

Combined Treatment with Interleukin-12 and Indomethacin Promotes Increased Resistance in BALB/c Mice with Established *Leishmania major* Infections

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Following infection of susceptible BALB/c mice with *Leishmania major*, early production of interleukin-4 (IL-4) is associated with the development of a nonprotective Th2 response and the development of progressive disease. Treatment of mice with IL-12 at the time of infection can promote the activation of a protective Th1 response; however, IL-12 treatment of mice with established infections has little effect on the progress of lesion development. This may be due to a down-regulation of the IL-12 receptor $\beta 2$ chain (IL-12R $\beta 2$) that accompanies the expansion of IL-4-producing Th2 cells. We have examined whether prostaglandins function to regulate in vivo responsiveness to IL-12. Mice treated with indomethacin are responsive to treatment with exogenous IL-12 through at least the first 2 weeks of infection and, unlike control mice treated with IL-12, develop an enhanced Th1-type response associated with increased enhanced resistance to infection. Cells from indomethacin-treated mice also exhibit enhanced production of gamma interferon (IFN- γ) following in vitro stimulation with IL-12. Although in vivo indomethacin treatment did not appear to influence IL-12 production in infected mice, cells from indomethacin-treated mice did express higher levels of IL-12R $\beta 2$, suggesting that prostaglandins may play a role in the loss of IL-12 responsiveness observed during nonhealing *L. major* infections.

Although a majority of inbred strains of mice develop small, self-healing lesions following infection with the protozoan parasite *Leishmania major*, a few mouse strains, such as BALB/c, develop large, nonhealing cutaneous lesions and ultimately succumb to disseminated disease (7, 16). Mice that spontaneously resolve their infections develop Th1-type responses characterized by heightened production of the macrophage-activating cytokine, IFN- γ , while nonhealing BALB/c mice develop Th2-type responses in which interleukin-4 (IL-4) is the dominant cytokine produced by a CD4⁺ effector cells (11, 22, 32). This differential development of Th1- versus Th2-type responses in infected mice has provided an excellent model for the study of factors that regulate the in vivo development of CD4⁺ subsets, and various immunological manipulations have been shown to alter the disease phenotype in susceptible mice. For example, BALB/c mice lacking the gene for IL-10 can control infection with *L. major* (18). In addition, treatment with recombinant IL-12 or with antibodies to IL-4, IL-2, CD4, or transforming growth factor β (TGF- β) will enable BALB/c mice to resolve *L. major* or *Leishmania amazonensis* infection, provided the treatments are administered at the time of parasite inoculation (2, 4, 10, 13, 30, 31, 35, 36). Presumably, these immunological interventions influence the initial pathway of CD4⁺ differentiation into Th1 or Th2 effector cells. In contrast, treatment of infected mice with IL-12 or anti-IL-4 anti-

body after they have developed dominant Th2-type responses does not promote a dominant Th1-type response or healing, suggesting that established effector cell populations are less amenable to immunological manipulation. However, we have shown that treatment with IL-12 or IFN- γ or with anti-IL-4 antibody, when combined with conventional antileishmanial drug therapy, can induce healing in chronically infected BALB/c mice and, importantly, that combined treatment results in a switch from a dominant Th2- to a Th1-type immune response (21, 24, 25).

How drug treatment of infected mice enables IL-12 or anti-IL-4 therapy to promote a Th2-to-Th1 shift in cytokine production is currently unclear. Drug treatment does lead to a marked reduction in parasite numbers that may alter the in vivo antigenic stimulus driving the expansion of a parasite-specific Th2 cell population. In addition, by reducing parasite numbers, drug treatment reduces the accumulation of inflammatory cells at the cutaneous site of infection and adjacent lymphoid tissues. Inflammatory cells, including macrophages, the host cells for *L. major*, are capable of producing multiple factors, such as IL-10, TGF- β , and prostaglandin E₂ (PGE₂), which may modify an immunological response. We have recently shown that treatment of *L. major*-infected CB6F1 mice with anti-TGF- β antibody leads to an increase in nitric oxide (NO) production and a decrease in parasite numbers within cutaneous lesions (20). In addition, combined treatment with IL-12 and anti-TGF- β antibody treatment significantly shifted the balance from Th2- to Th1-type cytokine production, showing that TGF- β may influence the in vivo response to IL-12 treatment (20).

