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The Role of IL-10 in Promoting Disease Progression in Leishmaniasis[1]

Margaret Mentink Kane and David M. Mosser[2]

To determine the role of IL-10 in cutaneous leishmaniasis, we examined lesion development following *Leishmania major* infection of genetically susceptible BALB/c mice lacking IL-10. Whereas normal BALB/c mice developed progressive nonhealing lesions with numerous parasites within them, IL-10$^{-/-}$ BALB/c mice controlled disease progression, and had relatively small lesions with 1000-fold fewer parasites within them by the fifth week of infection. We also examined a mechanism whereby *Leishmania* induced the production of IL-10 from macrophages. We show that surface IgG on *Leishmania* amastigotes allows them to ligate Fcγ receptors on inflammatory macrophages to preferentially induce the production of high amounts of IL-10. The IL-10 produced by infected macrophages prevented macrophage activation and diminished their production of IL-12 and TNF-α. In vitro survival assays confirmed the importance of IL-10 in preventing parasite killing by activated macrophages. Pretreatment of monolayers with either rIL-10 or supernatants from amastigote-infected macrophages resulted in a dramatic enhancement in parasite intracellular survival. These studies indicate that amastigotes of *Leishmania* use an unusual and unexpected virulence factor, host IgG. This IgG allows amastigotes to exploit the antiinflammatory effects of FcγR ligation to induce the production of IL-10, which renders macrophages refractory to the activating effects of IFN-γ. *The Journal of Immunology,* **2001, 166: 1141–1147.**

Leishmania are intracellular parasites that reside primarily within host tissue macrophages. The immunological response to *Leishmania* has been extensively characterized, and the importance of the activated macrophage in resolving infection has been unequivocally established (1, 2). In the *Leishmania major* model of cutaneous leishmaniasis, genetically inbred strains of mice exhibit polarized immune responses that can result in dramatic differences in the clinical outcome of infection. BALB/c mice mount an inappropriate Th2 response and succumb to progressive disease. In contrast, other strains such as C3H or C57BL/6 mice mount a Th1 response and control infections (3). There are, however, several species of *Leishmania* and many models of clinical leishmaniasis in which this immune deviation is not a true predictor of disease progression. In both humans and mice, for example, ample IFN-γ is produced during visceral leishmaniasis caused by *Leishmania donovani* (4, 5). Despite the presence of high levels of IFN-γ, infected hosts generally fail to control the infection and resolve their disease. In fact in humans, the severity of visceral leishmaniasis has been most closely associated with increased levels of IL-10 (5–7). IL-10 production also correlated with lesion progression in patients with cutaneous leishmaniasis (8). A similar correlation has recently been made in IL-10-transgenic mice, which are susceptible to progressive *L. major* disease despite producing IFN-γ (9). These and other studies point to an important role for IL-10 in regulating immune responses to this intracellular pathogen.

There are two developmental forms of *Leishmania*: the promastigote and the amastigote (10). The promastigote is introduced into the mammalian host when an infected sandfly takes a bloodmeal. This form is taken up by phagocytic cells and rapidly transforms into the amastigote form. Amastigotes replicate intracellularly within mononuclear phagocytes and are the only form found within the mammalian host following infection. The unexpected observation was made several years ago that *Leishmania* amastigotes have host-derived IgG on their surface (11, 12). This observation was recently confirmed, and the role of IgG as an opsonin for enhanced parasite adhesion to macrophages was proposed (13). We have previously shown that *Leishmania* amastigotes bind avidly to mammalian cell proteoglycans (14), and do not require opsonization for parasite adhesion to macrophages. We therefore began to look for alternative functions for Ig on the amastigote surface to explain the enhanced virulence of IgG-opsonized amastigotes.

We have recently demonstrated that the ligation of phagocytic receptors on macrophages can alter their cytokine profile when these cells are exposed to a variety of inflammatory stimuli (15, 16). We showed that the ligation of the FcγR by immune complexes was a particularly potent way to prevent the production of proinflammatory cytokines. The ligation of this receptor class not only inhibited the production of IL-12 (15), but unlike complement receptor ligation, FcγR ligation also induced the synthesis and secretion of IL-10 (16). IL-10 production occurred only in cells containing a functional FcR γ-chain, indicating that FcγR signaling through the γ-chain was required for IL-10 production. We proposed that this antiinflammatory cytokine milieu would have the potential to inhibit the production of a type 1 immune response and prevent macrophage activation. Consistent with this hypothesis is the observation by others that the administration of immune complexes to mice prevented effective cellular responses to *Listeria monocytogenes* and diminished bacterial clearance (17).

In the present study, we examined cytokine production by macrophages following their interaction with *Leishmania* amastigotes. We show that lesion-derived amastigotes induce the robust production of IL-10 from stimulated macrophages. The molecule responsible for this induction is host IgG on the amastigote surface, which ligates macrophage FcγRs. The IL-10 that is produced by this mechanism inhibits macrophage activation and contributes to

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parasite growth in lesions. Thus, we have identified an unexpected *Leishmania* virulence factor: host IgG.

