The Journal of Immunology



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*J Immunol* 2000; 165:969-977; ;
doi: 10.4049/jimmunol.165.2.969
http://www.jimmunol.org/content/165/2/969



*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

# A Natural Model of *Leishmania major* Infection Reveals a Prolonged "Silent" Phase of Parasite Amplification in the Skin Before the Onset of Lesion Formation and Immunity

**Yasmine Belkaid,* Susana Mendez,* Rosalia Lira,* Navin Kadambi,* Genevieve Milon,† and David Sacks[1]***

**A model of *Leishmania major* infection in C57BL/6 mice has been established that combines two main features of natural transmission: low dose (100 metacyclic promastigotes) and inoculation into a dermal site (the ear dermis). The evolution of the dermal lesion could be dissociated into two distinct phases. The initial "silent" phase, lasting 4–5 wk, favored establishment of the peak load of parasites in the dermis in the absence of lesion formation or any overt histopathologic changes in the site. The second phase corresponds to the development of a lesion associated with an acute infiltration of neutrophils, macrophages, and eosinophils into the dermis and was coincident with the killing of parasites in the site. The onset of immunity/pathology was correlated with the appearance of cells staining for IL-12p40 and IFN-γ in the epidermal compartment, and an expansion of T cells capable of producing IFN-γ in the draining lymph node. Parasite growth was not enhanced over the first 4.5 wk in anti-CD4-treated mice, SCID mice, or C57BL/6 mice deficient in IL-12p40, IFN-γ, CD40 ligand, or inducible NO synthase. These mice all failed to ultimately control infection in the site, but in some cases (anti-CD4 treated, IL-12p40$^{-/-}$, CD40 ligand$^{-/-}$, and SCID) high dermal parasite loads were associated with little or no pathology. These results extend to a natural infection model a role for Th1 cells in both acquired resistance and lesion formation, and document the remarkable avoidance of this response during a prolonged phase of parasite amplification in the skin.** *The Journal of Immunology,* **2000, 165: 969–977.**

Infection of mice with the protozoan parasite *Leishmania major* is a well-established model for the definition of factors that control CD4$^+$ T cell subset differentiation in vivo. Depending on the genotype of the mouse, *L. major* infection leads to the development of polarized Th1 or Th2 responses that control resistance or susceptibility, respectively, to this intracellular parasite. The resistant C57BL/6 mouse, in particular, is believed to be a relevant model of *L. major* infections in humans, which are characterized by the development of localized dermal lesions that spontaneously heal. The mouse model has typically employed high doses ($10^5$–$10^7$) of promastigotes inoculated into the footpad or other s.c. sites. In this model, lesion formation is generally correlated with an increasing number of parasites in the inoculation site, whereas healing is associated with immune activation of infected macrophages and the killing of parasites in the site. The model has been used to define in increasing detail the cells, cytokines, and effector molecules involved in acquired resistance, which to date has been shown to require CD4$^+$ T cells, activated in an IL-12-driven and CD40/CD40 ligand (CD40L)[2]-dependent manner, for production of high levels of IFN-γ, which mediates NO-dependent killing by infected macrophages (1–10).

The extent to which the high dose, s.c. inoculation model accurately reflects dermal leishmaniasis that develops following natural transmission of *L. major* by sand fly bite has not been carefully addressed. We previously established a model of natural infection that focused on the exacerbating effects of vector saliva on lesion development in a dermal site (11). In the present studies the coinoculation of sand fly saliva has been avoided because of recent findings that the salivary gland sonicate used to mimic natural transmission elicits powerful effects that are not seen following actual transmission by bite (S. Kamhawi et al., manuscript in preparation). The model retains two main features of natural transmission: low dose (100 metacyclic promastigotes) and inoculation into a dermal site (the mouse ear dermis). The model has been used to re-examine the basic relationship among parasite growth, lesion formation, and immunity. The studies have revealed two distinct phases in the pathogenesis of cutaneous leishmaniasis that have not been previously discerned: a remarkably silent phase, lasting 4–5 wk, favoring the amplification of parasites in the dermis without the formation of a lesion, followed by the development of a cutaneous lesion that is coincident with the killing of the parasite in the site.

## Materials and Methods

### *Mice*

C57BL/6 (B6) mice were purchased from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD). C57BL/6 SCID mice and knockout (KO) mice, deficient in IL-12 p40, iNOS, or IFN-γ, all generated on a C57BL/6 genetic background, were purchased from Taconic Farms (Germantown, NY). C57BL/6 CD40L$^{-/-}$ mice were provided by Dr. R. Seder. The IL-4-deficient mice were generated from an C57BL/6 embryonic stem cell line and were provided by Dr. N. Noben-Trauth. All mice were maintained in the National Institute of Allergy and Infectious Diseases animal care facility under pathogen-free conditions.

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Received for publication January 14, 2000. Accepted for publication April 28, 2000.

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[2] Abbreviations used in this paper: CD40L, CD40 ligand; KO, knockout; iNOS, inducible NO synthase; DC, dendritic cells; SLA, soluble *L. major* Ag.



0022-1767/00/$02.00

### *Parasite preparation and intradermal inoculation*

*L. major* clone V1 (MHOM/IL/80/Friedlin) was cultured in medium 199 with 20% HI-FCS (HyClone, Logan, UT), 100 U/ml penicillin, 100 $\mu$g/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 culture (5–6 days old) by negative selection using peanut agglutinin (Vector, Burlingame, CA). One hundred metacyclic promastigotes were inoculated intradermally into the ear dermis using a 27.5-gauge needle in a volume of 10 $\mu$l. 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, Swedesboro, NJ).

