Macrophage Microbicidal Mechanisms In Vivo: Reactive Nitrogen versus Oxygen Intermediates in the Killing of Intracellular Visceral *Leishmania donovani*

By Henry W. Murray*and Carl F. Nathan*‡

*From the *Department of Medicine and the ‡Beatrice and Samuel Seaver Laboratory, Division of Hematology-Oncology, Cornell University Medical College, New York 10021*

Summary

To determine the relative contributions of respiratory burst–derived reactive oxygen intermediates (ROI) versus reactive nitrogen intermediates (RNI) to macrophage-mediated intracellular host defense, mice genetically deficient in these mechanisms were challenged with *Leishmania donovani*, a protozoan that selectively parasitizes visceral tissue macrophages. During the early stage of liver infection at wk 2, both respiratory burst–deficient gp91$^{phox-/-}$ (X-linked chronic granulomatous disease [X-CGD]) mice and inducible nitric oxide synthase (iNOS) knockout (KO) mice displayed comparably increased susceptibility. Thereafter, infection was unrestrained in mice lacking iNOS but was fully controlled in X-CGD mice. Mononuclear cell influx into infected liver foci in X-CGD and iNOS KO mice was also overtly impaired at wk 2. However, granuloma assembly in parasitized tissue eventually developed in both hosts but with divergent effects: mature granulomas were functionally active (leishmanicidal) in X-CGD mice but inert in iNOS-deficient animals. These results suggest that (a) ROI and RNI probably act together in the early stage of intracellular infection to regulate both tissue recruitment of mononuclear inflammatory cells and the initial extent of microbial replication, (b) RNI alone are necessary and sufficient for eventual control of visceral infection, and (c) although mature granulomas have traditionally been associated with control of such infections, these structures fail to limit intracellular parasite replication in the absence of iNOS.

Key words: visceral leishmaniasis • protozoan • parasite • granuloma • macrophage

*L*eishmania donovani, a protozoan that causes visceral leishmaniasis, selectively parasitizes and initially replicates within resident macrophages in the liver, spleen and bone marrow. The host capable of controlling this disseminated intracellular infection develops an effective T cell–dependent immune response mediated by proinflammatory, largely Th1 cell–associated cytokines including IL-12 and IFN-γ (1–3; for review see reference 4). The net effect of this complex response, which involves granuloma formation (4, 5), is sufficient activation of both tissue macrophages and influxing blood monocytes to kill most intracellular *L. donovani* and induce quiescence in residual infection (4).

In vitro studies suggest that mononuclear phagocytes possess multiple leishmanicidal mechanisms. Cytokine-activated mouse peritoneal macrophages kill ingested *L. donovani* amastigotes by secreting either reactive oxygen intermediates (ROI; primarily H_2O_2 [6, 7]) or reactive nitrogen intermediates (RNI; derived from nitric oxide [8]). Inhibiting either pathway prevents mouse macrophage killing of *L. donovani* in vitro (6–8); thus, these mechanisms may also

act in concert (9, 10). In cytokine-activated human monocyte-derived macrophages, H_2O_2 also readily induces leishmanicidal effects (11); however, a similar role for RNI has been difficult to detect in cells from normal donors (12). In vitro–activated human macrophages can also limit *L. donovani* replication by degrading extracellular tryptophan (13).

Studies carried out to define the macrophage mechanisms of intracellular *Leishmania* killing in vivo have been more limited. Thorough observations in a separate mouse model of *L. major* cutaneous infection largely (14–17) but not entirely (18, 19) support a critical role for inducible nitric oxide synthase (iNOS)-generated RNI. RNI are not only leishmanicidal (8, 14–17) but also regulate immunologic pathways, including endogenous secretion of IL-12 and IFN-γ (15, 17). In *L. donovani*–infected BALB/c mice, iNOS mRNA is induced in parasitized tissues (2, 20) and presumed inhibition of iNOS by aminoguanidine (AG) treatment impairs host defense (2). However, iNOS mRNA induction (20) and generation of iNOS-derived products in serum and infected organs (21) do not necessarily correlate with control

over *L. donovani* (20, 21). In the one published study of parasitized tissue in human visceral leishmaniasis, splenic mononuclear phagocytes from each of 22 untreated Indian patients showed iNOS immunoreactivity (22) (36 ± 3% of cells were iNOS positive; Murray, H., A. Ding, S. Sundar, and C. Nathan, unpublished data).

