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Responses to *Leishmania donovani* in Mice Deficient in Interleukin-12 (IL-12), IL-12/IL-23, or IL-18

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**Interleukin-12 (IL-12) orchestrates acquired resistance in intracellular *Leishmania donovani* infection in the liver, inducing gamma interferon and, in turn, macrophage activation and parasite killing. Nevertheless, testing in IL-18−/− mice compared to wild-type mice and in IL-12p40−/− compared to IL-12p35−/− mice also suggested both early-acting (IL-18) and late-acting (IL-23) antileishmanial effects independent of IL-12.**

In experimental *Leishmania donovani* infection in the liver, granuloma assembly, activated macrophage parasite killing, and responses to conventional antimony (Sb) chemotherapy are regulated by mechanisms which govern gamma interferon (IFN-γ) production (7, 19, 21, 35). Although capable of IFN-γ-independent action (2, 18, 32), interleukin-12 (IL-12 [IL-12p70, composed of p35 and p40 subunits]) is a primary stimulus for *L. donovani*-induced IFN-γ (2, 15, 17, 32); thus, each of the preceding antileishmanial effects is also regulated by IL-12 (2, 17). Nevertheless, other cytokines, acting alone or with IL-12, also help to shape acquired resistance and/or promote IFN-γ secretion (3, 5, 8, 13, 14, 16, 18, 30, 31, 33, 34); IL-2, IL-4, and tumor necrosis factor, for example, have already been identified as being active in the initial defense against *L. donovani* (14, 20, 29).

To test roles of two other potential IFN-γ-inducing cytokines, IL-23 and IL-18−/− (8, 13, 25, 26, 31), the following types of female mice were infected with 1.5 × 107 intra-venously injected, hamster spleen-derived *L. donovani* amastigotes (1 Sudan strain): (a) wild type (WT), IL-12p35−/− (deficient in IL-12 alone), IL-12p40−/− (deficient in IL-12 and IL-23, the latter composed of p40 and p19 (25) and IFN-γ−/− (BALB/c background), and (b) WT, IL-18−/− and IFN-γ−/− (C57BL/6 background) (24). WT BALB/c and C57BL/6 mice respond similarly to *L. donovani* (21) and, as anticipated, showed initial susceptibility and then self-cure by week 8 (Fig. 1). This acquired resistance response is associated with induction of IL-12p40 and IFN-γ mRNA expression in liver tissue (2, 11, 22) and increases in IFN-γ and IL-12p40 and p70 in serum at weeks 2 to 4 (17, 23, 27, 29, 32). In livers of BALB/c mice, used here as representative of WT animals, p19 mRNA (IL-23 marker) was detected in uninfected mice, and expression was not increased by *L. donovani* infection (week 3, semiquantitative reverse transcription-PCR normalized to HRPT expression, not shown). While IL-18 mRNA was also expressed constitutively (10), its expression increased at weeks 2 and 4 in infected animals (Fig. 2).

IL-12p35−/− mice show enhanced susceptibility to *L. donovani* (18, 24) and, as illustrated in Fig. 1A, developed high-level, noncuring infection. Since IL-23 and IL-18 are preserved in p35−/− mice (1, 9, 13, 26), apparently, neither could compensate for IL-12's overall antileishmanial effect (2, 12, 17, 18). In addition, initial kinetics of parasite replication in IL-12p35−/− mice and IL-12p40−/− mice were similar, indicating little consequence from the additional absence of IL-23 in p40−/− mice. However, at week 12, a late-acting IL-23 effect was uncovered, as parasite burdens were appreciably higher in p40−/− mice (*P* < 0.05), reaching that level at week 12 in IFN-γ−/− mice (Fig. 1A).

In contrast, IL-18−/− mice controlled liver infection by week 12 (Fig. 1B). However, susceptibility to *L. donovani* was clearly increased initially, compared to WT animals (e.g., at week 4), and liver parasite burdens were still 13-fold higher at week 8 in IL-18−/− mice. These results point to a separate IL-12/IL-23-independent role for IL-18, since IL-12 and presumably IL-23 expression is intact in IL-18-deficient mice (12, 26). While the latter controlled infection at week 12, IFN-γ−/− mice (19, 24) infected in parallel did not (Fig. 1B), indicating stimuli other than IL-18 for IFN-γ production in this model (see the next paragraph).

Increased IFN-γ levels were detected in serum on days +14 and +21 in both infected BALB/c and C57BL/6 WT mice (Fig. 3). Attesting to IL-12's role in IFN-γ secretion in *L. donovani* infection (2, 15, 17, 28), serum IFN-γ was undetectable initially in both IL-12p35−/− and p40−/− animals, although low levels were detected on day +28 at 20 ± 19 and 8 ± 8 pg/ml, respectively (four mice per group). In contrast, IFN-γ levels in infected IL-18−/− mice were not different from WT controls (*P* > 0.05). While the measuring of IFN-γ in serum is a useful marker of Th1-cell-type responses, the physiologic implication of activity in serum (versus in situ IFN-γ expression at the infected tissue focus) is unknown. However, in view of initially enhanced susceptibility to *L. donovani* in IL-18−/− mice (Fig. 1B), preserved IFN-γ secretion suggested an early-acting, IFN-γ-indepen-

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dent antileishmanial effect for endogenous IL-18, as reported in other models (26). Such a mechanism might involve the effects of tumor necrosis factor (32).

In addition to macrophage activation and control of infection, the IFN-γ/H9253-mediated, IL-12-driven Th1-cell response also induces granuloma assembly in parasitized liver (17, 21, 28) and regulates the leishmanicidal activity of Sb (19). Therefore, seeking differences between IL-12p40/H11002/p40/H11002/mice and between IL-18/H11002/mice and WT mice, we examined histologic reactions (Fig. 4) and Sb-induced parasite killing (Table 1).

