Perforin and Gamma Interferon Are Critical CD8\(^+\) T-Cell-Mediated
Responses in Vaccine-Induced Immunity against
Leishmania amazonensis Infection

María Colmenares, Peter E. Kima,† Erika Samoff, Lynn Soong,‡ and Diane McMahon-Pratt*
Department of Epidemiology and Public Health, Yale University School of Medicine,
New Haven, Connecticut 06520-8034

Received 24 September 2002/Returned for modification 7 January 2003/Accepted 21 March 2003

Previous studies have demonstrated that protection against New World leishmaniasis caused by Leishmania amazonensis can be elicited by immunization with the developmentally regulated Leishmania amastigote antigen, P-8. In this study, several independent experimental approaches were employed to investigate the protective immunological mechanisms involved. T-cell subset depletion experiments clearly indicate that elicitation of CD8\(^+\) (as well as CD4\(^+\)) effector responses is required for protection. Further, mice lacking \(\beta_2\)-microglobulin (and hence deficient in major histocompatibility complex class I antigen presentation) were not able to control a challenge infection after vaccination, indicating an essential protective role for CD8\(^+\) T effector responses. Analysis of the events ongoing at the cutaneous site of infection indicated a changing cellular dynamic involved in protection. Early postinfection in protectively vaccinated mice, a predominance of CD8\(^+\) T cells, secreting gamma interferon (IFN-\(\gamma\)) and expressing perforin, was observed at the site of infection; subsequently, activated CD4\(^+\) T cells producing IFN-\(\gamma\) were primarily found. As protection correlated with the ratio of total IFN-\(\gamma\)-producing cells (CD4\(^+\) and CD8\(^+\) T cells) to macrophages found at the site of infection, a role for IFN-\(\gamma\) was evident; in addition, vaccination of IFN-\(\gamma\)-deficient mice failed to provide protection. To further assess the effector mechanisms that mediate protection, mice deficient in perforin synthesis were examined. Perforin-deficient mice vaccinated with the P-8 antigen were unable to control infection. Thus, the elicitation of CD8\(^+\) T cell effector mechanisms (perforin, IFN-\(\gamma\)) are clearly required in the protective immune response against L. amazonensis infection in vaccinated mice.

The feasibility of vaccination against cutaneous leishmaniasis is well established from practices in the Middle East (19) as well as more recent observations in humans and animal models of leishmaniasis (24, 49, 55). Cutaneous leishmaniasis can result from infection by several species of the genus Leishmania. In the Americas, members of the L. mexicana complex (including L. amazonensis and L. piñaii) and L. braziliensis complex are most often associated with cutaneous leishmaniasis. Each one of these species establishes a somewhat unique interaction with its host, leading at times to self-limiting disease or progressive-diffuse or mucocutaneous infections. In recent years, considerable diversity in immunological responses as well as in virulence factors have been determined among the species causing cutaneous leishmaniasis (26, 65). The pathogenesis caused by L. mexicana complex parasites follows a different pattern from that described for L. major. Notably, in contrast to L. major, C57BL/6, CBA, and C3H mice are susceptible to infection with L. amazonensis. Unlike L. major-infected mice, in which a polarized differentiation of Th1/Th2 CD4\(^+\) T cells occurs (48) in resistant and susceptible animals, susceptible L. amazonensis-infected mice do not consistently show an enhanced Th2 response (1, 56); further, L. amazonensis-infected C3H mice are unresponsive to treatment with interleukin-12 (IL-12) (27). In addition, T-cell responses to LACK antigen do not appear to play a critical role in determining susceptibility to L. mexicana (64). Moreover, in contrast to L. major, L. amazonensis infection in mice genetically deficient in T cells (more specifically, CD4\(^+\) T cells) or B cells fail to develop disease pathology (30, 54).

So a challenge in the study of the immunity to these diverse organisms is defining those immune responses to which most Leishmania species are susceptible. The contribution of CD4\(^+\) and CD8\(^+\) T cells to protection against infection with L. major has been assessed in both naive and vaccinated mice. The course of infection in nonvaccinated, resistant mice-depleted CD4\(^+\) T cells and challenged with L. major was profound, as these mice were rendered incapable of resolving infection (63). Interestingly, depletion of CD4\(^+\) T cells from susceptible BALB/c mice resulted in the control of the infection, which was reversed with depletion of CD8\(^+\) T cells (22, 43). However, studies that have assessed the role of CD8\(^+\) T cells in the control of infection in naive (nonvaccinated) mice have produced mixed results (7, 43, 63). Antibody depletion of CD8\(^+\) T cells from either naive CBA (resistant) or BALB/c (susceptible) mice subsequently challenged with L. major resulted in exacerbation of infection (63). However, in resistant C57BL/6 mice deficient in \(\beta_2\)-microglobulin expression (and hence functionally deficient in major histocompatibility complex [MHC] class I presentation), the course of a primary infection with L.
major was found to be unaltered with a high-dose challenge (45, 67) but was exacerbated in the case of a low-dose challenge infection (7).

The situation is less ambiguous when the roles of CD4^+ and CD8^+ T cells are investigated subsequent to vaccination. Depletion of CD8^+ T cells or CD4^+ T cells, from either CBA mice or BALB/c mice protectively immunized against L. major challenge, prevents the induction of a protective response (17). More recently, it has been shown that sustained immunity to L. major can be abolished by treatment with anti-CD8 antibody in mice vaccinated with LACK DNA. Here, CD8^+ T cells appear to have an immunoregulatory role in maintaining the frequency of CD4^+ T cells producing gamma interferon (IFN-\(\gamma\)) (21). Further support of a role for CD8^+ T cells in immunity has been shown in comparative studies of patients with New World human cutaneous leishmaniasis caused by L. braziliensis, before and after cure (16), as well as in human vaccine studies (39).

