Modulation of In Vitro Monocyte Cytokine Responses to Leishmania donovani
Interferon-γ Prevents Parasite-induced Inhibition of Interleukin 1 Production and Primes Monocytes to Respond to Leishmania by Producing Both Tumor Necrosis Factor-α and Interleukin 1

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Abstract

Cytokines produced by mononuclear cells are important regulatory and effector molecules and evidence has been presented to support a role at least for tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in host defense against Leishmania. In the present study, we examined the production of TNF-α and interleukin 1 (IL-1) by resting and IFN-γ-primed peripheral blood monocytes infected in vitro with Leishmania donovani. Monocytes produced neither IL-1 nor TNF-α during challenge with Leishmania. Cells primed with Leishmania synthesized normal amounts of TNF-α, but had diminished production of IL-1 in response to stimulation with either S. aureus or lipopolysaccharide (LPS). The induction by S. aureus or LPS of IL-1β mRNA accumulation in infected cells was normal despite diminished intracellular or supernatant IL-1 protein and bioactivity. Thus, inhibition of IL-1 production by Leishmania most probably reflected diminished translation of IL-1β mRNA. Pretreatment of cells with IFN-γ abrogated infection-induced inhibition of IL-1 production and primed cells for the production of both IL-1 and TNF-α upon subsequent exposure to Leishmania. These results indicate that L. donovani has evolved the capacity to infect mononuclear phagocytes, without stimulating the production of two potentially host-protective monokines. The ability of IFN-γ to prime monocytes to produce TNF-α and IL-1 in response to infection with Leishmania and to prevent inhibition of IL-1 production may have implications for immunotherapy with this lymphokine. (J. Clin. Invest. 1990. 85:1914–1924.) Leishmania • monocytes • monokines • interferon-γ

Introduction

Cells of the mononuclear phagocyte series perform a large array of functions that are crucial to the integrity of the immune system and for host defense (1–3). In addition to strictly cellular functions such as antigen processing and presentation, antibody-dependent and antibody-independent cellular cytotoxicity and killing of both intracellular and extracellular microorganisms, mononuclear phagocytes also produce and secrete a variety of cytokines that have potent, pleiotropic effects within the host (2). Important examples of the latter are interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α), 1 two cytokines which mediate a large and frequently overlapping repertoire of biological activities (4–8). In particular, IL-1 and TNF-α have been shown to be important elements in the host response to microbial invasion. For example, both IL-1 and TNF-α are produced in response to a variety of microbes and microbial products (4, 6) and in turn these cytokines have been shown to mediate antimicrobial activities both in vitro and in vivo (9–12).

Paradoxically, in spite of the prominent role of mononuclear phagocytes in host defense, a large number of diverse microorganisms have evolved the capacity to establish a state of intracellular parasitism within these cells. This suggests the possibility that in order to insure their own survival, these organisms may use strategies designed to either evade or subvert the regulatory or effector functions of their target cells. Protozoa of the genus Leishmania are an example of a group of organisms that have adapted remarkably well in respect to their ability to infect, propagate and persist within mononuclear phagocytes (13, 14). Given the pivotal roles of mononuclear phagocytes and of their cytokine products in mammalian homeostasis, it is important to understand how these functions may be perturbed during chronic intracellular infection. Moreover, with respect to Leishmania in particular, understanding how cytokine production is modulated in infected mononuclear phagocytes may be of fundamental importance to the immunobiology of the leishmaniasis. Support for this concept derives from recent findings that demonstrated a protective role for TNF-α in experimental leishmaniasis (15) and from studies in which interferon-γ (IFN-γ), a lymphokine that potentiates monocyte cytokine production (16–19), was shown to have therapeutic efficacy in human visceral leishmaniasis (20). Based upon these considerations, in the present study the modulation of IL-1 and TNF-α production by human monocytes during infection with Leishmania donovani (the causative agent of visceral leishmaniasis in the Old World) was examined. The question of whether IFN-γ could modulate human monocyte responses to L. donovani for the production of TNF-α and IL-1 was also studied.

Methods

Leishmania and leismania lysate. Amastigotes of the Sudan strain 2S of L. donovani used in this study were isolated from spleens of male

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1. Abbreviations used in this paper: TNF, tumor necrosis factor.
Syrian hamsters as described in detail previously (21). For use in some experiments, a soluble parasite lysate was prepared by suspending amastigotes in calcium- and magnesium-free Hank's balanced salt solution and subjecting the suspension to six freeze–thaw cycles. The suspension was then centrifuged at 12,500 g for 20 min, and the supernatant was recovered, filtered-sterilized, and adjusted to 1 mg/ml after protein determination by protein assay (Bio-Rad Laboratories, Richmond, CA).

