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Ranadhir Dey; ... et. al

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Characterization of Cross-Protection by Genetically Modified Live-Attenuated *Leishmania donovani* Parasites against *Leishmania mexicana*

Ranadhir Dey,*[,1] Gayathri Natarajan,[†,1] Parna Bhattacharya,* Hannah Cummings,[†] Pradeep K. Dagur,[‡] César Terrazas,[†] Angamuthu Selvapandiyan,[§] John P. McCoy, Jr.,[‡] Robert Duncan,* Abhay R. Satoskar,[†] and Hira L. Nakhasi*

Previously, we showed that genetically modified live-attenuated *Leishmania donovani* parasite cell lines (*LdCen*$^{-/-}$ and *Ldp27*$^{-/-}$) induce a strong cellular immunity and provide protection against visceral leishmaniasis in mice. In this study, we explored the mechanism of cross-protection against cutaneous lesion-causing *Leishmania mexicana*. Upon challenge with wild-type *L. mexicana*, mice immunized either for short or long periods showed significant protection. Immunohistochemical analysis of ears from immunized/challenged mice exhibited significant influx of macrophages, as well as cells expressing MHC class II and inducible NO synthase, suggesting an induction of potent host-protective proinflammatory responses. In contrast, substantial inhibition of IL-10, IL-4, and IL-13 expression and the absence of degranulated mast cells and less influx of eosinophils within the ears of immunized/challenged mice suggested a controlled anti-inflammatory response. *L. mexicana* Ag–stimulated lymph node cell culture from the immunized/challenged mice revealed induction of IFN-γ secretion by the CD4 and CD8 T cells compared with non-immunized/challenged mice. We also observed suppression of Th2 cytokines in the culture supernatants of immunized/challenged lymph nodes compared with non-immunized/challenged mice. Adoptively transferred total T cells from immunized mice conferred strong protection in recipient mice against *L. mexicana* infection, suggesting that attenuated *L. donovani* can provide protection against heterologous *L. mexicana* parasites by induction of a strong T cell response. Furthermore, bone marrow–derived dendritic cells infected with *LdCen*$^{-/-}$ and *Ldp27*$^{-/-}$ parasites were capable of inducing a strong proinflammatory response leading to the proliferation of Th1 cells. These studies demonstrate the potential of live-attenuated *L. donovani* parasites as pan–*Leishmania* species vaccines. ***The Journal of Immunology*****, 2014, 193: 3513–3527.**

Leishmania causes a spectrum of diseases ranging from cutaneous lesions to fatal visceral infections depending on the parasite species involved as well as on the host immune response (1). Leishmaniasis is reported in all five continents and is endemic in 88 countries (2). Available drugs are toxic, and the emergence of drug-resistant parasites makes *Leishmania* treatment challenging; there is no licensed vaccine available (3). In the past, several approaches have been tested for *Leishmania* vaccine development, including DNA vaccination, subunit vaccination, and heat-killed parasite vaccination with and without adjuvant (4–6). Most vaccination approaches have worked in animal models, but none has been successful in humans. With a better understanding of immunological correlates there is potential to predict the efficacy of a vaccine candidate.

Leishmanization is a process in which deliberate infections with a low dose of *Leishmania major* cause a controlled skin lesion and it has been shown to provide protection against reinfection (1, 7, 8). Furthermore, persons who recover from leishmaniasis develop protective immunity against reinfection, which altogether indicates that a vaccine is feasible. In the past, leishmanization was a common practice in *Leishmania*-endemic regions. However, under the current regulatory environment such practice may not be acceptable because there is the possibility of infection in naive populations. However, these studies suggest that for an effective vaccine against leishmaniasis, a controlled *Leishmania* infection that can provide a complete array of Ags of a wild-type parasite might be necessary for developing a protective immune response. Therefore, live-attenuated parasites that are nonpathogenic might induce the same protective immunity as leishmanization and thus would be ideal vaccines. Past experience with other pathogens such as viruses and bacteria has suggested that live-attenuated pathogens can be successful vaccines (9–11).

To test the hypothesis that live-attenuated parasites can be effective vaccines, previously we developed an amastigote-specific, replication-deficient, centrin gene–deleted *Leishmania donovani*

*Division of Emerging and Transfusion Transmitted Diseases, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD 20993; [†]Department of Pathology, The Ohio State University Wexner Medical Center, Columbus, OH 42310; [‡]National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892; and [§]Institute of Molecular Medicine, New Delhi 110020, India

[1]R. Dey and G.N. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Hira L. Nakhasi or Dr. Abhay R. Satoskar, Division of Emerging and Transfusion Transmitted Diseases, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Building 71, Room 4266, 10903 New Hampshire Avenue, Silver Spring, MD 20993-0002 (H.L.N.) or Department of Pathology, The Ohio State University Wexner Medical Center, 129 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 42310 (A.R.S.). E-mail addresses: Hira.Nakhasi@fda.hhs.gov (H.L.N.) or Abhay.Satoskar@osumc.edu (A.R.S.)

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; CL, cutaneous leishmaniasis; DC, dendritic cell; dLN, draining lymph node; DTH, delayed-type hypersensitivity; FTAg, freeze-thawed Ag; i.d., intradermal; iNOS, inducible NO synthase; LN, lymph node; L-NMMA, N^G-monomethyl-L-arginine; MHC-II, MHC class II; VL, visceral leishmaniasis.

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parasite cell line ($LdCen^{-/-}$) that was tested in a rodent model and was found to have limited persistence and induced a protective cellular immune response in immunized animals (12). Recently, we developed another *L. donovani* cell line devoid of the p27 gene ($Ldp27^{-/-}$), encoding an amastigote-specific cytochrome *c* oxidase component and demonstrated that these parasites persist longer and also induce lasting protective immunity (13, 14). From these studies, we observed that longer persistence of *Leishmania* Ags can produce robust protection. For example, the $Ldp27^{-/-}$ parasite cell line, which can persist for an extended period of time (>12 wk) without causing pathogenesis, elicits an effective cell-mediated effector protective immune response against visceral leishmaniasis (VL) (14). Other investigators have also tested genetically modified live-attenuated *Leishmania* parasites as vaccine candidates in animal models and demonstrated variable protective immunity against different forms of leishmaniasis (15–20).

