Modulation of T-Cell Costimulation as Immunotherapy or Immunochemotherapy in Experimental Visceral Leishmaniasis

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CD40 ligand (CD40L)-deficient C57BL/6 mice failed to control intracellular Leishmania donovani visceral infection, indicating that acquired resistance involves CD40-CD40L signaling and costimulation. Conversely, in wild-type C57BL/6 and BALB/c mice with established visceral infection, injection of agonist anti-CD40 monoclonal antibody (MAb) induced killing of ~60% of parasites within liver macrophages, stimulated gamma interferon (IFN-γ) secretion, and enhanced mononuclear cell recruitment and tissue granuloma formation. Comparable parasite killing was also induced by MAb blockade (inhibition) of cytotoxic T lymphocyte antigen-4 (CTLA-4) which downregulates separate CD28-CD80 CD40L-CD40 T-cell costimulatory pathways. MAb blockade of either T-cell-expressed CD40L or its B7 ligands on APC (10, 11) would enable expression of CD80 CD40-dependent IFN-γ production and IFN-γ-mediated events (20). While anti-CD40 and anti-CTLA-4 treatment produces variable effects and can exacerbate cutaneous visceral leishmaniasis, MAb targeting of T-cell costimulatory pathways (CD40L-CD40 and CD28-CD80) yields macrophage activation and immunotherapeutic and immunochemotherapeutic activity.

An alternative strategy to therapeutically harness the same Th1 cell mechanism has focused on T-cell costimulation (7, 11, 19, 20). Optimal T-cell activation, including induction of the antileishmanial Th1 response with secretion of IL-12, IL-2, and IFN-γ, requires second (costimulatory) signals likely delivered via interaction of surface molecules on T cells and antigen-presenting cells (APC) (7, 11, 19, 20, 39, 41). CD40 ligand [CD40L]-CD40 and CD28-CD80 represent two such signal-transducing receptor pathways (38, 40), active in various forms of experimental leishmaniasis (3, 4, 10, 11, 14, 15, 17, 32, 37, 38) and accessible to manipulation by MAb injection (7, 15, 18–20). Depending upon the model, the host, and the timing of MAb administration, receptor manipulation can stimulate Th1 type responses and enhance resistance.

For example, when given prophylactically, injections of agonist anti-CD40 MAb successfully curtail cutaneous L. major infection in susceptible BALB/c mice, an effect mediated by APC-secreted IL-12 and downstream T-cell-derived IFN-γ (7). Similarly, MAb-induced blockade of cytotoxic T lymphocyte antigen-4 (CTLA-4), an inhibitory receptor which limits CD28-CD80 costimulation (15, 19, 20, 40), can also enhance IL-12 production and IFN-γ-mediated events (20). While anti-CTLA-4 treatment produces variable effects and can exacerbate cutaneous L. major infection (10, 15), anti-CTLA-4 is active both prophylactically and therapeutically against visceral L. donovani infection (20). In vitro studies with L. chagasi, another agent of visceral leishmaniasis, also support the antileishmanial effect of MAb blockade of either T-cell-expressed CTLA-4 or its B7 ligands on APC (10, 11).

Increased levels of endogenous IL-12 and/or IFN-γ would

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be expected to enhance antileishmanial defense and strengthen the host response to Sb chemotherapy (20, 23). Therefore, in this study, anti-CD40 was used as an agonist to ligate CD40 and trigger IL-12 secretion (5, 14, 33, 39) while anti-CTLA-4 was used to block negative signaling and maintain CD28-B7-dependent T-cell activation and cytokine secretion (15, 20, 41, 43). We reasoned that in addition to producing activity by itself in the case of established *L. donovani* infection (20), MAb-induced modulation of T-cell costimulatory mechanisms could also be coupled with Sb in an immunochemotherapeutic regimen.

