The role of antigen and IL-12 in sustaining Th1 memory cells *in vivo*: IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge

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IL-12 plays a central role in both the induction and magnitude of a primary Th1 response. A critical question in designing vaccines for diseases requiring Th1 immunity such as *Mycobacterium tuberculosis* and *Leishmania major* is the requirements to sustain memory/effector Th1 cells *in vivo*. This report examines the role of IL-12 and antigen in sustaining Th1 responses sufficient for protective immunity to *L. major* after vaccination with LACK protein (LP) plus rIL-12 and LACK DNA. It shows that, after initial vaccination with LP plus rIL-12, supplemental boosting with either LP or rIL-12 is necessary but not sufficient to fully sustain long-term Th1 immunity. Moreover, endogenous IL-12 is also shown to be required for the induction, maintenance, and effector phase of the Th1 response after LACK DNA vaccination. Finally, IL-12 is required to sustain Th1 cells and control parasite growth in susceptible and resistant strains of mice during primary and secondary infection. Taken together, these data show that IL-12 is essential to sustain a sufficient number of memory/effector Th1 cells generated *in vivo* to mediate long-term protection to an intracellular pathogen.

Vaccines for a variety of bacterial and viral diseases have had a major global impact in reducing morbidity and mortality from disease. Primary immunity elicited by all current vaccines appears to be mediated by the humoral immune response. By contrast, for disease that may require a cellular immune response, such as malaria, leishmania, tuberculosis, and HIV, there are no currently available vaccines that are uniformly effective. Thus, understanding the mechanisms by which long-lived cellular immune responses are generated after vaccination will be important in the rational design of vaccines for those diseases requiring cellular immunity. In this report, the factors involved in the induction and maintenance of Th1 responses generated *in vivo* by two different vaccine formulations were studied.

Similar to other mouse models of intracellular infection, primary immunity to *Leishmania major* infection requires IL-12-dependent production of IFN-γ (1, 2). In addition, the mouse model of *L. major* is distinctive in that certain strains of mice (C57BL/6 and C3H) develop protective Th1 responses after infection whereas other strains (BALB/c) develop Th2 responses and succumb to infection (3, 4). This dichotomy has provided an important model to study Th1 and Th2 differentiation *in vivo* in a biologic model and to determine how these responses can be altered. Thus, in susceptible BALB/c mice after infection with *L. major*, the early production of IL-4 derives from a restricted population of $V_\beta4$ $V_\alpha8$ $CD4^+$ T cells in response to an immunodominant *L. major* protein called LACK (5). This rapid induction of IL-4 limits the ability to generate a protective Th1 response (6, 7). The functional importance of LACK-reactive $CD4^+$ IFN-γ-producing cells was demonstrated in two separate studies in which vaccination with either LACK protein (LP) and recombinant IL-12 (rIL-12) protein or plasmid DNA encoding the LACK antigen (LACK DNA) conferred protection when mice were challenged with *L. major* within 2 weeks after vaccination (5–8). Further, in a study to test the durability of Th1 immunity *in vivo*, it was demonstrated that vaccination with LACK DNA is more effective than vaccination with leishmanial protein plus rIL-12 protein in sustaining Th1 responses and controlling infection in BALB/c mice when challenged 12 weeks post-vaccination (9). These data were the first to show that Th1 responses generated *in vivo* were not sustained over a period (12 weeks) sufficient to mediate a biologic outcome. Furthermore, these data raised the issue as to the mechanism by which LACK DNA vaccination was able to provide long-term immunity and provided a model to determine how sustained Th1 immune responses could be achieved by using a protein-based vaccine.

First, mechanisms that could account for the enhanced efficacy of LACK DNA vaccination over leishmanial protein plus rIL-12 may include low levels of persistent antigen (10) and/or IL-12 induced by the CpG motifs contained within the plasmid DNA (11, 12). Second, with regard to how Th1 responses can be sustained after vaccination with leishmanial protein, we previously showed that vaccination with leishmanial protein plus IL-12 DNA but not rIL-12 protein could sustain Th1 responses and provide long-term control of infection (9). These data strongly suggested that persistence of IL-12 may be required to sustain a sufficient number of effector/memory Th1 cells to control infection when mice are challenged 12 weeks post-vaccination. The studies reported here further define the role of antigen and IL-12 in the induction and maintenance of Th1 responses after vaccination with either LACK DNA or LP plus rIL-12. In addition, the role of IL-12 in sustaining memory Th1 immunity and controlling parasite growth was determined in susceptible and resistant strains during the course of primary and secondary infection.

Materials and Methods

Mice. Female BALB/c and C57BL/6 mice were purchased from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD) and were kept in the National Institute of Allergy and Infectious Diseases Animal Care Facility under pathogen-free conditions. IL-12 (p40)-deficient ($IL\text{-}12^{-/-}$) BALB/c mice were kindly provided by Jeanne Magram (Roche).

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Abbreviation: LP, LACK protein.

