

ELIMINATION OF CD4⁺ SUPPRESSOR T CELLS
FROM SUSCEPTIBLE BALB/c MICE RELEASES CD8⁺
T LYMPHOCYTES TO MEDIATE PROTECTIVE IMMUNITY
AGAINST *LEISHMANIA*

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BALB/c mice, in contrast to most other mouse strains, are extremely susceptible to infection with the intracellular, protozoan parasites of the genus *Leishmania* (1). *L. major*, for example, multiplies progressively at the site of primary cutaneous infection in the footpad, and rapidly disseminates to visceral and distinct cutaneous sites (2). Several hypotheses have been put forward to explain the failure of BALB/c mice to generate a therapeutic level of anti-*Leishmania* immunity (3). It has been proposed (4), for example, that the protective immune response in the BALB/c host is downregulated by *Leishmania*-induced suppressor T (Ts) cells, via a mechanism similar to that which down-regulates the immune response to immunogenic murine tumors (5). An entirely different hypothesis that has been offered is that the susceptibility of BALB/c mice is caused by "disease-promoting" (6, 7) sensitized T cells that function to recruit too many mononuclear phagocytes to sites of infection, thereby providing the parasite with host cells within which it must reside and multiply.

It needs to be realized that, in spite of their innate susceptibility, BALB/c mice nonetheless possess the capacity to generate a protective immune response. It has been shown that immunizing BALB/c mice with attenuated parasites (8), or with live parasites (9) and parasite antigens (10, 11) admixed with adjuvant, renders the mice resistant to an otherwise lethal challenge infection. The immunity generated is T cell mediated (4), although the identity of the T cells involved has yet to be unequivocally established. Moll et al. (12) have presented evidence that L3T4⁺ (CD4⁺) T cells alone are sufficient to reconstitute immunity in athymic BALB/c mice. However, in contradiction to this, other investigators (13-15) have shown that BALB/c mice rendered deficient in CD4⁺ T cells by treatment with anti-L3T4 mAb paradoxically develop the capacity to resolve *Leishmania* infection. In an attempt to reconcile these contradictory findings, it has been argued (12, 15) that the immunity that develops in anti-L3T4 mAb-treated mice is mediated by residual CD4⁺ T cells. The obvious alternative explanation, that immunity in anti-L3T4 mAb-treated mice is mediated by Ly-2⁺ (CD8⁺) T cell (16), apparently has not been seriously consid-

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ered, because it would not be in keeping with the rule that CD4⁺ T cells are the only cells that mediate delayed-type hypersensitivity (DTH)¹ (15, 17, 18).

The purpose of this paper is to present evidence that CD4⁺ T cells are responsible for the susceptibility of BALB/c mice to *L. major* infection. It will demonstrate that eliminating CD4⁺ T cells with a single dose of anti-L3T4 mAb releases CD8⁺ T cells to mediate a protective immune response capable of eliminating *Leishmania* from the primary lesion, and of preventing the parasite from disseminating to visceral sites.

Materials and Methods

Mice. Male BALB/c and B6.PL-Thy-1^a/Cy (C57BL/6-Thy-1.1) mice, 7-10 wk old, were obtained from the Trudeau Institute Animal Breeding Facility. The mice were reared under barrier-sustained conditions and were shown to be free of common viral pathogens by serological testing (Charles River Technical Services, Wilmington, MA).

Parasite. Amastigotes of *L. major*, strain 173 (MHOM/IR/-/73) (19), were obtained from the footpads of BALB/c mice as previously described (20). Primary subcutaneous infections were initiated by injecting 10⁵ amastigotes in 50 μ l of PBS into the left hind footpad. To follow the development of disease, the size of the cutaneous lesion was determined by measuring the thickness of the foot at regular intervals. The numbers of viable parasites in the primary lesion (hind footpad), liver, and spleen were determined by plating appropriately diluted aliquots of homogenized tissues onto heart-infusion agar (20). After 5-7 d of incubation at 26°C, the promastigote colonies were counted, and the number of viable parasites (CFU) present in the tissues calculated.