**MATERIALS AND METHODS**

**Animals.** Twenty- to 30-g female C57BL/6 and BALB/c mice, purchased from the Jackson Laboratory (Bar Harbor, Maine) and Charles River Laboratories (Wilmington, Mass.), respectively, were used as wild-type controls. Pairs of gene-disrupted mice for breeding on a C57BL/6 background were originally obtained from the following sources: CD40L−/−, intracellular adhesion molecule-1 (ICAM-1)-deficient, and IFN-γ−/− mice were from Jackson (22, 27); inducible nitric oxide synthase (iNOS)−/− mice were from C. Nathan, Weill Medical College, New York, N.Y. (26); and respiratory burst (phagocyte oxidase [phox])-deficient gp91 phox−/− mice were from M. Dinauer, Indiana University Medical Center, Indianapolis, Ind. (26). IL-12p35−/− breeders on a BALB/c background were originally provided by J. Sypek (Genetics Institute, Andover, Mass.) (28). Mice were 6 to 12 weeks old when challenged with *L. donovani*; male and female gene-disrupted mice were used in a random fashion.

**Visceral infection and tissue response.** Groups of four to five mice were infected via the tail vein with 1.5 × 10⁷ hamster spleen-derived *L. donovani* amastigotes (1 Sudan strain) (30). Visceral infection was monitored microscopically using Giemsa-stained liver imprints in which liver parasite burdens were measured by blinded counting of the number of amastigotes per 500 cell nuclei and multiplication by the liver weight in milligrams (liver parasite burdens are measured by blinded counting of the number of amastigotes per 500 cell nuclei) (15, 20, 41, 43). We reasoned that in addition to producing activity by itself in the case of established *L. donovani* infection (20), MAb-induced modulation of T-cell costimulatory mechanisms could also be coupled with Sb in an immunochemotherapeutic regimen.

![FIG. 1. Course of *L. donovani* infection in livers of wild-type C57BL/6 (open circles) and CD40L−/− (closed circles) mice. Results shown are means ± standard errors of the means (SEM) of values from two experiments with 8 to 10 mice for each time point. *P* is <0.05 for control versus CD40L−/− mice at weeks 3, 4, and 8.](Image)

**RESULTS**

CD40L and anti-CD40 in defense against *L. donovani*. CD28-B7 signaling has already been shown to be active in experimental *L. donovani* infection since manipulation of this pathway by blockade of CTLA-4 or one of its B7 ligands increased Th1 type cytokine expression, promoted granuloma formation, and reduced liver parasite burdens (19, 20). To determine whether the CD40L-CD40 mechanism can mediate a similar effect, we first challenged CD40L−/− mice with *L. donovani* and then treated parasitized normal mice with agonist anti-CD40 MAb. In the latter experiments, anti-CTLA-4 was tested in parallel.

**CD40L-deficient mice.** As shown in Fig. 1, wild-type C57BL/6 mice controlled *L. donovani* infection after week 3, demonstrating a Th1 cell type-dependent response which requires IL-12 and IFN-γ (6, 27, 28, 34, 40). In contrast, CD40L−/− mice developed high-level, nonresolving liver infections. Since CD40L-CD40 interaction is a primary stimulus for IL-12 generation by APC (5, 14, 33, 39, 41), the failure to control *L. donovani* infection likely involved deficient Th1 cell type responses. In one of the experiments with results shown in Fig. 1, sera (from three to four mice per group) were available for cytokine measurement before (day 0) and on day 21 after infection. In wild-type C57BL/6 mice, levels of IL-12p40 increased from 3.0 ± 0.1 to 8.1 ± 0.4 ng/ml and those for IFN-γ increased from 0.01 ± 0.01 to 0.25 ± 0.11 ng/ml. In CD40L−/− mice, corresponding day 0 and day 21 values for IL-12p40 were 1.9 ± 0.3 and 2.8 ± 0.3 ng/ml and those for IFN-γ were 0 (<6 pg/ml) and 0.01 ± 0.01 ng/ml. Thus, on day 21, when liver parasite burdens in control and deficient mice had clearly diverged (Fig. 1), serum IL-12p40 and IFN-γ levels appeared to be lower in CD40L−/− mice. Uncontrolled *L. major* and *L. amazonensis* cutaneous infection in CD40L−/− and CD40−/−
mice has also been previously linked to defective IL-12 and IFN-γ generation (4, 17, 37).

