Reactivation of Latent Leishmaniasis by Inhibition of Inducible Nitric Oxide Synthase

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Summary

Nitric oxide (NO) generated by the inducible isoform of NO synthase (iNOS) is required for the resolution of acute cutaneous leishmaniasis in resistant C57BL/6 mice. As is the case in several other infections, the clinically cured host organism still harbors small amounts of live *Leishmania major* parasites. Here, we demonstrate lifelong expression of iNOS at the site of the original skin lesion and in the draining lymph node of long-term–infected C57BL/6 mice. iNOS activity in the lymph node was dependent on $CD4^+$, but not on $CD8^+$ T cells. By double labeling techniques, iNOS and *L. major* were each found in macrophages ($F4/80^+$, $BM\text{-}8^+$, and/or $MOMA\text{-}2^+$) and dendritic cells ($NLDC\text{-}145^+$), but not in granulocytes or endothelial cells. In situ triple labeling of lymph node sections revealed that ~30–40% of the *L. major* foci were associated with iNOS-positive macrophages or dendritic cells. The majority of the *L. major* foci (60–70%), however, was located in areas that were negative for both iNOS and the macrophage and dendritic cell markers. In *L. major*–infected C57BL/6 mice, which had cured their cutaneous lesions, administration of L-N^6-iminoethyl-lysine (L-NIL), a potent inhibitor of iNOS, led to a 10^4–10^5-fold increase of the parasite burden in the cutaneous and lymphoid tissue and caused clinical recrudescence of the disease. Persistent expression of iNOS and resumption of parasite replication after application of L-NIL was also observed in resistant C3H/HeN and CBA/J mice. We conclude that iNOS activity is crucial for the control of *Leishmania* persisting in immunocompetent hosts after resolution of the primary infection. Failure to maintain iNOS activity might be the mechanism underlying endogenous reactivation of latent infections with NO-sensitive microbes during phases of immunosuppression.

Cure of acute infections with intracellular microorganisms is dependent on the activation of static or cidal mechanisms in the infected host cells. The armamentarium, which is brought up by the host when encountering disease-mediating microbes, consists of constitutively expressed (lysosomal) proteases, peroxidative enzymes, cytotoxic polypeptides, and several cytokine-inducible antimicrobial effector pathways (1, 2). Among the latter, the high level synthesis of nitric oxide (NO)[1] from L-arginine by the inducible isoform of NO synthase (iNOS, NOS-2) has been shown to be indispensable for the defense against a growing spectrum of viruses, bacteria, protozoa, fungi, and helminths (3, and references therein). The antimicrobial role of NO (and related nitrogen intermediates) generated by iNOS was initially established in vitro where the killing of microbes by cytokine-activated macrophages correlated with the release of NO and was abrogated by nonselective inhibitors of NOS (which block the activity of both the constitutive and the inducible isoform of the enzyme) (3). In vivo, several infections took a more severe, nonhealing course, when iNOS was inhibited by isoform-selective or -nonselective antagonists (4, and references therein) or genetically deleted in mice (5, 6). Furthermore, in several disease models, enhanced expression of iNOS was paralleled by a reduced number of microorganisms in the tissue and vice versa (7–9). Although NO was reported to be directly toxic to some parasites, e.g., *Leishmania major* (10, 11), it should be mentioned that the vast majority of data published so far is also compatible with an indirect antimicrobial effect of NO, which, for example, could be exerted by its signal transduction function (12). This possibility, however, does not

[1] *Abbreviations used in this paper:* (i)NOS, (inducible isoform of) nitric oxide synthase (NOS-2; independent of exogenous calmodulin and $[Ca^{2+}]$ above the levels in resting cells); L-NAME, L-N^G-nitroarginine-methyl-ester; L-NIL, L-N^6-(1-iminoethyl)-lysine; L-NMMA, N^G-monomethyl-L-arginine; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); NO, nitric oxide; RT, room temperature.

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question the critical involvement of iNOS in the resolution of a large number of infectious diseases.

Whereas the function of iNOS in vitro and during the acute phase of infections with intracellular microorganisms is fairly well studied in the mouse system, little is known about its role during latent infections in vivo. Several bacteria or protozoa (e.g., *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Leishmania spp.*) continue to reside in small numbers within immunocompetent hosts after clinical cure of the disease, which bears the risk of endogenous reactivation of the infection later in life whenever cell-mediated immunity becomes suppressed (13–17). At least in mice, healing or control of these diseases is paralleled by the expression of iNOS in the tissue and clearly requires the presence of NO (7, 9, 10, 18, 19). Nevertheless, the host fails to completely eliminate the microorganisms. This suggests that during latent infection, the persisting microbes may acquire resistance to NO and that other antimicrobial mechanisms become activated and inhibit replication, but do not lead to eradication of the bacteria and/or parasites. We decided to test this possibility using the *L. major* mouse model, where resistant strains continue to harbor a small amount of parasites throughout life (16, 20–24) despite NO-dependent healing of the cutaneous lesions during the acute phase of infection (4, 10, 18). By in situ analysis and inhibitor experiments, we demonstrate that expression of iNOS is maintained lifelong in chronically infected but clinically healthy mice and is absolutely crucial for the continuous control of the *Leishmania*. Our data do not provide evidence that the persisting parasites are dormant or become resistant to NO, but rather suggest a dynamic process in the host tissue with alternating phases of parasite killing and evasion.

Materials and Methods

Animals, Parasites, and In Vivo Infection. Female BALB/c, C57BL/6, CBA/J, C3H/HeN, and CD1 mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany), kept on a normal rodent diet, and used for infection at 8–10 wk of age. Origin, in vivo passage, and in vitro propagation of the *L. major* isolate (MHOM/IL/81/FE/BNI) were as reported previously (9). Mice were routinely inoculated into the right hind footpad with 3×10^6 stationary phase *L. major* promastigotes (after two to four in vitro subcultures). In some experiments, mice were infected bilaterally into both hind footpads. This allowed processing of the footpad tissue from one animal for both immunohistology and PCR. At regular intervals after infection, the footpad swelling was measured with a metric caliper (9). The number of parasites in the tissue of infected mice was determined by limiting dilution analysis (with serial 10-fold dilutions of the tissue seeded into 96-well plates with Novy-Nicolle-MacNeal blood agar slants) applying Poisson statistics and χ^2-minimization method as published earlier (9, 22).

In Vivo Treatment of Long-Term–Infected, Clinically Cured Mice. Genetically resistant mice (C57BL/6, CBA/J, C3H/HeN) or susceptible mice (BALB/c) made resistant by application of anti-CD4 or IL-12 or low dose inoculation with *L. major* resolved their skin lesions within 50–70 d after infection. Treatment with NOS inhibitors or Abs was not started before day 103 after primary infection. The NOS inhibitors were added to the drinking water (adjusted to pH 2.7 to prevent microbial growth) and freshly provided every second day. Food and water uptake as well as body weight were recorded throughout the treatment. Monoclonal anti-CD4 (YTS 191.1) or anti-CD8 Ab (YTS 169.2), prepared by ammonium sulphate precipitation of rat ascites fluid and subsequent dialysis against PBS, were injected at 2–4-d intervals (200 or 500 μg i.p. per dose, respectively).

NOS Inhibitors and Other Reagents. The reduced form of nicotinamide dinucleotide phosphate (NADPH), flavin adenin dinucleotide (FAD), L-arginine hydrochloride, pepstatin A, chymostatin, and PMSF were obtained from Sigma Chemie (Deisenhofen, Germany). (6R-) tetrahydrobiopterin (BH_4) was purchased from Dr. Schircks Co. (Jona, Switzerland), the L and D analogues of N^G-monomethyl-arginine (L-NMMA, D-NMMA) and of N^G-nitro-arginine-methyl-ester (L-NAME, D-NAME) were from Alexis Co. (Läufelfingen, Switzerland). The L-N^6-(1-iminoethyl) lysine (L-NIL) was synthesized and kindly provided by Drs. Pam Manning, W.M. Moore, and M.G. Currie (G.D. Searle Research and Development, Monsanto Co., St. Louis, MO).

