Leishmania Promastigotes Selectively Inhibit Interleukin 12 Induction in Bone Marrow–derived Macrophages from Susceptible and Resistant Mice

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Summary

Leishmania major promastigotes were found to avoid activation of mouse bone marrow–derived macrophages (BMMø) in vitro for production of cytokines that are typically induced during infection with other intracellular pathogens. Coexposure of BMMø to the parasite and other microbial stimuli resulted in complete inhibition of interleukin (IL) 12 (p40) mRNA induction and IL-12 release. In contrast, mRNA and protein levels for IL-1α, IL-1β, tumor necrosis factor (TNF) α, and inducible NO synthase (iNOS) were only partially reduced, and signals for IL-10 and monocyte chemoattractant protein (MCP-1/JE) were enhanced. The parasite could provide a detectable trigger for TNF-α and iNOS in BMMø primed with interferon (IFN) γ, but still failed to induce IL-12. Thus IL-12 induction is selectively impaired after infection, whereas activation pathways for other monokine responses remain relatively intact. Selective and complete inhibition of IL-12(p40) induction was observed using BMMø from either genetically susceptible or resistant mouse strains, as well as IL-10 knockout mice, and was obtained using promastigotes from cutaneous, visceral, and lipophosphoglycan -deficient strains of *Leishmania*. The impaired production of the major physiologic inducer of IFN-γ is suggested to underlie the relatively prolonged interval of parasite intracellular survival and replication that is typically associate with leishmanial infections, including those producing self-limiting disease.

Leishmania sp. are obligate intracellular protozoa that colonize macrophages in the vertebrate host, and their control requires the induction of immune responses capable of activating macrophages to a microbicidal state. The most potent cytokine for the induction of leishmanicidal activity in macrophages is IFN-γ (1, 2). The sustained production of IFN-γ in response to infection is commonly associated with the development of specific Th1 and $CD8^+$ T cell responses, whereas NK cells have been identified as an important, though more transient source of this cytokine (reviewed in reference 3). Parasites that are able to compromise the induction or function of IFN-γ are undoubtedly favored for transmission since organisms able to attain high parasitemias and/or to persist in the vertebrate host will be more likely to encounter their phlebotomine vectors.

Considering the central role of macrophages in the initiation as well as effector functions of the immune response, infection of these cells might impair their ability to provide the appropriate regulatory cytokines for enhanced production of IFN-γ. Recent studies indicate that IL-12 plays an important role in potentiating cell-mediated immune responses during leishmanial infection. IL-12 is a heterodimeric cytokine produced primarily by monocytes, macrophages, and B cells, that stimulates growth of Th1 and NK lymphocytes and induces synthesis of IFN-γ by these cells (4–6). In models of murine leishmaniasis, treatment of susceptible BALB/c mice with rIL-12 rendered these animals resistant to *L. major* by upregulating IFN-γ and simultaneously inhibiting Th2 cytokine production (7, 8). In vivo administration of IL-12 in mice also potentiated the efficacy of a killed vaccine for *L. major* (9) and provided effective therapy for an established systemic infection with *L. donovani* (10). In vitro and in vivo experiments have demonstrated the crucial role of IL-12 in initiating and establishing both innate immunity and T cell–mediated resistance to other intracellular pathogens, including *Toxoplasma gondii*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis* (11–14). Exposure of macrophages to these organisms results in strong induction of IL-12 in vitro. In contrast, *L. major* fails to elicit

cytokine synthesis (including IL-12) by macrophages in vitro, (15–17), and this has been suggested as an important strategy to delay the onset of strong IFN-γ production and macrophage activation during the early stages of infection (15).

In the present studies, we ask whether cytokine responses by macrophages are induced, avoided, or actively impaired during in vitro infection with metacyclic promastigotes of different strains of *Leishmania*. Our approach was to follow the kinetics of cytokine responses by bone marrow–derived macrophages (BMMø)[1] exposed to *Leishmania* promastigotes alone, or in combination with microbial stimuli known to activate cytokine gene expression by these cells. The responses examined included the proinflammatory cytokines IL-1α, IL-1β, and TNF-α; the monocyte chemoattractant protein (MCP-1/JE); inducible NO synthase (iNOS); and the immunomodulatory cytokines IL-12 and IL-10. Our findings support the hypothesis that in contrast to other intracellular pathogens, *Leishmania* promastigotes fail to induce strong monokine responses generally, but that only the absence of IL-12(p40) mRNA expression and IL-12 release is due to an active and powerful inhibition of its induction. The selective impairment of IL-12 induction might underlie the early progression but eventual control that is characteristic of leishmanial infections in the vertebrate host.

Materials and Methods

Macrophages. Female BALB/c, C3H/HeN, and C57BL/6N (B6), mice 8–12-wk old, were obtained from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD). The B6 IL-10–deficient (knockout) mice have been previously characterized (18). BMMø were recovered following a previously published protocol (19), with minor modifications. Briefly, femurs were flushed with HBSS containing 25 mM Hepes and 50 μg/ml gentamycin sulfate, using a 27-in gauge needle. Bone marrow cells were washed once and resuspended in DMEM supplemented with 10% HI-FCS, 30% L929 cell-conditioned medium (source of M-CSF), 100 mM sodium pyruvate, 0.1 mM NEAA, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 μg/ml gentamycin. Differentiated MØ were allowed to adhere overnight in 75 cm^2 culture flasks. Nonadherent cells were removed and plated (3 plates/mouse) in 3.5 cm polystyrene culture-treated petri dishes and incubated for 7 d at 37°C and 5% CO_2. 1 d before infection, cells were washed twice and replenished with complete medium without L929 supernatants.

Parasites. *L. major* clone V1 (MHOM/IL/80/Friedlin) and *L. donovani* 1S strain (MHOM/SD/001S-2D) and its derived lipophosphoglycan (LPG)-deficient mutant, R2D2, (20) were cultured in 199 medium supplemented with 20% HI-FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 40 mM Hepes, 0.1 mM adenine (in 50 mM Hepes), 5 μg/ml hemin (in 50% triethanolamine), and 1 μg/ml 6-biotin (in 95% EtOH). Infective-stage promastigotes (metacyclics) of *L. major* were isolated from stationary cultures (5–6 d old) by their lack of agglutination by peanut agglutinin (PNA) (21). The *L. donovani* promastigotes were obatined from unselected stationary cultures. Before infection, promastigotes were opsonized with 5% C5-deficient serum obtained from B10.D2/OsNj mice, by incubation at 37°C for 30 min.