Reagents and chemicals. RPMI 1640 and Dulbecco's modified Eagle's medium (DME, 4,500 mg/liter glucose) were prepared in the media preparation facility at the Terry Fox Laboratory of the Cancer Control Agency of British Columbia, and were supplemented with L-glutamine (2 mM), 2-mercaptoethanol (5 x 10^−3 M), penicillin (100 U/ml), and streptomycin (100 µg/ml). In addition, DME was also supplemented with sodium pyruvate (0.01 mM), Hapes (10 mM), L-arginine-HCl (1.48 mM), folic acid (0.01 mM), and asparagine (0.27 mM). Hanks' balanced salt solution (HBSS), also prepared at the Terry Fox Laboratory, was supplemented with penicillin and streptomycin and Hapes (10 mM) and adjusted to pH 7.4 with 7.5% sodium bicarbonate. Histopaque (no. 1077) and lipopolysaccharide (LPS) (L-3129, Escherichia coli 0127:B8, lot no. 3123-25, B2F-4012) were from Sigma Chemical Co., St. Louis, MO. Fixed S. aureus cells of the Cowan I strain were from Calbiobem-Behring Corp., La Jolla, CA, as Pansorbin (no. 507858) as a 10% (wt/vol) solution in PBS. Methyl-tritiated thymidine was from Amersham, Oakville, Ontario with sp act 5 Ci/mmol. Recombinant human IFN-γ (lot no. K9079A, sp act >2.7 x 10^6 U/mg), recombinant human TNF-α (sp act > 5 x 10^5 U/mg), and the cDNA probes for human IL-1β (22) and human TNF-α (23) were generous gifts from Genentech Inc., South San Francisco, CA.

Cell preparation and culture. Peripheral blood mononuclear cells were isolated from buffycoats (obtained from normal human volunteers by the British Columbia and Yukon Division of the Canadian Red Cross) by centrifugation (800 g for 15 min) over Histopaque. After three washes in HBSS, the cells were resuspended in RPMI 1640 with 10% human serum at 1 x 10^6 viable cells per ml and dispersed into 150-cm² tissue culture flasks (20 ml per flask for the preparation of cellular RNA) and incubated at 37°C in a 5% CO₂/95% air, humidified atmosphere for 45 min. Nonadherent cells were removed by vigorous washing with divalent cation-free HBSS (37°C) and the flasks were repopulated with RPMI 1640 supplemented with 20% fresh human serum for culture with or without the addition of various stimuli. Adherent monolayers prepared in this manner were 89±5% monocytes by morphologic and phagocytic criteria as determined by microscopic examination of Diff-Quik (B4132-1, CanLab, Vancouver, BC) stained preparations.

Monocyte supernatants for IL-1 and for TNF-α assays were generated by culturing 1 x 10^6 peripheral blood mononuclear cells per chamber of eight-chamber Lab-Tek chamber slides (no. 4808, Miles Scientific, Inc., Naperville, IL) in DME/10% heat-inactivated fetal bovine serum (FBS) (lot 1111646, Hyclone Laboratories, Logan, Utah) for 60 min at 37°C in 5% CO₂/95% air. Nonadherent cells were removed by washing as above for flasks, and the chambers were repopulated with DME 10% FBS with or without stimulants. After overnight incubation (12–16 h), cell-free supernatants were collected and either assayed immediately or stored at −70°C. Monolayers prepared in this manner were 86±2% monocytes by both morphologic and phagocytic criteria as described above for flask cultures.

IL-1 assay. IL-1 activity in monocyte supernatants was assayed by the induction of [3H]thymidine incorporation into D10.G4.1 cells in the presence of a suboptimal concentration of concanavalin A (0.625 µg/ml, no. 234367, Calbiobem-Behring Corp.), (24). One unit of IL-1 activity was defined as that which stimulated 50% of maximal [3H]thymidine incorporation by D10.G4.1 cells. Results from individual experiments were normalized against a standard preparation of IL-1 produced from normal human monocytes stimulated with S. aureus as described (25). As used under the experimental conditions of this study, the D10.G4.1 assay was shown to be specific for IL-1. This was demonstrated by neutralization of monocyte supernatants by rabbit polyclonal antisera specific for IL-1α or IL-1β generously provided by Dr. Kenneth H. Grabstein of Immunex Corporation, Seattle, WA. Under the experimental conditions of this study, > 95% of the IL-1 activity detected in monocyte supernatants was neutralized by treatment with the anti–IL-1β serum. In selected experiments, the content of immunoreactive IL-1β in monocyte supernatants was determined by ELISA using an immunoassay kit (03-0096, Cistron Biotechnology, Pinebrook, NJ). The sensitivity of this assay system is 20 pg/ml and there is no cross-reactivity with IL-1α or TNF.

Assay for TNF-α. Immunoreactive TNF-α was measured by ELISA using a rabbit antibody to recombinant human TNF-α as the capture antibody and horseradish peroxidase conjugated mouse monoclonal anti–recombinant human TNF-α as the second antibody. Both antibodies were generously supplied by Genentech, Inc. The standard curve for detection of recombinant TNF-α was linear in the range of 25–1,000 pg/ml.

Northern blot analyses. Total RNA was isolated from cells as described by Chirgwin et al. (26) using cesium chloride gradients. RNA was denatured and size separated on agarose formaldehyde gels as described (27) and transferred to nylon membranes (Hybond-N, Amersham). Blots were prehybridized, hybridized and washed following the manufacturer's protocol. IL-1β and TNF-α mRNAs were detected using human cDNA probes generously provided respectively by Dr. Patrick Gray (22) and Dr. Dianne Pennica (23) of Genentech Inc. DNA was oligolabeled with [32P]dCTP using random primers as described (28).

Statistical analysis. Except where otherwise stated, the results shown are representative data from a minimum of three similar or identical experiments which yielded comparable results. Unless otherwise indicated, differences between experimental groups were examined using Student's t test for independent means.