Abs for FACS® Analysis, Immunofluorescence, and Immunohistology. FITC-conjugated rat mAb against CD4 (clone YTS191.1), CD8 (clone YTS169.4), B220 (clone RA3-6B2), Mac-1/CD11b (clone M1/70.15), and mouse mAb against Thy1.2 (clone 5a-8) were obtained from Medac Inc. (Hamburg, Germany). FITC-conjugated rat mAb against F4/80-antigen was from Serotec/Camon (Wiesbaden, Germany). A FITC-conjugated rat mAb NLDC-145 (IgG2a, dendritic cells) was obtained by protein G affinity chromatography of NLDC-145 hybridoma culture supernatants and subsequent FITC labeling (Boehringer, Mannheim, Germany) of the purified Ab following the manufacturer's protocol. A polyclonal rabbit anti–*L. major* antiserum (9) was generously supplied by Dr. H. Moll (Zentrum für Infektionsforschung, Würzburg, Germany). Human anti–*L. major* antiserum was from a patient with multilesional cutaneous leishmaniasis (25). Pilot experiments with serial sections from the skin lesions and lymph nodes of *L. major*–infected mice established that the rabbit and the human antiserum recognized *L. major* parasites equally well. A rabbit anti–mouse iNOS antiserum raised against an octapeptide derived from the COOH terminus of the mouse iNOS sequence (NOS-16) was kindly provided by Drs. Q.-w. Xie and C. Nathan (Cornell University Medical College, New York) and by Drs. J. Weidner and R. Mumford (Merck, Rahway, NJ). The rat mAbs BM-8 (IgG2a; mature tissue macrophages), M1/70.15 (IgG2b; CD11b antigen [Mac-1] on macrophages, granulocytes, and NK cells), C1.A.3-1 (IgG2b; F4/80 antigen on monocytes and tissue macrophages), MOMA-2 (IgG2b; monocytes, tingible body macrophages, and macrophages in T cell–dependent areas), RB6-8C5 (IgG2b; GR-1 antigen on granulocytes), and NLDC-145 (IgG2a; dendritic cells in skin, spleen, and lymph node) were all purchased from Dianova Inc. (Hamburg, Germany). A rat mAb (MECA-32) specific for murine endothelial cells (26) was kindly provided by Dr. R. Hallmann (Institut für Experimentelle Medizin, Erlangen, Germany).

Isolation of Cells and Tissue. For quantification of the parasite load with the limiting dilution technique, homogenates of the skin lesion or single cell suspensions of the draining lymph nodes were prepared as described (9, 22). For determination of the cellular composition of lymph nodes by flow cytometry using a FACScan® (Becton Dickinson & Co., Mountain View, CA), the organs were gently crushed between two glass slides in order to obtain single cell suspensions. For isolation of total RNA, organs were flash frozen in liquid nitrogen and stored at −70°C, while the contralateral tissue samples were embedded in specimen

molds (cryomold®) using optimal cutting temperature (OCT) compound (Diatec, Hallstadt/Bamberg, Germany) and stored frozen for later immunohistological analysis. For iNOS enzyme activity assays, the lymph nodes from *L. major*–infected mice were first flash frozen and later disrupted in lysis buffer (40 mM Tris, pH 8, 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 μg/ml aprotinin, 10 μM leupeptin, 100 μM PMSF) by sonication with a Sonifier B-12 (Branson Ultrasonics, Heinemann Company, Schwäbisch-Gmünd, Germany; 2 × 30 s at intensity 2).

Detection of iNOS by Double Immunofluorescence. Single cell suspensions were prepared from lymph nodes of long-term–infected C57BL/6 mice and, in some experiments, partially depleted of T and B cells by magnetic cell separation with a Macs® column (Miltenyi Biotech, Bergisch-Gladbach, Germany) after incubation with anti-Thy1.2 and anti-B220 Abs coupled to magnetic particles. Cells were allowed to attach for 30 min on adhesion slides (Bio-Rad, Munich, Germany). For detection of cell surface antigens, cells were incubated with FITC-conjugated F4/80 or NLDC-145 rat mAb diluted in PBS/0.1% BSA/0.01% NaN_3 (30 min, 4°C) before fixation with ice-cold acetone (7–8 min). Cells were then incubated with rabbit anti-iNOS peptide (NOS-16; 45 min at room temperature [RT]) followed by lissamine-rhodamine–conjugated $F(ab')_2$ fragments of donkey anti–rabbit IgG (Dianova; 30–45 min, RT), both diluted in PBS/0.1% BSA/1% saponin. Cells were examined by using an Axiophote microscope (Zeiss, Oberkochen, Germany) equipped with appropriate fluorescence filters.

Immunoenzymatic Staining of Tissue Sections. 5-μM tissue sections prepared with a cryostat (model HM500; Fa. Microm, Wallstadt, Germany) were thawed onto gelatin-coated slides, air-dried for 60 min, and fixed in acetone (for 10 min, at −20°C). The remaining OCT compound was washed off with PBS/0.05% Tween 20 and nonspecific binding sites were blocked by incubation in PBS containing 1% BSA and 20% FCS (for 30 min at room temperature).

Staining of a single antigen (iNOS, cell type or *L. major*) was performed exactly as described (9). For double labeling, a combination of gold-silver immunostaining (for detection of iNOS) and immunoenzymatic labeling (for detection of *L. major* or cell type) was performed exactly as published previously (9). In some cases we used streptavidin-biotin-peroxidase complex (with 3-amino-9-ethylcarbazole [AEC] as substrate) instead of streptavidin-biotin-alkaline phosphatase complex (with Fast blue BB salt as substrate) for the immunoenzymatic labeling (9). For triple labeling, gold-silver immunostaining (for detection of iNOS) was combined with peroxidase and alkaline phosphatase immunoenzymatic labeling (for detection of cell type or *L. major*, respectively). After blocking, the following sequence of treatments and incubations was used (3 × 10 min washing with PBS/0.05% Tween 20 between steps 1 and 2 and 4 and 8 or with 50 mM Tris, pH 8.2/0.05% Tween 20 between steps 2 and 4; brief washing with distilled water between steps 8 and 10): (step 1) simultaneous addition of all three primary Abs (rabbit anti-iNOS, rat anti–cell type, human anti–*L. major*; overnight, 4°C); (step 2) gold-conjugated goat anti–rabbit IgG (Dako, Hamburg, Germany; 1 h, RT); (step 3) affinity-purified alkaline phosphatase–conjugated $F(ab')_2$ fragment goat anti–human-IgG (1 h, RT); (step 4) development with Fast blue BB salt substrate solution (5–20 min, RT); (step 5) affinity-purified, biotin-conjugated $F(ab')_2$ fragment mouse anti–rat-IgG (1 h, RT); (step 6) streptavidin-biotin-peroxidase complex (1h, RT); (step 7) development with AEC substrate solution (5–10 min, RT); (step 8) postfixation with 2% glutaraldehyde in PBS (10 min, 4°C); (step 9) quenching of aldehyde groups with 0.05 M glycine in PBS (10 min, 4°C); and (step 10) treatment with immunogold-silver enhancement mixture (Amersham, Braunschweig, Germany) (one to five incubation periods of 3–10 min each, RT). After brief rinsing in distilled water, slides were mounted without counterstaining. In control experiments, no staining was obtained when any of the primary Ab was combined with an irrelevant secondary reagent used in the triple staining procedure, or when the primary Abs were replaced by preimmune sera or an irrelevant isotype-matched control Ab. NADPH diaphorase staining was performed as described (9).

