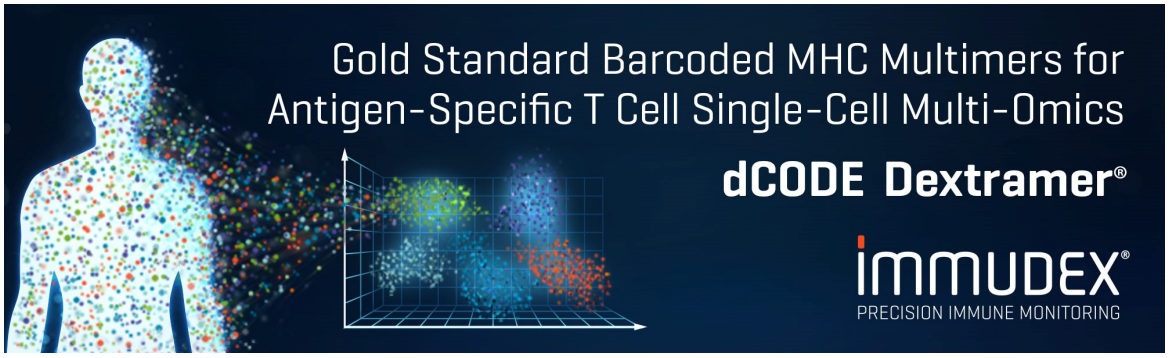

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Cutting Edge: Mice Defective in Fas Are Highly Susceptible to *Leishmania major* Infection Despite Elevated IL-12 Synthesis, Strong Th1 Responses, and Enhanced Nitric Oxide Production[1]

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MRL/MP-*lpr/lpr* (MRL/*lpr*) mice have a single mutation (*lpr*) of the *fas* apoptosis gene. The mutant mice developed significantly smaller lesions than the wild-type mice at the earlier stage of infection with the intracellular protozoan parasite *Leishmania major*. However, while all the wild-type mice achieved complete lesion resolution, the disease in the mutant mice progressed inexorably. The mutant mice had more IL-12 and nitrite/nitrate in the serum than wild-type mice following infection. Lymphoid cells from infected MRL/*lpr* mice produced more IFN-γ but less IL-4 and IL-5 than cells from MRL-+/+ mice. Peritoneal macrophages from the mutant mice also produced more IL-12 and NO after stimulation with LPS. Thus, Fas expression is essential for resistance against leishmaniasis, and Fas-mediated apoptosis may form an integral part of the Th1-mediated microbicidal function. ***The Journal of Immunology*, 1998, 160: 4143–4147.**

MRL/*lpr* mice have a single gene mutation (*lpr*) of the *fas* apoptosis gene on mouse chromosome 19 (1, 2) and background gene from the MRL strain (2, 3). These mice develop a spontaneous autoimmune disease and have been used extensively as a model for clinical systemic lupus erythematosus. The disease is characterized by lymphadenopathy, autoantibody production, and inflammatory manifestations such as nephritis, vasculitis, and arthritis (3, 4). We have previously reported that the mutant mice produced significantly higher levels of IL-12 compared with the wild-type MRL-+/+ mice. This led to an enhanced production of nitric oxide (NO) that mediates, at least in part, the autoimmune diseases (5). Since IL-12 (6, 7) and NO (8) are both essential for resistance against leishmanial infection, we therefore investigated whether the MRL-*lpr* mice are more resistant to *Leishmania major* infection compared with the wild-type mice.

Cutaneous leishmaniasis is the most polarized example of the differential roles of Th1 and Th2 subsets of $CD4^+$ T cells. An impressive range of clinical and experimental evidence supports the host-protective role of Th1 cells that produce IFN-γ, which activates macrophages to produce NO, which kills the intracellular parasite (8–10). IL-12 is the major inducer of Th1 cell differentiation (11). In contrast, Th2 cells driven by IL-4 and also producing IL-4 are disease promoting, via the inhibition of the expression of inducible nitric oxide synthase (iNOS)[5] by IL-4 (12, 13).

