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Live Attenuated Leishmania donovani p27 Gene Knockout Parasites Are Nonpathogenic and Elicit Long-Term Protective Immunity in BALB/c Mice

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Leishmaniasis causes significant morbidity and mortality worldwide, and no vaccines against this disease are available. Previously, we had shown that the amastigote-specific protein p27 (Ldp27) is a component of an active cytochrome c oxidase complex in Leishmania donovani and that upon deletion of its gene the parasite had reduced virulence in vivo. In this study, we have shown that Ldp27−/− parasites do not survive beyond 20 wk in BALB/c mice and hence are safe as an immunogen. Upon virulent challenge, mice 12 wk postimmunization showed significantly lower parasite burden in the liver and spleen. When mice were challenged 20 wk postimmunization, a significant reduction in parasite burden was still noted, suggesting long-term protection by Ldp27−/− immunization. Immunization with Ldp27−/− induced both pro- and anti-inflammatory cytokine responses and activated splenocytes for enhanced leishmanicidal activity in association with NO production. Protection in both short- and long-term immunized mice after challenge with the wild-type parasite correlated with the stimulation of multifunctional Th1-type CD4 and CD8 T cells. Adoptive transfer of T cells from long-term immunized mice conferred protection against virulent challenge in naive recipient mice, suggesting involvement of memory T cell response in protection against Leishmania infection. Immunization of mice with Ldp27−/− also demonstrated cross-protection against Leishmania major and Leishmania braziliensis infection. Our data show that genetically modified live attenuated Ldp27−/− parasites are safe, induce protective immunity even in the absence of parasites, and can provide protection against homologous and heterologous Leishmania species. The Journal of Immunology, 2013, 190: 000–000.

Leishmaniasis is an insect vector–borne disease caused by the protozoan parasite Leishmania. Approximately 350 million people living in tropical and subtropical regions throughout the world are under the threat of such infections. There are 12 million infections worldwide, with ~2 million new clinical cases reported annually and an estimated death toll of ~50,000 persons per year (1). The clinical manifestations of leishmaniasis depend mostly on the infecting Leishmania species as well as host factors that range from mild cutaneous leishmaniasis (CL) to potentially fatal visceral leishmaniasis (VL) (2–4). VL is one of the major health problems on the Indian subcontinent, East Africa, and most of the Latin American countries (5–7). Available drugs are toxic; moreover, emergence of drug-resistant parasites, as well as co-infection with HIV, makes the drug treatment regimen even more complex (8). Currently, no vaccines against Leishmania infections in humans are available. In leishmaniasis, the host defense against intracellular Leishmania is cell mediated, which involves Th1 responses owing to T cells primed mostly by dendritic and macrophage cells producing IL-12 (9–12). Production of IL-12 by APCs and IFN-γ by T cells is crucial for controlling parasite growth by enhancing NO generation (13). The Th2 cytokines, mainly IL-10, suppress host immunity and help parasite survival (13, 14); however, IL-10 also protects the host from tissue damage by excess inflammatory cytokines (15). Unlike CL, the Th1/Th2 dichotomy is not as clear in visceral infection of mice and even less so in human VL (16). The immune response and pathology of VL is complex, involving a number of genetic and cellular factors in the process of susceptibility or resistance to parasites (17).

In past years, several approaches have been tested for Leishmania vaccine development, such as DNA vaccination, subunit vaccination, and heat-killed parasite vaccination with and without adjuvant (8, 18, 19). Some of these worked in animal models; however, so far, none have been successful in humans. Leishmanization, a process in which deliberate infections with L. major cause a controlled skin lesion with very low numbers of parasites has been shown to provide protection against reinfection (20, 21). Immunity can also be acquired by pre-exposure to infection, as was demonstrated in individuals who migrated from an L. major–endemic region and who were reactive to Leishmania Ag, and had a lower risk of developing VL (22, 23). These studies suggest that an effective vaccine against leishmaniasis might require a controlled parasitic infection that can provide the complete array of

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Abbreviations used in this article: CL, cutaneous leishmaniasis; DXM, dexamethasone; FTAg, freeze-thaw L. donovani Ag; VL, visceral leishmaniasis; Wt, wild-type.
Ags of a wild-type (Wt) parasite needed for developing a protective immune response. Past experience with other pathogens has indicated that live attenuated pathogens can fulfill such a requirement (24–26).

Attempts to develop a live attenuated *Leishmania* vaccine, including chemical mutagenesis, long-term serial in vitro cultures, irradiation, temperature sensitivity, and targeted gene deletions of both alleles, have been made in the past (18, 27–33). However, most of the mutated parasite cell lines were developed in species causing CL, such as *L. major* or *L. mexicana*. Attempts were also made using partial targeted gene-deleted parasites for the A2-A2rel gene cluster in *L. donovani* (34) and SIR2 gene in *L. infantum* (35), to test as immunogens. However, such mutants developed in VL-causing parasites cannot be used as vaccine candidates because they still carry single alleles of the Wt gene that could revert to the wild genotype and regain virulence. Therefore, it is critical to develop attenuated parasites through complete gene knockouts in which all the alleles of a virulence gene are nonfunctional and hence not capable of reverting to cause the disease. *L. donovani* biotyper transporter (BT1) null mutant parasites with both alleles disrupted were tested in mice, showing reduced infectivity and induced protection against infection with WT parasites (36). However, this study did not address the issue of safety and correlates of immune protection for genetically modified live attenuated *L. donovani* parasites. To address this question, we previously developed an amastigote-specific replication-deficient Centrin gene–deleted *L. donovani* parasite cell line (LdCen⁻) that was tested in a rodent model, and was found to have limited persistence and an ability to induce a protective cellular immune response in immunized animals (37). Recently, we developed another *L. donovani* cell line devoid of the p27 gene, encoding an amastigote-specific cytokrome c oxidase component (38), and demonstrated that Ldp27⁻/⁻ parasites persist longer (>12 wk) than LdCen⁻ (5 wk) in mice (37, 38). We thus evaluated whether longer persistence of *Leishmania* Ags can produce better protection. In our current study, we showed that the Ldp27⁻/⁻ parasite cell line, which can persist for an extended period without causing pathogenesis, can elicit an effective cell-mediated effector protective immune response against homologous and heterologous *Leishmania* species.