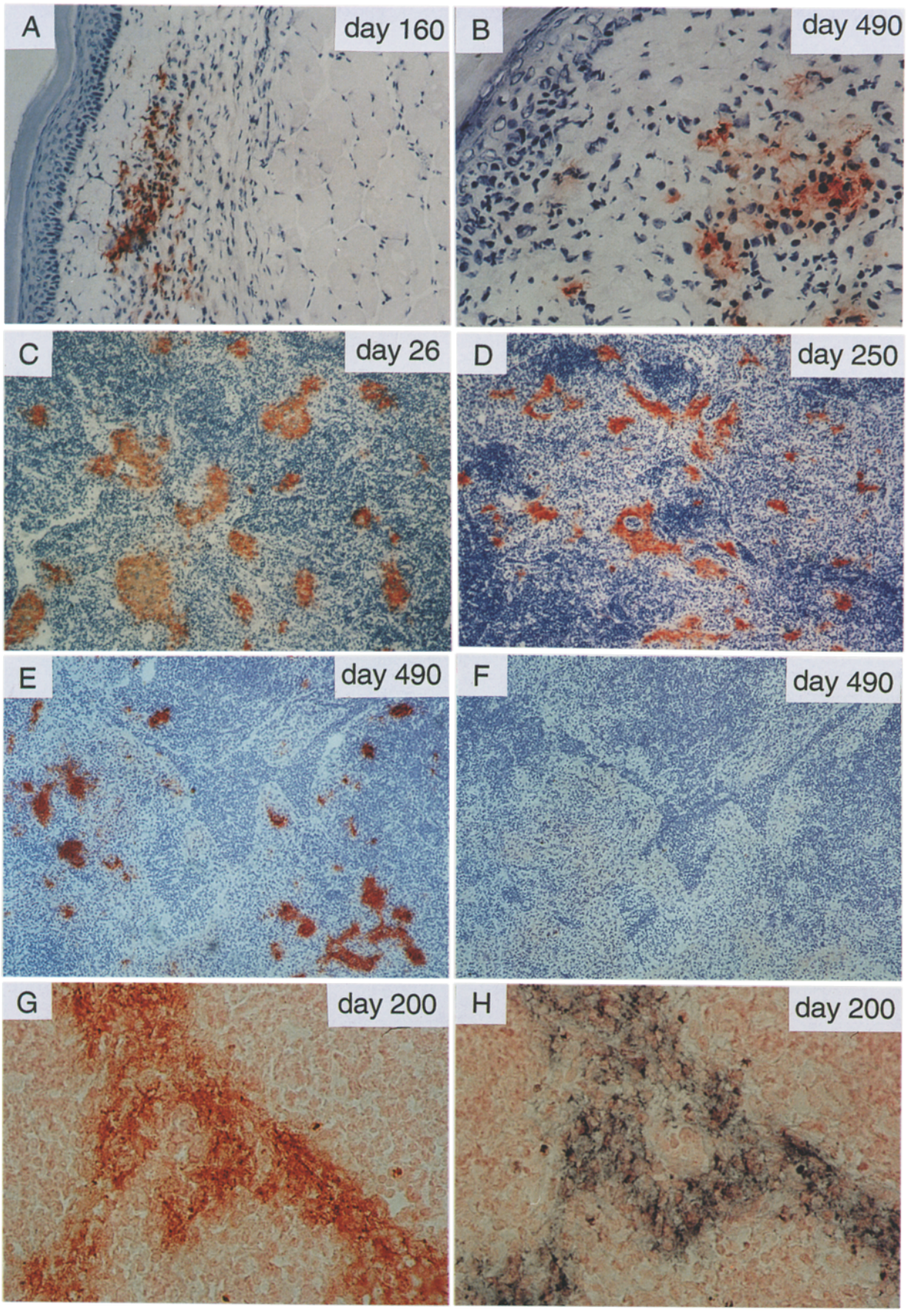
RNA Preparation and Competitive Reverse Transcriptase–PCR. RNA was prepared by the guanidinium isothiocyanate method and reverse transcribed as described (9). iNOS or β-actin cDNA was amplified and quantitated by competitive PCR technique using primers specific for iNOS or β-actin in the presence of defined dilutions of the respective competitor plasmids as reported recently (9).

Determination of iNOS Enzyme Activity. iNOS enzyme activity in total lymph node lysates (40–60 μg) was measured in a microplate assay exactly as described (27).

Results

Lifelong Expression of iNOS mRNA, Protein, and Activity in the Tissue of L. major–infected C57BL/6 Mice. In a previous study with *L. major*–infected, resistant C57BL/6 mice, we demonstrated that the onset of parasite reduction and healing of the cutaneous lesions coincided with an upregulation of iNOS in the dermis of the skin lesion and in the draining lymph node (9). Immunohistological analysis of the footpad (Fig. 1, *A* and *B*) and the popliteal lymph node from C57BL/6 mice (Fig. 1, *C–H*) at various time points after resolution of clinical disease revealed that the expression of iNOS protein remained at high levels, despite a 10^3–10^6-fold decrease of the parasite burden, respectively (Fig. 2 *A*). No staining for iNOS was obtained in the presence of 1–10 μg/ml of the iNOS peptide used for generating the polyclonal rabbit antiserum (Fig. 1, *E* and *F*) or when the tissue sections were incubated with the respective rabbit preimmune serum (not shown). Persistence of iNOS protein in long-term–infected C57BL/6 mice (>day 100 of infection) was found in 34 of 38 skin lesions and in all of 78 examined lymph nodes, which were derived from 20 independent time course experiments. 26 (skin) and 44 (lymph node) individual time points (>day 100) were investigated, the latest being day 766 after infection. Whereas in the draining lymph node virtually every 5-μM section was positive for iNOS, the expression was much more focal in the dermis of the former skin lesion. No iNOS protein was detected in the spleen of long-term–infected mice or in the lymphoid tissue of age-matched uninfected mice (not shown).

Three additional experimental approaches confirmed the persistent expression of iNOS in the tissue beyond the acute phase of infection. By competitive reverse transcriptase–PCR analysis, the amount of iNOS mRNA in the tissue remained at comparable levels between days 35 and 252 of infection (Fig. 2 *B*). Similarly, the specific activity of NOS in the lymph nodes of *L. major*–infected C57BL/6 mice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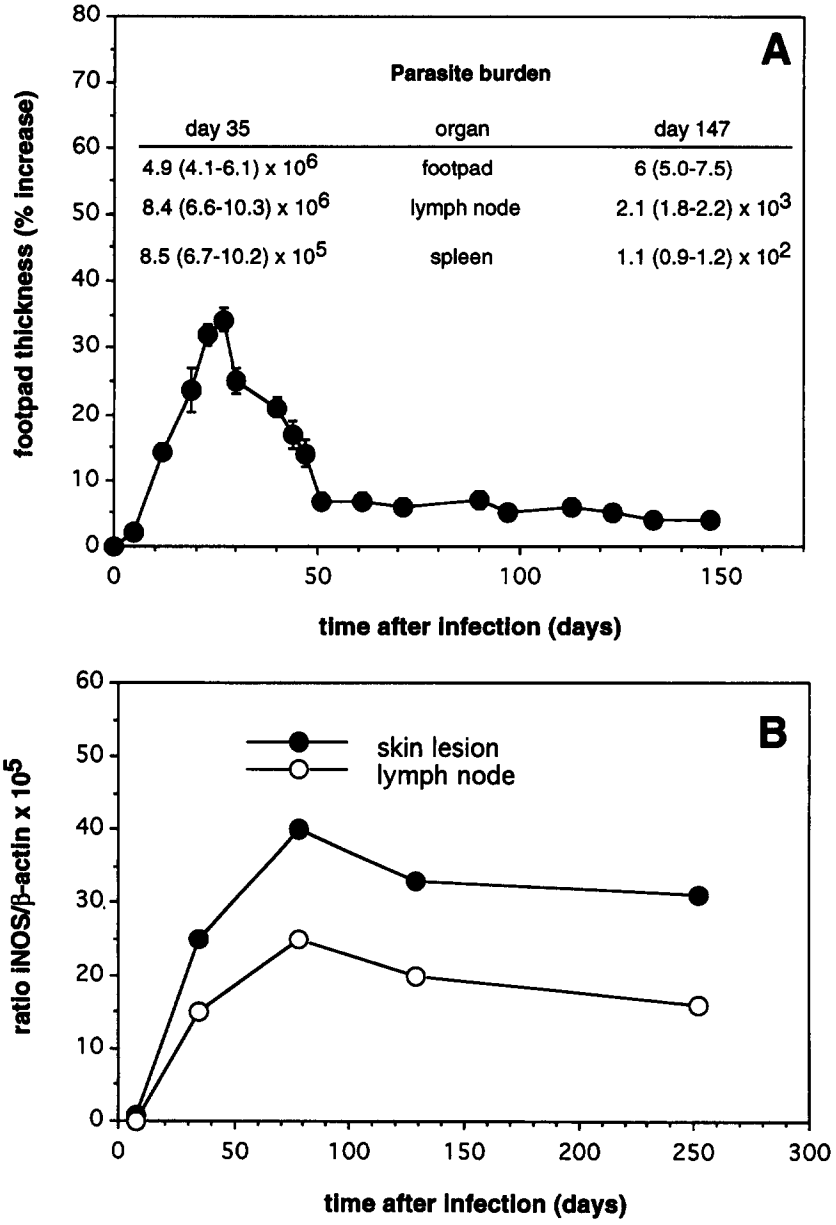