Because leishmaniasis is caused by several different species of *Leishmania* and each infection has a different clinical outcome, it would be ideal to have a vaccine that can afford protection across species. Toward this end, it has been previously observed that cross-immunity can be acquired by pre-exposure to infection as was demonstrated in individuals who migrated from an *L. major* endemic region and had a lower risk of developing VL (21, 22). Furthermore, in several animal model studies, cross-species protection has been reported between VL and cutaneous leishmaniasis (CL) using either crude or purified parasite Ags, DNA vaccines, or irradiated promastigotes (23–27). There are also reports of *L. donovani* DNA vaccine cross-protecting against cutaneous murine *Leishmania mexicana* infection (28, 29). Additionally, immunization with lower doses of infectious parasites also has been shown to provide cross-protection. For example, vervet monkeys infected with subclinical doses of *L. donovani* were cross-protected against *L. major* infection (23). Rhesus monkeys who recovered from a low-dose *L. major* infection showed significant protection against *Leishmania amazonensis* and *Leishmania Guyanenesis* but lacked protection against *Leishmania braziliensis* (30). Alternatively, monkeys recovered from *L. braziliensis* or *L. chagasi* infection were protected from challenge with *L. amazonensis* (30). Preliminary studies from our laboratory using genetically modified *L. donovani* live-attenuated parasites as immunogens also has shown to provide cross-protection against *L. major* and *L. braziliensis* infections, causative agents for CL and mucocutaneous leishmaniasis, respectively, in mice (14). However, in most of these studies a detailed analysis of immunological correlates of protection has not been well documented.

Therefore, in this study we have undertaken to analyze the mechanism of cross-protection by immunization with *L. donovani* live-attenuated parasites against *L. mexicana*, the causative agent of CL and diffuse CL. In murine models, *L. mexicana* causes a progressive disease in susceptible BALB/c mice. The Th2 response in BALB/c mice is responsible for disease progression whereas induction of Th1 cytokines leads to disease resistance (28, 31, 32). In this study we have demonstrated that live-attenuated *L. donovani* can provide long-term protection against *L. mexicana* infection. We also examined the type of immune cells involved in the wound healing process within the lesions and the cytokines produced by such cells. Protection against heterologous challenge occurs through robust host cellular immune responses, and both CD4 and CD8 T cells play an important role in cross-protection. Interestingly, we also observed important differences in the induction of immune response between the two live-attenuated parasite strains tested. Additionally, we also investigated the innate response in host bone marrow–derived dendritic cells (BMDCs) infected with $Ldp27^{-/-}$ and $LdCen^{-/-}$. Specifically, we found that both the live-attenuated parasite infection in DCs significantly enhanced production of IL-12 compared with the *LdWT* parasite, and we were able to promote proliferation of OVA-specific $CD4^+$ T cells and induce Th1-type immune responses in vitro. Additionally, the control of infection in DCs was independent of induction of innate response, suggesting gene-deleted live-attenuated parasites are safe as vaccine candidates.

Materials and Methods

Animals and parasites

Five- to 6-wk-old female BALB/c mice from the National Cancer Institute (Bethesda, MD) were used in the experiments. OVA peptide–specific DO11.10 TCR transgenic mice (BALB/c) were purchased from The Jackson Laboratory and were maintained in a pathogen-free animal facility at The Ohio State University in accordance with National Institutes of Health and institutional guidelines. Animal study procedures used were reviewed and approved by the Animal Care and Use Committee of the Center for Biologics Evaluation and Research, U.S. Food and Drug Administration. *Leishmania* parasites were cultured according to procedures previously described (33, 34). *L. mexicana* promastigotes were grown at 26°C in medium 199 supplemented (M199/S) with 20% FCS.

Cultivation of BMDCs

Female BALB/c mice (aged 6–8 wk) were sacrificed to excise femurs and tibias. Bone marrow was isolated and cultured with complete RPMI medium supplemented with 10% (v/v) FBS and 1% penicillin (20 U/ml)/streptomycin (20 μg/ml) and GM-CSF for 7 d to obtain >75% purity of $CD11c^+$ DCs. BMDCs were harvested and plated on 24-well tissue culture plates. For early infection studies, 5×10^7 *LdWT*, $Ldp27^{-/-}$, and $LdCen^{-/-}$ parasites were stained with 5 μM CFSE at 37°C for 10 min and DCs were infected with CFSE-stained parasites for 3 and 7 h. Parasite uptake was analyzed by flow cytometry by gating on $CD11c^+$ DCs ($CD11c^+/CFSE^+$ cells). For parasite clearance assays, cells were infected with the respective parasites (1:4) and, after 6 h, cells were washed with culture medium to remove the extracellular parasites. Then, infected cells were cultured for 24 and 72 h. At the respective time points, cells were washed with PBS, fixed with methanol, stained with Giemsa, and intracellular parasite numbers were evaluated microscopically. In another set of experiments DCs were treated with blocking Ab anti–TNF-α (1 μg/ml) or 0.5 mM N^G-monomethyl-L-arginine (L-NMMA) for 1 h and then cells were infected with the respective parasites and infected cells were cultured for 72 h. Cells were washed with PBS, fixed with methanol, stained with Giemsa, and intracellular parasite numbers were evaluated microscopically. For cytokine and NO measurements, DCs were infected with parasites and stimulated with LPS (1 μg/ml) for 24 h. Culture supernatants were collected at 24 h postinfection to evaluate cytokine production by ELISA and NO production by Griess assay as previously described (12). Capture and detection cytokine ELISA Abs for IL-12 and TNF-α were purchased from BioLegend.

In vitro DC and T cell coculture studies

For T cell coculture assays, DCs were harvested, pulsed with 2 μg/ml OVA peptide 323–339 (AnaSpec), and infected with *LdWT*, $Ldp27^{-/-}$, and $LdCen^{-/-}$ parasites for 18 h. Parasite-infected OVA-pulsed DCs were cocultured with $CD4^+$ T cells purified from spleens of DO11.10 transgenic mice for 7 d. T cells were stained with CFSE to evaluate T cell proliferation by flow cytometry. Culture supernatants were collected at day 7 to evaluate cytokines by ELISA. Capture and detection Abs for IFN-γ and IL-10 were purchased from BioLegend and for IL-4 from BD Biosciences.

Immunizations and challenge studies

Mice were immunized via tail vein with 3×10^6 stationary phase $Ldp27^{-/-}$ promastigotes or $LdCen^{-/-}$ promastigotes. In each study, at least four to five mice were used per group. Six weeks after immunization the mice were challenged with 750 metacyclic *L. mexicana* promastigotes by intradermal (i.d.) injection in the left ear. The numbers of *L. mexicana* parasites in infectious inoculum were determined by a titration study with different numbers of parasites, and from that study we found that 750 metacyclic parasites can cause reproducible pathology in BALB/c mice ear. Parasite loads were measured from the ears of *L. mexicana*–challenged mice by limiting dilutions as previously described (35).

Measuring the delayed-type hypersensitivity response and lesion development

Six week postimmunized mice were injected with *L. mexicana* freeze-thawed Ags (FTAg; 100 μg/ mice) in their ears. After 24 h, immunized mice developed redness around the injection site, and this was measured

48 h postinjection. Disease progression was monitored at weekly intervals by measuring the diameter of the infected ear minus the diameter of the uninfected ear. The ears were measured using a Mitutoyo pocket thickness gage (Mitutoyo American, Aurora, IL). Data are presented as mean lesion size in millimeters ± SE from three independent experiments with similar results (n = 20 mice/group).

