The Adjuvant Effect of Interleukin-12 in a Vaccine Against *Leishmania major*

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Protection induced by vaccination depends on the capacity of the vaccine to elicit an appropriate immune response. In leishmaniasis, protection requires leishmanial-specific CD4$^+$ T helper (T_H) cells. Vaccination of BALB/c mice with leishmanial antigens and interleukin-12 (IL-12) promoted the development of leishmanial-specific CD4$^+$ T_H1 cells. These mice were resistant to subsequent infection with *Leishmania major*. Thus, IL-12 is an effective adjuvant for the initiation of protective cell-mediated immunity against leishmaniasis and may be an important component in other vaccines that need to induce cell-mediated immunity.

Protective immunity induced by vaccination is dependent on the capacity of the vaccine to elicit the appropriate immune response to either resist, control, or eliminate the pathogen. Depending on the pathogen, this may require a cell-mediated or humoral immune response, which in turn is determined by the nature of the T cells that develop after immunization. The current paradigm is that CD4$^+$ T cells can be separated into subsets on the basis of the repertoire of cytokines produced and that the distinct cytokine profile observed in these cells determines their function. This model includes two major subsets: T_H1 cells that produce IL-2 and interferon γ (IFN-γ) and mediate cellular immune responses, and T_H2 cells that produce IL-4, IL-5, and IL-10 and augment humoral immune responses (*1*). An essential role of adjuvants in vaccines is to direct CD4$^+$ T cell subset differentiation, although how adjuvants perform this function is poorly understood.

The protozoan parasite *Leishmania major* causes either a healing or nonhealing cutaneous lesion in mice, depending on the CD4$^+$ T_H cell subset that dominates. In BALB/c mice, *L. major* infection usually leads to a dominant CD4$^+$ T_H2 response, uncontrolled lesion development, metastasis of the parasite, and eventual death of the animal, whereas many other mouse strains (for example, C3H/HeN and C57BL/6) develop a T_H1 response, control parasite multiplication, and heal (*2*). Interferon γ is required for T_H1 cell development in this model, since in vivo depletion of IFN-γ before infection abrogates T_H1 cell development in resistant C3H/HeN mice, leading to a dominant T_H2 response and susceptibility to *L. major* (*3*). However, IFN-γ by itself cannot induce T_H1 cell development. For example, when BALB/c mice were immunized subcutaneously with a soluble leishmanial antigen (SLA), inclusion of IFN-γ in the vaccine enhanced protective immunity, but the bacterial adjuvant, *Corynebacterium parvum*, was still required for protection, suggesting that other factors may be involved in the differentiation of T_H1 cells (*3*). Interleukin-12, which stimulates IFN-γ production by natural killer (NK) cells and T cells (*4*), may be the missing factor. When lymphocytes from atopic patients were cultured with IL-12 there was an increase in the number of T_H1 clones detected (*5*). Furthermore, in an in vitro system using T cells from T cell receptor (TCR) transgenic mice, IL-12 was shown to promote the development of a T_H1 cell phenotype from naïve CD4$^+$ T cells (*6*). We now show that IL-12 enhances T_H1 cell development in vivo and can replace both *C. parvum* and IFN-γ in a vaccine against leishmaniasis.