Prostaglandins, which may be produced by multiple cell

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types, including macrophages, also modify immunological responses (28). PGE₂ has been shown to inhibit T-cell proliferation as well as cytokine production by Th1-type cells (1, 3, 6, 13, 19, 23, 34). In addition, it may influence the differentiation of naive T cells to Th1 cells by suppressing IL-12 production and IL-12 receptor (IL-12R) expression (12, 13, 17, 37, 38). We have previously shown that PGE₂ production is elevated during *L. major* infection in BALB/c mice and that in vivo treatment with the cyclooxygenase inhibitor indomethacin can significantly slow the growth of parasitized lesions, although it does not, by itself, induce healing (8, 33). More recently, it has been shown that indomethacin treatment can enhance the production of Th1-type cytokines in *L. major*-infected mice, although the treated mice again did not resolve their infections (5). In addition, in vitro addition of indomethacin to cultures of cells from mice infected with *Leishmania mexicana* upregulates the production of IL-12 and IFN- γ (27).

In this study, we have examined the effects of combined treatment with indomethacin plus IL-12 on *L. major* infections in BALB/c mice. We show that mice treated with this combination therapy can control their infections, while treatment with either indomethacin or IL-12 alone fails to halt disease progression. In addition, we demonstrate that mRNA levels for IL-12R β 2 chain (IL-12R β 2) in lymph nodes (LNs) of indomethacin-treated mice are elevated over those in non-treated mice and that cells from indomethacin-treated mice produce elevated levels of IFN- γ following in vitro stimulation with IL-12. Together, these results suggest that heightened prostaglandin production may play a role in the rapid loss of IL-12 responsiveness that accompanies *L. major* infection in BALB/c mice.

MATERIALS AND METHODS

Parasites and animals. Female BALB/c mice were purchased from The Jackson Laboratories (Bar Harbor, Maine) and were 5 to 7 weeks of age at the time of infection. *L. major* (WHO MHOM/IL/80/Friedlin) was maintained in Grace's insect cell culture medium (GIBCO, Grand Island, N.Y.) containing 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μ g of streptomycin, and 100 U of penicillin G-potassium per ml. Metacyclic promastigotes were selected from stationary-phase cultures with *Arachis hypogaea* agglutinin as previously described (29). Soluble leishmanial antigen (SLA) was prepared from promastigotes (25).

Infections and treatment protocols. Mice were inoculated into one hind footpad with 5×10^5 metacyclic promastigotes. Lesion size was measured with a dial caliper (L. S. Starrett Co., Athol, Mass.) and expressed as the difference in thickness between the infected and the uninfected contralateral footpads. Parasites were enumerated by a limiting dilution assay as previously described in which homogenates of infected lesions were serially diluted in Grace's insect culture medium (Life Technologies, Grand Island, N.Y.) and observed 5 to 7 days later for growth of promastigotes. Parasite numbers are expressed as the negative log₁₀ dilution at which promastigote growth was observed. One day prior to infection, groups of mice were treated with indomethacin (Sigma, Saint Louis, Mo.) administered in drinking water (20 μ g/ml in 0.4% ethanol). Control mice received 0.4% ethanol in drinking water. Indomethacin-treated and untreated mice were inoculated with murine IL-12 (a gift from Genetics Institute, Cambridge, Mass.) delivered directly into the parasitized footpad. IL-12-treated mice received a total of six intralésional injections with 0.2 μ g of IL-12 given every 3rd day starting on day 15 of infection. Control mice received 0.4% ethanol in drinking water or were inoculated with phosphate-buffered saline (PBS) delivered into the parasitized footpad.