Macrophage Infections and Activation. For determination of cytokine responses in infected macrophages, parasites were used at a parasite/macrophage ratio of 20:1. LPS (Sigma Chemical Co., St. Louis, MO) was used in all the experiments at a concentration of 10 ng/ml. Recombinant mouse IFN-γ was used at 100 U/ml. Heat killed *M. tuberculosis* was kindly supplied by Drs. Patrick Brennan and Andrea Cooper (Colorado State University, Fort Collins, CO) and used at three different concentrations: 1,000, 100, and 10 bacteria/macrophage). Soluble antigen from *T. gondii* (STAg) was prepared as described (22) and used at 10 μg/ml. The stimuli were either added to macrophages alone, or simultaneously with parasites for 2, 6, and 8 h before harvesting supernatants or addition of RNAzol for extraction of mRNA.

mRNA Detection by RT-PCR. mRNA detection for the cytokines IL-1α, IL-1β, TNF-α, IL-10, and IL-12(p40), the β-chemokine MCP-1/JE, iNOS, as well as a housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), were performed using a semiquantitative RT-PCR method previously described (23), with minor modifications. Briefly, at various times after incubation with parasites or stimuli, RNAzol was added directly to the petri dishes, and RNA extracted from the BMMø following the manufacturer's recommendations. 1 μg of RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD). Samples were denatured at 70°C for 5 min before the addition of the enzyme and were then incubated 1 h at 60°C and 5 min at 90°C to inactivate the enzyme. After cooling on ice for 3 min, samples were diluted in distilled water to a final volume of 200 μl (1:8) and stored at −20°C. 10 μl of this solution was used for amplification in a semiquantitative PCR with Taq I Polymerase (Promega Corp., Madison, WI). Samples were incubated for 3 min at 95°C and were then amplified with an experimentally determined optimal temperature cycle number of (94°C for 1 min, 54°C for 1 min, and 72°C for 2 min) for each cytokine mRNA. 24 cycles were used for HPRT, IL-1α, IL-1β, TNF-α, and JE; 35 for iNOS; 40 for IL-12(p40); and 30 cycles for IL-10. PCR products were Southern blotted, transferred to Hybond$^+$ membranes (Amersham Corp., Arlington Heights, IL), and probed with cytokine-specific fluorescein-labeled oligonucleotides. The enhanced chemiluminescent (ECL) detection system (Amersham Corp.) was used for detection, exposing the membranes to hyperfilm (Amersham Corp.) at room temperature. The signals were analyzed using a densitometrical optical scanner (model 600ZS; Microteck, Torrance, CA) and Image software (National Institutes of Health, Bethesda, MD). Results were expressed as fold increase over control, considering the control value as one unit.

The primers and probes used for amplification and detection were as described for IL-1α, IL-1β, and TNF-α (23); for IL-10 and HPRT (24); for iNOS (22) and IL-12(p40) (25); and for JE (26): HPRT: 5′-GTTGGATACAGGCCAAGACTTTGTTG; 3′-GATTCAACTTGCGCTCA-TCTTAGGC; probe GTTGTTGGATTGCCCTTGAC; IL-1α: 5′-TGAGATTTTTAGAGTAACAGG; 3′-GGAAGATTGTCAAGAAGAGACGG; probe CCAGATCAGCACCTTACACC; IL-1β: 5′-GGGATGATGATGATAACCTG; 3′-TTGTCGTTGCTTGGTTCTCCT; probe CAGCTGCACTACAGGCTCCG; TNF-α: 5′-CTCCAGCTGGA-

[1]*Abbreviations used in this paper:* BMMø, bone marrow–derived macrophages; HPRT, hypoxanthine-guanine phosphoribosyl transferase; iNOS, inducible nitric oxide synthase; LPG, lipophosphoglycan; MCP-1/JE, monocyte chemoattractant protein; Mtb, *Mycobacterium tuberculosis*; PKC, protein kinase C; STAg, soluble antigen from *Toxoplasma gondii*.

AGACTCCTCCCAG; 3′-GATCTCAAAGACAACCAACTAGTG; probe CCCGACTACGTGCTCCTCACC; iNOS: 5′-CTGGAGGAGCTCCTGCCTCATG; 3′-GCAGCATCCCCTCTGATGGTG; probe CTGGATGAGCTCATCTTTGCC; IL-12 p40: 5′-CGTGCTCATGGCTGGTGCAAAG; 3′-CTTCATCTGCAAGTTCTTGGGC; probe TCTGTCTGCAGAGAAGGTCACA; IL-10: 5′-CGGGAAGACAATAACTG; 3′-CATTTCCGATAAGGCTTGG; probe GGACTGCCTTCAGCCAGGTGAAGACTTT; JE: 5′-TCAGCCAGATGCAGTTAACG; 3′-CAAGAAGGAATGGGTCCAGA; probe TCACCAGCAAGATGATCCCAATGAG.

Cytokine Detection Assays. TNF-α was determined by measuring the cytotoxicity to TNF-sensitive L929 cells of macrophage supernatants recovered 6 h after infection and/or treatment (25). Murine (Mu) rTNF-α was used as standard. IL-12 was assayed in 6-h supernatants by a two-site RIA. Briefly, 96-well plates were coated overnight with 5 μg/ml C15.1.2 Ab in sodium carbonate/bicarbonate buffer (pH 9.5). Plates were washed three times with PBS/0.05% Tween 20. Standard and samples were added to the plates, incubated overnight, washed again with PBS/Tween and incubated for 6 h with ^{125}I-labeled C15.6.7 Ab. Bound radioactivity was assessed by scintillation counting and the amount of IL-12 calculated by reference to a standard curve generated using rMuIL-12 (Genetics Institute, Cambridge, MA). IL-1 was measured as described (27) using the IL-1α/β–dependent cell line T1165.17, obtained from Dr. A. Glasebrook (Eli Lilly, Indianapolis, IN) and Dr. Richard Titus (Colorado State University, Ft. Collins, CO). The cells were maintained in DMEM plus 5% FCS supplemented with human rIL-1α (R&D Systems, Inc., Minneapolis, MN). Cells were plated at 5,000 per well in duplicate wells in 96-well flat bottom plates and test supernatant was added to a final volume of 200 μl. After a 42-h incubation at 37°C, 5% CO_2, the plates were pulsed for 6 h with 1 μCi methyl-[^{3}H]thymidine (5 Ci/mmol) (Amersham Corp.). Proliferation was determined by scintillation counting, and levels were calculated by reference to a standard curve constructed with rMuIL-1α.

