Antagonizing Deactivating Cytokines To Enhance Host Defense and Chemotherapy in Experimental Visceral Leishmaniasis

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In experimental visceral leishmaniasis, inhibition of interleukin 10 (IL-10) signaling enhances Th1-cell-associated responses, promoting gamma interferon (IFN-γ) secretion, granuloma assembly, macrophage activation with substantial liver parasite killing, and synergy with pentavalent antimony (Sb) chemotherapy. To determine if inhibiting other suppressive cytokines has similar therapeutic potential, Leishmania donovani-infected BALB/c mice were injected with anti-IL-4 monoclonal antibody or receptor fusion antagonists of IL-13 or transforming growth factor β (TGF-β). Targeting IL-13 or TGF-β enabled inhibition of L. donovani replication but little parasite killing; anti-IL-4 had no effect. None of the three antagonists promoted IFN-γ production, granuloma maturation, or Sb efficacy. Excess IL-13 and TGF-β exacerbated liver infection; however, effects were transient. Among IL-10, IL-4, IL-13, and TGF-β, cytokines capable of disabling Th1-cell mechanisms (including those which support chemotherapy), IL-10 appears to be the appropriate target for therapeutic inhibition in visceral L. donovani infection.

In visceral leishmaniasis, a disseminated protozoal infection, resident macrophages in liver, spleen, and bone marrow are preferentially targeted and support parasite replication. In the susceptible host, intracellular infection progresses until chemotherapy is given or, alternatively, a T-cell-dependent response emerges to induce acquired resistance (43). In experimental Leishmania donovani infection in susceptible mice, acquired resistance in the liver is initially regulated by multiple Th1- and Th2-cell-associated cytokines (11, 41, 43, 54, 57, 59). However, the mechanism is primarily driven to completion by Th1-type products, including interleukin 12 (IL-12) and IL-12-induced gamma interferon (IFN-γ), acting in concert with tumor necrosis factor (TNF) (11, 41–43, 54, 59). If unimpeded, the net result at infected liver foci is the assembly of epitheloid granulomas within which intracellular parasites are killed by IFN-γ and TNF-activated macrophages (44).

This same immunological mechanism also supports the efficacy of conventional antileishmanial chemotherapy—T cells and endogenous IL-12 and IFN-γ are required for expression of the visceral leishmanicidal action of pentavalent antimony (Sb) in experimental infection (12, 40, 41). As judged by results with additional cytokine gene-disrupted mice, TNF and IL-4 (Sb) in experimental infection (12, 40, 41). As judged by results of the visceral leishmanicidal action of pentavalent antimony (Sb) therapy is given or, alternatively, a T-cell-dependent response emerges to induce acquired resistance (43). In experimental Leishmania donovani infection in susceptible mice, acquired resistance in the liver is initially regulated by multiple Th1- and Th2-cell-associated cytokines (11, 41, 43, 54, 57, 59). However, the mechanism is primarily driven to completion by Th1-type products, including interleukin 12 (IL-12) and IL-12-induced gamma interferon (IFN-γ), acting in concert with tumor necrosis factor (TNF) (11, 41–43, 54, 59). If unimpeded, the net result at infected liver foci is the assembly of epitheloid granulomas within which intracellular parasites are killed by IFN-γ and TNF-activated macrophages (44).

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Efforts to take advantage of the preceding immunopharmacology have focused on IL-12 and IFN-γ and on raising the level of T-cell reactivity at the time of Sb treatment. Approaches in visceral infection have included coadministration of Sb (i) with exogenous IL-12 or IFN-γ (37, 41) or (ii) with induction of endogenous IL-12 and/or IFN-γ achieved by T-cell costimulation (46, 63), transfer of sensitized dendritic cells (16), or injection of IL-12 to induce endogenous IFN-γ (41). These experimental approaches enhance Sb’s initial efficacy and/or the durability of its effect.

