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Establishment of resistance to *Leishmania major* infection in susceptible BALB/c mice requires parasite-specific CD8$^+$ T cells

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Abstract

Although CD4$^+$ T cells are generally accepted to be responsible for the determination of resistance to infection in experimental murine cutaneous leishmaniasis, a contribution of CD8$^+$ lymphocytes to immunity can be demonstrated under certain well-defined conditions. Normally highly susceptible BALB/c mice can be rendered resistant to infection with *Leishmania major* promastigotes by a single injection of monoclonal anti-CD4 antibodies at the beginning of infection. Mice treated in such a way can heal their primary cutaneous lesions and acquire immunity to subsequent challenge infection. Both the resolution of the primary infection and the induced state of immunity to reinfection in these mice is shown to be dependent upon the anti-leishmanial effector functions of CD8$^+$ T cells. Furthermore, in contrast to control infected BALB/c mice, which are unable to mount a delayed-type hypersensitivity (DTH) response to viable parasites, mice cured as a result of treatment with anti-CD4 antibodies *in vivo* exhibit a strong DTH response, which can be significantly reduced by injection of either anti-CD4 or anti-CD8 monoclonal antibodies prior to antigenic challenge with viable promastigotes. Moreover, increased numbers of specific CD8$^+$ T cells, able to transfer *Leishmania*-specific DTH responses, were found in lymphoid organs of BALB/c mice rendered resistant to infection by immunointervention with anti-CD4 monoclonal antibodies at the beginning of infection. Neutralization *in vivo* of interleukin 4 during the course of infection in BALB/c mice also enables these otherwise susceptible mice to resolve their cutaneous lesions and to decrease the parasite burden in infected tissues. CD8$^+$ T cells are required for both of these beneficial effects. Taken together, these results indicate that in the immune BALB/c mouse, as in the normally resistant CBA mouse, CD8$^+$ lymphocytes are involved in the elimination of *L. major* and in the establishment and maintenance of immunity against infection with this parasite.

Introduction

Leishmania major, one of the causative agents of cutaneous leishmaniasis in man, is a protozoan parasite that infects mononuclear phagocytes in its vertebrate hosts. The various clinical manifestations of human leishmaniasis can be mimicked in mice from various inbred strains upon infection with *L. major*. The majority of inbred strains of mice are relatively resistant to infection with this parasite in that they develop only small lesions at the site of inoculation, which spontaneously resolve within a few weeks. In contrast, mice from a few strains, such as BALB/c, develop severe lesions at the site of parasite inoculation. These lesions show no tendency to resolve, and eventually fatal visceralization of the infection occurs (1,2).

The importance of CD4$^+$ T cells in resolution of cutaneous lesions induced by *L. major* in mice is firmly established (3–5). However, results are accumulating which indicate that CD8$^+$ T cells also participate in the resolution of *L. major*-induced lesions. A role for CD8$^+$ T cells in immunity to infection with this parasite was first suggested by observations showing the activation of larger numbers of parasite-specific CD8$^+$ T cells in resistant than in susceptible mice during the course of a primary infec-

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tion (6). Secondly, administration of anti-CD8 monoclonal antibodies was shown to enhance significantly the development of lesions in resistant mice, even though this treatment did not modify the outcome of infection (7). Thirdly, it has recently been shown that adult thymectomized BALB/c mice treated with α-CD4 mAb, which are thus virtually free of CD4+ T cells, are able to control infection induced by *L. major* amastigotes through the activity of CD8+ T cells mediating specific delayed-type hypersensitivity (DTH) reactions (8). Fourthly, resistance to infection, acquired by intravenous immunization of susceptible mice with killed promastigotes, was prevented by depletion of CD8+ T cells *in vivo* (9). Finally, recent results from our laboratory have shown that in resistant mice CD8+ T cells also appear to be important in controlling the lesions developing upon infectious challenge of immune mice, which had spontaneously cured their primary infections (10).

In the murine model of generalized visceral infection with *Leishmania donovani*, results show that the majority of specific T cells in the liver of resistant B10 mice, at the time when parasite load begins to decrease rapidly, express the CD8+ surface phenotype (11). Furthermore, CD8+ T cells have been demonstrated to contribute to immunity to reinfection of mice with *L. donovani* (12).

Several studies have demonstrated that susceptibility of normal BALB/c mice to infection with *L. major* can be overcome by various immunological interventions. For example, administration of α-CD4 mAb to normal BALB/c mice at the beginning of infection enables them to resolve their primary infection and render them resistant to reinfection (13–16). Treatment of BALB/c mice with α-interleukin 4 (IL-4) mAb has also been shown to reverse their extreme susceptibility to infection (17). In the present study, we show that the state of resistance to infection seen in BALB/c mice as a result of either of these two interventions on the immune system depends upon the triggering and the activity of CD8+ T cells.

Methods

Mice

Adult BALB/c mice were either obtained from the animal colony maintained at the Institut Pasteur Paris or purchased from IFFA-Credo, Saint-Germain-sur-l'Arbresle, France.

Parasites

L. major LV39 (MRHO/SU/59/P-strain) was kept virulent by continuous passage into mice. Parasites isolated from skin lesions of infected mice were grown at 26°C in Dulbecco's modified Eagle's medium (DMEM) on a solid layer of rabbit blood-agar as previously described (18).

Infection of mice and monitoring of lesions

Mice were infected s.c. in one hind footpad with 2×10^6 *L. major* promastigotes, obtained from cultures in the stationary growth phase, in a final volume of 50 μl. The development of lesions was monitored weekly by measuring the increase of footpad thickness compared to the uninfected contralateral footpad.

Determination of the number of viable L. major *parasites in infected mouse tissues*

Viable *L. major* parasites in infected tissues were enumerated using a limiting dilution assay described elsewhere (19). Briefly, dilutions of infected tissue homogenates were placed in the wells of microtiter plates which contained rabbit blood-agar slants. After 10–12 days incubation at 26°C, the wells containing growing parasites were identified by microscopic examination. Estimation of the frequency of *L. major* recovered from a given tissue was determined by minimum χ^2 analysis applied to Poisson distribution.

Delayed-type hypersensitivity (DTH)

DTH responses were elicited by s.c. injection of either 2×10^6 infective *L. major* promastigotes or frozen-and-thawed lysates of *L. major* promastigotes (L.m.Ag) in a final volume of 50 μl into one hind footpad. The doses of L.m.Ag employed are expressed in terms of the numbers of promastigotes used to prepare the antigen. The DTH reaction was quantitated by measuring at various times (6, 12, 24, and 48 h) the increase in footpad thickness, i.e. the difference between the thickness of the injected footpad and the control footpad by using a dial-gauge caliper (precision 0.05 mm. Kröplin, Schlüchtern, FRG).