We report here that contrary to expectation, MRL/*lpr* are highly susceptible to *L. major* infection. While all the *L. major*-infected wild-type MRL-+/+ mice achieved spontaneous lesion healing, the MRL/*lpr* mice were unable to restrict the disease development. This was so despite the fact that the infected mutant mice produced more IL-12, developed elevated Th1 responses, and synthesized more NO than the wild-type mice. These data therefore demonstrate that Fas expression is required in the resistance against leishmaniasis. This requirement can override the strong presence of IL-12, Th1, and NO activities, suggesting that the Fas-Fas ligand (FasL) pathway is an integral part of the Th1 microbicidal function.

Materials and Methods

Mice

Female MRL/*lpr* and age- and sex-matched control MRL/MP-+/+ (MRL-+/+) mice were obtained from Harlan U.K. (Bicester, U.K.). Some of the mice were bred in the animal facilities, University of Glasgow, from pairs obtained from Harlan U.K. They were housed under virus-free environment. The iNOS-deficient mice were derived as described previously (8). Disruption of the murine iNOS gene was achieved by homologous recombination in 129sv embryonic stem cells. The recombinant allele was passed through the germline following mating of embryonic stem cell chimeras with 129sv (Harlan U.K.). All the mice used were from littermate matings

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[5] Abbreviations used in this paper: iNOS, nitric oxide synthase; FasL, Fas ligand; L-NMMA, L-N^G-monomethylarginine.

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and were kept in a specific-pathogen-free environment. Extensive experiments using a variety of parameters demonstrated phenotypic similarity between the heterozygous and wild-type littermates. Peritoneal cells from the mutant mice did not produce iNOS protein following activation with IFN-γ and LPS in vitro, as judged by Western blot. They produced only background levels of nitrite up to 48 h of culture with IFN-γ and LPS.

Parasite and infection

The *L. major* isolate MRHO/SU/59/P, also known as LV39, was used throughout. The maintenance, cultivation, and isolation of the parasites have been described in detail elsewhere (14). Mice were injected s.c. in the right hind footpad with 1×10^6 stationary phase promastigotes, and the lesion development was measured at regular intervals (14). At the end of the experiments, mice were killed by cervical dislocation, and serum, spleen, and footpads were collected. Parasite loads in the infected footpad and draining lymph nodes were estimated by limiting dilution (15). Soluble antigen was prepared from promastigotes by five cycles of freezing and thawing (2×10^8 organisms/ml in PBS), followed by centrifugation at $8000 \times g$ for 10 min in 4°C. The supernatant was filtered (0.45-μm pore size filter) and stored at −70°C.

Cell culture

Resident peritoneal cells (2×10^5 cell/well) in 200 μl were cultured in 96-well culture plates (Nunc, Roskilde, Denmark) in complete culture medium (RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM 2-mercaptoethanol) at 37°C and 5% CO_2 for up to 6 d. To stimulate for IL-12 and NO production, LPS (100 ng/ml, *Salmonella enteritidis*, Sigma, Poole, U.K.) and IFN-γ (50 U/ml, a kind gift of Dr. G. Adolf, Bender, Vienna, Austria) were added. To stimulate for IFN-γ, IL-4, and IL-5 production, spleen cells (2×10^5 cells/well) were cultured with soluble leishmanial antigen (5×10^6 organism equivalent/ml) or Con A (2.5 μg/ml). Culture supernatants were collected at regular intervals and stored at −70°C. T cell proliferation was determined by [^{3}H]TdR incorporation, and results were expressed as mean ± SD in triplicate cultures.

Cytokine assays

These were conducted by ELISA in 96-well plates (Immulon 4, Dynatech, Billinghurst, U.K.). For IL-12, the capture Abs were C15.1.2 and C15.6 (kind gifts of the Genetic Institute, Boston, MA), and the detection Ab was a rabbit anti-IL-12 Ab (Rab 74.6). For IFN-γ, IL-4, and IL-5, paired mAbs from PharMingen (Cambridge Bioscience, Cambridge, U.K.) were used. Recombinant cytokines (IL-12, Genetic Institute; IFN-γ, Bender; IL-4/IL-5, Genzyme, West Malling, U.K.) were used as standards.