A separate immunochemotherapeutic strategy—inhibition of cytokines which deactivate the Th1-cell mechanism—has thus far been directed at two endogenous Th2-cell-type products, IL-4 and IL-10. In wild-type (WT) BALB/c mice with cutaneous Leishmania major infection, anti-IL-4 monoclonal antibody (MAb) injections restored the durability of the response to Sb by allowing Th1-cell-type responses to emerge (49). In WT BALB/c mice infected with visceral L. donovani, IL-4 is also induced (30) but IL-10 plays the primary deactivating role (35, 45). In these animals, IL-10 receptor (IL-10R) blockade by anti-IL-10R MAb injection enhanced Th1-cell-type responses and granuloma maturation, induced IFN-γ-dependent parasite killing in the liver, and synergistically increased Sb’s leishmanicidal effect (45, 47).

Like IL-10 and IL-4, IL-13 and transforming growth factor β (TGF-β) are also generated in experimental and human visceral, as well as cutaneous, leishmaniasis (4–6, 14, 15, 17, 20, 24, 25, 28–30, 33, 35, 43, 44, 49, 58, 60). While TGF-β and IL-13 induce certain immunomodulating effects (9, 18), both are also capable of deactivating Th1-cell and/or inflammatory macrophage responses and promoting intracellular Leishmania infection (5, 9, 10, 17, 24, 25, 52, 60). Therefore, in this study, we asked whether endogenous TGF-β and IL-13 or perhaps IL-4 (39) also represent targets worth inhibiting in L. donovani-infected mice to strengthen host defense and/or enhance chemotherapy efficacy. Animals with established visceral infection...
were injected with antagonists of TGF-β, IL-13, or IL-4 alone and in combination with Sb to determine if this immunotherapeutic approach can be extended beyond endogenous IL-10 (45, 47).

MATERIALS AND METHODS

Animals. Female BALB/c mice (each, 20 to 30 g) were purchased from Charles Rivers Laboratories (Wilmington, MA). IL-13Rα2−/− mice on a BALB/c background were produced at Wyeth Research (Andover, MA) (62) and bred at Well Medical College (New York, NY). These latter mice (females) were 6 to 11 weeks old when challenged with L. donovani. These studies were reviewed and approved by the Institutional Animal Care and Use Committee of Well Medical College.

Vesicular infection and tissue granuloma responses. Groups of three to five mice were injected via the tail vein with 1.5 × 10⁷ hamster spleen-derived L. donovani amastigotes (1 Sudan strain) (45). Vesicular infection was assessed microscopically by using Giemsa-stained liver imprints in which liver parasite burdens were measured by blinded counting of the number of amastigotes per 500 cell nuclei × liver weight in milligrams (Leishman-Donovan units [LDU]) (45). The histological response to infection was evaluated microscopically in liver sections stained with hematoxylin and eosin. The number of granulomas (injected Kupffer cells which attracted five or more mononuclear cells) (45) was counted in 100 consecutive 40× fields and, at 100 paraffinized sections, the granulomatous reaction was scored as none, developing, or mature (45). Mature granulomas consisted of a core of fused parasitized Kupffer cells surrounded by numerous mononuclear cells and showed epithelioid-type changes (44).

Anticytokine treatments. Cytokine antagonists were administered by intraperitoneal injection in 0.5 ml of saline starting 12 days after infection (day +12). All mice were sacrificed 9 days later on day +21. Day +21 liver parasite burdens (LDU) were compared to day +12 LDU to determine the percentage of parasite killing (45); differences between mean LDU values were analyzed by a two-tailed Student’s t test.