### *Analysis of the parasite-loaded dermis*

The leukocytes in the inflammatory ear dermis were recovered as previously described (12). Briefly, at different time points after intradermal inoculation of *L. major*, the ears were collected, and the ventral and dorsal dermal sheets were separated and immediately processed; the two leaflets were transferred, dermal side down, on culture medium into a six-well plate (nontissue culture treated, Greiner Labortechnik, Osterreich, Austria) for 6 h. Each well contained 4 ml of RPMI 1640, $NaHCO_3$ with 25 mM HEPES, 10% HI-FCS, and penicillin/streptomycin. The cell populations spontaneously emigrating from the dermis were recovered, filtered through a 70-$\mu$m pore size nylon cell strainer (Becton Dickinson, Mountain View, CA) and washed twice before being cytospun and stained with Diff-Quik (DADE, Miami, FL) or stained for flow cytometric analysis. For histologic studies, the complete ears were fixed in 10% phosphate-buffered formalin and embedded in paraffin, and 5-$\mu$m sections were stained using hematoxylin and eosin.

### *Immunolabeling and flow cytometry*

Before staining, the cells emigrating from the ear dermis were incubated with an anti-Fc$\gamma$III/II (PharMingen, San Diego, CA) receptor and 10% normal mouse serum in PBS containing 0.1% BSA and 0.01% $NaN_3$. The double or triple stainings were performed using directly conjugated Abs incubated simultaneously. Each incubation was conducted for 30 min on ice. The dermal cells were identified by characteristic size (forward scatter) and granulosity (side scatter) combined with two-color analysis, as previously described (11). The dendritic cells (DC) were identified as large cells, MHC class II (25-9-17, PharMingen) bright, and F4/80 (A3-1, Caltag, Burlingame, CA) and NLDC 145 (DEC-205) positive. The mononuclear phagocytes were identified as F4/80 positive, and MHC class II low or negative. The neutrophils were identified as small cells, Ly-6G bright (RB6-8C5, PharMingen), and negative for F4/80 (or MHC class II); the eosinophils were identified by their granulosity associated with F4/80 staining and the absence of MHC class II staining. The lymphocytes were identified by their small size, along with their TCR $\beta$-chain (H57-597) and CD3 (145-2C11, PharMingen) expression. The isotype controls used were rat IgG2b (A95-1, PharMingen) and rat IgG2a (R35-95, PharMingen). For each sample, 10,000 cells were analyzed. The data were collected and analyzed using CellQuest software and a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

### *Estimation of parasite load*

The two sheets of the infected ears were separated; deposited dermal side down in DMEM containing 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 1 mg/ml collagenase A (Sigma, St. Louis, MO); and incubated for 2 h at 37°C. The sheets were cut into small pieces and homogenized using a Teflon-coated microtissue grinder in a microfuge tube containing 100 $\mu$l of M199/S. The tissue homogenates were filtered using a 70-$\mu$m pore size cell strainer (Falcon Products, St. Louis, MO) and serially diluted in a 96-well flat-bottom microtiter plate containing biphasic medium, prepared using 50 $\mu$l of NNN medium containing 30% of defibrinated rabbit blood and overlaid with 50 $\mu$l of M199/S. The number of viable parasites in each tissue was determined from the highest dilution at which promastigotes could be grown after 7 days of incubation at 26°C .

### *Lymph node cell preparation and culture*

The retromaxillar draining lymph nodes were recovered and mechanically dissociated using a pellet pestle. Cell viability was assessed by trypan blue exclusion. For measurement of in vitro cytokine production, single-cell suspensions of lymph nodes were pooled from five animals, diluted to $4 \times 10^6$ cells/ml, and dispensed into 96-well plates without Ag or with soluble *L. major* Ag (SLA; 25 $\mu$g/ml) or Con A (2 $\mu$g/ml) in 100 $\mu$l of complete RPMI containing 2-ME. Cultures were incubated at 37°C in 5% $CO_2$. Supernatant fluids were harvested at 24 and 48 h and were assayed for IL-4 and IFN-$\gamma$, respectively, by ELISA as previously described (11).

### *Epidermal cell preparation*

The epidermal cells were recovered as previously described (11) with modifications; briefly, the ventral and dorsal ear sheets were separated and transferred dermal side down on DMEM-penicillin/streptomycin with 0.5% trypsin (U.S. Biochemical, Cleveland, OH) for the dorsal face and 1% trypsin for the ventral face. The sheets were incubated for 30 min at 37°C. The epidermis was separated from the dermis and deposited on a 70-$\mu$m nylon cell strainer (Becton Dickinson, Mountain View, CA), which was placed in a petri dish containing DMEM plus 20% FBS, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 0.05% DNase (Sigma). The filter was gently shaken for 2 min, and the cells passing through the filter were washed, plated, and incubated 6 h at 37 C in the presence of 10 $\mu$g/ml brefeldin A (Sigma). The cells were collected, fixed with 4% paraformaldehyde in PBS for 5 min, and washed with cold PBS containing 0.1% BSA before staining. The epidermal cells were incubated with anti-FcR Ab in PBS containing 10% normal mouse serum, 0.1% saponin (Fisher, Pittsburgh, PA), 0.1% BSA, 1 mM CaCl, 1 mM $MgSO_4$, and 40 mM HEPES (permeabilization buffer). After washing, the cells were incubated with the cytokine-reactive Abs or their corresponding isotype control. All Abs were obtained from PharMingen and were directly conjugated to PE: anti-IL-12p40 (C15.6), anti IFN-$\gamma$ (XMG1.2), anti-IL-4 (BVD4-1D11), and isotype control, rat IgG1 or rat IgG2b. After staining, the cells were fixed again with 1% paraformaldehyde and washed. For each sample, 40,000 cells were analyzed.

### *$CD4^+$ cell depletion or IL-12 treatment of mice*

For CD4 depletion the mice were inoculated weekly i.p. with 1 mg of anti-CD4 (GK1.5) or a rat isotype control (GL113). The IL-12 p40 KO mice were given 0.5 $\mu$g of recombinant murine IL-12 (provided by Genetics Institute, Cambridge, MA) i.p. during the first 3 days of infection.