Results

Human monocyte IL-1 production during infection with Leishmania. Supernatants of freshly isolated, normal peripheral blood monocytes infected with L. donovani for ~12 h showed no increase in IL-1 content when compared to control unstimulated cells (Fig. 1). In contrast, cells stimulated with heat-inactivated S. aureus or with LPS, released large amounts of IL-1 activity into their supernatants. The absence of increased IL-1 activity in the supernatants of leishmania-infected cultures appeared to be related to the failure of monocytes to accumulate IL-1 mRNA in response to infection (Fig. 2). Northern analysis showed that cells stimulated with S. aureus or with LPS for ~12 h accumulated readily detectable amounts of IL-1 mRNA, whereas Leishmania-infected cells, like unstimulated cells, did not.

Inhibition of monocyte IL-1 production by leishmania infection. To examine whether monocytes infected with L. donovani could respond to a second stimulus for the production of IL-1, cells were infected with Leishmania for 4 h and then secondarily stimulated with either S. aureus or with LPS. Supernatants were collected ~12 h after the addition of secondary stimuli and were assayed for IL-1 activity. The results shown in Fig. 1 demonstrate that supernatants of cells preinfected with Leishmania and secondarily stimulated with either S. aureus or with LPS contained significantly reduced IL-1 content when compared to supernatants of noninfected cells. This result was not likely to be related to suppressive factors in monocyte-conditioned media since, as shown in Table I, exactly parallel results were obtained when supernatants (derived from cells identically treated in separate experiments) were assayed for immunoreactive IL-1β by ELISA.
Reduced IL-1 content of supernatants derived from cells preinfected with Leishmania and secondarily stimulated with either S. aureus or with LPS was not related to altered kinetics of IL-1 release. Thus, as shown in Fig. 3, irrespective of the duration of incubation of Leishmania-infected cells with LPS, in comparison to LPS treated control cells, supernatants of infected monocytes had decreased IL-1 activity. In addition, reduced IL-1 content of supernatants derived from infected cells was shown (Table II) not to be related to intracellular accumulation of cytokine.

Additional experiments were carried out to determine whether inhibition of IL-1 production by Leishmania required infection with intact organisms. To examine this question, 4 h before stimulation with S. aureus, cells were either infected with Leishmania or treated with a soluble parasite lysate (prepared as described in Methods). As shown in Table III, cells preinfected with Leishmania had significantly decreased responses to S. aureus for IL-1 production. In contrast, in two of three experiments, pretreatment of cells with the parasite lysate (10 μg/ml) did not significantly affect the IL-1 response to

Table I. Content of Immunoreactive IL-1β in Supernatants of Monocytes Infected with L. donovani and Secondarily Treated with Either S. aureus or LPS

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Immunoreactive IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment No.</td>
</tr>
<tr>
<td></td>
<td>ng/ml</td>
</tr>
<tr>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>&lt;40*</td>
</tr>
<tr>
<td>Leishmania and S. aureus</td>
<td>7,920</td>
</tr>
<tr>
<td>LPS</td>
<td>1,424</td>
</tr>
<tr>
<td>Leishmania and LPS</td>
<td>17,504</td>
</tr>
<tr>
<td></td>
<td>25,696</td>
</tr>
<tr>
<td></td>
<td>6,240</td>
</tr>
<tr>
<td></td>
<td>8,960</td>
</tr>
</tbody>
</table>

Monolayers of human monocytes were prepared and treated as described in the legend to Fig. 1. Cells treated with Leishmania were ~70-80% infected with an average of three amastigotes per cell. 16 h after the addition of stimuli, cell-free supernatants were collected and assayed for IL-1β by ELISA.

* Average of duplicate measurements for each treatment group.
**Cell Treatment**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IL-1 Activity (U/ml $= 10^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td></td>
</tr>
<tr>
<td>-4</td>
<td></td>
</tr>
<tr>
<td>-4</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ld</td>
<td>[24 h]</td>
</tr>
<tr>
<td>LPS</td>
<td>[24 h]</td>
</tr>
<tr>
<td>Ld</td>
<td>[30 h]</td>
</tr>
</tbody>
</table>

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**Figure 3.** Kinetics of IL-1 release into supernatants of monocytes preinfected with *Leishmania*. Cells were prepared and treated as described in the legend to Fig. 1. After the indicated duration of exposure to LPS (1 μg/ml) supernatants were collected and assayed for IL-1 content using the D10.G4.1 cells. The results shown are from one of two identical experiments which yielded similar results, and represent the mean±SD of triplicate determinations for each experimental group. The differences between LPS vs. *Leishmania*-LPS at all time points were significant at $\leq$ 5% level.

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**Table II. Supernatant and Intracellular IL-1 Bioactivities Derived from Monocytes Infected with *L. donovani* and Secondarily Treated with Either *S. aureus* or LPS**

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>IL-1 bioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment No. 1</td>
</tr>
<tr>
<td></td>
<td>Supernatant/intracellular</td>
</tr>
<tr>
<td>Nil</td>
<td>23±15/3±2$^*$</td>
</tr>
<tr>
<td><em>Leishmania</em></td>
<td>19±15/6±5</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>1524±317/104±17$^*$</td>
</tr>
<tr>
<td><em>Leishmania</em></td>
<td>175±97/122±30</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>740±138/165±8$^*$</td>
</tr>
<tr>
<td>LPS</td>
<td>247±135/156±38</td>
</tr>
</tbody>
</table>

Monolayers of human monocytes were prepared as described in the legend to Fig. 1 using 96-well, flat-bottom tissue culture plates and were either untreated or infected with *L. donovani* at a parasite/cell ratio of 10:1. 4 h later cells were either untreated or treated with LPS of *S. aureus*. 16 h after the addition of stimuli, cell-free supernatants and cell lysates were collected as described previously (25) from all treatment groups and assayed for IL-1 activity using D10.G4.1 cells.