Figure 2. Lesion size, parasite burden, and tissue expression of iNOS mRNA during the course of *L. major* infection in C57BL/6 mice. 16 (*A*) or 15 (*B*) mice were infected bilaterally with *L. major* into the hind footpads. (*A*) Development of the footpad lesions (mean increase of footpad thickness ± SEM). Parasite burden in the pooled organs of two to three mice was determined by limiting dilution analysis at various time points after infection. The contralateral organs were processed for iNOS immunohistochemistry (not shown). (*B*) iNOS mRNA was quantitated in the pooled organs of three mice by competitive RT-PCR analysis.

was in the same order of magnitude before and after healing of the cutaneous lesions (Table 1). Finally, as previously shown for the acute stage of infection (9), we again found a perfect colocalization between iNOS (detected by anti-iNOS immunostaining) and NADPH-diaphorase activity in the skin (not shown) and draining lymph node of long-term–infected mice (Fig. 1, *G* and *H*). This further indicates the presence of functionally active iNOS.

Persistence of iNOS Is a More General Phenomenon Also Observed in other Mouse Strains. We envisaged the possibility that the continuous expression of iNOS, despite clinical healing of the disease, is unique for the C57BL/6 mouse strain, which shows an intermediate resistance to *L. major* (28) without complete clearance of the parasites from the tissue (23, and Fig. 2 *A*). Therefore, we decided to extend our analysis to resistant CBA and C3H mice, which were reported to develop less severe and more quickly resolving lesions than C57BL/6 mice after infection with *L. major* (28). By immunohistology, iNOS was found both during the acute stage of infection (day 29) and after healing of the skin lesions (days 146 and 201) in C3H and CBA mice (n = 4; not shown). There was, however, an inverse correlation

Figure 1. Expression of iNOS protein in the lesional skin (*A* and *B*) and the popliteal lymph nodes (*C–H*) from C57BL/6 mice at various time points after infection with *L. major* (3×10^6 into the footpad). The skin was analyzed for the presence of iNOS after clinical healing of the lesions. (*A–G*) Anti-iNOS immunoperoxidase labeling. In *F*, which is a consecutive section of *E*, the iNOS staining was blocked by the corresponding iNOS peptide (10 μg/ml). In *H*, which is a consecutive section of *G*, NADPH diaphorase activity was visualized histochemically. (*A*) ×200, (*B*) ×400, and (*C–F*) ×100, hematoxylin counterstain. (*G* and *H*) ×400, nuclear Fast red counterstain.

Table 1. *NOS Activity in the Lymph Node of Naive or L. major–infected C57BL/6 Mice*

C57BL/6 mice	Specific activity*	Total activity‡
Naive§	0.6 ± 0.2	0.5 ± 0.2
Acutely infected (days 28–46)‖	108.0 ± 16.6	176.0 ± 20.4
Chronically infected (days 164–344)¶	80.2 ± 24.3	250.0 ± 40.8

*pmol NO_2^-/mg protein/min in a 120-min assay.
‡pmol NO_2^-/min per total lymph node.
§Mean ± SEM of five lymph nodes from individual naive mice.
‖Mean ± SEM of 16 popliteal lymph nodes from 11 infected mice that were derived from eight independent time course experiments (28–46 d after infection).
¶Mean ± SEM of 13 popliteal lymph nodes from nine infected mice that were derived from five independent time course experiments (164–344 d after infection).

between the degree of clinical resistance (C3H > CBA > C57) and the amount of iNOS in the tissue of long-term–infected mice (C57 > CBA > C3H). In fact, no iNOS protein was detected in the footpads from clinically cured C3H mice. The implications of this finding will be discussed below. Persistence of iNOS was also observed in the lymph nodes of *L. major*–infected BALB/c mice (days 267, 292, or 366 after infection; not shown), which were rendered resistant by low dose infection or by treatment with anti-CD4 or IL-12 before infection, following published protocols (29–31). These findings demonstrate that tissue expression of iNOS beyond the acute phase of *L. major* infection occurs in a number of different mouse strains.

Persistence of iNOS Activity Is Dependent on CD4 T Cells. In cutaneous leishmaniasis of resistant mice, $CD4^+$ T cells are indispensable for the control of the primary acute infection (32), whereas both $CD4^+$ and $CD8^+$ T cells contribute to the resistance against reinfection (33). To define the T cell subpopulation that is required for maintaining the expression of iNOS, we applied anti-CD4 or anti-CD8 Abs to long-term–infected resistant mice. The data in Table 2 demonstrate that depletion of $CD4^+$ T cells caused a 80% reduction of the specific NOS activity in the lymph node. This was also confirmed by immunohistological analysis of the lymph nodes (not shown). In contrast, anti-CD8 treatment did not lead to a decrease, but rather to an increase of NOS activity (Table 2).