Assays for NO production

Total nitrate and nitrite concentration in serum was determined by the conversion of nitrate into nitrite following deproteination as described previously (16). Total nitrite content was then measured by the Greiss reaction (17), using $NaNO_2$ as standard with detection limit of 1 μM.

Leishmanicidal assay

This was conducted as described previously (18). Briefly, cells (5×10^5/well; 24-well plate) were washed with prewarmed (37°C) DMEM (Life Technologies) before addition of stationary phase promastigotes (1×10^7/ml) at a promastigote-macrophage ratio of 10:1 over an infection period of 18 h. Infection rate was estimated in Lab-Tek (Nunc, Roskilde, Denmark) incubation slides by May-Grünwald-Giemsa staining. Approximately 50% of the macrophages contained at least one parasite. Medium containing nonphagocytosed parasites was gently removed by washing. Cultures were then stimulated with LPS (500 ng/ml) alone or LPS plus L-N^G-monomethylarginine (L-NMMA) (10 mM). After 72 h, cells were washed with prewarmed DMEM and lysed using 0.01% SDS in 100 μl of prewarmed FCS-free DMEM for up to 30 min. This was assisted by pipetting the cells 10 times followed by 3 passages through a 26-gauge needle. Released amastigotes were resuspended in a total of 600 μl/well Schneider's *Drosophila* medium (Life Technologies) containing 30% FCS and cultured for a further 72 h. Aliquots of this culture (150 μl) were then transferred to quadruplicate wells of a 96-well plate for each sample and pulsed with [*methyl*-^{3}H]TdR (1 μCi/well) for a further 18 h. The cultures were then harvested and counted in a beta counter (Betaplate, LKB, Uppsala, Sweden).

Statistical analysis

Statistical significance (p value) was calculated by Student's t test.

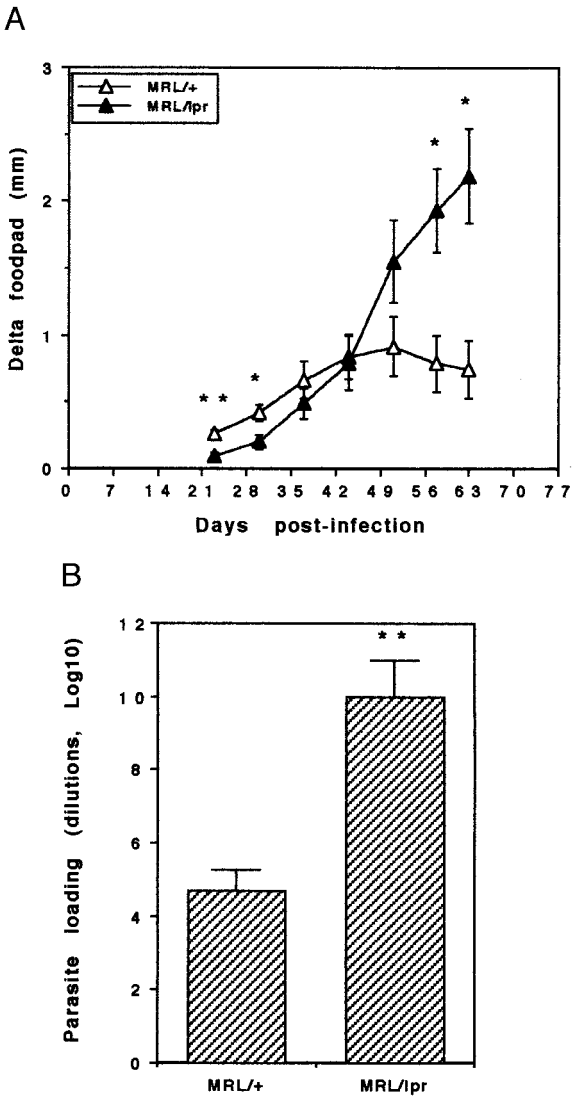

FIGURE 1. Infection of MRL/*lpr* and MRL-+/+ mice with *L. major*. Mice (13 wk old) were infected in the right hind footpad with 1×10^6 promastigotes, and the lesion development was measured at regular intervals (A). MRL/*lpr* mice developed smaller lesions than the MRL-+/+ mice for the first 3 to 5 wk after infection. Thereafter, the disease in the mutant mice progressed inexorably, while all the wild-type mice achieved eventual resolution of lesion. Experiments were terminated when the mutant mice developed autoimmune disease. Mice were killed 9 wk after infection and parasite loads in the footpad were estimated by limiting dilution (*B*). Data are mean ± SEM, $n = 7$. *$p < 0.05$, **$p < 0.01$. Similar results were obtained with mice ages 8 or 21 wk. Parasite loads in the draining lymph nodes were generally three orders of magnitude smaller than those of the corresponding footpads (data not shown).

