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$CD8^+$ T Cells Are Required for Primary Immunity in C57BL/6 Mice Following Low-Dose, Intradermal Challenge with *Leishmania major*

Yasmine Belkaid,* Esther Von Stebut,† Susana Mendez,* Rosalia Lira,* Elisabet Caler,* Sylvie Bertholet,* Mark C. Udey,† and David Sacks[1]*

Standard murine models of cutaneous leishmaniasis, involving s.c. inoculation of large numbers of *Leishmania major* promastigotes, have not supported an essential role for $CD8^+$ T cells in the control of primary infection. Recently, a *L. major* model combining two main features of natural transmission, low parasite dose and inoculation into a dermal site, has been established in resistant C57BL/6 mice. In the present studies, C57BL/6 mice with $CD8^+$ T cell deficiencies, including $CD8^{-/-}$ and CD8-depleted mice, failed to control the growth of *L. major* following inoculation of 100 metacyclic promastigotes into the ear dermis. The resulting dermal pathology was minor and delayed. Lesion formation in wild-type mice was coincident with the killing of parasites in the inoculation site. Both events were associated with the accumulation of $CD8^+$ T lymphocytes in the skin and with the capacity of $CD8^+$ T cells recovered from draining lymph nodes or infected dermis to release IFN-γ following coculture with infected dendritic cells. Reconstitution of resistance to *L. major* in $RAG^{-/-}$ mice using T cells from naive donors was optimal when both $CD4^+$ and $CD8^+$ T cells were transferred. Primed $CD8^+$ T lymphocytes obtained from C57BL/6 mice during the acute stage of infection were able to mediate both pathology and immunity when transferred alone. The low dose, intradermal challenge model reveals that $CD8^+$ T cells play an essential role in both pathogenesis of and immunity to primary infection with *L. major* in the skin. *The Journal of Immunology,* 2002, 168: 3992–4000.

Infection of mice with the protozoan parasite *Leishmania major* has been extensively used to define the factors controlling the differentiation of $CD4^+$ T lymphocyte subsets in vivo. Using the standard challenge model involving s.c. injection of high doses (10^5–10^7) of parasites in the footpad, and depending on the genotype of the mouse, *L. major* induces a Th1- or Th2-predominant response that respectively controls or promotes intracellular growth of the parasite in the inoculation site. Acquired resistance in this model has been shown to require $CD4^+$ T cells, activated in an IL-12-driven and CD40L-dependent manner, for the production of high levels of IFN-γ that induce NO-dependent killing by infected macrophages (1–10). Although $CD8^+$ T cells were shown to be essential to immunity to reinfection in resistant mice that healed their primary lesion (11–14), the role of $CD8^+$ T cells in the control of primary infection remains unclear. A higher frequency of parasite-specific $CD8^+$ T cells was found in resistant (C57BL/6) compared with susceptible (BALB/c) mice (2, 11). Furthermore, administration of anti-CD8 Ab to resistant mice exacerbated infection, although treated mice were still able to ultimately heal (2). C57BL/6 mice with β_2-microglobulin or CD8 deficiencies also maintained their ability to heal, strongly indicating that $CD8^+$ T cells are not required for control of primary infection (15–17).

In contrast to the conventional challenge models used in the studies described above, in natural transmission a low number (<1000) of metacyclic promastigotes (18) is delivered by the sand fly into the dermis of the mammalian host. We previously established a model of infection that combines two main features of natural transmission: low dose (100 metacyclic promastigotes) and cutaneous inoculation (the mouse ear dermis) (19). The model revealed two phases in the pathogenesis of dermal leishmaniasis that had not been distinguished previously: a remarkably silent phase, lasting 4–5 wk, favoring the uncontrolled growth of parasites in the skin without formation of a lesion, followed by development of a dermal lesion that is coincident with the onset of immunity and killing of the parasite in the inoculation site. A role for $CD4^+$ T cells in both pathogenesis and immunity was confirmed in the natural infection model.

In the present studies, $CD8^+$ T cells are reexamined in the context of the low dose, intradermal challenge model, including their homing to and accumulation in the skin, their capacity to produce IFN-γ in response to parasite Ag, their role in the pathogenesis of dermal lesions, and their requirement for the control of infection and the resolution of dermal disease.

Materials and Methods

Mice

C57BL/6 mice were purchased from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD). $RAG1^{-/-}$ mice (backcrossed for 10 generations to C57BL/6) were purchased from Taconic Farms (Germantown, NY). $CD8\alpha^{-/-}$ mice (backcrossed for at least five generations to C57BL/6) were kindly provided by A. Singer (National Cancer Institute, National Institutes of Health, Bethesda, MD). All mice were maintained in a National Institute of Allergy and Infectious Diseases animal care facility under specific pathogen-free conditions.

*Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, and †Dermatology Branch, Division of Clinical Sciences, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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[1] Address correspondence and reprint requests to Dr. David Sacks, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 4/126, 4 Center Drive MSC 0425, Bethesda, MD 20892-0425. E-mail address: dsacks@nih.gov

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Parasite preparation and intradermal inoculation

L. major clone V1 (MHOM/IL/80/Friedlin) was cultured in Medium 199 with 20% heat-inactivated FBS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 mg/ml hemin (in 50% triethanolamine), and 1 mg/ml 6-biotin (M199/S). Infective-stage metacyclic promastigotes of *L. major* were isolated from stationary cultures (4–5 days old) by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA). Metacyclic promastigotes (100) were inoculated intradermally into the ear dermis using a 27.5-gauge needle in a volume of ~5 μl. The evolution of the lesion was monitored by measuring the diameter of the induration of the ear lesion with a direct-reading vernier caliper (Thomas Scientific, Swedesboro, NJ).

CD8$^+$ cell depletion

Mice were inoculated i.p. with 1 mg of rat mAb anti-CD8 (2.43) or rat isotype control (GL113), purified from ascites by ammonium sulfate precipitation, at the time of the challenge and weekly thereafter until termination of the experiments.

Estimation of parasite load

Parasite titrations were performed as previously described (19). The two sheets of infected ears were separated, deposited dermal side down in DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 125 U/ml collagenase A (Sigma-Aldrich, St. Louis, MO), and incubated for 2 h at 37°C. Sheets were subsequently cut into small pieces and homogenized using a Teflon-coated microtissue grinder in a microfuge tube containing 100 μl of M199/S. Tissue homogenates were filtered using a 70-μm cell strainer (Falcon Products, St. Louis, MO) and serially diluted in 96-well flat-bottom microtiter plates containing biphasic medium prepared using 50 μl of NNN medium containing 30% of defibrinated rabbit blood overlaid with 50 μl of M199/S. The number of viable parasites in each sample was determined from the highest dilution at which promastigotes could be grown out after 7 days of incubation at 26°C.