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Injection of Mice. BALB/c mice (n = 6–8 per group) were injected in their hind footpad with 100 μg of plasmid DNA encoding LACK antigen (LACK DNA) or control DNA (empty vector) suspended in 50 μl of sterile PBS (8). For immunization with leishmanial protein, mice were injected as above with 50 μg of recombinant LP with 1 μg of rIL-12 protein (Genetics Institute, Cambridge, MA). In all experiments, mice were boosted 2 weeks later with their initial regimen. Throughout the text, "primary immunization" is defined as the initial immunization and boost 2 weeks later. Mice were then infected either 2 or 12 weeks after the primary immunization with 1×10^5 *L. major* (WHOM/IR/-/173) metacyclic promastigotes in their hind footpad as described (8). Weekly footpad swelling measurements were recorded by using a metric caliper.

In some experiments in which the primary immunization was done with LP plus rIL-12 protein, mice were given supplemental treatment every 2 weeks in the same footpad with either LP, rIL-12, or LP plus rIL-12.

Frequency of CD4$^+$ IL-4 and IFN-γ-Producing Cells by Intracellular Staining. Pooled lymph node cells from vaccinated mice (n = 6–8) either at various times after primary immunization or after infection were stimulated with LP for 4h at 37°C and then were processed for intracellular staining of cytokines as described (9). In the absence of LP stimulation, there are no detectable cytokine-producing cells. An isotype-matched control for IFN-γ and IL-4 was used to place statistical markers and stained less than 0.02% of cells.

Results

The Role of IL-12 and Antigen in Sustaining Long-Term Immunity After Vaccination. In a previous report, plasmid DNA vaccination encoding the LACK antigen (LACK DNA) was sufficient to confer protection to susceptible BALB/c mice challenged 2 or 12 weeks post-vaccination (9). By contrast, vaccination with various formulations of leishmanial protein plus rIL-12 protein protected mice challenged 2 weeks post-vaccination but failed to confer protective immunity when mice were challenged 12 weeks post-vaccination. In addition, in our previous report, although vaccination with leishmanial protein plus rIL-12 protein did not confer durable immunity, vaccination with leishmanial protein plus IL-12 DNA was sufficient to maintain Th1 immunity and control infection when challenge was done 12 weeks post-vaccination. These latter data suggested that continuous IL-12 was necessary and possibly sufficient to sustain long-term Th1 immunity induced by a protein-based vaccine in this model.

To further understand the specific role of antigen and IL-12 in sustaining Th1 immunity, mice were initially vaccinated and boosted 2 weeks later (primary immunization) with LP plus rIL-12. As noted above, this regimen is sufficient to induce protection when infectious challenge is done at 2 weeks but not 12 weeks after primary immunization. In addition, groups of mice vaccinated with LP plus rIL-12 were given supplemental treatment with either LP, rIL-12, or both every 2 weeks for an additional four cycles and then were infected 12 weeks after the primary immunization and 4 weeks after the last supplemental treatment. As shown in Fig. 1, mice vaccinated with LP plus rIL-12 controlled infection as assessed by footpad swelling when challenged at 2 weeks (Fig. 1*A*) but not 12 weeks (Fig. 1*B*) after primary immunization. By contrast, mice who received supplemental treatment with either LP or rIL-12 alone had a decrease in their footpad swelling when infection was done at 12 weeks (Fig. 1*B*). In addition, as a positive control, supplemental treatment with both LP and rIL-12 resulted in a further decrease in footpad swelling comparable to that seen in mice vaccinated with LP plus IL-12 DNA or with LACK DNA (Fig. 1*B*).

As an immune correlate of biologic protection, the frequency of antigen-specific CD4$^+$ IFN-γ-producing cells was determined

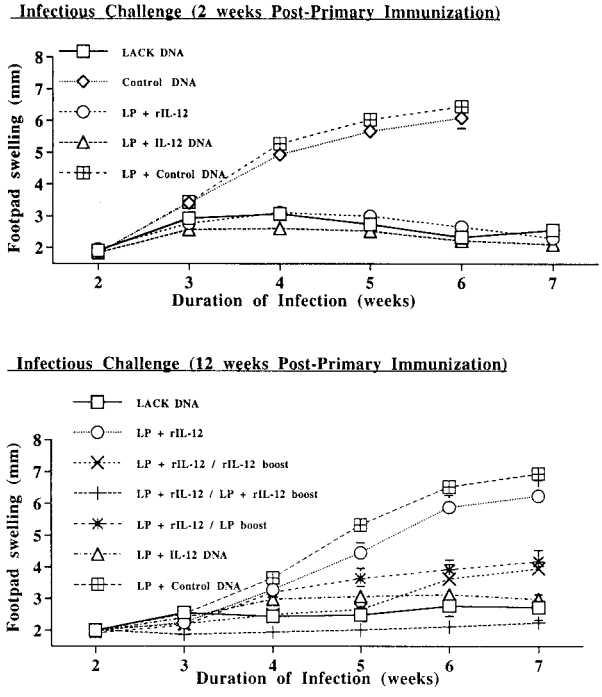

Fig. 1. The role of continuous IL-12 and LP in mediating long-term control of infection after primary immunization with LP plus rIL-12. BALB/c mice (n = 6–8 per group) were initially vaccinated in the footpad and were boosted 2 weeks later (primary immunization) with LACK DNA (100 μg), control DNA (100 μg), LP (50 μg) with IL-12 or control DNA (100 μg), or LP plus rIL-12 (1 μg). Mice were then challenged with 1×10^5 *L. major* (WHOM/IR/-/173) metacyclic promastigotes in their hind footpads 2 weeks (*A*) or 12 weeks (*B*) later, and weekly foot pad measurements were recorded by using a metric caliper. In addition, some of the mice (n = 6–8 per group) that were initially vaccinated and boosted with LP plus rIL-12 (primary immunization) were given supplemental treatment every 2 weeks in the same foot pad with either LP (50 μg), rIL-12 (1 μg), or LP plus rIL-12 (1 μg) for a total of four supplemental treatments. Infectious challenge was done 12 weeks after the primary immunization (4 weeks after the last supplementary treatment). Weekly footpad measurements were recorded by using a metric caliper. These data are representative of three independent experiments.