## Results

### *Evolution of the dermal lesion in C57BL/6 mice*

One hundred *L. major* metacyclic promastigotes were inoculated into the ear dermis (both ears) of C57BL/6 mice. The number of parasites was monitored every 2 wk, and the evolution of the lesion (measured by nodule diameter) was examined at least once a week over a period of 10.5 wk (Fig. 1*A*). During the first 2 wk, the number of parasites increased only slightly, followed by a dramatic increase during the third and fourth weeks, reaching a peak load of $\sim 3 \times 10^5$ parasites/ear. Surprisingly, this initial wave of parasite expansion was not accompanied by the appearance of a lesion in any of the mice. Two approaches were used to more carefully analyze the inflammatory response in the parasite-loaded dermis. The first was to analyze the cellular traffic through the dermis using a method based on the ability of leukocytes that are nonadherent or weakly adherent to the matrix (cells newly arrived or on their way to the draining node) to spontaneously sediment out of the dermal sheets (12). Second, histologic sections were prepared to observe the organization of both the trafficking and matrix-bound cells within the involved dermis. Following a transient cellular infiltrate due to the tissue damage associated with needle inoculation ($<$48 h), the types and the number of cells sedimenting out of the dermis remained unchanged from the steady state dermis during the first 4 wk (Fig. 1*B*). The tissue sections also failed to reveal any change in the cellularity or architecture of the parasite-loaded dermis during the first 4 wk of infection (Fig. 2).

The formation of a small nodular lesion was first observed during wk 5 and was coincident with a dramatic reduction in the parasite load in the site (Fig. 1*A*). Between the fifth and ninth weeks, the parasite load was reduced by $>$95%. The lesion size peaked at $\sim$1 mm 6 wk following the injection and resolved before the 12th week. Lesion development was characterized by a wave of cells invading the dermis beginning around the fifth week, comprised primarily of neutrophils, macrophages, and eosinophils

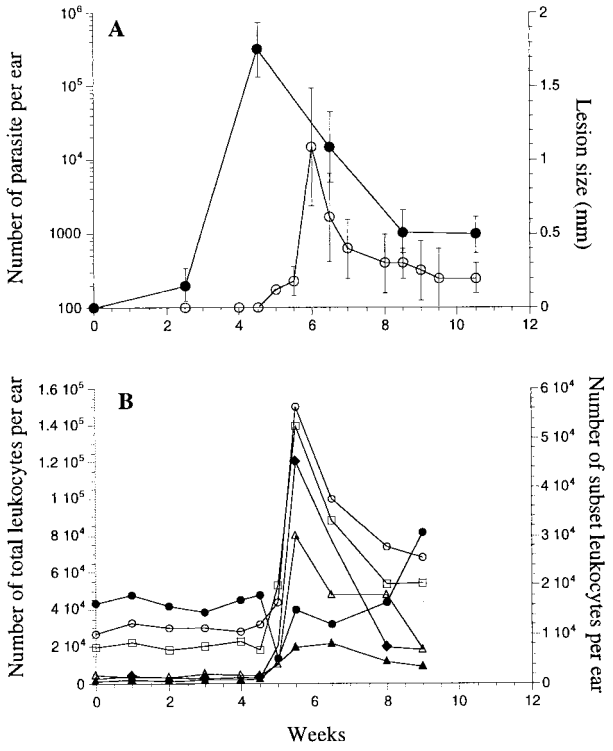


**FIGURE 1.** *A*, Number of parasites per ear (●) and diameter of induration (○) following intradermal inoculation of 100 *L. major* metacyclics. Values represent the mean induration (millimeters) ± 1 SD (4–10 mice/group) and the geometric mean parasite number per ear ± 1 SD (3 mice and 6 ears/group). *B*, Leukocytes trafficking in the dermal compartment during the course of the infection. Cells sedimenting out of the dermis (pooled from 5 mice and 10 ears at each time point) were analyzed by flow cytometry and monitored as the total number of leukocytes per ear (○) or as the total number of macrophages (□), eosinophils (△), neutrophils (♦), T lymphocytes (▲), or dendritic cells (●) per ear. The data shown are from a single experiment representative of two separate experiments.

(Fig. 1*B*). The number of T cells migrating through the site increased slightly during this time, as did the number of DC. The number of DC increased substantially during the latter stage of healing (8–10 wk). The tissue sections obtained between the fifth and seventh weeks revealed a large influx of cells into the dermal compartment (Fig. 2). The infiltrate was poorly organized, and by the ninth week had largely resolved.

The final phase of the infection is characterized by the persistence of 100–10,000 parasites in the site for up to 1 year following resolution of the cutaneous lesion (data not shown). The cells sedimenting from the ear dermis during the chronic stage of infection were primarily macrophages. Sections obtained after the 12th week showed an abundance of lymphatic and blood capillaries and a persistent mononuclear cell infiltrate embedded in a dense matrix (Fig. 2). High power magnification revealed heavily infected macrophages surrounded by uninfected mononuclear cells (not shown). Throughout the chronic phase, neither the parasite nor the cellular infiltrate induced any additional tissue damage, and this equilibrium between the parasite and the host persisted until the natural death of the animal.

### *Immune responses in the epidermis and draining lymph node*

The epidermis is in direct contact with the dermis, and the cells that constitute this site in the mouse, essentially keratinocytes, Langerhans cells, and dendritic epidermal T cells, are all characterized by their capacity to rapidly produce cytokines in response to tissue injury or microbial exposure (13). The epidermal cells were collected during the course of the infection and incubated for 12 h in the presence of Brefeldin A. The flow cytometric analysis of the cells staining intracellularly for IL-4, IL-12p40, or IFN-γ is shown in Fig. 3. The first stage of the infection, corresponding to the sustained period of parasite amplification in the underlying dermis, showed a consistent production of IL-4 that decreased after the peak of the lesion (sixth week). In contrast, IL-12p40 production in the epidermis was delayed until 4 wk and peaked at 6 wk with an impressive number of cells (~500,000) staining for IL-12p40. The kinetics of the IFN-γ response in the epidermal compartment were identical with those of IL-12p40, with a delay until wk 4 and a peak response at 6–8 wk involving up-regulated IFN-γ production by ~900,000 cells/ear.

