Infection with *Leishmania major* induces interleukin-12 production in vivo

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(Received 28 March 1994; accepted 30 March 1994)

1. Summary

Experimental infections of mice with the protozoan parasite *Leishmania major* provide an excellent model for defining the conditions required for generation of CD4⁺ Th1 and Th2 cells in vivo. Since interleukin-12 (IL-12) has been implicated in the development of Th1 cells, we investigated whether *L. major* stimulates IL-12 production in vitro or in vivo. Surprisingly, macrophages cultured in vitro failed to produce IL-12 following *L. major* infection. In contrast, lymph node cells from C3H mice infected for 2 days with *L. major* produced elevated levels of IL-12. In order to determine if the inability to stimulate IL-12 production was limited to in vitro infections, we infected macrophages in vivo by inoculating *L. major* into the peritoneal cavity. Peritoneal cells isolated 24 h later exhibited a significant increase in the number of cells producing IL-12. In addition, supernatants harvested from these cells following culture contained elevated levels of IL-12. These data indicate that *L. major* infection induces increased IL-12 production in mice.

2. Introduction

Interleukin-12 (IL-12) is produced by macrophages and enhances cytotoxicity and interferon-gamma (IFNγ) production by T and natural killer (NK) cells [1,2]. Recently it has been implicated as a critical cytokine in the development of Th1 cells both in vitro and in vivo [3–5]. Since we previously found that *L. major* infection in the resistant C3H mouse strain is associated with an early NK cell cytotoxic response, the production of IFNγ, early development of a CD4⁺ Th1 response, and early control of the parasites [6], we postulated that IL-12 might play a critical role in the immune response to this infection.

IL-12 is a heterodimeric cytokine. The mRNA for one chain of IL-12, p35, is constitutively produced by several cell types. Gene expression of the other chain, p40, is up-regulated in macrophages, and possibly other cell types such as B cells and neutrophils, following exposure to certain pathogens or their products [7]. To date, only the heterodimer has been found to have biological activity, although the presence of p40 correlates with increased levels of the heterodimer [7]. In the present study, we measured the levels of p40 as well as the presence of biologically active IL-12 in supernatants from cells that were infected in vivo. We found that *L. major* induces the production of both p40 and biologically active IL-12, but only when cells are infected in vivo. These data suggest that in vitro infection does not accurately mimic the events that are occurring in vivo.

3. Materials and Methods

3.1. Mice

Female C3HeB/FeJ mice of 6–8 weeks of age were purchased from Jackson Laboratories and maintained in a specific pathogen-free facility.
3.2. Parasites and infection

*Histoplasma capsulatum* WHO MHOM/IL/80/Friedlin was maintained in Grace’s insect tissue culture medium (Gibco, Grand Island, NY) with 20% fetal bovine serum and 2 mM glutamine. In all experiments, stationary phase promastigotes were harvested and the metacyclic forms were selected using *Arachis hypogaea* agglutinin (Sigma, St. Louis, MO). Mice were infected in the hind footpad with $2 \times 10^6$ metacyclic promastigotes, or intraperitoneally with $5 \times 10^7$ parasites. *Staphylococcus aureus* Cowan I strain (SAC) (Pansorbin; Calbiochem-Behring, La Jolla, CA), *Corynebacterium parvum* (Trudeau Institute, Saranac Lake, NY), and *Listeria monocytogenes* (strain 104035; generously provided by Dr. Daniel Portnoy, University of Pennsylvania School of Medicine) were used to stimulate macrophages in vitro. *L. monocytogenes* was heat-killed for 60 min at 60°C (HKLM).

3.3. Cytokine assays

IL-12 (p40) was measured in a 2-site ELISA using a pair of monoclonal antibodies (mAb) that recognize murine p40. Biologically active IL-12 was measured by a capture assay with a mAb directed against murine p40 bound to a 96-well plate. C3H spleen cells were added to these wells and incubated for 48 h after which IFNγ levels were measured. Values were derived from a standard curve performed with recombinant murine IL-12 (Genetics Institute, Cambridge, MA). The p40 ELISPOT assay was adapted from Morris et al. [8]. Briefly, monoclonal anti-murine p40 was bound to a 96-well plate. Peritoneal cells were added to the wells at various concentrations and incubated for 3 h, after which the plates were washed, and a biotinylated anti-p40 antibody added. After 1 h incubation, the plates were washed and alkaline phosphatase-streptavidin added. After 30 min, the substrate was added in agarose, and the plates were left to develop overnight. The number of spots were counted by visual examination on a dissecting microscope.

3.4. Macrophage cultures

Peritoneal exudate cells were harvested 5 days after injection of 2 ml of thioglycollate. Macrophages ($1 \times 10^6$) were incubated in 24-well plates for 18 h, after which the non-adherent cells were removed and bacteria or *L. major* promastigotes were added at various concentrations. After 24 h supernatants were collected, and the levels of p40 and IL-12 measured.

3.5. Statistical analysis

Each experiment was repeated at least 3 times. Significant differences between groups have been determined by Student’s *t* test.