**Materials and Methods**

**Animals and parasites**

Female 5- to 6-wk-old BALB/c mice from the National Cancer Institute, Bethesda, MD, were used in the experiments. Procedures used were reviewed and approved by the Animal Care and Use Committee, Center for Biologics Evaluation and Research, Food and Drug Administration. Among parasites, the WT *L. donovani* (Ld1S) maintained in golden Syrian hamsters and p27 gene–deleted (Ldp27⁻/⁻) line of *L. donovani* (Ld1S2D) (38) were used. The parasites were cultured according to the procedure previously described (39, 40). *L. major* (Friedlin) and *L. braziliensis* promastigotes were grown at 26°C in medium 199 supplemented with 20% FCS.

**Immunizations and challenge studies**

The mice were immunized via tail vein with 3 × 10⁶ stationary-phase Ldp27⁻/⁻ parasites. In each study, four or five mice were used per group. Immunized mice were challenged after different periods of immunization via tail vein with 10⁵ stationary-phase Wt *L. donovani* parasites. Age-matched naive mice used as controls were also similarly challenged with 10⁵ virulent stationary-phase Wt *L. donovani* parasites. In separate experiments, mice immunized (with Ldp27⁻/⁻) or not (naive with saline) were also challenged by injecting the left hind footpad s.c. or intradermally with 10⁵ stationary-phase Wt *L. major* or 10⁵ stationary-phase *L. braziliensis* parasites. After various periods postchallenge, parasite load was measured in spleens and livers from the L. donovani–challenged mice and in footpads and lymph nodes from the L. major– or *L. braziliensis*–challenged mice by limiting dilutions as previously described (41). As an additional confirmation of the presence of parasites in tissues, total DNA obtained from spleens of mice from certain groups, as specified in the Results, was also used as a template in real-time PCR. The real-time PCR was based on the target from the kinetoplast minicircle DNA, using primers and methods as described (42).

For the immune-suppression study, mice were infected with either Wt or Ldp27⁻/⁻ parasites, and at 20 or 25 wk postinfection, 2 mg/kg dexamethasone (DXM) sodium phosphate (Sigma-Aldrich) in PBS was administered s.c. three times per wk (43). At the end of this treatment, mice were sacrificed and their visceral organs evaluated for parasite burden.

**Multiplex cytokine ELISA**

Single-cell suspensions were prepared from splenocytes after lysis of RBCs using ACK (ammonium-chloride-potassium) lysis buffer (Lonza). Cells were washed with medium, plated in 24-well plates, and stimulated with either freeze-thawed *L. donovani* Ag (80 μg/ml FTAg) or without Ag in complete RPMI 1640 medium at 37°C in 5% CO₂, with a 95% humidity incubator. After 72 h of culture, cell supernatants were collected and stored at −80°C until analyzed using multiplex kits, MILLIPLEX Mouse Cytokine/Chemkine Magnetic Bead Panel (Millipore); then the plate was read in a Luminex-100 (LumineX) system using Bio-Plex Manager software 5.0. The cytokine analysis procedure has been performed according to the manufacturer’s instructions, and the level of cytokine concentration determined using a standard curve of each specific cytokine.

**Intracellular staining and flow cytometry**

Splenocytes were plated in 24-well plates in complete RPMI 1640 medium at 37°C and stimulated with or without FTAg (80 μg/ml) (44). After 48 h at 37°C, protein transport inhibitor (BD GolgiStop; BD Pharmingen) was added to the wells. At 6 h after, cells were blocked at 4°C with Rat anti-mouse CD16/32 (5 μg/ml) from BD Pharmingen for 20 min. Cells were surface stained with anti-mouse CD3 APC-eFluor®780, anti-mouse CD4 eFluor®450, anti-mouse CD8a eFluor®450, anti-mouse CD44 FITC, and anti-mouse CCR7 PE-Cy5 Abs (eBioscience) for 30 min (each with 1:200 dilution; 4°C). The cells were then stained with LIVE/DEAD Fixable Aqua dye (Invitrogen/Molecular Probes) to mark dead cells. Cells were washed with wash buffer and fixed with the Cytofix/Cytoperm Kit (BD Biosciences) for 20 min (room temperature). Intracellular staining was done with anti-mouse IFN-γ PE-Cy7, anti-mouse TNF-α PertCP-eFluor®710, anti-mouse IL-2 APC, and anti-mouse IL-10 PE (eBioscience) for 30 min (each with 1:300 dilution; 4°C). Cells were acquired on an LSRII (BD Biosciences) equipped with 405, 488, 532, and 638 laser lines using DIVA 6.1.2 software. Data were analyzed with FlowJo software version 9.1.5 (TreeStar). For analysis, first doublets were removed using width parameter; dead cells were excluded based on staining with the LIVE/DEAD Aqua dye. Lymphocytes were identified according to their light-scattering properties. CD4 and CD8 T cells were identified as CD3⁺ lymphocytes uniquely expressing either CD4 or CD8. Upon further gating, intracellular cytokines were measured in CD4⁺/CCR7⁺ cells. Fluorescence minus one controls were used for proper gating of positive events for designated cytokines.