Granuloma assembly in the liver correlated with outcome of infection: (i) BALB/c and C57BL/6 WT mice generated numerous mature-appearing granulomas by weeks 2 through 4 at >90% of parasitized foci, (ii) few granulomas developed in either IL-12p35−/− or p40−/− mice by weeks 8 through 12, and (iii) while initially suppressed at week 2, IL-18−/− animals expressed a granulomatous response by week 4 which was well developed by week 8 (Fig. 4). The tissue responses in IL-12p35−/− and p40−/− mice were not discernibly different. In both, heavily infected foci showed few recruited mononuclear cells, nearly indistinguishable from the absent inflammatory reaction in livers of BALB/c IFN-γ−/− mice 8 to 12 weeks after infection (not shown) (21). Similarly, and also akin to IFN-γ−/− mice (19), the parasite-killing response to Sb chemotherapy was comparably impaired in IL-12p40−/− and p35−/− mice (Table 1). In contrast, IL-18−/− animals responded normally to treatment, likely reflecting intact production of IFN-γ, which regulates the Sb effect (19).

While IL-12p40−/− mice are known to be susceptible to L. donovani (28, 36), this and a previous study with IL-12p35−/− mice (18) make it clear that the central position occupied by IL-12 in acquired resistance to L. donovani cannot be compensated for by other cytokines, including IL-18. At the same time, however, our results also suggest that the spectrum of cytokines which exert IL-12-independent regulatory roles in visceral infection (5, 10, 19, 20, 35) can be expanded to include IL-18 and probably IL-23. Since animals deficient in IL-23 alone (e.g., p19−/− mice) (8, 25) have not yet been tested, a role for IL-23 in the absence of IL-12 can only be inferred from the data derived in IL-12p40−/− mice. In this setting, IL-23 exerts an apparent antileishmanial effect in late-stage visceral infection. Since p35−/− mice, deficient in IL-12 alone, showed near-absent IFN-γ and granuloma responses, testing in p19−/− mice will be important to better clarify IL-23’s role and mechanism which may involve induction of cytokines other than IFN-γ (8). In contrast, the IL-18-dependent antileishmanial mech-

FIG. 1. Outcome of L. donovani infection in the liver measured microscopically in tissue imprints and expressed as Leishman-Donovan units (LDU) (24). Results are from two to four experiments in each group of mice and indicate mean ± standard error of the mean values for 7 to 14 mice per time point. At weeks 8 and 12, LDU results for WT mice were 112 ± 21 and 2 ± 1, respectively, in BALB/c mice (A) and 78 ± 15 and 28 ± 6, respectively, in C57BL/6 mice (B). For panel A, the P value is <0.05 for p40−/− and p35−/− mice versus WT mice at weeks 3 to 12. For panel B, the P value is <0.05 for IL-18−/− versus WT mice at weeks 2, 4, and 8.
anism identified here primarily acted early in infection before becoming dispensable, and influenced early granuloma assembly but not IFN-γ secretion. Eventual control of infection and intact IFN-γ secretion have also been reported in cutaneous Leishmania major infection in these same IL-18 /IL-11002/IL-11002 mice (12). IL-18 and IL-23 are pleiotropic cytokines (4, 9, 13, 25, 31); thus, multiple mechanisms may underlie their effects. In addition, the recent demonstration that IL-12p40/IL-11002/IL-11002/IL-11002/IL-11002 mice, deficient in IL-12, IL-23, and IL-18, retain IFN-γ-dependent intracellular antimicrobial activity (6) also suggests the presence of still other mechanisms for IFN-γ induction and macrophage activation.

TABLE 1. Leishmanicidal responses to chemotherapy a

<table>
<thead>
<tr>
<th>Type of Sb-treated mouse</th>
<th>Liver parasite burden (LDU) b</th>
<th>Sb-induced killing (%)</th>
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<tbody>
<tr>
<td>BALB/c WT</td>
<td>826 ± 71</td>
<td>89</td>
</tr>
<tr>
<td>IL-12p35−/−</td>
<td>1,444 ± 162</td>
<td>9°</td>
</tr>
<tr>
<td>IL-12p40−/−</td>
<td>1,085 ± 122.3</td>
<td>17°</td>
</tr>
<tr>
<td>C57BL/6 WT</td>
<td>1,055 ± 99</td>
<td>82</td>
</tr>
<tr>
<td>IL-18−/−</td>
<td>1,806 ± 116</td>
<td>86</td>
</tr>
</tbody>
</table>

a Two weeks after infection (day 14), liver parasite burdens were determined and mice were injected once with Sb (500 mg/kg of body weight). Results are from two to three experiments and indicate mean ± standard error of the mean values for 7 to 13 mice per group per time point. Day +21 results for untreated mice (0% killing in all groups) were omitted for brevity.

b LDU, Leishman-Donovan units.

c P value of <0.05 versus Sb-treated WT mice.

FIG. 2. IL-18 mRNA expression in WT BALB/c liver tissue on days +14 and +28 after infection. Real-time PCR results, normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression, are for three mice per group in the single experiment performed, and indicate mean n-fold increases (± standard errors of the means) relative to the day 0 result, arbitrarily assigned the value of 1. The P values were <0.03 at days +14 and +28 versus day 0.

FIG. 3. Serum IFN-γ levels before and 14 and 21 days after L. donovani infection. Enzyme-linked immunosorbent assay results in panels A and B indicate mean ± standard error of the mean values for four mice per group from the single experiment performed. In calculating mean values, an enzyme-linked immunosorbent assay result of <31 pg/ml (lower limit of detection) was arbitrarily assigned the value of 0.
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