To directly compare RNI and ROI as macrophage-derived antimicrobial mediators in vivo, we used *L. donovani* to challenge gene-disrupted iNOS knockout (KO) (23) and respiratory burst–deficient gp91*phox−/−* (X-linked chronic granulomatous disease [X-CGD]) mice (24).

Materials and Methods

Animals. Mice with a targeted disruption of the gp91*phox* subunit of the NADPH-oxidase complex (phox), derived from a C57BL/6 × 129/Sv background and backcrossed six times with C57BL/6 mice (24), were provided as breeders by Dr. M. Dinauer (Indiana University Medical Center, Indianapolis, IN). C57BL/6 mice (Charles Rivers Labs.) were used as controls. iNOS$^{-/-}$ KO mice (C57BL/6 × 129/Sv) were maintained as originally described (23); wild-type$^{+/+}$ littermates served as controls. Mice were 8–15 wk old when infected; both males and females were used randomly except for control C57BL/6 mice (all female).

Visceral Infection and Treatment. Groups of four to five mice were injected via the tail vein with 1.5×10^7 hamster spleen–derived *L. donovani* amastigotes (1 Sudan strain, provided by Dr. D. Sacks, National Institutes of Health, Bethesda, MD) (2, 3). Visceral infection was followed microscopically using Giemsa-stained liver imprints by counting the number of amastigotes per 500 cell nuclei × liver weight (g) (Leishman-Donovan units, or LDU) (2, 3). Granuloma formation was scored using formalin-fixed tissue sections stained with hematoxylin and eosin (1–3). In some experiments, starting 1 d after infection AG (Sigma Chemical Co.) was added at 1% (wt/vol) to acidified drinking water changed twice weekly; controls received acidified water alone (2). Differences between mean values were analyzed by a two-tailed Student's *t* test.
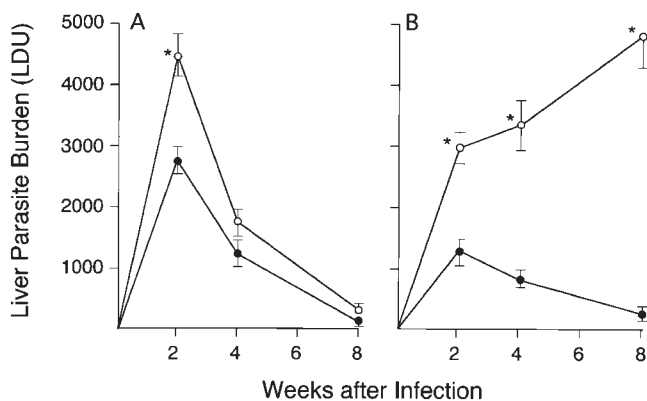
Table I. *Effect of Aminoguanidine Treatment in X-CGD Mice*

Wk after infection	AG	Parasite burden	Percentage of infected foci showing granuloma formation
		LDU	
2	−	3,065 ± 173	37 ± 5
	+	5,214 ± 251*	9 ± 2*
4	−	810 ± 146	88 ± 10
	+	2,550 ± 128*	93 ± 8

Starting 1 d after *L. donovani* infection, X-CGD mice received acidified drinking water alone or the same water containing AG at 1% (wt/vol) for 4 wk. Results indicate mean ± SEM values from two experiments, and for liver parasite burdens represent eight mice per group and for granuloma formation (percentage of liver-infected foci showing developing or mature granulomas; see Table II) represent six mice per group. *Significantly different ($P < 0.05$) from control mice.