**Ab responses**

Specific Ab responses were measured by conventional ELISA. Briefly, ELISA plates were coated overnight at room temperature with FTAg (15 μg/ml). A serial dilution of the sera was carried out to determine the titer, which is defined as the inverse of the highest serum dilution factor giving an absorbance of >0.2. Titers for the Abs were determined using the corresponding HRP-conjugated secondary Abs: Rabbit anti-mouse IgG (H+L)-HRP, Rabbit anti-mouse IgG1-HRP, Rabbit anti-mouse IgG2a-HRP (SouthernBiotech; all with 1:1000 dilutions). SureBlue (KPL) was used as a peroxidase substrate. After 15 min, the reaction was stopped by the addition of 100 μl 1 M H₂SO₄, and the absorbance was read at 450 nm.

**Histological staining**

Mouse livers were fixed in fixative solutions (10% buffered formalin phosphate solution) and sent to Histoserv (Gaithersburg, MD) for sectioning and H&E staining. Stained sections were analyzed under the microscope (Nikon Eclipse TE2000-U).

**NO quantification**

Splenocytes or macrophages obtained from peritoneal fluid (45) were cultured in complete RPMI 1640 medium in the presence or absence of FTAg (80 μg/ml) for 24 h at 37°C. NO (nitrite/nitrate) production was determined from the supernatants of the cultures by the Griess Reaction Kit (Sigma-Aldrich) (37).
**CFSE proliferation assay**

Proliferative capacity of T cells was assessed by a CFSE dilution assay in Ldp27\(^{-/-}\)-immunized mice before and after challenge with Wt parasites (46). Age-matched naive mice served as negative controls for Ag-specific proliferation. Splenocytes from different groups of mice were isolated, incubated in 5 μM CFSE (Molecular Probes/Invitrogen) for 10 min in RPMI 1640 without FCS, followed by 5 min of quenching in ice-cold RPMI 1640 plus 10% FCS, and subsequently washed thoroughly before plating in 96-well tissue culture plates at 2 × 10\(^5\) cells per well. Cells were cultured for 5 d at 37°C with 5% CO\(_2\) under stimulation with FTAg (50μg/ml) or without it. Cells were harvested, washed, and blocked with anti-CD16/32 (5 μg/ml) for 20 min (4°C); then they were cell surface stained with anti-mouse CD3 APC-eFluor@780, anti-mouse CD4 eFluor@450, anti-mouse CD8a eFluor@605NC (eBioscience, USA) for 30 min (each with 1:200 dilution; 4°C). Cells were acquired on an LSRII (BD Biosciences) equipped with 405, 488, 532, and 638 laser lines using DIVA 6.1.2 software. Data were analyzed with FlowJo software version 9.1.5.

**Adoptive cell transfer**

Total T cells or CD4 or CD8 T cells from mice 16 wk postimmunization with Ldp27\(^{-/-}\)-immunized or nonimmunized naive mice were isolated and transferred into naive mice. Purification of T cells was performed with the MidiMACS system (Miltenyi Biotec) using Pan T Cell, CD4 T Cell, or CD8 T Cell Isolation Kits, respectively, as recommended by the manufacturer. Then, 5 × 10\(^8\) total T cells or CD4 or CD8 T cells were transferred to recipient mice, and 24 h later all groups of mice were infected with L. donovani Wt parasites. The purity of the isolated T cell population was >95%, as observed by flow cytometry.

**Statistical analysis**

Statistical analysis of differences between means of groups was determined by unpaired two-tailed Student t test, using GraphPad Prism 5.0 software. A p value <0.05 was considered significant, and a p value <0.01 was considered highly significant.

**Results**

**Live attenuated Ldp27\(^{-/-}\) parasites disseminate into the visceral organs with limited persistence and are nonpathogenic**

We previously demonstrated that the Ldp27\(^{-/-}\) mutant is unable to sustain itself beyond 13 wk postinfection and that the defect is overcome by epismal expression of p27 in the Ldp27\(^{-/-}\) cell line (38). To further dissect the organ-specific survival of Ldp27\(^{-/-}\) parasites, mice were injected i.v. with stationary-phase Wt or Ldp27\(^{-/-}\) promastigotes, and the parasite burden was measured in the spleen and liver of Wt-infected mice up to week 16, reflecting the normal course of infection. However, Ldp27\(^{-/-}\) mutants showed a progressive decline from 4 wk postinfection in both spleen and liver. In both time points, 4 and 8 wk postinfection, Ldp27\(^{-/-}\)-infected mice had significantly (p < 0.05) less parasite burden than did Wt-infected mice in both organs (Fig. 1A, 1B). Of interest, in Ldp27\(^{-/-}\)-infected mice by week 16, the parasites were cleared from both organs, as we could not recover any live parasites from serial dilutions of cultured organ cell suspensions from these mice. Similar results were obtained in a confirmatory real-time PCR study using DNA from spleens of mice infected either with Wt or with Ldp27\(^{-/-}\) parasites (Supplemental Table I). Detection of the parasite-specific minicircle DNA target indicated the presence of a substantial number of parasites in Wt-infected (12 wk) and Ldp27\(^{-/-}\)-infected (12 wk) mice. However, 20 wk postinfection with Ldp27\(^{-/-}\), the mice no longer had detectable parasites, whereas significant parasite burden was noted in Wt-infected spleens, suggesting the absence of any residual attenuated parasite persistence. Taken together, these experiments suggested that deletion of the Ldp27 gene does not affect the visceralizing capacity of this cell line; however, parasite growth is severely impaired in host organs, leading to clearance by the host.