After subcutaneous injection of either live *L. major* parasites or SLA into BALB/c or C3H/HeN mice there is a rapid response in the draining lymph node (LN), characterized by a three- to fivefold increase in cell number and enhanced cytokine production. In BALB/c mice IL-4 is detected within 3 days, whereas in C3H/HeN mice the NK cell population expands and produces IFN-γ (*3*, *7*). To determine whether administration of IL-12 alters the initial response to SLA in BALB/c mice, we assessed cytokine production by popliteal LN cells taken 3 days after footpad injection with SLA or SLA + IL-12 (*8*). C3H/HeN mice were injected with SLA to provide a comparison of cytokine levels. As observed previously, LN cells from BALB/c mice injected with SLA produce IL-4 but little IFN-γ, whereas LN cells from C3H/HeN mice produce IFN-γ but little or no IL-4 (Fig. 1A). However, when BALB/c mice were injected with SLA + IL-12, no IL-4 was detected, and substantial amounts of IFN-γ, higher than those observed in the resistant C3H/HeN mouse strain, were detected. A similar effect was obtained with several doses of IL-12, including 1, 0.1, and 0.01 μg, although complete ablation of IL-4 was not achieved with the lowest dose (*9*). The source of the IFN-γ at this early time point has not been determined, although associated with the IFN-γ response was the induction of an NK cell cytotoxic response (Fig. 1B). However, administration of IL-12 alone enhanced NK cell cytotoxic function in the draining LN but failed to stimulate IFN-γ production (Fig. 1, A and B). This may be due to the capacity of SLA to stimulate the production of other cytokines, such as IL-2, which synergize with IL-12 to enhance IFN-γ production. This finding is compatible with our previous observation that the production of IFN-γ by NK cells during the first 3 days of *L. major* infection in C3H/HeN mice is dependent on IL-2 produced by CD4$^+$ T cells (*7*).

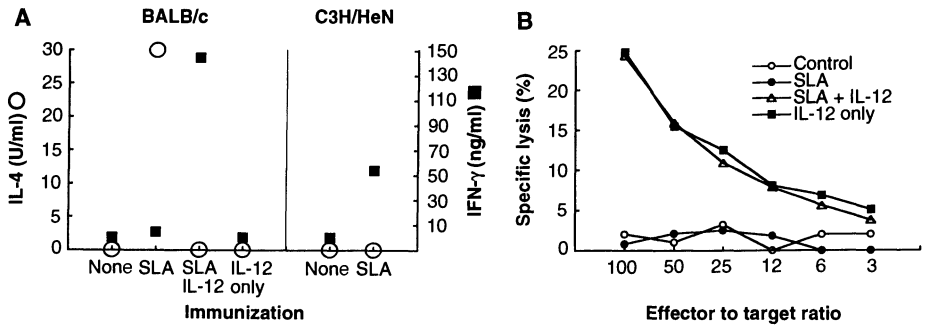

Fig. 1. Inoculation of SLA and IL-12 into BALB/c mice inhibits IL-4 production, promotes production of IFN-γ, and induces an NK cell response in the draining LN at 72 hours. (**A**) In vitro IFN-γ and IL-4 production by LN cells taken from normal mice or mice inoculated subcutaneously 3 days previously with SLA, SLA + IL-12, or IL-12 alone. (**B**) NK cell cytotoxic response of LN cells from immunized BALB/c mice against YAC-1 target cells. The cytokine responses depicted are from one representative experiment out of five, in which the mean IFN-γ levels in mice immunized with SLA and SLA + IL-12 were 4.6 ± 1.1 and 114 ± 20 ng/ml, respectively ($P < 0.005$). In five out of five experiments, IL-12 reduced IL-4 levels to less than 1 U/ml.

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To determine whether IL-12 induced the development of a CD4$^+$ T_H1 cell population, we collected popliteal LNs and spleens 10 days after the primary in vivo stimulation with SLA or SLA and IL-12, restimulated them in vitro with SLA only, and assessed the cytokine profile (*10*). As seen in Fig. 2, immunization of BALB/c mice with SLA primed for a dominant T_H2 type response characterized by high IL-4 production. Inclusion of IL-12 reversed this pattern, leading to a dominant T_H1 response characterized by elevated IFN-γ levels and little if any IL-4. A similar response was observed with both unfractionated lymphocytes and purified CD4$^+$ T cells, indicating that IL-12 was specifically influencing CD4$^+$ T_H1 development. In contrast, IL-12 administered without antigen had no effect on the capacity of either the LN or the spleen to produce IFN-γ.

