Effective and Long-Lasting Immunity against the Parasite *Leishmania major* in CD8-Deficient Mice

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The results of earlier investigations that tested whether CD8$^+$ T cells are required in the defense against *Leishmania major* have been inconsistent. We used CD8-deficient mice to directly address this issue. After primary infection with *L. major*, CD8-deficient mice controlled the infection for over 1 year and mounted strong T helper 1 cell responses. Thus, CD8$^+$ T cells are not required for the long-term control of a primary infection with *L. major*.

T-cell immunity mediated by both CD4$^+$ T cells and CD8$^+$ T cells plays a crucial role in the host defense against many intracellular microbes. The immune response to the intracellular protozoan *Leishmania major* has been extensively studied in the mouse model of cutaneous leishmaniasis. Ample evidence has demonstrated the central role of CD4$^+$ helper T (Th) cells in the control of this infection, including the findings that Th1 cells have been associated with cure and Th2 cells have been associated with progressive disease (18).

The role of CD8$^+$ cells in cutaneous leishmaniasis is less well defined. Although CD8$^+$ cells appear to be important for resistance to a secondary challenge with *L. major* (5, 15–17), their role in primary infection is not clear. By using antibody-mediated cell depletion (19), thymectomy plus cell depletion (8), or in vitro assays (4, 9), a number of studies have suggested a contribution of CD8$^+$ cells to the control of primary leishmaniasis.

In contrast, experiments with β2-microglobulin-deficient (β2-m$^{-/-}$) mice demonstrated the capacity of these mice to mount strong Th1 cell responses and to heal their primary infections with *L. major* (21). Because these mice carry no CD8$^+$ T cells, such data suggested no functional requirement for these cells in the control of an *L. major* infection. However, β2-m$^{-/-}$ mice lack not only CD8$^+$ T cells (24) but also a small population of CD4$^+$ T cells expressing the NK1.1$^+$ marker (NKT cells) (1, 2). NKT cells represent a unique lineage of cells capable of producing large amounts of interleukin 4 (IL-4) within hours after activation in vivo and are candidates for directing the immune response towards the Th2 cell type (22, 23). Consequently, the lack of these cells in β2-m$^{-/-}$ mice may influence the course of cutaneous leishmaniasis. Therefore, the interpretation of the importance of CD8$^+$ cells for the course of *L. major* infection may be limited by the types of experiments done with these mice. To circumvent this difficulty, we used CD8-deficient mice (6), which are not deficient in NKT cells, to further analyze the role of CD8$^+$ cells in the immunity to a primary infection with *L. major*.

Mice which had a C57BL/6 genetic background (seventh backcross) and which were either heterozygous (CD8$^{+/-}$) or homozygous (CD8$^{-/-}$) for the disrupted CD8α gene were infected subcutaneously in the right hind footpad with 2×10^7 stationary-phase promastigotes of the *L. major* strain MHOM/IL/81/FEBNI (13) in 50 μl of buffer. The course of the infection was monitored by weekly measurements of the thicknesses of the infected and the contralateral uninfected footpads for 70 weeks. CD8$^{+/-}$ and CD8$^{-/-}$ mice controlled the long-term infection with no discernible difference (Fig. 1). A similar course of disease occurred in wild-type C57BL/6 mice with intact CD8α loci (data not shown). After the 70-week test period, the animals were killed. A fluorescence-activated cell sorter analysis of lymph node (LN) and spleen cells confirmed that CD8$^+$ T cells were absent in CD8$^{-/-}$ mice and were present at normal levels in CD8$^{+/-}$ mice (data not shown). In contrast, the levels of NKT cells were comparable in CD8$^{-/-}$ and CD8$^{+/-}$ mice, while β2-m$^{-/-}$ mice contained no NKT cells, as expected (Fig. 2).

For direct quantification of the parasite burden in CD8$^{+/-}$ and CD8$^{-/-}$ mice, cultures were set up from the spleens and lesion-draining popliteal LNs 70 weeks after *L. major* infection. Tissues were homogenized, and serial dilutions (1:3) of single-cell suspensions obtained from individual mice were pipetted into 96-well flat-bottomed culture plates (24 wells per dilution), in medium generated according to a previously published formula (12). After 10 days, the growth of parasites was determined microscopically. In accordance with Poisson statistics (11), the cell dilution at which 37% of the wells were negative for parasite growth was taken to represent the original plating of one *Leishmania* organism. The number of parasites per LN or spleen was calculated by multiplying the frequency of parasites by the number of cells in each organ. The results are presented in Table 1. Consistent with their healed local lesions, CD8$^{-/-}$ mice carried small numbers of parasites within their LNs and spleens. These numbers were comparable to those obtained by measuring parasites carried in the respective organs of CD8$^{+/-}$ mice. Thus, CD8 deficiency had no effect on the clinical course of the infection with *L. major* and on the parasite burden in C57BL/6 mice.