For IL-10R blockade or IL-4 neutralization, the following were injected once on day +12: (i) 0.5 mg of rat immunoglobulin G (IgG) or anti-IL-10R MAb (1B13A; provided by A. Beebe, DNAx Research Institute of Molecular and Cellular Biology, Palo Alto, CA) (45) or (ii) 5 mg of rat IgG or anti-IL-4 MAb (11.B11; provided by C. Reynolds, Biologic Response Modifiers Program, National Cancer Institute, Frederick, MD) (30). For IL-13 inhibition, 0.2 mg of soluble IL-13 receptor-a2-IgG Fc (IL-13Rα2-Fc) (Wyeth Research) or human IgG (Wyeth Research) was injected every second day as in previous studies (8) and was given on days +12, +14, +16, and +18. Soluble chimeric TGF-β type II receptor-IgG-Fc (TGFB-RII-Fc) (Biogen Idec, Cambridge, MA) was used to inhibit TGF-β (34). Preliminary dose-response experiments (not shown) using a single injection on day +12 of 10 to 1 mg/kg of body weight (~25 to 250 μg) of TGFB-RII-Fc indicated no effect on day +21 for 1 mg/kg and maximal effects at 4 mg/kg (~100 μg). The latter dose was used with infected mice and was given once on day +12; controls received 4 mg/kg of mouse IgG2a (Biogen).

TGF-β treatment. Starting one day after L. donovani challenge, mice were given intraperitoneally thrice weekly for 4 weeks with 0.5 ml of saline alone or saline containing 5 μg of recombinant human TGF-β (5). Cytokine was generously provided by S. Reed (Corixa Corporation, Seattle, WA).

Chemotherapy. To demonstrate the effect of combination treatment, cytokine antagonist-treated and control IgG-treated mice were injected once on day +14 with suboptimal-dose pentavalent antimony (Sb) (50 mg/kg of sodium stibogluconate [Penstam]; Wellcome Foundation, Ltd., London, United Kingdom) (45). Based upon prior results (45, 47), suboptimal anti-IL-10R (0.1 mg) was used in these experiments, whereas the other cytokine antagonists were used as described above. Optimal-dose Sb was tested in IL-13Rα2−/− and TGF-β-treated WT mice by injecting 500 mg/kg once on day +12 (45).

TGF-β and IL-13 expression in liver tissue and serum IFN-γ assay. TGF-β1 and IL-13 mRNA expression was detected in liver lysates by semiquantitative reverse transcription-PCR (RT-PCR). The primers used for PCR were as follows: (i) for TGF-β1; AGGCCCAGGCCGACTTACAT (sense) and TTCACA TGTTGTCCTGCACT (antisense); (ii) for IL-13; AGGAGCTGAGCCACATCAC (sense) and GTGGTCTCGTGTTGGGACTTG (antisense); and (iii) for hypoxanthine guanine phosphoribosyltransferase, GTGGCGATACGCGGACATTGT (sense) and GAGGGTAGGTTGGGCTATGCTT (antisense). The PCR products were analyzed by gel electrophoresis, quantified using Quantity One 4.4.1 (Bio-Rad, Hercules, CA), and normalized to hypoxanthine phosphoribosyltransferase for equal loading. Immunolocalization of intracellular TGF-β1 protein was carried out as previously described (13) with liver sections and the polyclonal antibody LC 1-30-1 (6 μg/ml). Serum was assayed in duplicate at fourfold dilutions for IFN-γ activity by a murine IFN-γ enzyme-linked immunosorbent assay (BD-Pharmingen, set no. 555138) in which the lower limit of detection was 31 pg/ml.

RESULTS

IL-10R blockade in established infection. To provide a benchmark against which to compare the effects of antagonizing IL-4, TGF-β, and IL-13, WT BALB/c mice were injected once with anti-IL-10R MAb on day +12 after infection. As shown in Tables 1 and 2, anti-IL-10R induced liver parasite killing (63%) and at low doses enhanced the effect of suboptimal Sb, confirming the deactivating role of IL-10 in this model (45, 47). These effects were accompanied by elevated serum IFN-γ levels (Fig. 1) and a clear increase in liver granulomas scored as mature (68% ± 6% versus 18% ± 5% in