Results and Discussion

Initial Kinetics of Parasite Replication. *L. donovani* replicated in the livers of both strains of control mice during the first 2 wk after challenge (Fig. 1); thereafter, parasite burdens declined consistent with killing and a self-healing phenotype (1). At wk 2, liver burdens in both X-CGD and iNOS KO mice were significantly higher (by 1.6- and 2.3-fold, respectively) than in control animals. Since macrophages from X-CGD mice produce RNI normally (24) and macrophages from iNOS KO mice show intact respiratory burst activity (23), the results at wk 2 suggested that (a) neither mechanism by itself was sufficient to help limit

Table II. *Liver Histologic (Granulomatous) Response to L. donovani Infection in X-CGD and iNOS KO Mice*

	Weeks after infection											
	X-CGD			C57BL/6			iNOS KO			WT Controls		
Tissue reaction	2	4	8	2	4	8	2	4	8	2	4	8
None (%)	70	10	0	19	0	0	81	32	1	22	0	0
Granuloma												
Developing (%)	29	81	39	78	63	2	19	65	38	57	69	6
Mature (%)	1	9	33	3	37	32	0	3	61	21	31	43
Empty (%)	0	0	28	0	0	66	0	0	0	0	0	51

All infected foci in 50 consecutive ×63 microscopic fields were scored as follows for granuloma formation: none, single or fused parasitized Kupffer cells with no mononuclear cell infiltrate; developing, core of fused infected Kupffer cells with some cell infiltrate; mature, fused Kupffer cell core surrounded by a well-developed mononuclear cell mantle; and empty, granuloma devoid of amastigotes (1–4). See Figs. 2 and 3. Results are from two experiments, and indicate mean values for four to five mice at each time point. SEM, omitted for clarity, were all <12%.

Figure 1. Course of *L. donovani* liver infection in (A) X-CGD (○) and control C57BL/6 mice (●), and in (B) iNOS KO mice (○) and wild-type controls (●). Results are mean ± SEM values for 8–15 mice at each time point from two to four experiments. Asterisk, significantly higher ($P < 0.05$) than value in control mice.

early *L. donovani* replication and (b) phox and iNOS may act in concert to achieve optimal initial activity.

Acquired Resistance and Resolution of Infection. After wk 2, C57BL/6 and wild-type control mice acquired resistance and showed near-resolution of infection by wk 8 (Fig. 1). Although initially more susceptible, X-CGD mice also controlled infection after wk 2 (Fig. 1 A), indicating that the phox-derived ROI mechanism was dispensable. In contrast, liver infection was unrestrained in iNOS KO mice and increased to high levels by wk 8 (Fig. 1 B and see Fig. 3). Thus, although an intact respiratory burst was neither required (Fig. 1 A) nor sufficient (Fig. 1 B) for acquired resistance and near-resolution of visceral infection, these responses were iNOS dependent.

Antileishmanial Effect of iNOS in the Absence of Phox. To determine if the capacity of X-CGD mice to control *L. donovani* infection involved iNOS-derived products, AG was used as an iNOS inhibitor (2, 25). Continuous AG treatment enhanced initial susceptibility of X-CGD mice at wk 2 (Table I), pointing to an active iNOS-dependent mechanism. At wk 4, liver parasite burdens in AG-treated

X-CGD mice remained elevated; nevertheless, visceral infection began to come under control (Table I). Although this latter observation raised the possibility of an antimicrobial pathway independent of both phox and iNOS and although such pathways exist (9), we did not verify the degree of iNOS inhibition induced by AG. Since iNOS KO mice failed to control *L. donovani,* incomplete iNOS inhibition may well explain the decrease in parasite load at wk 4 in treated X-CGD animals.