Materials and Methods

Animals

C57BL/6, C3H/HeJ, and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-$10^{-/-}$ mice on a BALB/c background were kindly provided by Donna Rennick, DNAX (Palo Alto, CA). IL-$10^{-/-}$ mice were maintained under germfree conditions in the Barrier Animal Facility of Temple University in MicroFlow System ventilated cages (Allentown Caging Equipment, Allentown, PA). Breeding pairs of FcR γ-chain knockout mice ($\gamma^{-/-}$) (18) were purchased from Taconic Farms (Germantown, NY).

Parasites

A clone of *L. major* (WHO MHOM/IL/80/Friedlin) and the Josefa isolate of *Leishmania mexicana amazonensis* (14) were used for these studies. Promastigotes were grown in Schneider's insect cell culture medium (Life Technologies, Grand Island, NY) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Axenic *L. mexicana amazonensis* amastigotes were grown at 32°C, as previously described (19). Lesion-derived amastigotes were isolated from BALB/c mice infected 6–8 wk before as described previously (20).

Macrophages

Bone marrow-derived macrophages (BMMϕ)[3] were established as previously described (15). Murine peritoneal macrophages were washed from the peritoneal cavity of either C57BL/6 or BALB/c mice as described elsewhere (21). Cells were cultivated in DMEM containing 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin (complete medium) (D-10).

Macrophage stimulation and receptor ligation

BMMϕ were used to measure the production of cytokines. Cells were seeded overnight in 24-well plates in complete medium at a density of 2×10^5 cells/well. Cells were washed once with complete medium, and then stimuli were added to induce cytokine production. Lesion amastigotes and axenic amastigotes were added at a ratio of 10 amastigotes per macrophage. Amastigotes were added either alone or simultaneously with either LPS (*Escherichia coli* 0128.B12; Sigma, St. Louis, MO) or low molecular weight hyaluronic acid (HA; ICN Biomedicals, Costa Mesa, CA) at concentrations indicated in the figure legends.

Macrophage activation in vitro

BMMϕ were activated by pretreating them overnight with 250 U/ml IFN-γ (R&D Systems, Minneapolis, MN) and 100 ng/ml LPS. For in vitro leishmanicidal assays, peritoneal macrophages were pretreated with either 10 ng/ml rIL-10 (R&D Systems) or supernatants from stimulated macrophages infected with *Leishmania* amastigotes (infected macrophage supernatants) 3 h before activation with IFN-γ. Three hours later, *L. major* amastigotes were added to macrophage monolayers at a 3:1 (parasite:macrophage) ratio for 72 h at 35°C. Nonphagocytozed amastigotes were washed from the cultures at 24 h postinfection, and fresh medium was added to each well with the appropriate cytokine conditions for an additional 48 h. At the termination of the incubation period, the wells were washed once with complete medium, then fixed with 100% methanol at 4°C for 30 min. The monolayers were washed with PBS containing 5% FCS (PBS/FCS) and processed for immunofluorescent staining to visualize intracellular *Leishmania* amastigotes. Murine polyclonal anti-leishmania antiserum was used as the primary Ab, and goat α-murine-IgG conjugated with FITC was used as the secondary Ab, as described previously (20). Coverslips were counterstained with propidium iodide and examined by fluorescence microscopy.

Flow cytometry

Footpad lesion amastigotes were isolated from BALB/c mice infected 6–8 wk, as described previously (20). To directly stain murine IgG on the amastigote surface, 1×10^6 amastigotes were incubated on ice for 30 min with FITC-conjugated goat anti-murine (Fcγ chain-specific) IgG (Jackson ImmunoResearch,West Grove, PA) diluted 1/100 in PBS/FCS. Amastigotes were opsonized with IgG by incubating them on ice for 30 min with a 1/10 dilution of serum from a mouse infected with viable *Leishmania*. Following three washes in PBS/FCS to remove nonspecific IgG, FITC-conjugated goat anti-murine IgG was added on ice for an additional 30 min. The amastigotes were washed and fixed in 1% paraformaldehyde and immediately analyzed on an Epics Elite flow cytometer (Coulter Diagnostics, Hialeah, FL).

Cytokine ELISAs

Culture supernatants from monolayers of control and stimulated macrophages were assayed by ELISA for cytokine production 20–24 h after stimulation. Murine IL-10 production was measured as previously described (16) using mAbs to IL-10, JES5-2A5, and biotinylated JES5-16E3 (PharMingen, San Diego, CA). IL-12 (p70) levels were measured using mAbs C18.2 (IL-12 p35) and biotinylated C17.15 (IL-12 p40) as described elsewhere (16). TNF production was measured using mAbs G281-2626 and biotinylated MP6-XT3 (PharMingen).

Parasite quantitation

Mice were injected in the hind footpad with 2×10^6 *L. major* amastigotes. Parasite burdens in footpads were determined by a limiting serial dilution of single cell suspensions made from individual excised lesions as described reviously (22). Lesion size was determined by measuring the thickness of the footpad with a caliper, and subtracting the thickness of the uninfected contralateral footpad.