from total lymph nodes of infected mice challenged either 2 or 12 weeks after primary immunization from the same experiment as shown above. Similar frequencies of antigen-specific CD4$^+$ IFN-γ-producing cells were detected from mice infected 2 weeks after primary immunization with either LACK DNA (1.52%), LP plus rIL-12 (1.57%), or LP plus IL-12 DNA (1.11%) (Fig. 2*A*). These data are consistent with the ability of all of these groups to control infection as shown above (Fig. 1*A*). By contrast, mice vaccinated with LP plus control DNA that were susceptible to infection (Fig. 1) had a 3- to 5-fold reduction in the frequency of CD4$^+$ IFN-γ-producing cells compared with the other groups. In assessing the frequencies of CD4$^+$ IFN-γ-producing cells from mice infected 12 weeks after the primary immunization, mice vaccinated with LACK DNA, LP plus IL-12 DNA, or LP plus rIL-12 given supplemental treatment with both LP and rIL-12 had comparable frequencies of CD4$^+$ IFN-γ-producing cells approximating 4%. There was also a 2- to 3-fold increase in the frequencies of CD4$^+$ IFN-γ-producing cells from mice initially vaccinated with LP plus rIL-12 and given supplemental treatment every 2 weeks with either LP or rIL-12 alone compared with those from mice given only a primary immunization regimen with LP plus rIL-12. Similarly, in a second separate experiment, the frequencies of CD4$^+$ IFN-γ-producing cells from mice initially vaccinated with LP plus rIL-12 was enhanced after supplemental treatment with rIL-12 (Fig. 2*C*).