Tissue Granulomatous Response. In the liver, acquired resistance to *L. donovani* is expressed by a granulomatous response that encircles all initially parasitized resident macrophages (Kupffer cells) (1–4). This reaction is underway by wk 2 and fully evident at wk 4 as resistance develops. The surrounding mantle of the mature leishmanicidal granuloma is formed by influxing T cells and blood monocytes (4), a process governed by Th1 cell–derived cytokines (2–4). During resolution of visceral infection (wk 8), the tissue reaction involutes and most cellular foci are devoid of visible amastigotes and can be scored as in Table II as microscopically "empty" (5).
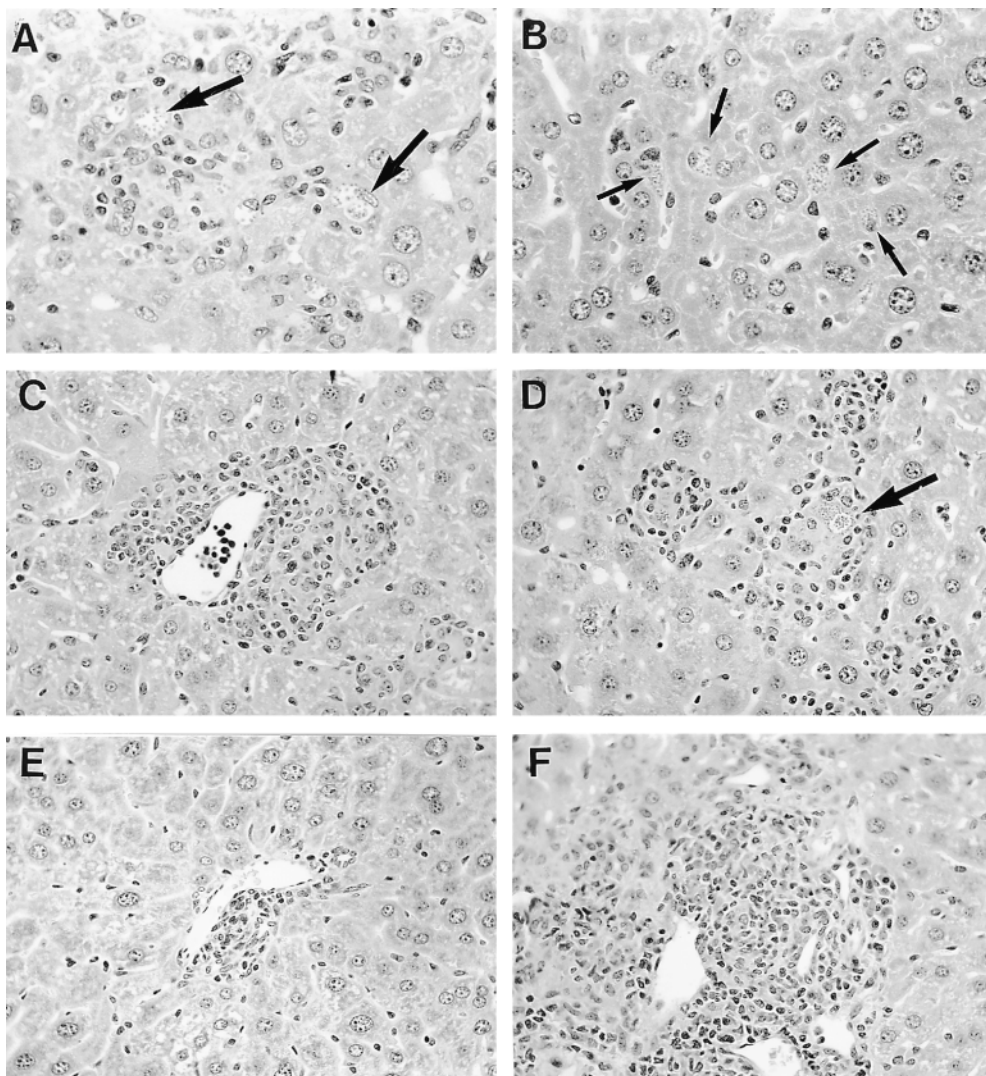

Figure 2. Histologic response to *L. donovani* infection in the liver in X-CGD versus C57BL/6 mice. 2 wk after challenge, granulomas are developing at infected foci (arrows) in C57BL/6 controls (A); in X-CGD mice (B), there is little or no reaction at well-parasitized foci (arrows). At wk 4, granulomas in C57BL/6 mice (C) in this field are mature and largely parasite free; (D) shows developing granulomas in X-CGD mice. At wk 8, (E) shows receding granulomas in C57BL/6 mice, whereas (F) illustrates intense, mature reaction in X-CGD mice. Original magnification: A and B, ×315; C–F, ×200.

