Visceral Leishmaniasis in Mice Devoid of Tumor Necrosis Factor and Response to Treatment

HENRY W. MURRAY,1* ACHIM JUNGBLUTH,2 ERIKA RITTER,2 CHRISTINA MONTELIBANO,1 AND MICHAEL W. MARINO2

Department of Medicine, Weill Medical College of Cornell University,1 and Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center,2 New York, New York 10021

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Tumor necrosis factor (TNF)-deficient mice were challenged with Leishmania donovani to characterize TNF in the response of visceral intracellular infection to antileishmanial chemotherapy. In wild-type controls (i) liver infection peaked at week 2 and resolved, (ii) discrete liver granulomas developed at weeks 2 to 4 and involuted, and (iii) leishmanial responses to antimony (Sb), amphotericin B (AmB), and miltefosine were intact. In TNF knockout (KO) mice (i) initial liver infection was unrestrained, plateaued, and then declined somewhat by week 6, (ii) an absent early granulomatous reaction abruptly accelerated with striking tissue inflammation, widespread hepatic necrosis, and 100% mortality by week 10, and (iii) while the initial response to AmB and miltefosine was intact, killing induced by Sb therapy was reduced by >50%. Although initial AmB treatment during weeks 2 to 3 killed 98% of liver parasites, 75% of AmB-treated KO mice subsequently relapsed and died by week 12; however, additional maintenance AmB preserved long-term survival. These results for a model of visceral infection indicate that endogenous TNF is required early on to control intracellular L. donovani, support granuloma development, and mediate optimal initial effects of Sb and prevent relapse after ordinarily curative AmB treatment. A compensatory, TNF-independent antileishmanial mechanism developed in TNF KO mice; however, its effect was an uncontrolled fatal inflammation. Chemotherapeutic elimination of the parasite stimulus reversed the hyperinflammatory response and preserved survival.

The pleiotropic cytokine tumor necrosis factor (TNF) appears to be a prominent component of a diverse spectrum of both beneficial and deleterious inflammatory responses (2, 34). Among the beneficial effects of endogenous TNF is its complex role in inducing macrophage activation and enhancing host antimicrobial defense, particularly against intracellular pathogens (2, 34). Such a role, initially demonstrated by the capacity of treatment with anti-TNF antibodies to exacerbate infection, has recently been confirmed in a number of models using TNF- and TNF receptor-deficient (knockout [KO]) mice (1, 4, 6, 7, 12, 21, 23, 26, 35, 36).

In a previous report, we illustrated the critical role of endogenous TNF in the multicytokine-mediated host defense response which controls experimental visceral leishmaniasis, a disseminated protozoal infection in which macrophages of the liver, spleen, and bone marrow are targeted (31, 33; reviewed in reference 13). Challenging normal BALB/c mice with Leishmania donovani induced TNF in infected liver and spleen, and increasing tissue TNF levels reflected both initial control over parasite replication and subsequent near resolution of visceral infection by week 8 (31, 33). Repeated injections of anti-TNF antiserum abolished acquired resistance, permitting intracellular amastigotes to replicate freely within visceral macrophages. At the same time, 8 weeks of anti-TNF treatment did not appear to interfere with the orderly assembly of inflammatory mononuclear cells into well-circumscribed granulomas at infected tissue foci (13) and did not cause death in treated animals despite high parasite burdens (33).

This report extends the analysis of endogenous TNF’s role in this model of visceral infection by asking whether this cytokine also acts with or regulates the in vivo response to antileishmanial chemotherapy (pentavalent antimony [Sb] or amphotericin B [AmB]). We posed this particular question for two reasons. First, gamma interferon (IFN-γ), another pivotal endogenous antileishmanial cytokine (13, 31) is required for the in vivo expression of Sb’s leishmanicidal action (16) and is closely intertwined with TNF in inflammatory events including macrophage activation and the generation of toxic intermediates for L. donovani killing (2, 11, 24, 25, 28, 30, 34). Second, both Sb and AmB (as well as miltefosine, a new antileishmanial agent [15, 29]) stimulate mononuclear phagocytes to secrete TNF (10, 32, 37), raising the possibility that induced TNF may act along with or enhance the local drug effect. To answer this question about endogenous TNF, we turned to well-characterized TNF KO mice (8, 12) for an in vivo test environment strictly free of the cytokine and characterized the host reaction and the behavior of visceral L. donovani in the absence of TNF and then the response to treatment.