To rule out the survival of any undetectable Ldp27\(^{-/-}\) mutant parasites in visceral organs beyond 20 wk postinfection, we treated Wt or Ldp27\(^{-/-}\)-parasite–infected mice with DXM, a known immune suppressor, to allow proliferation of any residual parasites. In the Wt-infected group, treatment with DXM enhances parasite growth in the spleen (Fig. 1C) and liver (data not shown) at 20 wk and 25 wk postinfection. In Ldp27\(^{-/-}\)-infected mice at 20 wk postinfection, only 2 of 12 (16%) mice showed a very low number

**FIGURE 1.** Avirulent properties of p27 knockout L. donovani (Ldp27\(^{-/-}\)) parasite in BALB/c mice. Survival of Wt or Ldp27\(^{-/-}\) parasites in liver (A) and spleen (B) of BALB/c mice. Mice were infected with Wt or Ldp27\(^{-/-}\) parasites, and at 4, 8, and 16 wk postinfection (PI) parasite load in infected mice was measured. (C) Effect of immunosuppressive drug DXM on infected mice. Parasite load was measured in spleen of mice infected with Wt or p27\(^{-/-}\) parasite for different weeks postinfection and treated with DXM. (D) Histopathology of liver (H&E stain) from Wt- or Ldp27\(^{-/-}\)-infected mice after 6 wk and 16 wk postinfection. Black arrows point to infection foci or intracellular parasites. The data presented are representative of three independent experiments with similar results and four mice in each group. Mean and SEM of each group are shown. *p < 0.05. ND, Not detected.
of parasites only in the spleen, not in the liver. However, at 25 wk postinfection, with or without DXM treatment we did not observe any Ldp27−/− parasites in the spleen, liver, or bone marrow. These observations confirm that Ldp27−/− is safe as an immunogen and does not persist for the long term.

To confirm that Ldp27−/− parasites do not cause any pathogenesis, we performed a histopathological study of the liver. Stained liver sections (Fig. 1D) showed that initially (6 wk postinfection) Ldp27−/− parasites generated an inflammatory response resulting in development of inflammatory foci. Unlike the Wt-infected mouse liver, all the inflammatory foci were devoid of parasites in Ldp27−/−-infected mice. Moreover, at a later stage of infection (16 wk postinfection), Ldp27−/−-infected mouse liver was completely free of any inflammatory foci, and the liver cells showed normal morphology. On the contrary, in the Wt L. donovani-infected mouse liver, some inflammatory foci were still present, although most of these foci were free of parasites. These observations confirm that Ldp27−/− causes initial mild pathogenesis in the host liver; however, eventually the host clears the parasite and restores the normal architecture of liver tissue.

**Immunization with Ldp27−/− protects against challenge with virulent L. donovani**

To determine the protective efficacy of Ldp27−/− immunization, naïve BALB/c mice were immunized with Ldp27−/− parasites or saline by i.v. injection. At 12 wk postimmunization, mice were challenged with Wt L. donovani parasites, and assessment of parasite burden in spleens and livers was done at three time points postchallenge, measured by limiting dilution. The results showed that immunization with Ldp27−/− significantly reduces the liver or spleen parasite burden 4, 8, or 12 wk postchallenge, compared with that in the nonimmunized mice group (Fig. 2A, 2B). However, at 12 wk postchallenge, immunized mice showed the most significant protection, having 2.5 log− and 4 log−fold reduction in parasite number in liver and spleen, respectively, compared with nonimmunized mice (Fig. 2A, 2B). Nonimmunized infected mice, compared with immunized infected mice, showed significant splenomegaly (data not shown). To evaluate the ability of Ldp27−/− immunization to confer long-term protection, mice were challenged with virulent L. donovani parasites 20 wk following Ldp27−/− immunization, and the parasite load was evaluated 12 wk postchallenge (Fig. 2C). Significant reduction in parasite burden was noted in the spleen and liver of mice challenged 20 wk postimmunization, compared with naïve challenged mice (Fig. 2C). However, the reduction in parasite burden was less in the 20-wk postimmunization group (2 log−fold in spleen and 1.5 log−fold in liver) than it was in the 12-wk immunized group (4 log−fold in spleen and 2.5 log−fold in liver; compare data in Fig. 2A, 2B with those in Fig. 2C). Overall, these data suggest that Ldp27−/− immunization confers significant sustained protection even at 20 wk postimmunization.

**Ldp27−/− immunization induces a mixed proinflammatory and anti-inflammatory cytokine response upon virulent challenge**

To characterize the immune response induced by live attenuated Ldp27−/− parasites, we analyzed Ag-specific cytokine secretion by splenocytes from naïve, immunized, nonimmunized challenged, and immunized challenged mice. There was a significant induction of Leishmania Ag-specific IFN-γ and IL-12p70 secretion in the splenocyte culture supernatants of 12-wk postimmunized mice (Fig. 3). IFN-γ and TNF-α were also induced in 20-wk postimmunized mice (Fig. 3). Of interest, following 3 wk of challenge with Wt L. donovani parasites, the 12-wk postimmunized mice showed significantly enhanced IFN-γ, TNF-α, and IL-12 secretion, whereas 20-wk postimmunized mice showed significantly enhanced IFN-γ and IL-12, but not TNF-α, secretion compared with nonimmunized challenged mice. In 12-wk postimmunized and challenged mice, compared with naïve challenged mice, significant induction of anti-inflammatory cytokines like IL-10, IL-4, and IL-13 was noted. Although there was significant induction of IL-10 secretion in 20-wk postimmunized mice and immunized challenged mice, compared with nonimmunized challenged mice, no difference was observed in the levels of IL-4 and IL-13 in 20-wk immunized challenged mice. Taken together, these results suggest that Ldp27−/− parasite immunization primes a selective long-term mixed pro- and anti-inflammatory immune response against Wt parasites.

**Resistance to infection induced by Ldp27−/− immunization is associated with induction of NO in splenocytes**

Most proinflammatory cytokines activate macrophages and induce NO production. NO is one of the macrophage-derived effector molecules and crucial for the control of intracellular Leishmania infections (47). A significant amount of Leishmania Ag–specific nitrite production was observed in both 12-wk and 20-wk postimmunized mice compared with nonimmunized mice splenocytes (Fig. 4). However a much greater amount of NO was observed in Ldp27−/−-immunized mice upon Wt L. donovani challenge than in naïve challenged mice, in 12- and 20-wk postimmunized mice (Fig. 4). Of note, the induction of nitrite concentration was similar in challenged animals immunized for 12 or 20 wk. Overall, the sustained nitrite secretion level by splenocytes indicates a proinflammatory cytokine–dominating milieu in immunized mouse spleens, which in turn favors the host in clearing parasites.

