Fibroblasts as Host Cells in Latent Leishmaniosis

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Abstract

Intracellular parasites are known to persist lifelong in mammalian hosts after the clinical cure of the disease, but the mechanisms of persistence are poorly understood. Here, we show by confocal laser microscopy that in the draining lymph nodes of mice that had healed a cutaneous infection with *Leishmania major*, 40% of the persisting parasites were associated with fibroblasts forming the reticular meshwork of the lymph nodes. In vitro, both promastigotes and amastigotes of *L. major* infected primary skin or lymph node fibroblasts. Compared with macrophages, cytokine-activated fibroblasts had a reduced ability to express type 2 nitric oxide synthase and to kill intracellular *L. major*. These data identify fibroblasts as an important host cell for *Leishmania* during the chronic phase of infection and suggest that they might serve as safe targets for the parasites in clinically latent disease.

Key words: *Leishmania major* • fibroblasts • persistent infection • nitric oxide • macrophages

Introduction

A hallmark of infections with certain viruses (e.g., herpesviruses), intracellular bacteria (e.g., *Mycobacteria, Coxiella, Chlamydiae*), or protozoa (e.g., *Trypanosoma cruzi, Leishmania*) is the long-term persistence of the pathogen after clinical cure of the disease. Based on in vitro results, modulation of host cell antimicrobial activities, synthesis of inhibitory cytokines, impairment of T cell activation, or retreat of the pathogen into cells that do not elicit an immune response have been proposed as viral or microbial survival strategies, but the mechanisms of persistence in vivo remain ill defined (1, 2). One example are infections with *Leishmania* parasites. *Leishmania* promastigotes are transmitted by sand flies to mammalian hosts, where they infect macrophages, granulocytes, and dendritic cells, transform into amastigotes, and cause cutaneous, mucocutaneous, or progressive visceral disease. In most strains of mice as well as in humans, infections with *Leishmania major* usually elicit skin swellings or single ulcers that are ultimately controlled by a CD4^{+} T cell response involving the production of IFN-γ

and the activation of antileishmanial effector mechanisms in macrophages (3–6). In mice, IFN-α/β and IFN-γ induced the production of nitric oxide (NO)[1] by the inducible (or type 2) NO synthase (iNOS or NOS2), which was shown to be indispensable for the healing of acute cutaneous lesions (7–10).

After spontaneous or chemotherapy-mediated healing of the infection, both mice and humans continue to harbor small numbers of alive *Leishmania* parasites in the lymphoid tissue (11–13). This was demonstrated most convincingly by the recrudescence of the disease after treatment with immunosuppressive drugs, depletion of CD4^{+} T cells, or inhibition of NOS2 activity (14–17). Although the components of the immune system that are responsible for the resolution of acute *Leishmania* infection are well defined, little is known about the mechanisms that allow the parasites to survive lifelong in the host. In genetically resistant mice that had resolved a skin infection with *L. major*, we found that NOS2 activity was indispensable for the long-term control of the remaining parasites. 30–40% of the parasites persisting in the draining lymph node colocalized with NOS2-positive macrophages or dendritic cells, whereas 60–70% of the parasites were located in NOS2-negative areas that could not be stained with known markers for mac-

[1]*Abbreviations used in this paper:* NO, nitric oxide; NOS2, type 2 NO synthase; PEM, peritoneal exudate macrophage; RPM, resident peritoneal macrophage.

rophages, dendritic cells, granulocytes, or endothelial cells and thus remained undefined (17). As fibroblasts had been reported to be susceptible to infection with various *Leishmania* species in vitro (18–20), and lymph nodes from chronically infected mice contained strongly increased amounts of fibrous tissue, we considered the possibility that *Leishmania* might also reside in fibroblasts in vivo. In this study, we identify reticular fibroblasts in lymph nodes as major host cells for *L. major* during latent disease and provide evidence that these cells might function as "safe targets" (21) for the parasites.

Materials and Methods

Parasites. The *L. major* strain MHOM/IL/81/FE/BNI (17) was propagated in vitro in RPMI 1640 plus 10% FCS on Novy-Nicolle-MacNeal blood agar slants for a maximum of six passages. Fresh *L. major* promastigotes were derived from amastigotes that were isolated from the ulcerated skin lesions or the spleens of BALB/c mice as described (22).

Mice. Female C57BL/6 mice, weighing 16–18 g, were purchased from Charles River, housed in our own facilities, and used at 8–12 wk of age. C57BL/6 mice were inoculated into the right hind footpad with 3×10^6 stationary phase *L. major* promastigotes. In some experiments, mice were infected bilaterally into both hind footpads. The footpad swelling was measured with a metric caliper. The mice resolved their skin lesions usually within 60–70 d after infection. For immunohistological analyses, the popliteal lymph nodes draining the site of infection were removed from mice that had been infected for at least 100 d.

Macrophages. Thioglycollate-elicited peritoneal exudate macrophages (PEMs) were prepared from the peritoneal cavity of C57BL/6 mice as described (23). Resident peritoneal macrophages (RPMs) were obtained from C57BL/6 mice by flushing the peritoneal cavity twice with 10 ml ice-cold PBS. The cells were resuspended in RPMI 1640 culture medium (supplemented as described [23] plus 1 or 2.5% fetal bovine serum [Sigma-Aldrich]), seeded into 24-well plates (10^6 cells/well in 500 μl) or 8-well LabTek® chamber slides (Permanox®; Nalge Nunc International), and cultured at 37°C in 5% CO_2/95% humidified air. After 90–120 min, nonadherent cells were washed off and the macrophage monolayers were further incubated as indicated.

Isolation of Fibroblasts. Murine skin fibroblasts were established from ear skin explants of C57BL/6 mice as described (24). In brief, after removal of the epidermis, the dermis was cut into 1×2 mm pieces, which then were allowed to attach to tissue culture petri dishes before addition of complete RPMI 1640 medium (see above) supplemented with 10–20% FCS. Nonadherent cells were removed by weekly medium exchanges. After 2–4 wk, fibroblasts grew out from the explants that were passaged after treatment with 0.25% trypsin-EDTA solution and further propagated in RPMI 1640 supplemented with 5% FCS by weekly 1:3 splitting.

Reticular fibroblasts were obtained from popliteal lymph nodes (~30–90 mg) of chronically infected mice (>200 d after infection with *L. major*). Collagenase D (100 U/ml in PBS; Boehringer) was injected into the lymph nodes that had been carefully prepared and freed of extracapsular tissue. The lymph nodes were opened and the cell suspension was harvested in PBS with 100 U/ml collagenase D. Finally, the lymph nodes were teased into small fragments that were further digested in PBS plus 400 U/ml

collagenase D at 37°C for 60–90 min. Undigested (capsular) fragments were removed and the cells were pooled with the primary suspension, centrifuged, washed, and seeded in small tissue culture flasks (Nalge Nunc International) in RPMI 1640 medium plus 10–20% FCS. Nonadherent cells were removed by weekly medium exchanges. After 2–4 wk, fibroblasts were growing in the cultures.