The presence of cells in the lymph node draining the ear that were reactive to SLA was monitored over a period of 21 wk. The production of IFN-γ and IL-4 in response to SLA was measured by ELISA (Fig. 4). Increased levels of IL-4 were detected as early as the first week, and small amounts of IFN-γ were detectable by wk 4. After the fourth week the production of both cytokines by lymph node cells increased along with development of the cutaneous lesion, with a peak at 6 wk ($2.3 \times 10^4$ and 97 pg/ml for IFN-γ and IL-4, respectively). As the dermal lesion resolved, the levels of IL-4 and especially IFN-γ fell sharply, although a residual production of both cytokines remained detectable during the chronic phase.

### *Evolution of the dermal lesion in immunodeficient mice*

A panel of immunodeficient mice, all on the C57BL/6 background, were challenged in the ear dermis using 100 metacyclic promastigotes, and the parasite loads, lesion development, and inflammatory infiltrate in the inoculation site were compared with those of wild-type mice throughout the first 10 wk postinfection. Surprisingly, the number of parasites did not differ significantly between

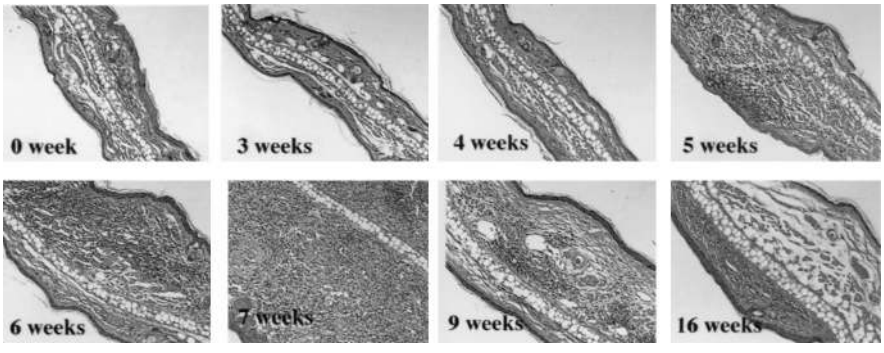


**FIGURE 2.** Hematoxylin and eosin-stained transverse sections of the ears of C57BL/6 mice at various weeks following intradermal inoculation of 100 *L. major* metacyclic promastigotes. Serial sections through the inoculation site were prepared, and the section showing the greatest transverse thickness at each time point was chosen for presentation. Magnification, ×100.

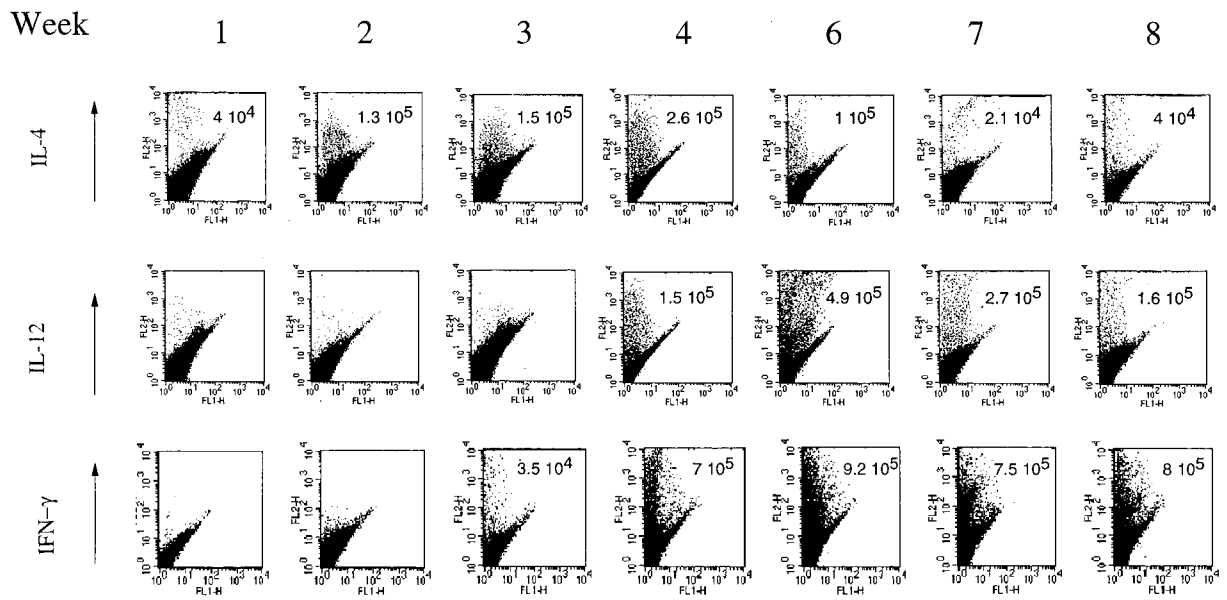


**FIGURE 3.** Two-parameter dot plots of epidermal cells recovered from C57BL/6 mice during the course of infection and stained for intracellular cytokines. The epidermal cells from six ears per group were pooled and preincubated with Brefeldin A for 6 h before staining. The numbers shown are calculated as the total number of cells per ear positive for IL-4, IL-12p40, or IFN-γ based on the frequency of positive signals at each time point established using the PE-isotype controls. The data shown are from a single experiment that is representative of three separate experiments.

the untreated, wild-type mice ($5 \times 10^5$) and any of the immunodeficient mice ($0.4-8 \times 10^5$) over the first 4.5 wk, with the exception of the CD40L KO mice, which had a 10-fold reduction in dermal parasite loads at 4.5 wk (Fig. 5). At each of the subsequent time points examined (6.5 and 8.5 wk), the anti-CD4-treated mice, SCID mice, and knockout mice deficient in IFN-γ, IL-12-p40, iNOS, or CD40L, all failed to reduce the number of parasites in the site (Fig. 5). Of these groups, the IFN-γ-deficient and the anti-CD4-treated mice appeared to be the most susceptible, yielding >100 million parasites/ear at 8.5 wk. The peak parasite loads observed in the IL-4-deficient mice were not significantly lower than those in the wild-type mice, and the number of dermal parasites persisting throughout the chronic phase was actually higher (data not shown). Although the overall results extend to a natural infection model the requirement for IL-12-driven Th1 responses in acquired immunity to *L. major*, the more important findings relate to the fact that for the first 4.5 wk the number of parasites in the ears of the immunodeficient mice was no greater than that in control mice, providing strong evidence that the activation or expression of cell-mediated immunity is effectively absent even in the immunocompetent mice throughout this period of parasite amplification in the skin.