Our previous studies have described leishmanial antigens (GP46/M2, P-8) that elicit significant protection against L. amazonensis (L. mexicana complex parasite) in several mouse strains (9, 55). In addition, using either IFN-\(\gamma\) production and/or T-cell cytolysis to assess CD8^+ T-cell activation, it has been shown that GP46/M-2-specific CD8^+ T-cell lines recognize macrophages infected with L. amazonensis (31). Therefore, it was of interest to determine the effect of both CD8^+ and CD4^+ T cells in protection against L. amazonensis challenge in vaccinated mice. In the present study, mice were immunized with the membrane-associated amastigote stage-specific antigen, P-8 (13, 55). T-cell subset depletion experiments and analyses of vaccinated genetically altered strains of mice consistently indicate an essential role at the site of infection for both CD8^+ and CD4^+ T cells in protection against cutaneous leishmaniasis caused by L. amazonensis. In addition to IFN-\(\gamma\), the protective CD8^+ T cells produce perforin at the site of infection, suggesting that both of these molecules are effectors that may be operative in vaccinated mice infected with L. amazonensis parasites.

**MATERIALS AND METHODS**

**Animals.** Female C57BL/6 mice were obtained from the National Cancer Institute (Frederick, Md.). Female C57BL/6-B2m^\(-/-\) (H-2^d\); Microglibelin; \(\beta_2\)-M \(-/-\) ), C57BL/6-Ilg^\(-/-\) (IFN-\(\gamma\)-r\(-/-\)), and C57BL/6-Pip^\(-/-\) (perforin; \(\beta_2\)-M \(-/-\)) mice were from the Jackson Laboratory (Bar Harbor, Maine). All animals were housed in the Yale University School of Medicine Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility. Sentinel mice were checked periodically for presence of viruses in the colony. All mice used were between 7 and 11 weeks of age.

**Parasites.** L. amazonensis MHOM/BR/77/LTB0016 promastigotes were cultured in Schneider's Drosophila medium as described previously (9). Parasites were passaged frequently through mice to maintain infectivity.

**Antibodies.** The hybridoma cell lines producing the following monoclonal antibodies were obtained from the American Type Culture Collection: GK1.5 (anti-LT4, CD4); 53-5-43 (anti-Lyt2, CD8); 15-5-5s (anti-H-2Dk). For immunohistochemical analyses, anti-mouse CD4 (clone H129.19) and anti-mouse CD8a (clone 53-6.7) were obtained from Life Technologies (GIBCO BRL, Rockville, Md.); biotin-conjugated anti-rat immunoglobulin G (IgG; mouse serum absorbed) was obtained from Kirkegaard & Perry Laboratories (Gaithersburg, Md.). The following antibodies for flow cytometry analysis (fluorescence-activated cell sorter [FACS]) were from Pharmingen (San Diego, Calif.): anti-mouse CD16/CD32 (Fc Block); anti-mouse CD8a (Ly-2) (clone 53-6.7; Cy-chrome conjugated); anti-mouse CD4 (L3T4) (clone RM4-5; fluorescein isothiocyanate [FITC] conjugated); anti-mouse Fas ligand (CD95 ligand; clone MFL3; R-phycocerythrin [PE] conjugated); anti-mouse IFN-\(\gamma\) (PE conjugated); anti-mouse IL-10 (PE conjugated); anti-Mac-1 (FITC conjugated; clone M1/ 70.15); anti-MHCII (PE conjugated; clone 2G9). Other antibodies used were anti-mouse peroxidase (clone P1.8; Kamiya Biomedical, Seattle, Wash.) and polyclonal goat anti-rat Ig (FITC conjugated, Kirkegaard & Perry Laboratories).

**Antigen preparation, vaccination, and T-cell subset depletion.** Affinity purification of the membrane amastigote complex antigen P-8 (55) has been described previously. When used for immunization, 5 \(\mu\)g of antigen was mixed with 50 or 100 \(\mu\)g of Propionibacterium acnes (formerly Corynebacterium parvum). Animals (six mice per experiment) were vaccinated three times intraperitoneally using either weekly (depletion experiments) or biweekly schedule. Mice were challenged 3 weeks (depletion experiments) or 6 weeks after the final immunization. In the right hind foot, with 10^5 stationary-stage L. amazonensis promastigotes. Control groups consisted of animals that received P. acnes (adjuvant control) or phosphate-buffered saline (PBS) alone (infection control). In T-cell subset depletion experiments, vaccinated animals received 500 \(\mu\)g of affinity-purified antibody in PBS (GK1.5 for depletion of CD4^+ T cells and 53.4.65 for depletion of CD8^+ T cells) on days 6, 1, 7 of infection. This schedule resulted in greater than 90% depletion of CD4^+ T cells and 60 to 75% depletion of CD8^+ T cells, as assessed by FACS analysis. Anti-H-2^D^d (15-5-5S) was used as a control in antibody depletion experiments employing C57BL/6 mice.

**Immunohistochemistry.** The infected foot tissue was excised and fixed in PBS containing 2% paraformaldehyde and 5% sucrose overnight. The tissue then was placed in 20% sucrose in PBS to 20% sucrose in OCT compound (Tissue Tek, Elkhart, Ind.). Frozen tissue sections (5 \(\mu\)m thick) were prepared as described earlier (4), blocked with 5% goat serum, and then incubated with anti-CD4 or anti-CD8 antibody, followed by biotin-labeled goat anti-rat Ig. Localization was determined using avidin-horseradish peroxidase provided in a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, Calif.) as suggested by the manufacturer. Coverslips were mounted over Permount (Fisher Scientific), and slides were then examined microscopically (model Orthoplan 2 fluorescence microscope; Leitz, Wetzlar, Germany). At least 250 nucleated cells were examined, and the number of positively stained cells was determined. Coverslips incubated with second antibody alone served as background reactivity controls. Data are expressed as the percentage of positively stained nucleated cells per tissue section. Scores from two experiments of two mice per experimental group for each sampling point were evaluated.