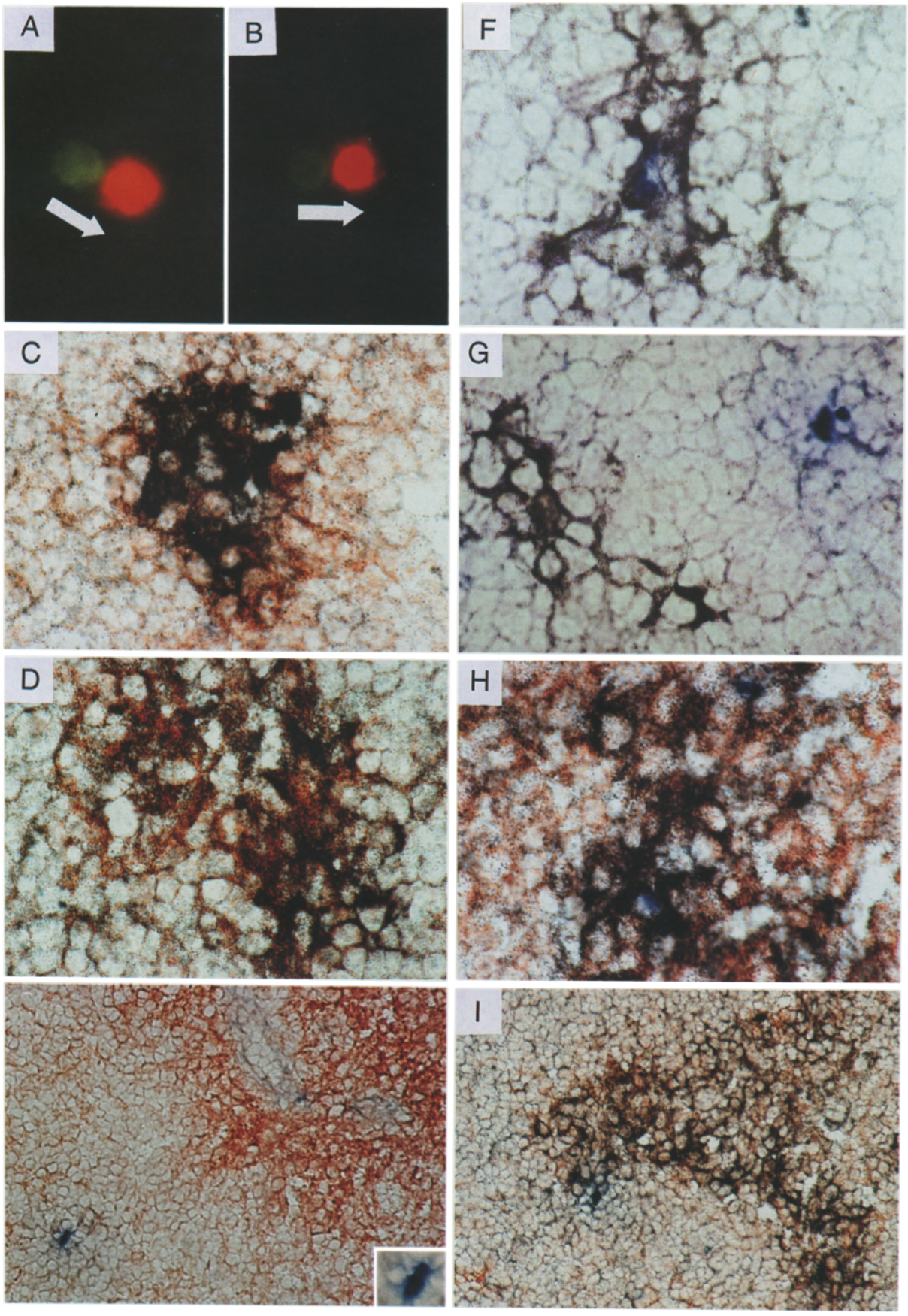
Table 2. *Effect of Anti-CD4 or -CD8 Treatment on the Cellular Composition and the NOS Activity in the Lymph Node of L. major–infected Mice*

	In vivo treatment*		
Parameter	PBS	Anti-CD4	Anti-CD8
Cellular composition			
Thy 1.2^+	32.0 ± 3.9	21.3 ± 2.8	21.5 ± 5.9
$CD4^+$	16.0 ± 1.5	2.5 ± 1.9	21.4 ± 4.6
$CD8^+$	13.4 ± 2.3	18.9 ± 3.3	1.4 ± 0.3
$B220^+$	57.1 ± 5.0	66.8 ± 2.3	66.5 ± 6
$F4/80^+$	1.4 ± 0.4	2.2 ± 1.3	1.5 ± 0.4
NOS activity			
Specific activity‡	43 ± 4	9 ± 2	75 ± 16
Total activity§	195 ± 47	13 ± 7	283 ± 46

*Long-term–infected C57BL/6 mice (day 147 or 220 after bilateral infection into the footpad; four mice per group) were injected intraperitoneally with PBS, anti-CD4, or anti-CD8 antibody (day ±0, +2, +6, +10). After 14 d of treatment, lymph nodes were subjected to FACS® analysis or NOS enzyme activity assays. Values denote mean ± SD from two independent experiments.
‡pmol NO_2^-/mg protein/min (120-min assay).
§pmol NO_2^-/min per total lymph node.

Identification of the Cells Expressing iNOS and/or Harboring L. major Parasites. In clinically healed resistant mouse strains, a small number of *Leishmania* continue to reside in the draining lymph node (16, 21–24) and at the site of the former skin lesion (33, and Fig. 2 *A*). Occasionally, parasites are also found to persist in the spleen (20) (see Fig. 2 *A*). To address the issue of how the parasites manage to survive despite the continuous presence of iNOS in the tissue, we determined the phenotype of the cells, which express iNOS and/or carry *Leishmania* during the chronic stage of infection. By immunofluorescence double staining of single cell suspensions and double labeling of tissue sections, iNOS was detected in distinct types of macrophages ($F4/80^+$, BM-8^+, and/or MOMA-2^+; Fig. 3, *A* and *C*) and in dendritic cells (NLDC-145^+; Fig. 3, *B* and *D*), but

Figure 3. Characterization of the cell types expressing iNOS and/or carrying *L. major* parasites/antigen in the popliteal lymph node of long-term–infected C57BL/6 mice. (*A* and *B*) Double immunofluorescence labeling of a lymph node single cell suspension (day 157 after infection) partially depleted of T and B cells as described in Materials and Methods. (*A*) Cell coexpressing the F4/80 marker (*green*) and iNOS (*red*); (*B*) a NLDC145^+ cell (*green*) that is also positive for iNOS (*red*). To visualize the differentially labeled markers on the same cell, slides were gently moved (direction indicated by *arrows*) before changing the fluorescence filters. Double (*C–G*) or triple (*H–I*) labeling of lymph node sections for iNOS (immunogold-silver staining, *black*), for cell-type marker (immunoperoxidase staining, *brown*), and/or for *L. major* (alkaline phosphatase staining, *blue*) without counterstain. (*C*) Colocalization of iNOS and MOMA-2 (macrophage), day 120. (*D*) Colocalization of iNOS and NLDC-145 (dendritic cell), day 147. (*E*) *L. major* and F4/80 plus NLDC-145, day 147. *L. major* (*blue*) is localized outside the F4/80- or NLDC-145–positive cells. (*F* and *G*) *L. major* and iNOS, day 378. *L. major* (*blue*) inside (*E*) or outside (*F*) of an iNOS cluster (*black*). (*H*) *L. major*, iNOS and F4/80, day 120, triple positive cluster. (*I*) *L. major*, iNOS and cell-type marker (Abs F4/80, NLDC-145, MOMA-2, and Mac-1 were combined), day 120. *L. major* (*blue*) is located outside the iNOS-positive macrophage/dendritic cell cluster. (*A–D*, *F–H*), and *inset*, *E* ×1,000. (*E* and *I*) ×400.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not in granulocytes (GR-1$^+$), endothelial cells (MECA-32$^+$), B cells (B220$^+$), or T cells (Thy1$^+$) (not shown). The same conclusions were reached by labeling of consecutive sections with anti-iNOS or the anti–cell type Abs, respectively (not shown). In accordance with previous data (24), *L. major* parasites were found in macrophages and dendritic cells (not shown). However, by combined staining of tissue sections for *L. major* and F4/80 plus NLDC-145, *Leishmania* were also identified in areas free of F4/80$^+$ macrophages and NLDC-145$^+$ dendritic cells (Fig. 3 *E*). No parasites were found in granulocytes or endothelial cells (not shown). In a series of 34 lymph nodes from long-term–infected mice (106–580 d after infection) derived from 12 independent time course experiments, 36% of 962 *L. major* foci detected colocalized with iNOS in the tissue (Fig. 3 *F*). The majority of parasites (64%), however, was clearly located outside the clusters of iNOS-positive cells (Fig. 3 *G*). Triple labeling experiments revealed that macrophages or dendritic cells, which harbored parasites or parasite antigens, were also positive for iNOS (Fig. 3 *H*). The *Leishmania* in the iNOS-negative areas, in contrast, did not colocalize with F4/80-, MOMA-2–, or Mac-1–positive macrophages or NLDC-145–positive dendritic cells (Fig. 3 *I*). From these data we conclude that *L. major* parasites might secure their survival in the host by entering an as yet unidentified type of cell in the absence of iNOS induction.