The tissue correlate of the IL-12- and IFN-γ-driven response, granuloma assembly (24), was also impaired in CD40L-deficient mice. In livers of wild-type C57BL/6 mice, granulomas were forming by week 2 at 89% ± 3% of parasitized foci and were developed (mature) at 92% ± 5% of foci by week 4 (Fig. 2A). In CD40L−/− mice, this response was essentially absent at week 2 and barely evident at week 4 (Fig. 2B), and then it emerged but remained incomplete. At week 8, 23% ± 6% of infected sites showed no mononuclear cell recruitment; the remainder showed developing (62% ± 7%) or morphologically mature (15% ± 2%) granulomas (Fig. 2C). However, virtually all of the latter contained large numbers of amastigotes (Fig. 2D), indicating that granulomas appearing to be structurally intact were nonfunctional in the absence of CD40L.

Anti-CD40-treated mice. To demonstrate the effect of CD40 ligation under intact conditions, wild-type BALB/c mice with established infection were injected with agonist anti-CD40 on day 12. Measurements made on day 21 showed that treatment with 0.25 to 0.5 mg of MAb induced leishmanicidal activity, with >50% reduction in liver parasite burdens (Fig. 3). Wild-type C57BL/6 mice responded in a similar fashion (Table 1). In addition, the antileishmanial effect of single-dose anti-CD40 persisted beyond day 21 (Fig. 4).

The anti-CD40 effect was also accompanied by increased IFN-γ production, as judged by cytokine levels in the serum of BALB/c mice on day 21 (Fig. 5). In the same mice, IL-12p40 was already readily detectable in sera on day 12, prior to MAb injection (8.1 ± 1.1 ng/ml; n = 10). Serum IL-12p40 levels increased in anti-CD40-treated mice at day 21; however, the day 21 level (11.1 ± 1.4 ng/ml; n = 10) was not significantly different from that in infected controls treated with rat IgG (7.8 ± 0.8 ng/ml; n = 6 [P = 0.11]). Since increases in serum IL-12 levels (as well as IFN-γ levels) appear to peak soon after (38) or within 3 to 5 days (F. Heinzel, unpublished data) of anti-CD40 injection, IL-12p40 levels prior to day 21 may have been higher in our experiments. In addition, it is also possible that examination at the infected tissue site (e.g., at developing liver granulomas) would have better demonstrated an anti-CD40 effect on IL-12 expression.

Anti-CD40 MAb treatment also induced the remarkable appearance of numerous, often large focal collections of mononuclear cells (primarily lymphocytes, morphologically) in livers of infected BALB/c (Fig. 6 and 7) and C57BL/6 (data not shown) mice. These collections, not present in infected rat IgG-injected controls, were both perivascular and parenchymal.
and were, in most instances, densely encased parasitized macrophages (Fig. 6D). Anti-CD40 injection also induced some mononuclear cell accumulations in livers of uninfected BALB/c mice (Fig. 7). However, the numbers and sizes of the collections were considerably greater in infected mice (Fig. 7B and D), suggesting that anti-CD40-mobilized cells were attracted to sites of developing inflammation and/or parasite replication. In infected mice examined 2 weeks after CD40 injection on day 12, the histologic reaction had begun to recede (data not shown).

Effect of anti-CTLA-4 MAb versus that of anti-CD40 MAb. In the experiments described above, wild-type BALB/c mice were also injected once on day 12 with anti-CTLA-4 MAb. Effects on day 21 mirrored those previously reported for anti-CD40 (20): (i) leishmanicidal activity, which also persisted on day 21. LDU values for untreated mice on day 14 were used for comparison with day 21 LDU values for treated animals. Treatment of the anti-CD40 group was as follows: rat IgG (0.5 mg; open bar) or MAb at 0.1 mg (diagonally hatched bar), 0.25 mg (closed bar), or 0.5 mg (horizontally hatched bar). Treatment of the anti-CTLA-4 group was as follows: hamster IgG (0.5 mg; open bar) or MAb at 0.1 mg (diagonally hatched bar), 0.3 mg (closed bar), or 0.5 mg (horizontally hatched bar). Results shown are means ± SEM of results from two to three experiments with 7 to 16 mice per group. *, P of <0.05 versus day 14 LDU value and versus day 21 LDU value for IgG-treated controls. **, P of <0.05 versus day 21 LDU value for IgG-treated controls.