For infection or phenotypic characterization, skin or lymph node fibroblasts were harvested with a rubber policeman, seeded, and used as confluent monolayers. To minimize growth, the serum concentration was reduced to 1–2.5%.

Infection of Macrophages and Fibroblasts with L. major Parasites. Monolayers of macrophages or fibroblasts in 24-well plates were incubated with a 3–10-fold excess of *L. major* promastigotes or amastigotes, either continuously (permanent infection) or for 4–14 h after which nonphagocytosed parasites were washed off (pulse infection). The cells were activated with recombinant murine (rm)IFN-γ (provided by Dr. G. Adolf, Ernst Boehringer Institut, Vienna, Austria) with or without rmTNF-α (R&D Systems) or LPS (*Escherichia coli* O111:B; Sigma-Aldrich). Culture medium was replaced every 24 h.

The infection rate and the number of intracellular parasites per infected cell were determined by immunoenzymatic or immunofluorescence staining of the monolayers. At least 300 cells were evaluated. The total number of intracellular parasites per culture was determined by two different methods that were previously shown to yield comparable results (22). In brief, infected macrophage or fibroblast monolayers were lysed in SDS (0.01% in serum-free RPMI) to release intracellular parasites. After addition of a twofold volume of modified Schneider's *Drosophila* medium (mSDM) (25), the cell lysates were spun and the pellets containing the amastigotes were resuspended in 500 μl modified mSDM. The parasite suspensions were seeded in triplicates into 96-well flat-bottomed plates for further incubation (24–48 h) and pulsed with 1 μCi (37 kBq)/well of [^{3}H]desoxy-thymidine (25 Ci/mmol; Amersham Pharmacia Biotech) for the last 12–18 h. Alternatively, the parasite suspensions were subjected to limiting dilution analysis using serial 2-fold dilutions and 12–24 individual wells per dilution step. After 7 d of culture, the number of wells negative for parasites was determined for each dilution and the number of viable *L. major* parasites per culture condition was calculated by applying Poisson statistics and the χ^2 minimization method (8, 22).

Cocultures of Macrophages and Fibroblasts. PEMs were allowed to adhere (2 h) to the outer side of the membrane (pore size 0.45 μM) of cell culture insets (Costar). The insets were then immerged in culture wells containing complete RPMI 1640 medium with 2.5% FCS. Reticular fibroblasts were added and, after adherence to the inner side of the membrane (6 h), were pulse-infected with *L. major* amastigotes (ratio 4–10:1) for 12 h. Thereafter, the insets were transferred to new wells containing culture medium with or without stimuli (see also Fig. 3 D).

Determination of Nitrite. As an indirect measurement for the production of NO, culture supernatants were analyzed for their content of nitrite (NO_2^-) using the Griess reaction (23).

Transmission Electron Microscopy. Incubation of fibroblasts with *L. major* parasites was stopped by adding an excess amount of cold Ito's fixative to the cells (26). After overnight fixation at 4°C, the samples were further processed according to established protocols (27). In brief, washes with 0.1 M cacodylate buffer, pH 7.4, were followed by postfixation in ferricyanide-reduced 1% osmium tetroxide, washes with 0.9% saline solution, encapsulation in 2% agar, en bloc staining with an alcoholic mixture of 0.5% phospho-

tungstic acid and 0.25% uranyl acetate, physical dehydration with a graded series of ethanolic solutions ending with pure acetone, and embedding in Epon 812 resin. Ultrathin sections were placed onto 200-mesh standard square copper grids, stained with a mixture of 10% uranyl acetate and 2.8% lead citrate, and viewed with a Zeiss type 906 transmission electron microscope.

Antibody Reagents. Rabbit antisera against human fibronectin, human laminin-1, human collagen VI (recombinant N9-N2 domain), mouse fibulin-2, or mouse perlecan (recombinant III-3 domain) were as described (28–31). The human or rabbit antiserum against *L. major*, the rabbit antiserum against mouse NOS2 peptide, the rat mAbs against macrophages (Mac-1, F4/80, BM-8, MOMA-2, and ER-MP-23), granulocytes (GR-1), dendritic cells (NLDC-145), or endothelial cells (MECA-32) were the same as used previously (17). The rat mAbs M5/114.15.2 (32) and ER-TR7 (33) were used for the detection of MHC class II antigens and mouse reticular fibroblasts, respectively. All rat mAbs as well as all of the secondary antibodies (affinity-purified biotin-conjugated donkey anti–rabbit IgG, mouse anti–rat IgG or goat anti–human IgG F[ab′]₂ fragments; affinity-purified Cy5- or lissamine rhodamine sulfochloride [LRSC]-conjugated donkey anti–rabbit IgG, Cy5- or dichlorotriazinyl aminofluorescein [DTAF]-conjugated donkey anti–rat IgG, or DTAF-conjugated goat anti–human IgG F[ab′]₂ fragments) were purchased from Dianova.

Immunoenzymatic Staining of Frozen Tissue Sections and of Fibroblasts and Macrophages. 5–6-μM tissue sections from embedded lymph nodes were prepared with a cryostat microtome (model HM 500 OM; Fa. Microm International GmbH), thawed onto slides coated with Fro-Marker® (Science Services), surrounded with PAP PEN® (Science Services), air-dried, fixed in acetone (for 10 min, at −20°C), and briefly washed in PBS/0.05% Tween 20. Monolayers of macrophages or fibroblasts on Permanox® chamber slides (Nalge Nunc International) were washed with PBS and fixed in acetone without prior air-drying. Nonspecific binding sites were blocked for 30 min with PBS/0.1% saponin/1% BSA/20% FCS. Immunoperoxidase staining (with 3-amino-9-ethyl-carbazole as a substrate), alkaline phosphatase immunoenzymatic labeling (with Fast Blue BB salt as a substrate), and hematoxylin counterstaining were performed as described previously (17, 23).

