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Infection of BALB/c Mice with the Filarial Nematode *Litomosoides sigmodontis*: Role of CD4$^+$ T Cells in Controlling Larval Development

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***Litomosoides sigmodontis* is the only filaria which develops from infective larvae into adults in immunocompetent laboratory mice. Depletion of CD4$^+$ T cells from infected BALB/c mice resulted in worm and microfilarial burdens significantly higher than those of infected controls. Th2 cytokines, eosinophilia, and immunoglobulin E, which were strongly induced in infected controls, were diminished in CD4-depleted mice.**

Filariasis is an arthropod-borne parasitism which affects animals and humans, debilitating more than 120 million people (26, 27). Infective third-stage larvae (L3 larvae) are injected into the host during a blood meal of the arthropod vector and develop into adult worms which release microfilariae (Mf) into either the skin (e.g., onchocerciasis) or the blood (lymphatic filarioses).

None of the murine models in which immunity to filarial infection has been analyzed (1, 2, 5, 13, 21) allows development of L3 larvae into adult worms in immunocompetent inbred animals. Therefore, we made use of the experimental infection of BALB/c mice with *Litomosoides sigmodontis* filarial nematodes, originally established by Petit et al. (16). This model accommodates the full cycle from L3 larvae to the release of Mf in the BALB/c mouse and thus allows study and modulation of immune responses during L3 larva maturation. We compared infected normal BALB/c mice with those whose CD4$^+$ T cells had been depleted by injection of anti-CD4 antibodies prior to infection and at weekly intervals thereafter.

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The *L. sigmodontis* strain was maintained by passage in cotton rats (*Sigmodon hispidus*) as described previously (28). The infection was done by a natural route (28), i.e., mites (*Ornithonyssus bacoti* [22]) were fed on microfilaremic (>2,000 Mf/μl of blood) cotton rats. After 10 days, the mites were allowed to feed on 5-week-old BALB/c mice (Charles River, Sulzfeld, Germany), thereby transmitting L3 larvae to the mice. To ensure comparability, equal numbers of anti-CD4-treated and control mice were always exposed to the same population of mites.

For depletion of CD4$^+$ T cells, mice were injected with anti-CD4 antibodies 1 day prior to *L. sigmodontis* infection and at weekly intervals thereafter until termination of the experiments. In five experiments, mice were injected with ascites as the antibody source, with 200 μg of anti-CD4 antibody per dose (four experiments with GK1.5 [8] ascites and one experiment using YTS 191.1 [6] ascites revealed consistent results). In a sixth experiment, mice were injected with a dose of 500 μg of GK1.5 monoclonal antibody (MAb) purified from culture supernatants by using a protein G-Sepharose Fast Flow column (Pharmacia, Freiburg, Germany). Control mice were injected with rat immunoglobulins. In all trials, the efficacy of anti-CD4 treatment was monitored by fluorescence-activated cell sorter analysis of peripheral blood as well as pleural exudate cells (PLEC) and splenic cells, with anti-CD4 as well as anti-CD3 antibodies used to exclude the possibility of a mere downregulation of the CD4 molecule instead of depletion.

Motile adult worms in the thoracic cavity, the representative site for assessment of worm numbers (15), were counted at day 28 postinfection (p.i.) after the cavity was flushed with phosphate-buffered saline–1% fetal calf serum. Microfilaremia was determined from peripheral blood treated with EDTA after staining with Hinkelmann's solution (11). Eosinophilia and eosinophils in PLEC were also determined by staining with Hinkelmann's solution (11).

Cells were cultured at 28 days p.i. Splenic cells were depleted from erythrocytes by incubation for 5 min with Tris-ammonium chloride at room temperature. PLEC were depleted from macrophages by adhesion on a petri dish at 37°C for 2 h. Depletion of macrophages was verified by fluorescence-activated cell sorter analysis. Cells were cultured separately for each animal at 37°C and 5% CO_2 in 96-well microtiter plates (total volume, 200 μl; cell density, 2×10^5 cells/well) in triplicate containing either RPMI medium–10% fetal calf serum alone, concanavalin A (ConA) (2.5 μg/ml), or *L. sigmodontis* antigen (LsAg) (25 μg/ml). Supernatants were removed 96 h after stimulation for cytokine analysis.