Continuous Activity of iNOS Is Required for the Control of L. major Parasites Persisting in Clinically Cured Mice. The findings presented so far raised the possibility that the persistence of iNOS is functionally linked to the control of the residual parasites in the tissue. To test this hypothesis, we applied L-NIL to long-term–infected mice. The inactive D-enantiomer of L-NIL is not yet available in amounts sufficient for in vivo experiments, but clearly fails to inhibit iNOS activity in vitro (4, 34). In contrast to the widely used L-NMMA, L-NIL is an inhibitor, which is 30–40 times more selective for the inducible than for the constitutive isoform of NOS. Furthermore, L-NIL is much more potent than L-NMMA and nontoxic in mice thus far tested (4). In five independent experiments, oral treatment of clinically cured C57BL/6 mice with L-NIL (4.5 or 9 mM in the drinking water, started at day 103, 123, 130, 139, or 244 of infection) led to a massive increase in the numbers of parasites in the footpad and popliteal lymph node as determined by limiting dilution analysis (Table 3) and immunohistology (Fig. 4, *A* vs. *B*). Most importantly, L-NIL caused the reappearance of cutaneous lesions in 13 out of 14 mice within 7–20 d of treatment (Fig. 5). Functional inactivation of iNOS was further demonstrated by a >90% suppression of enzyme activity (Table 4), and the colocalization of iNOS protein and clusters of parasites in the lymph node of L-NIL–treated mice. In serial sections from control lymph nodes, in contrast, only a few scattered *Leishmania* were present in areas of iNOS expression (Fig. 4, *A* vs. *C* and *B* vs. *D*). Despite the tremendous parasite burden in the skin and draining lymph node and the development of severe and eventually exulcerating lesions after treatment with L-NIL, dissemination into the spleen and/or reactivation of latent *Leishmania* in the spleen was observed in only two of five experiments and to a very limited extent (the increase of the parasite burden caused by L-NIL was <20-fold). When the application of L-NIL was discontinued, the lesions regressed and finally healed (not shown).

Table 3. *Effect of L-NIL on the Parasite Burden in the Tissues of L. major–infected C57BL/6, C3H, and CBA Mice as Determined by Limiting Dilution Analysis**

Group of mice	L-NIL	Parasites per organ‡	Parasites per 1,000 cells§
C57BL/6			
Footpad	−	6.1 (4.9–7.3) × 10	na
	+	3.4 (2.4–4.4) × 10^6‖	na
Lymph node	−	1.2 (0.9–1.3) × 10^3	1.7 (1.4–1.9) × 10^{-2}
	+	3.8 (2.6–5.0) × 10^7‖	3.2 (2.2–4.2) × 10^2
C3H			
Footpad	−	nd	na
	+	nd	na
Lymph node	−	1.5 (1.0–1.9) × 10^2	9 (6–11) × 10^{-3}
	+	1.5 (0.9–2.0) × 10^5‖	3.7 (2.3–5.1)
CBA			
Footpad	−	5.3 (3.1–7.5) × 10	na
	+	7.8 (6.7–8.8) × 10^5‖	na
Lymph node	−	4.7 (3.3–6.0) × 10^2	6 (4–8) × 10^{-2}
	+	1.5 (0.9–2.0) × 10^5‖	3.7 (2.3–5.1)

*Mice were infected with 3 × 10^6 parasites into the hind footpads. Groups of four mice were kept on normal water or treated with L-NIL (4.5 mM in the water) for 42 d starting at day 103 of infection when the primary lesions had healed (see Fig. 7).
‡95% confidence interval is given in parentheses. *ND*, no parasites detectable in the undiluted footpad homogenate (3 ml per footpad; 100 μl homogenate/well).
§Calculated from the total number of cells per lymph node.
‖Significant difference when compared with the corresponding control group (significance was assumed when the 95% confidence limits did not overlap).
NA = not applicable.

In parallel to L-NIL, we also tested the effect of L-NAME, an inhibitor with strong selectivity for the constitutive isoform of NOS, and of L-NMMA (which inhibits inducible and constitutive NOS) (35) in long-term–infected C57BL/6 mice. L-NAME and its inactive D-analogue failed to elicit recrudescence of the disease. Administration of L-NMMA (but not of D-NMMA) caused reappearance of leishmanial lesions similar to L-NIL. Both L-NAME– and L-NMMA–treated mice showed signs of severe drug toxicity (e.g., weight loss, reduced fluid, and food uptake) (Fig. 5). For L-NIL, in contrast, careful monitoring of body weight as

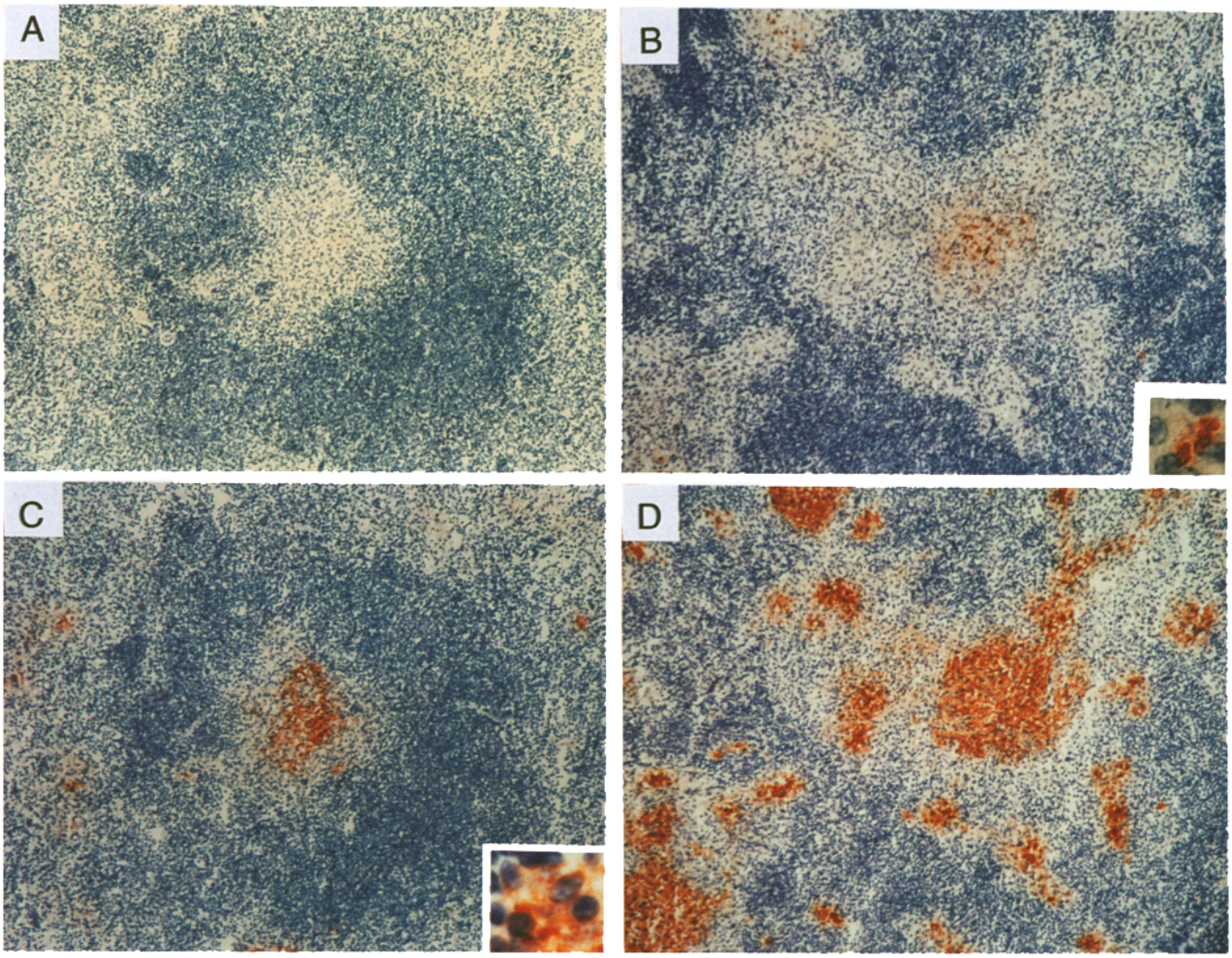

Figure 4. Effect of L-NIL on the expression of iNOS and presence of *L. major* parasites in the lymph node of C57BL/6 mice (day 168 after infection). (*A* and *C*) Consecutive sections from control mice kept on normal water. (*B* and *D*) Consecutive sections from mice kept on water with 9 mM L-NIL from days 139 to 168. (*A* and *B*) Anti–*L. major*; (*C* and *D*) anti-iNOS. (*A–D*) ×100; (*insets B* and *C*) ×1,000.

well as food and fluid uptake did not reveal any alterations compared with the control group kept on normal water (Fig. 5) which closely resembles our recently published results on the effect of L-NIL versus L-NMMA in C57BL/6 mice during the acute phase of *L. major* infection (4).