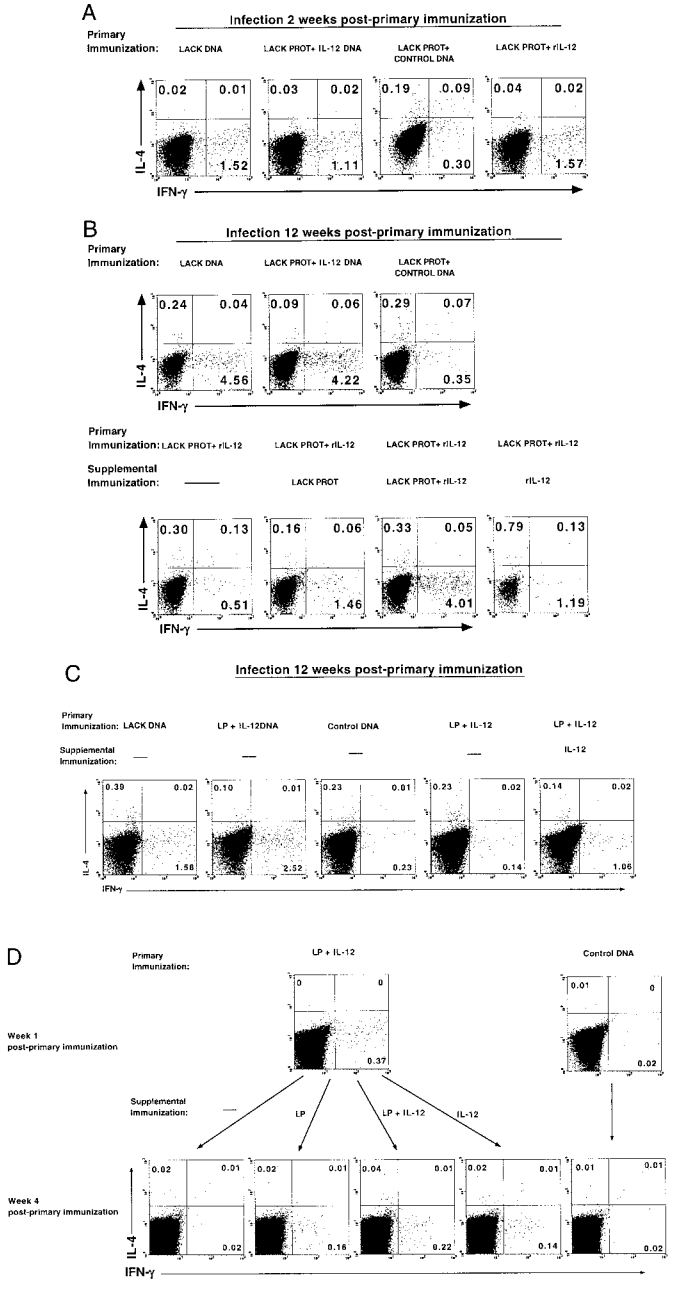

Fig. 2. The role of LP and rIL-12 in sustaining the frequency of CD4$^+$ IFN-γ-producing T cells after vaccination and infection. In the same experiment as in Fig. 1, draining lymph node cells were pooled from groups of vaccinated mice ($n = 6–8$ per group) that were challenged with *L. major* either 2 weeks (*A*) or 12 weeks (*B*) after primary immunization. Lymph node cells were harvested 6 weeks after infection. The frequency of LACK-specific IFN-γ- and IL-4-producing cells was assessed by intracellular cytokine as described in *Materials and Methods*. (*C*) In a separate experiment, draining lymph node cells were pooled from groups of vaccinated mice ($n = 6–8$ per group) challenged with *L. major* 12 weeks after primary immunization. In this experiment, supplemental treatment with rIL-12 was done in a manner similar to that described in Fig. 1. (*D*) Groups of mice ($n = 6–8$ per group) were initially vaccinated and boosted with LP plus rIL-12 (primary immunization), and the frequencies of CD4$^+$ IFN-γ-producing cells was determined 1 and 4 weeks after the primary immunization. In three groups of mice vaccinated with LP plus rIL-12, a single supplemental treatment with LP, rIL-12, or both was given 2 weeks after primary immunization, and the frequency of CD4$^+$ IFN-γ-producing cells was determined 2 weeks later (4 weeks post-primary immunization).

Again, these frequencies were less than those from cells of mice vaccinated with LP plus IL-12 DNA or LACK DNA.

As the previous experiments analyzed the frequencies of CD4$^+$ IFN-γ-producing cells after infectious challenge, it was of interest to determine the role of antigen and IL-12 in maintaining the frequencies of CD4$^+$ IFN-γ-producing cells after primary immunization but before infection. As shown in Fig. 2*D*, the frequencies of CD4$^+$ IFN-γ-producing cells 1 week after primary immunization with LP plus rIL-12 was 0.37%. By contrast, we failed to detect any CD4$^+$ IFN-γ-producing cells at 4 weeks after primary immunization with LP plus rIL-12. A single supplemental treatment with either LP, rIL-12, or both sustained the frequency of CD4$^+$ IFN-γ-producing cells at 4 weeks after primary immunization, consistent with the data above. It should be noted that, as a negative control, injection of LP or rIL-12 alone in nonimmunized mice does induce any CD4$^+$ IFN-γ-producing cells as assessed by intracellular cytokine staining (data not shown). Finally, in other experiments, the frequency of CD4$^+$ IFN-γ-producing cells in mice vaccinated with LP plus IL-12 DNA was detectable at both 1 and 4 weeks after primary immunization. These latter data suggest that low but continuous levels of IL-12 (as provided by IL-12 DNA) are sufficient to sustain Th1 responses induced by LP.

IL-12 Is Required for Induction and Maintenance of CD4$^+$ IFN-γ-Producing Cells in Mice Vaccinated With LACK DNA. As noted above, LACK DNA provides long-term Th1 immunity and control of infection in this model. One potential advantage of DNA vaccination over protein-based vaccines for diseases requiring cellular immunity is that CpG motifs contained within the DNA plasmid backbone can serve as its own adjuvant for inducing Th1 responses (11, 12). In this regard, the role of endogenous IL-12 was assessed on the induction, maintenance, and effector phase of the Th1 response after LACK DNA vaccination. First, the role of IL-12 in induction of the Th1 response after vaccination was assessed in two separate experiments. Treatment of mice with a single injection of anti-IL-12 at the time of primary immunization resulted in a striking decrease in the frequency of CD4$^+$ IFN-γ-producing cells when assessed at 1 week (Fig. 3*A*) or 2 weeks (Fig. 3*B*) after primary immunization. To assess the role of IL-12 in maintaining the post-vaccination Th1 response, mice were treated with a single injection of anti-IL-12 2 weeks after primary immunization with LACK DNA (Fig. 3*C*). The frequency of CD4$^+$ IFN-γ-producing cells from LACK DNA-vaccinated mice was 0.45 and 0.26 when assessed at 2 and 3 weeks after primary immunization, respectively. Mice vaccinated with LACK DNA and treated with anti-IL-12 2 weeks after primary immunization had a further decrease in the frequency of CD4$^+$ IFN-γ-producing cells (0.9%) when assessed 1 week later (3 weeks after primary immunization). These data suggest that neutralization of IL-12 *in vivo* limited the maintenance of CD4$^+$ IFN-γ-producing cells in a relatively short period. It should be noted that, although these frequencies of CD4$^+$ IFN-γ-producing cells after vaccination but before infection seem relatively small compared with other infectious models measuring CD8$^+$ T cell responses after viral infections, these data are representative of numerous experiments (>15) over a period of several years. Moreover, these data are among the first to analyze the frequencies of antigen-specific CD4$^+$ IFN-γ-producing cells generated *in vivo* after vaccination. Finally, to assess the role of IL-12 at the effector phase of the response, LACK DNA-vaccinated mice were treated with anti-IL-12 at the time of infection. As shown in Fig. 3*D*, anti-IL-12 treatment completely abrogated the frequency of CD4$^+$ IFN-γ-producing cells when assessed from lymph nodes of infected mice. Taken together, these data strongly suggest that the frequency of CD4$^+$ IFN-γ-producing cells induced by LACK DNA depends on IL-12 for the induction, maintenance, and effector phase of the response.