MATERIALS AND METHODS

Mice and visceral infection. Randomly selected male and female TNF KO mice (–/–) and their wild-type (WT) littermates (+/+), generated on a C57BL/6 × 129/Sv background (12), were used in these experiments. Groups of three to five mice were injected via the tail vein with 1.5 × 107 hamster spleen-derived L. donovani amastigotes (one Sudan strain) (33). Visceral infection was monitored microscopically using Giemsa-stained liver imprints, and liver parasite burdens were measured by counting in a blinded fashion the amastigotes per 500 cell nuclei and multiplying this number by the liver weight in milligrams (Leishman-Donovan units [LDUs]) (13). The histologic reaction in the liver was assessed using formalin-fixed tissue sections stained with hematoxylin and eosin. Granuloma formation at infected foci was scored as none, developing, or mature (13, 33).

Treatment. Two weeks after infection (day 0), liver parasite burdens were determined and mice then received no treatment, a single intraperitoneal injection of Sb, three alternate-day intraperitoneal injections of AmB, or five consecutive once-daily doses of oral miltefosine by gavage as in previous studies (15, 16). Optimal doses of each drug were administered: Sb (sodium stibogluconate, Pentostam; Wellcome Foundation Ltd., London, United Kingdom), 500 mg/kg...
Differences between mean values were analyzed by a two-tailed Student's t-test. LDUs were compared to day 0 LDUs to determine percent parasite killing (15). On day 7 (1 week after treatment was initiated), mice were sacrificed and liver parasite burdens were measured. Day +7 LDUs were compared to day 0 LDUs to determine percent parasite killing (15). Differences in mean LDUs for KO versus WT mice were significant ($P < 0.05$) at weeks 2 and 4 but not at week 1 or week 6 ($P < 0.05$).

In addition to reducing liver parasite burdens, treatment also blunted the emerging inflammatory response, presumably by decreasing the overall magnitude of (miltefosine, Sb) or nearly eliminating (AmB) the triggering stimulus, replicating intracellular parasites. In contrast to what was seen in untreated KO mice, there was little or no disordered inflammatory reaction at week 3 (day $+7$) in livers of AmB-treated animals (Fig. 2f); inflammatory changes in the livers of KO mice given either miltefosine or Sb were also reduced (not shown).

Durability of the response to treatment and effect on survival. To determine if the preceding initial response to chemotherapy was maintained in the absence of endogenous TNF and, at the same time, to test if antileishmanial therapy could prevent the subsequent death of KO mice due to inflammation, we used AmB because of its superior activity (98% killing; Table 1). Starting 2 weeks after infection, KO animals were randomly separated into three groups and received (i) no treatment (group A), (ii) three alternate-day 5-mg/kg injections of AmB during weeks 2 to 3 with no further treatment (as in Table 1) (group B), or (iii) the same three injections of AmB during weeks 2 to 3 followed by four maintenance injections (5 mg/kg) given once weekly at the beginning of weeks 4 to 7 (group C). Maintenance injections in group C were discontinued after week 7 at the time when 50% of untreated mice had died (Fig. 3B).

As shown in Fig. 3B, all untreated KO mice died by week 9, at which time 88 to 100% of AmB-treated mice in groups B and C were alive. However, group B animals (treated with AmB only during weeks 2 to 3) appeared ill after week 8 and only 25% eventually survived. In contrast, 88% of group C KO mice, which had received additional once-weekly maintenance AmB therapy to week 7, remained healthy at week 16. These mice were then sacrificed to examine their livers. Few amastigotes were present in 16-week liver imprints (42 ± 10 LDU; $n = 8$ mice examined), and the histologic appearance of this tissue was essentially unremarkable, with little evidence of either infection or inflammation (not shown).