Previous studies have shown a correlation between the production of Th1 cytokines by T lymphocytes and the control of *L. major* infection. At the same time, the appearance of Th2

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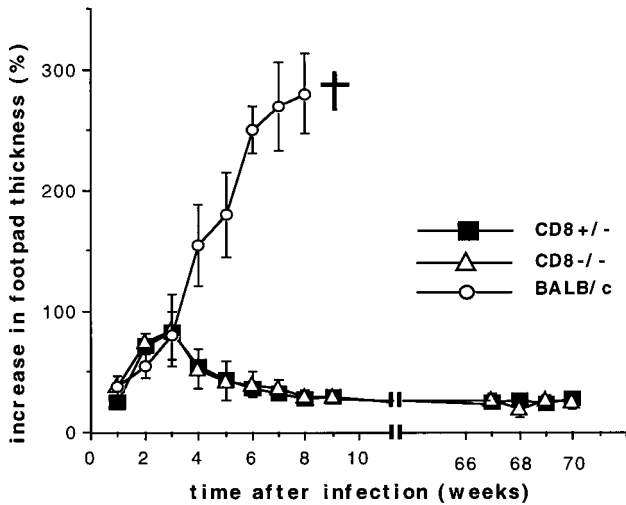
FIG. 1. Time-dependent lesion development in CD8$^{-/-}$ and CD8$^{+/-}$ mice after infection with *L. major*. Mice were infected in the right hind footpad with *L. major* promastigotes. At the indicated time points, the thicknesses of the infected and the contralateral uninfected footpads were measured with a vernier caliper, and the increases in footpad thicknesses were calculated as described previously (13). The bars denote the standard deviations. Results shown are representative of two experiments involving four CD8$^{-/-}$ mice, three CD8$^{+/-}$ mice, and four susceptible control BALB/c mice.

cytokines correlated with progressive disease (18). To assess the Th cell subset differentiation during an *L. major* infection in CD8-deficient mice, spleen and lesion-draining popliteal LN cells of CD8$^{+/-}$ and CD8$^{-/-}$ mice were analyzed 70 weeks after infection for cytokine production. Triplicate cultures of single-cell suspensions (2×10^5 cells/well) were incubated in 96-well plates together with uninfected, irradiated (20 Gy)
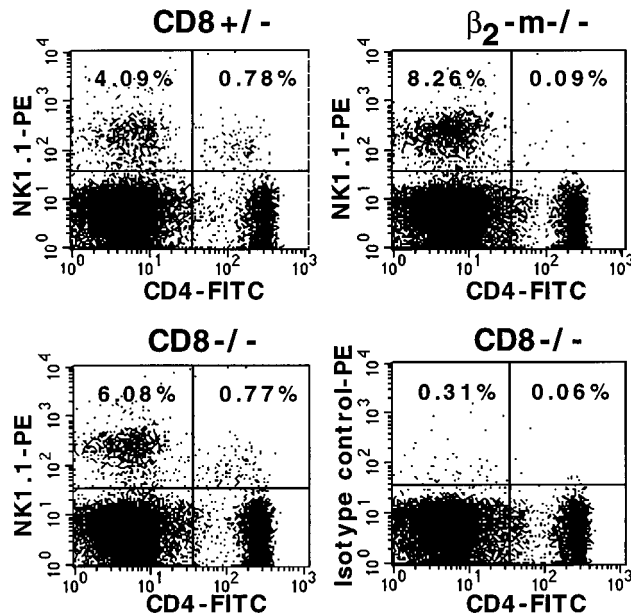

FIG. 2. Staining of NKT cells in the spleens of CD8$^{-/-}$, CD8$^{+/-}$, and β2-m$^{-/-}$ mice. Spleen cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD4 and phycoerythrin (PE)-labeled anti-NK1.1 or isotype control phycoerythrin-labeled mouse immunoglobulin G2a antibodies (all from Pharmingen). NKT cells are represented in the upper right quadrant. The numbers represent the percentages relative to the total splenocyte population.