Results

Kinetics of Cytokine mRNA Expression in BMMø from BALB/c Mice Stimulated with LPS, Mtb, or L. major Promastigotes. The kinetics of mRNA expression for TNF-α, IL-1α, IL-1β, IL-12(p40), MCP-1/JE, IL-10, and iNOS after exposure to either LPS, Mtb, or serum-opsonized *L. major* metacyclic promastigotes, are shown in Fig. 1, expressed as fold increase over untreated control. Both LPS and Mtb induced strong mRNA expression for each of the genes analyzed. The peak signal for each cytokine was always greater in response to Mtb, except IL-12(p40), for which the LPS- and Mtb-induced signals were roughly equivalent. Whereas positive signals were seen by 2 h, in each case the peak signals were seen at 6 h after treatment, with the exception of IL-1β and IL-10, which displayed equivalent or slightly stronger signals at the earlier time point. By 18 h, transcript levels of each of the genes had returned to just above baseline, again with the exception of IL-1β, which maintained strong expression at each of the time points studied. In contrast to LPS and Mtb, *L. major* promastigotes seemed to largely avoid activation of BALB/c BMMø, despite their efficient attachment to and uptake by these cells (more than 90% of cells infected, more than three promastigotes per cell). No positive signals were detected for either IL-1α, IL-1β, IL-12(p40), or iNOS at any of the time points studied. A weak signal for TNF-α, and

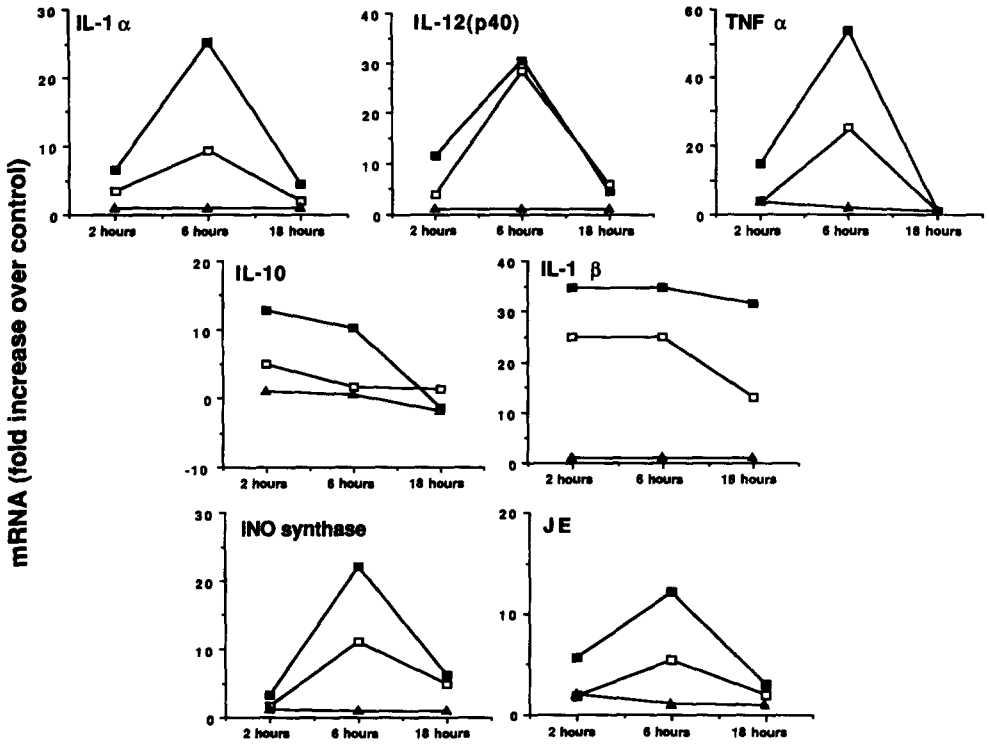

Figure 1. Kinetics of mRNA expression in BMMø from BALB/c mice exposed to LPS (*open squares*), high dose–killed Mtb (*closed squares*), or *L. major* metacyclic promastigotes (*closed triangles*). mRNA levels are expressed relative to levels in untreated control cells at each time point (given an arbitrary value of 1).

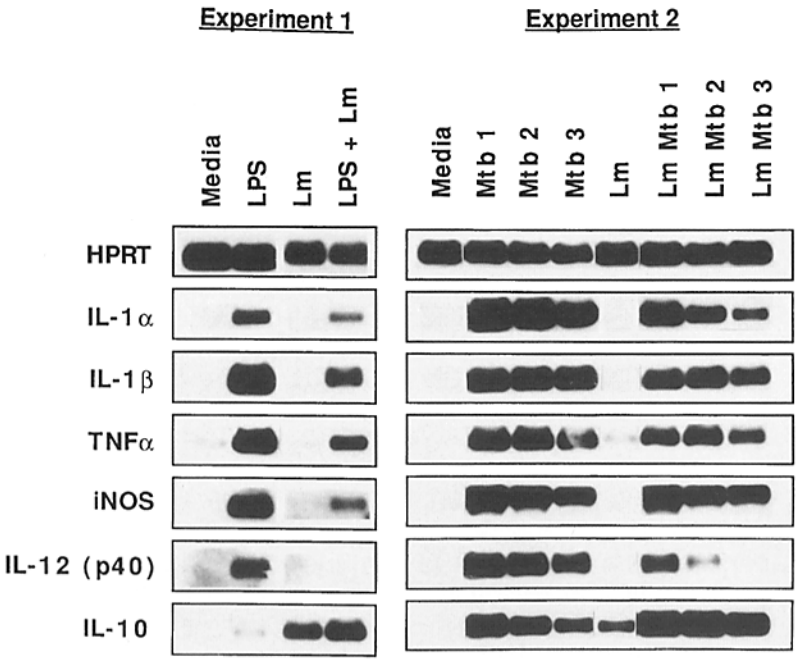

Figure 2. Effect of *L. major* metacyclic promastigotes on LPS- and Mtb-induced expression of cytokine mRNA. BMMø from BALB/c mice were exposed to *L. major* metacyclics (*Lm*), LPS, or killed Mtb at 1,000, 100, and 10 bacteria/macrophage (*Mtb 1*, *2*, and *3*), added either alone or at the same time, as indicated. Semiquantitative RT PCR was done on RNA from each sample, and the products were visualized after Southern transfer. mRNA levels obtained after 6 h of incubation are shown for each of the cytokines, except for IL-10, for which the 2-h time point is shown. Results are representative of six separate experiments.

weak but consistent signals for IL-10 and JE were observed at 2 h, declining to baseline by 6 h. These signals were no stronger when examined at 1 and 24 h (data not shown).

