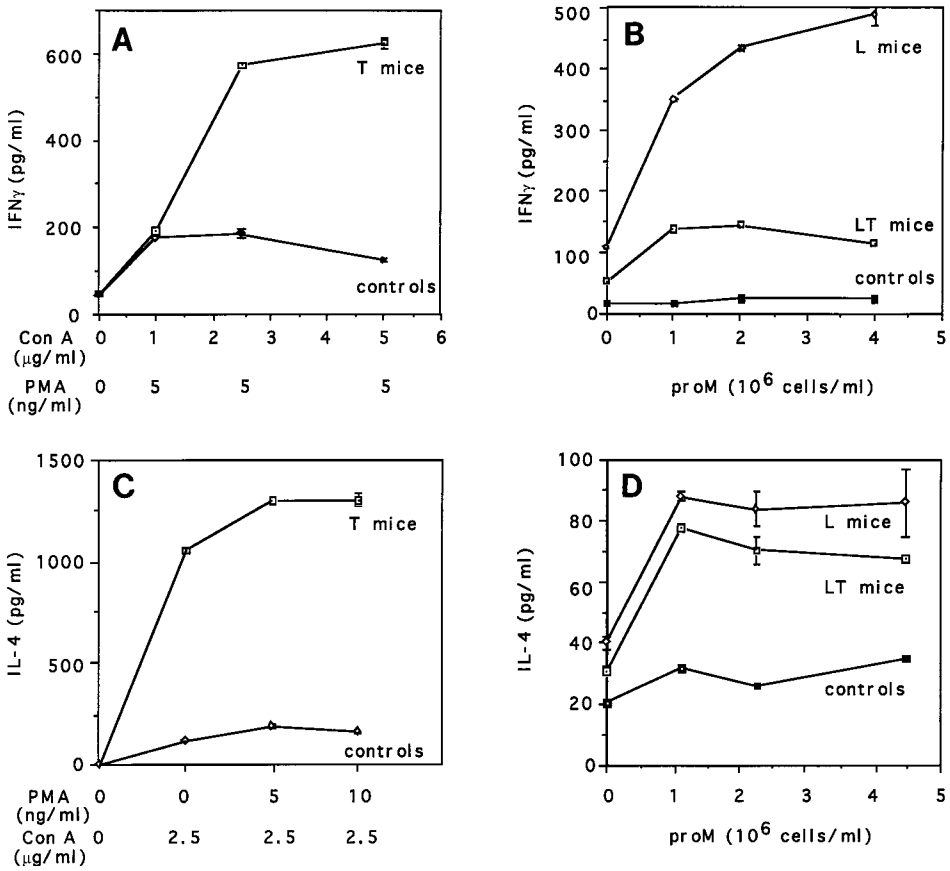

FIG. 3. Capacity of spleen cells to synthesize IFN-γ (A and B) and IL-4 (C and D) after polyclonal (A and C) or *Leishmania*-specific (B and D) in vitro stimulation. (A and C) Spleen cells isolated on day 0 from five T mice and five age-matched naive controls were activated by incubation for 48 h with the indicated concentrations of concanavalin A (Con A) and phorbol 12-myristate 13-acetate (PMA) (2.5×10^6 cells/ml), and cytokine synthesis was measured in duplicate in cell lysates by sandwich ELISAs. The results shown are from one representative experiment of the two performed and are means ± SDs. (B and D) Spleen cells were isolated from L, LT, and age-matched naive mice 70 days after *L. infantum* inoculation and activated by increasing concentrations of promastigotes (proM), as indicated. Sandwich ELISAs were carried out after 48 h of incubation; the results shown are from one representative experiment of the two performed and are means ± SDs of duplicate determinations.

ulomas did not. The authors concluded that the absence of IFN-γ in the liver correlated with high amastigote burden and that its presence in the spleen correlated with increased parasite killing. However, at the time of those analyses, although the hepatic load was higher than that in the spleen, it was in its decreasing phase, whereas the contrary was true for the splenic load (low but increasing). An interesting model (22), in which BALB/c mice were presensitized with injections of heat-killed *L. major* promastigotes, was recently reported. That manipulation induced a cross-reactive Th2 response which exacerbated visceral leishmaniasis (22), but our model of coinfection with two live unrelated pathogens enhanced both the Th1 and Th2 early environments and slowed the progression of the disease. The data reported here are relative to a particular protocol in which *T. spiralis* inoculation was carried out at the end of the intestinal phase and the beginning of the larva migration phase of trichinellosis (6). Whether the chronic phase of trichinellosis has an effect on the course of leishmaniasis is unknown. In yet another model of coinfection (1), *L. tropica*-infected BALB/c mice reportedly had the ability to partially inhibit *Toxoplasma gondii* replication in vivo.