Determination of parasite burden

Parasite burdens within the infected ears were determined by limiting dilution analysis. The infected ears were removed, separated, and passed through a 70-μm nylon cell strainer (BD Falcon, Bedford, MA). Cells were washed in PBS and serially diluted (1:10 dilutions) across a Costar 96-well flat-bottom tissue culture pate (Corning, Corning, NY) in M199 cell culture media in duplicate and incubated at 26°C without CO_2 for 5–10 d. At this time, the greatest dilution yielding viable parasites was recorded for each ear. Data are presented as mean parasite dilution ± SE from three independent experiments (n = 20 mice/group).

Splenocyte and lymph node culture

The draining lymph nodes (dLNs) and spleens were removed from *L. mexicana*–infected animals 7 wk postinfection. LN cells (3×10^5) or splenocytes (5.0×10^5) were added to the wells of a 96-well flat-bottom tissue culture plate. Cells were stimulated with 40 μg/ml freeze-thawed *L. mexicana* or *L. donovani* Ag or supplemented media as a control. Cells were incubated at 37°C with 5% CO_2 for 72 h. The supernatants were collected from parallel cultures for ELISA quantification of cytokine production. Splenocyte cultures were analyzed for IL-4 (BioLegend, detection limit 3 pg/ml) and IFN-γ (BioLegend, detection limit 20 pg/ml).

Multiplex cytokine ELISA

Cytokine analysis from the LN culture supernatants was performed using a Multiplex mouse cytokine/chemokine magnetic panel from Millipore and a Luminex 100 (Luminex, Austin, TX) system using Bio-Plex manager software 5.0. The cytokine analysis procedure was performed according to the manufacturer's instructions, and the level of cytokine concentration was determined by using a standard curve for each specific cytokine provided with the Milliplex kit (Millipore).

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. The Ab titers were determined using the following HRP-conjugated secondary Abs: rabbit anti-mouse IgG (H+L)–HRP, rabbit anti-mouse IgG1–HRP, and rabbit anti-mouse IgG2a–HRP (SouthernBiotech, Birmingham, AL; all with 1:1000 dilutions). SureBlue (KPL, Gaithersburg, MD) was used as a peroxidase substrate. After 15 min, the reaction was stopped by the addition of 100 μl 1 M H_2SO_4 and the absorbance was read at 450 nm.

Histological and immunohistochemical staining

Mouse ears were fixed in fixative solutions (10% buffered formalin phosphate solution). Paraffin-embedded sections were stained with H&E, Masson's trichrome staining for collagen, toluidine blue and Alcian blue/safranine staining for mast cells, and Luna stain for eosinophils. Immune staining was also done using an anti-macrophage Ab, anti–MHC class II (MHC-II) Ab, an anti–inducible NO synthase (iNOS) Ab (1:50 dilution, Abs from Abcam), and an anti–IL-10 Ab (1:50 dilution, Ab from BioLegend). All of the histochemical and immunohistochemical staining was done by Histoserv (Gaithersburg, MD). Stained sections were analyzed under an Olympus BX51 microscope with an Olympus DP73 camera using Olympus cellSens Dimension version 1.9 at the Infectious Disease Pathogenesis Section, Comparative Medicine Branch, Division of Intramural Research, National Institute of Allergy and Infectious Diseases/National Institutes of Health.

Real-time PCR

Total RNA was extracted from the ears using a PureLink RNA Mini kit (Ambion), which also eliminates any contaminating DNA by using on-column PureLink DNase treatment during RNA purification. Four hundred nanograms of total RNA was reverse transcribed into cDNA using random hexamers with a high-capacity cDNA reverse transcription kit (Applied Biosytems). Gene expressions were determined using TaqMan Gene Expression Master Mix and premade TaqMan Gene Expression assays (Applied Biosystems) using a CFX96 Touch real-time system (Bio-Rad, Hercules, CA) and the data were analyzed with CFX Manager software. The TaqMan Gene Expression Assay ID (Applied Biosystems) of different cytokines used were as follows: IL-4, Mm00445259_m1; IL-13, Mm99999190_m1; IL-10, mm00439614_m1; iNOS2, Mm00440502_m1; and GAPDH, Mm99999915_m1. Expression values were determined by the $2^{-\Delta\Delta Ct}$ method where samples were normalized to GAPDH expression and determined relative to untreated sample.

CFSE proliferation assay

The proliferative capacity of T cells was assessed by a CFSE dilution assay in *Ldp27*$^{-/-}$-immunized mice before and after challenge with wild-type parasites (36). Age-matched naive mice served as negative controls for Ag-specific proliferation. Lesion-derived lymphocytes 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/well. Cells were cultured for 5 d at 37°C with 5% CO_2 under stimulation with FTAg (50 μg/ml). Cells were harvested, washed, and blocked with anti-CD16/32 (5 μg/ml) for 20 min (4°C) and stained with anti-mouse CD3 allophycocyanin–eFluor 780 for 30 min (each with 1:200 dilution; 4°C). Cells were acquired on an LSR II (BD Biosciences) equipped with 405-, 488-, 532-, and 638-nm laser lines using FACSDiva 6.1.2 software. Data were analyzed with FlowJo software version 9.1.5 (Tree Star, San Carlos, CA).

Intracellular staining and flow cytometry

The dLNs of infected ear were removed from *L. mexicana*–infected animals 7 wk postinfection. LN cells (3×10^5) were added to the wells of a 96-well flat-bottom tissue culture plate. Cells were stimulated with 40 μg/ml freeze-thawed *L. mexicana* Ag (37) and incubated 12 h at 37°C with 5% CO_2. Then cells were stimulated with leukocyte activation cocktail (BD Pharmingen) at 37°C with 5% CO_2 for the final 4 h. For surface staining, cells were blocked at 4°C with rat anti-mouse CD16/32 (5 μg/ml) from BD Pharmingen for 20 min. Cells were then stained with anti-mouse CD3 allophycocyanin–eFluor 780, anti-mouse CD4 eFluor 450, anti-mouse CD8a eFluor 605NC (eBioscience) for 30 min (each with 1:200 dilution; at 4°C). The cells were then stained with Live/Dead fixable aqua (Invitrogen/Molecular Probes) to stain dead cells. Cells were washed twice with wash buffer and fixed with a Cytofix/Cytoperm kit (BD Bioscience) for 20 min at room temperature. Intracellular staining was performed with anti-mouse IFN-γ PE-Cy7, anti-mouse TNF-α PerCP–eFluor 710, anti-mouse IL-2 allophycocyanin and anti-mouse IL-10 PE (eBioscience) for 30 min (each with 1:300 dilution; at 4°C). Cells were washed twice with permeabilization buffer and acquired on an LSR II (BD Biosciences) equipped with 407-, 488-, 532-, and 633-nm laser lines using FACSDiva 6.1.2 software. Data were analyzed with FlowJo software version 9.7.5 (Tree Star). 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.