**Evaluation of infection.** Mice were infected with late-log, stationary-grow phase promastigotes of L. amazonensis. For certain experiments (as indicated), mice were infected in the cutaneous tissue on the top of the right rear hind foot. The course of infection was monitored by measuring the increase in footpad thickness, compared with that of the uninfected foot (ratio of infected foot/noninfected foot), with a dial gauge caliper. At the times designated, mice were sacrificed to determine the parasite burden in the infected tissue by using limiting dilution analyses and procedures reported previously (55, 62). Alternately, the ear pinnae of mice were infected and parasite burdens were determined by using limiting dilution analysis. It should be noted that the susceptibility of C57BL/6 mice to L. amazonensis infection was identical whether ear pinnae or the tops of the feet were used.

**Estimation of the frequency of P-8-responding cells by limiting dilution assay.** From popliteal and superficial inguinal lymph nodes, lymph node (LN) cells were obtained from two mice per group which had undergone various treatments: P-8 plus P. acnes immunized but not infected; P-8 plus P. acnes immunized and infected; or P. acnes injected and infected. These cells were dispensed in limiting numbers into 96-well round-bottom plates (40) which contained 2 \(\times\) 10^5 irradiated normal spleen cells and 4 \(\mu\)g of P-8/ML. Twelve replicate wells were set up for each LN cell concentration (ranging from 5 \(\times\) 10^3/well to 3 \(\times\) 10^5/well). Wells containing irradiated spleen cells and antigen alone were used as control. After 72 h of culture, supernatant fluid was obtained from each well, and an aliquot was used for the determination of IFN-\(\gamma\). Limiting dilution cultures were scored positive when the values exceeded the mean of the controls by 2 standard deviations. The minimal sensitivity of the enzyme-linked immunosorbent assay (ELISA) employed for the IFN-\(\gamma\)-assay was 0.5 U/mL. LN cells from P-8-immunized and infected mice were cultured in the absence of antigen to confirm that the IFN-\(\gamma\)-produced was indeed antigen specific. Minimal estimates of the frequency of P-8-responding cells were obtained by methods previously described (40).

**ELISA cytokine and antibody assays.** The level of IFN-\(\gamma\) or IL-4 in cell supernatant fluid was determined by a two-site ELISA, as previously described (55). The sensitivities of the IL-4 and IFN-\(\gamma\) assays were 2 and 0.5 U/mL, respectively. The relative antibody titers of P-8-vaccinated wild-type and perforin-/- mice were determined by ELISA. Briefly, sonicated amastigote membrane preparations were used as antigen; pooled mouse sera, collected 6 weeks after the final immunization, and a peroxidase-conjugated goat anti-

Downloaded from http://iai.asm.org/ on October 14, 2017 by guest
mouse IgG polyclonal antibody were employed. Titers were determined in duplicate at 50% maximal binding.

**Isolation of cells from Leishmania-infected ears and FACS analyses.** The cells from infected ear tissue were isolated as previously described (5). After 3 h of incubation (37°C) of the separated ear pinnae in RPMI 1640 medium supplemented with 10% fetal calf serum and 1 mg of collagenase (Boehringer/ml), single-cell suspensions of cells recovered were either not further treated (unstimulated; ex vivo) or stimulated. Cells were stimulated for 5 h with 5 ng of phorbol 12-myristate 13-acetate (Sigma)/ml together with 500 ng of ionomycin (Sigma)/ml for intracellular cytokine determinations or with L. amazonensis lysate antigen for intracellular perforin determinations. The last 3 h of culture were performed in the presence of 10 μg of brefeldin A (Golgi Plug; BD Pharmingen)/ml. Subsequently, cells were stained for CD4, CD8, Mac-1, MHC class II, and/or Fas ligand (using antibodies as indicated above), fixed, permeabilized, and stained for intracellular cytokine. The lymphocytes were identified by their small size, along with CD4 or CD8 expression. Monocytes and activated macrophage cells were identified as Mac-1 positive MHC class II low/negative by their small size, along with CD4 or CD8 expression. Monocytes and activated macrophage cells were identified as Mac-1 positive MHC class II low/negative and MAC-1 positive MHC class II positive, respectively, as previously described by others (6). All antibodies were used at 0.2 μg/106 cells. Intracellular perforin staining was performed as previously described (52). Cell surface antigen expression and intracellular cytokine staining data were acquired using a FACScan flow cytometer; data were analyzed employing a FlowJo software program.

**RESULTS**

**Effect of T-cell subset depletion on vaccination with the amastigote surface antigen P-8.** Immunization with the proteoglycolipid complex antigen P-8 has been previously demonstrated to protect C57BL/6, BALB/c, and CBA/J mice from challenge with L. amazonensis promastigotes (55); further, the P-8 antigen has been demonstrated to elicit CD4/CD8 Th1/Th1-like responses in leishmaniasis patients (15). Therefore, to further dissect the protective mechanism elicited by the P-8 antigen, susceptible C57BL/6 mice were employed to take advantage of the existence of genetically altered mice on this immunological background. First, the relative contributions of CD8+ and CD4+ T cells to vaccine-elicited protection were assessed in subset depletion experiments. P-8-protectively immunized C57BL/6 mice were rendered incapable of controlling a challenge infection when either CD4+ and/or CD8+ T cells were depleted. As shown in Fig. 1, while P-8-immunized mice had parasite burdens that were approximately 100-fold lower than their respective infection controls at the 8-week time point, CD4+ or CD8+ T-cell-depleted mice had parasite burdens comparable to adjuvant (P. acnes)-immunized control mice. Depletion of both T-cell subsets was not more effective in reversing protection than the depletion of either cell subpopulation, thus suggesting a potential collaborative mechanism between these two T-cell subsets. The specificity of the depletion was confirmed, since administration of an unrelated antibody (anti-H-2Dk) did not reverse protection conferred by P-8 immunization.

