Requirements for the Maintenance of Th1 Immunity In Vivo Following DNA Vaccination: A Potential Immunoregulatory Role for CD8+ T Cells


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Requirements for the Maintenance of Th1 Immunity In Vivo Following DNA Vaccination: A Potential Immunoregulatory Role for CD8+ T Cells

Sanjay Gurunathan,* Laura Stobie,*‡ Calmin Prussin,‡ David L. Sacks,§ Nicolas Glaichenhaus,¶ Deborah J. Fowell,‖ Richard M. Locksley,¶ John T. Chang,† Chang-You Wu,* and Robert A. Seder1* "Markers previous that depletion of CD8+ T cells in LACK DNA-vaccinated mice abrogated protection when infectious challenge was done 2 wk postvaccination. In this study, we extend these findings to study the mechanism by which CD8+ T cells induced by LACK DNA vaccination mediate both short- and long-term protective immunity against L. major. Mice vaccinated with LACK DNA and depleted of CD8+ T cells at the time of vaccination or infection were unable to control infection when challenge was done 2 or 12 wk postvaccination. Remarkably, it was noted that depletion of CD8+ T cells in LACK DNA-vaccinated mice was associated with a striking decrease in the frequency of LACK-specific CD4+ IFN-γ-producing T cells both before and after infection. Moreover, data are presented to suggest a mechanism by which CD8+ T cells exert this regulatory role. Taken together, these data provide additional insight into how Th1 cells are generated and sustained in vivo and suggest a potentially novel immunoregulatory role for CD8+ T cells following DNA vaccination. The Journal of Immunology, 2000, 165: 915–924.

Vaccination with plasmid DNA has been shown to induce protective immunity through both MHC class I- and class II-restricted T cell responses in a variety of experimental models of infection (reviewed in Ref. 1). By contrast, conventional protein vaccination in general induces MHC class II- but not class I-restricted responses. This distinction may have importance for designing vaccines for infections requiring cellular immunity (Leishmania major, Mycobacterium tuberculosis, and possibly HIV). For these infections in which IFN-γ and/or CTL responses may be required, the ability of DNA vaccination to elicit an MHC class I response may be advantageous over conventional protein vaccination in providing a more broad-based and potentially durable immune response.

The mouse model of L. major infection was among the first in vivo models to demonstrate that Th subsets existed in a biologic context (reviewed in Ref. 2). In this model, effective primary immunity in resistant mouse strains is due to development of a type 1 immune response characterized by the IL-12-dependent production of IFN-γ from NK cells and MHC class II-restricted CD4+ T cells (3–8). In contrast, disease susceptibility is correlated with early production of IL-4, which appears to inhibit IL-12R expression and the subsequent development of protective Th1-type cells (9, 10). In susceptible mice, this early production of IL-4 derives from a restricted population of Vβ4 Vo8 CD4+ T cells in response to an immunodominant L. major protein called LACK2 (for Leishmania homologue of receptor for activated C kinase). This 36-kDa protein is highly conserved among related Leishmania species and is expressed in both promastigote and amastigote forms of the parasite. The functional importance of LACK-reactive CD4+ cytokine-producing T cells in L. major infection has been demonstrated in several different studies. First, BALB/c mice made tolerant to LACK by transgenic expression of LACK in the thymus were found to be resistant to parasite infection, suggesting that the early activation of LACK-reactive T cells contributes to the initial cytokine milieu favoring a nonhealing Th2-type phenotype (12). These observations were further extended in a report showing that early IL-4 mRNA expression in response to LACK was diminished in Vβ4-deficient BALB/c mice (9). Finally, vaccination with LACK protein in the presence of rIL-12 protein has been shown to induce a protective Th1 response (11). In this last instance, redirecting the LACK-specific IL-4 response toward a Th1-type response confers protective immunity to susceptible mice. Similar results were obtained in a separate study in which effective vaccination in BALB/c mice was achieved using rIL-12 immunization with soluble Leishmania Ag (13). Thus, this model provides a way to study the requirements for generating and maintaining Ag-specific Th1 cells in vivo following vaccination.

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2 Abbreviations used in this paper: LACK, Leishmania homologue of receptor for activated C kinase; β2m, β2-microglobulin; TCR-Tg, TCR transgenic; TDS, T-depleted spleen cell.
In a previous study, we showed that s.c. administration of plasmid DNA encoding the LACK Ag was sufficient to confer protection to susceptible BALB/c mice challenged 2 or 12 wk postvaccination. These data suggested a fundamental difference in how LACK DNA and LACK protein plus IL-12 protein mediate long-term immunity. Although there are many mechanisms that could account for these differences, our previous report showed that LACK DNA but not LACK protein plus IL-12 induces Ag-specific CD8 T cells in a manner that is likely to be effective against L. major. In this study, we demonstrate that sustained immunity in mice vaccinated with LACK DNA is abrogated by treatment with anti-CD8 in vivo. Furthermore, data are presented to elucidate the mechanisms by which CD8 T cells mediate their effector function. Remarkably, these studies suggest that in addition to an effector role, CD8 T cells may have an immunoregulatory role in maintaining the frequency of CD4 IFN-γ-producing T cells.

