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Prevention of Relapse after Chemotherapy in a Chronic Intracellular Infection: Mechanisms in Experimental Visceral Leishmaniasis

Henry W. Murray

In visceral leishmaniasis, chemotherapy probably seldom eradicates all parasites in tissue macrophages; nevertheless, most T cell-intact patients show long-lasting clinical cure after treatment despite residual intracellular infection. To characterize prevention of posttreatment relapse, amphotericin B was used to kill ~90–95% of Leishmania donovani in livers of mice deficient in mechanisms of acquired antileishmanial resistance. Recrudescence subsequently developed 1) in animals deficient in both CD4 and CD8 T cells as well as CD40L-mediated T cell costimulation, but not in a) CD4 or CD8 cells alone, b) NK cell lytic activity, or c) ICAM-1-recruited monocytes; and 2) in mice deficient in IFN-γ, but not in the IFN-γ-inducing cytokines, a) IL-12, b) IL-12 and IL-23, or c) IL-18. Posttreatment recrudescence also did not develop in animals deficient in macrophage phagocyte NADPH oxidase (phox) or inducible NO synthase (iNOS) alone or, surprisingly, in those deficient in both phox and iNOS. Therefore, regulation of the intracellular replication of residual Leishmania donovani that escape chemotherapy evolves to a host mechanism distinguishable from initial acquired resistance at the T cell, cytokine, and macrophage levels. Posttreatment, either CD8 or CD4 cells can direct the response, IL-12 is not required, and iNOS and phox, the activated macrophage’s primary IFN-γ-inducible leishmanicidal pathways, both become dispensable. The Journal of Immunology, 2005, 174: 4916–4923.
mechanisms. AmB was used in this study because, unlike the experimental effect of Sh, AmB’s leishmanicidal activity is direct and fully expressed initially in both T cell- and cytokine-deficient animals (1, 31, 34–37, 40, 45, 46).

Materials and Methods
Mice
Twenty to 30 gram female C57BL/6 and BALB/c mice purchased from The Jackson Laboratory were used as controls. Twenty to 30 gram female athymic (nude), CD4−/−, CD8−/−, and bg/bg (beige) mice on a C57BL/6 background were also purchased from The Jackson Laboratory. Breeding pairs of gene-disrupted (knockout (KO)) mice on a C57BL/6 background were originally obtained from the following sources: CD40L−/−, ICAM-1-deficient, and IFN-γ−/− (The Jackson Laboratory); respiratory burst (phagocyte NADPH oxidase (phox))-deficient gp91phox−/−/− (double KO), Dr. M. Dinauer (Indiana University Medical Center, Indianapolis, IN) (35); inducible NO synthase (iNOS)−/− and gp91phox−/−/− iNOS−/− (double KO), Dr. C. Nathan (Weill Medical College, New York, NY) (35, 47); and IL-18/−/− (The Jackson Laboratory); athymic (nude) C57BL/6 mice, which did not control visceral infection (3988 ± 433 LDU at wk 12; two experiments; n = 8 mice), also showed an intact initial response to AmB, with 97% killing by day 21 (Fig. 1A). However, mirroring observations made in nude BALB/c mice (11), infection re-emerged in treated T cell-deficient animals at wk 12, yielding heavily parasitized liver foci with little inflammatory reaction (Figs. 1A and 2B).

Response to AmB in CD4 and CD8 cell-deficient mice
CD4 and CD8 KO mice were next tested in parallel with WT and nude animals to determine whether one T cell subset primarily acts to prevent relapse after chemotherapy. Consistent with findings in WT mice depleted of CD4 or CD8 cells by mAb injections (27), untreated CD4 and CD8 KO mice failed to control L. donovani replication. At wk 12, liver burdens were high (3103 ± 291 and 3610 ± 315 LDU, respectively, vs 98 ± 16 LDU in WT mice; two experiments; n = 8–12 mice/group), and granuloma assembly was impaired (not shown). In response to AmB given during wk 3, parasite killing in CD4 and CD8 KO mice was comparable and similar to that in nude mice (88–93% liver parasite killing by day 21; Fig. 1B). Thereafter, however, posttreatment outcome in these three groups of mice clearly diverged, because infection did not recur in livers of CD4- or CD8-deficient mice. This result indicated that either CD4 or CD8 cells could provide the required T cell mechanism. The wk 12 liver histologic appearance in treated CD4 and CD8 KO mice was also similar (Fig. 2, C and D), showing evidence of residual inflammation and few visible amastigotes.