**CD8+ T cells are required for protective immunity following vaccination with P-8.** The fact that elicitation of a CD4+ T-cell response is required for protection against leishmaniasis is an expected observation. In contrast, an essential protective role of CD8+ T cells in cutaneous leishmaniasis is still under debate. In order to confirm the observations obtained with T-cell subset antibody-depleted mice, genetically altered mice that lacked the β2-microglobulin (β2-M−/−) gene (47) were assessed for their capacity to be protectively vaccinated with P-8 against an L. amazonensis challenge. The results in Fig. 2 show the lesion development at different times postinfection for wild-type C57BL/6 mice (Fig. 2A) and β2-M−/− mice (Fig. 2B) and the parasite burden obtained after 10 weeks infection in wild-type (Fig. 2C) and β2-M−/− (Fig. 2D) mice. They demonstrate that β2-M−/− mice vaccinated with P-8 cannot control an L. amazonensis challenge. These experimental results complement observations in CD8+ T-cell antibody-depleted P-8-vaccinated mice and indicate an essential role for CD8+ T cells in protection against L. amazonensis infection in vaccinated mice. It was observed that the parasite burdens of the nonvaccinated β2-M−/− mice were lower at 10 weeks postinfection than that found for C56BL/6 wild-type mice. Previous experiments (reference 54 and data not shown) had indicated that lesion development in naive C57BL/6 mice and β2-M−/− mice is identical for the first 4 to 5 weeks postinfection; subsequently, β2-M−/− mice demonstrate a smaller lesion size. It is well established that β2-M−/− mice have reduced levels of antibody as a consequence of higher antibody catabolism (10, 35). Further, antibody has been demonstrated to be critical to L. amazonensis survival in the murine model (11, 30). At 10 weeks postinfection, the levels of IgG1, IgG2b, and IgG3 were elevated two- to fourfold in wild-type (P-8-vaccinated and nonvaccinated) mice in comparison to β2-M−/− mice (data not shown). Consequently, it is likely that later in infection the lower levels of antibody in the β2-M−/− mice contribute to the slower lesion development and lower parasite burdens, although a lack of protection is still evident. Overall, these results are consistent with previous studies where a protective role of CD8+ T cells has been demonstrated in vaccinated mice (17, 20, 21, 38, 42) infected with L. major.

**Enumeration of antigen-reactive T cells in LN of P-8-immunized mice.** Since immunization with P-8 failed to protect β2-
M\(^{-/-}\) mice from a subsequent \(L.\) \textit{amazonensis} challenge, it was of interest to determine whether this defect was due to an unsuccessfully elicited T-cell response. This possibility was initially assessed by using a limiting dilution assay of LN cells draining the site of infection. The frequencies of P-8-reactive T cells prior to and 12 days after infection were evaluated by determining the release of IFN-\(\gamma\) when LN cells were incubated together with P8 antigen and antigen-presenting cells. In both \(\beta_2\)-M\(^{-/-}\) and wild-type mice, there was an expansion of P-8-reactive cells producing IFN-\(\gamma\) in response to vaccination and/or infection. The frequencies of P-8-reactive cells in three experimental groups, P8 plus \(P.\) \textit{acnes}-immunized mice prior to infection (control), mice injected with \(P.\) \textit{acnes} alone and infected, and P-8 plus \(P.\) \textit{acnes}-immunized mice that had been infected, were 1/2,336,956, 1/230,414, and 1/80,000, respectively. There was a comparable expansion of P-8-reactive cells in \(\beta_2\)-microglobulin-deficient mice which were subjected to identical treatments: 1/800,000, 1/120,000, and 1/40,000, respectively. The production of IL-4 by these cells was also determined; however, no IL-4 production was observed. Further, LN cells obtained from P-8 plus \(P.\) \textit{acnes}-immunized \(\beta_2\)-M\(^{-/-}\) mice produced higher levels of IFN-\(\gamma\) in response to P-8 in bulk culture experiments, compared to similarly treated wild-type mice (data not shown). These results suggest that priming
of P-8-specific, IFN-γ-producing T cells in the nonprotectively vaccinated MHC class I-deficient mice was comparable to that in wild-type mice; consequently, a lack of priming could not account for the lack of protection found against *L. amazonensis* infection.

**Examination of T cells present at the cutaneous lesion site in P-8-vaccinated mice.** Recent data suggest that immune responses in the draining LN may not always be reflective of the host immune responses at specific tissue sites (36). Consequently, to further assess the participation of either T-cell subset in protection, the representation of CD8+ and CD4+ T cells at the cutaneous site of infection of protectively immunized mice versus unprotected animals was determined using an intradermal challenge in the ear. This site was employed as the ear allows the facile isolation of infiltrating cell populations (5). Further, infection of vaccinated mice in the ear, as found for the foot cutaneous site, resulted in protection against infection (Fig. 3); at 8 weeks postinfection, P-8-vaccinated mice had a 100-fold reduction in parasite burdens in comparison to control mice.