Adoptive cell transfer

Total T cells or CD4 or CD8 T cells from mice 6 wk postimmunization with *Ldp27*$^{-/-}$, *LdCen*$^{-/-}$, or from 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 cells, CD4 T cells, or CD8 T cells isolation kits as recommended by the manufacturer. Then, 10×10^6 total T cells or CD4 or CD8 T cells were transferred to recipient mice by tail vein injection, and 24 h later all groups of mice were infected with 750 metacyclic *L. mexicana* promastigotes by i.d. injection in the left ear. The purity of the isolated T cells population was >95% as observed by flow cytometry. In a separate set of experiments, total T cells were isolated from 6 wk postimmunized or nonimmunized naive mice using a Pan T cell isolation kit (Miltenyi Biotec). Cells were stained with CFSE as described before (14), and 20×10^6 cells were transferred to recipient mice by tail vein injection, and 24 h later all groups of mice were infected with 1×10^6 *L. mexicana* metacyclic parasites in the ear by i.d. injection.

Statistical analysis

Statistical analysis of differences between means of groups was determined by unpaired two-tailed Student t tests 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 L. donovani *parasites confer significant long-term protection against* L. mexicana *infection in BALB/c mice*

BALB/c mice were immunized with either *LdCen*$^{-/-}$ or *Ldp27*$^{-/-}$ parasites. Six weeks after immunization, immunized mice and also nonimmunized mice were challenged with wild-type *L. mexicana* parasites. We determined the minimum number of parasites to establish a reproducible lesion in the ear of infected mice, and based on this study we found a dose of 750 *L. mexicana* metacyclic parasites is required in the initial inoculum (data not shown). All nonimmunized animals developed lesions upon *L. mexicana* challenge (Fig. 1A, 1B), whereas the lesions were either absent or much reduced in size in the immunized animals (Fig. 1A, 1B). Parasite burdens in the ears were also significantly reduced in both immunized groups compared with nonimmunized animals (Fig. 1C). Animals immunized with *Ldp27*$^{-/-}$ also mounted a strong delayed-type hypersensitivity (DTH) induration response against *L. mexicana* freeze-thawed Ag (Fig. 1D), which correlated with the significantly higher percentage of *L. mexicana* Ag–specific proliferating T cells in the ear of *Ldp27*$^{-/-}$-immunized animals (Fig. 1E). Interestingly, *LdCen*$^{-/-}$-immunized mice failed to produce any significant DTH response even after 72 h of Ag stimulation, and there was no significant Ag-specific T cell proliferation.

Previously we found that even after 24 wk of immunization, mice were protected against a homologous *L. donovani* challenge (12). In the present study, we also observed that mice challenged with heterologous *L. mexicana* parasites in the ear are still protected after 30 wk of immunization, as indicated by significant reduction in lesion size and parasite burden within the ears (Supplemental Fig. 1). Overall, these results showed both *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ also induce long-lasting immunity against *L. mexicana*.

Histochemical analysis of the ear of live-attenuated, parasite-immunized mice postinfection with L. mexicana *showed a wound healing response*

Inflammatory cells recruited to the site of infection play an important role in the lesion healing response versus disease progression. The H&E-stained sections of infected ears from nonimmunized or *Ldp27*$^{-/-}$- or *LdCen*$^{-/-}$-immunized mice challenged with *L. mexicana* showed significant cellular infiltration into the lesion site (Fig. 2). The ears of nonimmunized mice showed moderate numbers of infiltrating mononuclear cells harboring significant numbers of parasites (Fig. 2D, red circle and arrow) and fewer lymphocytes in the dermis (Fig. 2A, 2D), whereas the ear sections of both immunized groups of mice showed moderate numbers of lymphocytes similar to nonimmunized mice but that contained significantly reduced numbers of *Leishmania* parasites compared with the nonimmunized group (Fig. 2E, 2F). Because collagen deposition is a marker for wound healing (38), we analyzed collagen deposition in the infected ears from all groups by Masson's trichrome staining (Fig. 2G–I). Ears from nonimmunized mice displayed very sparse and disorganized deposition of collagen fibers (Fig. 2G, lack of blue staining). However, deposition of collagen fibers in immunized groups was thick and