*Mean±SD of triplicate determinations for each experimental group.  
$^*$ The differences in IL-1 activities in supernatants of cells treated with *S. aureus* vs. *Leishmania*- *S. aureus* and LPS vs. *Leishmania*-LPS were significant at $\leq$ 2.5% level.

*S. aureus*. In fact, in one experiment pretreatment with the lyase actually augmented the IL-1 response to *S. aureus*. These findings indicated that inhibition of IL-1 production by *Leishmania* required infection with intact organisms.

To analyse further, the mechanism(s) of inhibition of IL-1 production by *Leishmania*, cells were treated exactly as described in Fig. 1, and after 11–12 h of incubation with secondary stimuli, total RNA was isolated and hybridized with a cDNA probe for IL-1β. As shown in Fig. 4, in these experiments cells treated with *S. aureus* (lane 3) or with LPS (lane 2) alone accumulated significant amounts of IL-1β mRNA. Of interest, monocytes preinfected with *Leishmania* and secondarily stimulated with LPS accumulated a level of IL-1β mRNA equal to that detected in cells stimulated with LPS alone (compare lanes 2 and 4), even though there was a significant reduction in the amount of IL-1 detected in the corresponding supernatants. In contrast, in the experiment shown here, monocytes preinfected with *L. donovani* and secondarily stimulated with *S. aureus* (lane 5) had no detectable IL-1β mRNA. A result identical to this was also obtained in a second experiment involving cells from another donor (data not shown). Inhibition of *S. aureus*-induced IL-1β mRNA accumulation in *Leishmania*-infected cells was not, however, a constant finding. Thus, in four additional experiments involving cells from separate donors (one of which is shown in Fig. 5 A) monocytes preinfected with *L. donovani* and secondarily stimulated with *S. aureus* had a level of IL-1β mRNA equal to that detected in cells stimulated with *S. aureus* alone. Again, this result was found despite a significant reduction in
Monolayers of human monocytes were prepared as described in the legend to Fig. 1 using Lab-Tek chamber slides. Cells were either untreated, infected with L. donovani at a parasite/cell ratio of 10:1 or treated with a soluble parasite lysate (10 μg/ml) prepared as described in Methods. After 4 h of preincubation under these conditions, cells were either untreated or treated with S. aureus and 16 h later cell-free supernatants were collected and assayed for IL-1 activity using D10.G4.1 cells.

* ND, not detectable.
† Mean±SD of triplicate determinations for each experimental group.
‡ The differences in IL-1 activities in supernatants of cells treated with Leishmania-S. aureus vs. S. aureus alone were significant at ≤ 1% level.
§ The difference in IL-1 activities in supernatants of cells treated with lysate and S. aureus vs. S. aureus alone was significant at the 2% level.

Table III. Comparative Effects of Infection with Leishmania or Treatment with a Soluble Parasite Lysate on Monocyte IL-1 Production

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>IL-1 bioactivity</th>
<th>Experiment No.</th>
<th>U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Leishmania</td>
<td>ND</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>Lysate</td>
<td>ND</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>S. aureus</td>
<td>8,690±1,487†</td>
<td>4</td>
<td>5,225±1,468</td>
</tr>
<tr>
<td>Leishmania and</td>
<td></td>
<td></td>
<td>1,134±120</td>
</tr>
<tr>
<td>S. aureus</td>
<td>3,511±836‡</td>
<td>5</td>
<td>641±525‡</td>
</tr>
<tr>
<td>Lysate and</td>
<td></td>
<td></td>
<td>535±76‡</td>
</tr>
<tr>
<td>S. aureus</td>
<td>13,692±4972</td>
<td>6</td>
<td>2,830±666</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2,006±357§</td>
</tr>
</tbody>
</table>

Figure 5. Northern blot analysis of IL-1β and TNF-α mRNA in human monocytes. Monocyte monolayers treated as indicated below were lysed in guanidium isothiocyanate ~ 12 h after the addition of S. aureus; RNA was isolated and analyzed as described in the legend to Fig. 2. (A) IL-1β cDNA probe; (B) TNF-α cDNA probe; (C) ethidium bromide stained gel. Lane 1, unstimulated monocytes; lane 2, Leishmania-infected monocytes; lane 3, monocytes stimulated with S. aureus; lane 4, cells infected with Leishmania for 4 h before stimulation with S. aureus.

Figure 4. Northern blot analysis of IL-1β mRNA in human monocytes. Monocyte monolayers treated as indicated below were lysed in guanidium isothiocyanate ~ 12 h after addition of S. aureus or LPS. RNA was isolated and analyzed for IL-1β mRNA (right) as described in the legend to Fig. 2. Lane 1, unstimulated cells; lane 2, LPS (1 μg/ml) stimulated cells; lane 3, S. aureus-treated cells; lanes 4 and 5, cells infected with Leishmania for 4 h before being stimulated with either LPS (lane 4) or S. aureus (lane 5). (Left) Gel stained with acridine orange.