### Table 1. Leishmanicidal effects of anti-CD40 and anti-CTLA-4 treatments

<table>
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<th>Mouse group</th>
<th>Treatment</th>
<th>Liver parasite burden (LDU) on day:</th>
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* Twelve days after infection, mice received one injection of either 0.25 mg of rat IgG or anti-CD40 or 0.5 mg of hamster IgG or anti-CTLA-4, and LDU values were determined on day 21. Day 14 values indicate LDU values for untreated mice. Results shown are means ± SEM of results from two to three experiments with 7 to 10 mice per group. **, P of <0.05 versus day 14 value.

** a, mice received indicated treatment.

### Treatment with anti-CD40 plus anti-CTLA-4. CD40L-CD40 and CD28-B7, linked by induction of B7 expression by CD40 stimulation (5, 8, 13, 39, 41), also converge functionally since both support Th1 cell-associated responses and stimulate IFN-γ secretion downstream (39, 41). Since the initial activation of cytokines primarily induced are thought to differ (i.e., IL-12 and IFN-γ secretions from CD40 versus IL-2 via CD28) (39, 41, 43), we tested whether leishmanicidal activity could be increased by triggering both pathways. Wild-type BALB/c mice were injected on day 12 with anti-CD40 (0.25 mg) and 2 h later with anti-CTLA-4 (0.5 mg). Combined treatment did not, however, increase parasite killing on day 21 nor yield additional histologic changes other than those induced by anti-CD40 alone (data not shown).

### Host mechanisms in response to anti-CD40 and anti-CTLA-4. In normal BALB/c and C57BL/6 mice, the IL-12-driven Th1 cell response activates macrophages for intracellular L. donovani killing within encircling granulomas (23, 24). The leishmanicidal mechanism requires IFN-γ induced by IL-12 and/or IL-2, influxing T cells and blood monocytes, and mononuclear phagocyte secretion of toxic products, largely iNOS-derived reactive nitrogen intermediates (6, 20, 22, 23, 24, 26, 28, 34, 40). To characterize these host components in responses to anti-CD40 and anti-CTLA-4, we tested mice deficient in cytokine production (IL-12p35−/− and IFN-γ−/−),
monocyte influx (ICAM-1 deficient), and reactive nitrogen intermediate secretion (iNOS−/−) (22, 26, 28, 40).

**Leishmanicidal responses.** Killing induced by both anti-CD40 and anti-CTLA-4 required endogenous IFN-γ (Table 1). Optimal killing also involved IL-12, although both MAbs induced a limited effect in IL-12p35 −/− mice. In contrast, anti-CD40 and anti-CTLA-4 were fully active in mice deficient in iNOS and/or ICAM-1, and killing was also retained in iNOS−/− animals. Mice deficient in the mononuclear phagocyte’s auxiliary leishmanicidal mechanism, respiratory burst (phox)-generated toxic oxygen intermediates (26), were tested next. Phox-deficient mice also showed intact responses to both MAbs (Table 1), leaving open the question of what macrophage leishmanicidal pathway is triggered by MAb-induced CD40 ligation or CTLA-4 blockade.

**Tissue granulomatous responses.** Expression of ICAM-1, an endothelial adhesion molecule, is required early on in *L. donovani* infection (weeks 1 to 4) for monocyte entry into and formation of liver granulomas (22). Nevertheless, ICAM-1-deficient mice treated on day 12 readily responded to both anti-CD40 and anti-CTLA-4 with mononuclear cell recruitment and assembly (Fig. 8A and B and data not shown). On day 21, the percentage of infected foci which showed mature granulomas increased from <10% in rat and hamster IgG-treated mice to 81% ± 4% and 84% ± 6%, respectively. This ICAM-1-independent tissue response may reflect activation of a compensatory mechanism previously demonstrated in these same ICAM-1-deficient mice (22).