NK cell-deficient mice
Recurrent infection in treated nude mice indicated that NK cells retained in these animals were not sufficient to suppress parasite replication. However, NK cells may help to control L. donovani in T cell-intact mice, albeit in a limited fashion (10, 49). Therefore, euthymic beige mice, deficient in NK cell lytic activity (10), were also treated on days 14, 16, and 18 after infection. In these mice, AmB induced 94% killing by day 21 (two experiments; not shown), and liver parasite burdens in treated as well as untreated beige mice were low at wk 12 (88 ± 21 and 136 ± 14 LDU, respectively; n = 7–9 mice/group). Thus, NK cell lytic activity did not appear to contribute to posttreatment parasite suppression.

FIGURE 1. Outcome of L. donovani infection after AmB treatment in WT and T cell- and CD40L-deficient C57BL/6 mice. Two weeks after challenge, all mice received AmB on days 14, 16, and 18; liver parasite burdens were determined on day +14, day +21, and 9 wk after treatment (wk 12). A, WT (○) vs nude mice (●). B, Nude (●) vs CD4 (●) and CD8 cell KO mice (●). C, WT (○) vs CD40L KO mice (●). Results are from two (A and B) or three (C) experiments and indicate the mean ± SEM value per group per time point for eight to 11 mice. *p < 0.05 vs wk 3 value.
Response in CD40L-deficient mice

CD40:CD40L T cell costimulation, which can be expressed in CD4 and CD8 cells (50), regulates antileishmanial defense by optimizing IL-12 production, shaping the Th1 cell-type response, and inducing macrophage activation via T cell secretion of IFN-γ (40, 51, 52). Not surprisingly, CD40L KO mice fail to control L. donovani replication at wk 8 (40), and in two new experiments extended in this study to wk 12, liver parasite burdens reached remarkably high levels (7636 ± 909 LDU; three experiments; n = 10 mice). Although these mice are AmB responsive (40) and showed 93% parasite killing on day 21 (Fig. 1C), relapse seemed assured in the absence of CD40L once the drug effect had waned. This prediction was borne out by the 20-fold increase in parasite burdens between wk 3 and 12 in treated CD40L KOs (Fig. 1C).

Infection recurred despite granulomatous responses at the majority of parasitized liver foci (Fig. 2E), indicating, as in untreated CD40L KO mice (40), that CD40L is required for control of L. donovani, but not for late-stage granuloma assembly.

Posttreatment recurrence in cytokine-deficient mice

IFN-γ and IL-12. Multiple cytokines participate in initial as well as memory responses in L. donovani infection (1, 10, 11, 28–34, 37, 39). Along with IL-12, for example, IFN-γ and TNF interdigitate prominently in macrophage activation and acquired resistance; if one of the three is deficient, visceral infection does not come under control (1, 31–34, 37, 53). Therefore, although AmB retains its initial leishmanicidal activity in the absence of IL-12, IFN-γ, or TNF (31, 34, 37), outgrowth of surviving parasites would be anticipated in animals lacking these cytokines. This expectation was recently confirmed in TNF KO mice (37).

The experiments in Figs. 3 and 4 in BALB/c KO mice extend this posttreatment analysis to IFN-γ and IL-12. In untreated IFN-γ and IL-12p35 KO mice, liver parasite burdens at wk 12 were high (5843 ± 621 and 3825 ± 222 LDU, respectively; two or three experiments; n = 9–12 mice), far exceeding wk 12 values in untreated WT BALB/c controls (81 ± 22 LDU; two experiments; n = 9 mice). Although both groups of cytokine-deficient mice also showed similar initial responses to AmB (91–98% killing by day 21; Fig. 3A), parasite burdens and histologic findings were strikingly different at wk 12 (Fig. 3A and Fig. 4, A–D). IFN-γ KO mice...
obviously relapsed after therapy, whereas IL-12p35 KOs did not, indicating posttreatment evolution to an IL-12-independent mechanism. The requirement for IFN-γ, also demonstrated in IFN-γ-deficient C57BL/6 mice (Fig. 3A), was underscored by reconstituting C57BL/6 nude mice with spleen cells from WT vs IFN-γ KO animals (Fig. 5). In contrast to WT spleen cells, which enabled suppression of parasite replication after AmB, transfer of IFN-γ-deficient cells provided little protective posttreatment effect.

**IL-18 and IL-23.** IL-12 is the primary IFN-γ-inducing cytokine in this L. donovani model (31–33). However, the preceding wk 12 observations pointed to an effect of other IFN-γ-inducing cytokines and prompted testing roles for IL-18 and IL-23 (48, 54, 55).