In Vivo Depletion of T Cell Subsets. The hybridoma GK1.5 (Dr. Frank Fitch, University of Chicago, Chicago, IL) secreting rat IgG2b anti-L3T4 mAb, and hybridomas 30-H12 and TIB-210 (American Type Culture Collection, Rockville, MD) producing IgG2b anti-Thy-1.2 and anti-Ly-2.2 mAb, respectively, were grown as ascites in pristane-primed, irradiated BALB/c mice. The IgG2b content of the ascites fluid was quantified by radial immunodiffusion, and the fluids were stored at -70°C until needed. To deplete T cell subsets in vivo, BALB/c mice were thymectomized at 7 wk of age and infused intravenously 1 wk later with a single 1-mg dose of the appropriate mAb, as previously described (21, 22). One group of control mice received 1 mg of normal, affinity-purified rat IgG (ICN Immunobiologicals, Lisle, IL). The extent of T cell depletion was determined 1 wk later, and at 5 wk of infection, by flow cytometric analysis with a FACScan cytofluorometer (Becton Dickinson & Co., Sunnyvale, CA). Spleen cells were stained with FITC-conjugated anti-Thy-1.2, anti-L3T4, and anti-Ly-2.2 mAbs (22), and the results are expressed as the number of CD4⁺ and CD8⁺ T cells per spleen.

Delayed-type Hypersensitivity. The capacity of *Leishmania*-infected mice to express a DTH response was examined using live amastigotes as the eliciting antigen. 72 h before a scheduled sacrifice, baseline measurements of footpad thickness were made, and 10⁶ amastigotes were implanted in the right hind footpad (contralateral to primary footpad lesion). At 3, 6, 12, 24, 36, 48, and 72 h, measurements of footpad thickness were made on individually numbered mice.

Results

Depletion of CD4⁺ T Cells Alone, or CD4⁺ plus CD8⁺ T Cells, Results in a Reduction in the Size of the Lesion that Develops at the Site of Leishmania Inoculation. Fig. 1 shows the effect of an intravenous infusion of mAbs specific for T cell subsets on the development of the cutaneous lesion in the *Leishmania*-inoculated footpad of BALB/c mice. It can be seen that an increase in the thickness of the infected footpad was first evi-

¹ Abbreviation used in this paper: DTH, delayed-type hypersensitivity.

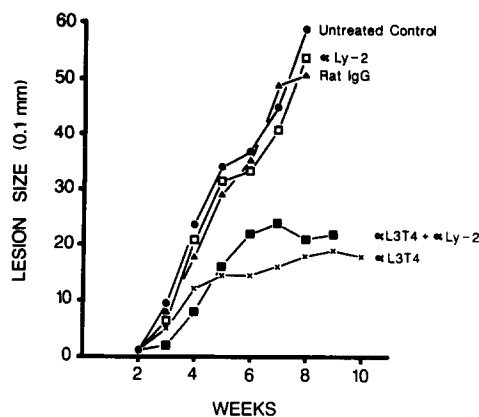


FIGURE 1. Development of primary lesions in the footpad of untreated and mAb-treated mice. Thymectomized mice were treated with 1 mg of anti-L3T4 (CD4) mAb, anti-Ly-2-2 (CD8) mAb, both mAbs, or 1 mg of affinity-purified rat IgG (treatment control). 1 wk later, these mice, along with thymectomized untreated controls, were infected in the left hind footpad with 10^5 *L. major* amastigotes. Lesion size is the difference between the thickness of the inoculated foot and that of the contralateral uninfected foot. Data are expressed as the mean of four to six mice per time point. The SD were always $\leq 20\%$ of the mean.

dent at 3 wk of infection in all experimental groups. In the control mice, and in mice treated with anti-Ly-2 mAb, footpad size continued to increase until 8 wk of infection, after which the entire foot became necrotic, and no further measurements were possible. In contrast, treatment with either anti-L3T4 mAb alone, or anti-L3T4 plus anti-Ly-2 mAb, prevented the lesion from increasing in size after 5 wk. This result would generally be taken to mean that the infection had failed to progress in these last mentioned mice.

