Brief Definitive Report

Interleukin 12 Is Effective Treatment for an Established Systemic Intracellular Infection: Experimental Visceral Leishmaniasis

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Summary

When administered at or near the initiation of experimental intracellular infection caused by *Leishmania major*, *Toxoplasma gondii*, or *Cryptococcus neoformans*, treatment with the immunoregulatory cytokine interleukin 12 (IL-12), induces protective antimicrobial activity. In contrast, once infections are established, IL-12 exerts considerably less or no effect in the face of a suppressive Th2 cell–associated response (*L. major*) or rapidly progressive fatal infection (*T. gondii*). To test the efficacy of IL-12 in an established intracellular protozoal infection but under quite different immunologic conditions (Th1 cell response, acquired resistance), *L. donovani*–infected BALB/c mice were treated starting 2 wk after challenge coincident with the onset of the Th1 cell response. In this environment, 7 d of IL-12 treatment reduced liver parasite burdens by 47%, an effect comparable to that induced by exogenous interferon (IFN) γ. The in vivo mechanism responsive to IL-12 was complex, and required both $CD4^+$ and $CD8^+$ T cells as well as natural killer cells and the action of multiple endogenous antileishmanial cytokines (IFN-γ, IL-2, tumor necrosis factor α). Early treatment with IL-12 before the expression of the Th1 cell response was also effective and induced an accelerated, near-cure response via an IFN-γ–dependent mechanism. These results extend the antimicrobial-inducing capacity of IL-12 beyond prophylaxis by indicating that IL-12 can exert clear-cut therapeutic activity in an established intracellular infection.

Numerous recent reports have identified the pleiotropic cytokine, IL-12, as a critical component of the early events that initiate successful cell-mediated antimicrobial defense (1–3). The role and/or effect of IL-12 has thus far been tested in four models of intracellular infection caused by *Leishmania major* (4, 5), *Toxoplasma gondii* (6–8), *Listeria monocytogenes* (9), and *Cyptococcus neoformans* (10). Results from these models suggest that the role of endogenous IL-12 and the treatment effect of exogenous IL-12 are both most prominent at or near the time infection is first introduced (4, 5, 7). Indeed, when tested after the initial stage of host–parasite interaction in two of these models, IL-12 in exogenous form exerted considerably less activity in *L. major* infection (4, 5) and no effect in acute toxoplasmosis (7). These latter results have prompted the impression that while IL-12 appears to be of clear-cut immunoregulatory importance (1–3) and effective early on or as prophylaxis (4–7, 10), there may nevertheless be a narrow therapeutic window for IL-12 in such infections.

Specific features of the preceding models of uncontrolled progressive infection (4, 5, 7), however, likely influenced the efficacy of delayed IL-12 treatment: (*a*) the rapid establishment of an overwhelming and uniformly fatal infection in the acute *T. gondii* model (7), and (*b*) the prompt emergence of a particularly suppressive Th2 cell–associated response which characterizes and propagates *L. major* infection in BALB/c mice (4, 5). The results in these selected models, therefore, have left open the question of whether IL-12 can act therapeutically in other settings, for example, in an established but not fatal intracellular infection and/or in an intracellular infection associated with acquired resistance and a Th1 cell– rather than a Th2 cell–associated response. Since our model of visceral leishmaniasis fulfills these latter criteria (11, 12), we treated *L. donovani*–infected BALB/c mice with IL-12 to test its in vivo activity under these quite different immunologic conditions.

Materials and Methods

Visceral Infection. 20–30-g female euthymic BALB/c and C57BL/6 beige mice (Charles River Breeding Laboratories, Wilmington, MA) and athymic (nude) BALB/c mice (Life Sciences, Inc., St. Petersburg, FL) were infected by tail vein with 1.5×10^7 *L. donovani* amastigotes maintained in hamsters (11). Visceral infection was assessed microscopically in liver imprints, and liver parasite burdens are expressed as Leishman-Donovan units (LDU) (11). Tissue granuloma development was examined using formalin-fixed, stained liver sections (11).