At both wk 2 and 4, liver parasite burdens were significantly higher in untreated C57BL/6 IL-18 KO mice vs WT animals; however, KO mice spontaneously controlled infection by wk 8 (two experiments; data not shown). At wk 12, liver burdens remained low in untreated IL-18 KO mice (164 ± 32 LDU) and were still lower in IL-18 KOs given AmB during wk 3 (24 ± 7 LDU; n = 8 mice/group).

To gauge the contribution of IL-23, BALB/c IL-12p40 KO mice (deficient in both IL-12 and IL-23) (38, 54–56) were tested in parallel with IL-12p35 KO mice (deficient in IL-12 alone) (31). Untreated IL-12p40−/− mice did not control L. donovani replication at wk 12 (6012 ± 649 LDU; two experiments; n = 9 mice). However, these KO animals responded to AmB (89% killing on day 21) and, like p35-deficient mice, did not show recurrent visceral infection at wk 12 (Fig. 3B and Fig. 4, E and F). Thus, by itself and akin to IL-18–IL-23 was not apparently required to prevent relapse after therapy, leaving open the question of what late-acting compensatory mechanism induces IL-12-independent IFN-γ in the posttreatment state.

Response to AmB in mice deficient in monocyte influx and macrophage leishmanicidal mechanisms. Blood monocytes, required effector cells in this model (57), initially use the endothelial cell adhesion molecule, ICAM-1, to enter infected liver foci and...

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**FIGURE 4.** Liver histologic appearance in cytokine-deficient mice 12 wk after infection. A, C, and E, Untreated mice; B, D, and F, mice given AmB 9 wk previously. A and B, IFN-γ-deficient BALB/c mice; C and D, IL-12p35-deficient BALB/c mice; E and F, IL-12p40-deficient BALB/c mice. All untreated mice (A, C, and E) show remarkably heavy infection (arrows) and little inflammatory response. Posttreatment, recrudescence developed only in IFN-γ KO mice (B), whereas IL-12p35 (D) and IL-12p40 (F) KO mice show few visible amastigotes (arrow in D) and little residual inflammatory response. Original magnification, ×200.

**FIGURE 5.** Response of reconstituted nude C57BL/6 mice to AmB given during wk 3. Conditions were as described in Fig. 1, except that 1 day before L. donovani challenge, nude mice received no cells or i.v. transfer of 5 × 10⁶ unfractionated spleen cells (27) from WT or IFN-γ-deficient C57BL/6 mice. Results are from two experiments and indicate the mean ± SEM values for six or seven mice per group per time point. *, p < 0.05 vs wk 3 value.
populate developing L. donovani-induced granulomas (36). Along with resident macrophages, influxing monocytes respond to IFN-γ and TNF activation with intracellular killing (57). The latter effect (and thus overall control of tissue infection) is achieved primarily via iNOS-derived reactive nitrogen intermediates; respiratory burst (phox)-derived reactive oxygen intermediates play an early, but transient, role (35). Therefore, to complete this analysis, we focused on mononuclear phagocyte mechanisms and tested the outcome of AmB treatment in mice deficient in ICAM-1 and in iNOS and/or phox.

**ICAM-1-deficient mice.** In ICAM-1 KO mice, initially unrestrained L. donovani replication is controlled after wk 4, but visceral infection does not resolve properly; wk 12 liver burdens are ~10-fold higher than in WT C57BL/6 mice (36). As reported previously (36) and observed in two new experiments, ICAM-1 KO mice treated during wk 3 responded to AmB (87% parasite killing; data not shown). At wk 12, liver burdens remained substantially elevated in untreated KO mice (969 ± 99 LDU; n = 9 mice); however, there was no recurrence of infection in AmB-treated animals (92 ± 38 LDU; n = 10 mice). The results from these two experiments suggested that the ICAM-1 mechanism had also become dispensable posttreatment.

**Phox-, iNOS-, and doubly-deficient mice.** In a prior study, L. donovani-infected phox KO and iNOS KO mice, treated with AmB during wk 3, showed near-identical initial (>90% parasite killing) as well as long-term responses (little or no increase in liver burdens at wk 12) (34). For phox-deficient mice, such a posttreatment outcome was not surprising, because these animals express iNOS activity (35, 58) and, after a transient increase in susceptibility, spontaneously resolve liver infection to low levels without treatment (35). In AmB-treated iNOS KO mice, however, the absence of relapse was entirely unexpected, because despite a retained phox mechanism and vigorous granuloma assembly (34, 35), untreated iNOS-deficient mice permit progressive parasite replication throughout 12 wk of infection (35).