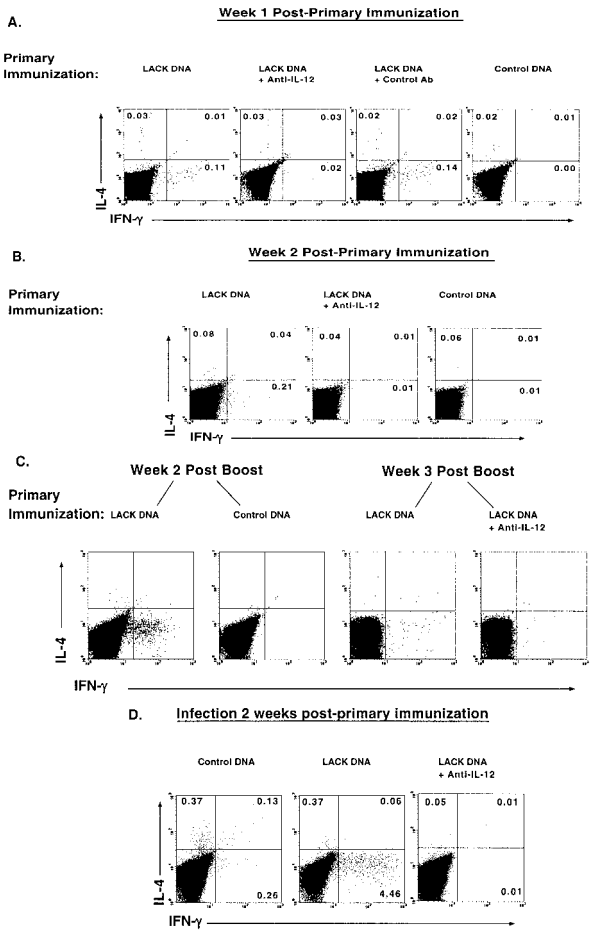

Fig. 3. The role of endogenous IL-12 in the induction and maintenance of CD4$^+$ IFN-γ-producing cells after LACK DNA vaccination. In two independent experiments, the frequency of LACK-specific CD4$^+$ IFN- or IL-4-producing cells was assessed from pooled (n = 6–8 per group) draining lymph node cells 1 week (*A*) and 2 weeks (*B*) after primary immunization with LACK DNA with or without treatment with a single dose of anti-IL-12 (1 mg) i.p. or isotype-matched control antibody. In a third experiment (*C*), the frequency of LACK-specific CD4$^+$ IFN-γ-producing cells was determined from draining lymph node cells of LACK- and control DNA-vaccinated mice 2 or 3 weeks after primary immunization. In this experiment, a single injection of anti-IL-12 was given to LACK DNA-vaccinated mice 2 weeks after primary immunization. (*D*) In a separate experiment, the frequency of LACK-specific CD4$^+$ IFN-γ- and IL-4-producing cells was determined from pooled lymph node cells from LACK DNA or control DNA (n = 6–8) vaccinated mice 6 weeks after infectious challenge. In this experiment, a group of LACK DNA-vaccinated mice were treated with a single injection of anti-IL-12 at the time of infection.

Continuous IL-12 Is Required to Sustain Immunity to *L. major* Infection. As the prior experiments focused on the role of IL-12 in maintaining CD4$^+$ IFN-γ-producing cells generated *in vivo* after vaccination, it was of additional interest to determine whether IL-12 was required to sustain Th1 responses using the best possible vaccine, which is primary infection. In this regard, susceptible wild-type or (IL-12$^{-/-}$) BALB/c mice were treated with rIL-12 for the initial 5 days of infection. This treatment regimen has been shown to be sufficient for wild-type BALB/c mice to control the infection in an IFN-γ-dependent manner (1, 2). In addition, a group of IL-12$^{-/-}$ mice that were treated for 5 days with rIL-12 were given supplemental boosting with rIL-12 once a week for the next 5 weeks. As shown in Fig. 4, wild-type but not IL-12$^{-/-}$ mice treated with IL-12 for 5 days at the onset of infection were able to control infection as assessed by footpad swelling. Remarkably, IL-12$^{-/-}$ mice treated with IL-12 for 5 days and then given supplemental injections with IL-12 once a week for 5 weeks had reduced footpad swelling compared with IL-12$^{-/-}$ mice treated for only 5 days. These data provide further evidence for a requirement of continuous IL-12 for effective control of infection to *L. major*. Moreover, these data suggest that, in wild-type mice, the 5-day treatment was sufficient to induce Th1 cells that could be maintained over the course of infection by endogenous IL-12. By contrast, in the IL-12$^{-/-}$ mice, although Th1 cells may have been generated after the initial 5-day treatment, they required additional IL-12 to be sustained over the course of infection. Evidence to support these mechanisms was determined in the following manner.