Results and Discussion

To address the role of Fas expression in the disease development in cutaneous leishmaniasis, MRL/*lpr* and the wild-type MRL-+/+ mice of various age groups were infected in the footpad with *L. major*. The mutant mice developed significantly smaller lesion compared with the wild-type mice during the first 3–5 wk after infection. Thereafter, the disease in the mutant mice progressed rapidly. While the wild-type mice eventually achieved complete lesion resolution, the disease in the mutant mice progressed inexorably (Fig. 1). By the time the experiments were terminated due to the spontaneous autoimmune disease normally developed in the

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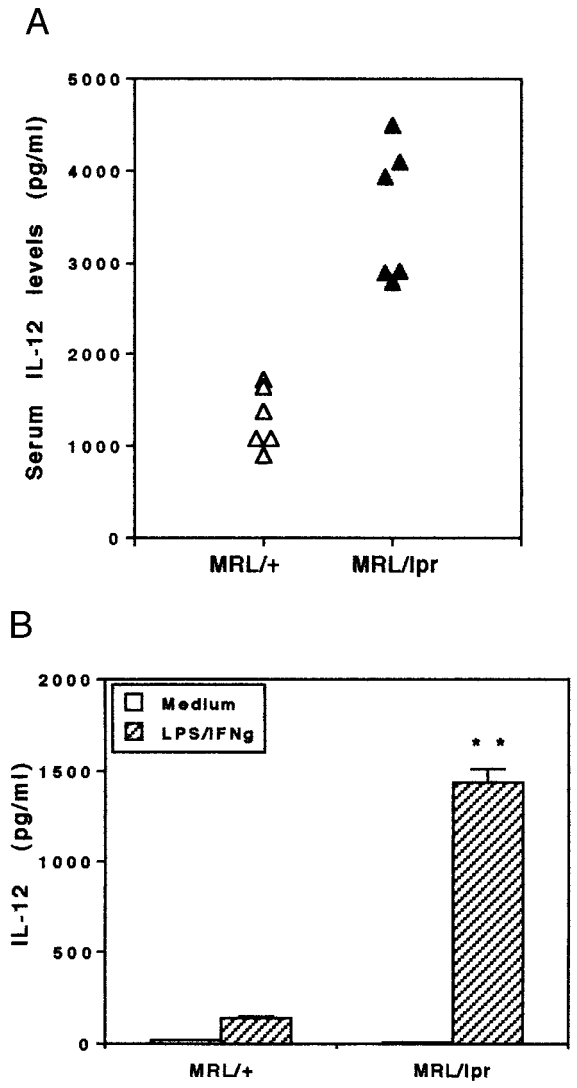

FIGURE 2. Production of IL-12 by infected MRL/*lpr* and MRL-+/+ mice. *A*, 8-wk-old mice were infected with *L. major*, and serum was collected 4 wk later by tail bleed. *B*, 12-wk-old mice were infected with *L. major* and resident peritoneal cells were harvested 9 wk after infection and pooled from groups of three mice. Cells were cultured with LPS (100 ng/ml) plus IFN-γ (50 U/ml) or medium alone. Culture supernatants were collected after 24 h. IL-12 concentration was determined by ELISA (p70 + p40). Data are shown as individual mice (*A*) or mean ± SD, $n = 6$. $^{**}p < 0.01$ (compared with MRL-+/+). Similar results were obtained in three independent experiments.

MRL/*lpr* mice, the infected mutant mice contained 5 orders of magnitude more parasites in the footpad than did the control wild-type mice (Fig. 1*B*).