Therefore, mice doubly-deficient in phox and iNOS (47) were examined to test the hypothesis that an entirely unrelated macrophage mechanism was active in preventing recurrent infection after treatment. As expected, liver parasite burdens in untreated animals at wk 12 were low in phox-deficient (77 ± 39) and high in both iNOS-deficient (3140 ± 291) and double-KO mice (4178 ± 636; two or three experiments; n = 7–17 mice/group; Fig. 6). In response to AmB treatment, initial leishmanicidal activity was similar in the three groups of deficient animals (97–98% killing; Fig. 7). At wk 12, infection did not recur in the absence of either phox or iNOS alone, as anticipated (34), but remarkably, also did not recur in mice lacking both pathways (Figs. 6D and 7). Thus, at the level of the parasitized macrophage, neither of this effector cell’s primary antileishmanial mechanisms was required to prevent relapse of chronic infection in the liver after chemotherapy.

**Discussion**

In the basic expressions of acquired resistance in this model of experimental visceral leishmaniasis, acquisition of initial resistance, resistance to reinfection, and maintenance of immunity after spontaneous self-cure (1, 10, 11, 27–42, 57), T cells, cytokines, and activated macrophages interdigitate as recognized cornerstones of host defense. However, in this spectrum of acquired responses, distinguishing features can be identified, including differing requirements for participation of particular T cells (e.g., CD4, CD8, IFN-γ, TNF). In this context, our findings suggest that a requirement for the iNOS pathway, in addition to the phox pathway, is necessary for the generation of acquired resistance to L. donovani.

**FIGURE 6.** Photomicrographs of liver sections from mice deficient in macrophage leishmanicidal mechanisms 12 wk after infection. A. Untreated phox KO mice have largely cleared infection, whereas untreated iNOS KO (B) and iNOS/phox double-KO mice (C) show heavily parasitized Kupffer cells (arrows) surrounded by intense, but ineffective, granulomatous inflammation. D. Double-KO mice treated 9 wk previously with AmB show only scattered residual granulomas and no recurrent infection. Original magnification, ×200.

**FIGURE 7.** Outcome of liver infection in mice deficient in macrophage leishmanicidal mechanisms after AmB treatment during wk 3 as described in Fig. 1 in iNOS KO, phox KO, and iNOS/phox double-KO (DKO) mice. Results are from two or three experiments and indicate the mean ± SEM values for six to 13 mice per group per time point. LDU values for phox, iNOS, and double KO mice were 41 ± 8, 49 ± 11, and 35 ± 11 at wk 3 and 6 ± 3, 9 ± 3, and 37 ± 14 at wk 12, respectively.
CD8, or both subsets) (10, 11, 27) and/or cytokines (e.g., IFN-γ is not required in resistance to rechallenge) (10). Similarly, regulation of the posttreatment response tested in this study also showed separate T cell and cytokine requirements compared with those, for example, of acquired resistance (Table I). Together, these observations underscore 1) the general flexibility of host antileishmanial programs, including the likely presence of multiple compensatory pathways, and 2) specific modification (evolution) of mechanisms in response to varying experimental conditions. Examples of the latter include the stage of visceral infection examined (acute vs chronic), the use of intentional rechallenge, and whether conversion to the chronic state develops spontaneously or is induced by chemotherapy as in the present study (10, 11, 27, 42).

Table I, which summarizes mechanisms in this model of initial acquired resistance vs prevention of recurrence after chemotherapy, should be viewed with some caution, however. First, different stages of infection were analyzed, and different experimental hosts were used (e.g., C57BL/6 and BALB/c mice and mAb-treated WT and KO mice). Second, different results might have been generated if chronically infected WT mice had been injected after treatment with cell-depleting or cytokine-neutralizing mAb or treated with an iNOS inhibitor (13), rather than studying genetically deficient KO mice. Third, the latter may express responses or mechanisms not ordinarily observed in or used in the same fashion by normal animals (37). Nevertheless, testing in cell-, cytokine-, or enzyme-deficient KO animals can readily demonstrate the presence of such compensatory-type mechanisms as well as identify dispensable factors. Fourth, the initial parasite-lowering effect of treatment should not be discounted because it may have provided the opportunity for alternative host immune responses to develop and be expressed during the posttreatment period. In addition, because responses to L. donovani can be organ specific (32), results for infection in the spleen or bone marrow in KO mice might also differ from those observed in this study.

To maintain the intracellular L. donovani amastigotes that escape chemotherapy in a long-term quiescent state, the chronically infected host may also have more than one basic mechanism upon which to draw. For example, humoral factors might play some protective role, although relapse in human infection occurs despite the presence of specific Ab (2). Much more likely, then, is an intracellular response, such as characterized in this report, which proved to be T cell-dependent and cytokine regulated, although independent of the activated macrophage’s primary leishmanicidal mechanisms.