First, antigen-specific production of IFN-γ after *in vitro* stimulation was assessed at two separate time points post-infection (Table 1). When examined at 1 week post-infection, antigen-specific production of IFN-γ was similar from total lymph node cells of wild-type or IL-12$^{-/-}$ mice that had each been treated with IL-12 for the first 5 days, demonstrating that induction of Th1 responses was established and comparable in both groups of mice. By contrast, at 6 weeks post-infection, production of IFN-γ was only detected in wild-type mice treated with IL-12 for 5 days or in IL-12$^{-/-}$ mice boosted with IL-12 continuously. Second, as an additional parameter for Th1 responses, IL-12Rβ2 receptor expression—which has been shown both to be a marker of Th1 cells (13) and to have an important functional role in regulating IL-12 responsiveness (6) in this model—was assessed. IL-12Rβ2 expression on CD4$^+$ T cells was comparable in wild-type or IL-12$^{-/-}$ mice treated with IL-12 for 5 days when assessed 1 week post-infection. By contrast, at 6 weeks post-infection, IL-12Rβ2 expression is only detected from CD4$^+$ T cells of IL-12$^{-/-}$ mice continuously treated with IL-12. Taken together, these data provide strong evidence that IL-12 is required to sustain CD4$^+$ IFN-γ-producing T cells (data not shown).

IL-12 Is Required to Control Infection in Resistant Strains of Mice. The previous experiments showing the requirement for IL-12 in sustaining Th1 immunity and the ability to control infection were carried out in the susceptible BALB/c strain of mice. Because BALB/c mice have been shown to have a bias toward Th2 production (14), which could affect the generation and maintenance of Th1 cells *in vivo*, it was of interest to verify the role of IL-12 in maintaining protection against *L. major* in a resistant strain of mice. To address this, C57BL/6 mice were initially infected and then allowed to resolve their infection over a period of 8 weeks. At this time point, there were very low but detectable numbers of parasites in the footpads (<100 parasites/g of tissue) and in the draining lymph nodes (100–1,000 parasites/g of tissue). Mice were then treated with anti-IL-12 or control antibody (no rechallenge group) to see whether IL-12 inhibition would lead to an increase in parasite burden after primary immunity had already been established. In addition, as a positive control, mice were also treated with anti-IFN-γ, because this is the effector cytokine mediating control of infection. As shown in Table 2, weekly treatment with anti-IL-12 (>30-fold) and anti-IFN-γ (>4-log) resulted in a striking increase in parasite load 5 weeks after treatment was initiated. Moreover, at the same time, previously infected mice (rechallenged group) were reinfected with *L. major* and were treated with anti-IL-12 or anti-IFN-γ antibodies. In some groups of mice, the effects of weekly treatment with anti-IL-12 was compared with that of a single treatment. Mice rechallenged and treated with a single injection of anti-IL-12 or anti-IFN-γ had approximately a 10-fold increase in parasite load 5 weeks post-infection in the draining lymph nodes. In addition, continuous treatment (weekly) with antibodies resulted in a further increase in the parasite load. Similar data were seen when parasite quantitation was assessed from lymph nodes 3 weeks post-treatment or when using another resistant strain of mice such as C3H (data not shown). These data provide further biologic

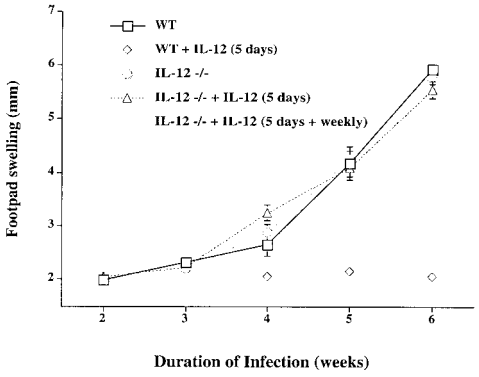

Fig. 4. The role of IL-12 in sustaining IL-12Rβ2 expression and control of infection in wild-type and IL-12$^{-/-}$ BALB/c mice after infection with *L. major*. Groups of wild-type BALB/c or IL-12$^{-/-}$ mice (n = 6–8 per group) were infected with *L. major* as described above. In some groups of mice, treatment with rIL-12 (1 μg) i.p. was initiated at the time of infection and was continued for the following 4 days for a total treatment of 5 days. A group of IL-12$^{-/-}$ mice initially treated with IL-12 for 5 days was given supplemental treatment weekly with rIL-12 (1 μg) i.p. for the next 5 weeks. Weekly footpad swelling measurements were recorded by using a metric caliper.

Table 1. The role of IL-12 in sustaining production of IFN-γ production in wild-type and IL-12$^{-/-}$ BALB/c mice after infection with *L. major*

	IFN-γ, pg/ml	
	Media	SLA
Week 1 post-infection		
Wild-type mice plus IL-12 (5 days)	<300	5,822 ± 349
IL-12$^{-/-}$ mice plus IL-12 (5 days)	<300	4,546 ± 227
Week 6 post-infection		
Wild-type mice	<300	<300
Wild-type mice plus IL-12 (5 days)	<300	2,076 ± 72
IL-12$^{-/-}$ mice	<300	<300
IL-12$^{-/-}$ mice plus IL-12 (5 days)	<300	<300
IL-12$^{-/-}$ mice plus IL-12 (5 days plus weekly)	<300	1,666 ± 49