Results

Mice lacking IL-10 have decreased lesion development and reduced parasite burdens

To determine the effect of IL-10 on disease progression in leishmaniasis, we infected mice deficient in IL-10 on a BALB/c background and compared them with wild-type mice. BALB/c mice are genetically susceptible to *L. major* infection (3), and therefore wild-type mice produce progressive nonhealing lesions (Fig. 1*A*) that increased in size until day 36, when the lesions began to ulcerate and metastasize. On day 36, there were in excess of 1×10^9 organisms per infected footpad (Fig. 1*B*). For humane reasons, these mice were euthanized at this time. In contrast to wild-type BALB/c mice, congenic mice lacking IL-10 were relatively resistant to infection, showing only modest increases in lesion size through the 11-wk observation period (Fig. 1*A*). At 2 wk postinfection, a time when footpad swelling in the two groups had not yet begun to exhibit differences, mice lacking IL-10 already had ~100-fold fewer parasites in their lesions than wild-type mice (Fig. 1*B*). By the fifth week, IL-$10^{-/-}$ mice had 1000-fold fewer organisms in their lesions, and by the 11 wk only ~100 organisms could be detected per infected foot in IL-$10^{-/-}$ mice (103 ± 128). Thus, mice lacking IL-10 are relatively resistant to *Leishmania* infection.

Lesion amastigotes are coated with surface Ig

Previous studies have demonstrated that lesion-derived amastigotes have host IgG on their surface (11–13). To confirm these observations, flow cytometry was performed to identify host IgG on the surface of lesion-derived amastigotes. Amastigotes were isolated from the footpads of infected BALB/c mice and directly stained with FITC-conjugated Ab to the Fc fragment of murine IgG (Fig. 2 , open histograms). By flow cytometry, lesion-derived amastigotes stained positively for murine IgG (Fig. 2, *right*). Virtually all of the organisms in the population were positive for surface Ig, and the majority expressed relatively high levels of IgG with a mean fluorescence intensity of over 10. In contrast, axenic amastigotes grown in vitro in the absence of IgG were devoid of surface IgG (Fig. 2, *left*). Their mean fluorescence intensity was not substantially different from unstained organisms (gray histograms). Preincubation of these organisms with antiserum to amastigotes as the primary Ab, followed by the FITC anti-IgG (filled histograms), resulted in axenic amastigotes staining positively for

[3] Abbreviations used in this paper: BMMϕ, bone marrow-derived macrophages; AA, axenically grown amastigotes; HA, hyaluronic acid.

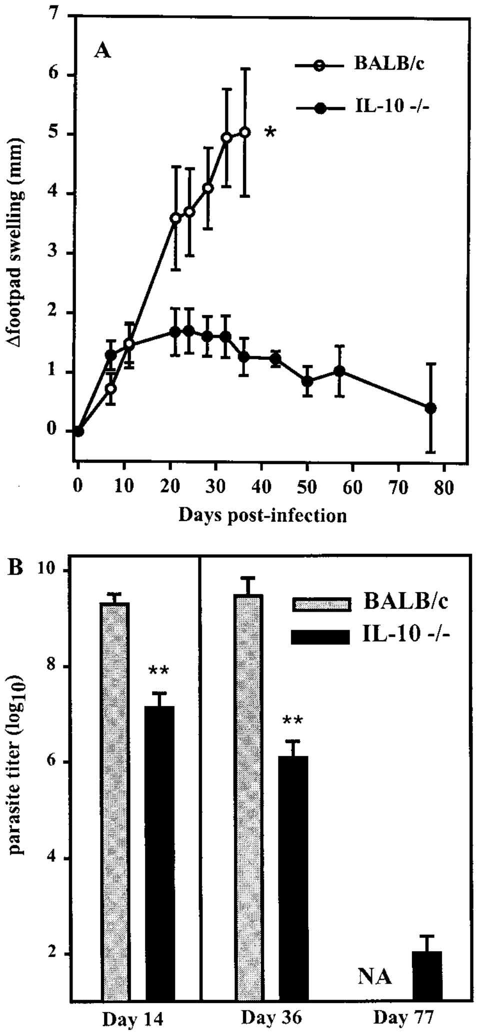

FIGURE 1. The growth of *L. major* in mice. *A*, Footpad swelling in infected wild-type BALB/c mice (open symbols) and IL-$10^{-/-}$ BALB/c mice (filled symbols) was compared following the injection of 2×10^6 *L. major* amastigotes. Data represent the mean ± SD from 13 mice/group. The two groups are statistically different from each other ($p \leq 0.05$) at day 21 and beyond. *, Wild-type mice were euthanized at day 36 of the experiment for humane reasons. *B*, Footpad parasite burdens (log_{10}) were measured by limiting dilution analysis at 14 days postinfection ($n = 5$/group) (*left*), and, in a separate experiment, on day 36 ($n = 5$) and day 77 ($n = 3$) postinfection (*right*). Wild-type BALB/c mice (gray bars) and IL-$10^{-/-}$ mice (filled bars). Double asterisks represent a difference of $p \leq 0.05$.

murine IgG (Fig. 1, *left*). Similarly, staining footpad amastigotes with primary Ab followed by FITC anti-IgG also resulted in positive staining. This staining was only slightly higher than lesion-derived amastigotes stained with secondary Ab alone. Thus, these data confirm previous observations (11) that lesion-derived amastigotes have host IgG on their surface, and they also demonstrate that axenic organisms do not.