The retention and activation of T cells at the site of infection was assessed using FACS analyses of P-8-vaccinated and control (*P. acnes*) mice (three mice/group). Analysis of ex vivo-isolated lesion cells revealed a correlation between the level of macrophages present at the site of infection and lesion development (Table 1). Macrophages are required for sustaining infection (59); thus, fewer potential target host cells appear to be available for the intracellular amastigotes in the protectively vaccinated mice. Overall, similar levels of lymphocytic cells were found in both vaccinated and control groups of mice at the three time points analyzed, with higher proportions observed early in infection (2 weeks). However, the level of the IFN-γ-producing cells varied between the protectively immunized and nonprotected groups of mice and suggested that IFN-γ was important for protection (Table 1). The total number of IFN-γ-producing cells in the infection site of P-8-immunized mice at 2 weeks postinfection was nearly twice that found for control mice receiving adjuvant alone. Further, the ratio of IFN-γ-producing lymphocytes to macrophages found at the site of infection was consistently higher (1.5- to 2.4-fold higher) (Table 1) in the protectively immunized mice than in control mice.

In addition, the relative T-cell subset contribution (CD4+ versus CD8+) in the production of IFN-γ was different between these two groups of mice. Initially in P-8-protectively vaccinated mice, mainly CD8+ T cells were responsible for cytokine production, while in nonprotected mice a lower proportion of CD8+ T cells were found to be producing IFN-γ. Over time, the proportion of CD4+ T cells producing IFN-γ increased in the P-8-vaccinated mice. In control mice (*P. acnes*) at all times postinfection (2 to 5 weeks), the number of IFN-γ-producing CD4+ T cells was 2.7 to 4.4 times that of the IFN-γ-producing CD8+ T cells. In contrast, in P-8-vaccinated mice the number of IFN-γ+ CD4+ T cells was 0.4 times that of CD8+ T cells at 2 weeks postinfection. Subsequently, at 3 weeks postinfection the level of IFN-γ-producing CD8+ T cells remained higher in the P-8-vaccinated mice than in the control mice. An increase in the proportion of IFN-γ-producing CD4+ T cells was also observed. At 5 weeks postinfection, the IFN-γ+ CD4+/IFN-γ+ CD8+ ratios were comparable in both groups of mice. These results suggest a changing cellular dy-

![Image](https://iai.asm.org/dv/a/91/8/pdf/10.1128%2Fiai.00110-07)</td>

<table>
<thead>
<tr>
<th>WK postinfection</th>
<th>% Macrophages P. acnes</th>
<th>% Macrophages P-8</th>
<th>% Lymphocytes P. acnes</th>
<th>% Lymphocytes P-8</th>
<th>% IFN-γ+ lymphocytes P. acnes</th>
<th>% IFN-γ+ lymphocytes P-8</th>
<th>% CD4, IFN-γ P. acnes</th>
<th>% CD4, IFN-γ P-8</th>
<th>% CD8, IFN-γ P. acnes</th>
<th>% CD8, IFN-γ P-8</th>
<th>IFN-γ+ CD4+/IFN-γ+ CD8+ ratio P. acnes</th>
<th>IFN-γ+ CD4+/IFN-γ+ CD8+ ratio P-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5.3 (±0.1)</td>
<td>4.4 (±0.1)</td>
<td>14.3 (±0.3)</td>
<td>10.2 (±0.2)</td>
<td>31 (±0.6)</td>
<td>17 (±0.34)</td>
<td>5.1 (±0.1)</td>
<td>4.8 (±0.3)</td>
<td>5.1 (±0.1)</td>
<td>1.6 (±0.1)</td>
<td>3.4 (±0.2)</td>
<td>4.4 (±0.2)</td>
</tr>
<tr>
<td>3</td>
<td>8.3 (±0.2)</td>
<td>6.1 (±0.1)</td>
<td>6.8 (±0.14)</td>
<td>7.8 (±0.16)</td>
<td>17.1 (±0.34)</td>
<td>19.8 (±0.4)</td>
<td>7.3 (±0.2)</td>
<td>5.1 (±0.1)</td>
<td>7.1 (±0.1)</td>
<td>1.1 (±0.1)</td>
<td>4.0 (±0.2)</td>
<td>4.4 (±0.2)</td>
</tr>
<tr>
<td>5</td>
<td>32.1 (±0.6)</td>
<td>9.2 (±0.2)</td>
<td>7.6 (±0.15)</td>
<td>6.6 (±0.13)</td>
<td>14.3 (±0.29)</td>
<td>11.2 (±0.22)</td>
<td>4.0 (±0.2)</td>
<td>4.0 (±0.2)</td>
<td>1.5 (±0.1)</td>
<td>7.4 (±0.2)</td>
<td>2.7 (±0.2)</td>
<td>2.2 (±0.2)</td>
</tr>
</tbody>
</table>

a P-8-immunized and adjuvant control mice (*P. acnes*) were infected with 10⁵ *L. amazonensis* parasites in both ears. Cells from the infection site were obtained at the indicated time postinfection (as indicated in Materials and Methods) and then processed for FACS analysis. The percentage of macrophages was calculated from total cells by gating on MAC-1⁺/MHC-II low or negative cells. Lymphocytes were defined as medium forward scatter and low side scatter and the expression of CD4 and CD8. For the quantification of IFN-γ-producing cells, isolated cells were initially reactivated in vitro with PMA plus ionomycin for 5 h, in the presence of brefeldin A, and then processed for FACS analysis as indicated in Materials and Methods. All results represent the mean values ± standard errors obtained from groups of three mice.
namic involved in the control of infection, with CD8$^+$ T cells having an important role in the early containment of infection. CD4$^+$ T cells, although involved in early stages of control, may be more critical in the latter stages of the response to infection. The increased level of IFN-$\gamma$-producing CD8$^+$ T cells at the site of infection in protectively vaccinated mice suggests that this may be one of the effector mechanisms.