Immunofluorescence and Laser Scanning Confocal Microscopy. For double and triple immunofluorescence, acetone-fixed fibroblast or macrophage monolayers or cryostat sections were blocked (see above) and simultaneously incubated (45 min, room temperature) with two or three different primary antibodies diluted in PBS/0.1% BSA/0.1% saponin. After washing with PBS/0.05% Tween 20, the fluorochrome (Cy5, DTAF, or LRSC)-conjugated secondary antibody reagents (diluted in PBS/0.1% BSA/0.1% saponin) were added sequentially for 30 min each, followed by extensive washing steps in between. In cases where two of the primary antibodies were derived from the same species, complexes of the first and secondary antibody were formed and free binding sites of the secondary antibody were saturated with the respective nonimmune serum (10%, 30 min on ice) before adding the complexes to the sections. The slides were finally mounted with Mowiol (Hoechst) containing 1,4-diazabicyclo-2,2,2-octane (DABCO; Sigma-Aldrich) as an antifading reagent. DTAF (green) was excited at 488 nm and collected using a 515–545-nm band pass filter. LRSC (red) was excited at 574 nm and collected with a 590-nm long pass filter. Cy5 (red) was excited at 651 nm and collected with a 665-nm long pass filter. Nuclei were visualized with the DNA stain TOTO-3 (red; Molecular Probes), which was excited at 642 nm and collected with a 665-nm long

pass filter. The slides were examined with a Leica laser confocal microscope equipped with an argon/krypton laser (laser lines of 488, 568, and 647 nm) using the Leica TCS NT software (v1.6.551). For the three-color presentation of the images, the far red emission of Cy5 or of TOTO-3 was turned into blue.

Results

Isolation and Phenotypic Characterization of Fibroblasts. Fibroblasts were isolated from the skin of naive mice and from the draining (popliteal) lymph nodes of mice that had healed a cutaneous infection with *L. major* (>day 200 after infection). Three skin fibroblast lines (CHF-1, CHF-2, and NOSS-1) and two lymph node reticular fibroblast lines (NOBO-1 and NOBO-3) were established and used for in vitro experiments at passage numbers <25. The cells were determined to be fibroblasts on the following grounds: (a) characteristic morphology, i.e., formation of monolayers consisting of cells that assumed a pavement-like and spindle-shaped appearance when confluent (see Fig. 2); (b) serum-dependent growth (data not shown); (c) absence of markers characteristic for macrophages (F4/80, Mac-1, BM-8, ER-MP-23), dendritic cells (NLDC-145, MHC class II), granulocytes (GR-1), T cells (Thy 1.2), B cells (B220), or endothelial cells (MECA-32) (data not shown); (d) production of a variety of known matrix proteins (fibronectin, laminin-1, fibulin-2, perlecan, and collagen VI) during in vitro culture for 1–6 d (Fig. 1, A and B, and data not shown); (e) positive staining with the rat mAb ER–TR7 (Fig. 1 C, and data not shown), which detects an intracellular component of mouse reticular fibroblasts but also reacts with an extracellular connective tissue product that is different from the matrix proteins laminin, fibronectin, types I–V collagen, heparan sulfate proteoglycan, entactin, and nidogen (33). RPMs or PEMs, in contrast, were negative for perlecan, fibulin-2, collagen VI, and ER-TR7 throughout a culture period of 6 d (Fig. 1, E–M) and showed only a very weak and transient staining with antilaminin or antifibronectin antiserum (not shown). Thus, ER-TR7, perlecan, and fibulin-2 (and in some experiments also laminin, fibronectin, and collagen VI) were used as primary markers for the detection of fibroblasts in lymph nodes.

Reticular Fibroblasts of Lymph Nodes or Skin Fibroblasts Phagocytose L. major Parasites. When skin fibroblast monolayers were continuously incubated with *L. major* promastigotes at a parasite/cell ratio of 3–5:1, the average infection rate at 48 h was 17.8 (± 9) and 24.7 (± 12.4)% in unstimulated or cytokine-stimulated cells, respectively (mean ± SD of three experiments; Fig. 1, A and B). Comparable results were obtained with lymph node fibroblasts and when *L. major* amastigotes were used for infection (Fig. 1, and data not shown). Most of the infected cells contained one or two parasites; rarely, three parasites were found within one cell. Phagocytosis and intracellular localization of pro- or amastigote *L. major* was confirmed by transmission electron microscopy (Fig. 2, D and E). These results demonstrate that both dermal and reticular fibroblasts are capable of taking up *L. major* pro- and amastigotes.
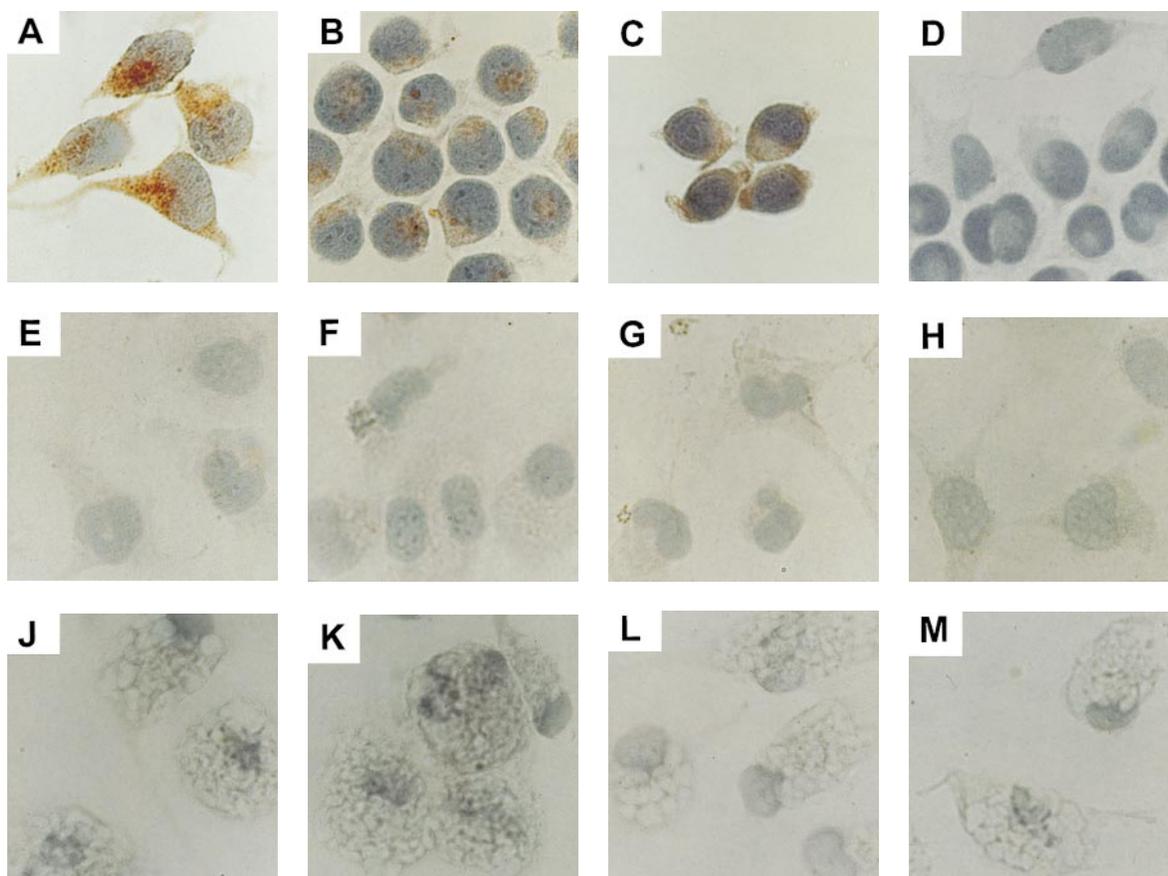
Figure 1. Expression of fibroblast markers by NOBO-1 cells (reticular fibroblasts isolated from the lymph node of a C57BL/6 mouse at day 239 of infection with *L. major*; A–D), RPMs (E–H), or thioglycollate-elicited macrophages (J–M). The cells were kept in cultures for 4–6 d and then analyzed for the expression of fibulin-2 (A, E, and J), perlecan (B, F, and K), or ER-TR7 (C, G, and L) by immunoperoxidase staining and hematoxylin counterstaining. Results similar to those shown for NOBO-1 cells were also obtained with primary skin fibroblasts of C57BL/6 mice (not shown). For control purposes, the primary antibodies were omitted and the cells were incubated with the biotinylated secondary reagent alone followed by streptavidin-peroxidase (D, H, and M). Original magnifications: ×1,000.
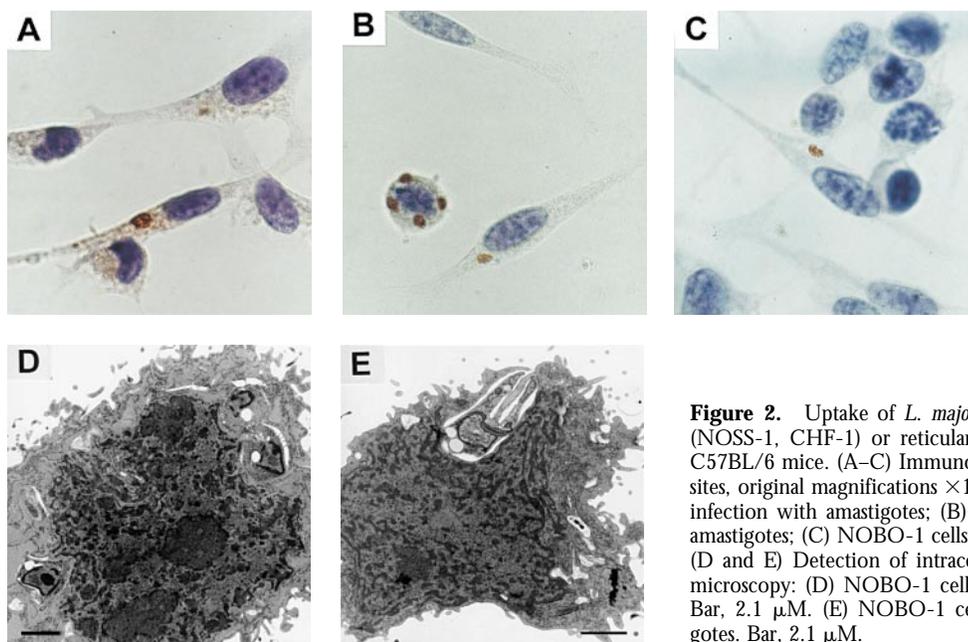