Finally, we investigated the effect of L-NIL on long-term–infected CBA and C3H mice, which had healed their cutaneous lesions (day 103 or 149 of infection). As was the case in C57BL/6 mice, a small amount of *L. major* parasites persisted in the draining lymph node of both strains, which strongly increased upon treatment with L-NIL (Table 3). In CBA mice, residual parasites were also detectable at the site of the former skin lesion (Table 3), which presumably are responsible for the recurrence of clinical disease (footpad swelling) after treatment with L-NIL in six of seven mice from two independent experiments (Fig. 6). In contrast, no *Leishmania* were grown from the footpad tissue of nine clinically cured C3H mice, individually tested by limiting dilution analysis at the end of a 42–52-d period during which the mice were either kept on normal drinking water or on water supplemented with L-NIL (Table 3). Consequently, reactivation of cutaneous leishmaniasis was only observed in one of eight C3H mice treated with L-NIL (Fig. 6, and data not shown). The spleens of control or L-NIL–treated CBA or C3H mice were routinely negative for parasites in our detection system (not shown).

Together, these results indicate that inducible, but not constitutive NOS, is required for the control of persisting *Leishmania*. Parasite replication and recrudescence of the disease after inhibition of iNOS seems to be confined to the sites where live parasite remained after resolution of the acute phase of infection.

Discussion

In a previous in vitro study, reactivation of Epstein-Barr virus replication in human B cell lines was shown to be inhibited by endogenous NO (36). In the present paper we

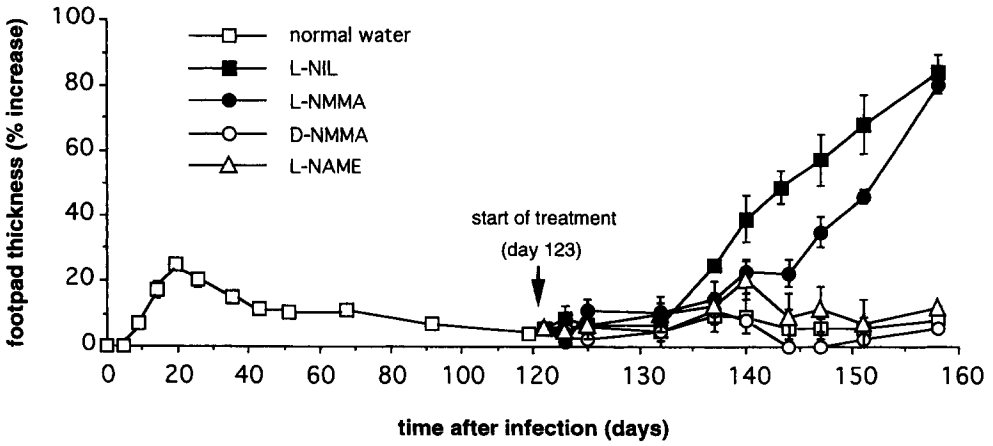

Figure 5. Reactivation of cutaneous lesions in C57BL/6 mice after treatment with inhibitors of iNOS. 15 mice were infected with *L. major* (3×10^6) into the right hind footpad. From day 123 of infection onwards, mice were either kept on normal water or treated for 36 d with L-NIL (4.5 mM), L-NMMA (50 mM), D-NMMA (50 mM), or L-NAME (50 mM). Lesion development (mean ± SEM, three mice per group; where error bars are not visible, they fall within the symbols) was monitored. The mean (± SD) food and water consumption and the mean (± SD) percent weight gain (= [weight at day 159 − weight at day 123] : weight at day 123 × 100%) during treatment is also given. Negative numbers denote weight loss. (* ‡) Significant decreases compared with control (Student's *t* test for unpaired samples; * $P < 0.001$, ‡ $P < 0.01$).

Additive to the drinking water	water consumption (ml/day per mouse)	food consumption (g/day per mouse)	weight gain (%)
none	3.2 ± 0.3	3.6 ± 0.4	0.9 ± 1.3
9 mM L-NIL	3.2 ± 0.5	3.5 ± 0.3	-0.7 ± 1.3
50 mM L-NMMA	1.0 ± 0.3 *	2.3 ± 0.4 §	-13.6 ± 3.4‡
50 mM D-NMMA	2.8 ± 0.2	3.2 ± 0.2	3.3 ± 0.1
50 mM L-NAME	1.3 ± 0.5‡	2.8 ± 0.5	-7.2 ± 2.3‡

provide the first direct in vivo evidence for NO-dependent control of an intracellular microogranism in a latently infected host organism. In clinically cured, *L. major*–infected resistant mice, small numbers of *Leishmania* are found in the draining lymph node (21–24) and, depending on the mouse strain, also at the site of the former skin lesion (33, and Fig. 2 *A* and Table 3). Persistence of parasites is accompanied by the continuous expression of iNOS in the respective tissue, which presumably lasts for life. Although the systemic urinary excretion of reactive nitrogen intermediates was reported to drop after healing of the cutaneous lesions in *L. major*–infected C3H mice (18), the results of our in situ analysis clearly demonstrate that the local expression of iNOS is maintained at levels similar to the acute phase of infection. By double labeling techniques, both macrophages and dendritic cells were found to express iNOS in the lymph node. The expression of iNOS in vivo by dendritic cells has not been reported before. Based on several observations, the iNOS protein in the tissue does not represent amyloid-like, functionally inactive debris left over from the previous inflammatory response. First, iNOS was not only detected by the Ab directed against the COOH terminus of the protein, but also by an Ab against amino acid 13-22 of the NH_2-terminal mouse iNOS sequence (Bogdan, C., and N. Donhauser, unpublished results). Second, persistence of iNOS expression was confirmed on the level of mRNA. Third, determination of NOS activity in the lymph node yielded values in the same order of magnitude during the acute and chronic stages of infection. Fourth, expression of iNOS protein always coincided completely with NADPH-diaphorase activity at early (9) and late time points after infection (Fig. 1). Finally, application of L-NIL, an inhibitor with relative selectivity for iNOS, led to the reactivation of parasite replication and resurgence of disease, whereas L-NAME, an inhibitor of constitutive NOS, was inactive in this respect. This indirectly proves the presence of functionally active iNOS. It is interesting to note that the reappearance of cutaneous lesions and/or the 10^3–

Table 4. *Suppression of NOS Activity by L-NIL in the Lymph Node of C57BL/6 Mice Chronically Infected with L. major**

Treatment	Specific activity‡	Total activity§
None‖	106 ± 37	304 ± 51
L-NIL‖	5 ± 1.8	30 ± 8

*Values denote mean ± SEM of eight popliteal lymph nodes from four *L. major*–infected mice per group that were derived from two independent in vivo experiments (day 277 or 344 after infection).
‡pmol NO_2^-/mg protein/min in a 120-min assay.
§pmol NO_2^-/min per total lymph node.
‖Mice were kept on drinking water with or without L-NIL (4.5 mM) for 9 d before analysis of the lymph nodes.