Estimation of the frequency of L. major specific T cells capable of mediating specific DTH reaction

The adoptive transfer of DTH reaction was performed by injecting cells from sensitized animals together with viable *L. major* promastigotes as antigen into one hind footpad of normal syngeneic mice. The limiting dilution analysis to determine the frequency of DTH-mediating T cells in lymphoid tissues has been described (20,21). In brief, three serial dilutions of lymphoid cells were mixed with viable *L. major* promastigotes, and 50 μl of cell suspensions were injected into one hind footpad of normal syngeneic mice (10–12 recipients for each dilution). The DTH reaction was quantitated by measuring the increase in footpad thickness 18 h later. Since the DTH-mediating activity is reflected by an increase in footpad thickness, the frequency determination of DTH-mediating T cells by limiting dilution assay implies the definition of values in which the swelling footpad response is scored as negative (negative transfer). The upper value of footpad thickness increase related to negative transfer was calculated from the mean (m) value of increase of footpad thickness measured after local injection of diluted suspensions of unsensitized cells (or sensitized cells against an unrelated antigen) mixed with 2×10^6 *L. major* promastigotes, into 10–12 naive recipients. To this value (m) was added the product $SD \times t_{0.05}$ in which SD represents the standard deviation and t is the value of Student's t for $P = 0.05$. The experimental values were always inferior to such a calculated value (0.20 mm) when cell suspensions and antigen were prepared under the appropriate conditions and mice were kept on a clean litter. The frequency of specific T cells was calculated as described (6).

mAbs

Rat mAb GK 1.5 (IgG2b, α-CD4) (22) and α-CD8 mAb 31M (IgM) (23) were kindly provided by Dr F. W. Fitch, University of Chicago, Chicago, IL. Rat mAb H35.17.2 (IgG2b, α-CD8) (24) was kindly supplied by Dr M. Pierres, INSERM/CNRS, Marseille, France. Rat mAb 11B11 (IgG1, α-murine-IL-4) (25) was a gift of Dr W. Paul, National Institutes of Health, Bethesda, Maryland, USA and rat mAb AN-18.17.24 (α-murine interferon (IFN-γ) was a gift of Dr S. Landolfo, University of Torino, Torino, Italy (26). Rat mAb R4-6A2 (IgG1, α-murine IFN-γ) (27) was obtained by Dr G. Spitalny. Rat mAb RL 172.4 (IgM, α-CD4) (28) was a gift of Dr

H. R. MacDonald, Ludwig Institute for Cancer Research, Epalinges, Switzerland. α-DNP mAb LO-DNP-11 (rat, IgG 2b) was kindly provided by Dr H. Bazin, Brussels, Belgium (29).

Treatment with mAb in vivo

Unless otherwise stated, semi-purified mAb (ammonium sulphate precipitation) from ascites fluid were injected i.p. or i.v. in phosphate-buffered saline at the indicated doses.

Preparation of T cell subset-depleted cell suspensions from lymphoid tissues

Before transfer, single-cell suspensions (5×10^7 nucleated cells/ml) were incubated with a 1/10 dilution of hybridoma culture supernatant containing mAb (IgM) directed against either the CD4 or CD8 T cell surface antigen and a 1/40 dilution of guinea pig serum as a source of complement. The T cell subset-depleted suspensions were washed twice before use in adoptive transfer experiments.

Two-color fluorescence staining and fluorescence-activated cell sorter (FACS) analysis

Samples of 2×10^6 cells were stained with a mixture of phycoerythrin-labeled α-CD4 mAb (GK 1.5, Becton Dickinson, Mountain View, CA) and 5-(4,6-dichlorotriazinyl) aminofluorescein-conjugated α-CD8 mAb (H35.17.2). Samples were analyzed on a flow microfluorometer (FACS II, Becton Dickinson, Mountain View, CA) gated by a combination of narrow-angle forward light scatter and perpendicular light scatter to exclude nonviable cells (30).

Lymphocyte cultures

Spleen cells (4×10^6/ml) were stimulated in the presence or absence of 1×10^6/ml live *L. major* promastigotes, inactivated by 2 – 5-min irradiation with UV light or L.m.Ag. at 37°C, 7% CO_2 in 24-well Costar plates in a final volume of 2 ml. DMEM (Seromed, Berlin, FRG) supplemented with 5% heat-inactivated FCS (Seromed), L-asparagine (36 mg/l), L-glutamine (216 mg/l), L-arginine (200 mg/l), 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin was used as culture medium. Twenty-four hours later, supernatants (SN) were removed and stored in small aliquots at −20°C until use to measure their content in various lymphokines.

Detection of IL-2, IFN-γ, and tumor necrosis factor (TNF) in SN

IL-2 activities were determined by measuring the lysosomal enzyme *N*-acetyl-β-D-hexosaminidase of an IL-2-dependent murine cytotoxic T cell line (CTLL) as described (31). The CTLL were kindly provided by Dr R. MacDonald, Ludwig Institute for Cancer Research, Epalinges, Switzerland. CTLL (4000 cells per well) were incubated with serially diluted ($\log_2$ dilutions) test samples in 96-well flat bottom plates (Costar). Recombinant mouse IL-2 was used as a positive control. Recombinant mouse IL-2 expressed in X63Ag8-653 plasmacytoma cells was a gift from Dr F. Melchers, Basel Institute for Immunology, Basel, Switzerland (32). After 48 h incubation at 37°C, 7% CO_2 the plates were centrifuged, the supernatants removed, the cells washed twice with PBS, and incubated with 60 μl of substrate. The chromogenic substrate for the hexosaminidase, *p*-nitro-phenyl-*N*-acetyl-β-D-glucosaminide was dissolved at 7.5 mM in 0.1 M citrate buffer, pH 5. The substrate solution was mixed with an equal volume of 0.5% Triton X-100 in water and added to the CTLL. After 16 – 20 h incubation at 37°C, 7% CO_2, the color reaction was developed and enzyme activity stopped by addition of 90 μl per well of 50 mM glycine buffer, pH 10.4, containing 5 mM ETDA. The absorbance was measured at 405 nm 10 min later.

IFN-γ activity was measured by ELISA as described (33). ELISA microtiter plates (96-well) (Dynatech, Alexandria, VA) were coated with 5 μg/ml of protein G-purified rat anti-mouse IFN-γ mAb R4-6A2, in PBS, pH 7.2. Free binding sites were blocked with 1% (w/v) BSA (Fluka, Buchs, Switzerland) for 2 h at 37°C. Plates were then washed three times with ELISA washing buffer. Different 2-fold dilutions (in PBS, 1% BSA) of the test samples were added to the plates, and incubated overnight at 4°C in a moist chamber. A standard curve was constructed by assaying serial dilutions of mouse r-IFN-γ at starting concentrations of 1600 U/ml in PBS, 1% BSA. After three washes, biotin-labeled protein G-purified rat anti-mouse IFN-γ mAb AN-18.17.24 at 1 μg/ml in PBS, 1% BSA, was added to each well and incubated for 2 h at 37°C. After three more washes, wells were incubated for 1 h at 37°C with horseradish peroxidase-conjugated biotin – streptavidin (BRL, Gaithersburg, MD) diluted in PBS. The enzymatic reaction was developed with *o*-phenylenediamine (BRL) as chromogen (1 mg/ml in 0.1 M sodium citrate buffer, pH 4.5, and 0.012% H_2O_2) for 10 – 15 min in the dark. The reaction was then stopped by addition of an equal volume of 2 M H_2SO_4. Absorbance was measured at 492 nm.