One of our present hypotheses on mechanism(s) of *T. spiralis*-induced modulation of the course of visceral leishmaniasis is based on a recent work by Vouldoukis et al. (44). They showed that ligation of the low-affinity receptor for IgE (FcεRII/CD23) induced the killing of *L. major* amastigotes due to the generation of nitric oxide in human macrophages in vitro. Moreover, induction of receptor CD23 by IL-4 on human monocytes has been previously shown (43). Whether these mechanisms hold in vivo and in other animal models and whether the ligation of CD23 induces the killing of *Leishmania* species other than *L. major* remain to be established. In our model, *T. spiralis*-specific IgE was produced quite early in infection. The concomitant presence in LT mice of specific IgE and the corresponding *T. spiralis* antigens could induce the formation of immune complexes, CD23 ligation, and subsequent amastigote killing. If this mode of action proves to be real and can be generalized, then coinfection with other helminths may also bring about modulation of the course of leishmaniasis.

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REFERENCES

1. **Aslan, G., K. Altintas, A. Seyrek, S. Tasci, and S. Atakan.** 1997. The effect of *L. tropica* on experimental toxoplasmosis in mice. Acta Parasitol. Turcica **21:**33.

2. **Bell, R. G.** 1996. IgE, allergies and helminth parasites: a new perspective on an old conundrum. Immunol. Cell Biol. **74:**337–345.
3. **Belosevic, M., D. S. Findbloom, P. H. Van Der Meide, M. V. Slayter, and C. A. Nacy.** 1989. Administration of monoclonal anti-IFNγ antibodies *in vivo* abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. J. Immunol. **143:**266–274.
4. **Bradley, D. J.** 1987. Genetics of susceptibility and resistance in the vertebrate host, p. 551–577. *In* W. Peters, and R. Killick-Kendrick (ed.), The leishmaniasis in biology and medicine. Academic Press, London, United Kingdom.
5. **Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.).** 1994. Current protocols in immunology, unit 10.23, supplement 11. John Wiley & Sons, Inc., New York, N.Y.
6. **Dupouy-Camet, J.** 1991. Conséquences physiopathologiques et immunologiques de l'infestation par *Trichinella*, p. 111–134. *In* C. Soulé and J. Dupouy-Camet (ed.) La trichinellose: une zoonose en évolution. Office International des Epizooties, Paris, France.
7. **Finkelman, F. D., E. J. Pearce, J. F. Urban, and A. Sher.** 1991. Regulation and biological function of helminth-induced cytokine responses. Immunoparasitol. Today **12/7:**A62–A66.
8. **Frydas, S., E. Karagouni, E. Dotsika, M. Reale, R. C. Barbacane, I. Vlemmas, G. Anogianakis, A. Trakatellis, and P. Conti.** 1996. Generation of TNFα, IFNγ, IL-6, IL-4 and IL-10 in mouse serum from trichinellosis: effect of the anti-inflammatory compound 4-deoxypyridoxine (4-DPD). Immunol. Lett. **49:**179–184.
9. **Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman, and R. M. Locksley.** 1989. Reciprocal expression of interferon γ or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. J. Exp. Med. **169:**59–72.
10. **Heinzel, F. P., M. D. Sadick, S. S. Mutha, and R. M. Locksley.** 1991. Production of interferon γ, interleukin 2, interleukin 4, and interleukin 10 by $CD4^+$ lymphocytes in vivo during healing and progressive murine leishmaniasis. Proc. Natl. Acad. Sci. USA **88:**7011–7015.
11. **Holaday, B. J., M. D. Sadick, Z. E. Wang, S. L. Reiner, F. P. Heinzel, T. G. Parslow, and R. M. Locksley.** 1991. Reconstitution of *Leishmania* immunity in severe combined immunodeficient mice using Th1- and Th2-like cell lines. J. Immunol. **147:**1653–1658.