That treatment with mAb as described above depleted mice of the appropriate T cell subsets is evidenced by the results of flow cytometric analysis of spleen cells shown in Table I. It can be seen that at 5 wk of infection, the absolute numbers of CD4⁺ and CD8⁺ T cells in mAb-treated mice were $<90\%$ of normal. Residual

TABLE I
Numbers of CD4⁺ and CD8⁺ Spleen Cells Present in mAb-treated
BALB/c Mice at 5 wk of *Leishmania major* Infection

Treatment	Total	Thy-1.2 ⁺	CD4 ⁺	CD8 ⁺
		$\times 10^{-6}$		
Untreated control	140	21.8	20.0	8.1
Rat IgG control	190	27.8	14.3	10.5
Anti-L3T4 mAb	260	13.5	<1.8	8.0
Anti-Ly-2 mAb	250	23.2	22.0	<2.0
Anti-L3T4 mAb + Anti-Ly-2 mAb	40	<0.4	<0.3	<0.2
Untreated C57BL/6-Thy-1.1 (negative control)	160	3.2	ND	ND

BALB/c mice were thymectomized at 7 wk of age and treated 1 wk later with 1 mg of the appropriate mAb. Groups of mice were infected 1 wk after treatment, and their pooled spleens were analyzed 5 wk later. Absolute numbers were calculated from the percentages obtained from flow cytometric analysis of spleen cells stained with FITC-conjugated anti-Thy-1.2, anti-L3T4 (CD4), and anti-Ly-2 (CD8) mAb. The numbers have not been corrected for background. Therefore, they represent the maximum number present in the organ. The background staining with anti-Thy-1.2 on spleen cells from B6.Thy-1.1 mice was 2%.

TABLE II
 Number (Log₁₀) of Viable Parasites in T cell-depleted BALB/c Mice at 5 and 10 wk
 of *Leishmania major* Infection

Treatment	Weeks of infection			
	5		10	
	Primary lesion	Liver	Primary lesion	Liver
	<i>log₁₀</i>			
Untreated control	8.01 ± 0.31	4.54 ± 0.78	-*	6.85 ± 0.71
Rat IgG control	7.46 ± 0.32	3.93 ± 0.44	-*	6.76 ± 0.71
Anti-L3T4 mAb	5.35 ± 0.57	2.65 ± 0.38	<2.30 [†]	<2.30 [†]
Anti-Ly-2 mAb	7.52 ± 0.35	4.23 ± 0.27	-*	6.54 ± 0.81
Anti-L3T4 mAb + anti-Ly-2 mAb	7.54 ± 0.40	4.15 ± 0.78	-§	-§

Thymectomized BALB/c mice were treated with 1 mg of appropriate mAb(s) and infected in the hind footpad with 10⁵ *L. major* amastigotes 1 wk later. Data are expressed as the mean ± SD of four to six mice.

* At 10 wk the entire foot of mice in these groups had become necrotic and had been lost.

[†] No viable parasites could be detected in these mice. The detection limit for viable parasites in this study was 200 (2.30 logs).

§ Mice in this group had died of systemic disease by 10 wk.

staining was below the background seen in C57BL/6-Thy-1.1 spleen cells stained with FITC-conjugated anti-Thy-1.2 mAb. It is possible, therefore, that the targeted T cell subset had been completely eliminated by mAb treatment.