Materials and Methods

Plasmid construction and purification

A cDNA encoding a truncated LACK protein (aa 143–312) was cloned in frame downstream to a Kozak consensus sequence and an initiation codon into a pECE vector (15). The insert was excised using HindIII and ligated into an expression vector pCDNA-3 downstream to the CMV promoter (Invitrogen, San Diego, CA). Plasmid DNA was purified by double banding cesium chloride gradient ultracentrifugation. The 260/280 ratios ranged from 1.8 to 2 and were endotoxin free.

Injection of mice

BALB/c mice were injected in their hind footpads with 100 μg of plasmid DNA encoding LACK Ag or control DNA (empty vector) suspended in 50 μl of sterile PBS. For immunization with protein, mice were injected as above with 50 μg of recombinant LACK protein with 1 μg of rIL-12 (Genetics Institute, Cambridge, MA). In all experiments, mice were boosted 2–3 wk later with their initial regimen. Mice were then infected 2 or 12 wk after the boost with 1 × 106 L. major (WHOM/IR/173) metacyclic promastigotes in their hind footpads. Weekly footpad swelling measurements were recorded using a metric caliper.

Frequency of IL-4- and IFN-γ-producing T cells by intracellular staining

Pooled lymph node cells from vaccinated mice (n = 6–8) either before or after infection were stimulated with LACK protein for 4 h at 37°C. Brefeldin A was added at 10 μg/ml and the cells cultured for an additional 2 h, at which time they were fixed in 4% paraformaldehyde. Cells were then permeabilized with 0.1% saponin and blocked with 5% nonfat dry milk for 1 h; stained simultaneously with Ab to CD4, CD8, IFN-γ, and IL-4 for 30 min; washed and analyzed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). The frequency of CD4+ and CD8+ T cells producing IFN-γ and IL-4 was determined by gating on the respective cell populations. An isotype-matched control for IFN-γ and IL-4 was used to place statistical markers and stained fewer than 0.02% of cells. To control for specificity, addition of unlabeled anti-IFN-γ abrogated all staining for IFN-γ. Power calculations were performed to determine the sample size required to detect the frequency of cytokine-producing cells with ±20% error with a confidence of 95% (p < 0.05) (16). Sufficient numbers of cellular events were collected to assure that the detected frequencies of cytokine-producing cells were within that limit. Typically, between 150,000 and 500,000 total cells were analyzed per sample. Specific confidence intervals at a p value of ≤0.05 are noted in the text.

Ab or cytokine depletion in vivo

Groups of mice vaccinated with LACK DNA or LACK protein plus rIL-12 were treated with 1 mg of anti-CD8 (24,34), anti-IL-12 (C17.8), or anti-IFN-γ (XMG1.2) Ab at the time of vaccination and weekly thereafter. In some experiments, LACK DNA-vaccinated mice were treated with anti-CD8 Ab at the time of vaccination and boost. Treatment with anti-CD8 Ab or cytokine depletion in vivo resulted in >95% depletion of CD8 T cells. In addition, CD8 depletion did not diminish the number of function (e.g., IL-12 production) of CD11c+ cells.

TCR transgenic (TCR-Tg) mice priming

CD4 T cells from mice transgenic for Vb4 specific (unpublished data) for LACK protein were isolated from pooled lymph node and spleen cells by positive selection using magnetic beads (Miltenyi Biotec, Cologne, Germany). The frequency of LACK-specific cells from these mice as assessed by tetramer staining is 1–2% (our unpublished data). Purity of CD4+ T cells was >99%. The percentage of CD4+ T cells Vβ8 >80%. Primary stimulation of transgenic T cells was conducted by adding 1 × 106 CD4+ T cells to individual wells of 24-well plates in a total volume of 1.5 ml together with T-depleted spleen cells (TDS; 2 × 105) with LACK protein (20 μg/ml). As a positive control for Th1 priming, IL-12 (5 ng/ml) and anti-IL-4 (10 μg/ml) were added to cultures. As a control for Th2 priming, IL-4 (1000 U/ml) and anti-IL-12 (10 μg/ml) were added to cultures. In addition, CD4+ T cells (1 × 106) from LACK DNA, control DNA, or infected mice treated with anti-IL-4 were isolated by positive selection (purification >90%), FACs sorted to >99.5% purity (CD8+/CD11+), and then added to primary cultures. After 5 days of primary stimulation, cells were extensively washed and restimulated with LACK protein (20 μg/ml) and TDS for 4 h. Brefeldin A was added at 10 μg/ml and the cells were cultured for an additional 2 h, at which time they were fixed in 4% paraformaldehyde. The frequency of CD4 IFN-γ- and IL-4-producing T cells was determined, as described above. Similar studies were done using CD4+ T cells from D011.10 TCR-Tg mice specific for OVA.