Cells of the fibrosarcoma line WEHI 164, clone 13 (34), a gift of Dr Espevik, University of Trondheim, Trondheim, Norway, were used for the detection of biologically active TNF. Duplicate samples were tested for cytotoxic activity by incubating serial dilutions with 6×10^5/ml WEHI 164 clone 13 cells and 0.5 mg/ml actinomycin D (Sigma, St Louis, MO) in a final volume of 0.2 ml in flat-bottom microtiter plates. After 24 h of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) was added (50 μl of MTT solution of 5 mg/ml in PBS) and incubated for 3 h at 37°C in a moist chamber. Plates were then centrifuged, supernatants removed, and 100 μl of 0.04 N HCl in isopropanol were added to each well. Plates were agitated for 5 min to dissolve the blue crystals, and absorbance was measured with a multi-scan ELISA spectrophotometer, using a 550 nm filter (35). A standard titration of recombinant murine TNF-α (r-TNF-α, kindly provided by Dr G. R. Adolf, Ernst-Boehringer-Institute, Vienna, Austria) at dilutions ranging from 653.3 pg/ml to 0.3 pg/ml was set up with each assay. Compared to the standard curve, the activity of each sample was expressed in pg/ml TNF. The detection threshold of the colorimetric assay for TNF activity was 0.9 pg/ml.

Results

Effect of administration of α-CD4 mAb on cutaneous leishmaniasis in BALB/c mice

The administration of α-CD4 mAb to BALB/c mice has been shown by several investigators to reverse the extreme susceptibility of these mice to infection with *L. major* (13 – 15, 36). Results in Fig. 1 confirm this observation and further demonstrate that this effect does not depend upon the continuous administration of the α-CD4 mAb during infection. Mice given 600 μg of αCD4 mAb over a 24-h period at the beginning of infection (10 days before or after infectious challenge) were capable of resolving their lesions around 60 days after infection. Interestingly, the

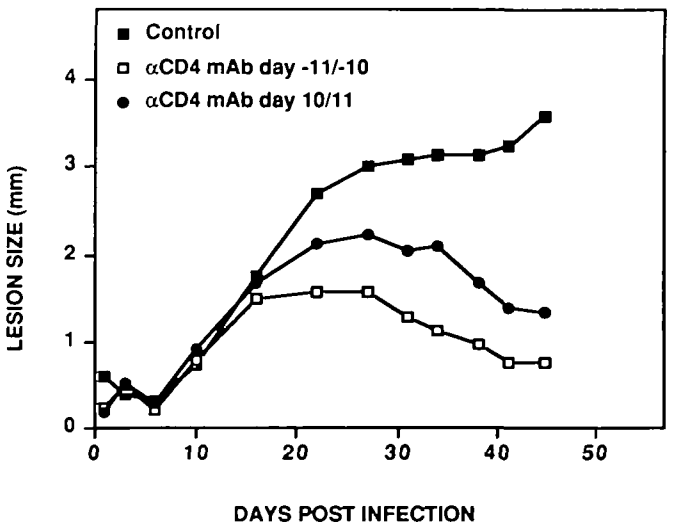

Fig. 1. Lesion development in BALB/c mice after treatment with α-CD4 mAb. Groups of susceptible BALB/c mice (10 mice per group) were infected s.c. in one hind footpad with 2×10^6 virulent *L. major* promastigotes. At the indicated times before or after infection, the animals received two injections of 300 μg α-CD4 mAb i.p. Lesion size was determined by subtracting the footpad thickness of the non-infected footpads from the infected ones

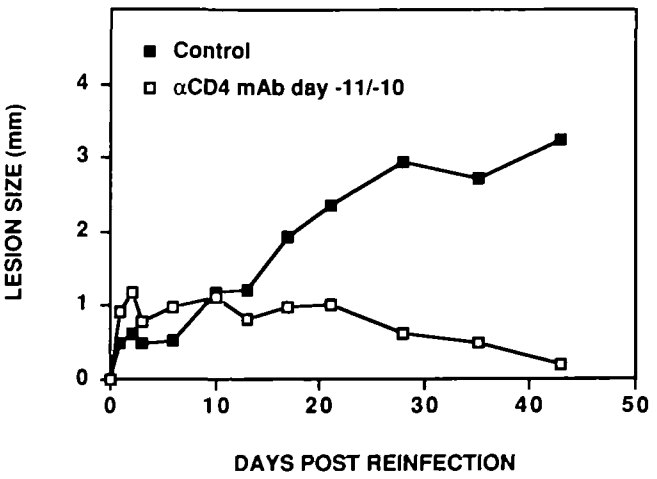

Fig. 2. Course of infection in cured BALB/c mice. Susceptible BALB/c mice, cured as a result of two injections of 300 μg α-CD4 mAb at day −11 and day −10 before infection, were reinfected 60 days later with 2×10^6 virulent *L. major* promastigotes in the footpad opposite to the site of primary infection. The development of lesions was determined by subtracting the footpad thickness of the healed footpads from the reinfected ones. Primary infected BALB/c mice were used as controls ($n = 7$).

administration of α-CD4 mAb at later times (20 days after infection) did not result in a beneficial effect on the course of disease (data not shown). Although the administration of mAb resulted in the elimination of more than 95% of CD4⁺ T cells 2 days later, 60 days after infection the percentage and the absolute number of CD4⁺ T cells in lymph nodes of cured mice was very similar to that seen in lymphoid tissues of normal BALB/c mice (data not shown). The challenge of BALB/c mice, cured as a result of administration of 600 μg α-CD4 mAb at the beginning of a primary infection led to the development of small lesions that resolved spontaneously within 5 weeks, indicating that these mice are immune to reinfection (Fig. 2).

CD8⁺ T cells play a role in the control of cutaneous leishmaniasis in BALB/c mice rendered resistant by the administration α-CD4 mAb at the beginning infection

Since previous results from this laboratory have shown that CD8⁺ T cells contribute to the resistance to infection in immune mice (9,10) it was of interest to determine whether or not CD8⁺ T cells also contribute to the ability of susceptible mice to resolve their primary lesions as a result of administration of 600 μg of α-CD4 mAb at the beginning of infection. BALB/c mice were infected s.c. in one hind footpad with 2×10^6 stationary phase *L. major* promastigotes and separated into four groups. One group was left untreated as a control. Mice from the second group received 600 μg of α-CD4 mAb GK 1.5 24 h after infection. Mice from the third group also received 600 μg of α-CD4 mAb 24 h after infection and weekly injections of 150 μg α-CD8 mAb H35, initiated 8 days after infection. Mice from the fourth group received only weekly injections of 150 μg α-CD8 mAb. The disease progression was monitored not only by measuring the size of cutaneous lesions but also by determination of the numbers of viable parasites in the lesions by limiting dilution analysis (19).