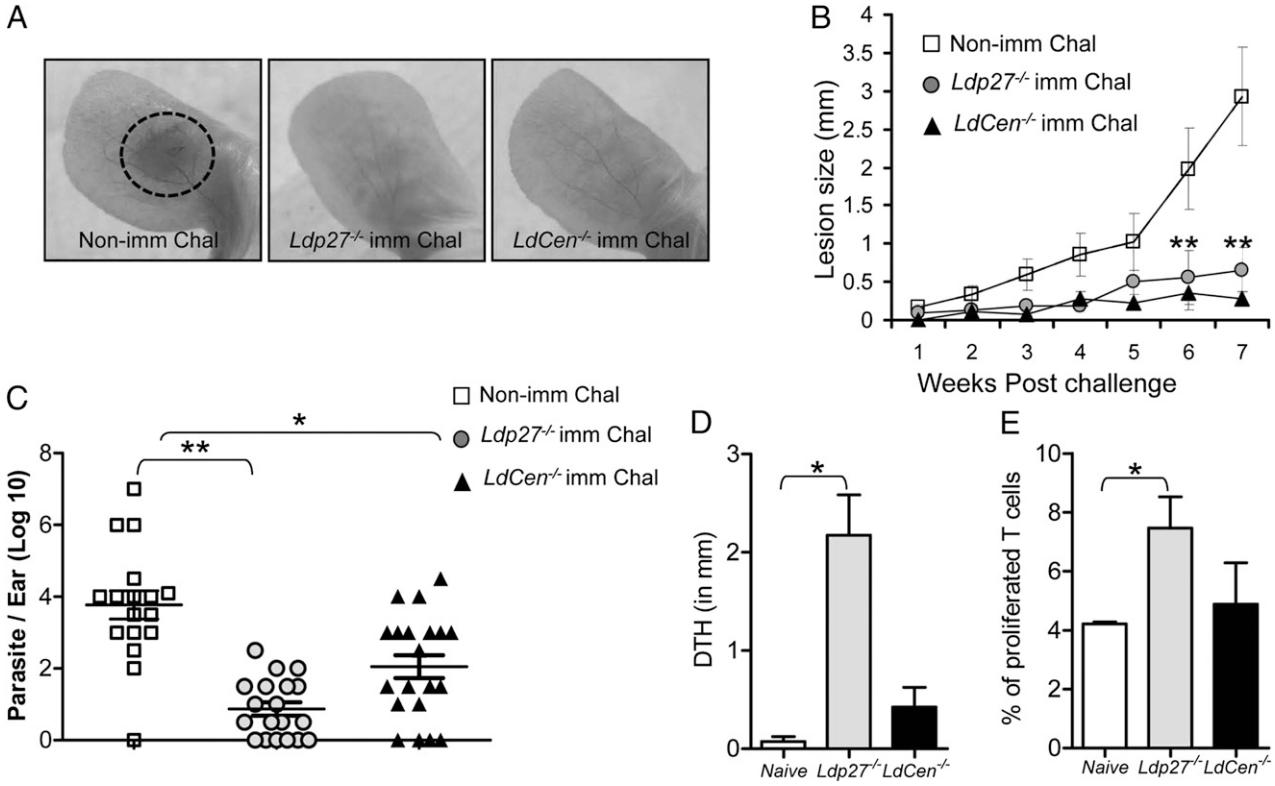

FIGURE 1. Immunization with genetically attenuated *L. donovani* parasites protects mice against wild-type *L. mexicana* challenge. Three different groups of BALB/c mice were inoculated i.v. with either *Ldp27*$^{-/-}$ or *LdCen*$^{-/-}$ or PBS as a naive control. Six weeks after immunization the mice were challenged with *L. mexicana* parasites in the ears. (**A**) Macroscopic images of ears from 7 wk postchallenged mice. (**B**) Ear lesion (black dotted circle) diameters were measured each week. (**C**) Parasite burdens at 7 wk postchallenge were measured by serial dilution of ear tissue homogenates. The data represented are cumulative of a least 15 mice per group from three independent experiments. (**D**) Attenuated parasite-immunized mice induce cell-mediated DTH responses against *L. mexicana* Ag. At 6 wk postinfection, 100 μg soluble *L. mexicana* FTAg was injected into the ears of all immunized and naive animals (n = 3/group). After 48 h, the circumference of the Ag injection site was measured. (**E**) The ears of immunized and nonimmunized mice were harvested, stained with CFSE, and stimulated with *L. mexicana* FTAg in culture for 5 d; cells were stained with anti-CD3e Ab and cell proliferations was measured by flow cytometry. Data are representative of two independent experiments (n = 4). *$p < 0.05$, **$p < 0.01$.

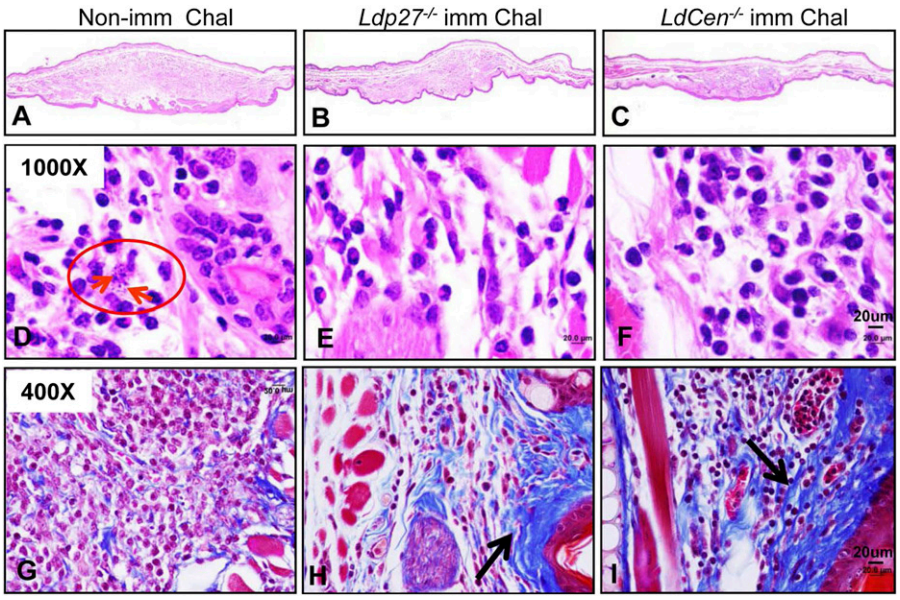

FIGURE 2. Histochemical analysis of the ear lesions of BALB/c mice immunized with live-attenuated parasites at 7 wk post-challenge with *L. mexicana*. Mouse ear samples were fixed in 10% neutral formalin and paraffin embedded. Four- to 5-μm sections were made and H&E stained (**A–F**). Red circle indicates a parasite focus, with a red arrow indicating intracellular amastigotes. Original magnification: *upper panel* (A–C), ×25; *lower panel* (D–F), ×1000. Masson's trichrome staining was performed on representative samples of each group to analyze deposition of organized collagen fibers (blue stain marked with arrow) present only in the immunized mice) (**G–I**) (original magnification, ×400).

highly organized (Fig. 2H, 2I, increased blue staining). These data suggest that immunized mice with smaller lesions and no parasites display a better wound healing response as compared with non-immunized challenged mice, which experience progressive disease.

Immunohistochemical analysis of infiltrating macrophages and cells expressing MHC-II and IL-10 in the ears of mice immunized with live-attenuated parasites after L. mexicana *infection*

Our observations from H&E-stained ear sections (Fig. 2) prompted us to investigate the nature of infiltrating cells at the lesions after infection with *L. mexicana* in nonimmunized as well as live–attenuated, parasite-immunized mice. We used immunohistological techniques to characterize the cellular distribution of APCs (macrophages and MHC-II–expressing cells) in *L. mexicana*–challenged mouse ears. Infected ears from immunized mice contained more macrophages than did nonimmunized mouse ears as indicated by the increased number of macrophage Ab stained cells (compare Fig. 3B and 3C with Fig. 3A). Similarly, staining with an anti–MHC-II Ab showed abundant numbers of APCs in the lesions of immunized/challenged mice compared with the non-immunized group (compare Fig. 3E and 3F with Fig. 3D).

IL-10 mediates anti-inflammatory effects during infection by inhibiting production of proinflammatory cytokines and Ag presentation, and previous studies have shown that IL-10 receptor blockade leads to increased monocyte recruitment in *L. mexicana*–induced mouse ear lesions (39, 40). Staining with an anti–IL-10 Ab revealed that the lesions from immunized mice contained fewer IL-10–producing cells compared with those from non-immunized mice (compare Fig. 3G with Fig. 3H and 3I). These data suggest that *L. donovani* live-attenuated, parasite-immunized mice recruited more APCs to the lesion site following *L. mexicana* challenge, and the observed protection correlates with reduced IL-10 secretion at the site of infection.

Parasite survival or killing is dependent on the ability of NO production by host macrophages (41). We observed that expression of iNOS was significantly higher in the lesions of immunized mice compared with nonimmunized mice (compare Fig. 3K and 3L with Fig. 3J). Interestingly, the level of iNOS expression was much higher in $Ldp27^{-/-}$ immunized mice (Fig. 3K) compared with mice immunized with $LdCen^{-/-}$ (Fig. 3L).