Groups of wild-type BALB/c or IL-12$^{-/-}$ mice (n = 8 per group) were infected with *L. major* and were treated with IL-12 (1 μg) at the time of infection (day 0) and for the following 4 days for a total treatment of 5 days. A group of IL-12$^{-/-}$ mice initially treated with IL-12 for 5 days was given supplemental treatment with rIL-12 (1 μg) i.p. once a week for the following 5 weeks. Pooled lymph nodes were harvested from groups of mice (n = 4) either 1 week or 6 weeks post-infection. Single-cell suspensions of total lymph node cells (3×10^5 cells/well) were stimulated *in vitro* in a 96-well microtiter plate in the presence of absence of 25 μg/ml of soluble leishmania antigen (SLA). Culture supernatants were harvested after 48 h, and production of IFN-γ was assessed by ELISA. The lower limit of detection for IFN-γ was 100–300 pg/ml. The standard error of the mean was <10%.

evidence that IL-12 is required to sustain protection by reactivation or reinfection even in a resistant background strain of mice.

Discussion

The Role of IL-12 in Maintaining Th1 Responses. One of the seminal findings in cytokine biology was the discovery that IL-12 was a potent inducer of IFN-γ from a variety of cell types (15). This discovery has been demonstrated to have widespread importance both in mouse models of intracellular infection and in human clinical disease. For CD4$^+$ T cells, IL-12 remains the most important cytokine in directing primary Th1 differentiation *in vitro* and *in vivo*. Once Th1 responses are established, the question arose as to the role of IL-12 in sustaining Th1 responses and protective immunity. This issue was initially studied in mouse models of *Listeria monocytogenes* (16), *Toxoplasma gondii* (17), and *Histoplasma capsulatum* (18) infections. In the Listeria model, it was shown that secondary antigen-specific IFN-γ responses were markedly inhibited by treatment with anti-IL-12 *in vivo* at the time of secondary challenge. Moreover, in the *T. gondii* and *H. capsulatum* models, injection of a single dose of anti-IL-12 at the time of secondary infection did not impair protective immunity. Based on these data, it appeared that IL-12 was not required to maintain immunity after secondary infection. This premise, however, needs to be reconsidered in the context of additional data. First, in all of the aforementioned studies, anti-IL-12 was given as single injection at the time of secondary infection. Results might have been different if continuous treatment with anti-IL-12 had been maintained throughout the course of secondary infection or if IL-12$^{-/-}$ mice had been used (see below). Second, in all of the aforementioned models, CD8$^+$ T cells have been shown to have a role in secondary infection. In this regard, it was recently reported that, although CD4$^+$ IFN-γ production was IL-12-dependent, CD8$^+$ T cell production of IFN-γ was induced in an IL-12-independent manner (19). Thus, in the absence of IL-12, CD8$^+$ T cells—through IFN-γ and/or other cytolytic mechanisms—could have a protective role. The most demonstrative evidence for a requirement of continuous IL-12 comes from recent experiments using IL-12$^{-/-}$ mice. In both the *T. gondii* (G. Yap, personal communication) and *H. capsulatum* (P. Zhou, personal communication) mouse models, it appears that IL-12 is required to maintain Th1 immunity and provide protection after primary infection and re-challenge. Taken together, these data strongly suggest that, for the aforementioned infectious disease models, the CD4$^+$ T cell production of IFN-γ is IL-12-dependent for both induction and maintenance of responses sufficient to mediate a biologic outcome. It should be noted, however, that, in mouse models of many viral infections, there is clear evidence that CD4$^+$ IFN-γ production is induced and sustained in an IL-12-independent manner (20, 21). These latter data raise interesting questions as to how viral pathogens differ from bacterial, fungal, and parasitic pathogens in their ability to induce Type 1 cytokine responses.

The clinical experience in humans with cutaneous Leishmania infection is that primary infection confers lifelong resistance to reinfection. Thus, as with other pathogens, live exposure represents the best vaccine in terms of long-term immunity and serves as the ultimate positive control in terms of establishing a protective immune response. In this regard, we show that, in the course of primary and secondary infection in both susceptible and resistant strains of mice, maintenance of IFN-γ production, IL-12Rβ2 expression, and immune protection requires continuous IL-12. Our findings that anti-IL-12 abrogated control of parasite growth in the lymph nodes of C57BL/6 mice is in contrast to a previous report in which anti-IL-12 treatment did not alter control of secondary infection (as assessed by footpad swelling) in a different resistant strain (C3H) of mouse (22). Of note, however, recent studies by the same group using IL-12$^{-/-}$ mice rather than neutralization with anti-IL-12 antibody have confirmed the requirement for IL-12 in this rechallenge model (P. Scott, personal communication).