Assessment of IL-12Rβ1 protein expression

Total lymph node cells of vaccinated mice following infection were first stained with anti-CD4 or anti-CD8 FITC Ab (PharMingen, San Diego, CA). Cells were then incubated with either isotype control biotinylated Ab (PharMingen) or biotinylated anti-IL-12Rβ1 mAb (generous gift of Dr. Maurice Gately, Roche Laboratories, Nutley, NJ). Cells were then stained with streptavidin-PE (PharMingen). The percentage of IL-12Rβ1+ T cells was based on the number of cells expressing IL-12Rβ1 compared with the isotype control.

mRNA expression for IL-12Rβ2

mRNA expression for IL-12Rβ2 was assessed, as previously described (17). Briefly, total mRNA was extracted from purified CD4+ T cells (1 × 106) from various groups of vaccinated mice following infection. RT-PCR analysis for IL-12Rβ2 subunit and βm was performed.

Measurement of IL-12 p40 production

Pooled (n = 6–8) draining lymph nodes from individual mice were harvested 6 wk postinfection challenge. Single cell suspensions from lymph nodes were plated at 3 × 105 cells/200 μl. Recombinant LACK protein (20 μg/ml) was added to cultures, and supernatants were collected 24 h later and assayed for IL-12 p40 by specific ELISA (PharMingen). The sensitivity of the assay was 125 pg/ml.

Results

CD8 T cells are differentially required for protective immunity following vaccination with LACK DNA vs LACK protein plus IL-12

In previous studies, we had shown that s.c. administration of plasmid DNA encoding the LACK Ag or LACK protein plus IL-12 protein was sufficient to confer protection to susceptible BALB/c mice challenged 2 wk postvaccination (14, 15). It is notable that in one of these studies, treatment of mice with anti-CD8 Ab at the time of infection (2 wk postvaccination) abrogated the protective efficacy of LACK DNA vaccination (15). This finding was in contrast to previous work showing only MHC class II CD4+ T cell responses to be critical in determining disease outcome in a primary L. major infection (4–6, 18, 19). We speculated, based on these data, that CD8+ T cell production of IFN-γ induced by DNA vaccination would have a selective role in maintaining immunity compared with that in mice vaccinated with LACK protein plus IL-12. To test this, mice vaccinated with LACK DNA or LACK protein plus IL-12 were able to control infection when infected 2 wk postvaccination (Fig. 1). Furthermore, mice vaccinated with LACK protein plus IL-12, but not LACK DNA, maintained effective immunity when treated with anti-CD8 at the time of vaccination. Similar data were seen if CD8 depletion was done at the time...
FIGURE 1. Depletion of CD8+ T cells at the time of vaccination abrogates protection to L. major following LACK DNA but not LACK protein plus IL-12 vaccination. Groups of BALB/c mice (n = 6–8 per group) were initially immunized in the footpad and boosted 2 wk later with LACK DNA (100 μg), control DNA (100 μg), or LACK protein (50 μg) with 1 μg of IL-12. Mice were then challenged with 1 × 10^6 L. major (WHOM/IR/1/173) metacyclic promastigotes in their hind footpads 2 wk after the boost, and weekly footpad swelling measurements were recorded using a metric caliper. At the time of each vaccination, mice were treated with 1 mg of anti-CD8 (2.43) Ab. This experiment is representative of three additional experiments.

of infection (our unpublished data). These data clearly demonstrate that CD8+ T cells have a differential role in mediating effective immunity induced by LACK DNA vs LACK protein plus IL-12 vaccination. As an extension to these data, β₂m−/− BALB/c mice vaccinated with LACK DNA remained susceptible to infection, providing additional evidence for a role of CD8+ T cells in vivo (our unpublished data).

CD8+ T cells are required for long-term protection and to sustain Th1 immunity in vivo following infection in mice vaccinated with LACK DNA

As noted above, while vaccination with either LACK DNA or LACK protein plus IL-12 could protect mice infected 2 wk postvaccination, our previous study showed that only vaccination with LACK DNA but not leishmanial protein plus IL-12 conferred durable immunity to L. major infection when challenged 12 wk postvaccination (14). Moreover, it was shown in that report that cells from LACK DNA but not leishmanial protein plus IL-12-vaccinated mice maintained their frequency of CD4+ IFN-γ-producing T cells over a prolonged period of time. Based on these data, because CD8+ IFN-γ-producing T cells are only detected from infected mice vaccinated with LACK DNA, it was of interest to determine the mechanism by which they were exerting their effector function and specifically whether they had a role in maintaining the frequency of CD4+ IFN-γ-producing T cells. Treatment of LACK DNA-vaccinated mice with anti-CD8, this time administered at the time of infection, abrogated protection in mice infected 2 and 12 wk postvaccination (Fig. 2A). These data demonstrate that CD8+ T cells may also be required in vivo for long-term protection of mice vaccinated with LACK DNA.