The kinetics of the granulomatous response and mature granuloma assembly were similar in control C57BL/6 (Fig. 2, A, C, and E) and wild-type C57BL/6 × 129/Sv (Fig. 3, A, C, and E, G) mice (Table II). In contrast, the tissue reaction in both X-CGD and iNOS KO animals was delayed and incomplete, particularly at the early stages. At wk 2, 70–81% of infected foci in X-CGD and iNOS KO livers had failed to attract any of the early mononuclear cell reac-

tion already evident at 69–81% of parasitized Kupffer cells in control mice (Table II and Figs. 2, A and B, and 3, A and B). Thus, both phox-derived ROI and iNOS-derived RNI, presumably acting together, appear to participate in the early recruitment of T cells and monocytes into infected tissue. This observation further supports the expanding immunoregulatory spectrum of these molecules, particularly NO (10, 26–28), and in iNOS KO mice may also
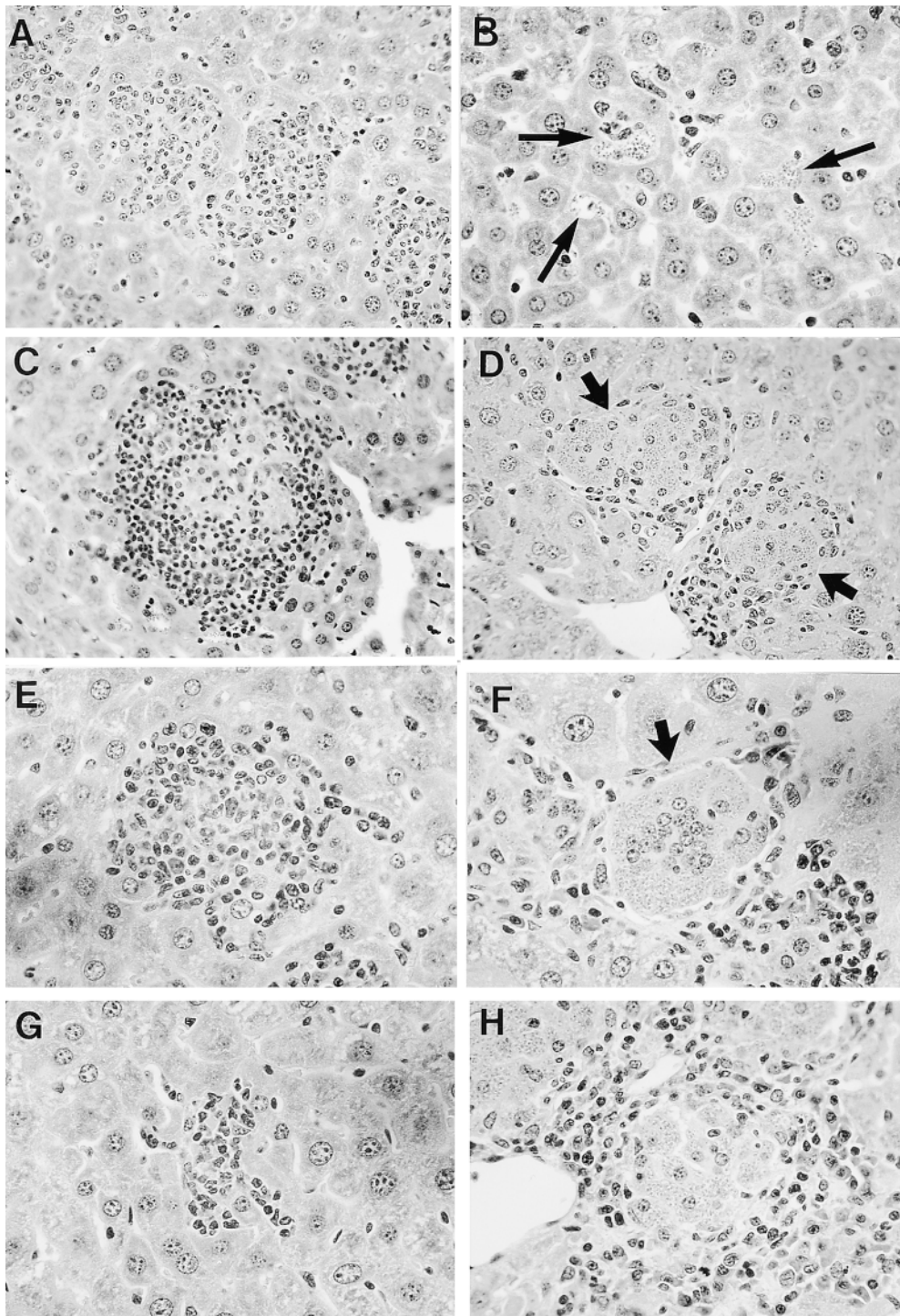