IFN-$\gamma$ is required for protective immunity following vaccination with P-8. As T-cell production of IFN-$\gamma$ induced by vaccination was high at early times postinfection by CD8$^+$ T cells, the role of this cytokine was also further evaluated using C57BL/6 IFN-$\gamma^{-/-}$ mice. Mice (wild type [C57BL/6] and C57BL/6 IFN-$\gamma^{-/-}$) were immunized with P-8 and 6 weeks after the last immunization were infected with L. amazonensis. A lack in the capacity to synthesize and secrete IFN-$\gamma$ abolishes the protection observed in wild-type C57BL/6 mice, as determined by parasite burden analyses at 10 weeks postinfection (Fig. 4A) and lesion development (Fig. 4B). These experiments, although not specifically demonstrating a role for CD8$^+$ T cells producing IFN-$\gamma$, show that IFN-$\gamma$ is critical for the resolution of disease caused by L. amazonensis infection in the vaccinated mice.

Interestingly, naïve C57BL/6 IFN-$\gamma^{-/-}$ mice do not show an increased susceptibility to L. amazonensis infection; this is in contrast to what has been observed for two other Leishmania species, L. major and L. donovani (44, 60, 68). However, it should be noted that in these infections, too, absence of IFN-$\gamma$ results in variable outcomes. In the case of L. donovani, the disease exacerbation is minimal (four- to fivefold increase in parasite burdens) and transitory (the mice eventually resolve their infections, as do wild-type mice); a more dramatic effect is seen for L. major. Hence, the differences in the level of effect of IFN-$\gamma$ deficiency on the course of infection may reflect differences in pathogenesis of these species of Leishmania (12).

Perforin is required for protective immunity following vaccination with P-8. As shown above, CD8$^+$ T cells played an important role in the protection conferred by P-8. However, as the total number of T cells producing IFN-$\gamma$ was similar 3 to 5 weeks postinfection in both the protectively and the nonprotectively vaccinated groups of mice (Table 1), it was possible that additional mechanisms might be involved in the control L. amazonensis infection. Consequently, the possibility of other mechanisms contributing to control of infection was initially explored by examining the phenotype of the CD8$^+$ T cells retained at the site of infection. The various groups of mice (adjuvant control and P-8 immunized; three mice/group) were infected intradermally, and the cells at the site of infection were examined by three-color flow cytometry for expression of CD8$\alpha$, intracellular perforin, and Fas ligand (Fas-L). Figure 5 shows representative results of perforin and Fas-L expression in CD8$\alpha$-gated cells recovered; CD8$^+$ T cells expressing intracellular perforin were detected in cells recovered from the infection site of P-8-immunized mice and not from the adjuvant control and infection control groups of mice. Such analyses showed that perforin-expressing cells were predominantly (90 to 100%), if not exclusively, CD8$^+$ from 2 to 9 weeks postinfection. While the level of expression of Fas-L was slightly increased in the protectively vaccinated mice, the expression of perforin was more evident. These data suggested the potential participation of a CD8$^+$ T-cell cytolytic mechanism at the infection site of P-8-protected mice.

As CD8$^+$ T cells with a cytolytic phenotype were induced by P-8 vaccination, the role of the pore-forming protein perforin was evaluated. C57BL/6 perforin$^{-/-}$ mice were immunized with P-8 and 6 weeks after the last immunization were infected with $10^5$ stationary-phase L. amazonensis promastigotes. The deficiency in the synthesis and secretion of perforin abolishes the protection observed in wild-type C57BL/6 mice, as determined by measuring lesion development (Fig. 6A) or parasite burdens (Fig. 6B); these results are representative of three separate experiments. In this experiment, P-8 plus P. acnes-vaccinated wild-type C57BL/6 mice (Fig. 6C and D) showed a 27-fold reduction in parasite burdens over nonvaccinated C57BL/6 mice at 11 weeks postinfection; comparable vaccination of perforin$^{-/-}$ mice did not alter the course of infection. These experiments show that for the vaccinated mice, perforin contributes to the resolution of disease caused by L. amazonensis. FACS analyses of cells isolated from the lesion or infection site of C57BL/6 perforin$^{-/-}$ mice at 3 weeks postinfection revealed a similar pattern of cell infiltration and activation in both P-8-vaccinated and control infected mice (P. acnes) (Table 2). Hence, the effect of perforin deficiency does not appear to operate or impact on the level of local cellular recruitment. Interestingly, a lower proportion of IFN-$\gamma$-producing CD8$^+$ T cells was recruited to the site of infection in the P-8-vaccinated perforin-deficient mice than in the P-8-vaccinated C57BL/6 mice (Tables 1 and 2). The ratio of CD4$^+$ to CD8$^+$ T cells producing IFN-$\gamma$ was comparable in P-8-vaccinated perforin$^{-/-}$ mice and control perforin$^{-/-}$ mice and/or nonprotected, P. acnes-immunized wild-type mice. Consequently, it appears that perforin deficiency may lead to a reduced activation or retention of CD8$^+$ T cells at the site of infection. Interestingly, naïve C57BL/6 perforin$^{-/-}$ mice do not show an increased susceptibility to L. amazonensis infection; this is similar to what has been observed for L. major infection in naïve mice (14). Hence, the role of perforin in the containment of L. amazonensis infection is apparent in vaccinated but not nonvaccinated mice.