Treatment with IL-12 and IFN-γ. Groups of three to five mice were treated for 7 d with 1 μg/d of murine rIL-12 (7.8×10^6 U/mg; Genetics Institute, Cambridge, MA) (4) and/or 2×10^5

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U/d of murine rIFN-γ (2×10^7 U/mg; Amgen Biologicals, Thousand Oaks, CA) suspended in saline containing 1 mg/ml of BSA (13). Treatment was given continuously via subcutaneously implanted osmotic pumps (Alzet model 2001; Alza Corp., Palo Alto, CA) (12) which deliver drug for 7 d (12a). Preliminary experiments indicated that IL-12 delivered at 1 μg/d by pump for 7 d was as effective as seven consecutive daily intraperitoneal injections of 1 μg of IL-12 (4, 5) (data not shown). Pumps were implanted 4 h after *L. donovani* challenge to test early (prophylactic) effects or 14 d after challenge to determine therapeutic activity in established infection. Treatment with pump-delivered saline/BSA alone had no effect as in prior studies (13).

Treatment with Cell-depleting and Anticytokine Antibodies. Using previously described preparations and administration schedules (14–16) (see legends to Tables 1 and 2 and Fig. 1), IL-12–treated mice were injected intraperitoneally with: (*a*) 1 ml of hybridoma culture supernatants containing rat anti-mouse mAb GK1.5 (anti-CD4, American Type Culture Collection [ATCC, Rockville, MD] TIB 207, 8 μg/ml of IgG), 53-6.72 (anti-CD8, ATCC TIB 105, 11.6 μg/ml of IgG), and S4B6.1 (anti-IL-2, 12.5 μg/ml of IgG), or 25 μg of normal rat IgG (Sigma Chemical Co., St. Louis, MO); (*b*) 40 μl of NK cell–depleting rabbit antiasialo GM1 antiserum (Wako Bioproducts, Richmond, VA) (15); or (*c*) 0.2 ml containing normal rabbit serum, rabbit anti-mouse TNF-α antiserum (1.5×10^5 neutralizing U/ml) (16), or rabbit anti-mouse IFN-γ antiserum raised against murine rIFN-γ. At a dilution of 1:10,000, the latter preparation neutralized the activity of 10 U of rIFN-γ in an ELISA (Endogen Inc., Boston, MA). Fluorescence analysis of spleen cells (14) after three consecutive daily injections of anti-CD4 or anti-CD8 mAb indicated depletion of the targeted T cell subset by 89–93% (data not shown).

Results and Discussion

Effect of IL-12 Treatment in Established Visceral Infection. In euthymic BALB/c mice, *L. donovani* multiplies logarithmically within macrophages of the liver and spleen during the first 2–4 wk after challenge (11). By week 4, T cell–dependent acquired resistance mediated by the Th1 cell–associated cytokines, IFN-γ and IL-2 (12, 15, 17), develops, visceral infection comes under control, and organ parasite burdens decline (11). To test the effect of IL-12 under these conditions in established visceral infection, pump treatment was begun 2 wk after *L. donovani* challenge and continued for 7 d. As shown in Table 1, IL-12 at 1 μg/d readily induced leishmanicidal activity, and liver parasite burdens decreased by 47% (day 14 vs. day 21). Treatment with 0.1 μg/d produced a similar effect, whereas 0.01 μg/d did not induce leishmanicidal activity (data not shown). The effect of pump IL-12 was comparable to the 40% killing achieved by 7 d of pump treatment with 2×10^5 U/d of IFN-γ (data not shown), a cytokine with already well-demonstrated antileishmanial activity (13). 2-wk infected mice were also treated for 7 d with IL-12 (1 μg/d) plus IFN-γ (2×10^5 U/d) by inserting two pumps. However, combination cytokine treatment did not achieve effects beyond those induced by IL-12 alone (two experiments, not shown) suggesting that exogenous IL-12 and IFN-γ may use or stimulate the same antileishmanial mechanism or target effector cells. The granulomatous response in the liver, the histologic correlate of acquired resistance in this model (11), can be upregulated by treatment with some

Table 1. *Effect of IL-12 Treatment in Established Infection and Role of T Cells and NK Cells*

Mice and treatment*	Liver parasite burden (LDU)		Percent killing‡
	day 14	day 21	
Euthymic BALB/c			
Untreated	1,219 ± 72 (16)	2,157 ± 155 (16)	0
IL-12		647 ± 51 (18)	47
+ anti-CD4		1,802 ± 115 (6)	0
+ anti-CD8		2,321 ± 147 (6)	0
+ rat IgG		810 ± 76 (9)	34
+ anti-asialo GM1		1,481 ± 110 (6)	0
+ rabbit serum		670 ± 65 (6)	45
Nude BALB/c			
Untreated	1,787 ± 118 (13)	3,483 ± 154 (13)	0
IL-2		3,964 ± 250 (12)	0
C57BL/6 Beige			
Untreated	2,105 ± 120 (6)	2,955 ± 145 (6)	0
IL-12		1,397 ± 104 (6)	34