Compared to control infected mice, both the time course of lesion development (data not shown) and the parasite numbers in the lesions of mice treated only with α-CD8 mAb once per week were not significantly different (for example, 9.25×10^7 parasites/lesion in control infected mice and 6.7×10^7 parasites/lesion in α-CD8 treated BALB/c mice on day 47 after infection). Thus, under the present experimental conditions, a protective effect of CD8⁺ T cells could not be revealed during the course of a primary infection in susceptible mice.

Results shown in Fig. 3 indicate that mice treated once with α-CD4 mAb at the beginning of infection and also administered α-CD8 mAb during the course of infection developed larger lesions than mice treated only with α-CD4 mAb at the beginning of infection. Moreover, compared to mice treated only once with α-CD4 mAb, the numbers of viable parasites in lesions of mice also given α-CD8 mAb were significantly higher as determined on day 88 after infection. It is important to stress that the therapeutic effect of a single injection of 600 μg α-CD4 mAb could not be completely reversed by weekly administration of the α-CD8 mAb, since the lesions of those mice never reached the size of the lesions of control infected mice, which lost their footpads around 55 days after infection. The degree of depletion of CD8⁺ T cells from lymphoid tissues after the administration of α-CD8 mAb following the regimen described above was documented by FACS analysis. For example, at day 88 the absolute number of CD8⁺ T cells in the spleens of mice administered α-CD8 mAb was reduced by 90% compared to mice receiving only α-CD4 mAb at the beginning of infection. More importantly, when enumerating the viable parasites by the quantitative limiting dilution analysis, the lesions of mice given a single injection of α-CD4 mAb and weekly injections of α-CD8 mAb contained about a million times more parasites than the lesions of mice treated with only a single dose of α-CD4 mAb. These data indicate that resistance to infection, which is seen

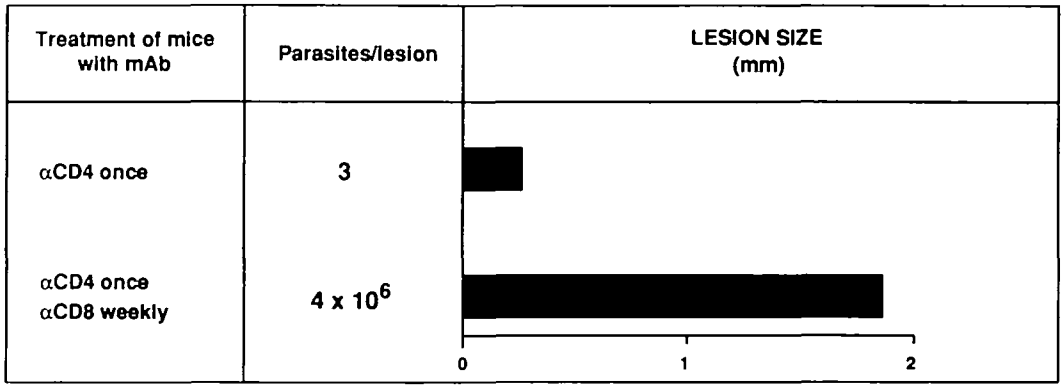

Fig. 3. Contribution of CD8⁺ T cells to the reduction of lesion-size and parasite load in susceptible BALB/c mice, rendered resistant by injection of α-CD4 mAb at the beginning of infection. BALB/c mice (4 groups of 6 mice) were infected s.c. in one hind footpad with 2×10^6 virulent *L. major* promastigotes. One day later, mice from two groups received a single injection of 600 μg α-CD4 mAb i.p. In one of the groups treated with a single injection of 600 μg α-CD4 mAb as well as in one control infected group, CD8⁺ T cells were depleted by weekly injections, initiated 8 days after infection, of 150 μg α-CD8 mAb throughout the entire course of the experiment. Lesion size was determined by subtracting the footpad thickness of the non-infected footpads from the infected ones. Parasite limiting dilution analysis was performed 88 days after infection. The control infected BALB/c mice and the infected mice which were treated only with α-CD8 mAb once per week lost their footpads around day 55 after infection and therefore the parasite burden in the lesions of these two groups was only determined on day 47 after infection. On that day, the size of lesions in these two groups of mice was > 4 mm.

in susceptible mice treated with α-CD4 mAb at the beginning of infection, depends—at least partly—upon the activity of CD8⁺ T cells.

Furthermore, the immunity to reinfection which is seen in BALB/c mice rendered resistant by the injection of 600 μg of α-CD4 mAb at the beginning of a primary infection was found to depend mainly upon the CD8⁺ T cell activity expressed early during reinfection. Indeed, a single i.v. injection of 100 μg of a α-CD8 mAb at the time of infectious challenge of cured BALB/c mice led to the development of severe lesions. In contrast, a similar treatment with α-CD4 mAb did not hamper the resolution of secondary lesions (Fig. 4).

Contribution of CD8⁺ T cells to lymphokine production by spleen cells of BALB/c mice rendered resistant by a single injection of α-CD4 mAb

It appears that the effect of T cells on the course of cutaneous leishmaniasis depends upon the activity of the lymphokines they secrete (16,17,37). Therefore, the production of several lymphokines was measured in the supernatants of specifically-activated spleen cells from mice of the different groups described above, at times when the lesions of α-CD4 mAb-treated mice were resolved. Results in Fig. 5 show that compared to control infected mice, the spleen cells from mice cured as a result of treatment with a single dose of α-CD4 mAb at the beginning of infection produced significantly higher amounts of IFN-γ in response to stimulation with live promastigotes or L.m.Ag when tested 88 days after infection. This difference in lymphokine production is not due to a different composition of the spleen cells put into culture, since the percentage of CD4⁺ and CD8⁺ T cells in the spleens of cured mice was restored to normal values. The spleens of mice cured by a single injection of α-CD4 mAb at the beginning of infection contained 16.5% CD4⁺ T cells and 6.3% CD8⁺ T cells and the spleens of control infected mice contained 11.7% CD4⁺ and 5.7% CD8⁺ T cells. Similar results were observed with IL-2 and TNF. In contrast, lymphokine

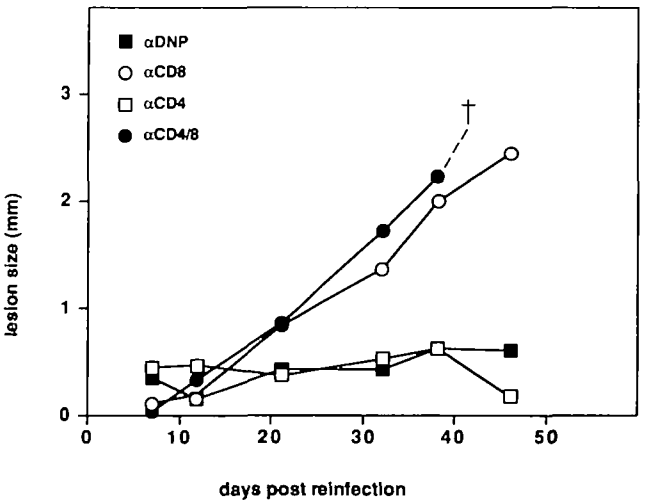

Fig. 4. CD8⁺ T cells contribute to immunity to reinfection in BALB/c mice, rendered resistant by the injection of 600 μg α-CD4 mAb at the beginning of infection. BALB/c mice were infected s.c. in one hind footpad with 2×10^6 virulent *L. major* promastigotes. At days 10 and 11 after infection the animals received two injections of 300 μg α-CD4 mAb i.p. After the primary lesions were healed, mice were divided into four groups (n = 6 mice per group) and injected i.v. with 100 μg of either α-DNP, α-CD4 or α-CD8 or a combination of α-CD4 and α-CD8 mAb. 6 h later, all mice were reinfected with 2×10^6 virulent *L. major* promastigotes in the footpad opposite to the site of primary infection. The course of reinfection was monitored by measuring the lesion development.