TABLE 1. Antileishmanial effect of cytokine antagonists in WT BALB/c mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver parasite burden (LDU)</th>
<th>% Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>876 ± 31</td>
<td>0</td>
</tr>
<tr>
<td>Anti-IL-10R</td>
<td>1,202 ± 60</td>
<td>63</td>
</tr>
<tr>
<td>Anti-IL-4</td>
<td>315 ± 36**</td>
<td>63</td>
</tr>
<tr>
<td>IgG</td>
<td>1,245 ± 92</td>
<td>0</td>
</tr>
<tr>
<td>TGF-βII-Fc</td>
<td>1,319 ± 101</td>
<td>100</td>
</tr>
<tr>
<td>IgG</td>
<td>1,141 ± 82</td>
<td>0</td>
</tr>
<tr>
<td>TGF-βII-Fc</td>
<td>753 ± 53***</td>
<td>14</td>
</tr>
<tr>
<td>IgG</td>
<td>1,327 ± 108</td>
<td>11</td>
</tr>
<tr>
<td>IL-13Rα2-Fc</td>
<td>780 ± 87***</td>
<td>11</td>
</tr>
<tr>
<td>IgG</td>
<td>1,458 ± 139</td>
<td>0</td>
</tr>
</tbody>
</table>

* 12 days after infection, liver parasite burdens were determined, and mice then received (i) no treatment, (ii) a single day +12 injection of anti-IL-10R or rat IgG (0.5 mg), anti-IL-4 MAb or rat IgG (5 mg) or TGF-βII-Fc or mouse IgG (4 mg/kg, ~100 μg), or (iii) injections of IL-13Rα2-Fc or human IgG (0.2 mg) on days +12, +14, +16, and +18. Results are from two to four experiments and indicate mean ± SEM values for 6 to 15 mice per time point.

a, b, c, d, and e, P < 0.05 versus day +12 value.

**b, P < 0.05 versus day +12 value.

***P < 0.05 versus day +14 value.

†***P < 0.05 versus day +16 value.

‡***P < 0.05 versus day +18 value.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Sb</th>
<th>Liver parasite burden (LDU)</th>
<th>% Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>−</td>
<td>989 ± 87</td>
<td>0</td>
</tr>
<tr>
<td>Anti-IL-10R</td>
<td>−</td>
<td>1,160 ± 67</td>
<td>0</td>
</tr>
<tr>
<td>IgG</td>
<td>+</td>
<td>851 ± 90</td>
<td>14</td>
</tr>
<tr>
<td>Anti-IL-4</td>
<td>−</td>
<td>820 ± 65</td>
<td>17</td>
</tr>
<tr>
<td>Rat IgG</td>
<td>+</td>
<td>217 ± 32**</td>
<td>72</td>
</tr>
<tr>
<td>Anti-IL-4</td>
<td>−</td>
<td>1,239 ± 147</td>
<td>0</td>
</tr>
<tr>
<td>TGF-βII-Fc</td>
<td>+</td>
<td>940 ± 119</td>
<td>5</td>
</tr>
<tr>
<td>IL-13Rα2-Fc</td>
<td>+</td>
<td>1,122 ± 121</td>
<td>0</td>
</tr>
<tr>
<td>Sb</td>
<td>−</td>
<td>890 ± 99</td>
<td>10</td>
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<tr>
<td>TGF-βII-Fc</td>
<td>+</td>
<td>930 ± 78</td>
<td>6</td>
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<tr>
<td>IL-13Rα2-Fc</td>
<td>+</td>
<td>771 ± 89</td>
<td>22</td>
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<tr>
<td>Sb</td>
<td>−</td>
<td>900 ± 106</td>
<td>9</td>
</tr>
<tr>
<td>TGF-βII-Fc</td>
<td>+</td>
<td>732 ± 66</td>
<td>26</td>
</tr>
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* Treatment starting day +12 as was in Table 1 legend, except a reduced dose (0.1 mg) of anti-IL-10R and rat IgG was used (see Materials and Methods). Suboptimal Sb (50 mg/kg) was given once on day +12. Control IgG for the other antagonists did not increase the effect of Sb, and data have been omitted for brevity. Results are from two to three experiments and indicate mean ± SEM values for 7 to 12 mice per time point.

b, c, d, e, and f, P < 0.05 versus day +14 value.
control IgG-treated mice; \( P < 0.05 \), two experiments). Figure 2 illustrates the histological response on day + 21. Anti-IL-10R-induced parasite killing and synergy with Sb require IFN-\( \gamma \); effects on granuloma assembly involve both IFN-\( \gamma \)-dependent and -independent pathways (45).