ELISA and ELISPOT assays. For determination of total cytokine production, draining LN cells were cultured at 5×10^6 /ml with SLA (50 μ g/ml) for 72 h. IFN- γ and IL-4 levels in supernatants were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (25). Recombinant mIFN- γ and mIL-4 used as standards were generously provided by Genentech (South San

Francisco, Calif.) and DNAX (Palo Alto, Calif.), respectively. NO production from the same supernatants harvested at 72 h was assessed with the Greiss reagent (9). Levels of PGE₂ production in cell supernatants were assayed by ELISA with a commercial kit purchased from Cayman Chemical Company (Ann Arbor, Mich.). The number of IL-12p40-secreting cells in LN and spleen cell suspensions was determined with an enzyme-linked immunospot (ELISPOT) assay. The monoclonal antibodies C17.8 and biotinylated C15.6 were generously provided by Christopher Hunter, University of Pennsylvania, Philadelphia. IL-12-secreting cells were determined in cell cultures following overnight stimulation with SLA (50 μ g/ml).

RPA. Total RNA was isolated from popliteal draining LNs with RNA STAT-60 (Tel-Test B, Friendswood, Tex.) as directed by the manufacturer. mRNA was quantified by RNase protection assay (RPA) with a Riboquant kit (PharMingen, San Diego, Calif.) as directed. A custom probe from PharMingen was prepared with [³²P]UTP and hybridized to 15 μ g of each sample RNA. The protected probe was purified and resolved on 5% denaturing polyacrylamide gel by using Ultra Pure Sequagel reagents (National Diagnostics, Atlanta, Ga.). Dried gels were exposed to a phosphorimaging screen, and protected fragments were visualized with a Phosphor-Imager GS-525 Molecular Imager system (Bio-Rad, Richmond, Calif.). Cytokine mRNA levels were normalized to level L32, and the results are expressed as the increase in message level in experimental mice compared to the level in control mice.

Statistical analysis. Data were analyzed for statistical significance with Student's *t* test. Results were considered significant at $P < 0.05$.

RESULTS

Indomethacin-treated mice control infection following in vivo treatment with IL-12. We have previously shown that combined treatment with IL-12 plus the antileishmanial drug sodium stibogluconate, initiated during the 3rd week of infection with *L. major*, induces healing and a switch from a Th2- to Th1-type response in BALB/c mice. Here, we have used a similar treatment protocol to test whether substitution of indomethacin for sodium stibogluconate could, when combined with IL-12, induce the same protective effect. The results shown in Fig. 1 demonstrate that it does. Although mice treated with IL-12 or indomethacin alone exhibited slowed lesion development, neither independent treatment dramatically altered the overall course of disease. However, when indomethacin and IL-12 treatment were combined, mice appeared capable of controlling disease through at least week 8 of infection. Analysis of parasite numbers at 8 weeks showed that only the group of mice treated with both IL-12 and indomethacin had significantly reduced levels ($P < 0.05$) of infection compared to those in control mice (Fig. 2).

Treatment with indomethacin and/or IL-12 alters the immunological response. Since treatment with IL-12 and indomethacin, either individually or in combination, slowed lesion development, we examined whether these treatments also altered the profile of Th1- or Th2-type cytokine production. As would be expected, draining LN cells from nonhealing control mice produced low levels of IFN- γ and high levels of IL-4, characteristic of a dominant Th2-type response (Fig. 3). Cells from mice treated individually with IL-12 or indomethacin produced higher levels of IFN- γ and lower levels of IL-4, consistent with their reduced lesion size, although these alterations in cytokine production were not sufficient to promote healing. The most dramatic shift in cytokine production was noted in cultures of cells from mice receiving the combined treatment. LN cells from mice treated with indomethacin and IL-12 produced high levels of IFN- γ and low levels of IL-4 compatible with the development of a dominant Th1-type response (Fig. 3). In addition, cells from these healing mice