**DISCUSSION**

These results for cytokine-deficient animals suggest several conclusions about the role of endogenous TNF in visceral antileishmanial resistance and its specific role in modulating the host response to antileishmanial chemotherapy.

First, in the absence of endogenous TNF, intracellular replication of *L. donovani* proceeded rapidly, reaching high levels 2 weeks after challenge. At this time, the early granulomatous tissue reaction, characteristic of this model (13), was largely absent at the majority of infected liver foci in KO mice. Both observations confirm the initial requirement for TNF in these
two visceral responses. Second, the response in KO mice spontaneously shifted such that, by weeks 3 to 4, unrestrained liver infection had plateaued, albeit at a high level. This response was accompanied by the delayed but intact emergence of normal early granuloma assembly. Yet, third, the apparently developing capacity in KO mice to control *L. donovani* (Fig. 1) and generate granulomas at infected foci, obviously mediated by a TNF-independent compensatory mechanism, was abruptly obscured by a florid, destructive inflammatory response. This response, well recognized in other microbial models for TNF-deficient mice (1, 4, 6, 8, 12, 21, 35), induced diffuse hepatic necrosis in *L. donovani*-infected mice. However, even though eventually uniformly fatal, this response was not uncontrollable once triggered. Thus, as judged by the partial action of short-term chemotherapeutic intervention with AmB and the curative effect when intervention is extended, inhibition or
removal of the inciting microbial stimulus can slow or terminate the progressive inflammatory response.

We did not formally determine the cause of death in TNF-deficient mice, nor did we examine responses in other parasitized organs such as the spleen. However, the level of hepatic inflammation was decreasing at week 6 (Fig. 1) as mice started to die, suggesting a role for factors other than progressive infection. Given the nature and extent of the histologic lesions in the liver, we suspect that death most likely resulted from inflammation-induced tissue destruction. Injecting these same TNF KO mice with a nonviable stimulus, killed *Corynebacterium parvum*, for example, produces a similarly intense, necrotic inflammatory tissue reaction in liver and spleen with near-100% mortality within approximately the same time period (12).

Paradoxically, the Th1 cell-dependent, interleukin-12 (IL-12)- and IFN-γ-mediated mechanism which successfully induces macrophage activation and acquired resistance to *L. donovani* (13, 14, 31) may well be responsible for this dysregulated, lethal inflammation (8). The generation of cytokine-induced inducible nitric oxide synthase-derived reactive nitrogen intermediates may also be involved (19, 21, 23). Alternatively, since TNF can induce IL-10 (3), deficient IL-10-related downregulation may play some role as well. Prior observations have well highlighted this unexpected but critical role for endogenous TNF in regulating and limiting the extent and duration of in vivo inflammatory reactions (1, 4, 6, 8, 12, 21, 35). Evaluation of TNF receptor KO mice with *Leishmania major* footpad infection has also shown a persistent local inflammatory response with failure of lesion healing (21, 35), which may reflect deficient apoptosis of infiltrating T cells (9). In this model, initial control over intracellular infection is also impaired; however, parasite clearance eventually develops (21, 35).

We believe that the presence and effects of this (or a similar) TNF-independent hypercompensatory mechanism in KO mice likely explains the differences we observed in our prior study of anti-TNF antiserum-treated animals in which there was no control over infection, no exaggerated inflammatory response, and no deaths (33). In antiserum-treated normal BALB/c mice, we suspect that either (i) sufficient endogenous TNF was still present to prevent aberrant inflammation (but not enough to control visceral infection) or (ii) there was no intrinsic compensatory mechanism poised to overshoot, thus preserving survival in the previous study (33).

Fourth, despite clearly heightened visceral infection at week 2 and the absence of the host defense effects of endogenous TNF, the short-term antileishmanial efficacy of AmB and miltefosine was maintained. Thus, the reported capacity of these two agents to induce macrophage secretion of TNF in vitro (32, 37) does not appear central to their antileishmanial action in vivo.