Effect of anti-IL-4 MAb. In addition to IL-10 mRNA, \( L. \) donovani-infected WT BALB/c mice also initially express IL-4 mRNA in parasitized liver and/or spleen (28, 30, 34). However, injection of anti-IL-4 MAb on day + 12 produced no therapeutic action on day + 21 (Tables 1 and 2), did not affect granuloma maturation, and decreased serum IFN-\( \gamma \)-levels (Fig. 1), although not significantly (\( P > 0.05 \)). Once-weekly injections of 5 mg of anti-IL-4 starting 1 day after \( L. \) donovani challenge also induced no antileishmanial activity on day + 21 or + 28 and did not enhance the efficacy of suboptimal Sb (50 mg/kg) given on day + 14. At the same time, this weekly anti-IL-4 treatment also did not promote parasite replication in WT mice or impair the response to Sb (50 or 500 mg/kg injected on day + 14) (two experiments; data not shown), effects reported with \( L. \) donovani-infected mice genetically deficient in IL-4 (57).

Detection of TGF-\( \beta \) and effect of soluble TGF-\( \beta \)RII-Fc. TGF-\( \beta \)-1 mRNA was detected by RT-PCR in livers of infected BALB/c mice (Fig. 3A); by day + 28, however, expression was not significantly different versus constitutive levels at day 0 in uninfected livers. Since TGF-\( \beta \) mRNA expression may not correlate with protein production (29), tissue sections were stained for immunoreactive cytokine using an antibody which detects TGF-\( \beta \)1 in active and latent form (13). In livers of uninfected mice, cell-associated reaction product was readily apparent and expressed within ~75% of hepatocytes (Fig. 4A). In infected livers, results were clearly different: few parenchymal cells showed immunoreactive TGF-\( \beta \)1 at either day + 14 (Fig. 4B) or day + 21 (not shown). In addition, within developing granulomas that encircle parasitized macrophages (Kupffer cells) (5, 52), few cells showed visible TGF-\( \beta \)-reaction product (Fig. 4B). Despite the preceding results, treatment with TGF-\( \beta \)RII-Fc, which interferes with cytokine-receptor binding and inhibits TGF-\( \beta \)-effects (34), altered the kinetics of \( L. \) donovani replication in WT mice. While a single injection of TGF-\( \beta \)RII-Fc on day + 12 primarily enabled inhibition of parasite replication, some parasite killing (14%) was also induced at day + 21 (Table 1). Administering an additional dose of TGF-\( \beta \)RII-Fc on either day + 14, +16, or +18 did not further enhance the effect. Although endogenous TGF-\( \beta \) appeared to actively promote infection, the antileishmanial effect of TGF-\( \beta \)RII-Fc was not sufficient to augment the action of low-dose Sb (Table 2) or to materially increase serum IFN-\( \gamma \)-granuloma maturation (Fig. 1 and 2).

Detection of IL-13 and response to soluble IL-13R\( \alpha \)2-Fc. \( L. \) donovani infection also stimulated IL-13 mRNA expression in livers of WT BALB/c mice (Fig. 3B), prompting the testing of IL-13R\( \alpha \)2-Fc, which inhibits IL-13-induced effects (8). Injections of IL-13R\( \alpha \)2-Fc on day + 12 or on days + 12 and + 14 produced no effect. However, four alternate-day treatments during days + 12 to + 18 clearly inhibited parasite replication (Table 1) and produced some enhancing effect on granuloma cellularity (Fig. 2F). Nevertheless, IL-13R\( \alpha \)2-Fc treatment did not promote Sb’s efficacy (Table 2), alter serum IFN-\( \gamma \) (Fig. 1), or significantly increase mature granuloma numbers in the liver (not shown).