Amastigotes from lesions induce macrophage IL-10 production

Macrophage IL-10 production was measured following infection of BMMϕ with *Leishmania* amastigotes. To induce cytokine production in these assays, BMMϕ were exposed in vitro to subnanogram amounts of bacterial LPS. The low levels of LPS used in these assays (125–500 pg/ml) did not induce detectable levels of IL-10. However, the simultaneous addition of *L. major* amastigotes to these cells induced the secretion of large amounts of IL-10 (Fig. 3*A*). The induction of IL-10 by lesion-derived amastigotes required the presence of a costimulus, such as LPS, since washed amastigotes alone, (even when added at high multiplicities of infection; not shown), were unable to induce significant IL-10 production from BMMϕ (Fig. 3*A*).

Because LPS would be present in lesions only during bacterial superinfections of cutaneous lesions (23), we chose another physiologically relevant stimuli to test for IL-10 induction. BMMϕ from C3H/HeJ mice, which are hyporesponsive to LPS, were exposed to low molecular weight HA, a matrix component that is present in inflamed tissue (24). These cells were infected with *Leishmania* amastigotes, and cytokine production was measured. Similar to LPS, HA alone induced little or no IL-10, but the combination of HA with amastigote infection induced a robust production of IL-10 (Fig. 3*B*). Thus, a stimuli that is present in inflamed lesions induces IL-10 production from macrophages when they encounter *Leishmania* amastigotes.

To determine whether the IgG on the surface of amastigotes was required for IL-10 induction, macrophages were infected with axenically grown amastigotes (AA) that lack surface IgG (see Fig. 2). These organisms failed to up-regulate macrophage IL-10 production from stimulated macrophages (Fig. 4*A*). The opsonization of axenic amastigotes with immune serum (IgG-AA), however, induced high levels of IL-10 from wild-type macrophages (Fig. 4*A*, filled bars). The failure of unopsonized axenic amastigotes to induce IL-10 was not due to a failure of these organisms to bind to or invade macrophages, since axenic amastigotes attach to and invade host macrophages nearly as well as IgG-opsonized organisms (data not shown). Thus, amastigotes of two different species of *Leishmania*, *L. major* (Fig. 3) and *L. amazonensis* (Fig. 4), induced IL-10 production from inflammatory macrophages.

Previous studies from our laboratory demonstrated that FcγR ligation could induce the secretion of IL-10 from stimulated macrophages (16). To show that the present effect was a result of FcγR ligation, cytokine production by macrophages from mice lacking the γ-chain of the FcγR ($\gamma^{-/-}$) was analyzed. Unlike wild-type cells, macrophages from $\gamma^{-/-}$ mice failed to up-regulate IL-10 when infected with axenic amastigotes opsonized with IgG (Fig. 4*A*, gray bars). The failure to produce IL-10 by $\gamma^{-/-}$ macrophages is consistent with our previous studies showing a requirement for γ-chain signaling in inducing macrophage IL-10 production following FcγR ligation (16). Similar studies were performed on $\gamma^{-/-}$ macrophages infected with lesion-derived amastigotes rather than axenic amastigotes (Fig. 4*B*). Amastigotes derived from lesions of infected mice induced some IL-10 production from stimulated $\gamma^{-/-}$ macrophages in vitro (Fig. 4*B*, gray bars). These levels, however, were much lower than those produced by parallel monolayers of wild-type macrophages (Fig. 4*B*, filled bars). Thus, maximal IL-10 production by macrophages infected with *Leishmania* amastigotes requires FcγR ligation along with a second costimulatory signal, such as bacterial products or components of the extracellular matrix.

IL-10 induced from infected macrophages suppresses the production of IL-12 (p70), and TNF-α by IFN-γ/LPS-activated macrophages

To examine the biological consequences of macrophage IL-10 production, supernatants from amastigote-infected macrophages were added to monolayers of uninfected BMMϕ, which were then stimulated with IFN-γ/LPS. Control monolayers of BMMϕ that were activated with IFN-γ/LPS secreted relatively large amounts of IL-12 (p70) (Fig. 5*A*) and TNF-α (Fig. 5*B*). The addition of supernatants from amastigote-infected monolayers to cells prevented