Analysis of intradermal lymphocytes

To characterize leukocytes in the inoculation sites, the ears were collected, and the ventral and dorsal dermal sheets were separated and incubated dermal side down on RPMI 1640, $NaHCO_3$, penicillin/streptomycin/gentamicin, containing 125 U/ml collagenase A (Sigma-Aldrich) for 2 h. The dermal sheets from three to five animals were pooled, cut into small pieces, and filtered through a 70-μm nylon cell strainer (BD Biosciences, Mountain View, CA) before being washed twice in RPMI 1640, $NaHCO_3$, penicillin/streptomycin/gentamicin, 10% FCS, and 0.05% DNase I (Sigma-Aldrich). Cells were analyzed by flow cytometry for surface markers and/or cytoplasmic staining for IFN-γ, as described below.

Preparation of APC, lymphocytes, and coculture

In some experiments, unfractionated lymph node (LN)[2] cells, purified lymphocyte subsets, or dermal cells were incubated with fetal skin-derived dendritic cells (FSDDC) prepared as previously described (20). Briefly, skin cells from day 16 fetal C57BL/6 or BALB/c mice were cultured in GM-CSF- and M-CSF-supplemented media and, after 2 wk, dendritic cell (DC) aggregates were isolated by serial 1-g sedimentation. DC aggregates were dissociated in trypsin/EDTA (0.25%/0.1 mM) as necessary to allow accurate determination of cell number. FSDDC aggregates were subcultured in growth media (5% FBS, GM-CSF- and M-CSF-supplemented RPMI 1640) in 24-well plates at 2×10^5 cells in 1 ml/well. *L. major* amastigotes, purified from footpad lesions and opsonized by incubation for 30 min at 37°C in 5% fresh normal mouse serum, were added at approximately five organisms per cell. FSDDC aggregates were completely dissociated in calcium- and magnesium-free HBSS containing 1 mM EDTA (30 min at 37°C). In some experiments, DC were generated from bone marrow in the presence of GM-CSF (BMDC), as previously described (21). Bone marrow-derived macrophages (BMDM) were cultured with 30% L929 supernatant for 5 days, and nonadherent cells were recovered and incubated for 18 h in 2.5% L929 supernatant. Adherent cells were recovered and infected for 18 h with serum-opsonized amastigotes (five amastigotes per macrophage). Aliquots of cells were prepared in a cytospin and stained using Diff Quick (Dade Behring, Dudinger, Switzerland) to estimate the level of infection. CD8$^+$ and CD4$^+$ T cells were isolated by positive selection using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD8$^+$ or CD4$^+$ T lymphocytes was >95%. Unfractionated LN or dermal cells were resuspended in complete RPMI 1640 at 2.5×10^6 cells/ml, and 1 ml of the cell suspension was incubated with or without soluble *Leishmania* Ag (SLA; 25 μg/ml) or with infected or uninfected FSDDC or BMDC at a ratio of 5:1 in 24-well plates at 37°C in 5% CO_2 for 18 h. The cells were harvested and analyzed for surface markers and intracytoplasmic staining for IFN-γ. Purified CD8$^+$ or CD4$^+$ T cells were cocultured with uninfected, infected, or Ag-loaded BMDM or FSDDC at a ratio of 5:1 (5×10^5 cells total) in U-bottom 96-well plates and incubated at 37°C in 5% CO_2 for 24 h. In some experiments, unfractionated LN cells were recovered and incubated for 48 h with SLA (25 μg/ml), and IFN-γ or IL-4 in culture supernatants was quantitated by ELISA, as previously described (22).

Immunolabeling and flow cytometry

Before staining, LN or dermal cells were incubated with an anti-Fc III/II receptor (BD PharMingen, San Diego, CA) mAb and 10% normal mouse serum in PBS containing 0.1% BSA and 0.01% NaN_3. The lymphocytes were identified by characteristic size (forward light scatter (FSC)) and granularity (side light scatter (SSC)), in combination with anti-TCRβ chain (H57-597, FITC conjugated; BD PharMingen) and anti-CD4 or anti-CD8 (CyChrome conjugated) surface staining. The staining of surface and intracytoplasmic markers was performed sequentially: the cells were stained first for their surface markers (TCRβ chain receptor, CD8 or CD4), followed by a permeabilization step and staining with anti-IFN-γ (XNG1.2; BD PharMingen). The isotype controls used (all from BD PharMingen) were rat IgG2b (A95-1), rat IgG2a (R35-95), and hamster IgG, group 2 (Ha4/8). For each sample, between 200,000 and 400,000 cells were analyzed. The data were collected and analyzed using CellQuest software and a FACSCalibur flow cytometer (BD Biosciences).

Reconstitution of C57BL/6 RAG$^{-/-}$ mice

Naive (4×10^6) or primed (2×10^6) CD4$^+$ and/or CD8$^+$ T cells were injected i.v. in 300 μl of PBS into RAG$^{-/-}$ mice. Unreconstituted and reconstituted RAG$^{-/-}$ mice were challenged the same day with 10^3 *L. major* metacyclic promastigotes in the dermis of both ears. Mice reconstituted with CD8$^+$ T cells were also inoculated the same day and 1 wk later with 1 mg of anti-CD4 (GK1.5). Parasite load and lesion size were followed, as previously described. Between 4 and 12 wk following infection, cells from the inoculation site and the draining LN were prepared as described above and incubated for 2 h in the presence of 10 μg/ml anti-CD28 (37.51; BD PharMingen), 5 ng/ml IL-2 (Endogen, Woburn, MA), and 25 μg/ml SLA, followed by a 4-h incubation with brefeldin A (Sigma-Aldrich). Cells were analyzed by FACS, as described.

Results

Parasite loads and dermal lesions in C57BL/6, CD8$^{-/-}$, and CD8-depleted mice following inoculation of 100 L. major *metacyclic promastigotes in the ear*

To characterize the role of CD8$^+$ T cells in the control of primary infection due to *L. major*, 100 metacyclic promastigotes were inoculated into the ear dermis (both ears) of wild-type C57BL/6 mice, CD8$^{-/-}$, or CD8-depleted C57BL/6 mice. The number of parasites in the inoculation sites was determined at different time points, and the evolution of the lesions (measured by nodule diameter) was monitored every week over a period of 17–24 wk (Fig. 1). In C57BL/6 wild-type mice or mice injected weekly with the anti-CD8 isotype control, the parasites grew to maximal numbers in the skin during the first 4–5 wk without inducing lesions (19). Control of parasite growth was apparent during wk 5 and 6, and was accompanied by the development of a small nodule that completely resolved by 9–10 wk postinjection (Fig. 1). There were no significant differences between the control groups and the various CD8-deficient groups in their dermal parasite loads up to wk 5. By 6–8 wk, when >95% of the parasites had been cleared from the site in the wild-type or control-treated mice, parasites continued to grow in the CD8-deficient mice such that by wk 10, the ears of CD8$^{-/-}$- and anti-CD8-treated mice contained, respectively, 2 and 3.3 log-fold more parasites compared with their respective

[2] Abbreviations used in this paper: LN, lymph node; DC, dendritic cell; BMDC, bone marrow-derived DC; BMDM, bone marrow-derived macrophage; FSC, forward light scatter; FSDDC, fetal skin-derived DC; SLA, soluble *Leishmania* Ag; SSC, side light scatter.