Effect of L. major Infection on LPS- and Mtb-induced BMMø Activation. The effect of leishmanial infection on either LPS- or Mtb-induced mRNA expression in BMMø from BALB/c mice was examined in separate experiments at 6 h after treatment for IL-1α, IL-1β, TNF-α, iNOS, and IL-12(p40), and at 2 h for IL-10. Again, the parasite alone induced no detectable proinflammatory cytokine mRNA, except for very weak expression of TNF-α (Fig. 2). In contrast, parasite activation of IL-10 message was relatively strong in these experiments. LPS and Mtb, at each of the three doses tested, induced strong expression of all cytokine genes, with the exception of IL-10, which was only weakly expressed after 2 h in response to LPS. The effect of the parasite on these responses was a partial inhibition of IL-1α, IL-1β, TNF-α, and iNOS, a strong inhibition of IL-12(p40), and potentiation of IL-10. The IL-12(p40) response to LPS was completely inhibited, as was the IL-12(p40) response to the lowest concentration of Mtb. The opposite effects that the promastigotes had on IL-12(p40) and IL-10 mRNA induction are more quantitatively represented in Fig. 3, which, after normalizing for input RNA, confirms the virtually complete inhibition of the IL-12(p40) response to LPS and lower doses of Mtb, and reveals in addition a strong inhibition of the response to even the highest concentration of Mtb. The upregulation of IL-10 is significant at the lower doses of Mtb.

L. major Promastigotes Evade Cellular Activation of BMMø from Genetically Resistant As Well As Susceptible Mouse Strains. The differential effects that the parasite has on cytokines known to regulate the development of distinct $CD4^+$ T cell subsets suggested a possible mechanism to explain the preferential activation of Th2 cells that occurs in response to *L. major* infection in susceptible BALB/c mice. To explore this possibility further, BMMø from BALB/c as well as genetically resistant B6 and C3H mice were compared for their responses to the parasite alone or in combination with LPS or Mtb. Infection with *L. major* promastigotes failed to induce expression of IL-1α, TNF-α, or IL-12(p40) in cells from either susceptible or resistant mice (Fig. 4). IL-10 mRNA also was not detected, although in these experiments only the 6-h time point was analyzed. Low levels of parasite-induced transcripts for JE were detectable in cells from BALB/c and B6 mice. The effects of the parasite on LPS- and Mtb-induced responses were also similar in the three mouse strains. Slight inhibition of IL-1α and TNF-α was accompanied by complete inhibition of both LPS- and Mtb-induced IL-12(p40) expression in all three mouse strains. Upregulation of IL-10 and JE also occurred in the three mouse strains.

The levels of IL-1α, TNF-α, and IL-12 contained in the BMMø culture supernatants were also determined for the three mouse strains (Table 1). TNF-α and IL-12 were measured in 6-h supernatants from the same cells used for mRNA analyses. Reliable assay of IL-1 required longer incubation, and was determined from a separate experiment in which supernatants were harvested at 18 h. The protein levels closely reflected the pattern of mRNA expression determined by RT-PCR. LPS and Mtb elicited good production of the three cytokines by cells from all three mouse strains. These cytokines were not detectable in supernatants from the cells exposed to *L. major* promastigotes alone, save for the relatively high levels of IL-1α/β produced by the infected cells from C3H mice. Furthermore, the parasite completely inhibited (to background levels) the LPS- and Mtb-induced IL-12 production by cells from all three

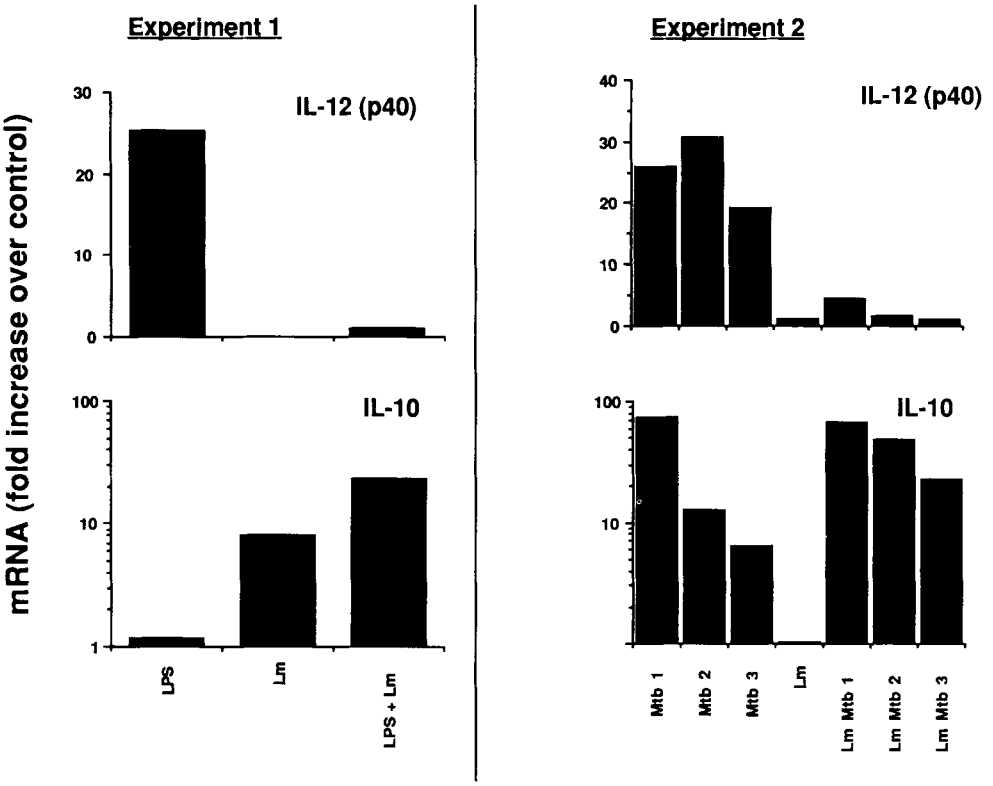

Figure 3. Chemiluminescent signals shown in Fig. 2 are expressed as fold increase over control.

mouse strains. In contrast, TNF-α production was only reduced by half or not at all, and IL-1 production was actually enhanced, again with comparable effects observed in all three mouse strains. Thus the selective effects that the parasite has on IL-12(p40) gene expression and production seem not to be related to the long-term outcome of infection in these mouse strains.