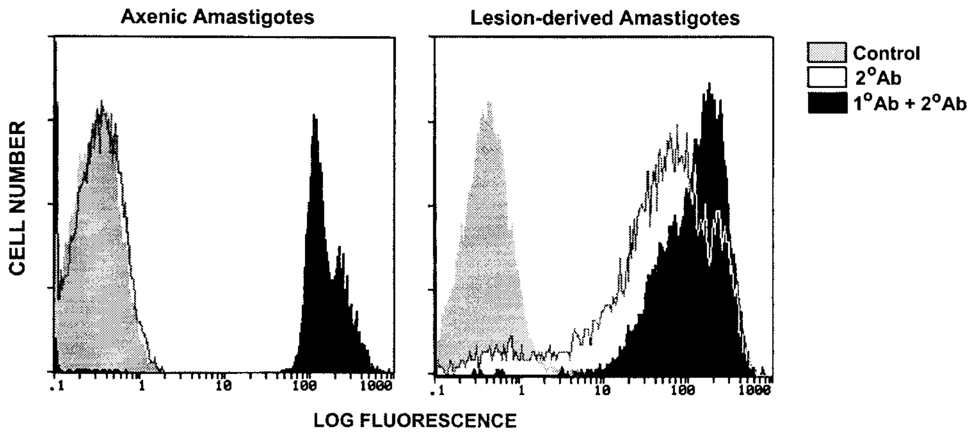

FIGURE 2. IgG on the surface of lesion-derived *Leishmania* amastigotes. Flow cytometry profiles of axenic amastigotes (*left*) or lesion-derived amastigotes isolated from BALB/c mice (*right*) directly stained with FITC-conjugated goat anti-murine Fcγ chain-specific Ab (open histogram). IgG-opsonized amastigotes (filled histograms) were made by incubating organisms in immune serum for 30 min before staining with FITC-conjugated goat anti-murine Fcγ chain-specific Ab. Control staining (gray histograms) utilized an irrelevant FITC-conjugated IgG (MOPC-21).

IL-12 production in a dose-dependent manner (Fig. 5*A*). Stimulated macrophages produced ~1 ng/ml of IL-12 (p70), and this production was inhibited to undetectable levels by the addition of 30% (v/v) amastigote supernatants (Fig. 5*A*). The inhibition of IL-12 (p70) depended on the presence of IL-10 in these supernatants because pretreatment of the supernatants with a blocking mAb to IL-10 completely abrogated this suppression, restoring IL-12 production to control levels (Fig. 5*A*). Parallel studies were performed to analyze TNF-α production by macrophages exposed to supernatants from infected macrophages. In vitro activation with IFN-γ/LPS caused a marked increase in TNF-α production by macrophages, and treatment of macrophages with either rIL-10 or supernatants from amastigote-infected monolayers dramatically inhibited macrophage TNF-α production (Fig. 5*B*). These results indicate that IL-10 produced by amastigote-infected inflammatory macrophages is adequate to inhibit the production of both IL-12 and TNF-α by stimulated macrophages.

Supernatants from amastigote-infected macrophages enhance the survival of Leishmania *in vitro*

BALB/c peritoneal macrophages were infected with *L. major* amastigotes in vitro, and their survival was measured over a 72-h interval. Parasite survival in resident (untreated) cells was compared with survival in activated cells. Some of the monolayers were pretreated with either rIL-10 or supernatants from amastigote-infected macrophages for 2 h before the addition of IFN-γ. Untreated cells, as expected, were unable to restrict parasite growth and allowed uncontrolled intracellular replication of amastigotes. By 72 h postinfection, the majority of infected cells had five or more organisms within them (Fig. 6, *A* and *E*). In contrast to the resident cells, macrophages activated in vitro with IFN-γ were able to restrict the intracellular growth of *Leishmania* (Fig. 6*B*). Most of the cells in the population had completely cleared their infection (Fig. 6*B*) and few if any of the cells contained five or more organisms within them (Fig. 6*E*). Pretreatment of cells with rIL-10 before the addition of IFN-γ prevented optimal activation (25, 26) and resulted in uncontrolled parasite replication (Fig. 6*B*). The majority of cells were infected and a significant percentage of the cells contained five or more parasites within them (Fig. 6*E*). Monolayers were also pretreated with 10% (v/v) supernatants from amastigote-infected macrophages. Similar to rIL-10, these supernatants prevented macrophage responses to IFN-γ and allowed uncontrolled intracellular replication of parasites (Fig. 6, *D* and *E*). Thus, pretreatment of macrophages with either IL-10 or supernatants from infected monolayers prevented them from responding to IFN-γ and restricting the intracellular growth of parasites.

Discussion

BALB/c mice are genetically susceptible to cutaneous *Leishmania* infection, mounting a Th2-type immune response that results in progressive lesion development and the widespread dissemination of parasites from the original inoculation site. In this study, we