Histologic inspection of livers from cytokine-deficient mice yielded additional information about the actions of anti-CTLA-4 and anti-CD40 and the ways in which their effects differ. In livers of IFN-γ−/− and IL-12p35 −/− mice, which fail to show granulomas or any tissue inflammatory reaction in response to *L. donovani* (28, 34, 39), anti-CTLA-4 treatment on day 12 had no effect (data not shown). In contrast, in the same IFN-γ−/− mice, anti-CD40 induced both the mononuclear cell accumulations seen in normal mice and developing granulomas at 79% ± 9% of infected liver foci on day 21 (Fig. 8C and D). Thus, while IFN-γ is strictly required for granuloma assembly in intact mice (40), CD40 ligation (but not CTLA-4 blockade) activated an apparently quiescent mechanism for mononuclear cell recruitment in IFN-γ−/− mice. IL-12 is one inducer of such a compensatory, IFN-γ-independent granuloma-forming pathway (40). In IL-12p35 −/− mice, anti-CD40 treatment had no effect on the absent histologic response (data not shown), suggesting that IL-12 may participate in the anti-CD40-induced IFN-γ-independent effect. Not surprisingly, however, in the absence of IFN-γ and macrophage activation (23, 24), granulomas induced by anti-CD40 in IFN-γ−/− mice remained heavily parasitized (Fig. 8E).

**Responses to antileishmanial chemotherapy.** In the case of *L. donovani* infection, the leishmanicidal efficacy of Sb chemotherapy also requires host T cells and an intact Th1 cell type response with IL-12 and IFN-γ secretion (23, 27, 28). Sb’s efficacy can be enhanced by IFN-γ either by coadministration in exogenous form or by injection of IL-12 to increase endogenous IFN-γ production (21, 25, 28). Therefore, we hypothesized that Sb’s effect would be (i) diminished in Th1 cell cytokine-deficient CD40L-deficient mice and (ii) enhanced in normal animals first treated with anti-CD40 or anti-CTLA-4.

The results presented in Table 2 confirm the first hypothesis. Wild-type C57BL/6 animals responded to optimal-dose Sb (500 mg/kg) with killing of 88% of liver amastigotes; identically treated CD40L−/− mice failed to show leishmanicidal activity.

![Graph showing extended antileishmanial effects of anti-CD40 and anti-CTLA-4.](image)

**FIG. 4.** Extended antileishmanial effects of anti-CD40 and anti-CTLA-4 MAb treatment. Wild-type BALB/c mice were injected once 12 days after infection with 0.25 mg of rat IgG (open circles) or anti-CD40 (closed circles) or 0.5 mg of hamster IgG (open squares) or anti-CTLA-4 (closed squares). Results shown are means ± SEM of results from two experiments with seven to eight mice per time point. *P* < 0.05 for results with anti-CD40 and anti-CTLA-4 at both days 21 and 28 versus the day 12 LDU value.

![Graph showing serum IFN-γ levels on day 21.](image)

**FIG. 5.** Serum IFN-γ levels on day 21, after MAb treatment on day 12. Wild-type BALB/c mice were injected once 12 days after infection with either 0.25 mg of rat IgG (horizontally hatched bar) or anti-CD40 (closed bar) or 0.5 mg of hamster IgG (diagonally hatched bar) or anti-CTLA-4 (open bar). Results shown are means ± SEM of results from two experiments with 6 to 10 mice. *P* of ≤0.05 versus day 21 value for IgG-treated controls.
To demonstrate responsiveness to directly acting chemotherapy which does not require the host Th1 cell response (23, 27, 28), animals were also treated with AmB. CD40L knockout mice responded normally to this agent (Table 2).