As an immune correlate of protection, the frequency of Ag-specific CD4+ and CD8+ IFN-γ-producing T cells was determined (Fig. 2B). Similar frequencies of Ag-specific CD4+ IFN-γ-producing T cells were detected from mice infected 2 wk postvaccination with either LACK DNA (0.87 ± 0.07%) or LACK protein plus IL-12 (0.99 ± 0.08%), correlating with the ability to control infection; however, only LACK DNA-vaccinated mice maintained their frequency of CD4+ IFN-γ-producing T cells when infected 12 wk postvaccination (0.92 ± 0.11%) (14). Remarkably, the frequency of CD4+ IFN-γ-producing T cells from LACK DNA-vaccinated mice treated with anti-CD8 was diminished ~4- and 2-fold following infection at either 2 (0.22 ± 0.035%) or 12 (0.44 ± 0.075%) wk postvaccination, respectively. These data suggest that CD8+ T cells could have an immunoregulatory role in maintaining the frequency of CD4+ IFN-γ-producing T cells during infection in LACK DNA-vaccinated mice.

Induction of CD8+ IFN-γ-producing T cells requires CD4+ T cell activation and/or IL-2 following in vitro stimulation with LACK protein

With regard to our analysis of cytokine-producing T cells in vivo using intracellular cytokine staining, it is important to note that we have not yet been successful in identifying a LACK-specific peptide capable of stimulating CD8+ T cells from LACK DNA-vaccinated mice. Thus, our assessment of the frequencies of both CD4+ and CD8+ IFN-γ-producing T cells is done by stimulation of total lymph node cells from vaccinated mice with LACK protein. In this regard, we have previously shown that bulk production of IFN-γ from supernatants of total lymph node cells stimulated in vitro for 48 h with LACK protein from mice vaccinated with LACK DNA was completely inhibited by addition of anti-MHC class II Ab and substantially inhibited by anti-MHC class I Ab (14). The latter result provided evidence that, following stimulation with LACK protein in vitro, some of the IFN-γ produced by total lymph node cells derived from CD8+ T cells. Moreover, the complete inhibition of IFN-γ in total lymph node cells by the presence of anti-MHC class II suggested that the IFN-γ response that includes CD8+ T cells was MHC class II dependent. Direct demonstration for the role of CD8+ T cells in regulating CD8+ IFN-γ production in response to stimulation with LACK protein was shown using intracellular cytokine staining (Fig. 3). In this experiment, the frequency of CD4+ and CD8+ IFN-γ-producing T cells from LACK DNA-vaccinated following stimulation with LACK protein in vitro was 3.05 ± 0.19% and 1.28 ± 0.14%, respectively. Addition of anti-CD4 Ab or anti-IL-2 to cultures substantially inhibited the frequency of CD8+ IFN-γ-producing T cells. Finally, production of IFN-γ from CD8+ T cells of infected lymph nodes stimulated in the presence of anti-CD4 could be substantially restored by addition of exogenous IL-2 (data not shown). Taken together, these data suggest that CD4+ T cell activation and/or induction of IL-2 are mechanisms by which CD4+ T cells are regulating the frequency of CD8+ IFN-γ-producing T cells in vitro in response to LACK protein. Thus, although these data suggest that the frequency of CD8+ IFN-γ-producing T cells from LACK DNA-vaccinated mice is determined in an indirect manner, their specificity is evidenced by the fact that they are only detected from mice vaccinated with LACK DNA and not LACK protein plus IL-12.

CD8+ T cells have a role in both the induction and expansion of CD4+ IFN-γ-producing T cells from LACK DNA-vaccinated mice

The previous figures suggest a potential immunoregulatory role for CD8+ T cells in regulating the frequency of CD4+ IFN-γ-producing T cells following infection from mice vaccinated with LACK DNA. To further elucidate the immunoregulatory role of CD8+ T cells induced by LACK DNA vaccination, the frequency of CD4+
IFN-γ-producing T cells was determined both before and at various times after infection from lymph node cells of LACK DNA-vaccinated mice depleted of CD8+ T cells at the time of vaccination.

First, to assess the effect of CD8+ T cell depletion on the induction of CD4+ IFN-γ-producing T cells, the frequency of CD4+ IFN-γ-producing T cells was determined in mice vaccinated with LACK DNA depleted of CD8+ T cells at the time of vaccination and before infection. As shown in Fig. 4A, LACK DNA-vaccinated mice treated with anti-CD8 at the time of vaccination had a reduced frequency of CD4+ IFN-γ-producing T cells (0.04 ± 0.014%) compared with cells from LACK DNA-vaccinated mice...
(0.11 ± 0.024%) when analyzed before infection. Similarly, in a separate experiment (Fig. 4C), the frequency of CD4⁺ IFN-γ-producing T cells depleted T cells at the time of vaccination was 0.09 ± 0.022% compared with 0.42 ± 0.052% from LACK DNA-vaccinated mice. Although the frequency of CD4⁺ IFN-γ-producing T cells was diminished from LACK DNA-vaccinated mice depleted of CD8⁺ T cells, the in vitro proliferative response from lymph node cells following stimulation with LACK protein was similar (~100,000 cpm) between the two vaccinated groups. Moreover, in the same experiment as above (Fig. 4C), total lymph node cells from mice vaccinated with LACK DNA induced ~300 pg of IFN-γ after 48 h of stimulation with LACK protein in vitro. By contrast, cells from LACK DNA-vaccinated mice that were depleted of CD8⁺ T cells had only 100 pg/ml of IFN-γ induced following in vitro stimulation with LACK protein. These data corroborate the reduction in the frequency of CD4⁺ IFN-γ-producing T cells from LACK DNA-vaccinated mice depleted of CD8⁺ T cells, as shown above. Taken together, these data suggest that depletion of CD8⁺ T cells at the time of vaccination in LACK DNA-vaccinated mice did not impair the activation of Ag-specific CD4⁺ T cells, but rather limited the differentiation of these cells toward a Th1 phenotype.