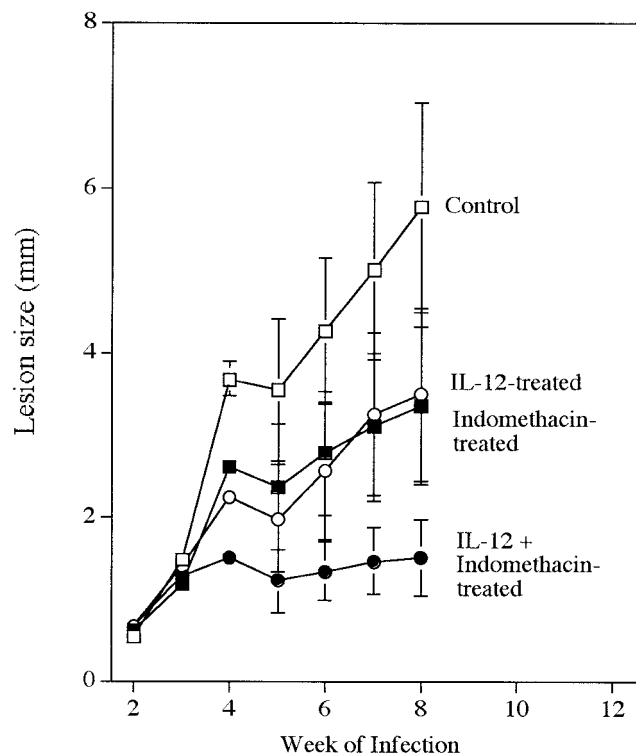


FIG. 1. Effect of indomethacin and/or IL-12 treatment on infection. BALB/c mice were given either indomethacin (20 μ g/ml in 0.4% ethanol) or 0.4% ethanol in drinking water starting 1 day before infection with 2×10^5 *L. major* metacyclic promastigotes. Treated and control mice received a total of six intralésional injections of 0.2 μ g of either IL-12 or PBS given every 3rd day starting on day 15 of infection. Lesion size was measured weekly for 8 weeks and expressed as the difference in thickness between the infected and the uninfected contralateral footpads. Values are the mean \pm standard deviation of four to five mice per group and are representative of results of two separate experiments.

produced elevated levels of NO₂, indicative of enhanced NO production required for macrophage microbicidal activity against intracellular parasites (Fig. 4).

We also examined cytokine message levels by RPA in draining LNs at weeks 2 to 3 of infection in an effort to understand how in vivo indomethacin treatment altered responsiveness to IL-12. Although we noted a general trend of increased message levels for inducible NO synthase (iNOS), IL-18, and IFN- γ , the results were variable, and none of the differences noted in treated and untreated mice were significant (Fig. 5). It should also be noted that IL-18 message levels do not reflect levels of active IL-18 protein production. No increases in message levels for IFN- γ or IL-12p40 over those in naïve mice were detected in either group of infected mice. However, a small, but significant, increase in mRNA levels for IL-12R β 2 was noted in mice receiving indomethacin (Fig. 5). We also enumerated IL-12-producing cells in LNs of infected mice. In keeping with our observation on IL-12p40 mRNA levels, indomethacin treatment did not appear to increase the number of IL-12-producing cells in infected mice (Fig. 6).

Cells from infected, indomethacin-treated mice produce more IFN- γ in response to in vitro stimulation with IL-12.

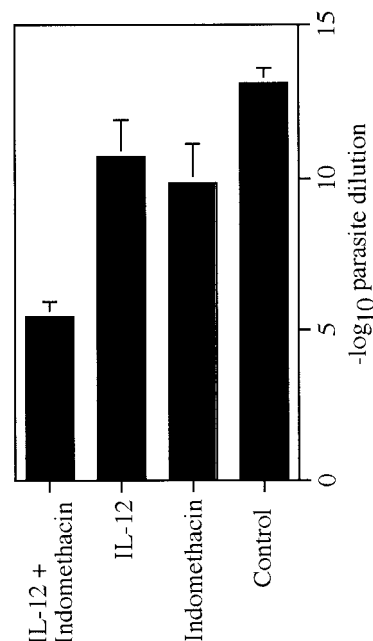


FIG. 2. Parasite burden in infected mice. Numbers of lesion parasites at week 8 of infection were determined by limiting dilution assay and expressed as the negative log₁₀ dilution at which promastigote growth was observed. Error bars represent the mean \pm standard deviation of four to five mice per group.