The likelihood that an alternate mechanism might account for the lack of protection found in perforin-deficient mice was considered. Perforin-deficient mice have been reported to produce higher levels antibodies than wild-type mice in response to vaccination (50). Antibody has been demonstrated to be important in the pathogenesis of L. amazonensis infection (11, 30). Further, elevated levels of the down-regulatory cytokine IL-10 have been associated with Fc receptor-mediated uptake and susceptibility to Leishmania infection (18, 28). Thus, the possibility that an elevated antibody response (to the amastigote surface antigen P-8) and consequent heightened IL-10 production contributed to the lack of protection in perforin-deficient mice was examined. The level of antigen-specific antibodies found for the P-8-vaccinated perforin$^{-/-}$ mice was not increased (titer of 1/1,369) over that found for wild-type mice (titer of 1/2,774); consequently, it appeared unlikely that changes in antibody levels could account for the differences observed in protection. However, as IL-10 production at the site of infection might still contribute to the lack of protection in perforin$^{-/-}$ mice, the relative number of cells able to synthesize this cytokine in the lesion site was also examined.
weeks postinfection, no significant differences in the levels of IL-10-producing cells were found between perforin−/− (Pfp−/−) and wild-type mice (8.3% in wild-type, P. acnes-immunized mice versus 9.6% in Pfp−/− P. acnes-immunized mice; 6.7% in wild-type P-8-immunized mice versus 6.5% in Pfp−/− P-8-immunized mice). Consequently, local IL-10 production and IL-10-mediated suppression do not appear to be contributing to the lack of protection found in the perforin-deficient mice.

FIG. 4. Comparative parasite burden analysis of wild-type C57BL/6 and IFN-γ−/− mice immunized with P-8 and challenged with L. amazonensis parasites. C57BL/6 and IFN-γ−/− mice were immunized with either P-8 plus P. acnes (adjuvant) or adjuvant alone, as indicated in Materials and Methods. The mice were then infected 6 weeks after the final immunization in the right hind foot with 10⁵ L. amazonensis parasites. (A) Parasite burdens were determined at 10 weeks postinfection and compared to immunization control mice that received adjuvant alone (P. acnes) and to control (untreated) mice. Each bar is the mean value from three mice. *, P < 0.005 compared with P. acnes control. (B) Lesion development in vaccinated and control groups of IFN-γ−/− mice with time postinfection. ○, control; □, P. acnes; ■, P-8 plus P. acnes. Lesion development (the ratio of infected foot to noninfected foot) was monitored as indicated in Materials and Methods. Each value is the mean of a group of five mice. Error bars represent standard errors of the means.
Therefore, these results indicate that a CD8-perforin-mediated mechanism(s) most likely contributes to the protection conferred by immunization with P-8 against *L. amazonensis* infection.

**DISCUSSION**

In our studies on immunity to cutaneous leishmaniasis, we have sought to determine the requirements for successful vaccination against *L. mexicana* complex parasites (*L. amazonensis* and *L. pifanoi*). In the case of *L. amazonensis*, previous studies with GP46/M2, including vaccination with recombinant vaccinia virus expressing GP46/M2 (37), adoptive transfer experiments of immune CD8$^+$ T cells (unpublished results), and recognition of *Leishmania*-infected macrophages by GP46/M2-specific CD8$^+$ T cells in vitro (31), had suggested a potential role for CD8$^+$ T cells in immunity to infection against *L. amazonensis*. In this study, it has been demonstrated by two approaches that CD8$^+$ T cells are required for protection against *L. amazonensis* infection after vaccination with the amastigote antigen P-8. The evidence presented here indicates that protection is dependent on the activation of both CD8$^+$ and CD4$^+$ T cells; further, protection requires the production of IFN-γ and perforin. Thus, defined developmentally regulated antigens from both the promastigote (GP46/M2) and the amastigote stages (P-8) appear to elicit similar mechanisms for protection against *L. amazonensis*.

In the present study, T cells (CD4$^+$ and/or CD8$^+$) producing IFN-γ have been shown to be important for protection against *L. amazonensis* infection. Experimental data indicated that P-8-vaccinated IFN-γ$^{-/-}$ mice were comparable to nonvaccinated IFN-γ$^{-/-}$ control mice in their susceptibility to infection. Further, in wild-type (C57BL/6) mice, although IFN-γ-producing T cells as well as macrophages were found at the lesion site of both protected and nonprotected mice (Table 1), the ratio of IFN-γ-producing cells and macrophages was consistently higher (1.5- to 2.4-fold) in the protectively immunized mice. These results are in agreement with the idea that the balance between macrophage recruitment and level of activation is critical for protection. In addition, increased numbers of IFN-γ-producing T cells were found early at the site of infection of the P-8-vaccinated and -protected versus nonvaccinated mice. The ratio of CD8$^+$ to CD4$^+$ IFN-γ-producing T cells observed indicated a preferential retention or expansion of CD8$^+$ T cells in the protectively vaccinated mice.

However, although a critical role for IFN-γ was evident, whether IFN-γ-producing T cells alone were required for protection was unclear. A higher level in the total IFN-γ-producing T cells was only observed at the earliest time point in the protected versus nonprotected mice (12 days postinfection with *L. amazonensis*). This result differs from observations of LACK DNA-vaccinated mice infected with *L. major*, where activated CD8$^+$ T cells, through the production of IFN-γ, significantly increased the frequency and number of antigen-specific IFN-γ-producing CD4$^+$ cells (20, 21) at 2 to 12 weeks postinfection. Further, it has been shown that *L. amazonensis* parasites are more resistant than *L. major* to in vitro killing by IFN-γ-activated macrophages (51). Consequently, it might be anticipated that a complementary mechanism(s) is involved in the control of the intracellular *L. amazonensis* amastigotes in the P-8-vaccinated mice.