Concentrations of cytokines gamma interferon (IFN-γ), interleukin 4 (IL-4), IL-5, and IL-10 in culture supernatants as well as of total serum immunoglobulin E (IgE) were determined by specific two-site enzyme-linked immunosorbent assays (ELISAs) using MAb pairs and protocols from Pharmingen (Hamburg, Germany). Recombinant cytokines (Pharmingen) and murine IgE protein (MAb clone SPE-7, anti-2,4-dinitrophenol [anti-DNP]; Sigma, Munich, Germany) were used as standards. Sensitivity was 1 pg/ml for all cytokines and 0.05 μg/ml for IgE.

L. sigmodontis-specific IgE was determined by coating with LsAg (5 μg/ml) and using anti-IgE MAb as a secondary reagent. To avoid competition by parasite-specific IgG antibody, serum samples diluted 1:25 with phosphate-buffered saline (final volume, 300 μl) were preabsorbed by overnight incubation

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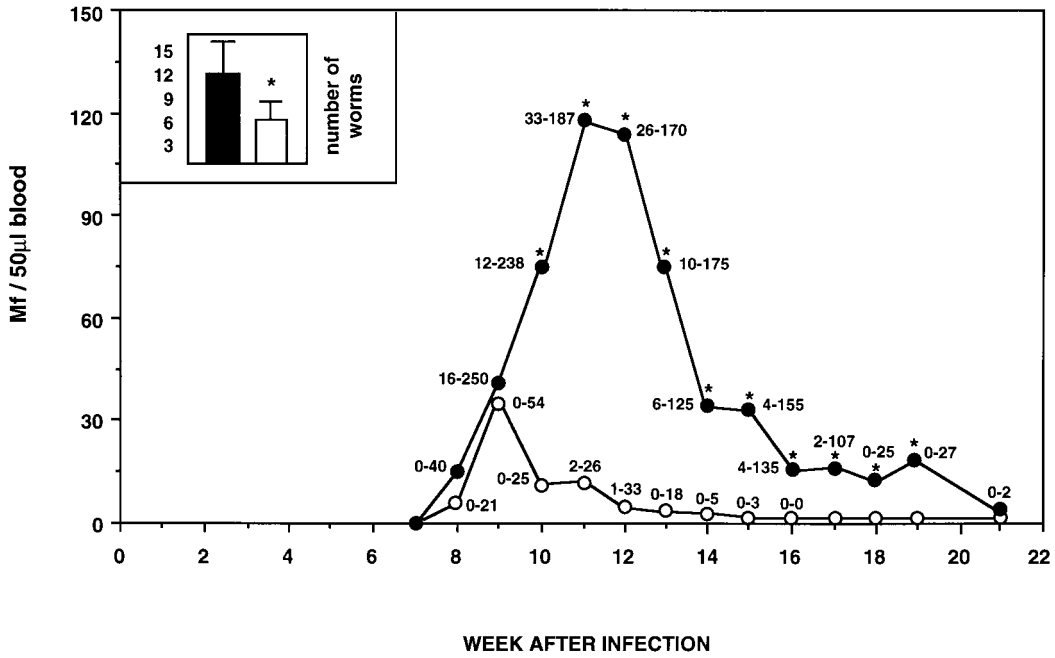

FIG. 1. Microfilaremia and worm burden (inset) in infected mice, either anti-CD4 treated (closed symbols) or control immunoglobulin treated (open symbols). The concentration of Mf in 50 μl of peripheral blood was determined weekly. The adult worm burden in mice sacrificed 28 days after *L. sigmodontis* infection was determined macroscopically. Statistical analysis was performed by using Student's *t* test for worm burdens and the Wilcoxon-Mann-Whitney U test for Mf counts. *, $P < 0.05$. Ranges are indicated on the graph. Data for worm burden are from one representative experiment of four; data for Mf are from one of two consistent experiments.

with an excess of protein G-Sepharose Fast Flow beads (Pharmacia). The absorbed sera were negative for IgG in a sandwich ELISA (not shown). For quantification of parasite-specific IgE, a serum pool obtained from 10 mice infected for 9 weeks with *L. sigmodontis* was used as a reference at a dilution within the linear part of the titration curve (1:200). Optical density (OD) means for individual serum samples (not further diluted after IgG depletion) from the control-infected and CD4-depleted group were determined. The index given in Table 1 was calculated by dividing the group OD means by the reference OD.