The amount of IL-1 activity detected in the corresponding supernatants. The explanation for the variable effect of Leishmania on IL-1β mRNA levels in these experiments involving secondary stimulation with S. aureus is not clear, but may be related to individual donor variability. Nevertheless, taken together the results of these experiments indicated that (a) inhibition of IL-1 production in response to either S. aureus or LPS was a constant finding in Leishmania-infected cells, (b) in the case of LPS, decreased IL-1 bioactivity and immunoreactive protein in monocyte supernatants was regularly associated with the normal accumulation of IL-1β mRNA in infected monocytes, and (c) with respect to S. aureus the predominant mechanism of inhibition of IL-1 synthesis in Leishmania-infected cells was also postmessage accumulation, but was variably associated with decreased IL-1β mRNA levels as well. Thus, inhibition by Leishmania of the IL-1 response to either S. aureus or LPS was likely related predominantly to mechanisms affecting translation of IL-1β mRNA or possibly to stability of translated protein.

Monocyte TNF-α production during infection with L. donovani. Infection of freshly isolated human monocytes with Leishmania did not result in the induction of TNF-α production. As shown in Fig. 6, supernatants of cells infected with L. donovani for ~16 h had no detectable increase in immunoreactive TNF-α content when compared with control cells. In contrast, stimulation of monocytes with either S. aureus or with LPS was associated with significant increases in TNF-α content. Monocyte supernatants were also assayed for TNF-α bioactivity in the L929 cytotoxicity assay. The results (data not shown) indicated that TNF-α bioactivity in monocyte supernatants paralleled exactly the levels of immunoreactive cytokine shown in Fig. 6.

In contrast to the findings with IL-1, monocytes preinfected with Leishmania responded normally to secondary stimuli for the production of TNF-α. As shown in Fig. 6, supernatants of cells infected for 4 h before exposure to either S. aureus or LPS had levels of immunoreactive cytokine that were equivalent to those found in supernatants of cells treated...
with either *S. aureus* or LPS alone. These results were also confirmed by measurements of TNF-α bioactivity in monocyte supernatants (data not shown). Consistent with these observations, was the finding that the steady-state level of TNF-α mRNA in *Leishmania*-infected, *S. aureus*-treated cells was equivalent to the level of TNF-α mRNA detected in *S. aureus*-induced normal monocytes (Fig. 5 B).

**Modulation by IFN-γ of *L. donovani* effects on monocyte IL-1 production.** The results of the experiments reported above, indicated that *L. donovani* was capable of infecting human monocytes without eliciting the production of either IL-1 or TNF-α. Furthermore, *Leishmania* infection selectively inhibited monocyte responses to secondary stimuli for cytokine production. Thus, preinfection with *Leishmania* inhibited monocyte responses to *S. aureus* and to LPS for the production of IL-1, but not for the synthesis of TNF-α. Because IFN-γ had been shown to upregulate IL-1 synthesis under certain conditions, we asked whether treatment with this cytokine could prevent parasite-induced inhibition of the IL-1 response to a secondary stimulus. As shown in Fig. 7, monocytes

![Graph showing cytokine production](image)

**Figure 6.** Human monocytes infected with *L. donovani* have normal TNF-α responses to *S. aureus* or LPS. Monolayers were prepared and treated, and supernatants were harvested exactly as described in the legend to Fig. 1. Immunoreactive TNF-α was measured by ELISA. The results shown represent the means±SD of values obtained from four identical experiments performed using cells from different donors. ND, not detectable.

![Graph showing IL-1 activity](image)

**Figure 7.** Interferon-γ treatment protects monocytes from inhibition of IL-1 production by *L. donovani*. Monolayers were prepared and treated, and supernatants were harvested and assayed as described in the legend to Fig. 1. Where indicated, cells were treated with IFN-γ (200 U/ml) at the designated time points. The results shown are from one of three identical experiments which yielded similar results. The values represent the mean±SD of triplicate determinations for each experimental group. The difference between *S. aureus* vs. *Leishmania*-infected *S. aureus* was significant at the 1% level. The IL-1 activity detected in the supernatants of cells treated with IFN-γ at -4 h (followed by Ld at 0 h) was not different from the activity in supernatants of cells treated with *S. aureus* alone. The difference in IL-1 activities in supernatants of cells treated simultaneously with IFN-γ and *Leishmania* in comparison to supernatants of cells receiving *Leishmania* (only) followed by *S. aureus* was significant at the 0.1% level. Untreated cells, or cells treated with IFN-γ alone had no detectable IL-1 activities in their supernatants.
preinfected with *Leishmania* for 4 h were markedly unresponsive to *S. aureus* for the production of IL-1. Treatment of cells with IFN-γ for 4 h before infection, however, abrogated *Leishmania*-induced inhibition of the IL-1 response to *S. aureus (n = 6)*. The protective effect of IFN-γ was found to be time dependent. Thus, as shown in Fig. 7, significant enhancement of the response to *S. aureus* (by *Leishmania*-infected cells) was also seen when IFN-γ and *Leishmania* were added simultaneously. On the other hand, when added at progressively later time points (relative to infection) the protective effect of IFN-γ was eventually lost.