Leishmania Promastigotes Evade Triggering IL-12 Production in BMMø Primed with IFN-γ. Macrophages are considered fully activated when they receive both "priming" and "triggering" stimuli, which in experimental systems typically have been provided by IFN-γ and LPS, respectively (28). If the parasite's inability to activate macrophages for cytokine expression were due to defects confined largely to macrophage priming, then provision of this signal might reveal a triggering stimulus associated with infection. This

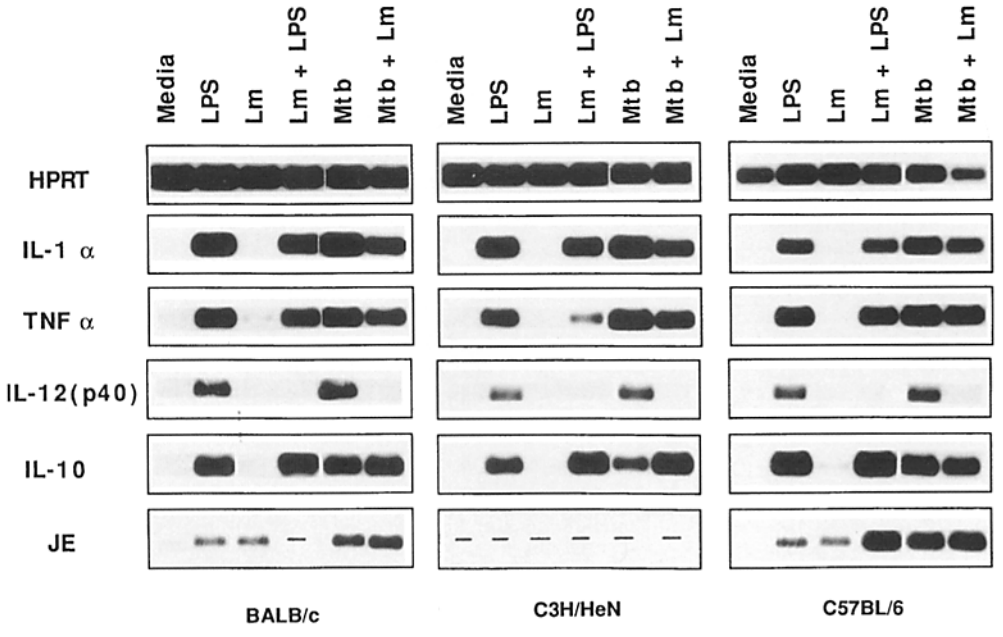

Figure 4. Effect of *L. major* metacyclic promastigotes on LPS- and Mtb- (100 bacteria/macrophage) induced expression of cytokine mRNA by BMMø from BALB/c, C3H/HeN, and C57BL/6 mice. Cells were harvested for RNA at 6 h. Dashes indicate samples for which no data was obtained. Results are representative of two experiments.

Table 1. *Effect of L. major Metacyclic Promastigotes on LPS- and Mtb-induced Cytokine Release by BMMø from Susceptible and Resistant Mice*

	TNF-α*			IL-1 α/β‡			IL-12*		
	C57BL/6	C3H	BALB/c	C57BL/6	C3H	BALB/c	C57BL/6	C3H	BALB/c
		U/ml			*U/ml*			*pg/ml*	
control	0	0	0	6	0	0	135	141	130
LPS	0	1,920	1,920	230	160	31	1,306	1,406	996
Lm	0	0	0	0	210	0	145	139	171
Lm/LPS	480	960	960	1,062	382	78	187	186	191
Mtb 1	480	1,920	1,920	ND	ND	ND	710	531	854
Lm/Mtb 1	480	960	1,920	ND	ND	ND	196	160	181
Mtb 2	480	960	1,920	400	30	52	1,109	724	1,039
Lm/Mtb 2	480	960	960	1,765	140	179	154	130	190

*6-h supernatants.
‡18-h supernatants.

possibility was investigated in BMMø from B6 mice exposed simultaneously to IFN-γ and various microbial stimuli. Levels of mRNA were determined at 6 h, and are shown in Fig. 5 for TNF-α, iNOS, and IL-12, each of which has been shown to be upregulated by IFN-γ (14, 22, 29). Costimulation with IFN-γ increased signals for TNF-α, iNOS, and especially IL-12(p40) in response to LPS, Mtb, and STAg. Costimulation with IFN-γ also upregulated mRNA expression for TNF-α and iNOS in cells infected with *L. major*, indicating that at least for some responses, the parasite can provide a measurable stimulus so long as the cells are adequately primed. In contrast, priming with IFN-γ had no effect on the IL-12(p40) response to the parasite, which remained undetectable. The protein data for this experiment is shown in Table 2. Priming with IFN-γ increased IL-12 production induced by LPS, Mtb, and STAg by approximately twofold. The IL-12 produced in response to *L. major* promastigotes remained at background levels even in the presence of IFN-γ. Preexposure of the BMMø to IFN-γ for 18 h before as well as during infection still failed to induce IL-12 (data not shown). The ability of IFN-γ to upregulate TNF-α production was apparent only for the response to Mtb, although the biological assay failed to detect TNF-α released in response to those stimuli for which the mRNA signals were weak.