Figure 2. Uptake of *L. major* pro- or amastigotes by skin fibroblasts (NOSS-1, CHF-1) or reticular lymph node fibroblasts (NOBO-1) of C57BL/6 mice. (A–C) Immunoperoxidase staining of intracellular parasites, original magnifications ×1,000: (A) NOSS-1 fibroblasts, 24 h after infection with amastigotes; (B) CHF-1 cells, 48 h after infection with amastigotes; (C) NOBO-1 cells, 24 h after infection with promastigotes. (D and E) Detection of intracellular *L. major* by transmission electron microscopy: (D) NOBO-1 cells, 20 h after infection with amastigotes. Bar, 2.1 μM. (E) NOBO-1 cells, 24 h after infection with promastigotes. Bar, 2.1 μM.

Fibroblasts Have a Reduced Capacity to Produce NO and to Kill Leishmania Parasites. As NOS2-derived NO has been shown to be indispensable for the control of *Leishmania* in phagocytes and in mice (8, 9, 34, 35), we analyzed the production of NO by fibroblasts in response to cytokines or microbial products. IFN-γ alone or IFN-γ plus TNF were sufficient to activate resting or inflammatory macrophages for high production of NO. In contrast, skin or lymph node fibroblasts required a microbial costimulus such as LPS (Fig. 3 A) or infection with *L. major* pro- or amastigotes (Fig. 3 B; see also Fig. 3 D). However, whereas >90% of infected macrophages stimulated with IFN-γ/LPS were positive for NOS2 protein by immunofluorescence analysis, the expression of NOS2 in *L. major*–infected and IFN-γ/LPS–stimulated CHF-1 or NOBO-1 fibroblasts was restricted to 33 (± 13) or 55 (± 14)% of the cells, respectively (mean ± SD of five culture wells from two experiments). Furthermore, in the same experiments, only 34 (± 14) or 62 (± 9)% of the intracellular parasites were localized in NOS2-positive fibroblasts.

In accordance with previous reports (22, 34), stimulation of amastigote-infected macrophages with IFN-γ or IFN-γ/TNF for 48–72 h caused a reduction of the total number of intracellular parasites by 20- to several hundredfold. In contrast, the average reduction of intracellular amastigotes was 1.2 (± 0.2) or 4.0 (± 1.8)-fold in reticular fibroblasts (NOBO-1) and 2.6 (± 1.1) or 5.65 (± 2.2)-fold in dermal fibroblasts (CHF-1) after stimulation with IFN-γ or IFN-γ/TNF, respectively (mean ± SEM of three to seven experiments; Fig. 3 C). However, coculture of infected fibroblasts with uninfected macrophages that were separated by a membrane significantly increased the killing of the intracellular amastigotes compared with cultures of fibroblasts alone (Fig. 3 D).