Because an NK cell cytotoxic response was observed with LN cells 2 days after injection with IL-12 (Fig. 1B), we tested whether the presence of NK cells contributed to the development of CD4$^+$ T_H1 cells after immunization with SLA and IL-12. Mice were depleted of NK cells by treatment with a rabbit polyclonal antibody to asialo GM1 (anti-asialo GM1) before immunization, which completely inhibited NK cell cytotoxic function (*9*). A comparison of the response in mice treated with anti-asialo GM1 before immunization with SLA + IL-12, and those not depleted of NK cells, indicated that the presence of NK cells during immunization significantly augmented subsequent T_H1 cell development (Fig. 2B). The requirement of NK cells is probably related to their ability to produce IFN-γ, since treatment of mice with anti-IFN-γ monoclonal antibodies also ablated the effects of IL-12 (day 10 spleen cell IFN-γ levels: SLA + IL-12, 58.7 ng/ml; SLA + IL-12 pretreated with anti-IFN-γ, 5.1 ng/ml) (*11*).

On the basis of the ability of IL-12 to direct the development of leishmanial-specific CD4$^+$ T_H1 cells, we assessed whether IL-12 could act as an adjuvant in a vaccine against *L. major* (*12*). Mice immunized with SLA + IL-12 were completely protected against disease (Fig. 3). The lesion size in these animals was as small as that produced in the resistant C3H/HeN mice (Fig. 3, inset). Cytokine analysis indicated that control mice produced small amounts of IFN-γ and IL-4, whereas mice immunized with SLA + IL-12 produced little IL-4 but amounts of IFN-γ that were comparable to those observed in resistant C3H/HeN mice (*9*). Lesions from unimmunized BALB/c mice or mice that had been immunized with SLA alone or IL-12 alone contained greater than 10^7 parasites, whereas BALB/c mice immunized with SLA + IL-12 contained 10^3 parasites (Table 1) (*13*).

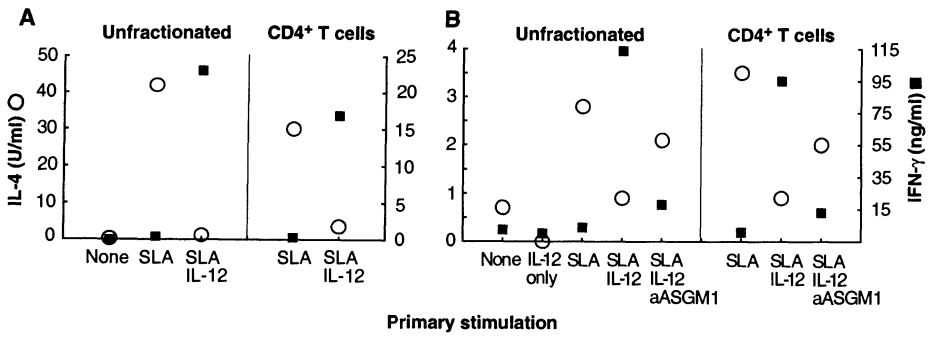

Fig. 2. IL-12 in the presence of SLA induces differentiation of CD4$^+$ T_H1 cells in the LN and the spleen. Cytokine responses of unfractionated or CD4$^+$-enriched popliteal LN (**A**) or spleen (**B**) cells harvested from BALB/c mice 10 days after immunization. Only the data from cells cultured with SLA (50 μg/ml) are shown since in the absence of antigen there was no response. One group of mice was depleted of NK cell activity before immunization with SLA + IL-12 by pretreatment with anti-asialo GM1 (aASGM1). The results presented are representative of two or more experiments.