**Immunization with Ldp27−/− induces a sustained level of Ag-specific T cell proliferation response**

Impairment of cell-mediated immune response is a hallmark of active VL in humans or in animals (48). Restoration of Ag-specific
immune response is crucial for effective vaccine-induced immunity. To investigate the proliferative potential upon re-encounter with a Leishmania Ag, spleen cells were isolated from naïve, naive challenged, 20-wk postimmunized, and immunized challenged mice. Splenocytes were CFSE labeled and stimulated in vitro with FTAg or medium alone. After 5 d of Ag stimulation, the cells were stained with anti-CD3, anti-CD4, and anti-CD8 Abs, and their CFSE profile was analyzed with the flow cytometer (Fig. 5A). FlowJo analysis showed that all CD3+ T cells, including CD4 and CD8 isolated from mice immunized with Ldp27^2/2, have significantly higher proliferative capacity in response to Leishmania FTAg than do T cells isolated from naive mice (Fig. 5B). Further, T cells isolated from mice immunized and challenged with virulent L. donovani parasites have significantly (*p, 0.05) higher proliferative capacity than do those of the naive challenged group (Fig. 5B). The significant proliferative capacity of T cells even in 20-wk postimmunized mice suggests that some of them may have derived from a memory response.

**Induction of multifunctional Ag–experienced Th1 effector cells correlates with Ldp27^2/2–induced immunity**

Having shown that immunization with the Ldp27^2/2– parasite establishes stable, long-lived immunological protection against the Wt Leishmania parasite, we subsequently characterized the phenotype and cytokine production of the Ag-experienced effector memory T cells CD44^hi/CCR7^low in 20-wk postimmunized animals (49, 50). With use of intracellular FACS staining, Ag-experienced CD4 and CD8 T cells were gated according to their surface expression of CD44 and CCR7 (Fig. 6A). The CD44^hi/CCR7^low cells were separated into seven distinct subpopulations based on their production of IFN-γ, TNF-α, or IL-2 in any combination within the pool of effector memory T cells (Fig. 6A). The results showed that at 20 wk postimmunization, in CD4 T cells, IFN-γ single cytokine–secreting cells are dominating, a significant number of IL-2 and TNF-α single–producing cells are present, as well as double IFN-γ^IL-2^ and IFN-γ^TNF-α^– producing cells (Fig. 6B). In CD8 T cells, although IFN-γ^+ cells are dominating, a significant number of IL-2 and TNF-α^+– producing cells are present, as well as double cytokine–producing IFN-γ^IL-2^, IFN-γ^TNF-α^, and TNF-α^IL-2^, or triple cytokine–producing IFN-γ^TNF-α^IL-2^ cells (Fig. 6C). Most importantly, the CD8 T cell compartment had higher percentages of double and triple cytokine–producing cells than did the CD4 T cell compartment (Fig. 6C versus Fig. 6B).

Having shown that Ldp27^2/2– immunization induces a long-term immune response characterized by a high level of multifunctional CD4 and CD8 T cells, we described the nature of the immune response after challenge with the Wt parasite. In CD4 and CD8 T cells from spleens of both 12-wk and 20-wk postimmunized and challenged mice, mostly IFN-γ cytokine–secreting cells were significantly higher than in the nonimmunized challenged mice (Fig. 7A–D). In addition, a significantly higher percentage of double or triple cytokine–producing CD4 (Fig. 7A, 7B) and CD8 cells is observed.

**FIGURE 3.** Leishmania Ag–stimulated cytokine profiles in splenocyte culture supernatants from naïve, Ldp27^2/2– immunized (Imm), naive challenged (Naive Chal), and Ldp27^2/2– immunized challenged (Imm Chal) mice. The 12- or 20-wk postimmunized mice were challenged with Wt parasite and 3-wk postchallenge mice were euthanized (shown below each bar diagram), and spleens were collected. Concentrations of cytokines in culture supernatants were measured using the multiplex mouse cytokine kit as described in the Materials and Methods section. The data presented are representative of two independent experiments with similar results. Mean and SEM of each group are shown. *p < 0.05, **p < 0.01.

**FIGURE 4.** Immunization with Ldp27^2/2– induces NO production by splenocytes. The activity of NO synthase is indicated by the amount of released nitrite in the Ag-stimulated splenocyte supernatants (48 h) and was measured by the Griess reaction. The data presented are representative of two experiments with similar results and four or five mice in each group. Mean and SEM of three or more mice in each group are shown. *p < 0.05, **p < 0.01. PC, Postchallenge; PI, postimmunization; ws, weeks.
T cells were observed for many cytokines in both 12-wk and 20-wk postimmunized and challenged mice compared with nonimmunized challenged mice. Particularly with CD4 T cells, IFN-\(\gamma\) + IL-2+–, IFN-\(\gamma\) + TNF-\(\alpha\)+–, and IFN-\(\gamma\) + IL-2+ TNF-\(\alpha\)+– producing cells were significantly higher in 12-wk postimmunized and challenged mice. Similarly, in the 20-wk immunized and challenged mice, TNF-\(\alpha\)+IL-2+ and IFN-\(\gamma\)+IL-2+ TNF-\(\alpha\)+– cells were higher than in nonimmunized challenged mice—albeit the percentage of such cells was smaller compared with that in 12-wk postimmunized and challenged mice. Of interest, in 20-wk immunized mice, the percentage of both double and triple cytokine–producing CD8 T cells was higher than that of CD4 T cells, similar to the results observed in immunized mice before challenge. In sum, these results indicate that Ldp27\(^{−/−}\)-immunized mice induce a strong Ag-experienced effector memory T cell–mediated immune response after 20 wk postimmunization, at a time point when the majority of mice have cleared the Ldp27\(^{−/−}\) parasite.