CD8$^+$ T cells can protect against pathogens by two important mechanisms: production of cytokines, such as IFN-γ, tumor necrosis factor alpha (TNF-α), and lymphotoxin-αβ, and the lysis of infected cells. Cytokine production can have both local and systemic consequences, whereas cytolytic activity is directed towards infected cells that are in contact with effector cells. Activated CD8$^+$ T cells are able to induce cytosis of infected cells by two distinct molecular pathways: the granule exocytosis pathway, dependent on the pore-forming molecule perforin, or by the up-regulation of Fas-L (CD95L), which may initiate programmed cell death through interaction with Fas (CD95) on target cells (8). The potential of cytotoxic T-lym-
phocyte (CTL)-mediated mechanisms in the regulation and control of *Leishmania* infection is largely unexplored. In vitro studies have indicated that CTL-mediated mechanisms may not play a significant role in regulating *Leishmania* parasite survival (53). In contrast, in vivo studies indicate that the Fas–Fas-L pathway of cytotoxicity contributes to healing of lesions induced by *L. major* infection (14, 25); Fas- and Fas-L-deficient mice are unable to control infection despite elevated levels of IL-12 and NO production. However, Fas-L expression was only somewhat elevated in P-8-vaccinated mice, suggesting that Fas and Fas-L interactions may not participate in the resolution of cutaneous infection in vaccinated mice infected with *L. amazonensis*.

In the present study, an examination of the events at the site of infection demonstrated that a specific and preferential activation of perforin- and IFN-γ-producing, CD8⁺ T cells occurs at the cutaneous site of infection of mice protectively immunized with P-8 antigen and challenged with *L. amazonensis*. Further, P-8-vaccinated perforin-deficient mice were unable to control *L. amazonensis* infection. Comparative FACS analysis suggests a defect in the recruitment and retention of CD8⁺ IFN-γ⁺ T cells at the site of infection in the perforin-deficient mice. Further, neither increased antibody levels as observed in earlier studies (50) nor consequent increased IL-10 production appeared to account for the lack of protection found in the vaccinated perforin-deficient mice. Therefore, direct perforin-mediated mechanisms appear to be critical for the control of infection with *L. amazonensis* in the vaccinated mice.
mice. Perforin can play a role in T-cell homeostasis as well as target cell lysis (3, 34). Further, it is known that perforin permits the release of other effector molecules, such as granulysin and granzyme, which have been shown to be responsible for the control of infection of intracellular microorganisms (23, 29, and 58). Consequently, the precise effector mechanism(s) by which perforin-CTL CD8+ T cells contribute to the control of L. amazonensis infection remains to be determined and is of further interest.

Altogether, we have demonstrated that CD8+ T cells are involved, together with CD4+ T cells, in the protective immune response against L. amazonensis infection. The immunological events within the first weeks following leishmanial infection are pivotal for the development of resistant or susceptible responses in the murine model (2, 32, 33, 59, 61, 66). Consequently, the early events within the cutaneous lesion site may have a strong bearing on the subsequent anti-Leishmania response in mice. Antigen-specific CD8+ T cells appear early at the site of infection, while CD4+ T cells predominate at the latter stages of infection. This suggests a possible role for CD8+ T cells early in infection. Evidence indicates that the CD8+ T-cell immune effector processes involved include IFN-γ secretion as well as perforin-mediated mechanisms. These results have potentially important implications in the design of vaccines against New World Leishmania parasites.

ACKNOWLEDGMENTS

We thank Karen Goldsmith-Pestana for technical assistance, Donna Barton for help with immunohistochemical analysis, and Phillip Scott and Nancy Ruddell for helpful discussions and reading of the manuscript. This work was supported by a grant from the National Institutes of Health (AI-27811).

REFERENCES


### Table 2: Enumeration of cells present within the cutaneous lesion site of wild-type and perforin−/− mice at 3 weeks postinfecțion

<table>
<thead>
<tr>
<th>Cell population (%)</th>
<th>Wild-type C57BL/6</th>
<th>P-8</th>
<th>Perforin−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>7.0 (±0.3)</td>
<td>4.6 (±0.2)</td>
<td>7.0 (±0.3)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>6.8 (±0.3)</td>
<td>7.7 (±0.4)</td>
<td>8.3 (±0.4)</td>
</tr>
<tr>
<td>IFN-γ-producing cells</td>
<td>6.1 (±0.3)</td>
<td>11.2 (±0.6)</td>
<td>14.3 (±0.7)</td>
</tr>
<tr>
<td>CD4⁺ IFN-γ</td>
<td>39.0 (±1.9)</td>
<td>10.5 (±0.5)</td>
<td>19.4 (±1.0)</td>
</tr>
<tr>
<td>CD8⁺ IFN-γ⁺</td>
<td>9.5 (±0.1)</td>
<td>15.8 (±0.8)</td>
<td>5.4 (±0.3)</td>
</tr>
<tr>
<td>CD4⁺ IFN-γ⁺/CD8⁺ IFN-γ⁺ ratio</td>
<td>4.0</td>
<td>0.67</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* P-8-immunized and adjuvant control mice (P-8) were infected with 10⁵ L. amazonensis parasites in both ears. Cells from the infection site were obtained at 3 weeks postinfection and then processed for FACS analysis. The percentage of macrophages was calculated from total cells by gating on MAC-1⁺/MHC-II low or negative cells. Intracellular IFN-γ expression and quantitation of lymphocytic cells were determined as indicated in the footnote for Table 1. Results were determined for pooled cells obtained from groups of three mice.

---

Downloaded from http://iai.asm.org on October 14, 2017 by guest
cytolytic component of CD8+ cell granules, restricts the spread of herpes simplex virus in the peripheral nervous systems of experimentally infected mice.


**Editor:** J. M. Mansfield