production by spleen cells of mice given 600 μg of α-CD4 mAb at the beginning of infection and in addition treated weekly with α-CD8 mAb was reduced to values below those seen in control infected mice. Compared to mice cured as a result of a single injection of α-CD4 mAb at the beginning of infection, the additional treatment with α-CD8 mAb resulted in a 75% reduction

of CD8⁺ T cells and in a 70% increase of CD4⁺ T cells in the spleen.

We observed that stimulation of immune spleen cells with live promastigotes as a source of antigen resulted in the release of higher amounts of lymphokines than stimulation with L.m.Ag. It is noteworthy that, using a very sensitive immuno-assay for the detection of IFN-γ, it was possible to detect significant amounts of IFN-γ in supernatants from spleen cells from infected BALB/c mice upon specific restimulation with live promastigotes *in vitro* (Fig. 5).

In none of the supernatants taken 24 h after stimulation of freshly isolated lymphoid cells could measurable amounts of IL-5 or IL-4 be detected.

DTH responses in BALB/c mice rendered resistant by the injection of α-CD4 mAb at the beginning of infection: analysis of the role of CD4⁺ and CD8⁺ T cells

The results described above indicate that the CD8⁺ T cell subset is involved in the state of resistance resulting from one injection of α-CD4 mAb in BALB/c mice. Therefore, experiments were devised to detect the actual triggering of *Leishmania*-specific CD8⁺ T cells directly *in vivo* by virtue of their ability to initiate a DTH reaction at the site of injection of viable promastigotes. After complete healing of the primary lesions (3 months after infection), mice were divided into three groups. The first group received an i.v injection of 100 μg α-DNP mAb, the second

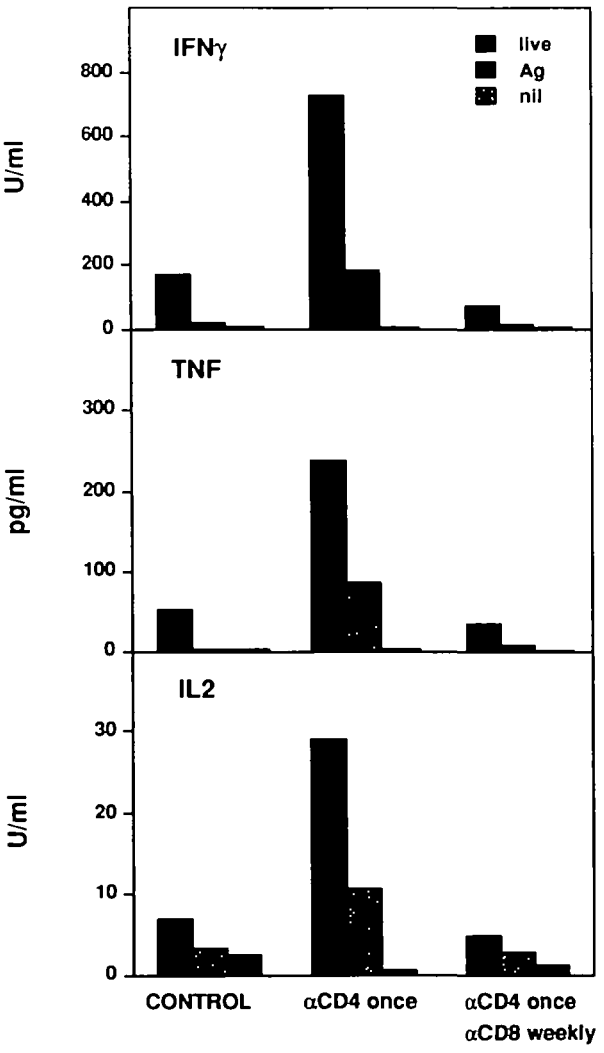

Fig. 5. Contribution of CD8⁺ T cells to lymphokine production by spleen cells from BALB/c mice rendered resistant by a single injection of α-CD4 mAb. Spleen cells (4×10^6/ml) of either control infected BALB/c mice, or BALB/c mice cured as a result of injection of 600 μg α-CD4 mAb 1 day after infection, or of mice injected 1 day after infection with 600 μg α-CD4 mAb and depleted of CD8⁺ T cells by weekly injections of 150 μg α-CD8 mAb, were stimulated *in vitro* with 1×10^6/ml live *L. major* promastigotes or 1×10^6/ml L.m.Ag. on day 88 after infection. 24 h later, supernatants were removed and tested for IFN-γ, IL-2, and TNF.

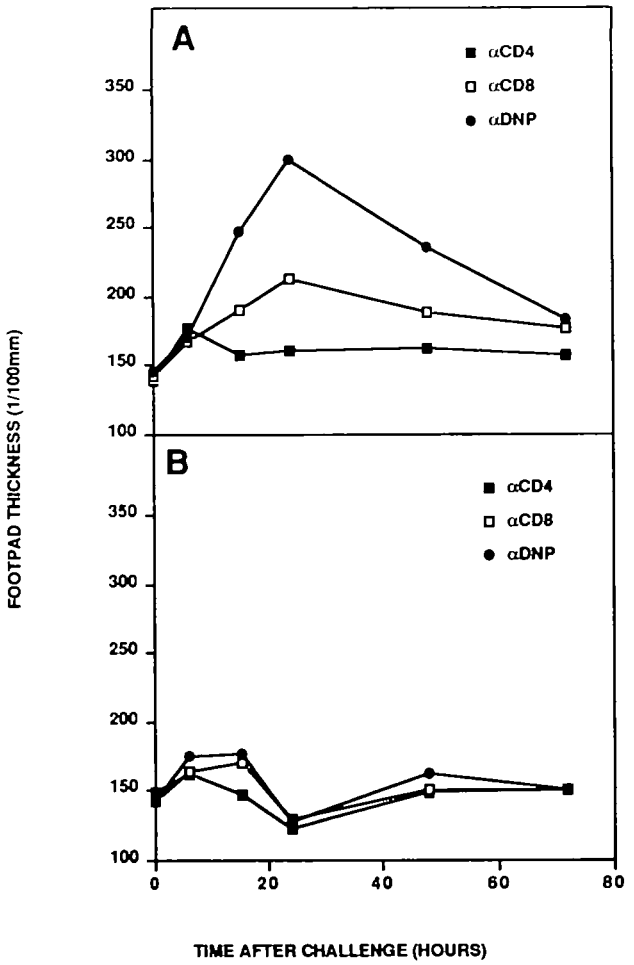

Fig. 6. Analysis of the role of CD4⁺ and CD8⁺ T cell in the development of DTH responses in BALB/c mice, cured as a result of injection of α-CD4 mAb at the beginning of infection. BALB/c mice were infected with 2×10^6 virulent *L. major* promastigotes in one hind footpad (**A**) At days 10 and 11 after infection, mice received two injections of 300 μg α-CD4 mAb i.p. After the primary lesions were healed (3 months after infection), mice were divided into three groups and injected i.v. with 100 μg of either α-DNP, α-CD4 or α-CD8 mAb. 6 h later, mice were challenged with 2×10^6 virulent *L. major* promastigotes in the footpad opposite to the site of primary infection and the DTH response was monitored by measuring the footpad thickness at different times after challenge. (**B**) Control infected BALB/c mice were treated in the same way as mice of group A, except that they were not injected with α-CD4 mAb at days 10 and 11.