Figure 3. Histologic response in livers of infected iNOS KO mice and wild-type controls. At wk 2, granulomas are widespread and developed in controls (A) but absent at infected foci (arrows) in iNOS KO mice (B). At wk 4, granulomas are well-established or empty in control mice (C and E); in iNOS KO mice (D and F), granulomas are developing but heavily parasitized. At wk 8, inflammatory reaction in controls has involuted (G); in iNOS KO mice (H), granulomas are well-formed (mature) but contain numerous replicating amastigotes (see also Fig. 4). Original magnification: A, C, and D, ×200; B and E–H, ×315.
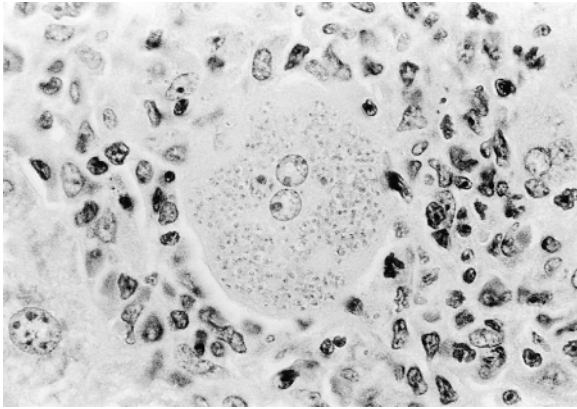
Figure 4. The ineffective granuloma. Photomicrograph shows well-developed tissue structure in 8-wk-infected iNOS KO liver containing a striking load of intracellular amastigotes. Original magnification: ×500.

reflect suppressed induction of IL-12 and IFN-γ (17), key antileishmanial cytokines involved in both mononuclear cell influx and macrophage activation (2–4).

At wk 4, granuloma formation emerged at 90% of infected sites in X-CGD mice (Fig. 2 D) and at 68% of foci in iNOS KO mice (Fig. 3, D and F, and Table II). However, further assembly into mature granulomas (present at 31–37% of foci in control mice; Figs. 2 C and 3, C and E) was retarded and <10% of infected foci in either group of mutant mice could be scored as mature granulomas (Table II). In X-CGD mice, AG treatment inhibited early granuloma assembly (Table I), supporting an active role for iNOS in the initial tissue response. However, granulomas developed by wk 4 despite AG treatment, suggesting a mechanism unrelated to phox or iNOS or suboptimal pharmacologic inhibition of iNOS.

Results at wk 8 indicated that neither phox nor iNOS alone was ultimately required for cell recruitment and assembly, since granulomas developed in both types of deficient mice (Table II). In X-CGD animals, capable of killing *L. donovani*, the tissue reaction was widespread, comprised of both mature granulomas and inflammatory foci devoid of parasites (Fig. 2 F), and as in a prior study (29) was exaggerated at some perivascular areas (data not shown). However, despite the presence of a similarly well-developed inflammatory reaction, mature-appearing granulomas in iNOS-deficient mice contained overwhelming numbers of replicating amastigotes defining a novel structure, the "ineffective granuloma" (Figs. 3 H and 4).