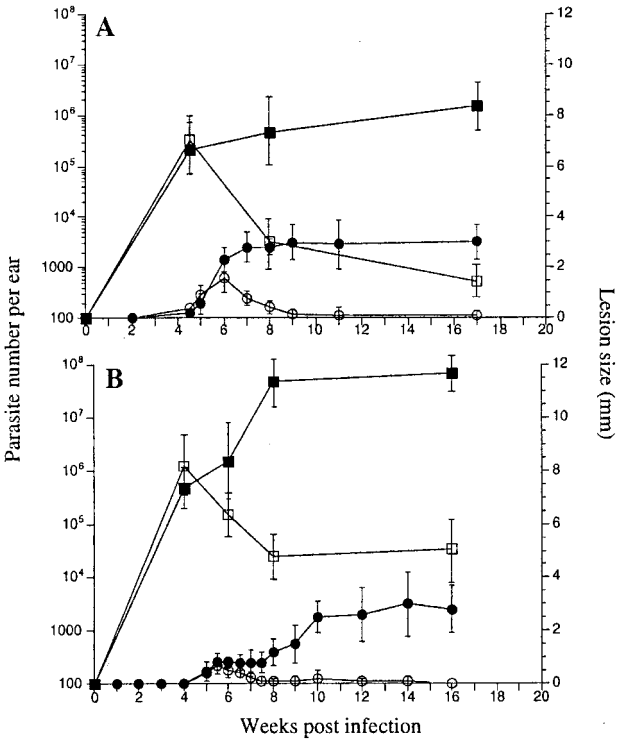

FIGURE 1. Number of parasites per ear (■ and □) and diameter of induration (● and ○) following intradermal inoculation of 100 *L. major* metacyclics in *A*, C57BL/6 mice (□ and ○) or in CD8$^{-/-}$ mice (■ and ●), and *B*, CD8-depleted (■ and ●) or isotype-treated (□ and ○) C57BL/6 mice. Values represent mean induration in mm ± SD, five mice per group, and geometric mean parasite number per ear ± SD, four mice and eight ears per group.

controls (Fig. 1). To confirm that this effect was not transient, all experiments were conducted over a period of 4–5 mo. No reduction in the total number of parasites in the inoculation site was observed in the CD8-deficient mice over this period. Parasite loads in the CD8$^{-/-}$ and the CD8-depleted mice stabilized at 10^6 and 10^8 parasites per ear, respectively, at about wk 16. These high parasite loads were associated with relatively minor dermal pathology, characterized by nonulcerative nodules that failed to heal (Fig. 1). The persistence of variable numbers of parasites in the skin of the wild-type and control-treated mice following healing of their dermal lesions has been reported previously, and is dependent on the production of IL-10 in the chronic site (23). The CD8 depletion was associated with a significant reduction in the amount of IFN-γ produced by LN cells in response to SLA 3.5 and 8 wk after infection (Table) compared with the isotype control. The production of IL-4 was slightly increased 8 wk postchallenge in anti-CD8-treated mice compared with the isotype control (305 ± 10 vs 123 ± 7).

T cell activation in the draining LN of C57BL/6 mice following low-dose, intradermal challenge

Naive T cells typically express high levels of CD62L (L-selectin), low levels of CD25 (IL-2Rβ chain), and low levels of the early activation marker CD69. We followed expression of these markers on CD4$^+$ and CD8$^+$ T cells present in draining LN during the course of the infection in the ear dermis (Fig. 2). The proportion of CD4$^+$ T cells expressing an activated phenotype increased as early as 1 wk postinfection, with CD25$^+$ cells increasing from 13 to 25%, CD62L^{low} from 9.5 to 18%, and CD69$^+$ from 13 to 17%. Expression of these activation markers on CD8$^+$ T cells was delayed until 4–5 wk, just before the onset of lesion formation and killing of parasites in the inoculation site.

Recruitment of CD8$^+$ and CD4$^+$ T lymphocytes to the inoculation site

The *L. major*-loaded dermis was analyzed for the presence of cells bearing TCRβ and either CD4 or CD8 by two-color FACS analysis of cells extracted from collagenase-treated skin (Fig. 3). A small number of CD4$^+$ T lymphocytes (4.5×10^3 cells/ear), but no CD8$^+$ T cells were found in the ear dermis of naive mice. These steady-state conditions were maintained until wk 4, at which time there was a substantial increase in CD4$^+$ T cells to 4.2×10^4/ear, and the onset of CD8$^+$ T cell recruitment to the site (7.2×10^3). At 6 wk, their cell numbers increased to 8.9×10^4 CD4$^+$ and 2.8×10^4 CD8$^+$ T cells/ear dermis.

APC requirements for Ag-specific IFN-γ production by CD4$^+$ and CD8$^+$ T cells in draining LN and dermis

Because IFN-γ production remains an absolute requirement for acquired immunity in the natural-challenge model (19), we examined the ability of CD4$^+$ and CD8$^+$ T cells present in draining LN 5 wk postinfection to produce IFN-γ in response to SLA (Fig. 4). An increased frequency (1.7%) of CD4$^+$ T cells, but not of CD8$^+$ T cells able to produce IFN-γ was observed. Because the APC and exogenous Ag present in the whole LN cultures may not have optimized the conditions necessary to reveal activation of CD8$^+$ T cells for production of IFN-γ, FSDDC, which have been shown to up-regulate MHC class I, class II costimulatory molecules, and IL-12 p40 in response to uptake of *L. major* amastigotes (24), were loaded with SLA or infected with amastigotes and added to the cultures. CD4$^+$ T cells were able to release IFN-γ when FSDDC alone were added to the LN cultures (1.8%), perhaps due to the presence of a small amount of Ag released by the infected LN cells. No IFN-γ-producing cells were seen when FSDDC were incubated with LN cells from uninfected mice (data not shown). The percentage of IFN-γ-producing CD4$^+$ T cells was slightly

Table I. *Effect of anti-CD8 depletion on* Leishmania*-specific cytokines production by LN cells draining the ear 3.5 and 8 wk postinfection*

	3.5 wk		8 wk	
	Isotype Control	Anti-CD8	Isotype Control	Anti-CD8
IFN-γ	726 ± 57[a]	12.6 ± 3.5[b]	8043 ± 321	3937 ± 176[b]
IL-4	47 ± 11	36 ± 2.3	123 ± 7	305 ± 10[c]

[a] Mean cytokine concentration (pg/ml) ± 1 SD produced by LN cells (four LN per group) assayed 48 h after stimulation with 25 μg/ml SLA.
[b] Significantly less than the IFN-γ produced by cells of the isotype control mice, $p < 0.001$.
[c] Significantly more than the IL-4 produced by cells of the isotype control mice, $p < 0.001$.