In the same experiment as in Fig. 4A, the frequency of CD4⁺ IFN-γ-producing T cells was also assessed at various times postinfection from LACK DNA-vaccinated mice depleted of CD8⁺ T cells at the time of vaccination (Fig. 4B). Cells from these CD8⁺-depleted mice had a marked reduction in the frequency of CD4⁺ IFN-γ-producing T cells at both 28 (0.09 ± 0.0022%) and 42 (0.17 ± 0.025%) days postinfection compared with cells from LACK DNA-vaccinated mice (1.45 ± 0.22% and 5.11 ± 0.36%, respectively). Taken together with the previous figures, these data show that treatment of mice with anti-CD8 at the time of either vaccination or infection abrogates protection and diminishes the frequency of CD4⁺ IFN-γ-producing T cells. Thus, these results provide additional evidence for an immunoregulatory role of CD8⁺ T cells for both the induction and maintenance of CD4⁺ IFN-γ-producing T cells.

**CD8⁺ T cells from LACK DNA-vaccinated mice enhance the frequency of CD4⁺ IFN-γ-producing T cells from LACK/TCRβ transgenic mice following in vitro stimulation**

Evidence for a direct role of CD8⁺ T cells on influencing the frequency of LACK-specific CD4⁺ IFN-γ-producing T cells was evaluated in an in vitro priming model using CD4⁺ T cells from Vβ4 TCR-Tg mice. CD4⁺ T cells from these mice have been shown to proliferate and secrete cytokines (e.g., IL-2, IL-4, and IFN-γ) in response to LACK protein in vitro (R. M. Locksley and D. J. Fowell, unpublished data). Thus, for these experiments, freshly isolated TCR-Tg CD4⁺ T cells from these mice were stimulated in vitro for several days with TDS from normal BALB/c mice and LACK protein (neutral conditions). As a positive control for Th2 priming, cells from LACK DNA-vaccinated mice (1.45 ± 0.025%) days postinfection compared with cells from LACK DNA-vaccinated mice (1.45 ± 0.22% and 5.11 ± 0.36%, respectively). Taken together with the previous figures, these data show that treatment of mice with anti-CD8 at the time of either vaccination or infection abrogates protection and diminishes the frequency of CD4⁺ IFN-γ-producing T cells. Thus, these results provide additional evidence for an immunoregulatory role of CD8⁺ T cells for both the induction and maintenance of CD4⁺ IFN-γ-producing T cells.

As shown in Fig. 5A, CD4⁺ T cells from LACK TCRβ-Tg mice stimulated under neutral conditions with TDS and LACK protein had a frequency of 4.2 ± 0.14% for IFN-γ and 0.3 ± 0.034% for IL-4 following restimulation (Fig. 5A). As a positive control for Th1 priming, CD4⁺ T cells cultured in the presence of IL-12 and anti-IL-4 (Fig. 5B) had a striking enhancement in the frequency of CD4⁺ IFN-γ-producing T cells compared with cells cultured under neutral conditions. As a positive control for Th2 priming, cells cultured in the presence of IL-4 and anti-IL-12 (Fig. 5C) had a

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**FIGURE 3.** The frequency of CD8⁺ IFN-γ-producing T cells following stimulation with LACK protein in vitro is inhibited by anti-CD4 or anti-IL-2. Pooled lymph node cells from LACK DNA or control DNA (n = 6–8)-vaccinated mice 6 wk after infection were stimulated in vitro with LACK protein for 4 h at 37°C in the presence or absence of 10 μg/ml of anti-CD4 (GK1.5) or anti-IL-2 (S4B6). The frequency of Ag-specific IFN-γ- and IL-4-producing T cells was assessed by intracellular cytokine, as described in Materials and Methods. This experiment is representative of two individual experiments.