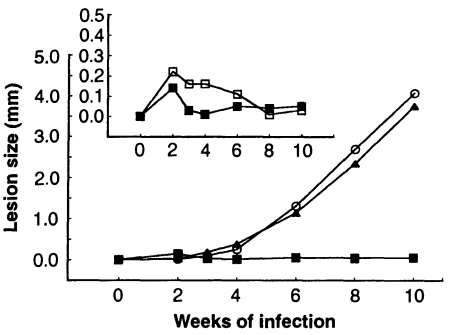

Fig. 3. BALB/c mice immunized with SLA + IL-12 are protected against infection with *L. major* (*12*). Unimmunized BALB/c mice (▲), BALB/c mice immunized with SLA (○) or SLA + IL-12 (■), and normal C3H/HeN mice (□) were injected in the left footpad with 1 × 10^5 purified metacyclic *L. major* promastigotes and the course of infection monitored by measurement of lesion size. (**Inset**) Course of infection in unimmunized C3H/HeN mice as compared with that in BALB/c mice immunized with SLA + IL-12. Each data point represents the mean lesion size of five mice. Differences in lesion size between control or SLA and SLA + IL-12 were significant from week 4 onward. Similar results were obtained in three experiments.

Table 1. Parasite quantitation in lesions of immunized mice 10 weeks after infection. Data represent mean ± SD of at least five animals per group.

Mouse strain	Immunization	−log (parasite titer)
BALB/c	None	8.1 ± 1.1
	SLA	7.7 ± 0.8
	IL-12	7.5 ± 0.3
	SLA + IL-12	3.2 ± 1.9*
C3H/HeN	None	1.1 ± 0.1

*P <0.001 as compared with other BALB/c groups (Student's t test).

Adjuvants are thought to function in several ways, including by slowing the release of antigen, targeting antigen to macrophages, and activating macrophages (*14*). Many of the most effective adjuvants include bacteria or their products, although the role that the bacteria play is ill-defined. The recent finding that *Listeria monocytogenes* enhances T_H1 cell development in vitro through IL-12 suggests that IL-12 production is one mechanism by which bacterial adjuvants function (*6*). This possibility is further supported by the finding that many bacteria or their products—lipopolysaccharide, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *C. parvum*—stimulate IL-12 production by macrophages (*15*). Our data show that IL-12 can substitute for bacterial adjuvants and suggest that the induction of this cytokine may be the key component to vaccine efficacy when cell-mediated immunity is required. Since many bacterial adjuvants have both immunostimulating properties as well as toxic effects, use of IL-12 as an adjuvant may be preferable. In our studies, local injection of IL-12 was not associated with any obvious adverse effects, and there was no appreciable alteration in cell populations within the draining LN after IL-12 injection. Finally, immunization against *L. major* infection by administration of the vaccine into the skin has been problematic, even when bacterial adjuvants were included (*3*). Our results suggest that IL-12 not only substitutes for bacterial adjuvants, but may be more efficacious.

REFERENCES AND NOTES

1. T. R. Mosmann, H. Cherwinski, M. W. Bond, M. A. Giedlin, R. L. Coffman, *J. Immunol.* **126**, 2348 (1986).
2. P. Scott, P. Natovitz, R. L. Coffman, E. Pearce, A. Sher, *J. Exp. Med.* **168**, 1675 (1988); F. P. Heinzel, M. D. Sadick, B. J. Holaday, R. L. Coffman, R. M. Locksley, *ibid.* **169**, 59 (1989).
3. P. Scott, *J. Immunol.* **147**, 3149 (1991).
4. S. H. Chan *et al.*, *J. Exp. Med.* **173**, 869 (1991).
5. R. Manetti *et al.*, *ibid.* **177**, 1199 (1993).
6. C.-S. Hsieh *et al.*, *Science* **260**, 547 (1993).