group received 100 μg of α-CD4 mAb i.v., and the third group 100 μg of α-CD8 mAb i.v. After 6 h, all mice were challenged in the footpad opposite to the site of the primary infection with 2×10^6 viable *L. major* promastigotes and the DTH response was assessed by measuring the increase in footpad thickness 6, 12, 24, and 48 h later. Results in Fig. 6 show that in contrast to control infected mice (panel B), mice cured as a result of immune intervention with α-CD4 mAb exhibited a strong DTH response (panel A). This delayed inflammatory reaction was abolished in mice given anti-CD4 mAb i.v. 6 h prior to challenge with viable promastigotes. Although an inflammatory response was still detectable in mice administered anti-CD8 mAb 6 h before antigenic challenge, the magnitude of the DTH response was significantly reduced. These results clearly indicate that, in addition to CD4⁺ T cells, CD8⁺ T cells are also mediators of this DTH response. The finding that deletion of CD4⁺ T cells before challenge with viable parasites abrogates the development of this DTH response strongly suggests that CD4⁺ T cells are required for CD8⁺ T cells to mediate a DTH inflammatory reaction in this experimental system. It is noteworthy that upon challenge with soluble parasite lysates (L.m.Ag), almost no DTH response was elicited in mice from either group (Fig. 7).

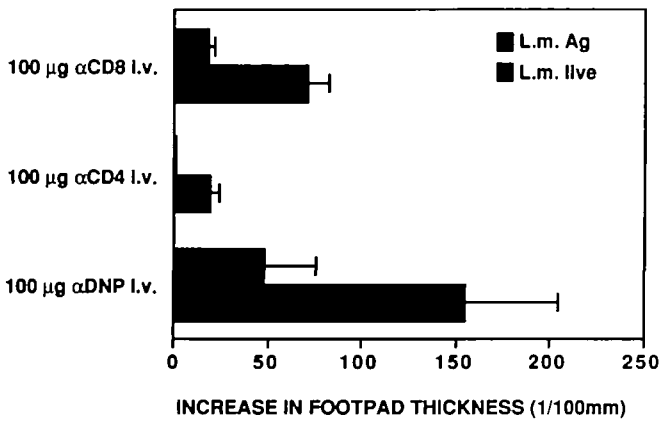

Fig. 7. Elicitation of DTH responses in cured BALB/c mice with viable *L. major* promastigotes or with soluble parasite lysates. After complete healing of the primary lesions (3 months after infection), induced by two injections of 300 μg α-CD4 mAb at days 10 and 11 after s.c. infection with 2×10^6 virulent *L. major* promastigotes, mice were divided into six groups and injected i.v. with 100 μg of either α-DNP, α-CD4 or α-CD8 mAb. 6 h later, DTH responses were induced by the injection of either 2×10^6 L.m.AG or 2×10^6 virulent *L. major* promastigotes in the footpad opposite to the site of primary infection. The DTH responses were determined by subtracting the size of the footpad before challenge from the footpad thickness after antigenic challenge.

Estimation of the number of parasite-specific CD4⁺ and CD8⁺ T cells in lymphoid organs of cured BALB/c mice

The delayed inflammatory reactions (peak at 24 h) mentioned above assess the presence of *Leishmania*-specific CD4⁺ and CD8⁺ T cells by their ability to be activated and mobilized to the skin site where viable promastigotes are injected and to recruit CR3⁺ blood cells (38). This DTH-effector function of both T cell subsets was used as a read-out system to determine the actual number of specific cells in lymphoid tissues. The frequencies of *L. major*-specific CD4⁺ and CD8⁺ T cells, able to transfer locally specific DTH responses, were determined in the draining lymph nodes, the spleens and the blood of either control infected animals, or BALB/c mice rendered resistant by administration of α-CD4 mAb 10 days after infection. Serially diluted cell suspensions, depleted or not of either CD4⁺ or CD8⁺ T cells, were mixed with viable *L. major* promastigotes and injected into the footpads of normal, syngeneic recipients. After 18 h mice expressing no increase in footpad thickness (DTH-negative transfers) were scored. The numbers of *L. major*-specific T lymphocytes were calculated from the frequency estimation and the numbers of nucleated cells in the lymphoid organs. Results in Fig. 8 confirm and extend previous observations that infec-

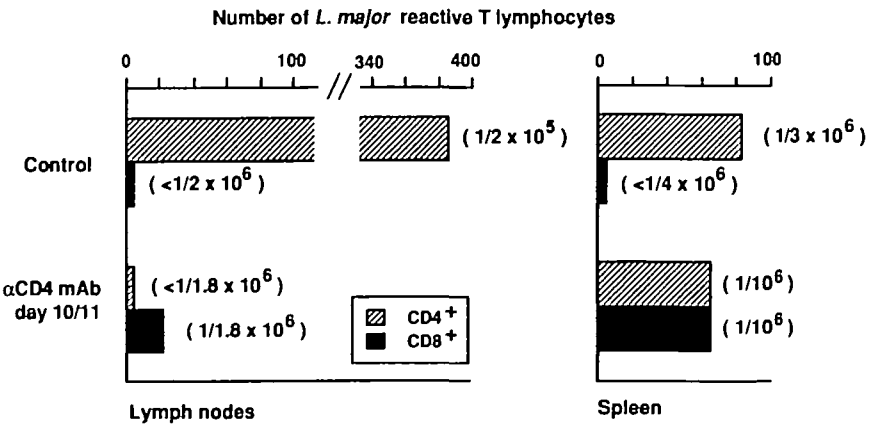

Fig. 8. Number of parasite specific CD4⁺ and CD8⁺ T cells in spleens and lymph nodes of infected and of cured BALB/c mice. BALB/c mice were infected s.c. in one hind footpad with 2×10^6 virulent *L. major* promastigotes. At days 10 and 11 after infection, one group of BALB/c mice received 300 μg α-CD4 mAb i.p. After healing of the primary lesion, (day 90) spleen and lymph node cell suspensions were prepared from cured and control infected BALB/c mice and depleted of either CD4⁺ or CD8⁺ T cells by treatment with mAb and complement. Serial dilutions of nucleated cells were transferred along with 2×10^6 viable *L. major* promastigotes into the footpads of naive syngeneic mice (10 – 12 mice/dilution). 18 h later, the negative DTH-transfers were scored and the frequency of specific T cells determined. Thereafter, the numbers of *L. major*-specific CD4⁺ and CD8⁺ T cells present in lymph nodes and spleens were calculated from the frequency estimation (given in parentheses) and the number of nucleated cells.