* 2 wk after *L. donovani* infection, liver parasite burdens were determined, and pumps delivering 1 μg/d of IL-12 were then inserted on day 14. Anti-CD4, anti-CD8, and control rat IgG injections were given on days 11, 12, 13, 17, and 19 (14, 15). Antiasialo GM1 antiserum and normal rabbit serum were injected on days 11 and 17 (14). Results are from two to four experiments, and indicate mean ± SEM values for (*n*) mice per group.
‡ Percent killing = day 14 LDU − day 21 LDU ÷ day 14 LDU × 100 (13).

Table 2. *Role of Endogenous Cytokines in IL-12–induced Antileishmanial Activity*

Treatment	Liver parasite burdens (LDU) day 14	Liver parasite burdens (LDU) day 21	Percent killing
None (control)	1,280 ± 46 (12)	2,488 ± 123 (12)	0
IL-12		771 ± 78 (12)	40
+ anti-IFN-γ		2,936 ± 160 (10)	0
+ anti-TNF-α		2,820 ± 125 (11)	0
+ rabbit serum		886 ± 86 (12)	31
+ anti-IL-2		1,624 ± 115 (6)	0
+ rat IgG		866 ± 106 (6)	32

* Pumps delivering 1 μg/d of IL-12 were inserted 2 wk after infection in euthymic BALB/c mice. Each of the indicated preparations were injected into IL-12–treated mice 2 h before pump insertion and repeated as follows: anti-IFN-γ on day 17 (17), and anti-TNF-α, normal rabbit serum, anti-IL-2, and rat IgG on days 17 and 20 (15, 16). Results are from two to three experiments, and indicate mean ± SEM values for (*n*) mice per group.

(12a, 15) but not all (13) exogenous cytokines that induce antileishmanial activity. Hepatic granuloma assembly, however, was not altered by IL-12 administration (data not shown).

Cells Required for In Vivo Responsiveness to IL-12. In contrast to euthymic animals, 2-wk infected nude BALB/c mice failed to respond to IL-12 (Table 1) indicating that host T cells were required and suggesting that NK cells (retained in nude mice [6]) were not sufficient by themselves to mediate the antileishmanial effects of exogenous IL-12. Treatment of euthymic BALB/c mice with T cell subset–depleting mAb indicated that both $CD4^+$ and $CD8^+$ cells were required for optimal IL-12–induced activity (Table 1). Although the results in nude mice suggested that NK cells alone were not sufficient to mediate the effect of IL-12, treatment of euthymic mice with NK cell–depleting antiasialo GM1 antiserum revealed clear-cut evidence (Table 1) that NK cells were indeed involved and interacted with $CD4^+$ and $CD8^+$ cells. Thus, NK cells represented a third host defense cell required for optimal in vivo responsiveness to exogenous IL-12. Since 2-wk infected C57BL/6 beige mice also responded to IL-12 (Table 1), the role identified for NK cells did not appear to involve cytotoxic activity (18).

Endogenous Cytokines Required for Response to IL-12. Previous studies in this model have demonstrated that *L. donovani* triggers the secretion of IFN-γ, IL-2, TNF-α, and that acquired resistance involves the participation and interaction of each of these endogenous cytokines (15–17). Since IL-12 can induce IFN-γ and IL-2 (1, 19) as well as interact with IL-2 and TNF-α to enhance IFN-γ secretion (1, 19), we treated mice with various anticytokine preparations at the time of IL-12 administration. The results in Table 2 indicate that the leishmanicidal activity induced by IL-12 is multi-cytokine dependent and in a likely complex fashion (1, 19, 20) involves endogenous IFN-γ, IL-2, and TNF-α.

