Increased Capacity for Interleukin-2 Synthesis Parallels Disease Progression in Mice Infected with Leishmania major

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Lymph node cells of BALB/c mice with progressive leishmaniasis produced sixfold more interleukin-2 (IL-2) in culture than those of healing C57BL/6 mice. IL-2 synthesis also increased in C57BL/6 mice made susceptible by IL-12 or gamma interferon deficiency. However, IL-2 mRNA levels in vivo did not reflect IL-2 production in vitro. Because IL-2 contributes to the pathogenesis of progressive leishmaniasis, the functional significance of these findings should be further explored.

The mouse model of cutaneous leishmaniasis provides a well-characterized system wherein spectral disease processes are mediated by distinct cytokine responses (13). Susceptible BALB/c mice fail to contain local cutaneous infection by Leishmania major due to biased expansions of interleukin-4 (IL-4)-producing CD4+ T cells that block gamma interferon (IFN-γ)-dependent parasiticidal responses (13), whereas resistant C57BL/6 mice produce IFN-γ without IL-4 and are self-healing. Progressive leishmaniasis is also dependent on the in vivo effects of IL-2, as anti-IL-2 monoclonal antibody (MAb)-treated BALB/c mice fail to make IL-4 and subsequently heal (5, 8). Because increased IL-2 production in susceptible hosts might further contribute to progressive leishmaniasis, we examined in vitro correlates of IL-2 synthetic capacity of the responding lymph node cells.

Only small amounts of IL-2 were present in the supernatants of antigen-stimulated lymph node cultures from healing mice during pilot studies, a finding compatible with cytokine neutralization and internalization by soluble and membrane-associated IL-2 receptors (IL-2R), respectively (6). This prompted us to examine whether the technique of antibody-mediated IL-2 receptor blockade would enhance steady-state levels of IL-2, control for the potential variability in cytokine sequestration by these receptors, and therefore provide a better measure of total cytokine production. A similar approach was previously used for measuring IL-4 synthesis in this model (7, 15). To test this concept first in uninfected animals, BALB/c and C57BL/6 mouse spleen cells (5 × 10^7/ml) were cultured for 48 h in Dulbecco's modified Eagle medium–10% fetal bovine serum media (BioWhittaker Inc., Walkersville, Md.) alone or in media containing mitogenic concentrations of concanavalin A (Con A) (2 µg/ml) or anti-CD3 MAb (2C11, 1 µg/ml). Replicate cultures contained MAb specific for the IL-2 receptor or IL-4 receptor (10 µg of MAb PC61 or M-1/ml, respectively) or for the IFN-γ receptor (5 µg of GR-20/ml). These concentrations of antibody were optimal in a dose-titration study (data not shown). Anti-IL-2 receptor antibody increased IL-2 levels three-fold in unstimulated splenocyte cultures and nearly 30-fold in response to Con A or anti-CD3 (Fig. 1), and IL-4 concentrations were similarly affected by specific receptor blockade, increasing three- to 10-fold when anti-IL-4 receptor MAb was added to Con A- or anti-CD3-activated cells. Although the GR-20 anti-IFN-γ receptor MAb is a well-characterized antagonist of the IFN-γ receptor (11), addition of 5 to 20 µg of purified antibody/ml failed to increase steady-state levels of IFN-γ by more than 10% (Fig. 1).

We next examined whether IL-2 production differed in response to progressive or healing leishmaniasis. Female C57BL/6 and BALB/c mice were infected subcutaneously in the feet with 2 × 10^6 promastigotes of L. major (World Health Organization strain WHOM/IR/-/173) prepared from stationary phase culture as described previously (14). After 4 weeks of infection, popliteal lymph node cells were harvested and cultured with 10 µg of soluble leishmania antigen/ml as described previously (7), in the absence or presence of anti-receptor antibodies. Steady-state levels of cytokine in the culture supernatant after 48 h were measured by specific enzyme-linked immunosorbent assay (Pharmingen, San Diego, Calif.) (Fig. 2). BALB/c mouse lymph node cells cultured with antigen alone accumulated three times more IL-2 than C57BL/6 mouse cultures, but this difference increased to fourfold in the presence of PC61. Although IL-2 production by uninfected lymph node cells was no different in the two mouse strains, infected BALB/c mouse lymph node cells produced threefold more IL-2 than those of C57BL/6 mice as early as 2 weeks after infection, a difference that increased to sevenfold at 4 weeks postinfection (Table 1). BALB/c mouse IL-2 levels remained proportionately increased following culture of lymph node cells with either PC61 or another anti-IL-2 receptor (alpha subunit) antibody, 7D4 (Table 1).