To test the second hypothesis and complete this analysis, we used wild-type BALB/c mice and injected suboptimal doses of MAb on day 12 and low-dose Sb on day 14. Injections were separated by 48 h since Sb’s efficacy appears to be enhanced if administered after cytokine effects are established (21, 28, 30). Results on day 21 indicated no parasite killing by low-dose Sb in animals pretreated with either rat or hamster IgG (Fig. 9). In contrast, in anti-CD40- or anti-CTLA-4-treated animals, Sb was significantly more active ($P < 0.05$) and induced killing of 40 to 50% of amastigotes. A portion of the effect (~10%) was

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**FIG. 6.** Liver histologic responses to anti-CD40 and anti-CTLA-4 MAb in infected wild-type BALB/c mice. Mice were injected on day 12 with 0.25 mg of rat IgG or anti-CD40 or 0.5 mg of hamster IgG or anti-CTLA-4, and sections were prepared on day 21. (A and C) Infected foci in rat IgG-treated mice show early, developing granulomas. (B and D) In contrast, in anti-CD40-treated mice, parasitized foci are encased by large accumulations of mononuclear cells (arrows). (E and F) Developing granulomas in hamster IgG-treated liver (E) versus more-mature-appearing granulomas in anti-CTLA-4-injected animal (F). Panels A and B, magnification of ×180; panels C to F, magnification of ×284.
due to MAb by itself (Fig. 9). However, four- to fivefold increases in killing induced by combination treatment indicated clearly enhanced responsiveness to Sb in the presence of either CD40 ligation or MAb-induced blockade of CTLA-4.

**DISCUSSION**

The findings in this report extend prior studies of the role and effect of the CD40-CD40L pathway in cutaneous *L. major* and *L. amazonensis* infection (3, 4, 7, 10, 14, 15, 17, 32, 37, 38) and of CTLA-4 blockade in *L. major* and *L. donovani* infection (10, 15, 20). Together, our results (i) demonstrate that CD40L is required for control of *L. donovani* infection, appropriate generation of IL-12p40 and especially IFN-γ/H9253, development of functional tissue granulomas, and response to Sb chemotherapy; (ii) identify mechanisms of the therapeutic effect of anti-CD40 MAb in the case of established visceral infection and show that anti-CD40 enhances IFN-γ and modifies mononuclear cell recruitment to parasitized sites; and (iii) confirm the antileishmanial action of CTLA-4 blockade in the case of *L. donovani* infection (20). In addition, our results indicate that both anti-CD40 and anti-CTLA-4 increase the efficacy of chemotherapy (Sb), consistent with effects on the Th1 type cytokine (e.g., IFN-γ) response which primarily regulates Sb’s leishmanicidal activity (1, 23, 27). In the assays we employed, the only meaningful difference between the actions of the two MAbs was the capacity of anti-CD40 to stimulate focal recruitment of inflammatory mononuclear cells and to accomplish this effect in an IFN-γ-independent fashion.

Although induction of granuloma assembly in IFN-γ−/− mice demonstrated an IFN-γ-independent action for anti-CD40, its leishmanicidal activity and that of anti-CTLA-4 required endogenous IFN-γ and, to a lesser extent, its primary inducer, IL-12 (28). While anti-CD40 and anti-CTLA-4 both stimulated IFN-γ production, likely enhancing downstream effector cell mechanisms, parasite killing triggered by these MAbs was nevertheless preserved in mice deficient in either iNOS or phox. This finding was unexpected since anti-CD40 induces macrophage iNOS (42) and iNOS in turn regulates the key leishmanicidal pathway in the IFN-γ-activated macrophage (26). While a prior study indicated that the phox mechanism by itself was not sufficient to control *L. donovani* in otherwise untreated iNOS−/− mice (26), gene-deficient animals, including iNOS−/− and phox−/− mice, may express heightened compensatory responses (29, 30, 31). Thus, it is possible that the efficacy of phox in iNOS−/− mice was enhanced by anti-CD40 or anti-CTLA-4 treatment leading to respiratory burst-mediated parasite killing. However, we have not tested anti-CD40 or anti-CTLA-4 in mice deficient in both iNOS and phox (36); therefore, it also remains possible that a
recently identified but undefined macrophage mechanism independent of both pathways may be involved (36). Given the intracellular location of *L. donovani*, the likelihood that anti-CD40 or anti-CTLA-4 stimulated an altogether separate, IFN-γ-dependent mechanism unrelated to macrophage activation seems low.