Effect of Early IL-12 Treatment. While *L. donovani* provokes a predominant Th1 cell–associated response in BALB/c mice, this response is not detectable until ≥ 10 d after infection as judged by the emergence of IFN-γ and IL-2 mRNA expression in the tissues (12). Therefore, we completed these treatment experiments by determining whether the presence of IL-12 at the initiation of *L. donovani* infection (before the induction of the Th1 cell response) could also modify outcome. Such early, essentially prophylactic treatment strikingly modifies the course of *L. major* infection apparently by inhibiting the development of suppressive Th2 cell–associated responses and promoting protective Th1 cell responses largely

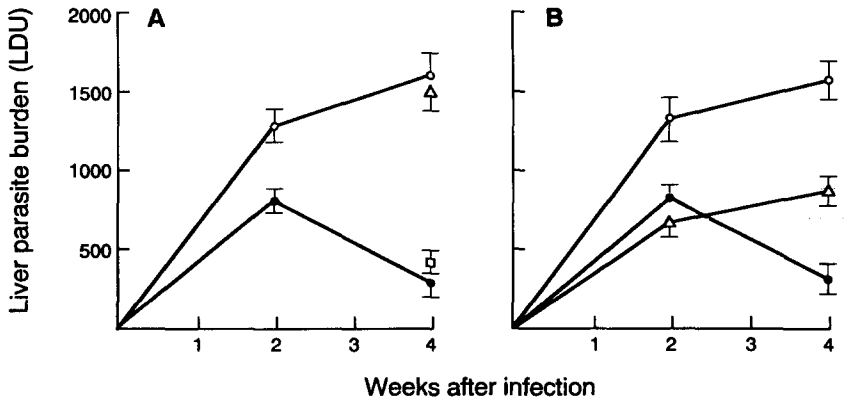

Figure 1. Effect of early IL-12 treatment during week 1 on the course of *L. donovani* infection in euthymic BALB/c mice. (*A*) Pumps delivering 1 μg/d of IL-12 for 7 d (●) were implanted 4 h after challenge; (○) untreated controls. IL-12–treated mice were also injected with either anti-IFN-γ antiserum (△) or normal rabbit serum (□) three times during week 1: 2 h, 3 d, and 7 d after pump insertion. (*B*) Effect of early treatment during week 1 with IL-12 vs. IFN-γ. 4 h after infection, pumps delivering 7 d of either IL-12 (1 μg/ml) (●) or IFN-γ (2×10^5 U/d) (△) were implanted; (○) untreated mice. Results in (*A*) and (*B*) are from two to four experiments, and indicate mean ± SEM values for 6–15 mice per group.

dependent on IFN-γ (4, 5). As shown in Fig. 1 *A*, early IL-12 treatment during week 1 only clearly enhanced control over visceral *L. donovani*; this effect was evident by week 2 and fully expressed by week 4. Although the effect of early IL-12 treatment was inhibited by simultaneous injections of anti-IFN-γ (Fig. 1 *A*), administration of exogenous IFN-γ alone during week 1 did not achieve similarly sustained antileishmanial activity (Fig. 1 *B*). This latter observation suggested effects of early IL-12 treatment in addition to the induction of endogenous IFN-γ.

Together, these results indicate that IL-12 is active as treatment in a firmly established systemic intracellular infection caused by a pathogen that exclusively resides within visceral macrophages (11). In view of the demonstrated role of endogenous IFN-γ in this model (17) and IFN-γ's direct macrophage-activating leishmanicidal effects (13), the capacity of IL-12 by itself or with IL-2 and TNF-α to induce T cells and NK cells to secrete IFN-γ (1, 19, 20) seems likely to represent a primary action for IL-12. This conclusion, however, does not exclude the possibility that in established infection IL-12 treatment also further promotes or accelerates other effects of the generalized Th1 cell–associated response which *L. donovani* infection provokes in this model (12, 15, 17). Indeed, the capacity of early IL-12 treatment to act before the Th1 cell response becomes detectable but still effectively influence the subsequent course of visceral infection supports other actions as well.

Controlled clinical trials have now shown IFN-γ to be an effective adjunct when used with conventional antimony chemotherapy in patients with visceral leishmaniasis (21, 21a). Thus, the present results suggest that by acting as an inducer of endogenous IFN-γ, IL-12 may have also a future role as an adjunct to chemotherapy in the management of this and perhaps other IFN-γ–responsive intracellular infections (22). Whether exogenous IL-12 can influence what appears to be a Th2 cell–associated response in human visceral leishmaniasis (23–25) and restore suppressed IFN-γ–generating capacity (23) are two important questions that remain to be tested.

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