12. **Jankovic, D., and A. Sher.** 1996. Initiation and regulation of CD4+ T-cell function in host-parasite models. Chem. Immunol. **63:**51–65.
13. **Kaye, P. M., A. J. Curry, and J. M. Blackwell.** 1991. Differential production of Th1- and Th2-derived cytokines does not determine the genetically controlled or vaccine-induced rate of cure in murine visceral leishmaniasis. J. Immunol. **146:**2763–2770.
14. **Kelly, E. A. B., E. S. Cruz, K. M. Hauda, and D. L. Wassom.** 1991. IFNγ and IL-5 producing cells compartmentalize to different lymphoid organs in *Trichinella spiralis*-infected mice. J. Immunol. **147:**306–311.
15. **Locksley, R. M., and P. Scott.** 1991. Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. Immunoparasitol. Today **12/7:**A58–A61.
16. **Miralles, C. D., M. Y. Stoeckle, D. Finkelman, and H. W. Murray.** 1994. Th1 and Th2 cell-associated cytokines in experimental visceral leishmaniasis. Infect. Immun. **62:**1058–1063.
17. **Moll, H., and M. Röllinghoff.** 1990. Resistance to murine cutaneous leishmaniasis is mediated by Th1 cells, but disease-promoting $CD4^+$ cells are different from Th2 cells. Eur. J. Immunol. **20:**2067–2074.
18. **Morris, L., A. B. Troutt, K. S. McLeod, A. Kelso, E. Handman, and T. Aebisher.** 1993. Interleukin-4 but not gamma interferon production correlates with the severity of murine cutaneous leishmaniasis. Infect. Immun. **61:**3459–3456.
19. **Mosmann, T. R., and S. Sad.** 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today **17:**138–146.
20. **Murray, H. W., J. S. Cervia, J. Hariprashad, A. P. Taylor, M. Y. Stoeckle, and H. Hockman.** 1995. Effect of granulocyte-macrophage colony-stimulating factor in experimental visceral leishmaniasis. J. Clin. Invest. **95:**1183–1192.
21. **Murray, H. W., and J. Hariprashad.** 1995. Interleukin 12 is effective treatment for an established systemic intracellular infection: experimental visceral leishmaniasis. J. Exp. Med. **181:**387–391.
22. **Murray, H. W., J. Hariprashad, and R. L. Coffman.** 1997. Behavior of visceral *Leishmania donovani* in an experimentally induced T helper cell 2 (Th2)-associated response model. J. Exp. Med. **185:**867–874.
23. **Murray, H. W., H. Masur, and J. S. Keithly.** 1982. Cell-mediated immune response in experimental visceral leishmaniasis. Correlation between resistance to *L. donovani* and lymphokine-generating capacity. J. Immunol. **129:**344–349.
24. **Murray, H. W., C. D. Miralles, M. Y. Stoeckle, and D. F. McDermott.** 1993. Role and effect of IL-2 in experimental visceral leishmaniasis. J. Immunol. **151:**929–938.
25. **Murray, H. W., K. E. Squires, C. D. Miralles, M. Y. Stoeckle, A. M. Granger, A. Granelli-Piperno, and C. Bogdan.** 1992. Acquired resistance and granuloma formation in experimental visceral leishmaniasis. Differential T cell and lymphokine roles in initial versus established immunity. J. Immunol. **148:**1858–1863.
26. **Murray, H. W., J. J. Stern, K. Welte, B. Y. Rubin, S. M. Carriero, and C. F. Nathan.** 1987. Experimental visceral leishmaniasis: production of interleukin-2 and interferon γ, tissue immune reaction, and response to treatment with interleukin-2 and interferon γ. J. Immunol. **138:**2290–2297.
27. **Noben-Trauth, N., P. Kropf, and I. Müller.** 1996. Susceptibility to *Leishmania major* infection in interleukin-4-deficient mice. Science **271:**987–990.
28. **Ramaswamy, K., D. Negrao-Correa, and R. Bell.** 1996. Local intestinal immune responses to infections with *Trichinella spiralis*. Real-time, continuous assay of cytokines in the intestinal (afferent) and efferent thoracic duct lymph of rats. J. Immunol. **156:**4328–4337.
29. **Reed, S. G., and P. Scott.** 1993. T-cell and cytokine responses in leishmaniasis. Curr. Opin. Immunol. **5:**524–531.