Together, these results demonstrate clear-cut, probable interdigitating roles for both the respiratory burst and endogenous iNOS–derived RNI as macrophage antimicrobial mechanisms in the initial host defense response to *L. donovani*. However, the activity of the respiratory burst in inflammatory cell recruitment and in limiting intracellular replication is early and transient, since granulomas formed and infection resolved in X-CGD mice. Similar findings of enhanced susceptibility followed by control in X-CGD mice have been reported in a short-term model of *Listeria monocytogenes* infection (30).

It is possible that mechanisms related to neither phox nor iNOS, such as other sources of ROI and RNI, also emerge to complete the induction of the tissue cellular immune response to *L. donovani* and contribute additional antileishmanial effects. However, our results clearly illustrate an obligatory role for iNOS in intracellular killing in vivo and resolution of visceral infection. The presence in iNOS-deficient livers of numerous granulomas, well-established but heavily-laden with intracellular parasites (Fig. 4), graphically illustrates the tissue consequences of the absence of this specific macrophage antimicrobial mechanism.

Address correspondence to Henry W. Murray, Cornell University Medical College, 1300 York Ave., Box 130, New York, NY 10021. Phone: 212-746-6330; Fax: 212-746-6332; E-mail: hwmurray@mail.med.cornell.edu

References

1. Tumang, M., C. Keogh, L.L. Moldawer, R.F. Teitelbaum, J. Hariprashad, and H.W. Murray. 1994. The role and effect of tumor necrosis factor–alpha in experimental visceral leishmaniasis. *J. Immunol.* 153:768–775.

2. Taylor, A., and H.W. Murray. 1997. Intracellular antimicrobial activity in the absence of interferon-γ: effect of interleukin 12 in experimental visceral leishmaniasis in interferon-γ

gene-disrupted mice. *J. Exp. Med.* 185:1231–1239.

3. Murray, H.W. 1997. Endogenous interleukin 12 regulates acquired resistance in experimental visceral leishmaniasis. *J. Infect. Dis.* 175:1477–1479.

4. Murray, H.W. 1999. Granulomatous inflammation: host antimicrobial defense in the tissues in visceral leishmaniasis. *In* Inflammation: Basic Principles and Clinical Correlates. J.I.

Gallin, R. Snyderman, D.T. Fearon, B.F. Haynes, and C.F. Nathan, editors. Lippincott-Raven, Philadelphia. In press.

5. Wilson, M.E., M. Sandor, A.M. Blum, B. Younbag, A. Metwali, D. Elliot, R.G. Lynch, and J.V. Weinstock. 1996. Local suppression of IFN-γ in hepatic granulomas correlates with tissue-specific replication of *Leishmania chagasi*. *J. Immunol.* 156:2231–2239.

6. Murray, H.W. 1982. Cell-mediated immune response in experimental visceral leishmaniasis. II. Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. *J. Immunol.* 129:351–357.

7. Murray, H.W. 1990. Effect of continuous administration of interferon-gamma in experimental visceral leishmaniasis. *J. Infect. Dis.* 161:992–994.

8. Roach, T.I., A.F. Kiderlen, and J.M. Blackwell. 1991. Role of inorganic nitrogen oxides and tumor necrosis factor-alpha in killing *Leishmania donovani* amastigotes in gamma-interferon lipopolysaccharide-activated macrophages from *Lsh^s* and *Lsh^r* congenic mouse strains. *Infect. Immun.* 59:3935–3944.

9. Shiloh, M.U., J.D. MacMicking, S. Nicholoson, J.E. Brause, S. Potter, M. Marino, F. Fang, M. Dinauer, and C. Nathan. 1999. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity.* In press.

10. Fang, F.C. 1997. Mechanisms of nitric oxide-related antimicrobial activity. *J. Clin. Invest.* 99:2818–2825.

11. Murray, H.W., and D.M. Cartelli. 1983. Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes: evidence for oxygen-dependent and -independent leishmanicidal activity. *J. Clin. Invest.* 72:32–39.

12. Murray, H.W., and R.F. Teitelbaum. 1992. L-arginine-dependent reactive nitrogen intermediates and the antimicrobial effect of activated human mononuclear phagocytes. *J. Infect. Dis.* 165:513–517.