Reticular Fibroblasts as Host Cells for Persisting Leishmania In Vivo. Next, we addressed the question whether fibroblasts represent (part of) the hitherto undefined NOS2-negative host cell population that harbors 60–70% of all parasites persisting in the lymph nodes of clinically cured mice (17). Initial experiments using immunoperoxidase/al-
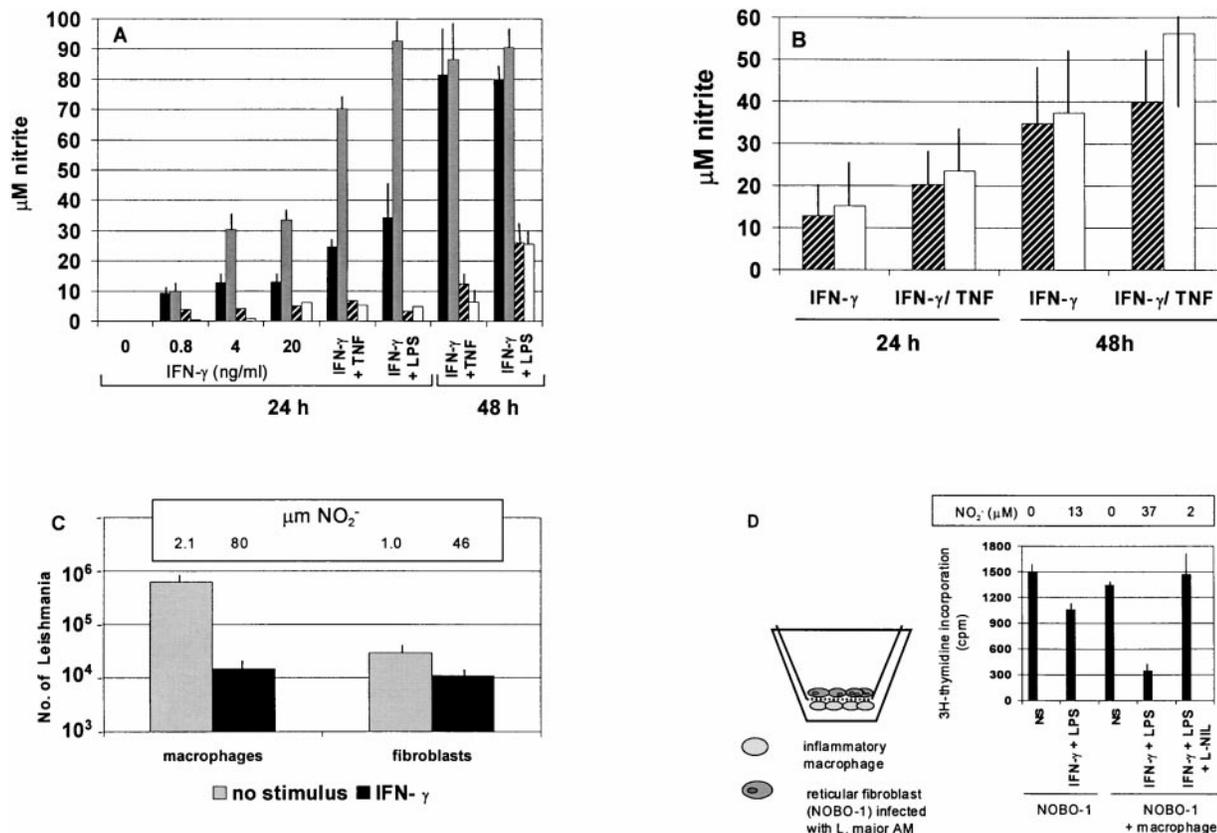

Figure 3. NO production and killing of intracellular *L. major* by RPMs, PEMs, skin fibroblasts (CHF-1), and reticular lymph node fibroblasts (NOBO-1). (A) Nitrite accumulation at 24 or 48 h of stimulation with IFN-γ, IFN-γ (20 ng/ml) plus TNF (10 ng/ml), or IFN-γ (20 ng/ml) plus LPS (20 ng/ml) by equal numbers of uninfected macrophages (RPMs, black bars; PEMs, gray bars) or fibroblasts (CHF-1, hatched bars; NOBO-1, white bars) (mean ± SEM of three to six experiments). (B) Nitrite accumulation in CHF-1 (hatched bars) and NOBO-1 (white bars) fibroblast cultures infected with *L. major* parasites and stimulated with IFN-γ or IFN-γ plus TNF (20 ng/ml each) (mean ± SD of 7–16 experiments). (C) Killing of intracellular *Leishmania* by PEMs and CHF-1 infected with a fourfold excess of amastigotes (6-h pulse infection) and cultured with or without IFN-γ (20 ng/ml) for 54 h. The total numbers of cell-associated parasites were determined by limiting dilution analysis (one of four experiments). (D) Killing of *L. major* amastigotes by NOBO-1 fibroblasts cultured in the absence or presence of PEMs and stimulated with IFN-γ/LPS (20 ng/ml each) with or without L-N^6-iminoethyl-lysine (L-NIL; 1 mM) for 72 h as determined by [3H]thymidine incorporation into *Leishmania* recovered from host cells after SDS lysis (one of two experiments).

2125   Bogdan et al.

kaline phosphatase double labeling revealed parasites closely associated with the reticular fibroblast marker ER-TR7 (Fig. 4 A) or various matrix proteins (not shown). Subsequently, >200 sections of 5 lymph nodes derived from 5 independent time course experiments (day 145–530 after infection) were analyzed by confocal laser microscopy. In a first series, 332 *L. major* amastigotes were analyzed for their colocalization with NOS2 and extracellular matrix proteins (fibulin-2, perlecan, laminin, or collagen VI). 256 (77%) of all parasites were NOS2-negative, confirming our previous findings (17). 102 of the NOS2-negative *Leishmania* (i.e., 30% of the total parasites) were tightly embedded in or closely surrounded by matrix. Only 8% of the total parasites colocalized with matrix and NOS2. Similar results were obtained in a second series of sections, in which 824 *L. major* amastigotes were assessed for their association with the ER-TR7 marker, NOS2, and host cell nuclei. 549 (66%) of all parasites were found within NOS2-negative areas, of which 358 (i.e., 43% of all parasites and 65% of the NOS2-negative parasites) colocalized with ER-TR7 (Fig. 4 C). Only 20% of all parasites colocalized with ER-TR7 and NOS2 (Fig. 4 D). At least 50% of the ER-TR7–positive amastigotes were located in the close vicinity of host cell nuclei, which further demonstrates that fibroblasts harbor *Leishmania* in vivo (Fig. 4 B). By similar triple immunofluorescence labeling, we found parasites not only within macrophages (Fig. 4 E) and dendritic cells (not shown), but also in areas devoid of host cell nuclei and fibroblasts (Fig. 4 F). Thus, fibroblasts are host cells for persisting *L. major* along with other cell types, but *Leishmania* can also be detected in necrotic areas of the chronically infected lymph nodes.
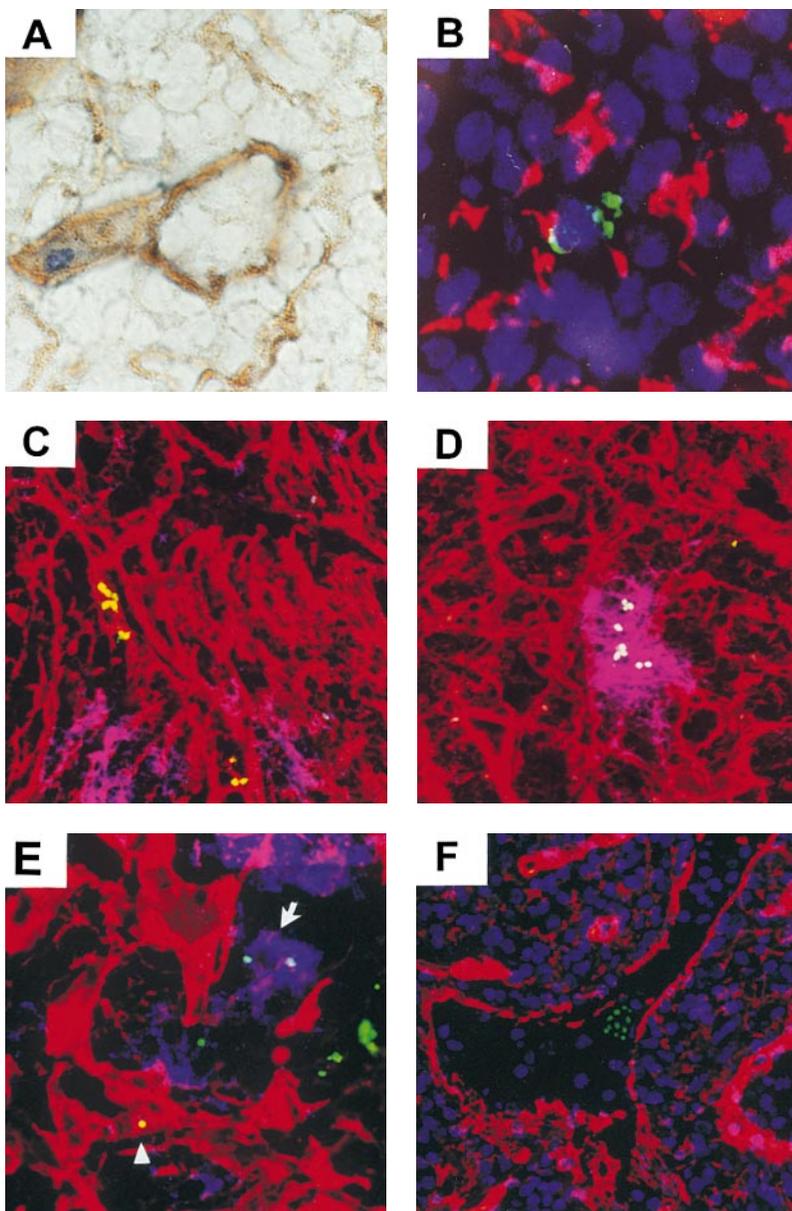