30. **Reiner, S. L., and R. M. Locksley.** 1995. The regulation of immunity to *Leishmania major*. Annu. Rev. Immunol. **13:**151–177.
31. **Sadick, M. D., R. M. Locksley, C., Tubbs, and H. V. Raff.** 1986. Murine cutaneous leishmaniasis: resistance correlates with the capacity to generate interferon-γ in response to leishmania antigens *in vitro*. J. Immunol. **136:**655–661.
32. **Sadick, M. D., N. Street, T. R. Mosmann, and R. M. Locksley.** 1991. Cytokine regulation of murine leishmaniasis: interleukin 4 is not sufficient to mediate progressive disease in resistant C57BL/6 mice. Infect. Immun. **59:**4710–4714.
33. **Scott, P.** 1991. IFNγ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. J. Immunol. **147:**3149–3155.
34. **Scott, P., A. Eaton, W. C. Gause, X. D. Zhu, and B. Hondowicz.** 1996. Early IL-4 production does not predict susceptibility to *Leishmania major*. Exp. Parasitol. **84:**178–187.
35. **Sher, A., and R. L. Coffman.** 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. Annu. Rev. Immunol. **10:**385–409.
36. **Smelt, S. C., C. R. Engwerda, M. McCrossen, and P. M. Kaye.** 1997. Destruction of follicular dendritic cells during chronic visceral leishmaniasis. J. Immunol. **158:**3813–3821.
37. **Stauber, L. A.** 1958. Host resistance to the Khartoum strain of *Leishmania donovani*. Rice Inst. Pamphlets **45:**80–83.
38. **Stern, J. J., M. J. Oca, B. Y. Rubin, S. L. Anderson, and H. W. Murray.** 1988. Role of $L3T4^+$ and $LYT\text{-}2^+$ cells in experimental visceral leishmaniasis. J. Immunol. **140:**3971–3977.
39. **Suffia, I., J. F. Quaranta, M. C. Eulalio, B. Ferrua, P. Marty, Y. Le Fichoux, and J. Kubar.** 1995. Human T-cell activation by 14- and 18-kilodalton nuclear proteins of *Leishmania infantum*. Infect. Immun. **63:**3765–3771.
40. **Swihart, K., U. Fruth, N. Messmer, K. Hug, R. Behin, S. Huang, G. D. Guidice, M. Aguet, and J. A. Louis.** 1995. Mice from genetically resistant background lacking the interferon-γ receptor are susceptible to infection with *Leishmania major* but mount a polarized T helper cell 1-type $CD4^+$ T cell response. J. Exp. Med. **181:**961–971.
41. **Tumang, M. C. T., C. Keogh, L. L. Moldawer, D. C. Helfgott, R. Teitelbaum, J. Hariprashad, and H. W. Murray.** 1994. Role and effect of TNFα in experimental visceral leishmaniasis. J. Immunol. **153:**768–775.
42. **Urban, J. F., K. B. Madden, A. Svetic, A. Cheever, P. P. Trotta, W. C. Gause, I. D. Katona, and F. Finkelman.** 1992. The importance of Th2 cytokines in protective immunity to nematodes. Immunol. Rev. **12:**205–220.
43. **Vercelli, D., H. H. Jabara, B. W. Lee, N. Woodland, R. S. Gha, and D. Y. M. Leung.** 1988. Human recombinant interleukin 4 induces FCRE/CD23 on normal human monocytes. J. Exp. Med. **167:**1406–1417.
44. **Vouldoukis, I., V. Riveros-Moreno, B. Dugas, F. Ouaaz, P. Bécherel, P. Debré, S. Moncada, and M. D. Mossolayi.** 1995. The killing of *Leishmania major* by human macrophages is mediated by nitric oxide induced after ligation of the FcεRII/CD23 surface antigen. Proc. Natl. Acad. Sci. USA **92:**7804–7808.
45. **Wilson, M. E., M. Sandor, A. M. Blum, B. M. Young, A. Metwali, D. Elliott, R. G. Lynch, and J. V. Weinstock.** 1996. Local suppression of IFNγ in hepatic granulomas correlates with tissue-specific replication of *Leishmania chagasi*. J. Immunol. **156:**2231–2239.
46. **Zwingenberger, K., G. Harms, C. Pendrosa, S. Omena, B. Sandkamp, and S. Neifer.** 1990. Determinants of the immune response in visceral leishmaniasis: evidence for predominance of endogenous interleukin 4 over interferon γ production. Clin. Immunol. Immunopathol. **57:**242–249.

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