13. Murray, H.W., A. Szuro-Sudol, D. Wellner, M.J. Oca, A.M. Granger, D.M. Libby, C.D. Rothermel, and B.Y. Rubin. 1989. Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. *Infect. Immun.* 57:845–849.

14. Evans, T.G., L. Thai, D.L. Granger, and J.B. Hibbs. 1993. Effect of in vivo inhibition of nitric oxide production in murine leishmaniasis. *J. Immunol.* 151:907–915.

15. Wei, X.Q., I.G. Charles, A. Smith, J. Ure, G.J. Feng, F.P. Huang, D. Xu, W. Muller, S. Moncada, and F.Y. Liew. 1995. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature.* 375:408–411.

16. Stenger, S., H. Thuring, M. Rollinghoff, 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.

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

18. Evans, T.G., S.S. Reed, and J.B. Hibbs. 1996. Nitric oxide production in murine leishmaniasis: correlation of progressive infection with increasing systemic synthesis of nitric oxide. *Am. J. Trop. Med. Hyg.* 54:486–489.

19. Huang, F.-P., D. Xu, E.-O. Esfandiari, W. Sands, X. Wei, and F.Y. Liew. 1998. Mice defective in Fas are highly susceptible to *Leishmania major* infection despite elevated IL-12 synthesis, strong Th1 responses, and enhanced nitric oxide production. *J. Immunol.* 160:4143–4147.

20. Melby, P.C., Y.-Z. Yang, J. Cheng, and W. Zhao. 1998. Regional differences in the cellular immune response to experimental cutaneous or visceral leishmaniasis with *Leishmania donovani. Infect. Immun.* 66:18–27.

21. Bories, C., E. Scherman, and P.N. Bories. 1997. Serum and tissue nitrate levels in murine visceral leishmaniasis correlate with parasite load but not with host protection. *Trans. R. Soc. Trop. Med. Hyg.* 91:433–436.

22. Nicholson, S., M. da G. Bonecini-Almeida, J.R. Lapa e Silva, C. Nathan, Q.W. Xie, R. Mumford, J.R. Weidner, J. Calaycay, J. Geng, N. Boechat, et al. 1996. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J. Exp. Med.* 183:2293–2302.

23. McMicking, J.D., C. Nathan, G. Hom, N. Chartrain, D.S. Fletcher, M. Trumbauer, K. Stevens, Q. Xie, K. Sokol, N. Hutchinson, et al. 1995. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell.* 81:641–650.

24. Pollock, J.D., P.A. Williams, G. Gifford, L.L. Li, X. Du, J. Fisherman, S.H. Orkin, C.M. Doershuck, and M.C. Dinhauer. 1995. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat. Genet.* 9:202–208.

25. Chan, J., K. Tanaka, D. Carroll, J. Flynn, and B.R. Bloom. 1995. Effect of nitric oxide synthase inhibitors on murine infection with *Mycobacterium tuberculosis. Infect. Immun.* 63:736–740.

26. Nathan, C. 1997. Inducible nitric oxide synthase: what difference does it make? *J. Clin. Invest.* 100:2417–2423.

27. Hogaboam, C.M., S.W. Chensue, M.L. Steinhauser, G.B. Huffnagle, N.W. Lukacs, R.M. Strieter, and S.L. Kunkel. 1997. Alteration of the cytokine phenotype in an experimental lung granuloma model by inhibiting nitric oxide. *J. Immunol.* 159:5585–5593.

28. Bogdan, C. 1998. The multiplex function of nitric oxide in (auto)immunity. *J. Exp. Med.* 187:1361–1365.

29. Morgenstern, D.E., M.A.C. Gifford, L.L. Li, C.M. Doerschuk, and M.C. Dinauer. 1997. Absence of respiratory burst in X-linked chronic granulomatous disease mice leads to abnormalities in both host defense and inflammatory response to *Aspergillus fumigatus. J. Exp. Med.* 185:207–218.

30. Dinauer, M.C., M.B. Deck, and E.R. Unanue. 1997. Mice lacking reduced nicotinamide adenine dinucleotide phosphate oxidase activity show increased susceptibility to early infection with *Listeria monocytogenes. J. Immunol.* 158:5581–5583.