We also quantified Ag-experienced CD4 and CD8 T cells that produce IL-10, a crucial anti-inflammatory cytokine in the pathogenesis of VL (16, 37, 51). In Ag-stimulated CD4 and CD8 T cells from the spleen, the IFN-\(\gamma\)-IL-10 ratio was significantly higher in the 12-wk immunized mice after challenge than in the nonimmunized challenged mice (Fig. 8A). These results indicate that increased IFN-\(\gamma\)-secretion and simultaneously decreased IL-10 production by Ag-experienced CD4 and CD8 T cells produce a strong Th1 response that could translate into protective immunity. In addition, IL-10 has been shown to be a critical immune-regulatory molecule and is necessary to shape the amplitude of immune response as well as to prevent infection-associated lesions (15, 52). Particularly, cells coproducing IFN-\(\gamma\) and IL-10 play an important role in preventing collateral tissue damage due to a high level of inflammatory cytokines (52). We analyzed the percentage of IFN-\(\gamma\)- and IL-10-coproducing Ag-experienced CD4 and CD8 T cells. In mice challenged 12 wk postimmunization, compared with naive challenged mice, there were significantly higher percentages of both the CD4 and CD8 T cells that were positive for both IFN-\(\gamma\) and IL-10 (Fig. 8B). Interestingly, the percentage of IFN-\(\gamma\)/IL-10-coproducing CD8 T cells was significantly higher than the percentage of coproducing CD4 T cells. Overall, these data clearly indicate that Ldp27\(^{−/−}\) immunization induces a host-protective cell-mediated controlled proinflammatory immune response against challenge with Wt infection.

Adoptive transfer of isolated T cells from Ldp27\(^{−/−}\)-immunized mice confers protection to recipient mice

To confirm that T cell–mediated immunity is sufficient to protect against Wt parasite infection, we performed adoptive transfer of T cells. The recipient mice were infected with Wt parasites for 10 wk, and parasite load was measured. Mice that received T cells from immunized mice showed significant reduction in parasite burden, compared with recipients of T cells from nonimmunized mice. There was 4 log–fold, 2 log–fold, and 3.5 log–fold reduction of parasite burden in spleens of recipients of Ldp27\(^{−/−}\)-immunized total T cells, CD4 T cells, or CD8 T cells, respectively, compared with mice receiving nonimmunized T cells (Fig. 9). These results...
clearly indicate that attenuated parasite-induced immunity is T cell–mediated immunity and that both CD4 and CD8 T cells are major effector T cell populations that are important in controlling the parasite burden in immunized mice.

**Ldp27⁻/⁻ immunization cross-protects mice against challenge with other Leishmania species**

We further investigated whether immunization with Ldp27⁻/⁻ could provide protection against heterologous species of *Leishmania*. Mice immunized (i.v.) with Ldp27⁻/⁻ parasites for 12 wk were infected (in footpad) with either *L. major* or *L. braziliensis*. The naive mice infected with *L. major* started developing progressive lesions from 3 wk postchallenge, and by 10 wk postchallenge they developed a large swollen footpad with lesions. On the contrary, Ldp27⁻/⁻-immunized mice, compared with naive mice, developed significantly smaller lesions that did not continue to swell (Fig. 10A). The immune-challenged mice, compared with the naive challenged mice, also showed significantly lower parasite burden in both the footpads and lymph nodes (Fig. 10B). Similarly, we found a significantly reduced footpad swelling in Ldp27⁻/⁻-immunized mice postinfection with *L. braziliensis*, compared with nonimmunized mice (Fig. 10C). The parasite burden in the footpad and draining lymph nodes was also significantly reduced in immunized mice compared with nonimmunized mice (Fig. 10D). These results suggest that immunization with live attenuated *L. donovani* parasites can control parasitemia from nonvisceralizing *Leishmania* infections.

**Induction of humoral response in the immunized mice**

Although no specific evidence exists for a role of *Leishmania*-specific Abs in determining the outcome of VL (53), from the dominance of Ig subtype one can predict the outcome of the immune response in vivo. Mouse sera were assessed for *L. donovani*-specific IgG1 and IgG2 isotypes, surrogate markers of Th2 and...
Th1 CD4 T cell differentiation, respectively. In Ldp27\(^{-/-}\)-immunized mice, the levels of IgG2a and IgG1 were not significantly different at 8 wk postinfection. However, the IgG2a levels were progressively higher in the 12- and 16-wk postimmunization groups of mice (Supplemental Fig. 1A), which correlated with the clearance of Ldp27\(^{-/-}\) parasites from the visceral organs (Fig. 1A, 1B). On the contrary, in mice infected with Wt parasites, the IgG2a/IgG1 ratio was lower, suggesting a Th2-type immune response that correlates with disease progression, as is evident by the increased parasite burden (Fig. 2A, 2B). Of interest, in 20-wk postimmunized and challenged mice, the IgG2a/IgG1 ratio is significantly higher than in the nonimmunized and challenged mice, suggesting a host-protective Th1-type immune response (Supplemental Fig. 1B). The increased serum IgG2a levels observed in immunized and challenged mice correlate with the increased Th1 response.