Figure 4. Association of *L. major* parasites with fibroblasts in popliteal lymph nodes of C57BL/6 mice during the chronic (latent) phase of infection (A, day 206; B–D and F, day 530; E, day 452). (A) *L. major* amastigote (alkaline phosphate staining, blue) tightly associated with the ER-TR7 fibroblast marker (immunoperoxidase staining, brown; original magnification: ×1,000). (B–F) Triple immunofluorescence overlay images obtained by confocal laser microscopy (acquired with a 40× objective lens, zoom factor 1.0–1.74). (B) Three *L. major* parasites (DTAF, green) in a ER-TR7–positive cell (nucleus, blue [TOTO-3]; ER-TR7 marker, red [LRSC]). The left of these *Leishmania* directly colocalizes with the ER-TR7 marker. A fourth parasite on the right is presumably located in a neighboring cell. (C) Seven to eight *L. major* parasites (DTAF, green; arrow) that colocalize with ER-TR7 (LRSC, red) and therefore appear yellow, but are clearly outside an NOS2-positive area on the bottom of the image that is stained with Cy5 (blue) and appears purple due to colocalization with ER-TR7. (D) Nine *L. major* parasites (DTAF, green) colocalizing with ER-TR7 (LRSC, red) and NOS-2 (Cy5, blue), therefore appearing white. (E) *L. major* parasites (DTAF, green) colocalizing with a Mac-1–positive cell (Cy5, blue, arrow; two intracellular turquoise parasites) or with matrix (laminin/perlecan-III, LRSC, red; one yellow parasite [arrowhead]). (F) Cluster of 10 *L. major* parasites (DTAF, green) located in a (presumably) necrotic area of the lymph node largely devoid of nuclei (TOTO-3, blue) and fibroblasts (ER-TR7, LRSC, red).

Discussion

Fibroblasts as Host Cells for Leishmania. Although macrophages are considered to be the most important host cell for *Leishmania* parasites, several other cell types have been shown to endocytose *Leishmania* in vitro or in vivo. These include neutrophils (36–38), eosinophils (39–41), dendritic cells (42, 43), and epithelial cells (44). Throughout the 20th century, a possible infection of fibroblasts or fibroblast-like mesenchymal cells with *Leishmania* species other than *L. major* has been sporadically witnessed by some researchers in animal models or in (experimentally) infected humans (45–49). In vitro, several authors demonstrated the uptake of pro- or amastigotes of *L. donovani, L. mexicana amazonensis,* and an *L. braziliensis*–like species by human skin fibroblasts (18–20). However, none of the above-mentioned studies provided any evidence that the infection of fibroblasts with *Leishmania* might be relevant for the survival of the parasites in vivo; investigated the expression of NOS2 by fibroblasts and analyzed the antileishmanial activity of fibroblasts after cytokine stimulation; or determined the actual frequency of *Leishmania* residing in professional phagocytes versus fibroblasts in vivo. Our present study shows that the majority of the parasites that are found in NOS2-negative areas of latently infected lymph nodes and do not colocalize with known macrophage and dendritic cell markers (∼60–70% of the total parasites [17]) are tightly associated with reticular fibroblasts. Thus, fibroblasts represent a hitherto unrecognized important host cell for *Leishmania* persisting in vivo.

Fibroblasts as Safe Targets for Leishmania. Previous reports on the interaction of unstimulated human skin fibroblasts with *Leishmania* species other than *L. major* provided evidence that the parasites can persist within these cells in vitro for prolonged periods of time (3–7 d), although replication of the parasites did not occur (18–20). Our present analysis shows that cytokine-stimulated fibroblasts have a limited capacity to kill intracellular *L. major* parasites in vitro. This is not due to a principal inability to produce NO. However, adherent fibroblasts stimulated with IFN-γ (with or without TNF-α) released much less NO than macrophage monolayers, and even after stimulation with IFN-γ plus LPS the expression of NOS2 protein was restricted to 30–50% of the fibroblasts (depending on the cell line). Similar observations were previously made with embryonic fibroblasts (50). After infection, production of NO was enhanced but still considerably lower than in macrophages. Furthermore, only 35–60% of the parasites were localized in NOS2-positive fibroblasts. Both factors might support the survival of *L. major* parasites within fibroblasts. Importantly, parasites residing in fibroblasts remained susceptible to the NO produced by neighboring macrophages (Fig. 3 D). Thus, macrophage-derived NO is able to control the *Leishmania* in nearby NOS2-negative fibroblasts, which might help to maintain a stable balance of parasite killing and evasion in the chronically infected lymph nodes.