The Downregulation of IL-12 Induction by L. major Promastigotes Is Not IL-10 Dependent. IL-10 was originally described as a factor produced by Th2 cells that inhibited the synthesis of cytokines by Th1 cells (30). It has since been shown to be produced by other cell types, including Ly-1 B cells, mast cells, and macrophages, and its effects on IFN-γ production have been shown to be through an inhibition of macrophage accessory cell function rather than a direct interaction with IFN-γ–producing cells (31). IL-10 has been found to inhibit the production of proinflammatory

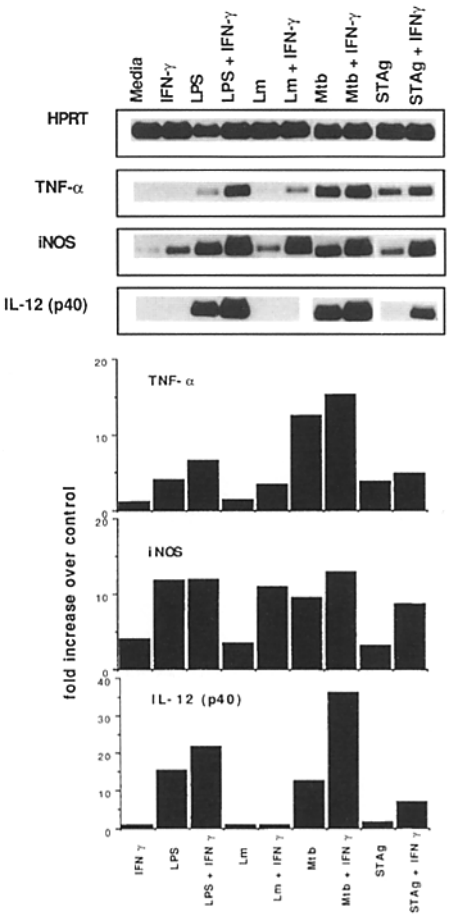

Figure 5. Induction of cytokine mRNA in BMMø from C57BL/6 mice exposed to *L. major* metacyclic promastigotes, Mtb (100 bacteria/macrophage), or soluble antigen from *T. gondii* (*STAg*), during priming with IFN-γ. RNA was harvested for RT-PCR 6 h after treatment. Results are representative of two experiments.

Table 2. *Effect of Priming with IFN-γ on Cytokine Release by BMMø from C57Bl/6 Mice Exposed to L. major Metacyclic Promastigotes and other Microbial Stimuli*

	Media	IFN-γ	LPS	IFN-γ/LPS	Lm	IFN-γ/Lm	Mtb	IFN-γ/Mtb	STAg	IFN-γ/STAg
IL-12 (pg/ml)	172	132	4,079	8,940	259	171	2,055	3,733	289	594
TNF-α (U/ml)	0	0	240	240	0	0	480	960	0	0

cytokines by macrophages, including TNF-α, IL-1, IL-6, and IL-12 (32). As IL-10 mRNA expression was stimulated early in infection (2 h) by *L. major* promastigotes, and its induction by LPS or Mtb was also upregulated by the parasite, the possibility that IL-10 might be responsible for the downregulation of IL-12 synthesis during infection was assessed by analysis of IL-12(p40) mRNA induced in BMMø from IL-10 knockout C57BL/6 mice (Fig. 6). The lack of detectable IL-10 transcripts in the activated cells from the knockout mice is consistent with the fact that the 5′ primer sequence used to amplify IL-10 cDNA binds within the first exon, which has been replaced by a neomycin gene in the knockout mice (18). In both the knockouts and the wild-type controls, no IL-12(p40) mRNA was seen in response to the parasite alone, and in both strains the LPS- and Mtb-induced expression of the gene was strongly inhibited by the parasite (Fig. 6). The apparent greater intensity of the residual IL-12(p40) signals observed in the knockout cells was found to be comparably reduced when normalized and expressed as fold increase over control. The lack of any difference between the strains is reinforced by the IL-12 levels in the culture supernatants. For the wild-type cells, LPS- and Mtb-induced IL-12 production was reduced by the parasite from 2,892 and 1,413 pg/ml, respectively, to 263 and 202 pg/ml. However, for the IL-10 knockout cells, the values for IL-12 were reduced from 3,982 and 2,300 pg/ml to 165 and 97 pg/ml.

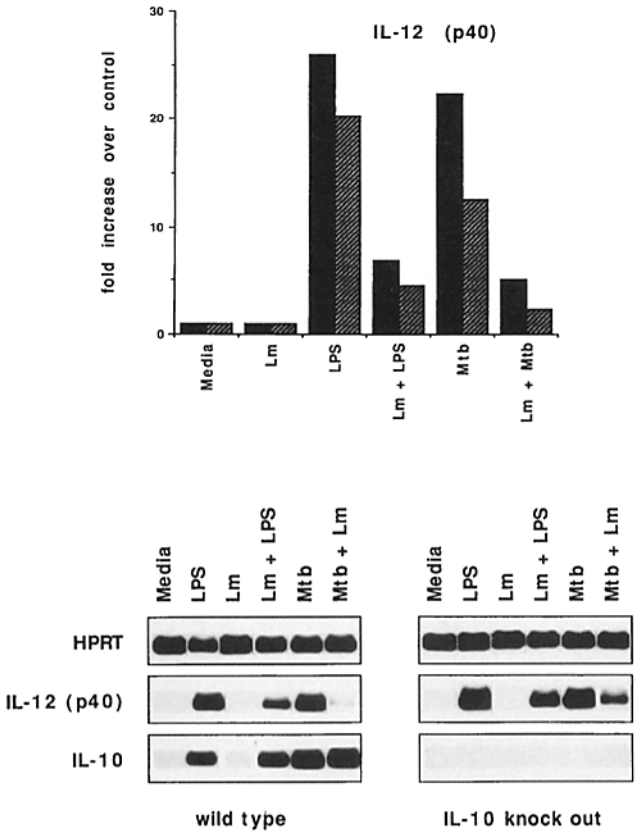

Figure 6. Effect of *L. major* metacyclic promastigotes on LPS- and Mtb- (100 bacteria/macrophage) induced expression of IL-12 and IL-10 mRNA by BMMø from wild-type C57BL/6 and IL-10 knockout mice. RNA was harvested for RT-PCR 6 h after treatment. The signals expressed as fold increase are shown for IL-12 only (*solid bars, IL-10 knockouts; hatched bars, wild-type*). Results are representative of three experiments.

The Ability of Promastigotes to Inhibit IL-12 Activation Is Not Dependent on Expression of the Abundant Surface Lipophosphoglycan. LPG is the major surface glycoconjugate of *Leishmania* promastigotes and has been implicated in the downregulation of protein kinase C (PKC)–mediated signal transduction in macrophages (33). To determine whether LPG plays a role in the inhibition of activation signals leading to IL-12(p40) expression, the Mtb-induced mRNA responses in BMMø from BALB/c mice were examined in the presence of stationary phase promastigotes of *L. major*, *L. donovani*, and an LPG-deficient mutant of *L. donavani*, termed R2D2. The effects of these parasites on cytokine gene expression were similar in each case (Fig. 7). The responses induced by the parasites themselves were negligible, save for the low expression of TNF-α. Their inhibitory effects on Mtb-induced IL-1, TNF-α, and iNOS expression were slight in each case (the absence of an iNOS signal was not observed in a subsequent experiment), whereas each was able to completely inhibit expression of IL-12(p40). Finally, each strain was able to significantly upregulate the IL-10 response. Thus, the requirement for LPG in the differential effects on cytokine gene expression can be ruled out, although the data do not preclude a requirement for glycolipids structurally related to LPG which are still expressed by the R2D2 mutant.