**Discussion**

In *Leishmania* infection, individuals who recover from natural infection are protected from reinfection and develop life-long protection, suggesting that infection may be a prerequisite for immunological memory. Genetically altered live attenuated parasites with controlled infectivity could achieve such immunological memory (37). In addition, live attenuated parasites can provide a broad spectrum of parasite Ags to generate a diverse Ag-specific memory immune response that is important for protection against infection. Live attenuated vaccination, such as with bacille Calmette-Guérin, polio vaccine, and smallpox vaccine, is an old and widely accepted method used against a broad spectrum of diseases (24, 25). In this study, we evaluated the safety and potency of the *Leishmania* live attenuated vaccine candidate Ldp27\(^{-/-}\) parasite against homologous and heterologous *Leishmania* species. We observed that host protection correlates with the induction of a *Leishmania* Ag–specific cell-mediated immune response.

Safety of live attenuated parasites as a vaccine is essential because such parasites should not regain virulence when used as human immunogens. Therefore we first tested Ldp27\(^{-/-}\) parasites for virulence in mice. Ldp27\(^{-/-}\) parasites had no reduced growth during the promastigote stage; however, their growth was attenuated during the intracellular amastigote stage. These parasites do viscerализe to spleen and liver in mice but are cleared after 20 wk of infection, as is demonstrated by the absence of parasites in immunosuppressed mice. At 6 wk after immunization with the Ldp27\(^{-/-}\) parasite, mice recruit infiltrating mononuclear cells and form foci in the liver, similar to Wt infection. However, these foci lack parasites, unlike Wt infection–induced foci. Furthermore, 16 wk postimmunization, liver cell morphology returns to normal, as in the uninfected condition. These findings suggest that Ldp27\(^{-/-}\) parasites are safe and illicit a host response without causing pathogenesis.

**FIGURE 7.** Ag-specific intracellular cytokine secretion analysis of CD4 and CD8 T cells from Ldp27\(^{-/-}\)-immunized and nonimmunized mice after virulent challenge. The 12-wk or 20-wk postimmunized or nonimmunized mice were challenged for 3 wk with Wt parasites. Intracellular cytokine analysis was done as shown in Fig. 6A and divided into seven distinct subpopulations. Cytokine analysis of CD4 T cells from 12-wk PI and 3-wk PC mice (A) and from 20-wk PI and 3-wk PC mice (B). Cytokine analysis of CD8 T cells of 12-wk PI and 3-wk PC (C) and 20-wk PI and 3-wk PC (D). Gray bars represent nonimmunized and challenged mice, and colored bars represent immunized and challenged mice. The data presented are representative of two experiments with similar results. Mean and SEM of four mice in each group are shown. *p < 0.05, **p < 0.01. PC, Postchallenged; PI, postimmunization.

**FIGURE 8.** IFN-γ- and IL-10–producing CD4 and CD8 T cells. Splenocytes were cultured from either nonimmunized challenged or 12-wk immunized challenged mice. (A) Ratio of IFN-γ- and IL-10–producing CD4 and CD8 T cells from spleens of either nonimmunized and 3-wk challenged or 12-wk Ldp27\(^{-/-}\)-immunized mice and 3-wk challenged. (B) CD4 or CD8 T cells coproducing IFN-γ and IL-10 from splenocytes of 12-wk immunized and 3-wk challenged mice. Mean and SEM of four mice in each group are shown. **p < 0.01.
is correlated with the induction of both pro- and anti-inflammatory postinfection at both early and late stages of immunization. It is confirmed by significant parasite control in immunized mice.

Recipient mice included four or five animals. Mean and SEM of four mice in each group of recipients mice that received T cells from immunized mice. Each group of recipient mice included four or five animals. Mean and SEM of four mice in each group are shown. * p < 0.05.

The efficacy of the Ldp27−/− parasites as immunogens was confirmed by significant parasite control in immunized mice postinfection at both early and late stages of immunization. It is well established in the literature that in VL control of parasitemia is correlated with the induction of both pro- and anti-inflammatory cytokine response (54, 55). Such a correlation was observed using Ldp27−/− parasites as immunogens, as indicated by significant enhanced secretion of proinflammatory cytokines like IFN-γ, TNF-α, and IL-12, as well as significant induction of anti-inflammatory cytokines like IL-10, IL-4, and IL-13. However, a balance must be achieved between the proinflammatory and anti-inflammatory cytokines to avoid tissue injury. Recent studies suggested that an exacerbated response to infections may result in deleterious lesions and extensive tissue damage, and IL-10 prevents the development of immunopathological tissue damage (15). Therefore, lack of liver tissue damage and the absence of splenomegaly in live attenuated parasite–immunized mice could be the outcome of induction of IL-10 secretion by Ldp27−/− parasite, balancing the effect of the high level of proinflammatory cytokines such as IFN-γ. In addition, IL-4 and IL-13 have been shown to be crucial for the clearance of L. donovani parasite from liver and spleen (56–58). Therefore, enhanced IL-4 and IL-13 levels observed during the early stage of Ldp27−/− parasite immunization in our study may be important for the development of hepatic granuloma maturation, with leishmanicidal activity resulting in enhanced NO production, which in turn leads to significant reduction of parasites in visceral organs. Taken together, these results clearly suggest that Ldp27−/− parasites induce a mixed Th1 and Th2 response without causing tissue damage and control the parasite burden by enhancing leishmanicidal activity.