Because IL-12 is an initiating cytokine for Th1 responses, the ability of the parasite to grow in the skin without inducing IL-12 production might underlie the delayed onset of immunity in this model. In a preliminary experiment to address this issue, IL-12p40 KO mice were injected with rIL-12 during the first 3 days following low dose challenge, and their dermal parasite loads were determined. Although the wild-type and IL-12p40 KO mice again showed comparable parasite loads at wk 3 ($9.6 \times 10^3 \pm 7.1 \times 10^3$ vs $1.4 \times 10^4 \pm 6.2 \times 10^3$), the IL-12-treated mice had a 10-fold reduction in the number of parasites in the site (925 ± 525).

Each of the groups of immunodeficient mice was also comparable to the untreated, wild-type mice in their absence of lesion

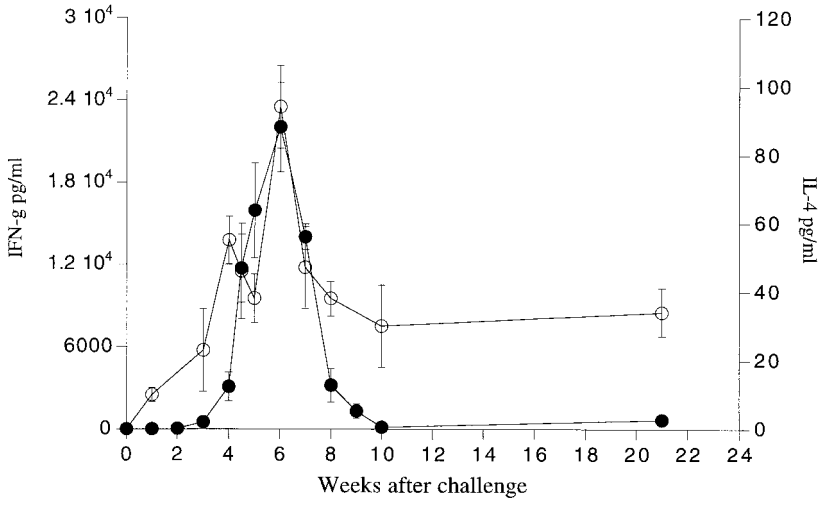


**FIGURE 4.** Production of cytokines by draining LN cells in response to stimulation with SLA. Cells were pooled from four mice and eight lymph nodes per group, and the supernatants were collected at 24 and 48 h for determination of IL-4 (○) and IFN-γ (●), respectively, by ELISA. Values represent the mean cytokine concentration of four separate experiments ± 1 SD.

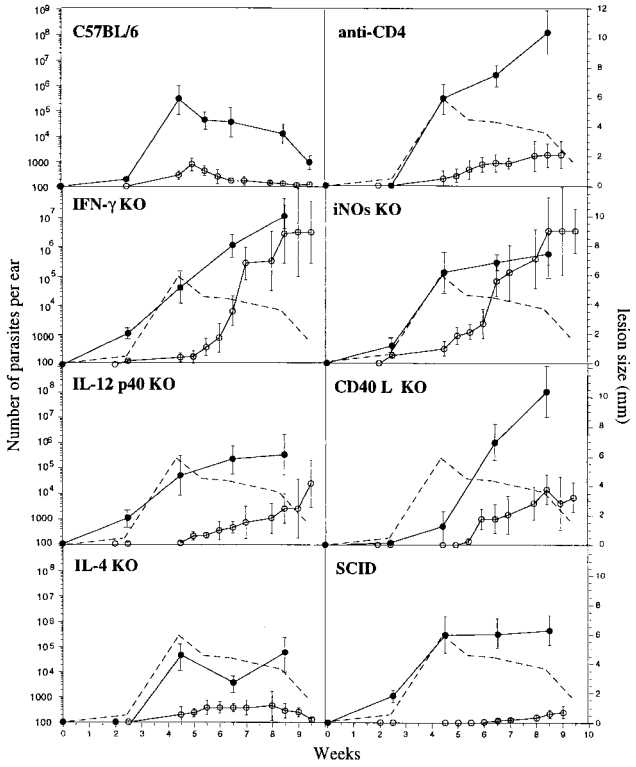


**FIGURE 5.** Number of parasites per ear (filled symbols) and diameter of induration (open symbols) following intradermal inoculation of 100 *L. major* metacyclics in untreated, wild-type C57BL/6, and various C57BL/6 immunodeficient mice. The dotted line in each panel shows the parasitic load in untreated, wild-type mice for comparison. Values represent the mean induration (millimeters) ± 1 SD (4–10 mice/group) and the geometric mean parasite number per ear ± 1 SD (3 mice and 6 ears/group).