Only Mice Depleted of CD4⁺ T Cells Alone Were Capable of Destroying Parasites in the Primary Lesion and in Visceral Metastatic Foci. The foregoing results suggest that if lesion size is taken as an indicator of disease status, mice depleted of CD4⁺ T cells alone, or of both CD4⁺ and CD8⁺ T cells, are resistant to cutaneous *L. major* infection. Indeed, enumeration of the parasite in the footpads and livers (Table II) revealed that mice depleted of CD4⁺ T cells were able to prevent the parasite from multiplying progressively at the primary site of infection. At 5 wk, the footpad of anti-L3T4 mAb-treated mice had <1% of the number of parasites found in the footpad of untreated control mice. In fact, by 10 wk, the parasites had been completely eliminated from the primary lesion, and the small number of *Leishmania* that had disseminated to the liver had been destroyed.

However, the situation in mice treated with anti-L3T4 plus anti-Ly-2 mAbs was entirely different. In spite of the fact that the primary lesions of mice depleted of both subsets of T cells were small, these mice proved to have progressive disease. At 5 wk of infection their cutaneous lesions contained up to 10⁸ viable parasites, and by the ninth week, they began to die of systemic disease. These results show, therefore, that treatment with anti-Ly-2 mAb completely negated the therapeutic effect of treatment with anti-L3T4 mAb. It follows, therefore, that CD8⁺ T cells are essential for a successful protective immune response against *Leishmania* in BALB/c mice.

BALB/c Mice Depleted Only of CD4⁺ T Cells Acquire a Sustained Capacity to Express a Cutaneous DTH Response to Leishmania Antigens. At 5 and 10 wk of the *Leishmania* infection, control and mAb-treated mice were tested for their capacity to express a DTH response to *Leishmania* amastigotes implanted in the foot contralateral to that containing the primary lesion. The criterion for a positive DTH was an increase

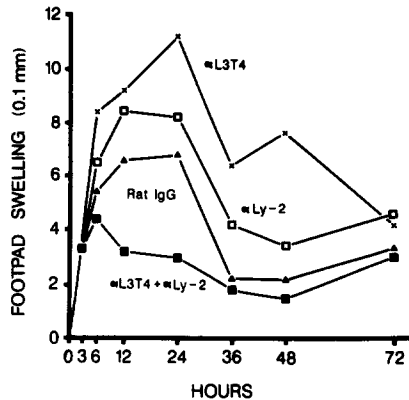


FIGURE 2. Kinetics of DTH response elicited at 5 wk of *L. major* infection in BALB/c mice depleted of L3T4⁺ (CD4⁺) T cells, Ly-2⁺ (CD8⁺) T cells, or both T cell subpopulations. Thymectomized mice were treated with 1 mg of anti-L3T4 mAb, anti-Ly-2 mAb, both mAbs, or 1 mg of affinity-purified rat IgG (treatment control). 1 wk later, these mice, along with thymectomized untreated controls were infected in the left hind footpad with 10⁵ *L. major* amastigotes. To elicit DTH, after taking baseline measurements of the right hind footpad (contralateral to primary lesion) of four to six mice from each treatment group, 10⁶ amastigotes were implanted in that site. Data are expressed as footpad swelling (0.1 mm), i.e., the increase in the thickness of the inoculated foot at the stated times after challenge.

TABLE III
Magnitude of the DTH Response Elicited by an Intrafootpad Injection of
Leishmania major Amastigotes in BALB/c Mice
Depleted of CD4⁺ or CD8⁺ T Cells

Treatment	Week of infection	
	5	10
Untreated control	5.2 ± 2.8	4.0 ± 2.1
Anti-L3T4 mAb	11.2 ± 1.5	16.8 ± 3.2
Anti-Ly-2 mAb	8.2 ± 3.4	4.5 ± 1.3
Anti-L3T4 mAb + anti-Ly-2 mAb	3.0 ± 1.6	-*
Uninfected, untreated controls	3.2 ± 1.2	4.3 ± 0.6

Thymectomized BALB/c mice were treated with 1 mg of appropriate mAb(s) and infected in the hind footpad with 10⁵ *L. major* amastigotes 1 wk later. Data are expressed as the mean (± SD) increase in thickness (0.1 mm) of the footpads of four to six mice at 24 h after injection of 10⁶ *L. major* amastigotes.