7. T. M. Scharton and P. Scott, *J. Exp. Med.* **178**, 567 (1993).
8. BALB/c mice were inoculated in the right and left footpad with SLA (25 μg), SLA + IL-12 (0.5 μg), or IL-12 alone, and the popliteal LNs were harvested 3 days later. C3H/HeN mice were inoculated with SLA alone. LN cells were assayed for cytotoxic potential against ^{51}Cr-labeled YAC-1 cells in a 4-hour Cr-release assay at several effector to target ratios. For cytokine analysis, cells were cultured in 24-well plates at 5 × 10^6 per milliliter per well in tissue culture medium [Dulbecco's minimum essential medium with glucose (4.5 mg/ml), 10% fetal bovine serum, 2 mM glutamine, penicillin 6-phosphate (100 U/ml), streptomycin sulfate (100 μg/ml), 25 mM Hepes, 50 μM 2-mercaptoethanol] with or without SLA (50 μg/ml). Only the data from cells cultured with SLA are shown. Supernatants were harvested at 72 hours and IFN-γ and IL-4 measured by enzyme-linked immunosorbent assay (*7*). SLA was prepared from *L. major* promastigotes as described [P. Scott *et al.*, *J. Immunol.* **139**, 221 (1987)].
9. L. C. C. Afonso *et al.*, unpublished data.
10. BALB/c mice were immunized as described (*8*). CD4+ T cells were cultured at 2 × 10^6 per milliliter with irradiated (3300 rad) normal spleen cells (3 × 10^6 milliliter) as antigen-presenting cells. CD4+ T cells were positively selected on a magnetic cell separator (MACS; Miltenyi, Biotec, Sunnyvale, CA) as described (*7*). The CD4+ T cell–enriched population contained greater than 98% CD4+ T cells as assessed by cytofluorometric analysis. To deplete NK cells, we treated mice with anti–asialo GM1 (ASGM1) on day −4 (1.5 mg intravenously) and day 0 (350 μg intravenously) (*7*).
11. A requirement for the presence of IFN-γ for the in vitro development of T_H1 cells in response to IL-12 has been reported [S. E. Macatonia, C.-S. Hsieh, K. M. Murphy, A. O'Garra, *Int. Immunol.* **5**, 1119 (1993)], although in another in vitro system the capacity of IL-12 to initiate T_H1 cell development was found to be independent of IFN-γ [R. A. Seder, R. T. Gazzinelli, A. Sher, W. E. Paul, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10188 (1993)]. Our data suggest that NK cells may be one source of IFN-γ in vivo that augments T_H1 cell development. Similarly, recent findings in the SCID model with intracellular pathogens indicate that a common pathway for IFN-γ production is through the ability of IL-12 to activate NK cells [C. S. Tripp, S. F. Wolf, E. R. Unanue, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3725 (1993); R. T. Gazzinelli, S. Hieny, S. Wynn, S. Wolf, A. Sher, *ibid.*, p. 6115].
12. BALB/c mice were immunized in the right footpad with SLA (25 μg) or SLA + IL-12 (1 μg). Ten days later mice were given an intradermal injection in the flank with SLA (10 μg) or SLA + IL-12 (1 μg). Two weeks later mice were challenged in the left footpad with 10^5 purified metacyclic *L. major* promastigotes (*7*). In three experiments the mean lesion size of mice immunized with SLA + IL-12 at 10 weeks was 0.26 ± 0.59 mm, while unimmunized mice, SLA-immunized mice, or mice receiving only IL-12 exhibited lesions of 3 to 5 mm. One group of mice immunized with SLA + IL-12 was maintained for 20 weeks with no signs of resumption of disease.
13. Interleukin-12 when administered multiple times systemically to BALB/c mice during the infection enhances their resistance to *L. major* [F. P. Heinzel, D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, M. K. Gately, *J. Exp. Med.* **177**, 1505 (1993); J. P. Sypek *et al.*, *ibid.*, p. 1797]. This effect is distinct from the adjuvant effects of IL-12 observed in these studies since IL-12 by itself failed to exhibit any protective effects (Table 1).
14. H. S. Warren, F. R. Vogel, L. A. Chedid, *Annu. Rev. Immunol.* **4**, 369 (1986).
15. A. D'Andrea *et al.*, *J. Exp. Med.* **176**, 1387 (1992).
16. We thank L. Taylor for technical assistance and J. Farrell and S. Carding for comments on the manuscript. We thank S. F. Wolf and J. P. Sypek of Genetics Institute, Inc. (Cambridge, MA) for providing recombinant murine IL-12 for these studies. Supported in part by grants from the NIH (AI-30073 and CA-20833) and the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (TDR).