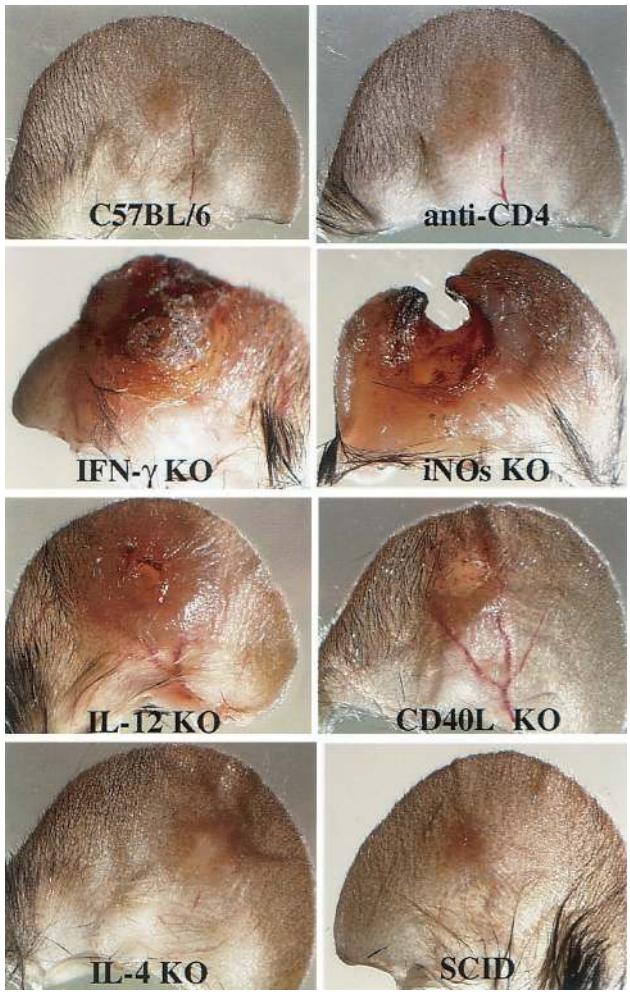


**FIGURE 6.** Ear lesions in C57BL/6 and immunodeficient mice 8.5 wk following intradermal inoculation of 100 *L. major* promastigotes. At this time point, C57BL/6 and IL-4 KO mice present healing lesions, while the uncontrolled infections in the other groups present with either large necrotic lesions with dermal erosion (IFN-γ KO and iNOS KO), nodular lesions with little or no ulceration (CD40L KO, IL-12 p40KO, and anti-CD4 treated), or no nodule (SCID).

formation during the first 4–5 wk (Fig. 5). Thereafter, three patterns of lesion evolution were associated with the inability to reduce the parasite burden in the site. In the anti-CD4-treated mice, CD40L KO mice, and IL-12p40 KO mice, small nodules began to appear at the same time as in the wild-type mice, and while the lesions failed to resolve, the rate of lesion progression (Fig. 5) and the extent of tissue destruction (Fig. 6) remained relatively benign given the numbers of parasites that were accumulating in the ear. In contrast, the infections in the IFN-γ KO and iNOS KO mice were associated with rapidly progressing dermal lesions (Fig. 5) that became ulcerative and necrotic by 8.5 wk (Fig. 6). At the other extreme, in SCID mice no clinical pathology was ever detected until wk 9 despite the high parasite numbers in the site (Figs. 5 and 6). Thereafter, nonulcerative nodular lesions progressed slowly in these mice. These outcomes confirm a role for $CD4^+$ T cells in lesion formation and development. Finally, the dermal lesions in the IL-4 KO mice, while resolved by wk 10, were not moderated in size or duration compared with those in wild-type mice (Fig. 5).

The leukocytes that were recovered from the 5-wk-infected dermis, corresponding to the onset of lesion formation in the control mice, were stained for flow cytometric analysis to determine the size and composition of the cellular infiltrate (Table I) or were fixed on a slide and stained for microscopic evaluation of the cells harboring amastigotes (Table I, expressed by the numbers in parentheses as a percentage of the total population of infected cells). Mice depleted of $CD4^+$ cells showed an increased cellular infiltrate compared with untreated, wild-type mice ($6.25 \times 10^5$ vs $4.3 \times 10^4$ cells/ear), but there was little change in cellular composition. The infiltrate was hardly altered in the IL-4 KO mice, apart from a much lower number of eosinophils, presumably reflecting an impaired regulation of IL-5. The types of inflammatory cells recovered from the other immunodeficient mice could be divided into three groups that correlated precisely with the three patterns of lesion development described above. The first group displayed an increased cellular infiltrate with a preponderance of neutrophils (IFN-γ KO and iNOS KO). In the second group the infiltrating cells were primarily eosinophils, with fewer numbers of neutrophils and macrophages (IL-12p40 KO and CD40L KO). The accumulation of neutrophils led to the rapid development of necrotic lesions in the iNOS and IFN-γ KO mice (by wk 7 and 9, respectively), whereas the necrotic process was delayed by 3–4 wk in the IL-12p40 and CD40L KO mice. The third group corresponds to the mice with little or no dermal pathology up to 9 wk postinfection (SCID), in which there was little increase in the total number of leukocytes in the site, and the sedimenting cells were predominantly macrophages and dendritic cells, with few eosinophils or neutrophils.

Table I. *Number and types of infected and uninfected leukocytes sedimenting out of the dermal site 5 wk postinfection in C57BL/6 and immunodeficient mice*

| | C57BL/6 | Anti-CD4 | IL-4 KO | IFN-γ KO | iNOs KO | IL-12 KO | CD40L KO | SCID |
|---|---|---|---|---|---|---|---|---|
| Total no. of cells/ear | $4.3 \times 10^4$ | $6.25 \times 10^5$ | $8 \times 10^4$ | $1.25 \times 10^5$ | $7 \times 10^5$ | $1.25 \times 10^5$ | $3.9 \times 10^5$ | $6 \times 10^4$ |
| Macrophages | 28[a] (100[b]) | 40 (63) | 31 (65) | 25 (37) | 38 (74) | 31 (55) | 7 (34) | 46 (57) |
| DC | 21 | 23 (2) | 37 | 13 (2) | 12 | 4 | 7 (3) | 41 (17) |
| Neutrophils | 11 | 21 (35) | 27 (35) | 58 (53) | 49 (26) | 13 (45) | 35 (34) | 5 (26) |
| Eosinophils | 40 | 16 | 5 | 4 (8) | 1 | 52 | 51 (29) | 8 |

[a] Percent of total sedimenting cells.
[b] Numbers in parenthese represent the number of each leukocyte subset harboring amastigotes as a percent of the total number of infected sedimenting cells.