* Mice in this group had died of systemic disease by 10 wk.

in footpad swelling, which reached a maximum at 24 h, and declined to baseline by 72 h. The time courses of the DTH responses are shown in Fig. 2, where it can be seen that all mice, except those depleted of both CD4⁺ and CD8⁺ T cells, were capable of expressing a delayed inflammatory response at 5 wk of infection. Importantly, the largest response was expressed by CD4⁺ T cell-depleted mice. Furthermore, CD4⁺ T cell-depleted mice were capable of expressing DTH at 10 wk of infection (Table III), whereas the capacity of the other groups to express a DTH response had waned. These results therefore show that eliminating CD4⁺ T cells not only allows CD8⁺ T cells to mediate protective immunity, but that acquisition of CD8⁺ T cell-mediated protective immunity correlates with the sustained capacity of the mice to express a DTH response to amastigote antigens.

Discussion

This paper confirms the results of others (13) by showing that treatment of *Leishmania*-susceptible BALB/c mice with anti-L3T4 mAb converts them to resistant mice capable of eliminating *Leishmania* from the site of primary infection and of preventing the

dissemination of the parasite to the viscera. This effect of anti-L3T4 mAb treatment is somewhat paradoxical, considering the generally held view that CD4⁺ T cells are responsible for mediating anti*Leishmania* immunity by way of their ability to activate the antimicrobial capacity of macrophages via the secretion of lymphokines (4). Indeed, in order to conform to this view, some (12, 15) have attempted to explain the therapeutic effect of anti-L3T4 mAb treatment by suggesting that immunity is mediated by CD4⁺ T cells that survive mAb treatment. However, the recent results of Titus et al. (16) challenge the belief that anti*Leishmania* immunity resides exclusively within the CD4⁺ T cell subpopulation. These workers found that anti-Ly-2 mAb treatment can exacerbate disease in susceptible BALB/c mice, and also delay, to a certain extent, healing of lesions in the genetically resistant CBA host. The results presented here clearly show that the CD8⁺ T cell subset is essential for anti*Leishmania* immunity. They show that the parasite multiplies progressively in anti-Ly-2 mAb-treated mice, and in mice treated with anti-Ly-2 plus anti-L3T4 mAb. In contrast, mice treated with the anti-L3T4 mAb alone acquired the ability to control *Leishmania* multiplication and to eliminate the parasite in <10 wk. Therefore, there can be little doubt that CD8⁺ T cells are key protective cells, and that CD8⁺ T cells can perform their protective role in the virtual absence of the CD4⁺ T cell subset. This central role for CD8⁺ T cells in immunity is in agreement with the interpretation of a published (23) immunohistochemical study of *Leishmania*-induced granulomas in the livers of BALB/c mice. This published study shows that CD8⁺ T cells predominate at a time when the parasite is being eliminated from foci of infection.

As to the reason why CD4⁺ T cells need to be eliminated in order for CD8⁺ T cells to perform their protective function, the results presented do not support the view that BALB/c mice normally generate too many *Leishmania*-sensitized CD4⁺ T cells and, consequently, express disease-promoting levels of DTH (6, 7). The notion that DTH-mediated accumulation of too many mononuclear phagocytes at the site of infection provides *Leishmania* with cells for it to enter and multiply in is difficult to reconcile with the evidence that mononuclear phagocytes are the cells that are eventually responsible for destroying the parasite. The results presented here indicate that DTH, per se, is not a disease-promoting mechanism. On the one hand, susceptible BALB/c mice not depleted of CD4⁺ T cells actually had a reduced capacity to express DTH. On the other hand, depletion of CD4⁺ T cells allowed mice to generate higher levels of DTH. Moreover, the acquisition of DTH was associated with the generation of an immune response that prevented rather than promoted the multiplication of the parasite.