Since IL-12 therapy promoted resistance in indomethacin-treated mice, we next examined whether cells from these mice were more responsive to IL-12 stimulation in vitro. LN cells from 4-week-infected mice were cultured with SLA and IL-12, indomethacin, or a combination of IL-12 plus indomethacin, and culture supernatants were assayed for levels of IFN- γ production. The results in Fig. 7 show that IL-12 stimulation induced higher levels of IFN- γ production by cells from indomethacin-treated mice. In addition, addition of indomethacin to in vitro cultures further enhanced IFN- γ production, particularly by cells from indomethacin-treated mice. The effects of in vitro indomethacin treatment on IFN- γ production, even in cultures of cells from mice receiving in vivo indomethacin therapy, are not surprising, since in vivo treatment did not totally block PGE₂ production. At week 3 of infection, levels of PGE₂ in cultures of draining LN cells from three untreated, infected mice were 820 ± 80 pg/ml, compared to 448 ± 72 pg/ml in cultures of cells from three indomethacin-treated mice. Addition of indomethacin to in vitro cultures reduced PGE₂ levels to approximately 200 pg/ml in all cultures. The ability of IL-12 to promote enhance IFN- γ production by cells from indomethacin-treated mice was noted in cultures of cells from mice infected for 2 weeks; however, by week 6 of infection, cells from both control and indomethacin-treated mice responded poorly to IL-12, and no differences in IFN- γ production were noted in cultures of treated versus nontreated animals (data not shown). These results suggest that the positive effects of in vivo indomethacin treatment were eventually lost with increased time of infection.

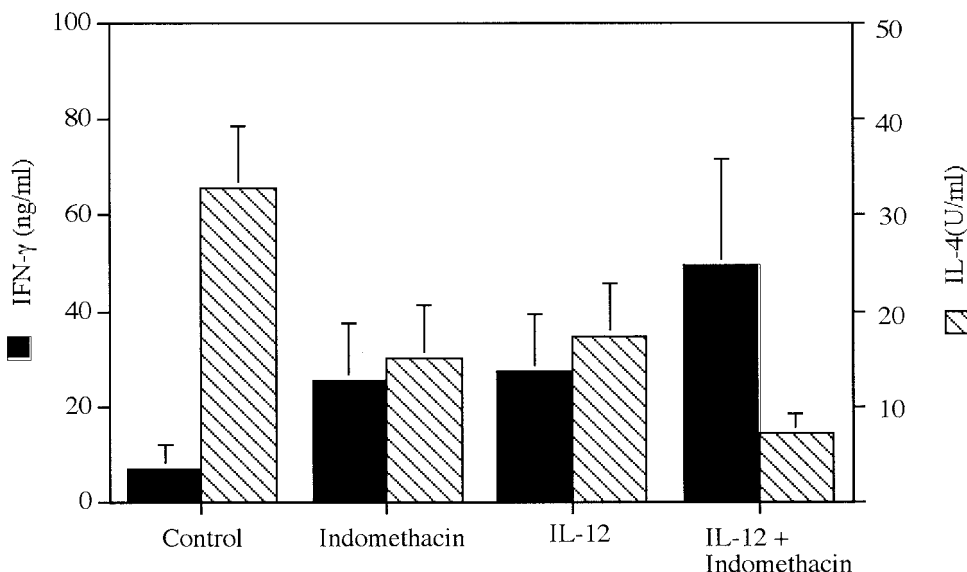


FIG. 3. Cytokine production in LN cells. LN cells from the mice in Fig. 1 were assayed for cytokine production at week 8 of infection. IL-4 and IFN- γ levels were measured in cultures by ELISA following in vitro stimulation of cells with SLA. Error bars represent the mean \pm standard deviation of four to five mice per group and are representative of results from two studies.

DISCUSSION

The events that influence the activation of a Th1- versus Th2-type response during *L. major* infection are complex. BALB/c mice infected with *L. major* develop a dominant Th2-type response in which the population of antigen-specific CD4⁺ effector cells produces high levels of IL-4 and low levels of IFN- γ . Message levels for IL-4 are rapidly elevated after infection in BALB/c mice, and compared to resistant strains of

mice, cells from infected BALB/c mice produce small amounts of IFN- γ , presumably because early production of IL-4 suppresses the expression of the IL-12R β 2 (14, 15). Loss of expression of the IL-12R, in turn, could block IL-12 signaling required for development of a population of Th1-type cells (14). Still, mice engineered to express the transgene for IL-12R β 2 still fail to control infection and develop a Th2-type response, suggesting that the loss of IL-12R β 2 expression by T cells is not the reason, per se, that infected mice fail to develop a protective Th1-type response (26). However, this study is complicated by the observation that continued expression of