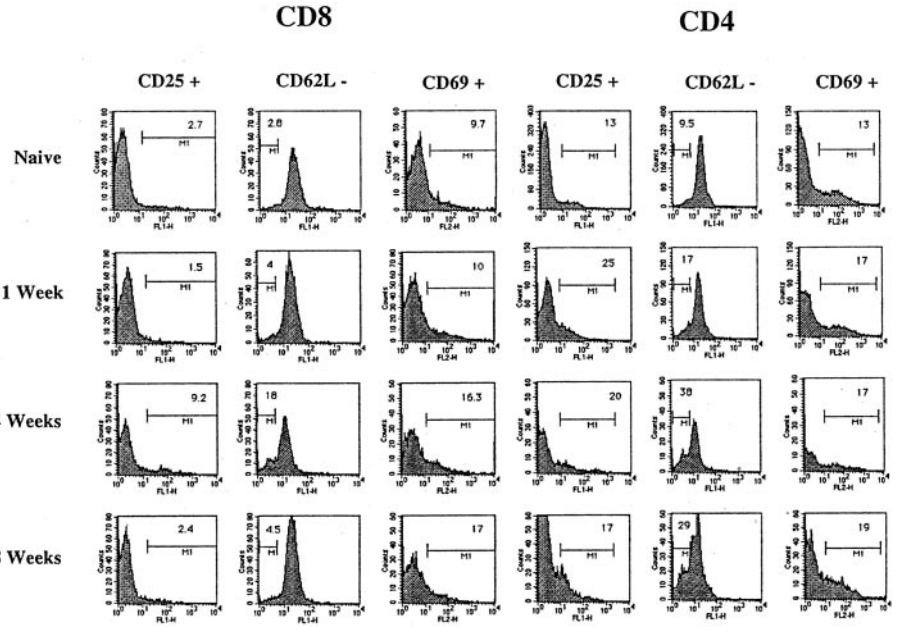

FIGURE 2. FACS analysis of surface activation markers expressed by $CD4^+$ or $CD8^+$ T cells in the draining LN following intradermal inoculation of 100 *L. major* metacyclics in C57BL/6 mice. For each marker, the histograms of gated $TCR\beta^+/CD4^+$ or $TCR\beta^+/CD8^+$ cells are shown. For CD25 and CD69, the numbers represent the percentage of cells with FL1 signals greater than that detected with the appropriate isotype control. For CD62, the numbers represent the percentage of cells that stained negative for the marker based on a comparison with isotype control. The draining LN from four mice were pooled for each time point. The experiment is representative of two separate experiments.

increased when the LN cells from infected mice were incubated with FSDDC loaded with SLA (1.8%) or live parasites (2.3%). In contrast, only FSDDC infected with live *L. major* induced substantial production of IFN-γ by $CD8^+$ T cells (3.2%).

To determine whether the $CD8^+$ T cells extracted from skin were also able to release Ag-specific IFN-γ, dermal cells were purified from 6-wk-infected mice and incubated with either *L. major* infected or noninfected BMDC. A small percentage of $CD4^+$ (0.7%) or $CD8^+$ T cells (0.2%) produced IFN-γ when exposed to noninfected BMDC. When exposed to infected DC, a high percentage of both $CD4^+$ (18%) and $CD8^+$ T cells (4.5%) was able to produce IFN-γ (Fig. 4*B*).

To confirm that the primed $CD8^+$ T cells can secrete IFN-γ and to investigate further their selective reactivation requirements, positively selected $CD4^+$ and $CD8^+$ cells prepared from LN of C57BL/6 mice 5 wk postchallenge were incubated with infected or SLA-pulsed macrophages or FSDDC and assayed for IFN-γ release by ELISA (Fig. 5). Although $CD4^+$ T cells released high and comparable amounts of IFN-γ regardless of the source of Ag or APC used for their activation, $CD8^+$ T cells released IFN-γ only when incubated with Ag-loaded FSDDC (5.2 ng/ml) or with FSDCC infected with *L. major* amastigotes (10 ng/ml). In addition to BMDM, resident peritoneal macrophages or inflammatory macrophages obtained from nonimmune granulomas also failed to activate $CD8^+$ T cells for IFN-γ production (data not shown).

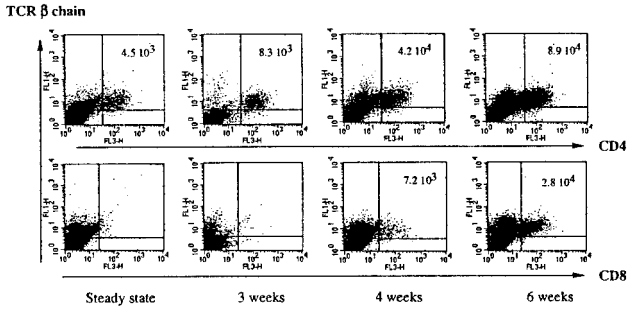

FIGURE 3. Dot plots of $TCR\beta^+/CD4^+$ and $TCR\beta^+/CD8^+$ cells present in the ear dermis following intradermal inoculation of 100 *L. major* metacyclic promastigotes in C57BL/6 mice. The numbers represent the absolute number of cells per ear positive for each of the two markers. Ten ears from five mice were pooled for each time point. The experiment is representative of three separate experiments.

Immunity in $RAG^{-/-}$ mice reconstituted with naive $CD8^+$ and/or $CD4^+$ T cells

To assess the effector capacity of $CD8^+$ lymphocytes in vivo, and to determine whether these cells could mediate protection and pathology, C57BL/6 $RAG^{-/-}$ mice, deficient in the recombinase-activating gene required for development of mature B or T cells, were reconstituted with $CD4^+$ and/or $CD8^+$ T cells recovered from naive wild-type mice. The recipient and unreconstituted mice were challenged in the ear with 1000 metacyclic promastigotes. The transferred $CD8^+$ cells did not contain $CD8^+$ DC, as assessed by the absence of MHC class II-positive cells. The purified $CD8^+$ population did, however, contain small numbers (<1%) of $CD4^+$ lymphocytes, which in an initial study led to the accumulation of large numbers of $CD4^+$ T cells in inoculated ears (55% of $CD3^+$ cells) and in draining LN (30% of $CD3^+$ cells) at 6 wk postinfection. To avoid this problem, the $CD8^+$-reconstituted $RAG^{-/-}$ mice were also treated with anti-CD4 Ab at the time of, and 1 wk after, adoptive transfer.