Treatment of BALB/c mice with a dose of 500 μg of anti-CD4 antibodies per week led to an elimination of peripheral blood $CD4^+$ T cells within 2 days, with $CD4^+$ T cells comprising 0.3% ± 0.2% of all CD4-depleted lymphoid cells versus 51% ± 5.5% in control antibody-treated mice (values are means ± standard deviations). On day 28, at necropsy (i.e., 7 days after the last antibody injection), $CD4^+$ T cells in the spleens and PLEC of anti-CD4-treated mice constituted 0.8% ± 0.7% and 0.3% ± 0.1% of lymphoid cells, versus 31% ± 1.0% and 13% ± 1.8% in control-infected littermates (not shown). The efficacy of CD4 depletion was also confirmed by the absence of an IgE response in these mice as measured during the first 28 days p.i., in contrast to control-infected animals (see below and Table 1). When a 200-μg weekly dose of antibody was used, depletion of $CD4^+$ T cells was just as effective in the peripheral blood, but $CD4^+$ T cells remained detectable in spleens and PLEC (sixfold and fourfold lower, respectively, than in control-infected littermates; data not shown). In addition, there was a slight elevation of IgE serum levels in the mice treated with 200 μg/dose in the first 28 days p.i. (Table 1).

These results indicate that elimination of $CD4^+$ T cells was complete only when a 500-μg dose was used. However, with the two antibody regimens, the worm loads in anti-CD4-treated mice were similar. Thus, *L. sigmodontis* infection in control mice was found to result in an average worm burden of 6 (Fig. 1), similar to the amount described for subcutaneous injection of L3 (16). However, in anti-CD4-treated mice, the worm numbers were twice those of control-infected mice (Fig. 1). In addition, maximum Mf levels fourfold above those of control-infected animals were detected ($P < 0.05$; Fig. 1). Maximum differences in microfilaremia between the groups were >10-fold. In anti-CD4-treated mice, microfilaremia persisted twice as long as in control animals (Fig. 1). Mf were detected in 18 of 18 anti-CD4-treated mice (100%) but in only 11 of 17 control mice (65%). These data show that $CD4^+$ T cells are involved in parasite control during *L. sigmodontis* infection.

Analysis of cytokine production by PLEC revealed that *L. sigmodontis* infection in control-infected animals resulted in the presence of cells producing IL-4, IL-5, and IL-10 in large amounts compared to those in noninfected animals. This was observed after stimulation either by ConA (Fig. 2A) or by LsAg (Fig. 2B). In particular, the fact that ConA-induced Th2 cytokine production was detected only in infected animals (Fig. 2A) suggests that the cells producing Th2 cytokines are not present in the thoracic cavity of noninfected mice but are induced by parasitic antigens to proliferate or to migrate into this compartment. This predominant Th2 cytokine production was also seen 80 days p.i. (not shown). Antigen-specific IFN-γ was not produced in detectable amounts by PLEC of infected mice (Fig. 2B).

When the effect of CD4 depletion on cytokine production was analyzed 28 days p.i., it was observed that the levels of both ConA-induced (Fig. 2A) and parasite-specific (Fig. 2B) IL-4 and IL-5 production and ConA-induced IL-10 were significantly lower in cells from CD4-depleted mice than in cells from control-infected mice. These data indicate that (i) anti-CD4 treatment of infected mice resulted in depletion primarily of those $CD4^+$ T cells which produce Th2 cytokines after stimu-