**In Vivo Vaccination:**

- **LACK DNA**
- **Control DNA**

**In Vitro Priming Conditions:**

- **LACK DNA**
- **LACK Protein + anti-CD4**
- **LACK Protein + anti-IL-2**

**CD4⁺ T cells from LACK DNA-vaccinated mice enhance the frequency of CD4⁺ IFN-γ-producing T cells from LACK/TCRβ transgenic mice following in vitro stimulation**

Evidence for a direct role of CD8⁺ T cells on influencing the frequency of LACK-specific CD4⁺ IFN-γ-producing T cells was evaluated in an in vitro priming model using CD4⁺ T cells from Vβ4 TCR-Tg mice. CD4⁺ T cells from these mice have been shown to proliferate and secrete cytokines (e.g., IL-2, IL-4, and IFN-γ) in response to LACK protein in vitro (R. M. Locksley and D. J. Fowell, unpublished data). Thus, for these experiments, freshly isolated TCR-Tg CD4⁺ T cells from these mice were stimulated in vitro for several days with TDS from normal BALB/c mice and LACK protein (neutral conditions). As a positive control for Th2 priming, cells from LACK DNA-vaccinated mice (1.45 ± 0.025%) days postinfection compared with cells from LACK DNA-vaccinated mice (1.45 ± 0.22% and 5.11 ± 0.36%, respectively). Taken together with the previous figures, these data show that treatment of mice with anti-CD8 at the time of either vaccination or infection abrogates protection and diminishes the frequency of CD4⁺ IFN-γ-producing T cells. Thus, these results provide additional evidence for an immunoregulatory role of CD8⁺ T cells for both the induction and maintenance of CD4⁺ IFN-γ-producing T cells.

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marked increase in the frequency of IL-4-producing cells compared with cells primed under neutral conditions.

To test whether CD8+ T cells from DNA-vaccinated mice could influence the frequency of LACK-specific CD4+ IFN-γ-producing T cells, CD8+ T cells were isolated from mice that had been vaccinated with LACK DNA or control DNA following infectious challenge. CD4+ TCRβ-Tg cells cultured with TDS, LACK protein, and sorted CD8+ T cells from LACK DNA-vaccinated mice (Fig. 5D) had a 3-fold increase in the frequency of CD4+ IFN-γ-producing T cells compared with cells primed under neutral conditions. By contrast, the presence of CD8+ T cells isolated from infected mice that had been vaccinated with control DNA (Fig. 5E) did not appreciably alter the frequency of Ag-specific CD4+ IFN-γ-producing T cells following restimulation.

As noted below, one mechanism by which CD8+ T cells from LACK DNA-vaccinated mice could be influencing the frequency of CD4+ IFN-γ-producing T cells is through production of IFN-γ. In this regard, the immunoregulation by a soluble mediator such as IFN-γ would not necessarily be restricted to LACK-specific CD4+ T cells. Thus, once these CD8+ T cells isolated following infection from only LACK DNA-vaccinated mice can influence the frequency of LACK-specific CD4+ IFN-γ-producing T cells in vitro.
CD4+ T cells from LACK DNA-vaccinated mice enhance the frequency of CD4+ IFN-γ-producing T cells. These include a cell surface interaction and/or through soluble factors such as cytokines. It is clear that in the L. major model, IL-12-dependent production of IFN-γ from CD4+ T cells is critical in maintaining primary immunity (20, 21). IL-12 expression and subsequent IL-12 responsiveness have also been shown to be important in protective immunity in this model (10).

First, with regard to IL-12R expression, IL-12R expression was assessed on CD4+ and CD8+ T cells. It should be noted that while IL-12R expression is critical in maintaining primary immunity (20, 21), IL-12-dependent production of IFN-γ and IL-12 responsiveness (25, 26). Furthermore, the availability of an Ab against IL-12R expression (24) in mice, we postulated that CD8+ T cells are affecting the frequency of CD4+ IFN-γ-producing T cells through these respective pathways could be regulating the frequency of CD4+ IFN-γ-producing T cells.

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also evaluated to provide additional evidence for a role of CD8+ T cells and IL-12R expression in a progressive reduction of IL-12 p40 in response to LACK protein, which could both affect priming for CD8+ T cells. Cells of mice treated with anti-CD8 had a decrease in IL-12R expression. As shown in Fig. 6, mice vaccinated with LACK DNA had increased mRNA expression for IL-12Rβ2 compared with control mice. Treatment of LACK-vaccinated mice with anti-CD8 or anti-IFN-γ diminished IL-12Rβ2 mRNA expression compared with LACK DNA-vaccinated mice. Moreover, treatment with anti-IL-12 completely abrogated IL-12Rβ2 expression.

Finally, IFN-γ from CD8+ T cells could function by enhancing production of IL-12 protein, which could both affect priming for IFN-γ and directly regulate IL-12R expression. To directly test this, in vitro production of IL-12 p40 was assessed from cells of vaccinated mice following infection. As shown in Table II, only cells from LACK-vaccinated mice had detectable IL-12 p40 induced by stimulation with LACK protein in vitro. Moreover, depletion of anti-CD8 cells, anti-IFN-γ, or anti-IL-12 in vivo resulted in a progressive reduction of IL-12 p40 in response to Staphylococcus aureus Cowan I bacteria stimulation in vitro. Similar qualitative results among the groups were obtained measuring IL-12 p70, although the amounts produced were substantially less.