We then investigated the immunologic parameters of the infected mice. Serum from MRL/*lpr* mice contained significantly higher concentrations of IL-12 at 4 and 9 wk postinfection than those from the MRL-+/+ mice (Fig. 2*A*). Resident peritoneal cells from the mutant mice infected with *L. major* 9 wk previously also produced markedly higher concentrations of IL-12 than those from similarly infected wild-type mice when stimulated with IFN-γ and LPS in vitro (Fig. 2*B*).

Spleen cells from the infected mutant mice produced markedly higher concentrations of IFN-γ than cells from the wild-type mice when stimulated with either soluble leishmanial antigen or Con A (Fig. 3*A*). In contrast, cells from the wild-type mice produced large amounts of IL-4 and IL-5, whereas these cytokines were not detectable in the supernatants of cells from the mutant mice (Fig. 3, *B* and *C*). However, spleen cells from the two strains of mice produced similar proliferative responses against leishmanial antigens or Con A in vitro (data not shown).

Serum of the infected mutant mice also contained significantly higher concentrations of nitrate/nitrite than that of the wild-type mice (Fig. 4*A*). Furthermore, peritoneal cells from the infected MRL/*lpr* mice produced more NO than those of the MRL-+/+ mice when stimulated with LPS and IFN-γ in vitro (Fig. 4*B*).

These results demonstrate that Fas expression is essential for the resistance against *L. major* infection and that this may override

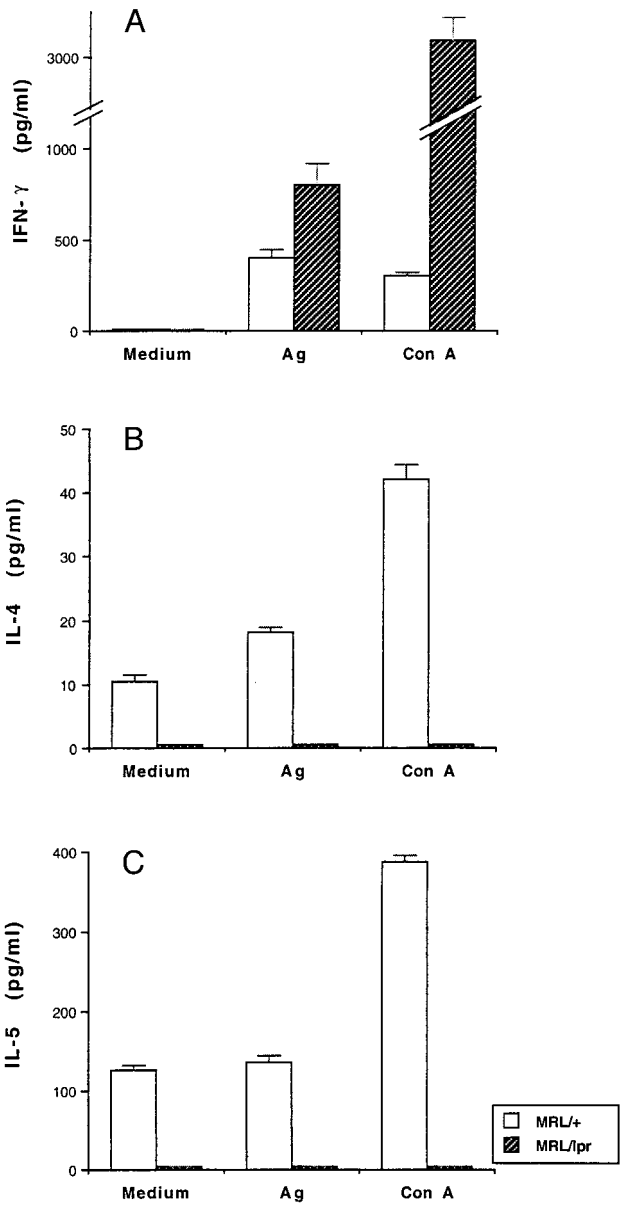

FIGURE 3. Infected MRL/*lpr* developed elevated Th1 cell responses. Mice (8 wk old) were infected with *L. major*, and spleen cells were collected 11 wk after infection. Cells pooled from groups of four mice were cultured with soluble leishmanial antigen (5×10^6 parasite equivalent/ml) or Con A (2.5 μg/ml). Supernatants were harvested at 72 h, and cytokine concentrations were determined by ELISA. Data are mean ± SD ($n = 6$) and are representative of three experiments.