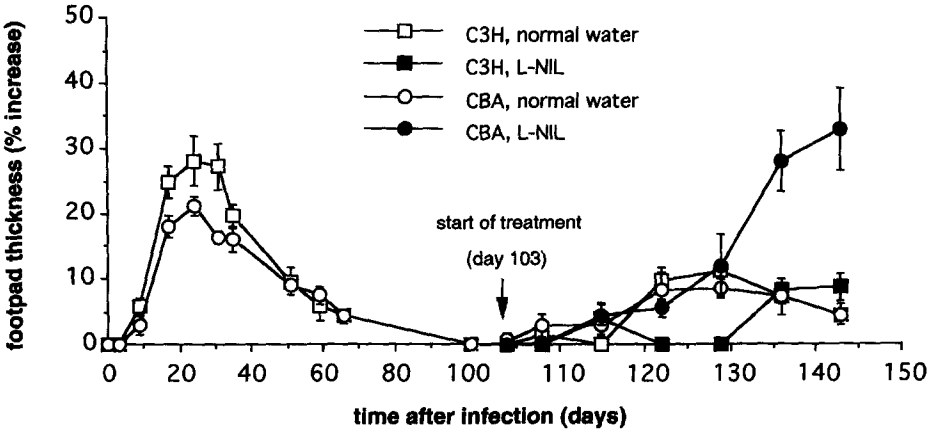

Figure 6. Effect of L-NIL on the healed cutaneous lesions of CBA and C3H mice. Eight C3H and eight CBA mice were infected with *L. major* (3×10^6) into the right hind footpad. From day 103 after infection onwards, mice were either kept on normal water or treated for 42 d with L-NIL (4.5 mM). Lesion development (mean ± SEM, four mice per group) was monitored. Weight development, as well as food and water consumption, was not altered by L-NIL (not shown).

10^4-fold increase of the numbers of *Leishmania* in the regional lymph nodes of C57BL/6, CBA, and C3H mice after treatment with L-NIL never led to overwhelming visceral leishmaniasis with a high parasite burden in the spleen as seen during the progressive infection of highly susceptible BALB/c mice. As iNOS mRNA and protein were only weakly expressed in the spleen of long-term–infected mice (not shown), our findings suggest the existence of NO-independent control mechanisms in this organ.

The persistence of iNOS in the lymph node of clinically cured mice raises questions about the stimuli underlying the continuous upregulation of iNOS. Our data demonstrate that $CD4^+$ but not $CD8^+$ T cells are required for maintaining expression of iNOS. Although the depletion of $CD4^+$ T cells led to a 75–80% decrease of the specific NOS activity (Table 2) and to an increased number of parasites in the respective lymph node sections (not shown), this was not yet sufficient to cause reappearance of cutaneous lesions within the 10–14 d of Ab application and observation. Previous studies, however, have already shown that prolonged treatment with anti-CD4 (>20–25 d) will reactivate latent leishmaniasis in resistant mice (37). Considering the pivotal role of IFN-γ for the induction of iNOS in vivo (38, 39) and the exacerbative effect of anti-IFN-γ during acute leishmaniasis and in clinically cured *L. major*–infected mice (40, 41), it is likely that the reduction of NOS activity by anti-CD4 at least partially results from a lack of IFN-γ. As cells other than $CD4^+$ T cells are known to produce IFN-γ (e.g., NK or $CD8^+$ T cells), and multiple cytokines cooperate with IFN-γ for the induction of iNOS (for a review see reference 42), it is not surprising that inhibition of iNOS by L-NIL is by far more efficient in reactivating clinical disease than anti-CD4 treatment.

Given the susceptibility of *L. major* promastigotes (10, 11) and amastigotes to native NO or NO donors (Bogdan, C., A. Diefenbach, and M. Röllinghoff, manuscript in preparation), it is unexpected that some parasites survive despite the abundance of iNOS in the tissue. So far, there is no evidence for irreversible genetic alterations or for an increase of virulence during latency (23). The recrudescence of leishmaniasis seen after inhibition of iNOS by L-NIL further demonstrates that persistent *L. major* obviously do not acquire overt resistance to NO, but remain susceptible to the direct or indirect antileishmanial effects of NO. Our in situ analysis, however, offers some ideas of how *Leishmania* might manage to survive in the tissue. 60–70% of the parasites persisting in the lymph node were located in areas that were negative for iNOS and for known markers of macrophages (F4/80, BM-8, MOMA-2, Mac-1), dendritic cells (NLDC-145), granulocytes (GR-1), or endothelial cells (MECA-32). Within the inherent limits of immunohistological analyses (e.g., it is impossible to recognize a double positive cell when two colors are exactly superimposed), our findings suggest the existence of a novel type of host cell, which either intrinsically or due to suppression by parasite products (43) lacks the production of NO and therefore might serve as a "safe target" (44) for *L. major*. It is possible that the *Leishmania* reside in a subtype of tissue macrophages (histiocytes) that is not picked up by the tested Abs. Alternatively, the parasites might infect fibroblasts. The latter possibility is suggested by the large amounts of collagen fibers and extracellular matrix components in the chronically infected lymph nodes (Bogdan, C., N. Donhauser, and S. Stenger, unpublished results). In vitro, *L. braziliensis* efficiently entered fibroblasts, but became lodged in vacuoles that did not fuse with secondary lysosomes and thereby could evade killing by the host cell (45).

Approximately 30–40% of the *L. major* (antigen) foci in the lymph nodes of long-term–infected mice colocalized with iNOS-positive macrophages or dendritic cells. Whereas we cannot prove NO-mediated killing of the parasites in situ, this observation indicates an intimate interaction between iNOS-positive (host) cells and *L. major*. Our results support the idea that parasite killing and evasion are permanently ongoing in the lymph nodes of long-term–infected mice. The CD4-dependent persistence of iNOS, the concurrent expression of cytokines (IL-4, IFN-γ, TGF-β) in the latent lesions (Stenger, S., and C. Bogdan, unpublished results) and the presence of *L. major* parasites or antigen in both iNOS-negative and -positive areas strongly suggest a dynamic, long-lasting inflammatory process and argue against a static dormancy of the microorganisms.

From a teleological point of view one might wonder why the host organism allows persistence rather than clear-

ance of parasites with the result of a lifelong inflammation in the tissue. It has been proposed that the presence of a few viable *Leishmania* might be required to maintain protective immunity to reinfection (33), following the notion that T cell memory is short-lived in the absence of antigen (46). It is also intriguing to speculate that the sustained levels of iNOS after infection with an intracellular microorganism might enhance the resistance of the host against unrelated, but NO-sensitive pathogens. In this context it is worthwhile to reconsider earlier studies that demonstrated resistance to bacterial or fungal infections (e.g., *Listeria monocytogenes*, *Cryptococcus neoformans*) or to malignant tumors in mice latently infected with *Toxoplasma gondii* (47–49).

Reduced activity of iNOS might be the mechanism underlying the reactivation of leishmaniasis in immunosuppressed mice and humans. Cutaneous lesions reappeared in clinically cured C57BL/6 mice after a 3-wk treatment with cyclophosphamide or hydrocortisone (16). Glucocorticoids have been shown to suppress the cytokine-induced induction of iNOS in macrophages and other cells (50 and references therein). Several cases of visceral leishmaniasis have been noted in immunosuppressed persons many years after they have moved from endemic areas, which strongly suggests that endogenous reactivation of *Leishmania* replication also occurs in humans (17, 51, 52). In addition, evidence has emerged that human monocytes/macrophages control *L. major* parasites in an NO-dependent manner (53).

In conclusion, the experiments presented here demonstrate that the expression of iNOS by macrophages and dendritic cells is crucial for maintaining a state of long-term resistance against latent *Leishmania*. We believe that our findings will also apply to infections with other NO-sensitive intracellular microbes including viruses that persist in the host after resolution of the acute disease.

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