The enhanced IL-2 productive capacity of mice with non-healing leishmaniasis was not strain specific, as IFN-γ deficient C57BL/6 mice also produced markedly greater amounts of IL-2 with disease progression (Fig. 2). As previously reported (16), IFN-γ knockout (KO) and BALB/c mice also produced 20-fold more IL-4 than infected wild-type C57BL/6 mice, although these differences were only evident following the addition of anti-IL-4 receptor MAb to culture (7). Addition of anti-IFN-γ receptor antibody had no significant effect on steady-state levels of this cytokine. The high spontaneous release of cytokines by heavily infected BALB/c and IFN-γ KO mice has been attributed to the abundance of endogenous leishmania antigen in the lymph node preparations (7). In another experiment, eightfold increases in IL-2 productive capacity were observed in C57BL/6 mice with progressive disease.
following treatment with neutralizing anti-IL-12 MAb (IL-2 levels of 0.16 ± 0.11 and 1.42 ± 0.19 ng/ml following culture with PC61 MAb for control and anti-IL-12-treated mice, respectively).

Despite the increasing and outcome-disparate production of IL-2 protein in culture over the course of infection, lymph node IL-2 mRNA expression failed to change significantly after infection in either BALB/c or C57BL/6 mice (Fig. 3A). Furthermore, lymph node IL-2 mRNA levels were no different in C57BL/6, IFN-γ KO, and BALB/c mice infected for 4 weeks (Fig. 3B) despite changes in IL-4 mRNA and IFN-γ expression that closely paralleled spontaneous or antigen-induced IL-4 production in culture.

**FIG. 1.** Anti-IL-2R and anti-IL-4R MAb s enhance enzyme-linked immunosorbent assay quantitation of IL-2 and IL-4 in mitogen-stimulated cultures of normal spleen cells. Splenocytes from uninfected BALB/c (B) and C57BL/6 (C) mice were cultured in triplicate with unsupplemented media or media supplemented with mitogenic concentrations of Con A or anti-CD3 MAb. Anti-receptor antibodies specific for the IL-2 receptor (PC61, 10 μg/ml), the IL-4 receptor (M-1, 10 μg/ml), or the IFN-γ receptor (GR-20, 5 μg/ml) were added to replicate cultures. Increasing GR-20 concentrations to 20 μg/ml had no additional effect on IFN-γ levels. Data represent the mean ± standard error of the mean concentrations (in nanograms per milliliter) of IL-2, IL-4, and IFN-γ in the absence (open bars) or presence (filled bars) of their respective anti-receptor antibodies.

**FIG. 2.** Progressive leishmaniasis in susceptible BALB/c or C57BL/6 IFN-γ KO mice is associated with markedly increased production of IL-2. C57BL/6 mice, IFN-γ KO C57BL/6 mice, and BALB/c mice (n = 5 mice per group) were infected with L. major for 4 weeks before harvest of draining lymph nodes. Lymph node cells were cultured in media alone or media containing soluble leishmania antigen (SLA). Replicate cultures contained anti-IL-2R, anti-IFN-γR, or anti-IL-4R MAb at 10 μg/ml. Data represent the mean ± standard error of the mean concentrations of each cytokine present in supernatants after 48 h of culture in the presence or absence of their respective anti-receptor antibodies. The findings obtained for BALB/c and C57BL/6 are representative of three additional studies.
These studies demonstrate the increased capacity of lymph node cells from mice with progressive leishmaniasis to produce IL-2 in culture compared to self-healing mice. Enhanced IL-2 synthesis was dependent on disease outcome and not strain background, as IL-2 production similarly increased in C57BL/6 mice rendered susceptible by anti-IL-12 treatment or IFN-γ deficiency (7, 16). This suggests that IL-2 production was regulated in response to increased parasite load or local cytokine environment (9). Consistent with this, preliminary studies demonstrate that IL-2 responses are disproportionately enhanced only in lymph nodes draining the infected lesions and not in cultures of spleen cells (data not shown). The presence of IL-2-producing cells in BALB/c mice progressively infected with L. major had been previously determined by limiting dilution analysis of secreting cells (12), but this is the first report to show a specific increase in IL-2 productive capacity relative to healing mice.