4. Results and Discussion

In order to determine if metacyclic promastigotes induce IL-12 production following infection of C3H mice, we measured p40 levels in supernatants taken from cultures of lymph node cells harvested 2 days after infection. The production of p40 was significantly higher by cells from infected mice when compared with normal animals, ranging from a 2- to 70-fold increase (Table 1). This increase correlated with our previously reported findings that within the first 2 days of infection there is increased IFNγ production and a significant NK cell cytotoxic response by lymph node cells [6]. These data indicate that *L. major* can induce p40 production in vivo. Since macrophages are the host cell of *L. major*, and also produce IL-12, they would be the likely candidate for the in vivo source of IL-12. To test this possibility directly, macrophages were harvested from the peritoneal cavity of C3H mice and cultured in 24-well plates. *S. aureus* Cowan strain I (SAC), *C. parvum*, *L. monocytogenes* (HKLM) or metacyclic *L. major* promastigotes were added to the macrophages, and supernatants harvested 24 h later. Different concentrations of each pathogen were added to the macrophages; only the data for the concentration that induced optimal IL-12 production are shown (Fig. 1). Increased levels of both p40 and biologically active IL-12 were present in supernatants from macrophages incubated with all of the bacteria. In contrast, when metacyclic promastigotes were added to the macrophages, no

<table>
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<tr>
<th>Experiment No.</th>
<th>p40 (pg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Control mice</td>
</tr>
<tr>
<td>1</td>
<td>418</td>
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<tr>
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<td>270</td>
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</tr>
<tr>
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<td>215</td>
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<td>standard deviation</td>
<td>161</td>
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*p < 0.05
significant p40 or IL-12 was detectable in supernatants from these cultures. We failed to observe IL-12 production under several conditions, including different infection ratios, different sources of macrophages, and opsonization of the parasites with complement (data not shown). There are several possible explanations for the discrepancy between our in vivo and in vitro results. For example, the cells in the lymph node-producing IL-12 might not be macrophages, or the lymph node macrophages might differ in their responsiveness to L. major infection. Alternatively, undefined differences between the in vitro and in vivo conditions of infection might have an influence.

To begin to address this issue, we investigated whether peritoneal macrophages infected in vivo would produce IL-12. L. major metacyclic promastigotes ($5 \times 10^7$) were inoculated into the peritoneal cavity and 24 h later the peritoneal cells were harvested. The cell population included approximately 50–60% macrophages and 20% neutrophils; the remainder were lymphocytes, mast cells and eosinophils. The composition was similar following inoculation of phosphate-buffered saline (PBS). Approximately 25% of the macrophages contained an average of 2.5 parasites. Some cells were immediately tested for p40 production in an ELISPOT assay. Others were cultured in vitro for an additional 24 h, after which the supernatant was collected and p40 and IL-12 measured. As seen in Fig. 2A, there was a dramatic increase in the number of peritoneal cells producing p40 after in vivo infection with L. major. As might be expected, following 24 h of incubation, supernatants from these cells contained significantly more p40 and IL-12 than control supernatants (Fig. 2B–C).

Taken together these data suggest that L. major promastigotes stimulate the production of IL-12 soon after infection in C3H mice. Given the early IFNγ and NK cytotoxic response observed in these animals, this might be expected [6]. However, our in vitro experiments, and those of others [9], indicate that L. major promastigotes fail to stimulate IL-12 production in vitro. It is possible that this inability may be an in vitro artifact, since following in vivo infection there was an increase in the number of cells producing p40 and an increase in the levels of both p40 and IL-12. Alternatively, it is possible that macrophages are not the source of IL-12 in vivo following L. major infection. However, although we have not yet identified the cells within the peritoneal cavity that are producing IL-12, we have found that IL-12 production is associated with the adherent population, implicating macrophages as the probable IL-12 source. In addition, in preliminary experiments we have found that peritoneal cells from scid mice also produce IL-12 following infection, ruling out B cells as an IL-12 source (data not shown). Efforts in our laboratory are underway to confirm that macrophages are the IL-12 producers in this infection.

The differences between the in vivo and in vitro infections that account for our results are unknown. Assuming that macrophages are the IL-12 producers in vivo, it is possible that accessory factors are pre-
sent in vivo that are required for IL-12 production induced by \( L. \text{major} \). Alternatively, a different form of the parasite may be inducing IL-12 in vivo. \( \textit{Leishmania} \) enters the vertebrate host as a metacyclic promastigote which invades macrophages and transforms to an amastigote. Within the macrophage these amastigotes multiply, eventually rupture the cell, and re-invade other cells. It has been suggested that upon re-invasion amastigotes, rather than promastigotes, stimulate IL-12 production in vitro [9]. However, since relatively low numbers of parasites were present within macrophages following in vivo infection in the peritoneal cavity, it is unlikely that within 24 h amastigotes have ruptured macrophages and re-invaded other cells. A more likely explanation may involve the accumulation of a parasite-derived lipophosphoglycan (LPG) within the in vitro macrophage cultures. LPG is a molecule present on the surface of \( \textit{Leishmania} \) promastigotes that helps anchor the parasite within the sandfly vector [10]. The molecule is secreted by the parasites when they are grown in culture and can be detected in vitro by immunofluorescence on the surface of both infected and non-infected macrophages (authors' unpublished observations). Interestingly, it has been reported that LPG is capable of inhibiting macrophage protein kinase C-mediated protein phosphorylation [11]. Following infection of macrophages in vitro a significant number of parasites remains extracellular and secretes LPG which accumulates in the culture. In contrast, it is less likely that LPG accumulates in high concentrations in the peritoneal cavity.

In summary, our data demonstrate that \( L. \text{major} \) infection is accompanied by enhanced production of IL-12. It has been reported that treatment of mice normally resistant to \( L. \text{major} \) with a polyclonal anti-IL-12 antisera enhances susceptibility [12] which we have recently confirmed using mAbs directed
against IL-12. Taken together, these observations indicate that the increased IL-12 production observed in these mice may be critical both for the development of CD4\(^+\) Th1 cells following *L. major* infection and for the development of resistance to infection.

**Acknowledgements**

This work was supported in part by grants from NIH (AI-30073, CA-20833, CA-09140) and the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR). L.Q.V. was partially supported by CNPq, Brazil, on leave from the Department of Biochemistry and Immunology, UFMG, Brazil.

**References**