Effect of raising the levels of IL-13 or TGF-\( \beta \). The preceding results indicated a regulatory role for endogenous TGF-\( \beta \) and IL-13, albeit limited, but none for IL-4. These findings were generated in the presence of counterbalancing and IL-12- and IFN-\( \gamma \)-mediated Th1-type responses, which are also expressed in BALB/c mice by the second week of infection (30, 45). Thus, the observations did not exclude the possibility that IL-13 or TGF-\( \beta \) might act in a more suppressive fashion under different conditions. For example, although anti-IL-4 has no effect in this \( L. \) donovani model (30), administration of IL-4 or manipulation that provokes endogenous IL-4 readily enhances visceral parasite replication in these same BALB/c mice (39). Therefore, to complete this analysis, we examined the consequences of raising IL-13 or TGF-\( \beta \) levels rather than inhibiting their effects.

In the absence of the decay function of IL-13R\( \alpha \)2, IL-13R\( \alpha \)2\( ^{-/-} \) mice show high IL-13 levels in the tissues including liver (62) and in this way are thought to phenotypically resemble IL-13 transgenic mice (26). The following results were observed with IL-13R\( \alpha \)2\( ^{-/-} \) versus WT BALB/c mice, respectively. (i) Serum IFN-\( \gamma \) was reduced at day + 14 (76 ± 23 versus 205 ± 34 pg/ml, six to seven mice per group; \( P > 0.05 \)) and day + 21 (72 ± 18 versus 351 ± 53 pg/ml, six to eight mice per group; \( P < 0.05 \)). (ii) Initial granuloma assembly and maturation were impaired (Fig. 5). (c) Liver parasite burdens were increased (Fig. 6A). These findings are consistent with a suppressive effect for IL-13. However, deficient tissue inflammation and susceptibility were not sustained in IL-13R\( \alpha \)2\( ^{-/-} \) mice, as after week 4, the granulomatous response evolved and...
liver burdens declined by ~60% (Fig. 6A). IL-13Ra2−/− mice also demonstrated an intact response to optimal-dose Sb (500 mg/kg injected once on day + 14) with 88% killing of liver amastigotes by day + 21 versus 93% in WT controls; responses to suboptimal Sb (single injections of 50 or 100 mg/kg) were preserved as well (two experiments).

Raising the level of TGF-β by injecting WT BALB/c mice with high-dose human TGF-β also increased susceptibility but in a similarly transient fashion. Thrice-weekly treatment starting 1 day after infection with the same cytokine preparation that promoted *L. braziliensis* infection (5) increased liver parasite burdens on day + 14, but continued injections did not maintain the effect (Fig. 6B). In these same mice, exogenous TGF-β did not impair either the liver granulomatous response...
In the spectrum of experimental leishmaniasis, IL-10, IL-4, IL-13, and TGF-β are generally but not invariably (22, 31, 57) recognized for derailing Th1-cell-associated mechanisms and deactivating macrophages, thereby dampening inflammation but fostering progressive intracellular infection (17–19, 24, 25, 32, 35, 49, 51, 52). In the \( L. \) donovani model, results with IL-10 transgenic and -deficient mice have already assigned such a role to IL-10 (35, 45). However, other suppressive-type cytokines, acting alone, with IL-10 or via crossregulation (4, 28, 29, 33, 39, 51, 55, 59, 60) may also promote visceral infection and therefore represent separate potential targets for therapeutic inhibition. Such inhibition, which can raise the level of Th1-cell reactivity and strengthen host defense in general (1, 24, 32, 35, 43, 45, 47, 49, 51, 52) can also specifically enhance chemotherapy efficacy. In addition to combining Sb or amphotericin B with targeting IL-10 in \( L. \) donovani infection (45, 47, 48), this anticytokine strategy has also been successfully directed at IL-4 in cutaneous \( L. \) major infection (anti-IL-4 MAb plus Sb) (49) and in systemic \( C. \) albicans infection (antagonistic soluble IL-4 receptor plus antifungal agents) (7).