$RAG^{-/-}$ mice were incapable of controlling parasite growth in the site (Fig. 6*A*). At 12 wk, these mice harbored 10^6 parasites per ear compared with 5×10^3 in the wild-type mice. Naive $CD8^+$ T cells failed to transfer resistance and, at 8 or 12 wk postchallenge, the number of parasites was comparable with the number estimated in the $RAG^{-/-}$ control. At 12 wk postinfection, $RAG^{-/-}$ mice reconstituted with $CD4^+$ T were able to maintain a small number of parasites in the site (9.1×10^3/ear), while all the mice cotransferred with naive $CD4^+$ and $CD8^+$ T cells were able to clear the parasite from the skin (Fig. 6*A*) and the LN (data not shown). The high tissue parasite burden in the $RAG^{-/-}$ mice was associated with little dermal pathology (Fig. 6*B*). Surprisingly, mice reconstituted with $CD8^+$ T cells alone developed early (3–4 wk) and severe pathology that by 10–12 wk postchallenge resulted in the complete destruction of the tissue in six of eight infected ears. Mice reconstituted with naive $CD4^+$ T cells developed small nodules that resolved 11–12 wk postinfection. Mice reconstituted

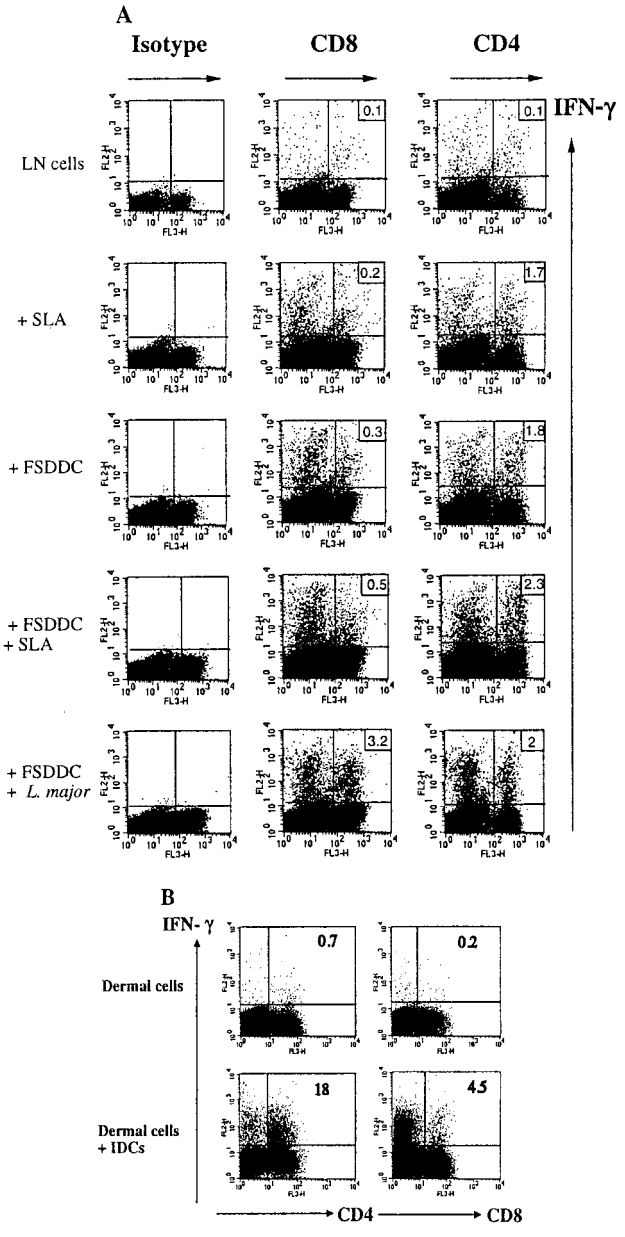

FIGURE 4. Intracytoplasmic staining for IFN-γ in $CD8^+$ or $CD4^+$ T cells from draining LN (*A*) or dermis (*B*) 6 wk following intradermal inoculation of 100 *L. major* metacyclic promastigotes in C57BL/6 mice. Whole LN cells were cultured alone or with SLA, SLA plus FSDDC, or FSDDC infected with *L. major* amastigotes. Dermal cells were cultured with noninfected or infected BMDC. Events were initially gated on size and granulosity, then on TCRβ expression. The numbers represent the percentage of $CD4^+$ or $CD8^+$ T cells positive for IFN-γ, based on comparison with FL2 isotype control. The experiment is representative of two separate experiments.

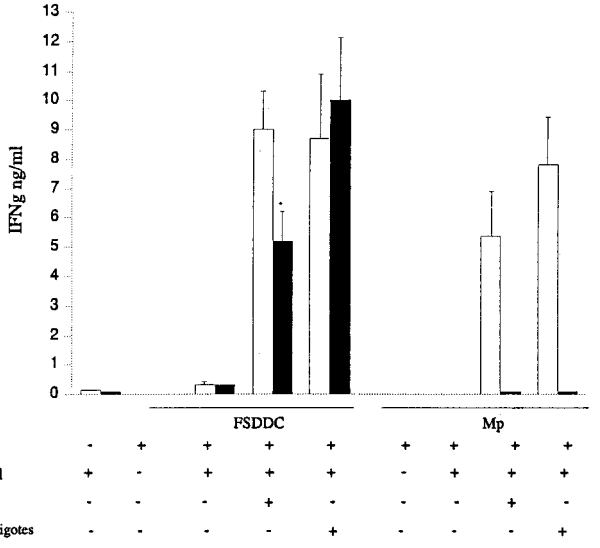

FIGURE 5. IFN-γ production by $CD8^+$ or $CD4^+$ T cells from draining LN 6 wk following intradermal inoculation of 100 *L. major* metacyclic promastigotes in C57BL/6 mice. $CD4^+$ (□) and $CD8^+$ (■) T cells were purified and incubated with noninfected, infected, or SLA-loaded FSDDC or BMDM. IFN-γ levels in culture supernatants were determined by ELISA after 24-h incubation. The data shown are the mean ± SD of three replicates, and the experiment is representative of three separate experiments.

with both $CD4^+$ and $CD8^+$ T cells produced only a minor and transient pathology.

Immunity in $RAG^{-/-}$ mice reconstituted with primed $CD8^+$ and/or $CD4^+$ T cells

To determine whether primed $CD8^+$ T cells could mediate protection independent of other lymphocyte populations, C57BL/6 $RAG^{-/-}$ mice were reconstituted with $CD4^+$ and/or $CD8^+$ T cells recovered from the draining LN of wild-type mice during the acute phase of infection in the skin (wk 5), and were challenged with 1000 *L. major* promastigotes. In each case, cell reconstitution was able to transfer resistance. At 6.5 wk, the number of parasites in the ear was reduced by 3.6 logs for $CD4^+$-reconstituted mice and 3.7 logs for $CD8^+$-reconstituted mice (Fig. 7*A*). One-half of the mice reconstituted with both $CD4^+$ and $CD8^+$ T cells did not harbor parasites in the inoculation site 6.5 wk postchallenge. $RAG^{-/-}$ mice reconstituted with either primed $CD8^+$ and/or $CD4^+$ T cells developed nodules at sites of the infection (Fig. 7*B*). Lesions were evident at 3.5 wk, progressed, and by 6.5 wk began to resolve in the mice transferred with $CD4^+$ T cells alone or with $CD8^+$ and $CD4^+$ T cells. Mice transferred with primed $CD8^+$ T cells developed a more severe lesion, but they nonetheless showed signs of healing when the experiment was terminated for parasite quantitation at 7.5 wk. In an additional experiment, it was confirmed that $RAG^{-/-}$ mice reconstituted with primed $CD8^+$ T cells fully resolved their lesions by 8–10 wk.