To examine whether there were conditions under which monocytes would respond to *Leishmania* alone by producing IL-1, cells were primed with IFN-γ before infection. The results shown in Fig. 8 indicate that pretreatment of freshly isolated monocytes with IFN-γ could prime cells to respond to *Leishmania* with the synthesis of IL-1. Supernatants of cells incubated with IFN-γ for 4 h before the addition of *Leishmania* (for an additional 16 h) demonstrated a significant increase in IL-1 content when compared to supernatants of control nonstimulated cells. Treatment of monocytes with IFN-γ alone did not result in increased supernatant IL-1 activity in comparison to conditioned medium from unstimulated control cells.

The ability of cytokine treatment to prime cells to respond to *Leishmania* by producing IL-1 was also time-dependent. As shown in Fig. 8, the simultaneous addition of *Leishmania* and IFN-γ resulted in significantly less priming for IL-1 production when compared to cytokine pretreatment. When leishmania infection was followed either 2 or 4 h later by the addition of IFN-γ, the ability to convert cells from being IL-1 nonresponders to IL-1 responders was progressively lost. The priming effect of IFN-γ for monocyte IL-1 synthesis in response to *Leishmania* was found to vary amongst monocyte donors. Thus, significant priming was observed in six out of nine experiments using cells from distinct donors. This variability may be related to heterogeneous responses to IFN-γ.

IFN-γ primes monocytes to produce TNF-α in response to *L. donovani*. As shown in Table IV, monocytes primed with IFN-γ produced large amounts of immunoreactive TNF-α in response to infection with *Leishmania* (priming was observed in three out of five separate experiments using cells from individual donors). Treatment of cells with either IFN-γ or *Leishmania* alone resulted in minimal or negligible amounts of TNF-α production (when compared to unstimulated cells). On the other hand, the content of immunoreactive TNF-α detected in supernatants of cells (from three of five donors) primed with IFN-γ for 4 h before the addition of *Leishmania* was increased significantly (from 7- to 30-fold) in comparison to supernatants of cells treated with *Leishmania* alone.

**Discussion**

The results of the present study demonstrate that amastigotes of *L. donovani* are capable of infecting human monocytes without stimulating these cells to synthesize IL-1 or to secrete IL-1 activity into their supernatants. This finding is in agreement both with previous experiments from this laboratory which examined the interaction of this organism with murine macrophages (25) and with the data of Crawford et al. (29).

The latter workers reported that human monocytes infected with *Leishmania major* also did not produce IL-1. On the other hand, these findings are in direct contrast to those recently reported by Cillard and co-workers demonstrating that murine macrophages responded to infection with *L. major* by producing IL-1 (30). The explanation for the divergent findings of these two studies that examined *L. major* is not known. The potential difference, however, between *L. donovani* and *L. donovani*...
Table IV. IFN-γ Primes Human Monocytes to Produce TNF-α in Response to L. donovani

<table>
<thead>
<tr>
<th>Immunoreactive TNF-α</th>
<th>Experiment No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>pg/ml</td>
<td></td>
</tr>
<tr>
<td>Nil</td>
<td>&lt;50</td>
</tr>
<tr>
<td>L. donovani</td>
<td>145</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>36</td>
</tr>
<tr>
<td>IFN-γ + L. donovani†</td>
<td>2,070</td>
</tr>
</tbody>
</table>

Monolayers of human monocytes, prepared as described in the legend to Fig. 1, were either untreated or infected with L. donovani at a parasite/cell ratio of 10:1. Supernatants were removed after 16 h of culture and analyzed for immunoreactive TNF-α by ELISA. For the five experiments shown the infection rate was 88.9±7.8% (mean±SD) with a mean of 8.1±2.9 amastigotes per cell.

* IFN-γ was used at 200 U/ml.
† Monolayers treated with both IFN-γ and L. donovani were incubated with lymphokine for 4 h before infection. The values for immunoreactive TNF-α in supernatants of cells treated with L. donovani alone and those detected in supernatants of cells treated with IFN-γ + L. donovani were log transformed and compared by Student's paired t test (two-tailed). The mean increase in TNF-α content associated with IFN-γ pretreatment was significant at P = 0.04.

The data presented above furthermore establish that cellular unresponsiveness to Leishmania donovani with respect to IL-1 synthesis is related to the absence of IL-1β mRNA accumulation following monocyte infection (Fig. 2). This finding is consistent with and provides an explanation for our prior inability to detect either intracellular, membrane or secreted IL-1 activities associated with leishmania-infected murine macrophages (25).

The data presented herein also indicate that in addition to infecting human monocytes without inducing IL-1 production, L. donovani is capable of inhibiting monocyte IL-1 responses to other stimuli. Inhibition by Leishmania of the IL-1 response to either S. aureus or LPS was likely related predominately to mechanisms affecting translation of IL-1β mRNA or possibly to stability of translated protein. This conclusion is supported by several findings including (a) normal steady-state levels of either S. aureus or LPS was likely related to the presence of transporter mechanisms (26) and (b) decreased supernatant content of IL-1 was not explained by altered kinetics of IL-1 release induced by infection (Fig. 3), and (c) the absence of the intracellular accumulation of IL-1 in infected cells (Table II). Although the predominant mechanism of inhibition of S. aureus-induced IL-1 synthesis in Leishmania-infected cells was post-IL-1β message accumulation, these particular experimental conditions were consistent with the presence of an IL-1β mRNA levels as well (n = 2 out of 6 experiments). The finding of diminished steady-state IL-1β mRNA levels under these conditions may have been related to either decreased gene transcription or accelerated mRNA degradation. The observation that L. donovani inhibited IL-1 synthesis at different levels along the induction pathway, according to the secondary stimulus involved (i.e., S. aureus vs. LPS) is consistent with previous studies in which stimulus-specific regulation of IL-1 production by mononuclear phagocytes was shown. For instance, Kunkel et al. (31) found IL-1 production by murine macrophages to be differentially regulated according to whether the inducing stimulus used was phagocytic or soluble. Furthermore, Fenton and co-workers (32) demonstrated that independent mechanisms regulated phorbol ester vs. LPS induction of IL-1 production by THP-1 cells, by human monocytes and by the HL-60 myelocytic cell line.