The technique of receptor blockade to enhance the measurement of cytokine production had been previously reported for analysis of human IL-2 (1) and murine IL-4 (7). Although differences in IL-2 accumulation between healing C57BL/6 and nonhealing BALB/c mice were apparent without receptor blockade, the addition of anti-receptor antibody provides important information indicating that these differences were not caused by enhanced cytokine uptake or neutralization in C57BL/6 mice related to disproportionate receptor function and/or synthesis. These antibodies presumably disrupt cytokine binding to soluble IL-2R and IL-4R alpha subunits produced in abundance during leishmaniasis (2, 3) or by cultured lymphocytes (4, 6). Alternatively, a similar increase in IL-2 steady-state concentrations provided by 7D4 MAb, which disrupts receptor function and does not block IL-2 binding, may instead favor internalization as the major mechanism for cytokine sequestration in these studies (10). The inability of anti-IFN-γ R MAB to affect IFN-γ levels, despite potent antagonism of receptor-ligand binding (11), is unexplained but suggests that this technique cannot be extended reliably to all cytokines. Regardless of mechanism, the technique of receptor blockade provided novel and significant insights into the biology of IL-2 in murine leishmaniasis and might be generally applicable for the study of selected cytokines in other disease processes.

We conclude that the capacity of lymph node cells to produce IL-2 is increased in response to progressive infection with L. major. This is of interest because IL-2 is necessary for progression of this disease in susceptible hosts (5, 8), and because it demonstrates unexpected heterogeneity in the T-cell phenotype of an otherwise Th2-dominated host response. However, conclusions regarding correlative differences in the in vivo production of this cytokine cannot be reached without further study. In particular, the constitutive expression of IL-2 mRNA in lymph nodes through infection, regardless of disease outcome or strain background, needs to be reconciled with the

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>IL-2 production (ng/ml) in cultures of lymph node cells stimulated with:</th>
<th>Soluble leishmania antigen</th>
<th>Soluble leishmania antigen plus antibody</th>
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</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>Media</td>
<td>0.05 ± 0.03</td>
<td>0.13 ± 0.09</td>
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<tr>
<td>BALB/c</td>
<td></td>
<td>0.13 ± 0.03</td>
<td>0.13 ± 0.11</td>
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<tr>
<td>C57BL/6</td>
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<td>0.24 ± 0.02</td>
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<tr>
<th>Infected (weeks postinfection)</th>
<th>IL-2 production (ng/ml)</th>
<th>Soluble leishmania antigen</th>
<th>Soluble leishmania antigen plus antibody</th>
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<tbody>
<tr>
<td>BALB/c (2)</td>
<td>0.39 ± 0.05a</td>
<td>0.47 ± 0.05b</td>
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<td>C57BL/6 (2)</td>
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<td>BALB/c (4)</td>
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<tr>
<td>C57BL/6 (4)</td>
<td>0.04 ± 0.03</td>
<td>0.18 ± 0.08</td>
<td>0.71 ± 0.07</td>
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* Data represent the means and standard errors of the means for quadruplicate cultures. Poptical lymph nodes were harvested at 14 days after infection of mice (n = 4) in the hind feet with L. major promastigotes. Inguinal, axillary, and submandibular lymph nodes were pooled from uninfected mice (n = 4). ND, not done.

# Data significantly different (P < 0.05) from values for infected C57BL/6 cultures.
marked increases in IL-2 synthesis observed in culture. The regulation and extent of the IL-2 response in leishmaniasis need further study to determine if this cytokine serves only as a required cofactor for IL-4 synthesis, regardless of the amount of IL-2 present, or if expanded IL-2 production in vivo proportionately augments deleterious Th2 responses that mediate the immunopathology of this infectious disease.

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REFERENCES