Discussion

Cells of the macrophage lineage play a pivotal role in the development of immune responses to microorganisms, in part through the processing of microbial antigens for presentation to T cells, through the expression of cell surface costimulatory molecules, and through the release of immunoregulatory cytokines. The ability of infected macro-

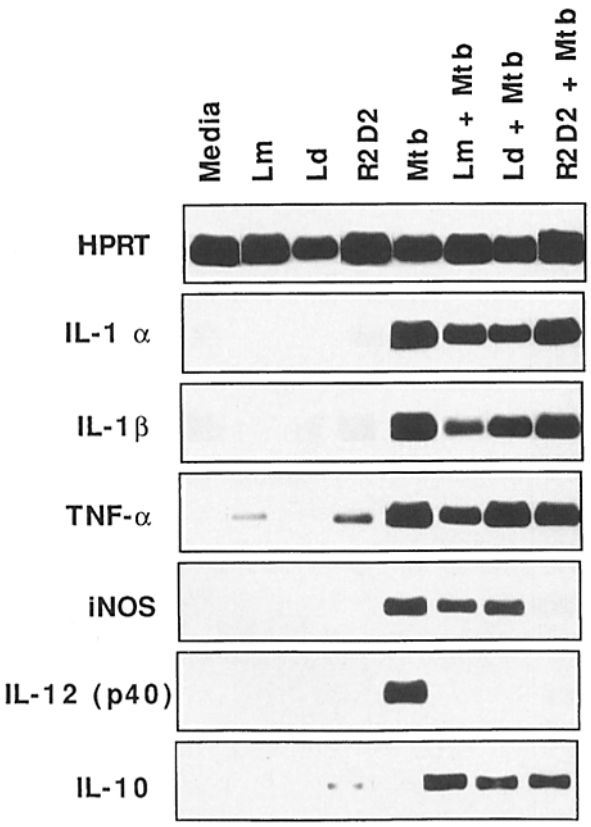

Figure 7. Effect of stationary phase promastigotes of *L. major* (*Lm*), *L. donovani* (*Ld*), or the LPG-deficient *L. donovani* mutant (*R2D2*) on LPS- and Mtb- (100 bacteria/macrophage) induced expression of cytokine mRNA by BMMø from BALB/c mice. RNA was harvested for RT-PCR 6 h after treatment.

phages to present leishmanial antigens to T cells and to drive their activation is well supported (34–36). The present data argue that infected macrophages will drive early T cell activation in the absence of IL-12, thereby avoiding the major physiologic inducer for Th1 development, elevated production of IFN-γ, and activation of macrophages to a microbicidal state. The findings are essentially in agreement with those of Reiner et al. (15), who proposed that *Leishmania* promastigotes avoid initiating activation signals for inflammatory and immunodulatory cytokines generally, and IL-12 in particular, and in this way the survival of the parasite in the vertebrate host is prolonged. The present studies extend these observations by demonstrating that whereas the attachment to and entry of *Leishmania* promastigotes into macrophages proceeds in a remarkably silent manner, of the various cytokine responses examined, only the induction of IL-12(p40) was found to be actively impaired.

Infection of BMMø with serum-opsonized *L. major* metacyclic promastigotes failed to induce detectable levels of mRNA for most of the cytokine genes studied. Weak but positive signals for IL-10 and JE were consistently observed, especially when the response was examined early. Infection of BMMø during exposure to microbial stimuli known to initiate strong activation signals resulted in three general effects: (*a*) strong, and in most cases, complete inhibition of IL-12(p40) mRNA and protein synthesis; (*b*) partial, and in most cases, weak inhibition of mRNA and/or protein for IL-α, IL-1β, TNF-α, and iNOS; and (*c*) upregulation of mRNA for IL-10 and MCP-1/JE. These outcomes suggest that IL-12 production is tightly regulated by an activation pathway(s) that is selectively and efficiently compromised by *Leishmania* promastigotes during infection. The involvement of several alternative induction pathways for TNF-α and IL-β (28, 37, 38) suggests a basis for the partial inhibition of these cytokines during leishmanial infection, in which only the pathway(s) common to IL-12(p40) induction might be impaired.

Selective inhibition of IL-12 synthesis was also observed when BMMø were treated with IFN-γ, which revealed that the parasite could provide a detectable stimulus for TNF-α and iNOS gene expression in primed cells, but still failed to provide a trigger for IL-12. In contrast, exposure of the cells to LPS, killed Mtb, or STAg during priming with IFN-γ resulted in significantly upregulated synthesis of TNF-α, iNOS, and especially IL-12. The ability of *Leishmania*-infected cells to be activated by IFN-γ for intracellular killing, either alone or in combination with other stimuli, is well described (1, 2, 29). The data suggest that the inability of the parasite to induce certain cytokines during infection is due less to the fact that relevant induction pathways are disrupted than to the fact that the parasite alone is unable to provide both the priming and triggering stimuli required for their expression. Other investigators have reported that even primary exposure of macrophages to *Leishmania*, either amastigotes or promastigotes, results in release of significant levels of IL-1 or TNF-α (27, 29, 39, 40). It is not clear why in our studies and that of others (41) the parasite alone failed to stimulate production of these cytokines, although differences in the subpopulation of the macrophages used and/or LPS contamination are possible explanations. These data, nonetheless, reinforce the conclusion that *Leishmania*-infected cells retain the potential to produce most of the cytokines involved in inflammatory and effector functions, including those mediated by IFN-γ.

The reciprocal effects that the parasite has on IL-12 and IL-10 production by BMMø does not seem to explain the preferential activation of Th2 cells during infection of genetically susceptible BALB/c mice, since identical outcomes were observed using BMMø from resistant C57BL/6 and C3H mice. In addition, these effects were not confined to *Leishmania* species with particular clinical associations, since they were seen using both *L. major* and *L. donovani* promastigotes. The avoidance of IFN-γ induction pathways might, instead, underlie the relatively prolonged duration of parasite survival and amplification that is characteristic of leishmanial infections generally, whether they be self-healing or progressive, cutaneous or visceral. Unlike the rapidly self-limiting infections produced by other intracellular pathogens, such as *Mycobacteria*, *Listeria*, *Cryptosporidia*, or *Toxoplasma*, the self-limiting infections caused by *Leishmania* require weeks or months to resolve. *Leishmania* strains able to amplify their numbers by delaying the onset

of host control mechanisms clearly will be favored for transmission. This may be especially true for parasites producing localized cutaneous or mucosal disease, which will have infrequent encounters with their phlebotomine vectors.