Inhibition of monocyte IL-1 production by Leishmania at both the level of mRNA accumulation (in response to S. aureus) as well as posttranscriptionally (in response to LPS or S. aureus), is also consistent with data demonstrating that IL-1 expression is subject to regulatory control at each of these levels. Thus, glucocorticoids have been shown to suppress IL-1β gene transcription and mRNA accumulation in cells of the promonocyte cell line U937 (33, 34). Lew and co-workers also reported suppression of LPS-induced IL-1α and IL-1β mRNA accumulation in adherent human monocytes treated with prednisolone (35), although Kern et al. noted only post-transcriptional suppression of IL-1 production in dexamethasone treated human monocytes in suspension culture (36). Furthermore, both glucocorticoids (33, 36) and elevated levels of prostaglandins (34) have been shown to downregulate IL-1 expression posttranscriptionally through a cAMP-dependent pathway (34, 37). In regard to the latter, we have previously shown that macrophages respond to infection with L. donovani with increased synthesis of prostaglandin E2 (38) and such a mechanism could explain a predominantly posttranscriptional regulation of the IL-1 response to either LPS or S. aureus in Leishmania-infected cells. Attempts to demonstrate such a relationship by treating cells with cyclooxygenase inhibitors such as indomethacin or sodium meclofenamate prior to and during Leishmania infection, did not result in the restoration of the IL-1 response to LPS (data not shown). A negative result from such an experiment does not, however, provide definitive evidence to exclude a role for prostaglandins. Thus, as has been shown by us (38) and by others (39, 40) these putative cyclooxygenase inhibitors lack specificity in that they also inhibit the metabolism of arachidonic acid via the lipoxigenase pathway. This consideration is of particular importance since evidence has been presented to indicate that lipoxigenase products of arachidonic acid promote monocyte IL-1 synthesis (41, 42). The failure of putatively specific cyclooxygenase inhibitors to restore the IL-1 response to LPS in Leishmania-preinfected cells could thus be related to countervailing effects of inhibitors on these dual pathways of arachidonic acid oxygenation.

As was the case for IL-1, the results of the present study furthermore indicate that infection of human monocytes with L. donovani did not result in increased TNF-α activity in monocyte supernatants. This result may be interpreted to indicate that Leishmania did not induce cytokine synthesis, since >90% of TNF-α produced by fresh monocytes is found extracellularly (16). This interpretation is supported by the finding that TNF-α mRNA accumulation was not induced in monocytes in response to Leishmania alone (Fig. 5).

These findings demonstrate, therefore, that neither IL-1 nor TNF-α are produced by human monocytes in response to Leishmania. Of particular interest, however, was the observa-
tion that these two cytokines were not coordinately regulated during infection with *Leishmania*. Thus, in response to sec-
ondary stimuli the production of IL-1 was found to be inhib-
ited in leishmania infected cells, but the production of TNF-α
was unaffected. This selective inhibitory effect of *Leishmania*
on the production of these two cytokines is consistent with the
findings of Burchett et al., indicating that the synthesis of IL-1
and TNF-α in human monocytes are not coordinately regu-
lated (16).

The finding that human monocytes were unresponsive to
*Leishmania* with respect to cytokine production, and that
infection inhibited IL-1 induction in response to secondary
stimuli, prompted examination of whether these events could
be modified by IFN-γ. This approach was based upon data
demonstrating the capacity of this lymphokine to upregulate
IL-1 production by both freshly isolated human monocytes
and by in vitro monocyte-derived macrophages (16–19).
Treatment of cells with IFN-γ before infection, primed cells
to respond to *L. donovani* by producing both IL-1 (*n = 6 out of 9
experiments*) and TNF-α (*n = 3 out of 5 experiments*). Com-
pared to cells that were not treated with lymphokine, the
priming effect of IFN-γ was greater (in absolute, but not rela-
tive terms) for monocyte production of TNF-α (in response to
*Leishmania*) than it was for the synthesis of IL-1 (compare Fig.
8 and Table IV). This result is in agreement with previous
results (16) indicating a quantitatively greater priming effect
of IFN-γ on monocyte production of TNF-α in comparison
to IL-1.

In addition to converting cells from a nonresponsive state
to a responsive one, with respect to cytokine production upon
exposure to *Leishmania*, IFN-γ was also able to abrogate para-
histate-induced inhibition of the IL-1 response to a second stimu-
lus (Fig. 7). Both in the case of priming for cytokine produc-
tion (Fig. 8) and also in preventing inhibition of the IL-1 re-
sponse (Fig. 7), the efficacy of lymphokine treatment was time
dependent. Treatment with IFN-γ before exposure to *Leish-
mania* was required for maximal responses. When lympho-
kine treatment was initiated as little as 2 h after leishmania
infection efficacy was lost.