We considered the possibility that the localization of *Leishmania* in fibroblasts might also protect the parasites against the activity of standard antileishmanial drugs and thereby account for the persistence of *Leishmania* after chemotherapy. When we treated long-term–infected, clinically cured C57BL/6 mice with a combination of pentostam and amphotericin B (Ambisome®), there was a strong (∼900-fold) reduction of the parasite burden. However, we did not observe an increase in the number of parasites associated with fibroblast markers. Thus, to date we do not have evidence for a preferential survival of *L. major* parasites after chemotherapy in fibroblasts.

Extracellular Matrix and Leishmania. Although our confocal laser microscopy analysis strongly suggests that *L. major* parasites are located within fibroblasts in vivo (Fig. 4 B), we cannot exclude that some of the persisting *Leishmania* amastigotes are located extracellularly tightly embedded by matrix (Fig. 4, A and C, and data not shown). In addition, in the chronically infected lymph nodes, *Leishmania* amastigotes were also seen in necrotic areas lacking nucleated cells (Fig. 4 F). In this context, it is important to note that *L. mexicana* amastigotes express surface molecules that were shown to function as ligands for certain (extracellular) proteoglycans (51). Extracellular amastigotes, surrounded by connective matrix, have also been seen in acute leishmanial skin lesions of humans infected with *L. braziliensis guyanensis*, but the finding was not discussed (52). Whether a possible extracellular localization of the parasite in a connective tissue matrix contributes to the control (i.e., killing) of the parasite or to its long-term survival in the host is unknown to date.

In conclusion, we have demonstrated that ingestion of *Leishmania* by fibroblasts is a frequent event during latent disease. Our data suggest that fibroblasts might form a less hostile environment for *L. major* than macrophages and thereby allow for the persistence of the parasites. Parasite survival and replication, however, are subject to control by neighboring macrophages that are effective against *Leishmania* residing in fibroblasts and thereby help to maintain a stable host–parasite relationship. Whether such a mechanism also operates in latently infected humans remains to be established.

References

1. Murray, P.J. 1999. Defining the requirements for immunological control of mycobacterial infections. *Trends Microbiol.* 7:366–372.
2. Bogdan, C., and M. Röllinghoff. 1999. How do protozoan

parasites survive inside macrophages? *Parasitol. Today.* 15:22–28.

3. Titus, R.G., C.M. Theodos, A. Shankar, and L.R. Hall. 1993. Interactions between *Leishmania major* and macrophages. *In* Macrophage–Pathogen Interactions. T. Zwilling and T. Eisenstein, editors. Marcel Dekker, Inc., New York. 437–459.

4. Pearson, R.D., and A. de Queiroz Sousa. 1996. Clinical spectrum of leishmaniasis. *Clin. Infect. Dis.* 22:1–13.

5. Reiner, S.L., and R.M. Locksley. 1995. The regulation of immunity to *Leishmania major. Annu. Rev. Immunol.* 13:151–177.

6. Solbach, W., and T. Laskay. 2000. The host response to *Leishmania* infection. *Adv. Immunol.* 74:275–317.

7. MacMicking, J., Q.-w. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15:323–350.

8. Diefenbach, A., H. Schindler, N. Donhauser, E. Lorenz, T. Laskay, J. MacMicking, M. Röllinghoff, I. Gresser, and C. Bogdan. 1998. Type 1 interferon (IFN-α/β) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity.* 8:77–87.

9. Niedbala, W., X.-Q. Wei, D. Piedrafita, D. Xu, and F.Y. Liew. 1999. Effects of nitric oxide on the induction and differentiation of Th1 cells. *Eur. J. Immunol.* 29:2498–2505.

10. Diefenbach, A., H. Schindler, M. Röllinghoff, W. Yokoyama, and C. Bogdan. 1999. Requirement for type 2 NO-synthase for IL-12 responsiveness in innate immunity. *Science.* 284:951–955.

11. Aebischer, T. 1994. Recurrent cutaneous leishmaniasis: a role for persistent parasites. *Parasitol. Today.* 10:25–28.

12. Ramirez, J.L., and P. Guevara. 1997. Persistent infection by *Leishmania (Viannia) braziliensis. Mem. Inst. Oswaldo Cruz.* 92:333–338.

13. Schubach, A., F. Haddad, M.P.-O. Neto, W. Degrave, C. Pirmez, G. Grimaldi, and O. Fernandes. 1998. Detection of *Leishmania* DNA by polymerase chain reaction in scars of treated human patients. *J. Infect. Dis.* 178:911–914.

14. Ma, D.F.F., A.J. Concannon, and J. Hayes. 1979. Fatal leishmaniasis in renal-transplant patient. *Lancet.* 2:311–312.

15. Badaró, R., E.M. Carvalho, H. Rocha, A.C. Queiroz, and T.C. Jones. 1986. *Leishmania donovani*: an opportunistic microbe associated with progressive disease in three immunocompromised patients. *Lancet.* 1:647–649.

16. Müller, I., J.A. Garcia-Sanz, R. Titus, R. Behin, and J. Louis. 1989. Analysis of the cellular parameters of the immune responses contributing to resistance and susceptibility of mice to infection with the intracellular parasite, *Leishmania major. Immunol. Rev.* 112:95–113.

17. Stenger, S., N. Donhauser, H. Thüring, M. Röllinghoff, and C. Bogdan. 1996. Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase. *J. Exp. Med.* 183:1501–1514.

18. Chang, K.-P. 1978. *Leishmania* infection of human skin fibroblasts in vitro: absence of phagolysosomal fusion after induced phagocytosis of promastigotes, and their intracellular transformation. *Am. J. Trop. Med. Hyg.* 27:1084–1096.

19. Dedet, J.P., A. Ryter, E. Vogt, P. Hosli, and L. Pereira da Silva. 1983. Uptake and killing of *Leishmania mexicana amazonensis* amastigotes by human skin fibroblasts. *Ann. Trop. Med. Parasitol.* 77:35–44.

20. Schwartzman, J.D. 1985. The interaction of *Leishmania donovani* promastigotes and human fibroblasts in vitro. *Am. J.*

Trop. Med. Hyg. 34:850–855.

21. Mirkovich, A.M., A. Galelli, A.C. Allison, and F.Z. Modabber. 1986. Increased myelopoiesis during *Leishmania major* infection in mice: generation of "safe targets," a possible way to evade the effector immune mechanism. *Clin. Exp. Immunol.* 64:1–7.

22. Bogdan, C., S. Stenger, M. Röllinghoff, and W. Solbach. 1991. Cytokine interactions in experimental cutaneous leishmaniasis. Interleukin 4 synergizes with interferon-γ to activate murine macrophages for killing of *Leishmania major* amastigotes. *Eur. J. Immunol.* 21:327–333.