SB also stimulates macrophages to produce TNF (10), and the response to this agent, conventional therapy for human visceral leishmaniasis, was clearly diminished in KO mice. Such a finding, indicating that SB and TNF interact in vivo, is consistent with SB’s specific requirement for endogenous host immune mechanisms for expression of its leishmanicidal activity in vivo (16, 20). (Neither AmB nor miltefosine has this requirement, and both appear to initially act directly in vivo without immunologic cofactors [15–17].) Our ongoing analysis indicates that host regulation of the response to SB is strictly T cell dependent and requires two Th1 cell cytokines (IFN-γ and IL-12) intertwined with the expression in tissue of at least one adhesion molecule, ICAM-1 (16, 20; unpublished data). (ICAM-1 itself is also upregulated by IFN-γ and/or TNF [5, 27].) Although clearly present, the defect in SB responsiveness seen here in TNF KO mice is not as complete as it is in parasitized IFN-γ or IL-12-deficient mice. In these KO animals, similar SB treatment induces 0% *L. donovani* killing (16; unpublished data).

In addition to demonstrating that the hyperinflammatory response triggered in TNF KO mice was not a runaway process but rather one which can be successfully terminated, the treatment studies using AmB also yielded a final conclusion: that compensatory, TNF-independent antileishmanial mechanisms were not sufficient to prevent reemergence of residual post-treatment infection. Thus, while the three-injection AmB regimen during weeks 2 to 3 initially eradicated 98% of liver amastigotes in KO mice (Table 1), residual infection nevertheless recurred, as judged by the eventual death of 75% of these animals not further treated. Therefore, endogenous TNF also appears to be a critical determinant of the relapse-free state in the treated host with *L. donovani* infection. Providing AmB-treated KO mice with additional short-term maintenance therapy enhanced survival. Thus, continued pharmacologic inhibi-

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### TABLE 1. Response to antileishmanial treatment*

<table>
<thead>
<tr>
<th>Mouse type</th>
<th>Treatment</th>
<th>Liver parasite burden (LDU) at day:</th>
<th>% Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>WT controls</td>
<td>None</td>
<td>825 ± 113</td>
<td>1,137 ± 264</td>
</tr>
<tr>
<td></td>
<td>SB</td>
<td>825 ± 113</td>
<td>140 ± 87</td>
</tr>
<tr>
<td></td>
<td>AmB</td>
<td>825 ± 113</td>
<td>78 ± 25c</td>
</tr>
<tr>
<td></td>
<td>MILT</td>
<td>825 ± 113</td>
<td>148 ± 71c</td>
</tr>
<tr>
<td>TNF KO</td>
<td>None</td>
<td>1,332 ± 358</td>
<td>1,690 ± 365</td>
</tr>
<tr>
<td></td>
<td>SB</td>
<td>1,332 ± 358</td>
<td>906 ± 25f</td>
</tr>
<tr>
<td></td>
<td>AmB</td>
<td>1,332 ± 358</td>
<td>26 ± 10c</td>
</tr>
<tr>
<td></td>
<td>MILT</td>
<td>1,332 ± 358</td>
<td>342 ± 99c</td>
</tr>
</tbody>
</table>

*Two weeks after *L. donovani* challenge (day 0), LDUs were determined and mice received no treatment, a single dose of SB (500 mg/kg), three injections of AmB (5 mg/kg) on alternate days, or five once-daily oral doses of miltefosine (MILT) (25 mg/kg). One week after treatment was started (day + 7), all mice were sacrificed. Results are from two to four experiments and represent means ± standard errors of the means for LDUs from 7 to 14 mice per group at each time point.

% Killing = (day 0 LDU − day + 7 LDU)/day 0 LDU × 100.

*Significantly lower (*P* < 0.05) than day 0 value.
tion of residual parasite replication (or perhaps driving the parasite number even lower) appears to permit TNF-independent mechanisms, likely mediated by T cells (18, 22), time to develop sufficiently to preserve long-term survival.

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REFERENCES


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