In mice, vaccine-induced T cell response has been shown to correlate with protection against leishmaniasis (37, 59–61). Therefore, we analyzed CD4 and CD8 T cells that are CD44highCCR7low populations and represent Ag-experienced effector memory T cells (61, 62) to determine correlates of immune protection for Ldp27−/− parasites. In the current study, the Ldp27−/− immunization induced a significantly high frequency of Ag-experienced CD4 and CD8 T cells that could be detected even after 20 wk of postimmunization and that conferred a significant level of protection. In addition, the level of Ag-specific T cell recall response was significant, as indicated by T cell proliferation even after 20 wk postimmunization in the absence of parasite persistence, suggesting generation of Ag-specific memory cells. IFN-γ and TNF-α are the two major cytokines involved in the clearance of intracellular pathogens like Leishmania, and multifunctional cytokine-producing cells are much more effective than the single cytokine secretory cells (37, 46, 61, 63). In the current study, immunization with Ldp27−/− increased the percent of Leishmania-specific CD4 and CD8 cells expressing Th1 cytokines (IFN-γ, TNF-α, and IL-2) either single or in multiple combinations in response to WT parasite challenge. Importantly, IFN-γ/"TNF-α" is one of the major double cytokine–producing cells in Ldp27−/−-immunized and challenged mice, as was observed with protection against L. major infections (60, 61). From the previous studies, it was well established that CD8 T cells play a potential role in the cure of leishmaniasis, particularly VL (5, 37, 64–66). In this study, we also observed significant numbers of single or multiple cytokine–producing CD8 T cells in immunized and challenged mice, confirming that in immunization with live attenuated Ldp27−/− parasites, CD8 T cells play an essential role in host protection. Furthermore, significantly more Leishmania Ag–responsive CD8 T cells are present in immunized mice than in naive mice. The protective function of these cells may derive from their cytokine secretion as well as cytotoxic activity. However, at this time we have not analyzed the cytotoxic function of these cells; that will be the focus of future studies.

We also found a significantly increased IFNγ/IL-10 ratio in both CD4 and CD8 T cells in immunized and infected mice compared with nonimmunized infected mice, suggesting polarization toward Th1-type cell development, as was observed in L. infantum–infected mice after immunization with SIR2−/− (35). Further, co-production of IFN-γ and IL-10 by both Ag-experienced CD4 and CD8 T cells in immunized and infected mice suggests a desirable balance in the immune response, allowing brief parasite persistence that facilitates protection without causing host damage (37).
fore, production of both multiple cytokine–producing effector memory T cells and T cells coproducing IFN-γ and IL-10 upon immunization with Ldp27−/− parasites indicates that these could be used as markers of immune correlates of protection for the Ldp27−/− vaccine candidate.

It has been debated whether persistence of parasites or a pool of central memory T cells is essential for protective immunity against reinfection with Leishmania (67–69). In CL it has been reported that anti-Leishmania memory cells develop postinfection and are maintained in the absence of parasites; however, the quality of memory cells maintained in the presence of live parasites differs from that in their absence (68, 70). Our studies showed that strong recall of cellular immune response occurred both during the persistence of attenuated parasites and in the absence of attenuated parasites. However, the observation that short-term immunized mice show a higher level of protection than long-term immunized mice suggests that with persistence a pool of effector cells arises that rapidly respond to infection, whereas in the absence of parasite persistence, which serves as a source of Ag, a small pool of central memory cells respond slowly to infection by transitioning into effector memory cells that eventually help in controlling the parasite burden. Therefore, persistence of parasite Ag and Ag-specific effector T cells are probably more effective than central memory cells alone. Further adoptive transfer experiments confirm the generation of Ag-specific CD4 and CD8 T cells in immunized mice that confer protection during reinfection with WT parasites. Future studies are needed to analyze the type of T cells (potentially memory T cells) and their specific role in live attenuated parasite immunization.

Various Leishmania species have different tissue tropism resulting in different phenotypes. An ideal Leishmania vaccine would be the one that will control most Leishmania infections. Several reports have shown cross-protection against L. major infections using either whole-parasite lysates or defined proteins from L. donovani (71, 72). In addition, L. donovani DNA vaccines and L. infantum sterol 24-c- methyltransferase partial knockout parasites induced a strong protection against L. mexicana and L. major infections, respectively (60, 73). Similarly, in our previous studies we showed that LdCen−/− parasite–immunized mice are cross protected against L. braziliensis infection (37). In this study, we found that Ldp27−/−–immunized mice were cross-protected against L. major (CL) as well as L. braziliensis (mucocutaneous leishmaniasis) infections. All these studies suggest that the efficacy of a vaccine candidate will depend on the quality of cellular immunity and the Ag conservation among species. Therefore, it is possible to develop Leishmania vaccines, especially the live attenuated parasites that can provide an opportunity for a broad-based Ag repertoire to which the immune system can respond and hence cross protect from infections by other Leishmania species.

Overall, this study established that the Ldp27−/− live attenuated parasite could be a potential vaccine candidate against VL, CL, and mucocutaneous CL. However, host immunity differs significantly, depending on the mode of primary infections like needle inoculums versus sand fly–mediated infections. Particularly in CL, it has been reported that vaccine-induced immunity confers protection against needle challenge but fails to protect mice against sand fly–mediated infection (69, 74). These studies strongly suggest testing the efficacy of all Leishmania vaccines against sand fly–mediated infections. Recently, we have developed a rodent VL model with sand fly vector–mediated transmission (75). Vector transmission generated a slower progression of VL that resembled the chronicity of the disease following natural transmission in the field. The slower progression of disease may be relevant to the evolution of immunity to infection and to pathogenesis. Therefore, the slow progression of vector-initiated VL may be more appropriate for studies of early immune events, parasite establishment, and the screening of drugs and vaccines. Such a model will allow us to test the live attenuated vaccine candidates against sand fly–mediated L. donovani infections.

In summary, our results demonstrate that immunization with Ldp27−/− parasites provides significant protection against infection with homologous as well as heterologous species of Leishmania parasites. The immunization induced Ag-specific strong multi-functional CD4 and CD8 T cells as correlates of immune protection. Ldp27−/− parasites elicit memory response in both the present and the absence of parasite persistence, and the Ag-specific cell-mediated immunity correlated with robust NO generation and humoral response. Further, Ldp27−/− parasites are safe because they do not persist for a long time, hence reducing the chance of reversion to WT, and do not cause pathogenesis. Taken together, these studies strongly support the view that the Ldp27−/− mutant parasite is a safe and effective immunogen and has the potential to be a vaccine against a broad spectrum of leishmaniasis.

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Disclosures

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