Furthermore, to quantify the expression level of different cytokines in the lesions of immunized and nonimmunized mouse ears, we performed RT-PCR analysis. In nonimmunized mouse ear lesions high expression of IL-10, IL-4, and IL-13 and reduced expression of iNOS2 were observed (Fig. 3M). However, in immunized mouse ear lesions, reduced expression of IL-10, IL-4, and IL-13 and a higher tendency of expression of iNOS2 were observed. Interestingly, there are differences in the induction of iNOS2 between the $Ldp27^{-/-}$-immunized mice ear lesion, which had significantly higher expression of iNOS2, compared with the nonimmunized group; additionally, the $LdCen^{-/-}$-immunized mouse ear lesions had a slight (but not significant) increase in iNOS2 expression compared with the nonimmunized lesions. Note that the quantitative expression levels of cytokines correlate with the qualitative immunohistochemistry analysis of the ear lesions.

The ears of live-attenuated, parasite-immunized mice show an influx of mature and active mast cells upon L. mexicana *challenge*

A previous study found that several immune mediators, including granules released by mast cells, play an important role in the progression of *L. mexicana*–induced lesions in BALB/c mice (42). Our earlier observation (Fig. 2) of increased cellular infiltration in both immunized and nonimmunized infected ears prompted us to analyze mast cells in the lesions of these mice. Alcian blue/safranin staining (Fig. 4A–F) was used to assess the level of mast cell activation, and toluidine blue (Fig. 4G–L) was used for general morphological observations and to reveal mast cells with cytoplasmic granules based on metachromatic staining. A substantial infiltration of mast cells was observed in the ear lesions of nonimmunized mice (Fig. 4A, red-stained cells; Fig. 4G, blue-stained cells), and most mast cells are metachromatic-positive, granule-containing mature and active cells (Fig. 4D, 4J). In contrast, the ear lesions from immunized-challenged mice had reduced infiltration of mast cells (Fig. 4B, 4C, 4H, 4I) and most of them were metachromatic-negative, granule-containing cells (Fig. 4E, 4F, 4K, 4L). Interestingly, substantial infiltration of eosinophils was observed in the ear lesions of nonimmunized mice (Fig. 4M), and there were fewer eosinophils in the lesions of immunized mice (Fig. 4N, 4O).

Immunization with $Ldp27^{-/-}$ *or* $LdCen^{-/-}$ *parasites drives a robust mixed Th1/Th2-type immune response in the spleen*

Our previous studies showed that $Ldp27^{-/-}$ and $LdCen^{-/-}$ parasites both induce a mixed pro- and anti-inflammatory response

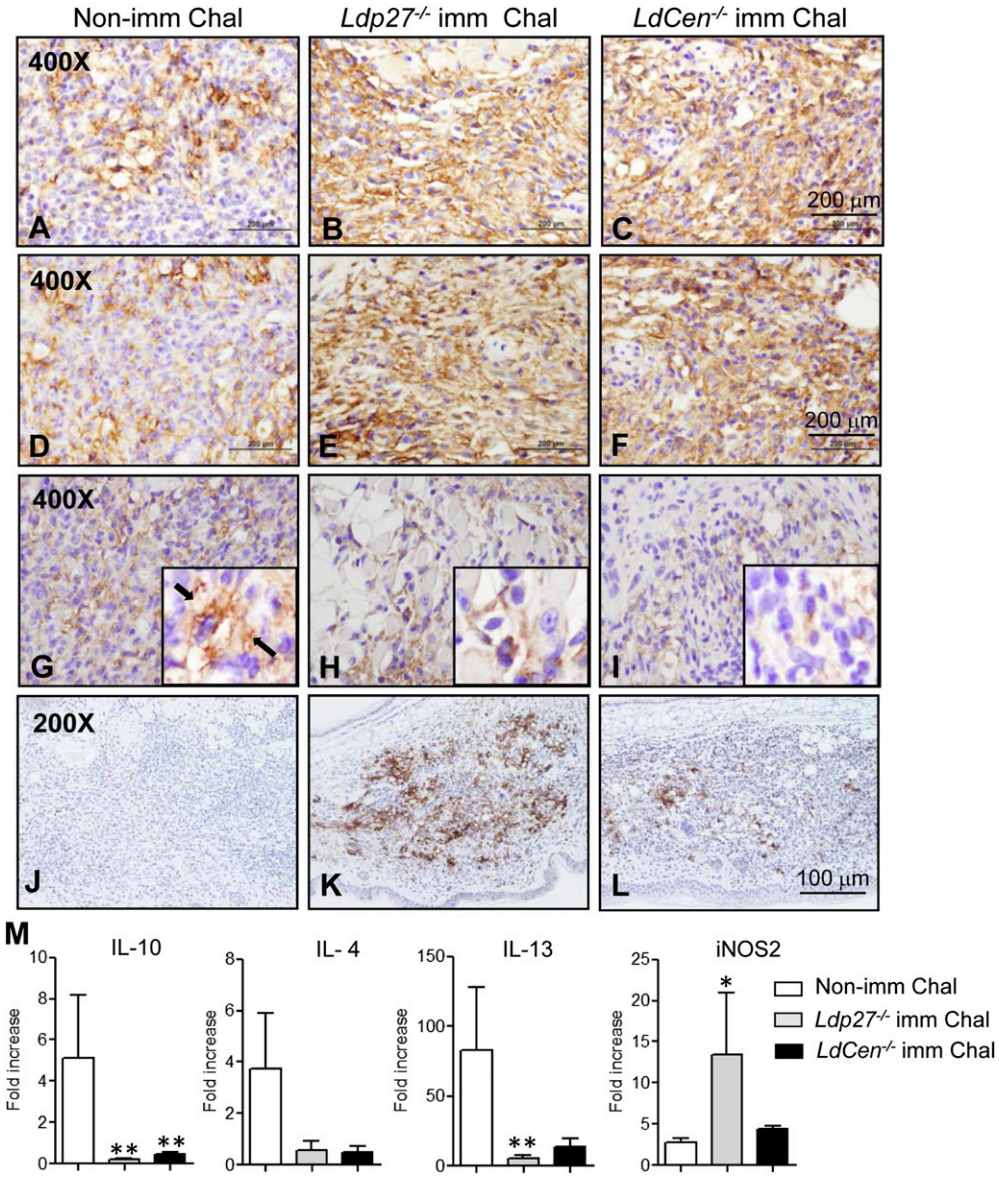

FIGURE 3. Ear lesions of immunized/challenged mice showed host-protective cellular response. Immunohistochemical analysis of paraffin-embedded ear lesions of BALB/c mice immunized with live-attenuated parasites and 7 wk postchallenge with *L. mexicana*. (**A**–**C**) Four- to 5-μm sections were stained with anti-macrophage Ab, indicated by brown color; (**D**–**F**) staining with anti–MHC-II Ab; (**G**–**I**) staining with anti–IL-10 Ab; and (**J**–**L**) staining with anti-iNOS Ab. Brown color represents respective Ab-stained cells. *Inset* shows higher magnification, with black arrow pointing to IL-10–producing cells. (**M**) Expression levels of IL-10, IL-4, IL-13, and iNOS2 were measured by RT-PCR analysis after extracting RNA from 6 wk immunized/7 wk postchallenged mice ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$.