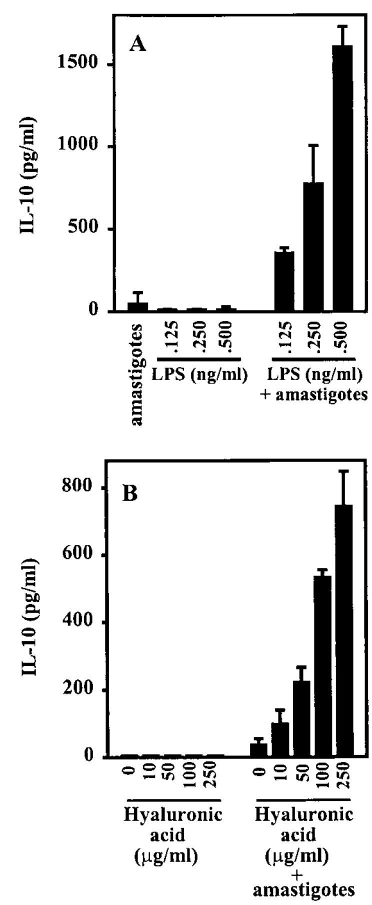

FIGURE 3. The induction of IL-10 by *Leishmania* amastigotes. *A*, Parallel monolayers of BMMϕ were exposed to *L. major* amastigotes alone, to increasing concentrations of LPS, or to amastigotes in the presence of increasing amounts of LPS. After 20 h, supernatants were harvested and IL-10 levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the means ± SD. Results are representative of three separate experiments. *B*, Monolayers of C3H/HeJ BMMϕ were stimulated as described in *A*; however, low molecular weight HA was used instead of LPS. Results are expressed as in *B* and are representative of two experiments.

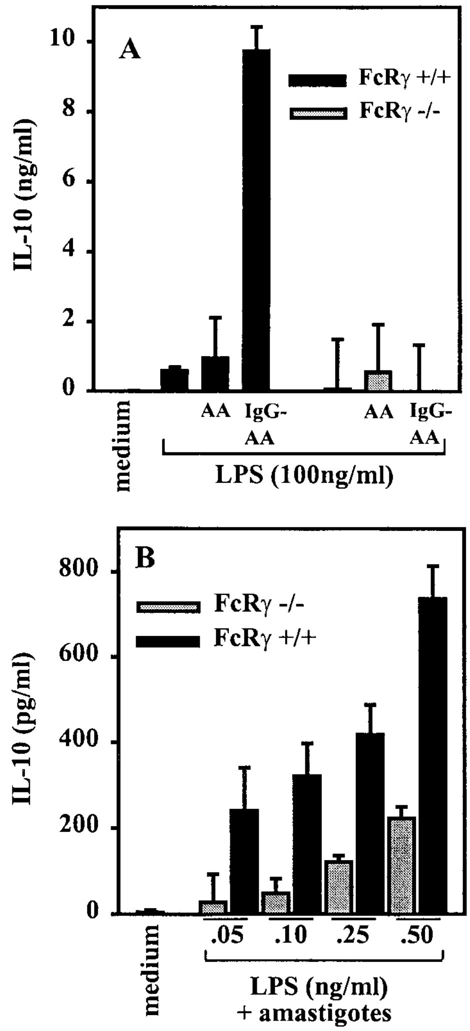

FIGURE 4. The role of host IgG and FcγR ligation in IL-10 induction. *A*, BMMϕ from wild-type (filled bars) or FcR$\gamma^{-/-}$ (gray bars) mice were incubated with 100 ng/ml LPS alone or with AA of *L. amazonensis* in the presence of 100 ng/ml LPS. Some amastigotes were opsonized with IgG (IgG-AA). After 20 h, supernatants were harvested and IL-10 levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the means ± SD. Results are representative of three separate experiments. *B*, Similar to *A*, except that macrophages were incubated with lesion-derived *L. major* amastigotes. The production of IL-10 in wild-type macrophages (filled bars) is compared with parallel monolayers of macrophages from FcR $\gamma^{-/-}$ mice (gray bars).

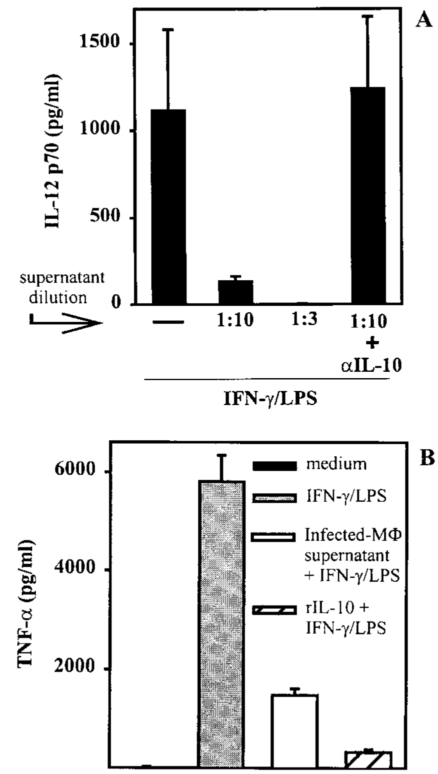

FIGURE 5. The suppression of IL-12 (p70) and TNF-α by amastigote-induced IL-10. *A*, Supernatants from BMMϕ stimulated with amastigotes in the presence of LPS for 20 h (infected macrophage supernatants) were harvested and diluted 1/3 or 1/10 (v/v) with complete medium. Diluted supernatants were then added to BMMϕ that were subsequently activated with IFN-γ/LPS. After 20 h, supernatants were harvested, and IL-12 (p70) levels were determined by ELISA. Anti-IL-10 Ab (JES5-2A5) was added to wells at 20 μg/ml to reverse the supernatant inhibition. *B*, Similar to *A*, cells were stimulated with IFN-γ/LPS (gray bars) rIL-10 was added to IFN-γ/LPS-treated monolayers at 10 ng/ml (striped bars), and supernatants from infected monolayers (open bars) were added at 10% (v/v). TNF-α levels were determined by ELISA.