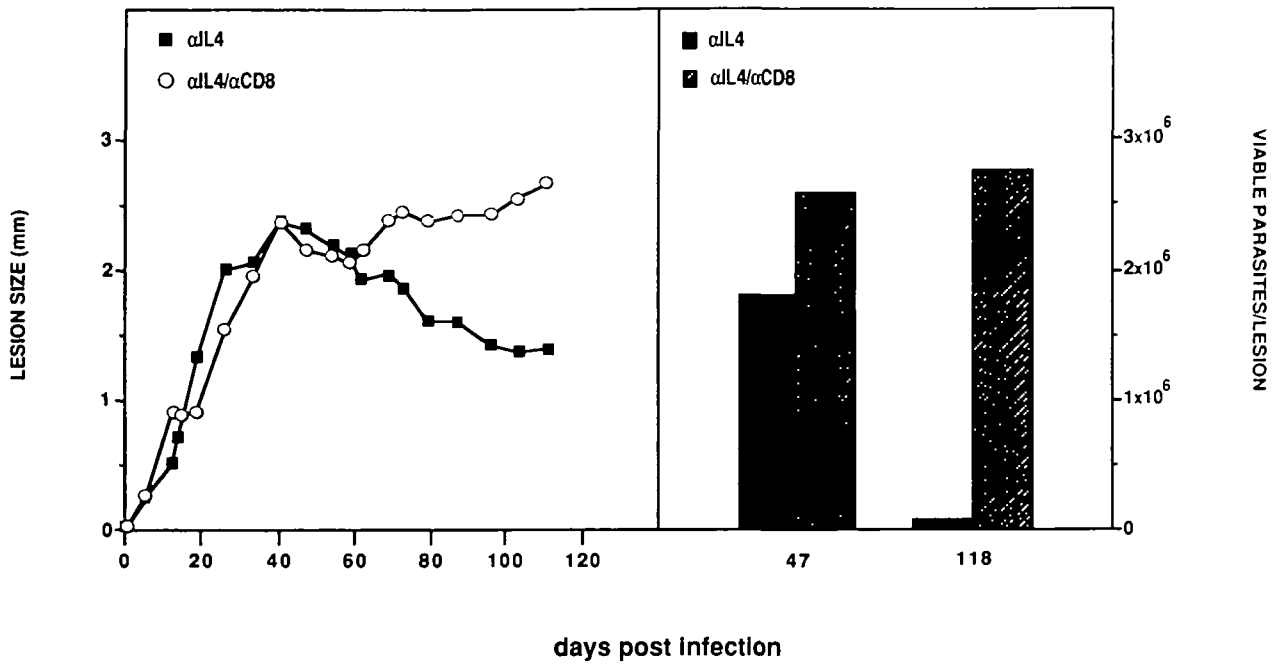

Fig. 9. Contribution of CD8⁺ T cells to the reduction of lesion size and parasite burden in susceptible BALB/c mice, rendered resistant by weekly injections of α-IL-4 mAb. BALB/c mice were infected s.c. in one hind footpad with 2×10^6 virulent *L. major* promastigotes. One group of mice (n = 6 per group) received weekly injections of 500 μg α-IL-4 mAb, starting 2 days before infection. The other group of mice was also treated with α-IL-4 mAb following the same regimen, and received in addition weekly injections of 150 μg α-CD8 mAb. Lesion size was determined by subtracting the footpad thickness of the non-infected footpads from the infected ones. The number of viable parasites present in the lesions were determined by limiting dilution analysis at days 47 and 118 after infection.

tion of BALB/c mice leads to the expansion of high numbers of specific CD4⁺ T cells capable of mediating DTH in response to viable *L. major* promastigotes (6). In contrast, compared to control infected mice, the number of specific CD4⁺ T cells in draining lymph nodes of cured BALB/c mice was drastically reduced (Fig. 8). An expansion of specifiic CD8⁺ T cells was observed in the spleens of cured BALB/c mice (Fig. 8).

Using this test system, it was possible to detect *Leishmania*-specific T cells in the blood of mice of both groups. In untreated blood of infected control mice, we found that $1/3 \times 10^5$ nucleated cells was able to transfer specific DTH responses. After treatment of blood cells with α-CD4 mAb and complement, the capacity to transfer specific DTH reaction was abrogated. In blood of cured BALB/c mice we found that $1/1 \times 10^5$ nucleated cells was able to transfer adoptively specific DTH responses. Interestingly, while elimination of CD8⁺ T cells abrogated the capacity of nucleated blood cells to transfer *Leishmania*-specific DTH, the removal of CD4⁺ T cells had no influence on this effect.

The direct demonstration of the DTH-mediating capacity of CD8⁺ T cells using this experimental transfer system possibly stems from the fact that these cells were injected into normal recipient mice i.e. with an intact CD4⁺ T cell compartment.

Contribution of CD8⁺ T cells in the control of cutaneous leishmaniasis in BALB/c mice rendered resistant by the administration of α-IL-4 mAb

Elegant studies by Heinzel *et al.* (39) have demonstrated that progression of infection in BALB/c mice is related to the presence of high amounts of IL-4 mRNA in lymphoid organs. In addition, a causal role for IL-4 in susceptibility to infection with *L. major* has been demonstrated by observations showing that the injection of specific IL-4 neutralizing mAb rendered BALB/c mice resistant to infection (17). The following experiment was performed to determine whether or not CD8⁺ T cells contribute to the resolution of lesions in those mice. BALB/c mice were infected s.c. into one hind footpad with 2×10^6 stationary phase promastigotes. Mice from the first group were left untreated and used as controls. Mice from the second group received weekly injections of 500 μg α-IL-4 mAb, starting 2 days before infection. Mice from the third group were treated with mAb following the same regimen and in addition, received weekly injections of 150 μg α-CD8 mAb, also initiated 2 days before infection. Disease progression was evaluated by measuring lesion size and determining the numbers of parasites in lesions. The results in Fig. 9 show, in confirmation with published results (17), that BALB/c mice administered α-IL-4 mAb are able to control their infection. Interestingly, the combined treatment of α-IL-4 and α-CD8 mAb led to the development of more severe lesions. Determination of parasite numbers at two time points during infection confirm this contention: mice receiving α-IL-4 and α-CD8 mAbs harboured ~40 times more parasites in their lesions than mice treated with α-IL-4 mAb only, indicating that the ability to control infection induced in susceptible mice by treatment with α-IL-4 mAb requires the participation of CD8⁺ T cells. The effect of CD8⁺ T cell depletion in mice treated with α-IL-4 mAb on the control of infection could only be seen at late times during infection.