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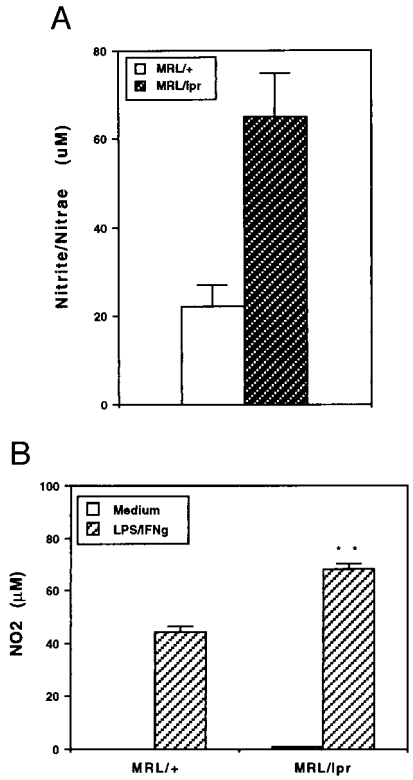

FIGURE 4. Infected MRL/*lpr* mice produced more NO than MRL-+/+ mice. *A,* Total nitrite and nitrate concentration in the serum of mice infected as described in Figure 2*B*. *B,* Nitrite concentration in the culture supernatant of resident peritoneal cells stimulated with LPS + IFN-γ or medium alone. Cells were the same as those shown in Fig. 2*B*. Data are mean ± SD, $n = 3$, $^{**}p < 0.01$. Data are representative of three experiments.

the NO-mediated leishmanicidal function. We therefore investigated whether macrophages from the MRL/*lpr* can kill the parasite in an NO-dependent manner. Resident peritoneal macrophages from the MRL/*lpr* and MRL-+/+ mice were infected with *L. major* promastigotes and stimulated with LPS in vitro in the presence or absence of an NOS inhibitor, L-NMMA. Cells from the mutant mice were as efficient as the cells from the wild-type mice in killing the parasites (Fig. 5*A*). Furthermore, the leishmanicidal activity was completely abolished by L-NMMA, which significantly enhanced the survival of the parasites, indicating that NO produced endogenously by macrophages could inhibit the replication of the parasites. We then investigated the role of NO in leishmaniasis using a strain of iNOS-deficient mice of the highly resistant 129 background. Consistent with the earlier report (8) that iNOS-deficient mice of the MF1 background was highly susceptible to *L. major* infection, the iNOS-deficient 129 mice developed significantly larger lesions compared with the wild-type mice (Fig. 5*B*). However, both the mutant and the wild-type mice eventually achieved complete lesion resolution. These results therefore indicate that NO contributes to, but is not sufficient to account for, the resistance against this intracellular parasitic infection. This is consistent with our observation that the infection progressed more slowly in the MRL/*lpr* mice than in the MRL-+/+ mice at the early stage of infection (Fig. 1). This is probably due to the larger amounts of NO produced by the mutant mice than by the wild-type mice. NO may be able to contain the initial replication of a small number of parasites, thereby delaying the early onset of the disease. However, in the absence of Fas expression, NO alone was not able to control the eventual disease progression.

The precise mechanism by which Fas expression contributes to the resistance against intracellular parasitic infection is currently unclear. It is well established that Fas-FasL interaction leads to apoptosis (19). We have confirmed that in contrast to cells from the MRL-+/+ mice, macrophages from *L. major*-infected MRL/*lpr* mice showed little or no apoptosis when incubated with anti-Fas Ab (data not shown). Furthermore, activated Th1, but not Th2, cells preferentially express FasL, which induces apoptosis in target cells expressing the Fas protein (20–22). Thus, interaction of Th1 cells (expressing FasL) and macrophages (expressing Fas) could lead to macrophage apoptosis, which would deprive the parasites of their natural habitat. Furthermore, release of the parasites into the extracellular milieu would also contribute to their destruction.