Although the exact mechanism(s) whereby IFN-γ modu-
lated monocyte IL-1 and TNF-α responses to *Leishmania* are
as yet unknown, this lymphokine has been shown to increase
the duration of transcription of the genes encoding these cyto-
kines when cells were activated by LPS (16). Enhancement by
IFN-γ of cytokine mRNA stabilities were also described (16).
In addition, priming of cells by IFN-γ for increased IL-1 re-
sponses to various stimuli may be related to modulation of
monocyte arachidonic acid metabolism by this lymphokine.
Prostaglandins of the E series inhibit monocyte IL-1 produc-
tion (31, 37) and IFN-γ has been shown to downregulate
monocyte PGE2 synthesis in response to a variety of agonists
(43–46). Modulation by IFN-γ of monocyte PGE2 production
in response to *Leishmania*, therefore, could potentially
explain its capacity to upregulate cytokine production during in
vitro infection.

Data from both the present and from previous experiments
(25) clearly show that the direct interaction of *L. donovani*
with mononuclear phagocytes does not result in the produc-
tion of IL-1. In addition, here we present evidence that TNF-α
is also not produced under these conditions. Considering that
IL-1 and TNF-α represent two of the three (interferon-α being
the third) currently recognized endogenous pyrogens (47),
these data must be viewed in light of the fact that fever is nearly
a constant feature in patients with visceral leishmaniasis (13,
14). At least two explanations may account for this apparent
paradox. First, patients with visceral leishmaniasis have been
found to have circulating immune complexes (48, 49) and the
latter have been shown to induce monocyte IL-1 production
(29, 50). Secondly, it has recently been reported (51) that splenic macrophages obtained from Syrian hamsters infected
with *L. donovani*, when cultured in vitro, produce increased
amounts of IL-1 and TNF-α. In light of the findings of the
present study, it is reasonable to postulate that the production
of IFN-γ locally within the spleen may prime tissue macro-
phages to produce TNF-α or IL-1 or both in response to
*Leishmania*.

Previously, we reported that *L. donovani* inhibited the accu-
mulation of MHC class II mRNA in infected macro-
phages in response to IFN-γ (52). Recent data indicates that
this effect is related to decreased transcription of MHC class II
gen (P. Kwan, W. R. McMaster, W. Ng, and N. Reiner,
manuscript in preparation). These observations, together with
the finding of selective, post-transcriptional inhibition by
*Leishmania* of stimulus-induced IL-1 production, raise ques-
tions regarding the effects of intracellular parasitism on
mRNA translation, the stabilities of translated proteins and the
integrity of signal transduction mechanisms in mononu-
clear phagocytes. Studies which are now underway are directed at
examining these questions including defects in intracellular
signalling which may contribute to abnormal responses ob-
served in infected cells.

When considered in the context of accruing data, the find-
ings of the present study may be of fundamental importance to
the immunobiology of leishmaniasis infections. Thus, based
upon the ability of IFN-γ to promote mononuclear phagocyte
killing of *Leishmania* and a variety of other intracellular path-
genists, clinical trials of immunotherapy with recombinant
IFN-γ in human visceral leishmaniasis were recently under-
taken. Results of these studies indicated that this lymphokine
was of therapeutic benefit in this disease (20). The mechanism
accounting for the efficacy of IFN-γ in this setting may have
been related to the direct induction of monocyte killing of
*Leishmania*. On the other hand, the findings of the present
study, that IFN-γ primes monocytes to produce both TNF-α
and IL-1 in response to *Leishmania*, suggest a potential alter-
avative mechanism by which IFN-γ may mediate its therapeu-
tic effect. Thus, both IL-1 and TNF-α have been shown to
mediate antimicrobial effects against intracellular pathogens
(3, 9–12). Moreover, and of direct relevance to the results of
the present study, are the recent findings of Titus and co-
workers that demonstrated a protective role for TNF-α in ex-
perimental leishmaniasis (15). This suggests the possibility
that priming by IFN-γ for monocyte TNF-α production (and
perhaps IL-1) in response to *Leishmania* is a potential mecha-
nism by which IFN-γ mediates its therapeutic effect in vivo.
Although direct evidence that IL-1 plays a role in the pathogene-
sis of leishmaniasis infections has not yet been presented, it is
reasonable to consider this a likely possibility given the consid-
erable extent to which the biological activities of IL-1 and TNF
overlap (4–8). Furthermore, the ability of IFN-γ to promote
monocyte IL-1 production in response to *Leishmania* may
have other indirect effects on the immune response. Thus,
numerous investigators have demonstrated cellular immune
defects and deficiencies of lymphokine production in visceral

1922  Reiner, Ng, Wilson, McMaster, and Burchett
leishmaniasis (21, 53–55). The precise relationship of these immune deficiencies to inhibition of IL-1 production (which plays a central role in the evolution of a cellular immune response) by Leishmania remains to be defined. Nevertheless, the ability of mononuclear phagocytes primed in vivo with IFN-γ to respond to Leishmania by producing IL-1 could potentially reverse some of these immune defects. Such an effect would favor the evolution of a protective, antileishmanial cell-mediated immune response, which is critical to ultimate cure (13, 14).

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References