upon homologous virulent *L. donovani* challenge (12, 14). To characterize the immune response induced by live-attenuated parasites in response to heterologous *L. mexicana* challenge, we analyzed *L. donovani* Ag–specific cytokine secretion by splenocytes from nonimmunized challenged and immunized challenged mice at week 7 after *L. mexicana* challenge. Following in vitro stimulation with *L. donovani* Ag, spleen cells from *Lpd27*$^{-/-}$- or *LdCen*$^{-/-}$-immunized *L. mexicana*–challenged mice produced significantly more Th1-associated IFN-γ and Th2-associated IL-4 cytokine compared with those from nonimmunized mice (Fig. 5A and 5B, respectively). Secretion of IFN-γ, as well as the ratio of IFN-γ/IL-4, was much higher in *LdCen*$^{-/-}$-immunized mice splenocytes s than in splenocytes of *Ldp27*$^{-/-}$ mice (Fig. 5C). These results indicate that immunization with *L. donovani* parasites (visceral species) induces a robust mixed immune response in the spleen of *L. mexicana* (cutaneous species)–infected mice.

Ldp27$^{-/-}$- *or* LdCen$^{-/-}$*-immunized mice challenged with* L. mexicana *show preferential induction of Th1 and suppression of Th2-type cytokines in the dLNs*

Next we determined the inflammatory response in the LNs draining the parasite-induced lesion. The dLN cells of immunized and nonimmunized mice were collected 7 wk after challenge with *L. mexicana* stimulated in vitro with freeze-thawed *L. mexicana* Ag, and cytokine production was measured by ELISA. Cytokine analysis from the culture supernatants of dLN cells revealed significant induction of Th1-type cytokine (IFN-γ) secretion by both *Ldp27*$^{-/-}$- and *LdCen*$^{-/-}$-immunized/challenged mice compared with nonimmune/challenged mice (Fig. 6A). Furthermore, we observed significantly less secretion of Th2-type cytokines (IL-4, IL-5, IL-6, IL-10, and IL-13) from immunized/challenged mice compared with nonimmunized challenged mice with the exception of IL-5 levels in *LdCen*$^{-/-}$-immunized/challenged mice, which

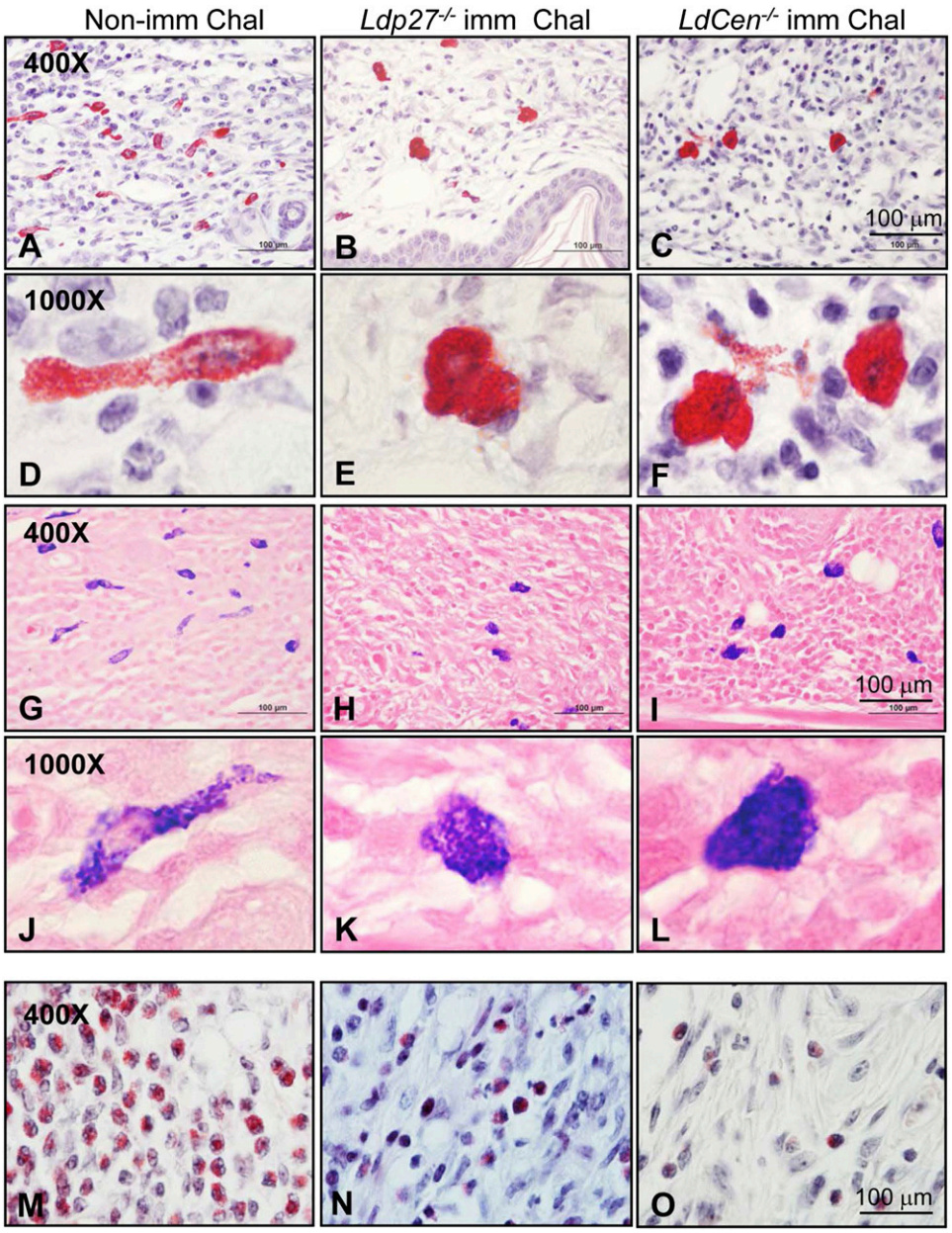

FIGURE 4. Connective tissue from the ears of immunized mice contains low numbers of active and mature mast cells. Histochemical analysis of paraffin-embedded ear lesions from BALB/c mice were immunized with live-attenuated parasites at 7 wk post-challenge with *L. mexicana*. (**A**–**F**) Alcian blue/safranin staining, (**G**–**L**) toluidine blue staining, and (**M**–**O**) Luna staining for eosinophil of consecutive paraffin-embedded section of the ear from one representative mouse from each group as indicated.