Lymphocyte recruitment and IFN-γ response in reconstituted $RAG^{-/-}$ mice following low-dose, intradermal challenge

At 6.5 wk following transfer of primed $CD8^+$ T cells, the draining LN of these reconstituted mice contained a population of $TCR\beta^+$ cells that were 91% $CD8^+$ and <1% $CD4^+$ (Fig. 8*A*). In the mice reconstituted with $CD4^+$ cells, 95% of the TCRβ chain-positive cells were $CD4^+$, and <2% were $CD8^+$. The ability of the LN-reconstituted cells to produce IFN-γ in response to Ag was investigated by a short restimulation in vitro using SLA, anti-CD28, and IL-2, conditions that appear to at least partially overcome the requirement for addition of infected DC to whole LN cultures. In the $CD8^+$ T cell-reconstituted mice, 0.5% of the $CD8^+$ cells stained for IFN-γ, while 2.5% of the $CD4^+$ T cells stained for IFN-γ in the $CD4^+$-reconstituted mice. By comparison, 1% of the $CD4^+$ and 1.3% of the $CD8^+$ T cells stained for IFN-γ in the wild-type mice. Analysis of cells from the dermis 6.5 wk after challenge (Fig. 8*B*) revealed that both $CD4^+$ and $CD8^+$ cells were able to migrate to the site of parasite inoculation. A total of 4.2×10^4 $CD8^+$ T cells per ear was recovered from the $CD8^+$-reconstituted mice. A total

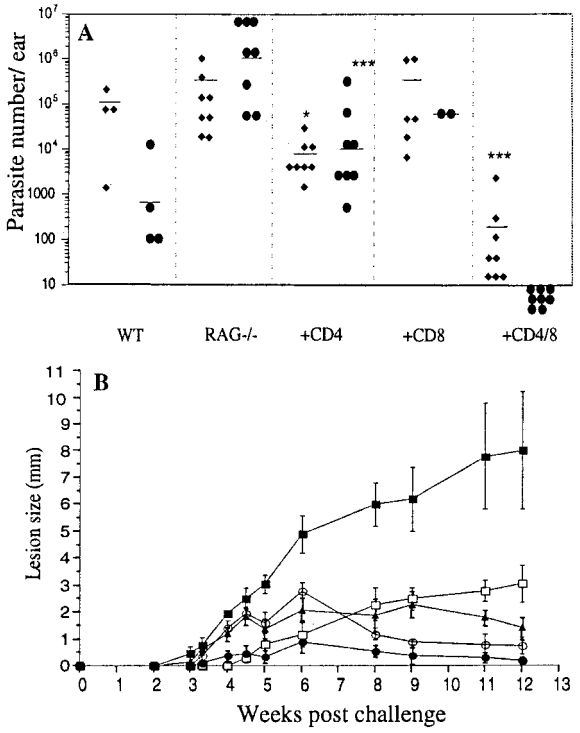

FIGURE 6. Number of parasites per ear (*A*) at 8 (♦) or 12 (●) wk, and diameter of induration (*B*) following ear challenge with 1000 *L. major* metacyclic promastigotes in wild-type (○), $RAG^{-/-}$ (□), CD8-reconstituted (■), CD4-reconstituted (▲), or CD4 plus CD8-reconstituted (●) $RAG^{-/-}$ mice. Cells were purified from naive C57BL/6 mice, and were i.v. transferred at the time of ear challenge. Values represent number of parasites per individual ear, with bar indicating geometric mean, 4 mice and 4–8 ears per group; and mean induration in mm ± SD, 4–16 ears per group. Values are significantly reduced (**, $p < 0.001$; *, $p < 0.05$) compared with the parasite number in the $RAG^{-/-}$-unreconstituted controls. The values of only two ears are shown for the CD8-reconstituted mice at 12 wk because of the severity of the dermal erosion at this time point.

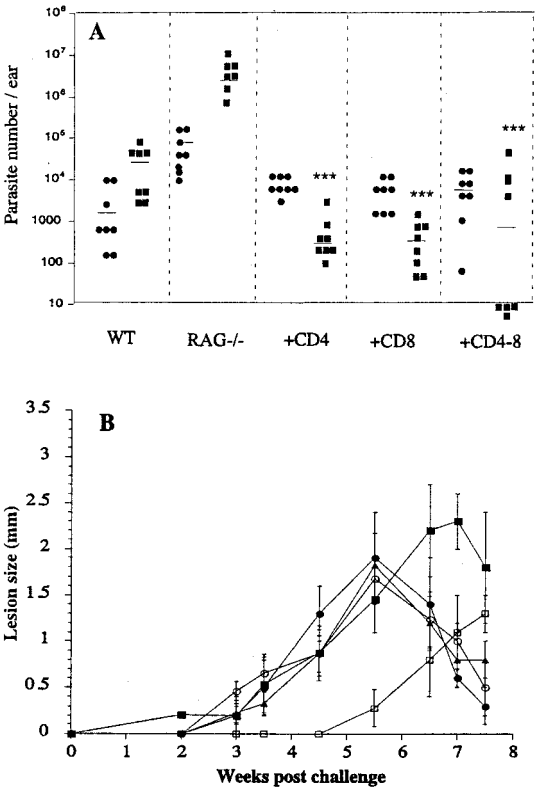

FIGURE 7. Number of parasites per ear (*A*) at 4 (●) or 6.5 (■) wk, and diameter of induration (*B*) following ear challenge with 1000 *L. major* metacyclic promastigotes in wild-type (○), $RAG^{-/-}$ (□), CD8-reconstituted (■), CD4-reconstituted (▲), or CD4 plus CD8-reconstituted (●) $RAG^{-/-}$ mice. Cells were purified from LN of 6-wk-infected C57BL/6 mice, and were i.v. transferred at the time of ear challenge. Values represent number of parasites per individual ear, with bar indicating geometric mean, 4 mice and 8 ears per group; and mean induration in mm ± SD, 8–16 ears per group. Values are significantly reduced (***, $p < 0.001$) compared with the parasite number in the $RAG^{-/-}$-unreconstituted controls. The experiment is representative of two separate experiments.

of 2.5×10^5 $CD4^+$ T cells was present in the ears of the $CD4^+$-reconstituted mice, and 5.3% of these cells stained for IFN-γ following restimulation in vitro. Mice cotransferred with $CD4^+$ and $CD8^+$ T cells contained both populations in the dermis (respectively, 1.3×10^5 and 2.4×10^4 cells/ear), and of these, 4.1% of the $CD4^+$ and 8% of the $CD8^+$ T cells were able to produce IFN-γ.