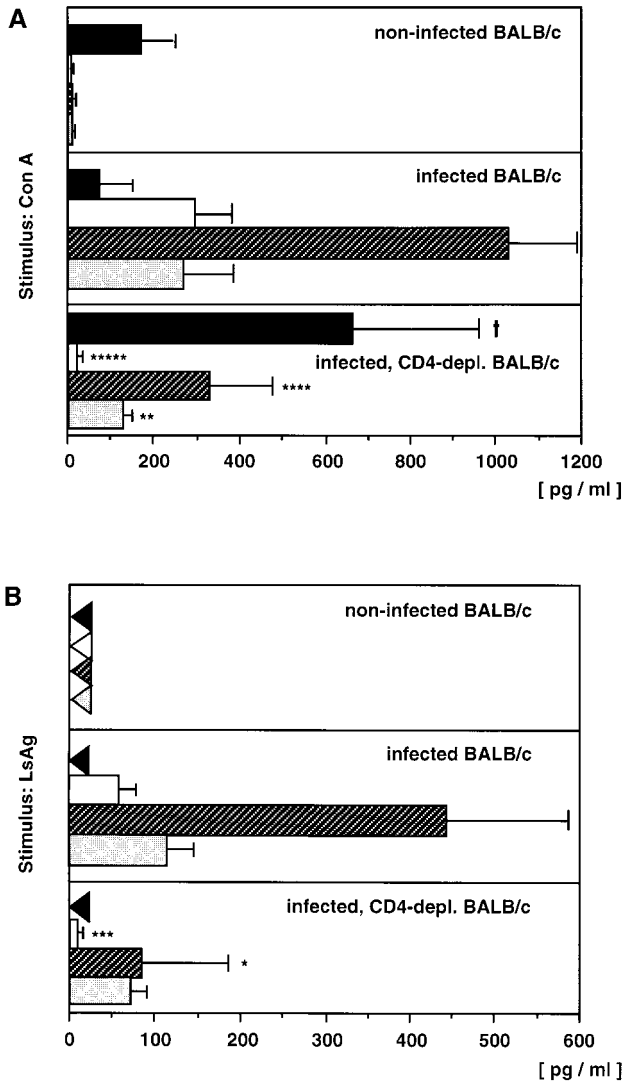

FIG. 2. Production of IFN-γ (black bars), IL-4 (white bars), IL-5 (hatched bars), and IL-10 (gray bars) by PLEC (2×10^5/well) in response to ConA (2.5 μg/ml) (A) and LsAg (25 μg/ml) (B) 28 days after *L. sigmodontis* infection (before microfilaremia). Supernatants were removed 96 h after stimulation and examined by ELISA. Values are mean cytokine concentrations obtained individually for each animal ± standard deviations. Triangles denote values below the detection limit. Statistical analysis was performed by using Student's *t* test (differences between control-infected and CD4-depleted [CD4-depl.] animals: †, $P < 0.025$, elevation in CD4-depleted animals; *, $P < 0.05$; **, $P < 0.03$; ***, $P < 0.01$; ****, $P < 0.005$; *****, $P < 0.001$, reduction in CD4-depleted animals). There was no detectable cytokine production in the absence of a stimulus, except for 103 ± 110 pg of IL-5 per ml produced by PLEC from CD4-depleted mice. Data are from the experiment using 500 μg of anti-CD4 MAb per dose; similar results were obtained when anti-CD4 ascites was used, with the exception that small amounts of IFN-γ were specifically produced in some experiments by both groups of infected mice.

lation in vitro and (ii) residual production of IL-4, IL-5, and IL-10 is not CD4 derived. Conversely, upon ConA stimulation, IFN-γ was produced in greater amounts by PLEC from CD4-depleted mice (Fig. 2A). A pattern of cytokine production highly consistent with that of PLEC was detected in supernatants of cultured splenic cells, including increased ConA-induced IFN-γ production by cells from CD4-depleted mice (not shown).

The increase in non-antigen-specific IFN-γ production of cells from CD4-depleted animals compared to that of control-infected animals implies that this cytokine has a non-CD4 source. It may reflect both the proportional increase of non-CD4 lymphocytes and a lack of inhibition of these cells by Th2 cells. Nevertheless, it is also possible that during infection IFN-γ is produced by Th1 cells that are lost by CD4 depletion. However, the main observation remains that the two cytokines which are preferentially produced by control-infected mice and reduced in CD4-depleted animals are IL-5 and IL-4. Therefore, it was next analyzed if these differences in IL-5 and IL-4 production would result in consistent differences in eosinophilia and IgE levels between the two groups of infected mice.

In CD4-depleted mice, both eosinophilia and eosinophils in the thoracic cavity were significantly reduced compared to those of control-infected mice, consistent with the reduction of IL-5 production in these animals (Table 1). $CD8^+$ T cells, B cells, and macrophages in the thoracic cavity showed compensatory relative increases in CD4-depleted mice (not shown).

Two reports on infection of mice with L3 larvae of other filarial species attribute a critical role to eosinophils in immunity to L3 larvae (4, 12). However, L3 larva survival, not adult worm development, had to be used as a parameter since maturation of L3 larvae into adult worms does not occur in these models, and therefore no conclusions on the role of eosinophils in the inhibition of larval development into adults can be drawn. It is possible that eosinophils, which are operative against a variety of other nematodes (11, 19, 20), are also important effector cells in *L. sigmodontis* infection, and our data show an inverse correlation of eosinophil numbers with the worm load. Future direct-intervention studies using anti-IL-5 antibodies will clarify if eosinophils are involved in reducing larval survival time and/or in inhibiting maturation into adults, a process tightly regulated in nematodes.

When IgE levels in serum were analyzed, an elevation was observed in control-infected mice and also in mice treated with ascites containing 200 μg of anti-CD4 antibody per dose (Table 1). However, IgE levels in mice treated with 500 μg of purified anti-CD4 MAb per dose did not differ from those in noninfected mice (Table 1; values similar to those in reference 14, where IgE was measured with the same reagents), confirming that IgE production is dependent on the presence of $CD4^+$ T cells (10). Moreover, after preabsorption of IgG, an elevated level of *L. sigmodontis*-specific IgE was detected in control-infected mice but not in CD4-depleted mice (Table 1). The potential for IL-4 and IgE in protection will be assessed in future studies with anti-IL-4 antibodies and anti-IgE treatment (3).