In wild-type mice, intracellular amastigotes were found exclusively in macrophages. In contrast, the infected leukocytes recovered from the immunodeficient mice were remarkably heterogeneous. For all immunodeficient mice, a high proportion of the infected cells were neutrophils (26–53%). The CD40L and IFN-γ KO mice had a significant number of infected eosinophils (29% and 7% of infected cells). The infected neutrophils and eosinophils had a mean of six amastigotes per cell, suggesting that the parasites persisted and even replicated within these cells. Infected DC made up a small proportion (1–2%) of the infected leukocytes recovered from the anti-CD4, IFN-γ KO, and CD40L KO mice and a high proportion of the infected cells from SCID mice (17% and 50%). The average number of amastigotes per infected DC was low (1.6/DC).

## Discussion

*Leishmania* parasites are transmitted by the bites of infected female sand flies, which inject low numbers of infectious stage, metacyclic promastigotes into the skin. Although the vast majority of experimental models of *L. major* infection have employed high doses of parasites inoculated into s.c. sites, the current model focuses on two key features of natural transmission: low dose (100 metacyclic promastigotes) and intradermal inoculation. Although the average number of promastigotes delivered into the skin by an infected sand fly remains unknown, data from forced feeding experiments suggest that as few as 100 parasites is not an underestimate (14). The ear dermis was chosen as the inoculation site because, in addition to being a common transmission site in rodent reservoirs, it offers the advantage that all the dynamic events occurring at the site of infection as well as the interconnected compartments of the epidermis and draining lymph node can be carefully monitored (11, 12). Finally, C57BL/6 mice were chosen as the host genotype because this strain appears to reproduce the self-limiting infections with *L. major* that occur in natural reservoirs and human hosts. The studies reported here extend our prior use of the natural infection model by providing the most comprehensive kinetic and quantitative analysis to date of the *L. major*-loaded dermis in terms of parasites, inflammatory leukocytes, and cytokine-producing cells. The analysis has revealed two underlying aspects of *L. major* pathogenesis and immunity that have not been generally appreciated: 1) the peak parasite load in the skin is achieved in a remarkably silent manner, without any accompanying clinical/histological changes; and 2) lesion formation is coincident with the onset of immunity and the reduction of the parasite load in the site.

The ability of a challenge inoculum as low as 100 *L. major* promastigotes to initiate the development of small, healing footpad lesions in genetically resistant mice has been described previously (15, 16). In these studies, however, the number of parasites present in the inoculation site was not monitored over time, so that the relationship between parasite growth and lesion development could not be evaluated. In the few studies that have carefully monitored the parasite load in the footpad following high dose challenge, the early stage of lesion formation was associated with an increasing number of viable parasites in the site (17–19). In the present studies, by quantifying the parasite load at multiple time points, especially during the subclinical stage of infection, it was revealed that the maximum number of parasites in the site occurs before the onset of lesion formation. The quiescence of the parasite-loaded site was supported by the finding that apart from a transient increase in neutrophils immediately after needle injection, the number and types of leukocytes sedimenting out of the dermis remained unchanged during the first 4 wk postinfection. Presumably macrophages were recruited from the tissue or from blood to support the growth of amastigotes in the skin. These cells may have been sufficiently adherent to the matrix that they were missed in the restricted analysis of sedimenting cells. Nonetheless, analysis of tissue sections through the intact ear also failed to reveal any overt inflammatory process associated with the peak load of parasitized macrophages in the skin.

The dermal lesions that began to appear around wk 5 were associated with an acute infiltration of uninfected leukocytes, primarily neutrophils, macrophages, and eosinophils. The cell types in the inflammatory dermis are consistent with the few histological descriptions of human dermal lesions (20–22). The novel finding presented here is that the onset of this inflammatory process coincides with a reduction in the number of parasites in the site. Prior studies have noted the concurrence of pathology and immune response (23, 24), and others have determined that the number of parasites in the site does not necessarily correlate with the size of the lesion, particularly during the healing stage of disease (17, 19). So far as we are aware, however, there has not been any prior demonstration that lesion formation is so clearly timed with the onset of parasite clearance from the site.

The data suggest that induction of host responses that promote immunity/pathology is avoided during a relatively sustained period of intracellular growth in the skin. Parasite growth during the first 4–5 wk correlated with the absence of IL-12 and IFN-γ production by potentially responsive cells in the epidermal compartment and with the absence of Ag-reactive cells producing IFN-γ in the lymph node draining the site. Because immune responses below the detection limits of these assays might still be expected to moderate the early growth of the parasite in the site, the finding that there was no enhancement of parasite growth over the first 4.5 wk in any of the immunodeficient mice, including the IL-12p40 KO and IFN-γ KO mice, is perhaps the strongest evidence that in the intact mice the induction and expression of host control mechanisms are effectively absent

during this time. The presence of IL-12 would appear to be a sufficient condition to initiate a host protective response during the normally silent phase of infection, because provision of exogenous IL-12 to IL-12p40 KO mice during the first 3 days following inoculation resulted in a 10-fold reduction in the numbers of parasites present in the site at 3 wk. These data establish the in vivo relevance of a large series of in vitro observations indicating that *Leishmania* avoid or actively inhibit the immune initiation functions of their host macrophages, including Ag presentation (25–27), expression of MHC class II and costimulatory molecules (28–30), and especially production of IL-12 in response to infection (31–35). It is important to note that the significance of these in vitro studies has remained controversial, in so far as *L. major*-driven immune processes in vivo, including IL-12 and IFN-γ production, have in many instances been rapidly and efficiently induced following parasite delivery (36–39) .