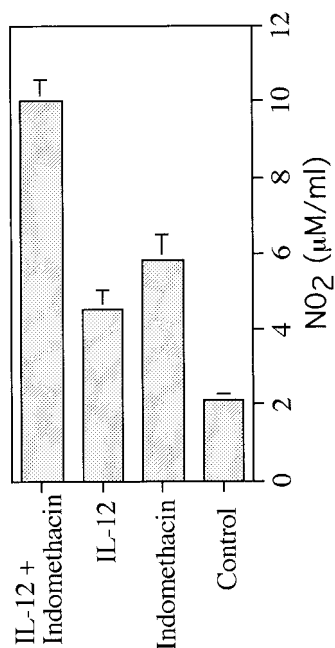


FIG. 4. NO₂ production by LN cells from mice at week 8 of infection as determined by the Greiss assay.

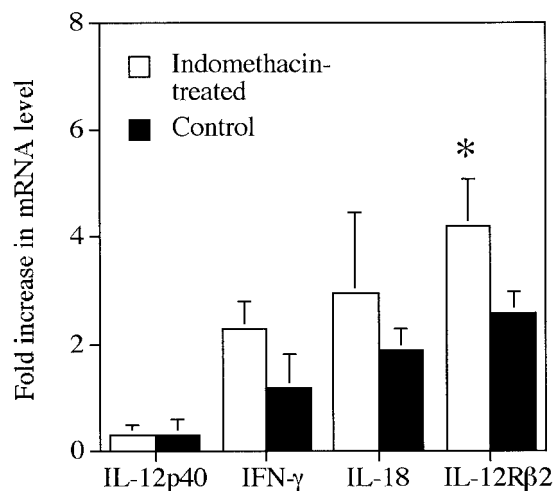


FIG. 5. Cytokine mRNA levels in LNs at week 3 of infection. Message levels were determined by RPA analysis and are expressed as the fold increase over mRNA levels in LNs of naive mice. Values represent the mean \pm standard deviation of three mice. *, significantly different from the value in control mice ($P < 0.05$).

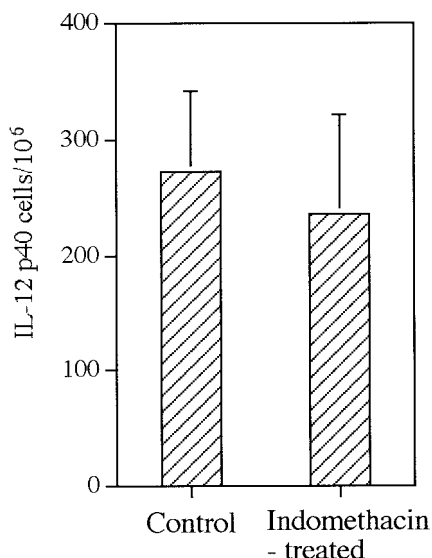


FIG. 6. IL-12p40 production in LNs from control and indomethacin-treated mice. LN cells from control and indomethacin-treated mice were assayed at week 4 of infection for IL-12p40 production by direct ELISPOT assay. The data are expressed as mean frequency per 10⁶ LN cells ± standard deviation of three or more mice per group.

the IL-12R on Th2 cells may actually promote enhanced IL-4 production (26). Thus, loss of response to IL-12 still provides a plausible explanation as to why IL-12 treatment fails to promote healing if given to mice with established infections.

Although IL-4 production during early stages of infection appears to be central to the down-regulation of IL-12Rβ2 expression on CD4⁺ T cells resulting in a state of unrespon-