A key finding from this study is that both DTH and protective immunity to *Leishmania* are mediated by CD8⁺ T cells. Both mechanisms were expressed in the virtual absence of CD4⁺ T cells, but not in the absence of CD8⁺ T cells. Even so, these results do not rule out a possible role for CD4⁺ T cells in the DTH response to *Leishmania*. Indeed, mice treated with the anti-Ly-2 mAb alone also acquired a short-lived capacity to mount a response to an intrafootpad injection of *Leishmania* amastigotes. It has been suggested, however, that this inflammatory reaction in susceptible BALB/c mice may not be a true DTH reaction (24). Until this possibility is investigated, the results can be interpreted to mean that CD8⁺ as well as CD4⁺ T cells can mediate DTH in *Leishmania*-infected BALB/c mice.

Given what is currently known about the response of BALB/c mice to *Leishmania* infection, the most plausible explanation for the impressive therapeutic effect of anti-L3T4 mAb treatment is that the mAb eliminates CD4⁺ T cells, thereby releasing *Leishmania*-sensitized CD8⁺ T cells to mediate protective immunity. It is apparent that there has been a reluctance to invoke T cells to explain the effect of anti-L3T4 mAb treatment, in spite of the fact that there are several publications (25–27) showing that *Leishmania*-infected mice generate CD4⁺ T cells capable, on passive transfer, of suppressing anti-*Leishmania* immunity in BALB/c mice. It should be pointed out, moreover, that CD4⁺ T cells have been shown to mediate unresponsiveness to a number of antigens (28–31). In this regard, the results presented here are strikingly similar to those recently obtained from a study of the effect of deleting T cell subsets on the growth of an immunogenic tumor. It was shown (22) that deleting CD4⁺ T cells resulted in complete regression of the tumor and in long-term host survival. In contrast, deleting CD8⁺ T cells resulted in enhanced tumor growth and in shorter host survival time.

The last finding deserving of discussion is that the size of the primary lesion need not be a reflection of the parasite load in the primary lesion, nor of the severity of systemic disease. On the contrary, the results show that the largest numbers of *Leishmania* were present in some of the smallest lesions, namely, in the footpads of mice depleted of both T cell subsets. Thus, the increased thickness of an infected footpad does not represent the space occupied by parasites. Rather, it probably represents mostly edema caused by the host's inflammatory response. It is possible, therefore, that much of the past confusion about both the nature of underlying anti-*Leishmania* immunity in the susceptible host, and the mechanism that suppresses it, has been caused by too heavy a reliance on lesion size measurements to monitor the progress of infection. The current studies serve to emphasize that the only acceptable way to determine the disease status of the host at any one time is to enumerate the parasites in the primary lesion and elsewhere.

Summary

This study examined the capacity of BALB/c mice that had been depleted of T cell subpopulations to generate a protective immune response to *Leishmania major*. Thymectomized mice were depleted of either L3T4⁺ (CD4⁺) T lymphocytes, Ly-2⁺ (CD8⁺) T lymphocytes, or both, by treatment with appropriate mAbs. It was found that susceptible mice were rendered resistant to *Leishmania* by an intravenous infusion of anti-L3T4 mAb. These mice generated an immune response that destroyed the parasite in the primary lesion and in visceral metastatic foci. CD4⁺ cell-depleted mice also acquired a capacity to mount a sustained delayed-type hypersensitivity (DTH) response to parasite antigens, indicating that DTH, per se, is not a disease-promoting mechanism in the susceptible murine host as has been suggested. Depleting BALB/c mice of CD8⁺, as well as CD4⁺ T cells, left them highly susceptible to *Leishmania* infection, thereby indicating that CD8⁺ lymphocytes are key protective cells. Our results can be interpreted as showing that the susceptibility of BALB/c mice is due to the generation of CD4⁺ cells that suppress either the generation or expression of CD8⁺ T cell-mediated anti-*Leishmania* immunity.

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Note added in proof: Since this manuscript was submitted, Scott et al. (32) reported the isolation of CD4⁺ Th₂ T cell lines that, upon infusion into BALB/c mice, exacerbate *L. major* infection.

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