examined the role of IL-10 in cutaneous leishmaniasis, and found that mice deficient in IL-10 controlled *Leishmania* infection. Following the injection of a large dose of *L. major* amastigotes, IL-10-deficient mice demonstrated minimal footpad swelling and limited parasite growth in lesions. IL-10$^{-/-}$ mice had 100-fold fewer parasites by 2 wk postinfection, and by 5 wk postinfection they had 1000-fold fewer parasites than controls. At the final time point (11 wk), IL-10$^{-/-}$ mice had almost completely resolved their infection, with only 103 (±128) parasites on average per infected foot. These data argue for an important role for IL-10 in progressive cutaneous leishmaniasis.

These results differ from the phenotype observed in mice treated with anti-IL-10 Ab during infection with *L. major* (27), which showed only a minimal phenotype. Another study used an IL-10-transgenic mouse model in which the IL-10 transgene was under the control of the IL-2 promoter (28), and these IL-10-transgenic mice had no change in their response to *Leishmania* infection compared with control mice. These and other studies suggested that IL-10 was not a key regulator in *Leishmania* infection, and that IL-10 did not play a role in T cell subset development (27, 29). Recent studies (9), however, have examined the role of IL-10 in IL-10-transgenic mice, in which the IL-10 gene was under the control of the MHC class II Ea promoter. These mice had a profound phenotype and were highly susceptible to *L. major* infection. The susceptible phenotype of these transgenic mice indicates that the immunosuppressive activity of IL-10 on the macrophage/monocyte population contributes to disease progression in leishmaniasis. Our model using IL-10 knockout mice supports these later observations and further clarifies the role of IL-10 in contributing to uncontrolled intracellular parasite growth. Studies to identify alterations in macrophage function in response to IL-10 are ongoing.

Our in vitro data indicate that macrophage IL-10 is being turned on by the amastigote itself. We have previously demonstrated that the ligation of FcγR on stimulated macrophages can induce the production of IL-10 in vitro (16). We now show that *Leishmania* amastigotes exploit this mechanism to produce IL-10 production from infected macrophages. There are several lines of evidence that indicate that IL-10 production was a consequence of the ligation of macrophage FcγRs by amastigotes. First, axenic amastigotes grown in the absence of IgG failed to induce IL-10 unless