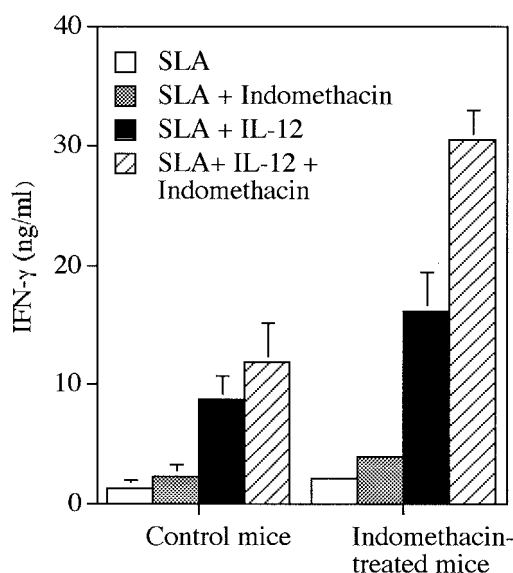


FIG. 7. Effects of IL-12 and/or indomethacin on in vitro IFN-γ production by cells from control and indomethacin-treated mice at week 4 of infection. Cells were stimulated with SLA in the presence or absence of indomethacin (2 μg/ml) and/or IL-12 (1 ng/ml) for 72 h. Values represent the mean ± standard deviation of three to four mice per group.

siveness to IL-12, other factors, such as PGE₂, may play a role in this process. PGE₂ is known to suppress multiple responses, including T-cell proliferation and Th1 cell differentiation (28). It can also suppress the production of IL-12 by human monocytes and dendritic cells and inhibit the expression of IL-12R by human monocytes (37, 38). We have previously shown that cells from BALB/c mice infected with *L. major* produce large amounts of PGE₂ that can suppress in vitro proliferation by parasite-specific T cells, an effect that could be reversed by culturing cells with the cyclooxygenase inhibitor indomethacin (33). Addition of indomethacin to cultures of antigen-stimulated cells from *L. mexicana*-infected mice has been shown to promote increased production of both IL-12 and IFN-γ (27). Importantly, in vivo administration of indomethacin to *L. major*-infected mice can moderate the course of lesion development and promote an enhanced Th1-type response, although indomethacin treatment by itself does not induce BALB/c mice to control infection (5, 8).

In this study, we confirm our previous observation that indomethacin treatment during *L. major* infection can slow the expansion of parasitized lesions and also confirm the observation of others that cells from treated mice produce increased amounts of IFN-γ and NO and reduced amounts of IL-4. We also show that IL-12 treatment initiated during the 3rd week of infection can promote enhanced resistance and the development of a dominant Th1-type response in mice given indomethacin, but not in untreated control mice. The capacity of in vivo IL-12 treatment to promote a more vigorous Th1-type response in indomethacin-treated mice is mirrored by the fact that cells from indomethacin-treated mice produce increased amounts of IFN-γ following in vitro stimulation with parasite antigen and IL-12 compared to cells from untreated mice. Enhanced in vitro responses to IL-12 were observed in cultures of cells from mice infected for 2 or 4 weeks. However, the differential capacities of cells from treated and untreated mice to respond to IL-12 in vitro were lost by week 6 of infection, suggesting that indomethacin treatment may slow, but not prevent, the loss of responsiveness to IL-12.

The reason that in IL-12 promotes resistance in indomethacin-treated mice or that cells from indomethacin-treated mice exhibit enhanced responsiveness to IL-12 in vitro is unclear. Although PGE₂ can suppress IL-12 production, we could find no evidence that either the number of IL-12p40-producing cells or the level of mRNA for IL-12p40 was increased in mice following indomethacin treatment. However, cells from treated mice are clearly more responsive to exogenous IL-12, and we did observe a small, but significant, increase in message levels for IL-12Rβ2 following indomethacin treatment. As noted above, IL-12R expression during infection is presumably maintained on IFN-γ-producing Th1 cells, but lost in Th2 cells by a mechanism that appears to involve IL-4 signaling. Since PGE₂ can directly suppress IL-12Rβ2 expression on T cells, it is possible that inhibition of PGE₂ production by indomethacin directly enhances IL-12R on antigen-activated cells. Alternatively, inhibition of PGE₂ production, by promoting a modest increase in the Th1-type response, may help maintain IL-12 responsiveness during infection by altering the balance of positive (IFN-γ) and negative (IL-4) regulators of the IL-12R expression. Increased production of IFN-γ following IL-12 stimulation may thus reflect increased numbers of Th1-type

cells in cultures of cells from indomethacin-treated mice rather than any direct effect of indomethacin on IL-12R β 2 expression. Either way, this study demonstrates that PGE₂ plays a role in the activation and maintenance of a Th2-type response and, importantly, illustrates that inhibition of endogenous PGE₂ production may have beneficial effects on immunotherapeutic treatments for leishmaniasis.

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