TABLE 1. Parasite burden in organs of CD8$^{+/-}$ and CD8$^{-/-}$ mice 70 weeks after infection with *L. major*

Organ	CD8 genotype	No. of *Leishmania* parasites/organ[a]
LN	+/−	4.63 ± 0.95
	−/−	3.75 ± 0.65
Spleen	+/−	<2
	−/−	<2

[a] Parasite numbers in the popliteal LNs draining the infected footpads or in the spleens of CD8$^{+/-}$ and CD8$^{-/-}$ mice were determined 70 weeks after *L. major* infection by limiting dilution assays. Results are given as the logs of parasites (means ± standard deviations for three to four mice per group) in total LNs and spleens of infected animals. The absolute cell numbers of LNs and spleens were comparable in CD8$^{+/-}$ and CD8$^{-/-}$ mice. The data are representative of two separate experiments.

syngeneic spleen cells (3×10^5/well) and with or without *L. major* antigens (freeze-thawed lysates of 3×10^5 promastigotes/well). After 48 h, culture supernatants were harvested and tested for IL-4 and gamma interferon (IFN-γ) by enzyme-linked immunosorbent assay with commercial antibody pairs (Pharmingen, San Diego, Calif.). The measured values were standardized with recombinant IL-4 and IFN-γ. The results (Table 2) show that cells from CD8$^{-/-}$ mice as well as from CD8$^{+/-}$ mice produced the typical low-IL-4 and high-IFN-γ profile characteristic of healer mice. The levels of IL-4 and IFN-γ were comparable in both CD8$^{-/-}$ and CD8$^{+/-}$ mice. These data demonstrate a strong Th1 cell response against *L. major* and the long-term maintenance of this response in CD8-deficient mice.

A number of reports have suggested a role for CD8$^+$ cells in mediating successful immunity against a primary infection with *L. major* in mice. For example, it has been shown that antibody-mediated depletion of CD8$^+$ cells resulted in increased footpad swelling and parasite burden in genetically resistant and susceptible mice (19). Further, there have been several in vitro analyses of the capacity of CD8$^+$ cells isolated from mice after primary infection to be activated by or to lyse *Leishmania*-infected macrophages. The conclusions of these in vitro

TABLE 2. Cytokine production by total LN and spleen cells of CD8$^{+/-}$ and CD8$^{-/-}$ mice 70 weeks after infection with *L. major*[a]

Type of cell	Genotype	Stimulus	Amt of IL-4 in culture supernatant (ng/ml)	Amt of IFN-γ in culture supernatant (ng/ml)
LN	CD8$^{+/-}$	None	<0.05	<0.05
		LmAg	<0.05	13 ± 7.3
	CD8$^{-/-}$	None	<0.05	<0.05
		LmAg	<0.05	27 ± 18
Spleen	CD8$^{+/-}$	None	<0.05	<0.05
		LmAg	0.35 ± 0.06	21 ± 6.5
	CD8$^{-/-}$	None	<0.05	<0.05
		LmAg	0.56 ± 0.09	15 ± 2

[a] Amounts of IFN-γ and IL-4 secreted in response to *L. major* antigens (LmAg) (freeze-thawed lysates of 3×10^5 promastigotes/well) in the presence of irradiated (20 Gy) syngeneic spleen cells (3×10^5/well) by total popliteal LN and spleen cells (2×10^5 cells/well) of CD8$^{+/-}$ and CD8$^{-/-}$ mice were determined 70 weeks after infection with *L. major*. Results are representative of two separate experiments and give the means of three to four individually tested mice per group ± standard deviations.

studies were inconsistent. Some reports showed that $CD8^+$ cells derived from mice with resolved primary *L. major* infections efficiently lysed sensitized syngeneic macrophages (4, 9). In contrast, other reports failed to demonstrate lytic activity of $CD8^+$ cells against macrophages infected with *Leishmania* (3, 7, 14). In accordance with these negative results, the experiments with $\beta2\text{-m}^{-/-}$ mice suggested no functional requirement for $CD8^+$ cells in the control of an *L. major* infection (21). Our results show that *L. major*-infected CD8-deficient mice developed a typical healing Th1 cell response with small, transient footpad lesions: even after long-term infection, small numbers of parasites were detectable in the LNs and spleens of infected animals, while LN cells and spleen cells produced large amounts of IFN-γ after antigen-specific stimulation in vitro. Therefore, $CD8^+$ T cells are not required for the long-term maintenance of a Th1-cell-mediated immunologic control of an infection with *L. major*. These findings extend the results reported for *L. major*-infected $\beta2\text{-m}^{-/-}$ mice by demonstrating that even in the presence of NKT cells, which could provide IL-4 for Th2 cell differentiation, $CD8^+$ cells are not decisive in the maintenance and control of Th1 cell immunity to *L. major* in C57BL/6 mice. Our results are also consistent with those of von der Weid et al. (20), who found that, in BALB/c mice, the presence or lack of NKT cells does not affect the generation of Th2 cell responses against *L. major*. Accordingly, it has been shown by Launois et al. (10) that NK1.1$^-$ CD4$^+$ T cells rather than NKT cells are responsible for the early burst of *L. major*-induced IL-4 mRNA expression in BALB/c mice.

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