Discussion

Using a challenge model that more closely reproduces the conditions of natural sand fly challenge, in which 100-1000 metacyclic promastigotes were inoculated into the ear dermis of genetically resistant C57BL/6 mice, a requirement for $CD8^+$ T cells in the control of primary infection by *L. major* was revealed. C57BL/6 $CD8^{-/-}$ and CD8-depleted mice failed to control the growth of *L. major* in the skin. The control of infection in wild-type C57BL/6 mice was associated with a high number of $CD8^+$ T cells in the dermis, a high frequency of $CD8^+$ T cells in the draining LN able to produce IFN-γ in response to parasite Ag, and the generation of primed $CD8^+$ T cells that were able to transfer immunity to $RAG^{-/-}$ mice. Furthermore, reconstitution of resistance in $RAG^{-/-}$ mice using T cells from naive C57BL/6 mice was optimal only when both $CD4^+$ and $CD8^+$ T cells were transferred.

In the conventional high dose, s.c. infection model, a role for $CD8^+$ T cells in resistance to reinfection by *L. major* has already been established (12). $CD8^+$ T cells from healed mice were able to transfer *Leishmania*-specific delayed-type hypersensitivity to syngeneic recipients (11, 14) to express cytotoxic activity (25), and to release IFN-γ in the presence of irradiated promastigotes (13, 14). The role of $CD8^+$ T cells in the control of primary infection in genetically resistant mice remains controversial. *Leishmania*-reactive $CD8^+$ T cells were found in higher numbers in resistant mice than in susceptible mice (2, 11). Depletion of $CD8^+$ T cells increased the severity of footpad lesions in mice inoculated with a high dose of parasites; however, the mice remained able to control the infection (2). More recent studies in β_2-microglobulin- or CD8-deficient mice, again using a high-dose footpad challenge, showed that the mice were able to heal their footpad lesions. Taken together, these studies provide a sound basis for concluding that $CD8^+$ T cells have little or no role in the control of primary infection by *L. major* (15–17).

The different outcomes obtained in these previous studies compared with the results presented in this work raise the possibility that the inoculation of an excessive dose of parasites (10^5-10^7) in an unnatural site may mask or subvert the factors responsible for the control of infection resulting from sand fly challenge. The exogenous parasite Ag provided by high-dose inocula might elicit a level of $CD4^+$ reactivity adequate to control infection in the site, whereas intracellular infections evolving from low-dose challenge might generate a relatively poor source of Ag for class II presentation, and a requirement for both $CD4^+$ and $CD8^+$ T cells is

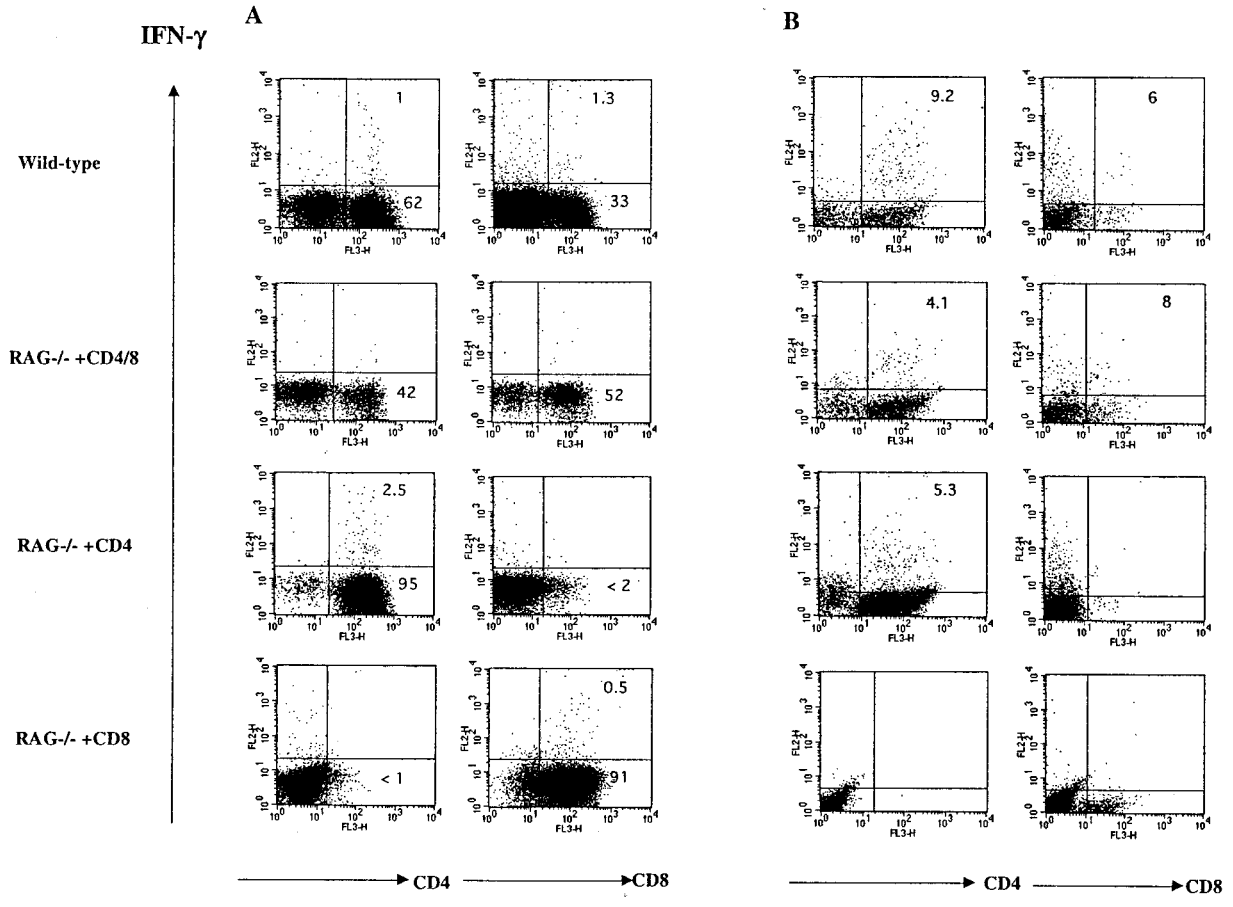

FIGURE 8. IFN-γ production by lymphocytes present in the draining LN (*A*) or ears (*B*) of RAG$^{-/-}$ mice 6.5 wk following transfer of primed CD4$^+$, CD8$^+$ T cells, or CD4$^+$ plus CD8$^+$ T cells. Cells were purified from LN of 6-wk-infected C57BL/6 mice and were i.v. transferred at the time of ear challenge with 1000 *L. major* metacyclics. Cells were restimulated in vitro using SLA, anti-CD28, and IL-2. Dot plots of FSC/SSC and TCRβ$^+$-gated cells are shown. Values in *lower quadrant* refer to percentage of the FSC/SSC TCRβ-chain-gated events positive for CD4 or CD8 markers; values in *upper quadrant* refer to percentage of CD4$^+$ or CD8$^+$ T cells positive for IFN-γ, based on comparison with FL2 isotype control. The experiment is representative of two separate experiments.

revealed. Furthermore, the skin may be especially well suited for driving CD8$^+$ T cells in response to intradermal challenge. FSDDC (26) and Langerhans cells (27) have been shown to effectively prime CD8$^+$ T cells, and immature DC (e.g., Langerhans cells) have the capacity to induce class I-restricted CTL via presentation of Ag through an exogenous pathway (28, 29) or via presentation of peptides acquired from uptake of apoptotic cells (30).