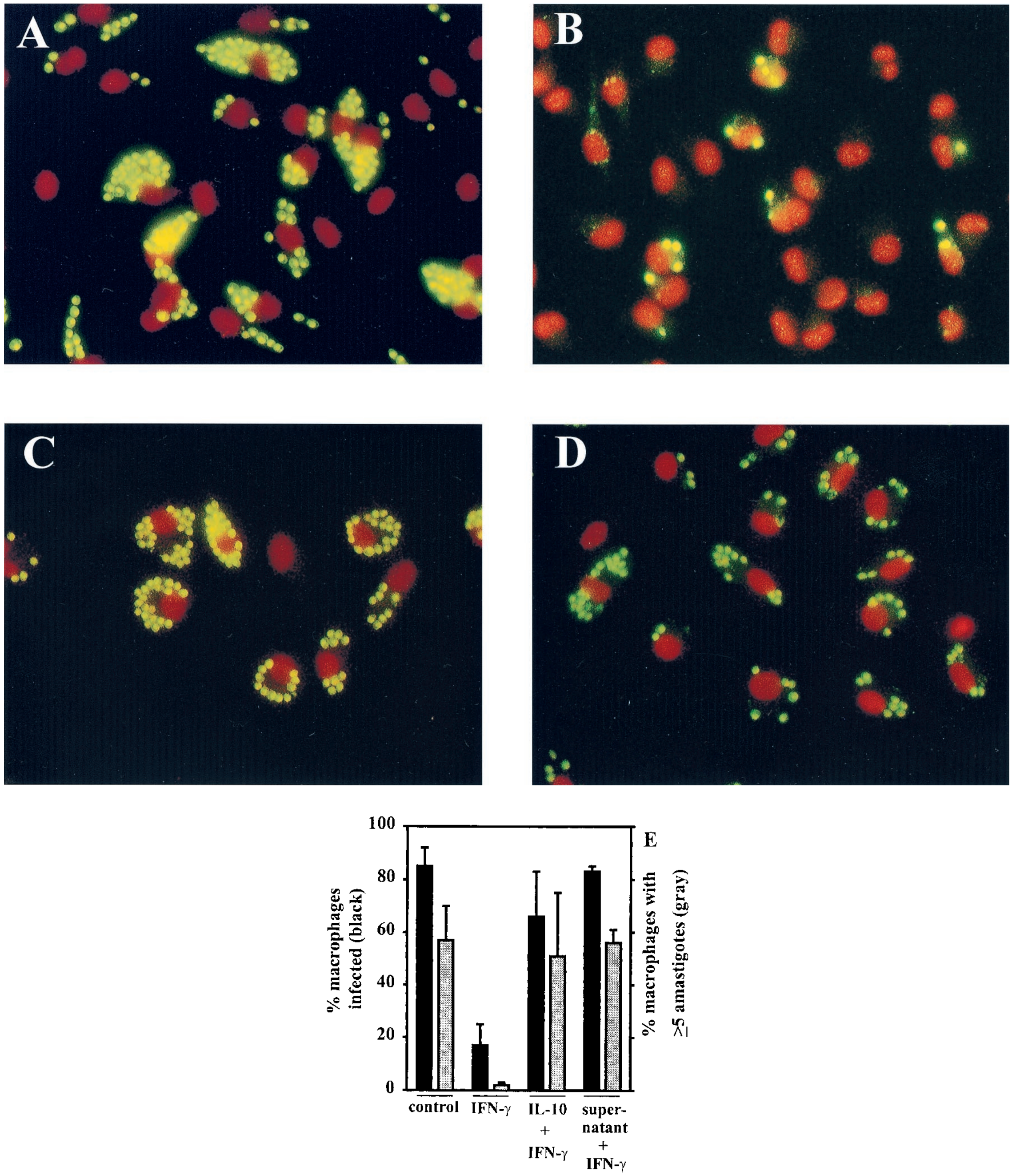

FIGURE 6. The in vitro growth of *L. major* amastigotes in macrophages. Equal numbers of amastigotes of *L. major* were added to parallel monolayers of peritoneal macrophages that were: *A*, untreated; *B*, activated in vitro with 250 U/ml IFN-γ; *C*, pretreated with 10 ng/ml rIL-10 3 h before IFN-γ; or *D*, pretreated with 10% (v/v) supernatants from infected monolayers 3 h before IFN-γ. Monolayers were fixed and stained by immunofluorescence 72 h following infection. In these micrographs, amastigotes are stained with FITC and appear green, whereas macrophage nuclei are stained with propidium iodide and appear red. *E*, The percentage of macrophages infected with one or more amastigotes (black bars), and the percentage of macrophages with five or more parasites within them (gray bars).

they were opsonized with immune IgG, in which case their inducing capacity was fully restored. Second, macrophages lacking the common γ-chain of the FcγRs produced less IL-10 following infection than did parallel monolayers of normal macrophages. Thus, optimal IL-10 production in this system depended on FcγR ligation. We note that the low levels of IL-10 induced by lesion-derived amastigotes from $\gamma^{-/-}$ macrophages (Fig. 4*B*) suggest that the ligation of other macrophage receptors by amastigotes may (minimally) also contribute to IL-10 induction. Thus, although FcγR ligation may not be required for IL-10 production, it is a major contributing factor.

Receptor ligation alone, however, was not sufficient to induce IL-10 production. Low levels of costimulation with either low molecular weight HA or LPS were also required. These costimuli may be physiologically relevant because both have the potential to be present in *Leishmania* lesions. Cutaneous lesions in patients and experimentally infected animals are frequently superinfected with bacteria (23), and HA is ubiquitous in inflamed tissue (24). Current studies are underway to define other costimulatory stimuli, such as chemokine stimulation, that may cooperate with receptor ligation to induce IL-10 production.

The present studies may provide a partial explanation for two recent observations showing that mice lacking IgG or FcγRs are actually more resistant to *Leishmania* infection. Working in a cutaneous model of *L. amazonensis* infection, Kima and colleagues (30) showed that the common γ-chain of the FcγR was required for optimal lesion progression in mice. These results support our hypothesis that IgG-opsonized amastigotes use Fc receptors during infection to enhance macrophage IL-10 production. Smelt and colleagues (31) have shown that visceral infection with *L. donovani* was diminished in mice lacking IgG. This observation would also be consistent with a role for IgG-induced IL-10 in contributing to lesion progression during leishmaniasis.

We examined the consequences of macrophage IL-10 production by adding supernatants from amastigote-infected macrophages to naive monolayers, which were then exposed to IFN-γ/LPS. Supernatants from infected monolayers inhibited the activation of macrophages exposed to IFN-γ/LPS. These treated macrophages produced significantly less TNF-α, and they were virtually unable to produce IL-12. Importantly, these pretreated monolayers failed to control *Leishmania* infection. The majority of the cells in the monolayer were infected, and most of the cells had multiple organisms growing within them (Fig. 6, *D–E*). Thus, a prior encounter with IL-10 renders macrophages refractory to the activating effects of IFN-γ and prevents them from eliminating intracellular parasites, as previously reported (26).

In summary, we have examined the interaction of *Leishmania* amastigotes with tissue macrophages and have identified an unexpected role for host IgG. Rather than simply acting as a classical opsonin to accelerate parasite phagocytosis, an additional role of surface IgG is to induce the production of IL-10 by macrophages. This induction prevents these cells from responding to IFN-γ and eliminating intracellular parasites. This work suggests that an important way that *Leishmania* parasites modify the host immune response is by exploiting the antiinflammatory effects of FcγR ligation to induce the production of IL-10.

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