Discussion

The results presented in this paper strongly suggest that parasite-specific CD8⁺ T cells contribute to the host's defense against infection with the obligate intracellular parasite *L. major*. The protective effect of CD8⁺ T cells has been revealed in susceptible BALB/c mice rendered resistant as a result of two different interventions on the immune system. First, evidence has been presented that CD8⁺ T cells are important for the control of infection occurring in BALB/c mice as a result of an early treatment with a single dose of α-CD4 mAb. Secondly, the establishment of a state of immunity to reinfection developing in those mice was shown to be dependent upon an anti-leishmanial effector function of CD8⁺ T cells, since the administration of 100 μg α-CD8 mAb prior to infectious challenge allowed unrestricted disease progression in these otherwise immune mice (Fig. 4). Thirdly, CD8⁺ T cells are also involved in the resolution of lesions and the reduction of parasite burden which occurs in susceptible BALB/c mice treated with α-IL-4 mAb. This last observation is supported by results showing that CD8⁺ lymphoid cells from α-IL-4 treated, cured BALB/c mice have the capacity to transfer adoptively partial protection into 200 R-irradiated syngeneic recipients (17). The beneficial effect of the administration of α-IL-4 mAb on the course of cutaneous leishmaniasis in susceptible BALB/c mice is somewhat surprising, since, in our hands, no release of IL-4 was detectable upon specific activation *in vitro* of freshly isolated spleen and lymph node cells from infected BALB/c mice, using HT2 cells as detection system (I. Müller and J. Louis, unpublished observations). However, it is possible that IL-4-producing cells are concentrated at the site of infection, where this lymphokine could be neutralized by specific antibodies.

It has been suggested that CD4⁺ T cells need to be permanently eliminated in order for CD8⁺ T cells to be activated and to perform a protective function in experimental leishmaniasis (8). The results presented in this paper clearly indicate that the beneficial effect of CD8⁺ T cells on the development of lesions can occur in susceptible mice with a normal number of CD4⁺ T cells in their lymphoid tissues. Indeed, the percentage of CD4⁺ T cells in lymphoid organs of α-IL-4 treated mice was normal, and the proportion of CD4⁺ T cells in the spleen and lymph node of mice treated with a single dose of α-CD4 mAb at the beginning of infection was restored to normal values at the time when the protective role of CD8⁺ T cells was evident. Furthermore, the weekly treatment of mice with α-CD8 mAb did not adversely affect the numbers of CD4⁺ T cells as compared to the controls. Under the two experimental conditions described here, the protective effect of CD8⁺ T cells could be detected by measuring the size of the lesion; however, this protective effect was much more striking when the number of viable parasites in the lesions were enumerated (Figs 3 and 9). Thus, the results presented here demonstrate again that, since progression of the lesion size does not necessarily correlate with the number of parasites in the lesions, the precise quantitation of parasite numbers in infected tissues represents the more reliable means to evaluate any protective effect of the immune system on experimental leishmaniasis (8,40).

Using a very sensitive limiting dilution approach (21), which allows the detection of a single, specific T cell that is able to mediate DTH, increased numbers specific CD8⁺ T cells were found in lymphoid organs of BALB/c mice rendered resistant to infection as a result of immunointervention with α-CD4 mAb (Fig. 8). The presence of specific CD8⁺ T cells in the blood of such mice shows moreover that these cells are able to circulate. In order to express their anti-*Leishmania* effector functions, these specific CD8⁺ T cells must be mobilized from the recirculating pool to the infected site where they are specifically activated. Results obtained suggest that CD4⁺ T cells are required for CD8⁺ T cells to fully express their capacity to mediate DTH responses, a conclusion currently being investigated in more detail. It is well documented that the elicitation of DTH responses depend upon the mobilization of circulating sensitized T cells to the site of antigenic challenge and their subsequent ability to recruit CR3⁺ blood cells (38). Thus, these results showing that the administration of α-CD8 mAb prior to antigenic challenge with viable *L. major* promastigotes significantly impairs parasite-specific DTH responses strongly suggest that specific CD8⁺ T cells are recruited to the site of lesion where they restrict parasite growth.

The expansion of parasite-specific CD8⁺ T cells in these mice indicates that parasite antigens are presented in the context of MHC class I molecules. In this respect it is of interest that in all the experiments described in this report, the optimal stimulation of effector functions of CD8⁺ T cells (DTH and lymphokine production) was dependent upon the use of living promastigotes as a source of antigen. Thus, it seems that some antigens of this intracellular microorganism, which lives exclusively in the phagosome-lysosome compartment, may, after processing, somehow associate with class I MHC products of their host cells. A hypothetical leak of some parasite products from those vesicles to the cytosol would make parasite antigens available for the MHC class I pathway of presentation (42). It is also possible that peptides from *Leishmania* parasites are released from infected cells and are able to bind to MHC class I molecules expressed on the surface of neighboring uninfected cells. However, our observations that CD8⁺ T cells are required for the elimination of parasites in infected tissues suggest that CD8⁺ T cells recognize antigens expressed at the surface of parasitized macrophages. It is worth mentioning that in a variety of infections with intracellular microorganisms, such as mycobacteria and *Listeria*, the development of protective immunity also depends upon the triggering of CD8⁺ T cells, and that vaccination with viable microorganisms confers optimal protection (41,42).

Several mechanisms could account for the protective effect of CD8⁺ T cells in experimental cutaneous leishmaniasis. Activation of parasitized macrophages by T cell-derived lymphokines is considered to be the essential mechanism by which parasites are destroyed in infected tissues. Thus, it is possible that the protective function of CD8⁺ T cells is expressed through production of macrophage-activating cytokines. Results in Fig. 5 indicate that the release of IFN-γ, IL-2 and TNF by specifically-activated spleen cells from BALB/c mice, cured as a result of treatment with α-CD4 mAb at the initiation of infection, was drastically reduced after depletion of CD8⁺ T cells *in vivo*. These results clearly indicate that CD8⁺ T cells participate in the production of cytokines which have been ascribed anti-leishmanial effector functions (16,43–45). A direct cytotoxic effect of CD8⁺ T cells on parasitized macrophages cannot, however, be excluded.

A final point which merits emphasis is the fact that significant amounts of IFN-γ were measured in supernatants of specifically-

activated lymphoid cells from infected, susceptible BALB/c mice (Fig. 5 and manuscript in preparation). Inasmuch as release of IFN-γ is significantly higher upon stimulation of lymphoid cells from infected BALB/c mice with living parasites, it would be of interest to determine the phenotype of the T cells from these mice capable of releasing these lymphokines. Thus, our results further indicate that the susceptibility of BALB/c mice to infection with *L. major* cannot simply be ascribed to an inability to produce IFN-γ.

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Abbreviations

CR 3	type 3 complement receptor
CTLL	cytotoxic T cell line
DNP	2,4-dinitrophenyl
DTH	delayed-type hypersensitivity
IFN	interferon
IL	interleukin
L.m.Ag	*Leishmania major* antigen
LNC	lymph node cells
SN	supernatant
TNF	tumor necrosis factor

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