Careful analysis of the inoculation site and draining LN in the wild-type mice provided further evidence in favor of a role for CD8$^+$ T cells in the control of primary infection. There was a clear temporal association between reduction of the parasite load and the accumulation of CD8$^+$ T cells in the skin able to produce IFN-γ, and the presence of CD8$^+$ T cells in draining LN expressing an activated phenotype and also able to produce IFN-γ. Our results indicated that the addition of infected DC as APCs was necessary to reveal CD8$^+$ T cell effector activity. However, the direct restimulation of the cells by anti-CD28, IL-2, and SLA could partially overcome this requirement. In cocultures involving purified populations of primed T cells, either macrophages or FSDDC were efficient APC for activation of CD4$^+$ T cells for the release of IFN-γ, while only infected or SLA-loaded DC induced the release of IFN-γ by CD8$^+$ T cells. The delayed in vivo priming of CD8$^+$ T cells during infection may be due to the requirement for infection of DC by amastigotes released by heavily infected macrophages in the skin. These data are consistent with previous studies indicating that, in contrast to macrophages, DC can efficiently process exogenously delivered Ag for presentation by MHC class I molecules (29, 31), and that, in contrast to infected macrophages, *L. major*-infected DC up-regulate MHC class I and II molecules, costimulatory molecules, and IL-12 (24, 32), and effectively prime T cells in vivo (24, 33).

The experiment in RAG$^{-/-}$ mice reconstituted with naive lymphocytes showed that CD4$^+$ T cells conferred a partial resistance, while CD8$^+$ T cells transferred no protection at all. It is possible that the few contaminating CD8$^+$ T cells contributed to the partial resistance transferred by the purified CD4$^+$ T cells, although there was no expansion of the CD8$^+$ cells following transfer, nor did they appear to accumulate in the site of inoculation (Fig. 8). Regardless, it was only when both CD4$^+$ and CD8$^+$ T cells were present that the parasite was cleared from the site, consistent with the fact that both populations are necessary for the expression of acquired immunity following natural challenge in intact C57BL/6 mice. The inability of naive CD8$^+$ T cells transferred alone to develop into immune effectors suggests that CD4$^+$ Th cells are needed to regulate their activation and/or differentiation. In contrast, primed CD8$^+$ T cells, obtained from wild-type mice 4–5 wk following intradermal challenge, were by themselves able to transfer partial resistance to RAG$^{-/-}$ mice. These results formally demonstrate that effector CD8$^+$ T cells are generated during the acute

phase infection in the skin, and that they can traffic to the inoculation site and provide effector function even in the absence of $CD4^+$ T cells. Since their capacity to produce IFN-γ in response to Ag was also confirmed, this is likely to be the primary effector mechanism used by $CD8^+$ T cells to mediate resistance. A role for $CD8^+$ T cells producing IFN-γ has been implicated in resistance to other protozoan parasites such as *Toxoplasma gondii* (34), *Plasmodium berghei* (35, 36), and *Plasmodium yoelii* (37). Nonetheless, a role for cytotoxic activities that might damage amastigotes (38) or release them for uptake by other cells (e.g., activated macrophages or DC) should not be ruled out. The presence of class I-restricted cytotoxic $CD8^+$ T cells was revealed in mice that healed their primary lesion (25).

The fact that in both the naive and primed cell reconstitution experiments the most powerful resistance was observed when both populations were transferred suggests that $CD4^+$ and $CD8^+$ T cells provide complementary and not simply redundant effector and/or regulatory functions. For example, $CD8^+$ T cells, via release of IFN-γ or provision of CD40L, may play a regulatory role in the induction or the maintenance of a population of Th1 cells, as was recently suggested in the context of a vaccination model using LACK DNA (39). In the present studies, it is interesting that LN cells from anti-CD8-treated mice stimulated with SLA produced significantly less IFN-γ than the isotype control-treated mice. Because exogenous SLA Ag added to whole LN cultures is a sufficient condition for activation of $CD4^+$ T cells to produce IFN-γ (Fig. 4), this result may indicate that the $CD4^+$ T cell priming/function is impaired following CD8 depletion. Furthermore, $CD8^+$ T cells, which have the capacity to release various chemokines (40–42), might contribute to the recruitment of $CD4^+$ T cells to the inoculation site. It is important to note that in our preliminary studies involving adoptive transfer of positively selected $CD4^+$ and $CD8^+$ cells to $RAG^{-/-}$ mice, a minor population of CD4 cells ($<1\%$) in the CD8 transfers resulted in a striking expansion of this population following challenge. This experience revealed a clear need to include anti-CD4 treatment in the recipient mice to maintain the purity of the $CD8^+$ T cell reconstitution.

The delayed onset of lesion formation and slow lesion progression associated with the high dermal parasite loads in the mice with $CD8^+$ T cell deficiencies suggests that these cells also play an important role in pathogenesis. The $RAG^{-/-}$ mice also had delayed lesions despite a high parasite burden in the skin. When these mice were reconstituted with $CD4^+$ T cells, they developed a nodule at the challenge site similar to that seen in wild-type mice. These results confirm previous findings that $CD4^+$ T cells help to mediate the pathology induced by *Leishmania* (19, 43). $RAG^{-/-}$ mice reconstituted with naive $CD8^+$ T cells developed a severe pathology that rapidly led to the destruction of the tissue. The mechanism by which naive $CD8^+$ T cells, when transferred alone, can aggravate pathology is not clear, but may be associated with their release of high amount of chemokines and the absence of IL-10-producing regulatory $CD4^+$ T cells that have been shown to dampen the T cell-dependent inflammatory response to *L. major* in wild-type mice (44).

Our conclusions regarding the role of $CD8^+$ T cells in pathogenesis and in the control of primary infection by *L. major* are consistent with clinical studies reporting high numbers of intralesional $CD4^+$ and $CD8^+$ T cells present during the acute stage of lesion formation and during the healing process (45–50). The finding that $CD8^+$ T cells are essential for development of naturally acquired immunity to primary infection has important implications regarding vaccine design. These studies provide a sound rationale for DNA vaccines, which in contrast to protein-based vaccines, have already been shown to elicit strong and durable $CD8^+$ T cell-dependent immunity in mice (51–53).

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