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Phase Shifting of the Circadian Clock by Induction of the *Drosophila period* Protein

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Virtually all organisms manifest circadian (24-hour) rhythms, governed by an ill-defined endogenous pacemaker or clock. Several lines of evidence suggest that the *Drosophila melanogaster period* gene product PER is a clock component. If PER were central to the time-keeping mechanism, a transient increase in its concentration would cause a stable shift in the phase of the clock. Therefore, transgenic flies bearing a heat-inducible copy of PER were subjected to temperature pulses. This treatment caused long-lasting phase shifts in the locomotor activity circadian rhythm, a result that supports the contention that PER is a bona fide clock component.

A wide variety of organisms exhibit daily fluctuations in many biochemical, physiological, and behavioral phenomena that are governed by an endogenous circadian (≈24 hours) pacemaker or "clock" (*1*). Although no bona fide components of a circadian oscillator have been identified, a candidate is the *period* (*per*) gene product from *Drosophila melanogaster* (*2*, *3*). In the absence of *per* activity (per^{01} nonsense mutation), there is no observable rhythmicity of eclosion or of locomotor activity (*2*). Moreover, missense mutations shorten (per^s) to 19 hours or lengthen (per^L) to 29 hours the free-running periods of both rhythms (*2*). Consistent with the possibility that *per* is directly involved in the generation of these rhythms is the observation that PER itself is subject to circadian regulation; both *per* protein (PER) (*4*) and *per* mRNA (*5*) undergo daily fluctuations in the heads of adult flies. These two oscillations are connected, as PER may have a role in the circadian regulation of its own transcription (*5*, *6*).

If PER oscillations contribute directly to the clock mechanism, transient perturbations of its level or activity would cause a phase shift in the oscillator (Fig. 1A). Because identical stimuli applied at different phases in a circadian cycle can give rise to shifts with different magnitudes and directions, PER increases might advance or delay the rhythm of locomotor activity in an a priori unpredictable fashion (Fig. 1A).

To test this prediction, we generated transgenic flies bearing a heat-inducible copy of *per*, termed *hspcper* (Fig. 1B) (*7*). Several independent lines were obtained, and we assayed the biological activity of *hspcper* in a per^{01} arrhythmic background. As in previous results (*8*), some lines were rhythmic at 25° or 29°C, whereas others were not (*9*). We chose one completely arrhythmic line (per^{01};*hspc*-23a) (*10*) and reasoned that in a wild-type strain with this insert (per^+;*hspc*-23a), only the per^+ gene should contribute significantly to locomotor activity rhythms at 25°C. Indeed, the rhythms of per^+;*hspc*-23a flies were indistinguishable from those of control wild-type flies, including in their periods (*11*) and in their phases (*9*). With this strain, we then induced PER at various times throughout a circadian cycle and measured the phase of the resultant locomotor activity.

Because temperature pulses elicit phase shifts in many organisms (*12*), we first determined the effects of temperature on wild-type *D. melanogaster* locomotor activity rhythms. We administered short pulses of the heat stimulus throughout the circadian cycle and determined the magnitude and direction of the ensuing phase shift; plotting the average phase shift (*13*) as a function of the circadian time of the stimulus yielded a phase response curve (PRC) (Fig. 2A). This heat PRC is similar to results obtained with light pulses as the stimulus for phase-shifting *D. melanogaster* locomotor activity (*14*). Depending on when the temperature pulse was given, the activity phase was delayed, advanced, or unchanged. Both delays and advances reached a new steady state within one circadian cycle after treatment with heat (*9*).

We applied the identical temperature-pulse regime to per^+;*hspc*-23a flies and a different PRC was generated (Fig. 2, A and B). Expression of heat-induced PER (HSP-PER) converted the phase delay region

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