Discussion
Our previous study showed that LACK DNA but not leishmanial protein plus IL-12 protein vaccination selectively induced CD8+ T cells and provided long-term protective immunity to L. major infection. Moreover, immunity induced by LACK DNA was shown to be CD8+ T cell dependent in vivo. This study focuses on the mechanism by which CD8+ T cells induced by LACK DNA vaccination were required for long-term protection.

CD8+ T cells from LACK DNA-vaccinated mice have a critical role in regulating the frequency of Ag-specific Th1 cells
CD8+ T cells have been shown to play a critical role in protective immunity to a variety of intracellular infections. With regard to L. major infection, while previous reports have shown that CD8+ T cells may not be essential for primary immunity, there are several studies showing that CD8+ T cells have a role in secondary responses (27–31). Our previous work, as well as what is reported in this work, showed that CD8 depletion at the time of infection abrogated protection in LACK DNA-vaccinated mice. These data provided the first evidence that CD8+ T cells have an important role in primary immunity in this model. Although not shown in this study, we have additional evidence that CD8+ T cells from LACK DNA-vaccinated mice are functional effectors in vivo against L. major using an adoptive transfer model (manuscript in preparation). Moreover, the surprising finding that LACK DNA-vaccinated mice depleted of CD8+ T cells in vivo had diminished frequencies of CD4+ IFN-γ-producing T cells suggested that in addition to an effector role for CD8+ T cells, they also could have an immunoregulatory function. Evidence to support an immunoregulatory role for CD8+ T cells from LACK DNA-vaccinated mice was obtained by measuring the frequency of CD4+ IFN-γ-producing T cells from LACK DNA-vaccinated mice following infection (Table II).

Table II. Depletion of CD8+ T cells in vivo decreases IL-12Rβ1 expression, IL-12 production, and the frequency of CD4+ IFN-γ-producing T cells from LACK DNA-vaccinated mice following infection at 12 wk

<table>
<thead>
<tr>
<th>12-wk Postinfection</th>
<th>Frequency of IFN-γ-Producing T Cells (%)</th>
<th>% of CD4 T Cells Expressing IL-12Rβ1</th>
<th>IL-12 Production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LP</td>
<td>SAC</td>
</tr>
<tr>
<td>LACK DNA</td>
<td>3.98 ± 0.35</td>
<td>54.14</td>
<td>1060 ± 73</td>
</tr>
<tr>
<td>LACK DNA + anti-CD8</td>
<td>0.21 ± 0.04</td>
<td>37.92</td>
<td>&lt;125</td>
</tr>
<tr>
<td>LACK DNA + anti-IFN-γ</td>
<td>0.25 ± 0.04</td>
<td>24.31</td>
<td>&lt;125</td>
</tr>
<tr>
<td>LACK DNA + anti-IL-12</td>
<td>0.04 ± 0.026</td>
<td>19.03</td>
<td>&lt;125</td>
</tr>
<tr>
<td>LACK protein + control DNA</td>
<td>0.20 ± 0.07</td>
<td>25.58</td>
<td>&lt;125</td>
</tr>
</tbody>
</table>

See legend to Table I.
mice is based on the following: 1) CD8 depletion at the time of vaccination limited the generation of CD4<sup>+</sup> IFN-γ-producing T cells even before infection (Fig. 4). These data serve to underscore the potential importance of CD8<sup>+</sup> T cells absent of any effector function or other complicating variables related to the infection. 2) CD8 depletion at the time of vaccination or infection inhibited the frequency of CD4<sup>+</sup> IFN-γ-producing T cells when assessed at both 28 and 42 days postinfection (Figs. 1 and 4). It should be noted that at 28 days postinfection, parasite loads in the lymph node of LACK DNA-vaccinated mice are similar to those of LACK DNA-vaccinated mice in whom CD8<sup>+</sup> T cells are depleted (data not shown). These data suggest that differences in parasite burden between LACK DNA-vaccinated mice and mice depleted of CD8<sup>+</sup> T cells at 28 days postinfection cannot fully account for the changes in the frequency of CD4<sup>+</sup> IFN-γ-producing T cells. 3) CD8<sup>+</sup> T cells from LACK DNA-vaccinated mice increased the frequency of CD4<sup>+</sup> IFN-γ-producing T cells in vitro using LACK-specific or OVA TCR-Tg mice (Fig. 5). These latter data provide the most direct evidence, in a well-controlled in vitro model, that CD8<sup>+</sup> T cells from infected mice that had been vaccinated with LACK DNA can influence the frequency of CD4<sup>+</sup> IFN-γ production.