23. Stenger, S., H. Thüring, M. Röllinghoff, and C. Bogdan. 1994. Tissue expression of inducible nitric oxide synthase is closely associated with resistance to *Leishmania major. J. Exp. Med.* 180:783–793.

24. Werner-Felmayer, G., E.R. Werner, D. Fuchs, A. Hausen, G. Reibnegger, and H. Wachter. 1990. Tetrahydrobiopterin-dependent formation of nitrite and nitrate in murine fibroblasts. *J. Exp. Med.* 172:1599–1607.

25. Lima, H.C., J.A. Bleyenberg, and R.G. Titus. 1997. A simple method for quantifying *Leishmania* in tissues of infected animals. *Parasitol. Today.* 13:80–82.

26. Ito, S., and M.J. Karnovsky. 1968. Formaldehyde-glutaraldehyde fixatives containing trinitro compounds. *J. Cell Biol.* 39:168a–169a.

27. Glauert, A.M. 1975. Fixation, dehydration, and embedding of biological specimens. *In* Practical Methods in Electron Microscopy. Vol. 3, Part I. A.M. Glauert, editor. North Holland Publishing Co., Amsterdam. 133–162.

28. Timpl, R., H. Rohde, P. Gehron-Robey, S.I. Rennard, J.M. Foidart, and G.R. Martin. 1979. Laminin—a glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933–9937.

29. Specks, I., U. Mayer, R. Nischt, T. Spissinger, K. Mann, R. Timpl, J. Engel, and M.-L. Chu. 1992. Structure of recombinant N-terminal globule of type VI collagen α3 chain and its binding to heparin and hyaluronan. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4281–4290.

30. Pan, T.-C., T. Sasaki, R.-Z. Zhang, R. Fässler, R. Timpl, and M.-L. Chu. 1993. Structure and expression of fibulin-2, a novel extracellular matrix protein with multiple EGF-like repeats and consensus motifs for calcium-binding. *J. Cell Biol.* 123:1269–1277.

31. Schulze, B., K. Mann, R. Battistutta, H. Wiedemann, and R. Timpl. 1995. Structural properties of recombinant domain III-3 of perlecan containing a globular domain inserted into an epidermal-growth-factor-like motif. *Eur. J. Biochem.* 231:551–556.

32. Battacharya, A., M.E. Dorf, and T.A. Springer. 1981. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol.* 127:2488–2495.

33. van Vliet, E., M. Melis, J.M. Foidart, and W. van Ewijk. 1986. Reticular fibroblasts in peripheral lymphoid organs identified by a monoclonal antibody. *J. Histochem. Cytochem.* 34:883–890.

34. Green, S.J., R.M. Crawford, J.T. Hockmeyer, M.S. Meltzer, and C.N. Nacy. 1990. *Leishmania major* amastigotes initiate the l-arginine-dependent killing mechanism in IFN-γ stimulated macrophages by induction of tumor necrosis factor-α. *J. Immunol.* 145:4290–4297.

35. Murray, H.W., and C.F. Nathan. 1999. Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania*

donovani. J. Exp. Med. 189:741–746.

36. Chang, K.P. 1981. Leishmanicidal mechanisms of human polymorphonuclear phagocytes. *Exp. Parasitol.* 55:377–385.

37. Pearson, R.D., and R.T. Steigbigel. 1981. Phagocytosis and killing of the protozoan *Leishmania donovani* by human polymorphonuclear leukocytes. *J. Immunol.* 127:1438–1443.

38. Pearson, R.D., J.A. Sullivan, D. Roberts, R. Romito, and G.L. Mandell. 1983. Interaction of *Leishmania donovani* promastigotes with human phagocytes. *Infect. Immun.* 40:411–416.

39. Grimaldi, G., Jr., M.J. Soares, and P.L. Moriearty. 1984. Tissue eosinophilia and *Leishmania mexicana mexicana* eosinophil interactions in murine cutaneous leishmaniosis. *Parasite Immunol.* 6:735–739.

40. Pearson, R.D., I.L. Uydess, S.W. Chapman, and R.T. Steigbigel. 1987. Interaction of human eosinophils with *Leishmania donovani. Ann. Trop. Med. Parasitol.* 81:735–739.

41. Oliveira, S.H.P., S.G. Fonseca, P.R.T. Romao, F. Figueiredo, S.H. Ferreira, and F.Q. Cunha. 1998. Microbicidal activity of eosinophils is associated with activation of the arginine-NO pathway. *Parasite Immunol.* 20:405–412.

42. Williams, R.O. 1988. Invasion of murine dendritic cells by *Leishmania major* and *L. mexicana mexicana. J. Parasitol.* 74:186–187.

43. Moll, H., S. Flohé, and M. Röllinghoff. 1995. Dendritic cells in *Leishmania major*-immune mice harbor persistent parasites and mediate an antigen-specific T cell immune response. *Eur. J. Immunol.* 25:693–699.

44. Belle, E.A. 1958. Cultivation of *Leishmania donovani* in human amnion epithelial cell tissue cultures: a preliminary report. *Can. Med. Assoc. J.* 79:726–728.

45. Adler, S. 1940. Attempts to transmit visceral leishmaniasis to man. Remarks on the histopathology of leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg.* 33:419–437.

46. Pai, H.C., and C.H. Hu. 1941. Attempts to grow *Leishmania donovani* in tissue cultures. *Proc. Soc. Exp. Biol. Med.* 46:606–608.

47. Hawking, F. 1948. Growth of protozoa in tissue culture. V. *Leishmania donovani. Trans. R. Soc. Trop. Med. Hyg.* 41:545–554.

48. Zuckerman, A. 1953. Initial reaction to the subcutaneous inoculation of cultures of *Leishmania tropica* in the hamster. *Acta Medica Orientalia.* 12:238–240.

49. El-Shoura, S.M., A.K. Sheikha, K.A. Bahamdan, T.M. Tallab, and O.A. Hassounah. 1995. Visceral and cutaneous leishmaniasis: comparative ultrastructure of host-parasite interactions. *J. Egypt. Soc. Parasitol.* 25:861–876.

50. Lavnikova, N., and D.L. Laskin. 1995. Unique patterns of regulation of nitric oxide production in fibroblasts. *J. Leukoc. Biol.* 58:451–458.

51. Love, D.C., J.D. Esko, and D.M. Mosser. 1993. A heparin-binding activity on *Leishmania* amastigotes which mediates adhesion to cellular amastigotes. *J. Cell Biol.* 123:759–766.

52. Esterre, P., J.P. Dedet, S. Guerret, M. Chevallier, C. Frenay, and J.A. Grimaud. 1991. Matrix remodelling and fibroblast phenotype in early lesions of human cutaneous leishmaniasis. *Pathol. Res. Pract.* 187:924–930.