There is considerable evidence that the delayed onset of healing typically seen in self-limiting forms of leishmanial disease is due to the slow development of cell-mediated immune response (i.e., delayed production of IFN-γ), rather than, as has been suggested (42), the inability of infected cells to be activated for killing (that is, impaired response to IFN-γ). For example, patients are susceptible to reinfection during the active stages of cutaneous disease, whereas they are resistant after healing has commenced (43, 44, and reviewed in reference 45). Experimentally, evidence for defective IL-12 induction by *Leishmania* is suggested by studies in *scid* mice, which are capable of producing a T cell–independent, IL-12-dependent IFN-γ response to a variety of intracellular bacteria and parasites, including *L. monocytogenes*, *Corynebacterium parvum*, *Salmonella*, *Mycobacteria*, and *T. gondii* (12, 25, 46). In contrast, *L. donovani* promastigotes failed to initiate an NK cell–derived IFN-γ response in spleen cells from *scid* mice, either in vivo or in vitro (47).

Direct evidence for delayed induction of IL-12(p40) transcripts during *L. major* infection was reported by Reiner et al. (15) for both susceptible and resistant mouse strains). In addition, anti-IL-12 antibodies could be administered to C57BL/6 mice as late as 2 wk after infection and still exacerbate disease, consistent with delayed production of IL-12 (48). In more recent studies (49, 50), delayed production of IL-12 in response to *L. major* was confined to C57BL/6 mice, whereas C3H and, surprisingly, BALB/c mice, produced an elevated IL-12 response within 1 d of infection. Whereas the early C3H response was associated with rapid control of the parasite, the delayed response in C57BL/6 mice was associated with larger lesions requiring longer to heal. For BALB/c mice, the induction of Th1 cells and elevated levels of IFN-γ by IL-12 was thought to be prevented by the simultaneous production of inhibitory cytokines.

To what extent do the in vitro outcomes using BMMø grown in M-CSF conditioned medium and used after 6–7 d of adherence, which represent relatively heterogeneous populations of differentiated cells, reflect the in vivo experience with high doses of subcutaneously inoculated parasites? The response of BMMø to infection is certainly consistent with the in vivo outcomes in C57BL/6 mice, which arguably model most closely the course of cutaneous leishmaniasis in natural hosts. In contrast, the early IL-12 response in C3H and BALB/c mice might be explained by strain polymorphisms in the number and tissue distribution of distinct macrophage subpopulations, or cell types such as B cells, keratinocytes or neutrophils, that are each capable of producing IL-12, but that might respond quite differently to the parasite or its products. Recent observations bear indirectly on this point. When leishmanial antigens were presented by GM-CSF–derived macrophages as opposed to M-CSF macrophages, they were able to induce high levels of IFN-γ and resistance to infection in BALB/c mice (51). There is growing evidence from in vitro studies that differences in antigen-presenting cell populations can influence the pattern of $CD4^+$ subset development (52). The ability of a high dose inoculum to stimulate an early IL-12 response in certain mouse strains may reflect events which are more typically delayed during infection in C57BL/6 mice and natural hosts. As the parasite load increases, the parasite or its products might no longer be confined to the same cell types targeted by metacyclic promastigotes at the initiation of infection, such that different patterns of regulatory cytokines emerge.

Although IL-10 was first described as a cytokine that inhibits macrophage-dependent cytokine synthesis by Th1 cells (30), it does not appear to be involved in downregulating IL-12 synthesis by infected macrophages since cells from IL-10 knockout mice still failed to produce IL-12 in response to promastigotes, and were also markedly inhibited by the parasite for expression of LPS- and Mtb-induced IL-12(p40) transcripts and protein. It is possible that IL-10 production by infected cells might impair other accessory cell functions. IL-10 has been found to downregulate expression of B7 and other costimulatory surface molecules (53), and this might be related to the recent observation that B7 is downregulated on *Leishmania*-infected cells (54). Furthermore, since B7/CD28 interactions synergize with IL-12 to induce high levels of IFN-γ (55, 56), the coordinate inhibition of both molecules, whether by related or independent mechanisms, might ensure that IFN-γ induction pathways are avoided. The previously unreported possibility that the parasite can directly activate an IL-10 response in macrophages, might explain the source of IL-10, which has been found to be associated with certain chronic leishmanial infections (57, 58), especially since other Th2-associated cytokines were not also seen.

The possible role of TGF-β in IL-12 downregulation has not been investigated in these studies but should be considered, given the ability of this mediator to potentiate *Leishmania* intramacrophage survival in vitro and in vivo (59, 60). TGF-β is a suppressor of many known macrophage responses, including the oxidative burst (61), microbicidal response to IFN-γ (59), as well as IL-12–initiated IFN-γ production by human neonatal $CD4^+$ cells (62). Its pleiotropic activities may not, therefore, be consistent with the selective effects that the parasite has on IL-12. Furthermore, TGF-β–mediated suppression of LPS-induced TNF-α and IL-1 release by macrophages was found to be late acting (12–16 h; 63); this is not consistent with the defects associated with IL-12(p40) induction in infected cells.

The alternative to agonist-mediated suppression of IL-12 induction is that the parasite or its products directly interfere with specific signal-transducing events. There is substantial evidence that leishmanial infection selectively impairs PKC-dependent signal transduction in host macrophages (64, 65), suggesting that this may be an essential pathway for induction of IL-12. *Leishmania*-infected cells or macrophages treated with promastigote LPG display defective membrane translocation of PKC or defective phosphoryla-

tion of endogenous PKC substrates, respectively. It is clear that since the LPG-deficient mutant, R2D2, was as inhibitory for IL-12 induction as the wild-type strain, LPG is not required for this effect. This result does not rule out the possible role of LPG-related structures, that is, the glycosylinositolphospholipids (66), which are still abundantly expressed by R2D2. The involvement of PKC-dependent signal transduction in IL-12 upregulation and its specific disruption by the parasite are currently being explored.

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