Although the previous discussion has focused on the role of CD8<sup>+</sup> T cells in regulating the frequency of CD4<sup>+</sup> IFN-γ-producing T cells, an alternative mechanism to explain some of the in vivo data would involve a role for CD8<sup>+</sup>/CD11c<sup>+</sup> dendritic cells. In this regard, the priming of immune responses by DNA vaccination occurs in large measure by direct transfection of dendritic cells. Moreover, because the CD8<sup>+</sup>/CD11c<sup>+</sup> subset of lymphoid dendritic cells is enriched in their IL-12-producing capacity, it was possible that treatment of LACK DNA-vaccinated mice with anti-CD8 at the time of vaccination depleted both CD8<sup>+</sup> T cells and CD8<sup>+</sup>/CD11c<sup>+</sup> lymphoid dendritic cells, resulting in a decrease in the frequency of CD4<sup>+</sup> IFN-γ-producing T cells. In a series of experiments to address this issue, we found that only CD8<sup>+</sup> T cells and not CD8<sup>+</sup>/CD11c<sup>+</sup> dendritic cells were depleted from mice treated with anti-CD8. Moreover, anti-CD8 treatment did not impair the IL-12-producing capacity of CD8<sup>+</sup>/CD11c<sup>+</sup> cells as assessed in vitro or in vivo (data not shown). Taken together, these data would appear to exclude this as a potential mechanism for our findings and provide further support for an immunoregulatory role of CD8<sup>+</sup> T cells.

Mechanisms to account for the ability of CD8<sup>+</sup> T cells to regulate the frequency of CD4<sup>+</sup> IFN-γ-producing T cells

Because regulation of IL-12 expression and subsequent IL-12 responsiveness on CD4<sup>+</sup> T cells has been shown to be a critical factor in protective immunity in this model, it is likely that CD8<sup>+</sup> T cells are exerting their effects through this pathway. In mice, IFN-γ regulates both IL-12R expression (24, 32) and IL-12 transcription (22, 23), while IL-12 up-regulates its own receptor (17) as well as enhancing production of IFN-γ. The data shown in Tables I and II substantiate a critical role for endogenous IL-12 and IFN-γ in maintaining IL-12R expression, IL-12 production, and the frequency of CD4<sup>+</sup> IFN-γ-producing T cells. Furthermore, the data showing that CD8 depletion (which removes a source of IFN-γ) decreased IL-12 production and IL-12R expression provide strong correlative evidence that CD8<sup>+</sup> T cells, via the production of IFN-γ, help maintain the frequency of CD4<sup>+</sup> IFN-γ-producing T cells by these mechanisms.

An additional soluble mediator that could play an important role in the selective generation of these CD8<sup>+</sup> T cells induced by LACK DNA vaccinations is IFN-α. In this regard, IFN-α can be induced by DNA vaccination (33, 34). IFN-α has also been implicated in the generation of CD8<sup>+</sup> memory T cells (35) as well as having a specific role in regulating the early events of Th1 generation (specifically regulating IL-12 production) following L. major infection (36). In ongoing work, we found that depletion of IFN-α at the time of infection abrogates protection in LACK-vaccinated mice; this is associated with a decreased frequency of both CD4<sup>+</sup> and CD8<sup>+</sup> IFN-γ-producing T cells.

CD4<sup>+</sup> T cells are also required for protective immunity following vaccination

While this study has had a focus on the role of CD8<sup>+</sup> T cells, the immunity conferred by LACK DNA vaccination must also be viewed in the context of the critical role that CD4<sup>+</sup> T cells have in primary immunity to L. major. In other models of L. major infection, CD4<sup>+</sup> T cell production of IFN-γ has been shown to be both necessary and sufficient for primary immunity (4, 5, 18). As shown in this work and consistent with our previous studies, mice vaccinated with LACK protein plus IL-12 protein are able to control parasite growth when infected within 2 wk postvaccination. In addition, mice vaccinated with leishmanial protein and IL-12 DNA have sustained immunity that can protect mice when infected 12 wk postvaccination. Using either of these vaccine approaches, IFN-γ is detected from CD4<sup>+</sup> but not CD8<sup>+</sup> T cells. Thus, in this study, while we have not directly shown that CD4<sup>+</sup> T cell depletion in vivo abrogates protection in LACK DNA-vaccinated mice, we provide in vitro evidence that addition of anti-CD4 Ab completely inhibits production of IFN-γ from total lymph node cells, which includes CD8<sup>+</sup> T cells. Thus, in this DNA vaccine model, there is a bidirectional interaction in which CD4<sup>+</sup> T cell activation is required for CD8<sup>+</sup> production of IFN-γ, which in turn helps to maintain the frequency of CD4<sup>+</sup> IFN-γ-producing T cells.

To conclude, these experiments clearly show that CD8<sup>+</sup> T cells have a critical role in mediating long-term protection in vivo induced by LACK DNA vaccination. Thus, for diseases requiring cellular immunity in which Th1 responses may be important (e.g., L. major, M. tuberculosis, and malaria), these studies provide a potentially novel mechanism by which DNA vaccination not only generates CD8<sup>+</sup> effector cells, but also can have an additional immunoregulatory role in sustaining Th1 responses.
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References