In these experiments, prevention of posttreatment recurrence 1) was directed equally well by CD4 or CD8 T cells, 2) involved CD40L signaling, and 3) required IFN-γ (and TNF (37)), presumably to maintain a certain level of macrophage activation. Although CD4 cells probably exert multiple effects (27, 59), the role of CD8 cells in macrophage activation in this L. donovani model has been less well appreciated, albeit previously noted (27, 60). Nevertheless, like CD4 cells, CD8 cells express CD40L and secrete IFN-γ, TNF, and a variety of chemokines (27, 50, 60, 61). Thus, the effects of both cytokines, possibly mediated by CD40L:CD40 signaling (62), may underlie the capacity of either T cell subset to successfully orchestrate protective posttreatment responses. Antileishmanial effects of CD8 cells might also relate to separate pathways, including, perhaps, the granzyme-perforin mechanism (60, 61, 63).

Our results in cytokine-deficient mice suggest that although IFN-γ (present report) and TNF (37) probably act in concert after treatment to suppress the replication of residual intracellular liver parasites, this effect does not require IL-12, the primary IFN-γ-inducing cytokine in this model (31). IL-23 and IL-18 also induce or enhance IFN-γ secretion (48, 54, 55); however, neither cytokine was by itself required to prevent posttreatment relapse. These observations may well reflect an alternative IFN-γ-producing pathway, apparently dependent on TNF, but independent of IL-12, IL-23, and IL-18, recently described in IL-12p40−/−/IL-18−/− double-KO mice infected with Mycobacterium tuberculosis (55). Other IFN-γ-inducing stimuli, IL-2 (28), IL-15 (64), IL-27 (65), or...
possibly IL-21 (66), may also be worth investigating in the chronically infected host in this posttreatment model.

Because *L. donovani* resides within tissue macrophages in all stages of visceral infection, the logical target and role for IFN-γ and TNF in maintaining a quiescent posttreatment state is the parasitized macrophage and its activation. Our results in livers of mice doubly-deficient in phox and iNOS demonstrate, however, that neither of these leishmanial pathways is required to prevent posttreatment recurrence. Because prevention of relapse requires inhibition of replication, but not necessarily intracellular parasite killing, it is possible that in this particular setting, IFN-γ and TNF evolve to regulate a leishmanistic, rather than a leishmanical, mechanism. Either way, the results in double-knockout mice clearly uncover the presence and activity of a novel, compensatory antileishmanial mechanism not induced by *L. donovani* alone, but in this case triggered by AmB. Whether any form of effective antileishmanial chemotherapy can induce this macrophage mechanism (see below) will be clarified by testing Sb and/or miltefosine in iNOS/phox double-knockout mice. These same animals also show spontaneous macrophage antibacterial activity (47). Although the basis of this latter effect in doubly-deficient mice has not yet been identified, p47 GTPases have recently been implicated in a separate IFN-γ-inducible, iNOS- and phox-independent, macrophage antimicrobial mechanism (67, 68). This mechanism, which is active against intracellular pathogens (68), might also operate in *L. donovani* infection.

Although not investigated in this study, default to a suppressive-type cytokine response, mediated by IL-10 (41, 69) or, perhaps, IL-4, IL-13, or TGF-β (1, 2, 38, 70, 71), might possibly have fostered posttreatment relapse in IFN-γ- or CD40L-deficient animals (51, 52, 72). However, outcomes in AmB-treated TNF and IL-12 KO mice are at odds with this consideration, because TNF KO mice are not known to default to a Th2 cell-type response, but relapse after AmB (37), and IL-12p35 KO mice show evidence of default (31), but do not relapse after treatment (Fig. 3). In addition, WT BALB/c mice, induced by immunization to respond to *L. donovani* with an IL-4/IL-10-mediated noncure phenotype (70), also fostered posttreatment relapse in IFN-γ after miltefosine therapy also requires T cells (77). In preliminary experiments in which IFN-γ and IL-12p35 KO mice were treated during wk 3 with miltefosine (44), liver infection recovered at wk 12 in IFN-γ-deficient, but not IL-12-deficient, mice (not shown), similar to the findings in AmB-treated animals. These results with other forms of antileishmanial chemotherapy suggest that the mechanisms described in this report may be generally applicable to prevention of posttreatment relapse in experimental visceral infection.

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Disclosures

The authors have no financial conflict of interest.

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