In cats and jirds, protective immunity against filarial nematodes was shown experimentally to be directed against L3 and L4 larval stages (7, 9). Infection of BALB/c mice with *L. sigmodontis* (16) is the only murine model of filarial infection which allows, in immunocompetent mice, analysis and modification of immune reactions during larval development and which uses recovery of adult, fertile worms instead of larval survival to assess protective immunity. So far, no data on T-cell-dependent immune responses in this model have been published. One crucial prerequisite for future investigation in the role of T helper cell-derived cytokines for host protection is the answer to the question whether $CD4^+$ T cells are involved in protection against larval development of this filarial worm. Therefore, it is of importance that our data show a clear dependency on $CD4^+$ T cells for the control of maturation of L3 larvae into adult, Mf-producing worms. This is also novel information on filarial infection in mice in a more general sense, given that the data available from the surrogate murine models of *Brugia* infection show permissiveness to full worm

TABLE 1. Eosinophilia and IgE production[a]

BALB/c mice	% Eosinophils in:			Total serum IgE (μg/ml) on the following day p.i.:			*L. sigmodontis*-specific serum IgE (index) on day 28 p.i.[b]
	Peripheral blood		Pleural cavity, day 28 p.i.	14	28	28[c]	
	Day 3 p.i.	Day 14 p.i.					
Infected and untreated	1.5 ± 0.7	11.9 ± 5.3	29.5 ± 13.8	1.2 ± 0.6	6.2 ± 2.2	11.3 ± 3.5	0.6 ± 0.4
Infected and treated with anti-CD4	1.4 ± 1.1	3.2 ± 2.6[d]	11.8 ± 4.0[d,e]	0.8 ± 0.3	1.2 ± 0.3[f]	3.8 ± 1.2[e,g]	0.1 ± 0.04[g]
Noninfected	1.8 ± 0.5	2.1 ± 0.9	0.9 ± 1.0	0.9 ± 0.4	0.8 ± 0.5	0.7 ± 0.4	0.2 ± 0.07

[a] Except where indicated otherwise, data are from experiments using 500 μg of anti-CD4 MAb per dose. Data are means ± standard deviations.
[b] Determined by using a reference pool (OD = 0.092 in the experiment whose results are shown); see text.
[c] Data are representative for experiments using 200 μg of anti-CD4 MAb per dose.
[d] Significantly different ($P < 0.05$) from value for infected normal mice (Student's *t* test).
[e] Significantly different ($P < 0.01$) from value for noninfected mice (Student's *t* test).
[f] Significantly different ($P < 0.002$) from value for infected normal mice (Student's *t* test).
[g] Significantly different ($P < 0.02$) from value for infected normal mice (Student's *t* test).

development in the absence of all T cells (23–25) but not in the absence of either $CD4^+$ (17) or $CD8^+$ (18) subsets alone. Furthermore, there was no greater recovery of L3 larvae from T-cell-deprived mice than from normal CBA mice into which *Onchocerca volvulus* L3 larvae had been transplanted (5). Although in this system greater L3 larva recovery from preimmunized mice after IL-4 or IL-5 depletion indicates a possible involvement of Th2 cells in L3 larval attack, this was not directly shown (12). In addition, since nematodes control their development tightly, one cannot directly conclude from higher L3 larva recoveries seen after immunomodulation (4, 12) that these measures would also result in a higher adult worm or microfilarial load. Concluding from the above, this is the first study in which anti-CD4 treatment resulted in enhanced survival and maturation of a filarial worm in a naive (i.e., not preimmunized) mouse.

Taking these findings together, we have shown that infection of normal BALB/c mice with *L. sigmodontis* filarial nematodes results in a Th2 response which is accompanied by specific IgE and eosinophilia. In anti-CD4-treated mice, a dose-dependent reduction of Th2 cytokines and eosinophils as well as suppression of the IgE response is seen. These immunological parameters correlate inversely with the enhanced worm load in anti-CD4-treated mice. Since anti-CD4 treatment facilitated development of fertile adult filarial worms, this new model appears suitable for analyzing the relevance of T-cell-dependent cytokines, as well as of other $CD4^+$ T-cell-mediated components of the immune system, by using direct intervention and knockout technology.

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