The ability of the natural infection model to establish such a discreet silent phase of infection is not dependent on intradermal challenge, since we have observed that low dose challenge in the footpad produces up to 6 wk of *L. major* growth without footpad swelling, followed by lesion formation that is associated with a reduced parasite burden in the site (R. Lira et al., unpublished observations). The critical feature of the model seems to be dose. We suggest that high dose inocula will undermine the ability of the parasite to initiate infection in a quiescent manner, which may depend on selective targeting of infectious stage parasites to macrophages in the inoculation site. The excessive parasites and released Ags present in high dose inocula, particularly inocula containing a high proportion of noninfective forms, will probably be taken up by other cell types, including dendritic leukocytes, which, in contrast to macrophages, have been shown to produce IL-12 in response to *Leishmania* infection (40–42) and to provide a potent source of APC for T cell activation (42–44). In the present analysis, low dose challenge in the dermis appeared to avoid activation of epidermal cells for IL-12 production until wk 4, at which time the activation of these cells, requiring perhaps a threshold level of infected macrophages or released parasites in the dermis, correlated with the strong up-regulation of IFN-γ production by T cells in the draining node and the onset of both parasite killing and lesion formation. The identity of the epidermal cells that stained positively for IL-12p40 could not be confirmed due to defective expression of surface markers following the trypsin treatment. Nonetheless, it is likely that they are Langerhans cells based on their forward scatter and the IL-12-producing capacity of these cells following *L. major* infection in vitro (40).

Of interest was the increased frequency of IL-4-producing cells observed in both the epidermis and the draining node as early as 2 wk postinfection. Transient IL-4 responses following *L. major* infection in resistant mouse strains have been described previously (35, 45), although this is the first report that IL-4-producing cells are detectable with such high frequency in the epidermal compartment. The role that the early IL-4 response plays in promoting the sustained growth of *Leishmania* in the dermis is not clear, however, because the infections became just as well established following low dose inoculation in IL-4-deficient mice. Nonetheless, a role for additional type 2 cytokines that might be induced early on needs to be considered in the light of recent findings that IL-13 and IL-10 can also promote *L. major* infections in conventional mouse footpad models (46, 47). The inability of the IL-4-deficient mice to as efficiently control infection in the chronic stage as wild-type mice was a consistent finding. Similar findings were reported for IL-4-deficient mice infected with *Candida albicans* (48) or with *Toxoplasma gondii* (49) along with the surprising result that IL-4 was required to sustain IFN-γ production by activated $CD4^+$ T cells (48).

Although the initial silent phase of infection was unaltered in the immunodeficient mice, these mice ultimately confirmed that the immune mechanisms responsible for control of *L. major* infection in the conventional model continue to operate in a natural infection model, i.e., a requirement for IL-12-driven, CD40/CD40L-dependent $CD4^+$ T cell activation, IFN-γ production, and NO-mediated killing. The immunodeficient mice also confirmed a role for T cells in pathology, with some interesting comparisons. Lesion development, especially necrosis, was related primarily to an acute infiltration of neutrophils, and secondarily macrophages and eosinophils, into the parasite-loaded dermis, and this response was at least in part $CD4^+$ T cell dependent, because each of the mice with more global defects in $CD4^+$ T cell activation and function (i.e., anti-CD4, CD40L KO, and IL-12p40 KO) had far less severe pathology than the mice for which the defect was confined to effector molecules involved in macrophage activation and intracellular killing (i.e., IFN-γ KO and iNOS KO mice). The complete absence of lesions in SCID mice up to 9 wk postinfection despite the high parasite burden suggests a role for other T cell subsets and/or B cells in dermal pathology. Delayed development of footpad swelling in SCID mice (50) and in MHC class II-deficient mice (7, 8, 51) has been observed previously. The absence of pathology in these studies was thought to be due to the lack of T cells that are required for the recruitment of macrophages to the inoculation site (i.e., a lack of host cells available for infection). It should be emphasized that in our analysis the reduced pathology in the ears of the T cell-deficient mice was in no case associated with a deficit in parasite growth or host cells available for infection in the site. Instead, the prolonged absence of lesions in these mice seems to reflect a maintenance of the conditions associated with the initial silent phase of infection that occurs in intact mice, in which the accumulation of infected macrophages in the site, in the absence of an accompanying acute inflammatory process, appears to have few pathological consequences. The chronic phase also reflects the absence of an active inflammatory process despite the persistence of infected macrophages in the site. The persistence of *L. major* in the footpad and draining node has been noted previously following the resolution of footpad lesions in resistant mice (19, 52–54). The natural infection model indicates that *L. major* persistence in resistant mice is not an artifact of an inappropriate inoculation site or high dose challenge and may prove a useful model to study the mechanisms underlying low level, chronic infection and the events associated with reactivation disease.

Another surprising aspect of the dermal pathology in the immunodeficient mice was the heterogeneity of the cells harboring parasites that were recovered from the infected dermis. In contrast to the wild-type mice, for which amastigotes were found only in macrophages, the intracellular amastigotes in the immunodeficient animals were variably associated with neutrophils, eosinophils, and dendritic leukocytes, which in the case of neutrophils and eosinophils harbored large numbers of parasites. Dendritic cells recovered from lymphoid tissue have been found to harbor persistent *L. major* (55), and transient or low multiplicity infections in neutrophils and eosinophils have been noted within cutaneous lesions (56, 57). Our results in the immunodeficient mice extend these findings by suggesting that neutrophils and eosinophils are under some circumstances capable of supporting sustained, productive

intracellular infections. This would suggest that the cells are themselves long lived, due perhaps to the absence of cytokines or chemokines involved in their cell death signaling pathways. The data imply that infection outcomes in animals with specific immune defects need to be interpreted with care, since in addition to a compromised ability to activate infected macrophages for killing, the number and types of leukocytes permissive to infection might be significantly expanded.

In conclusion, the present studies have revealed an underlying feature of *L. major* infection in vivo that has been obscured by experimental approaches designed primarily to study parasite-driven Th1 and Th2 subset development. Our analysis of a natural infection model promotes the concept, raised repeatedly in the context of in vitro studies, that sustained intracellular growth in the absence of a parasite-driven host response is a hallmark of leishmanial infection and one that we believe is central to the maintenance of its transmission cycle in nature.

## Acknowledgments

We thank Sandra Cooper for help with the mouse care and breeding, Drs. R. Seder and N. Noben-Trauth for kindly providing the gene knockout mice, and the Genetics Institute for supplying the recombinant mouse IL-12.

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