BALB/c mice express multiple deactivating cytokines in response to \( L. \) donovani (28, 30, 33), and our results indicate regulatory (suppressive) roles for both endogenous TGF-β and IL-13. That endogenous IL-4 is expressed but does not exert a measurable effect in \( L. \) donovani-infected WT mice has also been made clear in previous studies (22, 27, 28). The effects of inhibiting endogenous TGF-β or IL-13 in the midst of progressive infection were modest and limited primarily to induction of leishmanistatic activity. In contrast, anti-IL-10R treatment stimulated IFN-γ secretion and granuloma maturation, induced high-level parasite killing in the liver, and supported the efficacy of chemotherapy. These effects were not detected in mice treated with TGF-βRII-Fc or IL-13Rα2R-Fc (or anti-IL-4), emphasizing in a comparative fashion IL-10’s prominent handcuffing effect in visceral infection (35, 45, 47).

Since there may be organ-specific responses in the \( L. \) donovani model (11, 36), cytokine inhibition might produce different effects on infection in spleen or bone marrow. In addition, we have not yet examined how targeting IL-13 or TGF-β enables inhibition of parasite replication, although in view of the intracellular location of \( L. \) donovani, enhancing effects on macrophage activation seem likely. Given our objective—a practical therapeutic strategy for use alone or in combination with chemotherapy in established infection (e.g., a single injection of anti-IL-10R (45, 47, 48)—these experiments were intended to determine effects of acute rather than chronic cytokine inhibition. Thus, it also remains possible that for TGF-β and IL-13 (but not IL-4) inhibition, more time is required for the expression of additional antileishmanial effects. However, we tested chronic TGF-β antagonism in one experiment by injecting TGF-βRII-Fc (4 mg/kg) thrice weekly, starting 1 day after infection. In mice treated for 4 weeks, liver parasite replication was inhibited but still proceeded, granuloma formation was...

FIG. 3. Semiquantitative RT-PCR results for TGF-β (A) and IL-13 (B) mRNA expression in livers of uninfected WT BALB/c mice (day 0) and mice infected for 14 or 28 days. Results show mean ± SEM values for five mice per group per time point. *, \( P < 0.05 \) versus day 0 value.

FIG. 4. Immunocytochemical detection of TGF-β1 in liver tissue. (A) Uninfected WT BALB/c mice show prominent reaction product at most hepatocytes (arrows). (B) At 14 days after \( L. \) donovani infection, TGF-β1 expression is near absent at hepatocytes and developing granulomas; arrows indicate few positive-staining cells. Peroxidase with Carazzi hematoxylin counterstain. Magnification, \( \times 180 \) (original magnification, \( \times 200 \)).
unaffected at weeks 2 and 4, and the effect of suboptimal Sb injected on day +14 was not increased on day +21 (four mice; data not shown).

The limited effects of inhibiting endogenous TGF-β as well as IL-13 in WT mice might also reflect other factors. Both cytokines may preferentially target an auxiliary (leishmanicidal) rather than a primary killing mechanism, and such a secondary mechanism might also not be involved in supporting Sb’s leishmanicidal efficacy. For example, neither antagonist of TGF-β or IL-13 provoked IFN-γ, the cytokine required for *L. donovani* killing, as well as the key endogenous cofactor in the host response to Sb (40, 43). IL-10-induced deactivation in this model may also be sufficient to mask competing, suppressive effects stimulated by IL-13 or TGF-β; future experiments will test the effect of simultaneously inhibiting IL-10, TGF-β, and/or IL-13.