Anti-CD40 was used here to ligate CD40 and primarily trigger IL-12 secretion by APC, a well-defined effect of signaling through CD40 in dendritic cells (5, 14, 33, 39). Anti-CTLA-4 was employed to block negative signaling and maintain CD28-B7-dependent T-cell activation and cytokine (e.g., IL-2) secretion (15, 20, 41, 42). Despite differing modes of

FIG. 8. Liver histologic response to anti-CD40 on day 21 in granuloma-deficient ICAM-1−/− and IFN-γ−/− mice. Mice were injected on day 12 after infection with 0.25 mg of rat IgG or anti-CD40. (A and B) In ICAM-1−/− mice, IgG had no effect on deficient granuloma formation (arrow) (A), while anti-CD40 induced a granulomatous reaction at parasitized foci (B). (C to E) Rat IgG-treated IFN-γ−/− deficient mice show no tissue inflammatory response at infected foci (arrows) (C), while anti-CD40-induced granulomas (D) remain heavily parasitized (arrow) (E). Panels A and B, magnification of ×290; panels C and D, magnification of ×184; panel E, magnification of ×460.
action, both MAb-directed immunomodulations would be expected to enhance IFN-γ production and generally raise the level of T-cell reactivity (9, 15, 20, 34, 37, 38, 40, 42). Such reactivity, expressed by increased Th1 cell type cytokine activity, regulates the outcome of visceral *L. donovani* infection and in vivo responsiveness to Sb chemotherapy (6, 23, 24, 27, 28, 30, 34, 40). Thus, in the case of established visceral infection, manipulation of CD40L-CD40 or CD23-B7 costimulatory mechanisms, conveniently achieved by a single injection of MAb, appears to represent a viable immunotherapeutic or immunochemotherapeutic strategy. Triggering other costimulatory pathways may also have similar treatment potential (8, 12, 32, 35, 43).

Since the preceding experiments were carried out in normal animals in the midst of developing a Th1 cell response, the therapeutic usefulness of anti-CD40 or anti-CTLA-4 is likely predicated on host capacity to satisfactorily produce IL-12 and IFN-γ. In addition, it is also worth noting that CD40 activation and blockade of CTLA-4 have the potential to induce the release of multiple proinflammatory cytokines (7, 16, 39, 41); thus, although not seen in our model, MAb treatment, especially if given repeatedly (16), could induce an undesirable inflammatory cytokine cascade. Nonetheless, from the perspective of treatment intended to activate macrophages, modulation of Th1 cell responses via costimulation pathways may well be an alternative to the administration of exogenous cytokines (23).

**TABLE 2. Response of CD40L knockout mice to chemotherapy**

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Treatment</th>
<th>Liver parasite burden (LDU) on day 14</th>
<th>% Killing</th>
<th>Liver parasite burden (LDU) on day 21</th>
<th>% Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>None</td>
<td>1,168 ± 122</td>
<td>0</td>
<td>1,315 ± 92</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sb</td>
<td>140 ± 37α</td>
<td>88</td>
<td>191 ± 16α</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>AmB</td>
<td>91 ± 16α</td>
<td>92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40L−/−</td>
<td>None</td>
<td>1,785 ± 201</td>
<td>0</td>
<td>2,911 ± 270</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sb</td>
<td>1,948 ± 194</td>
<td>0</td>
<td>2,911 ± 270</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AmB</td>
<td>52 ± 33α</td>
<td>97</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Two weeks after infection (day 14), C57BL/6 wild-type and CD40L−/− mice received no treatment, a single injection of 500 mg of Sb/kg, or three every-second-day injections of 5 mg of (AmB/kg). Results shown are means ± SEM of results from two experiments with six to seven mice per group.

b *P* <0.05 versus day 14 value.

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