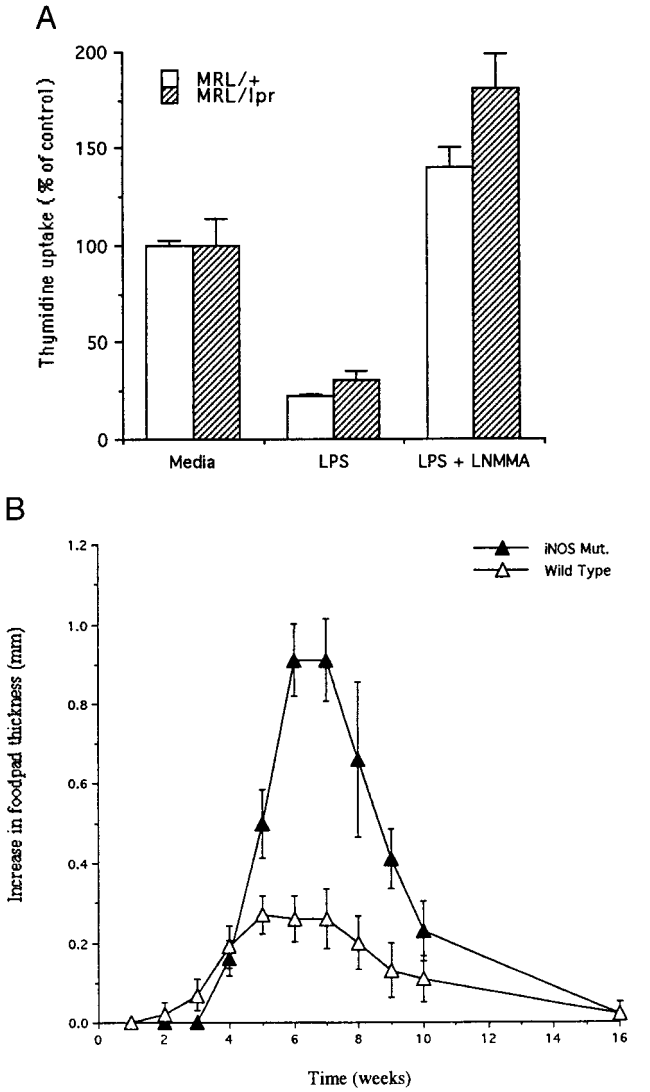

FIGURE 5. NO contributes toward the host resistance against *L. major* infection. *A,* Resident peritoneal cells from uninfected mice (12–16 wk old) were collected and pooled. Cells were infected with *L. major* promastigotes and cultured with medium alone, LPS (500 ng/ml) alone, or LPS + L-NMMA (10 mM). Surviving parasites at the end of 3-day culture were estimated by incorporation of [^{3}H]TdR. Similar results were obtained in two experiments. *B,* The course of infection in iNOS-deficient (Mut.) and wild-type mice of the 129 background. Mice were injected in the right hind footpad with 1×10^6 *L. major* promastigotes and disease progression followed by lesion development at regular intervals. Data are mean ± SEM, $n = 7$.

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Thus, resistant strains of mice preferentially produce IL-12 (23), which induces Th1 cell differentiation (11). Th1 cells mediate resistance to leishmanial infection, at least, via the following two pathways: 1) induction of macrophage apoptosis by the FasL-Fas pathway; and 2) production of IFN-γ, which activates macrophages to produce NO that kills the parasites directly. Also NO can induce macrophage apoptosis (24). We have also found that NO inhibits the expression of Bcl2 (F.-P. Huang and F. Y. Liew, unpublished observations), an inhibitor of apoptosis (25). Therefore, NO may contribute to the host resistance via the induction of macrophage apoptosis.

In conclusion, we demonstrate here the crucial role of Fas expression in resistance to leishmanial infection. Furthermore, the interaction of NO and the Fas-FasL pathway of apoptosis may be required for the effective resistance against intracellular pathogens.

Note added in proof. Recently, Fatima Conceicao-Silva et al. (1998, *Eur. J. Immunol. 28:237*) also reported that the resolution of lesions induced by *L. major* in mice requires a functional Fas (APO-1, CD95) pathway of cytotoxicity.

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