Three additional observations are also worth comment. First, like IL-10 (32), endogenous IL-4 and IL-13 can also suppress Th1-cell-type mechanisms, indirectly and/or directly deactivate macrophages, and therefore foster intracellular *Leishmania* infection (9, 25, 35, 37, 47, 49, 50, 51, 53). However, neither IL-4 nor IL-13 plays a consistently suppressive role; their effects appear model and/or *Leishmania* species dependent (22, 23, 27, 30, 31, 49, 53, 56, 57). Adding to this complexity, IL-4 and IL-13 can also act to support host defenses. Results with *L. donovani*-infected IL-4−/− and IL-4Rα−/− mice indicate roles in initial Th1-cell development, IFN-γ regulation, and control of early *L. donovani* replication (27, 57), and for IL-4, in responsiveness to Sb treatment (2). In our experiments, these IL-4-associated effects were not apparent under the quite different conditions of treating WT mice once weekly with anti-IL-4 MAb starting 1 day after infection. In a separate setting, however, with WT BALB/c mice manipulated (immunized) to cross-react to *L. donovani* with a predominant Th2-type response, IL-4 readily emerges as a suppressive cofactor acting with IL-10 to impair granuloma assembly and induce the noncure phenotype (39).

Second, TGF-β, which can produce upregulatory effects, is also fully capable of impairing Th1-cell responses and macrophage activation (10, 18). In experimental *Leishmania* infection, exogenous TGF-β promotes cutaneous as well as visceral parasite replication (5, 60) (Fig. 6B) and, in endogenous form, stimulates initial progression or subsequent persistence of cutaneous infection (3, 24, 52). Inferential results also suggest that endogenous TGF-β fosters the visceral replication of *L. chagasi* (14) and persistence of *L. infantum* (17); our findings with *L. donovani*-infected, TGF-βRII-Fc-treated mice support such a role in vivo.

Nevertheless, in most but not all (28) models of visceral infection in WT mice, TGF-β activity either does not increase
during the initial period in which liver parasite replication is unrestrained (33, 61, 63) or increases but then decreases at the peak of hepatic infection (17). These results, reflected in vitro and in vivo TGF-β protein production (Fig. 4) (17, 61, 63) and in vivo mRNA expression (Fig. 3A) (33), may help to explain the comparatively limited effect of TGF-βRII-Fc treatment given during week 2 (Tables 1 and 2). However, TGF-β activity can also reemerge or increase at a later stage of infection (28, 33, 61) and might take on other roles: curtailing the inflammatory response after liver infection is largely controlled, regulating conversion of residual liver infection to a chronic, persistent state (3, 17), or fostering the delayed but progressive infection in the spleen reported in some models of visceral infection (21, 33).

Finally and third, the intact response to Sb in the presence of excess IL-13 or TGF-β adds to prior observations indicating that suppression-type cytokines do not impair the initial in vivo response to chemotherapy (45, 49). Optimal-dose Sb (500 mg/kg) is also fully active against L. donovani in the face of either sustained IL-10 in transgenic BALB/c mice (45) or raised levels of both IL-4 and IL-10 in heat-killed L. major-immunized BALB/c mice (39). In both of these latter noncuring groups of mice, as well as in IL-4 transgenic, IL-13Ra2−/−, and TGF-β− treated animals, an injection of 500 mg/kg of Sb on day +14 is as active as in control mice, killing ~90% of liver L. donovani by day +21 (39, 45; the present report and unpublished data). Thus, although the leishmanicidal efficacy of Sb in the liver is dependent upon and can be amplified by the host Th1-cell-type response (12, 16, 37–41, 46, 47, 63), this particular IFN-γ- and IL-12-mediated Th1-cell action paradoxically escapes the deactivating effects of IL-10, IL-4, IL-13, or TGF-β. Conversely, however, only inhibition of IL-10 synergistically enhanced the effect of Sb in this comparative analysis, strengthening the appeal of IL-10 as the appropriate cytokine target for therapeutic inhibition in experimental visceral infection.

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FIG. 6. Course of L. donovani liver infection in IL-13Ra2−/− and TGF-β-treated BALB/c mice. (A) WT BALB/c (open circles) versus IL-13Ra2−/− (closed circles) mice. (B) Starting 1 day after infection, WT mice were injected with saline (open circles) or 5 µg of TGF-β (closed circles). Results indicate mean ± SEM values from three experiments (9 to 13 mice per time point) (A) and two experiments (6 to 7 mice per time point) (B). * P < 0.05 versus control value.


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