The exact mechanism and point in the pathway of Th1 memory at which IL-12 is exerting its effect remain open questions. First, is IL-12 is required to sustain "resting" and/or "effector" memory T cells? Second, is IL-12 continually required to instruct and/or select out a population of antigen-specific CD4$^+$ T cells that have not fully committed to IFN-γ production? Third, is IL-12 required to enhance the magnitude of a few effector CD4$^+$ T cells and/or to maintain a sufficient number of Th1 cells to allow for control of infection? Based on current available data, there are some clues to the mechanism by which IL-12 sustains Th1 responses. In the course of Th1 differentiation, production of IFN-γ is enhanced and IL-2 is diminished. Because IL-2 is a potent growth factor and IFN-γ is antiproliferative, IL-12 could function to sustain cell growth and viability (23) or to prevent apoptosis of CD4$^+$ IFN-γ T cells (24, 25). Moreover, recent experiments studying how CD8$^+$ memory T cells

Table 2. IL-12 is required to maintain control of parasite growth in previously infected resistant strains of mice

Intervention	Parasite quantitation	Fold increase
Primary infection		
No treatment	73,100	
Anti-IFN-γ	7,812,500	106
Anti-IL-12	7,812,500	106
Control antibody	125,000	1.7
No rechallenge		
No treatment	2,236	
Anti-IFN-γ (weekly)	28,718,384	12,843
Anti-IL-12 (weekly)	73,100	32
Rechallenge		
No treatment	8,549	
Anti-IFN-γ (single treatment)	125,000	14
Anti-IL-12 (single treatment)	75,000	9
Control antibody (single treatment)	5,000	0
Anti-IFN-γ (weekly)	1,827,511	213
Anti-IL-12 (weekly)	213,746	25
Control antibody (weekly)	5,000	0

C57BL/6 mice were challenged with 1×10^5 *L. major* (WHOM/IR/−/173) metacyclic promastigotes and then were allowed to resolve their infection over a period of 8 weeks. At that time, groups of mice (no rechallenge group) were treated weekly with 1 mg i.p. of anti-IL-12 (C17.8), anti-IFN-γ (XMG1.2), or isotype control antibody. At the same time, groups of mice were rechallenged with 1×10^5 *L. major* metacyclic promastigotes and were treated with anti-IL-12, anti-IFN-γ, or control antibody as described above. In some groups of mice, the effects of weekly treatment with anti-cytokine antibodies were compared to that of a single treatment given at the time of reinfection. As an additional control, naive mice (primary infection) were infected with *L. major* and were treated with anti-cytokine antibodies at the same time that previously infected mice were rechallenged. Five weeks after infection, parasite quantitation from the draining lymph nodes was performed as described (8). The data shown represent the number of viable parasites/lymph node at the highest dilution at which promastigotes could be grown after up to 7 days of incubation at 26°C. These data are representative of two independent experiments.

are controlled show that IL-15 is required to maintain cell division (26). In this regard, it is possible that IL-12 has an analogous role in sustaining memory Th1 cells. The notion that Th1 cells are continually regulated and that IL-12 could play a role in the regulation is also consistent with previous *in vitro* experiments showing a requirement for cytokines on maintaining differentiated Th1 but not Th2 cells (27). Additional mechanisms for IL-12 could include a role in augmenting and/or maintaining expression of T-bet, a specific Th1 transcription factor (28). Finally, IL-12 may also be required to sustain responsiveness of both IL-12 (29) and IL-18 (30). In fact, some have suggested that IL-18 may be the critical cytokine for sustaining Th1 responses (31, 32) and that inhibiting IL-12 may have a downstream effect on IL-18, as well.

Role of Antigen in Maintaining Th1 Responses *in Vivo*. In addition to cytokines, the role of antigens in sustaining memory cellular immune responses has been intensely studied for both $CD8^+$ and, more recently, $CD4^+$ T cells. For $CD4^+$ T cells, adoptive transfer studies showed that activated $CD4^+$ T cells from TCR transgenic mice primed *in vitro* under polarizing Th1 or Th2 conditions persisted for long periods of time in the absence of antigen in immunodeficient or irradiated hosts (33, 34). Thus, these data clearly establish that a population of activated effector cells *in vitro* can revert to resting memory $CD4^+$ T cells *in vivo* and persist in an antigen-independent manner. In this report, we address the role of antigen in sustaining Th1 cells that have been generated *in vivo* after vaccination with LP plus rIL-12. In mice originally vaccinated with LP plus rIL-12, additional boosting with LP enhanced the frequency of $CD4^+$ IFN-γ-producing cells and reduced the degree of footpad swelling after infection with *L. major*. Thus, antigen appears necessary but not sufficient to maintain memory/effector $CD4^+$ IFN-γ-producing cells sufficient to fully control infection in this model. Remarkably, mice vaccinated with LP and IL-12 DNA did have sustained Th1 responses and were able to control infection 12 weeks post-immunization. These data suggest that, once Th1 cells are generated with antigen and IL-12, continuous IL-12 as provided by IL-12 DNA is necessary and possibly sufficient to sustain Th1 immunity even in the absence of continuous antigen. Finally, it should be noted that persistence of antigen can be detected in other types of cutaneous leishmania infection well after the resolution of primary infection. These data provide evidence that antigen can persist *in vivo* well after primary infection has resolved and suggest that it may be required to maintain long-term protective immunity.

To conclude, for Th1 memory responses, the issue of cellular memory shifts more specifically to what is required for "immune protection" rather than what is required to maintain resting memory cells. In this regard, while IL-12 may not be absolutely essential to sustain all Th1 memory cells, our data clearly show that, in this model, IL-12 is required in the memory Th1 pathway to mediate a successful biologic outcome.

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