were not significantly reduced (Fig. 6A). Overall, the immune response from dLN cells from immunized mice indicated the correlates of control of *L. mexicana* growth in the lesions. In CL both CD4 and CD8 T cells are crucial in controlling disease progression (43). In this study we measured specific cytokine secretion from ear dLN–derived CD4 and CD8 T cells in response to *L. mexicana* Ags by flow cytometry (Fig. 6B; gating strategy in Supplemental Fig. 2). Percentages of IFN-γ–producing CD4 and CD8 T cells were significantly higher in both immunized (*Ldp27*$^{-/-}$ or *LdCen*$^{-/-}$) groups compared with the nonimmunized

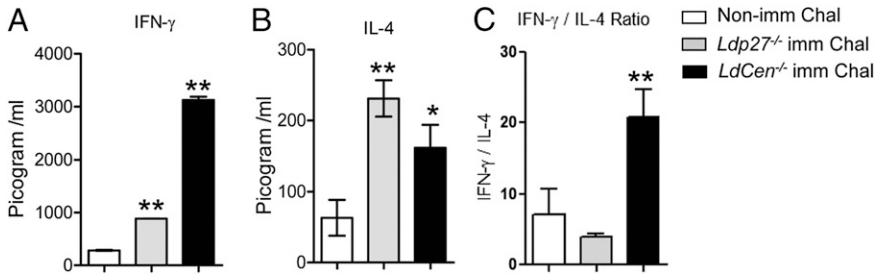

FIGURE 5. Cytokine analysis of supernatants from splenocytes of nonimmunized and live–attenuated, parasite-immunized mice 7 wk after *L. mexicana* challenge. Splenocytes were prepared from the each group of mice and stimulated with *L. donovani* Ag. Culture supernatants were collected after 72 h of stimulation, and cytokines were measured by ELISA. (**A**) IFN-γ, (**B**) IL-4, and (**C**) ratio of IFN-γ/IL-10–producing CD4 and CD8 T cells are shown. Data are representative of two independent experiments ($n = 9$). *$p < 0.05$, **$p < 0.01$.

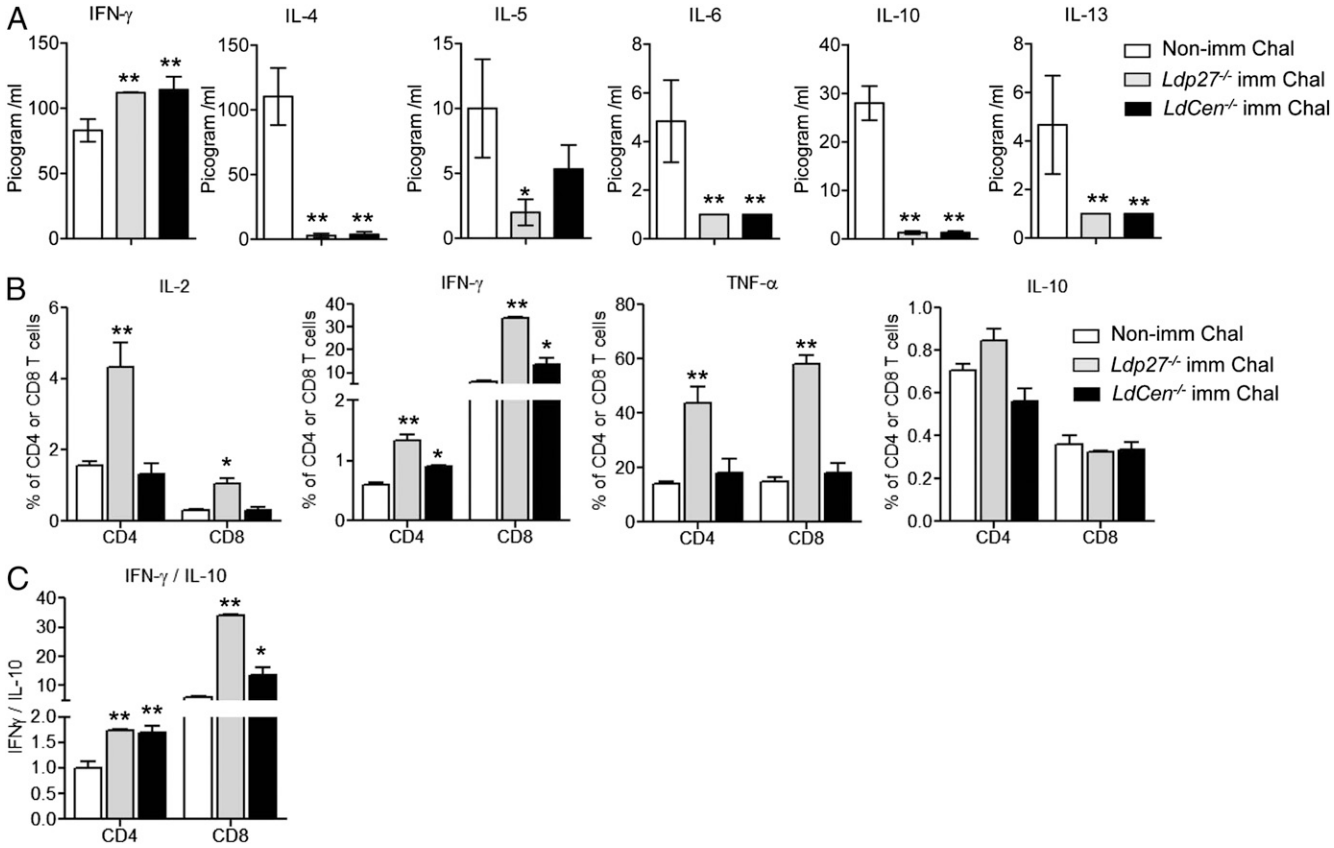

FIGURE 6. Cytokine analysis from the culture supernatants as well as intracellular expression level of *L. mexicana* Ag–stimulated dLN cells from live-attenuated, parasite-immunized mice and 7 wk after mice were challenged with *L. mexicana*. (**A**) dLN cells were collected from different groups of mice (LNs from three individual mice in the same group were polled together), stimulated with *L. mexicana* FTAg, and after 72 h the supernatants were collected and cytokines were measured with a Multiplex ELISA kit. Data are representative of two independent experiments (n = 9). (**B**) Bar diagram represents the percentage of CD4 or CD8 T cells expressing either IL-2, IFN-γ, TNF-α, or IL-10. Stained cells were analyzed by flow cytometry. (**C**) Ratio of IFN-γ/IL-10–producing CD4 and CD8 T cells. Data are representative of two independent experiments (n = 6). $^*p < 0.05$, $^{**}p < 0.01$.

group (Fig. 6B). Interestingly, in the *Ldp27*$^{-/-}$-immunized group there was a significant increase of IL-2 and TNF-α from both CD4 and CD8 T cells, but not much of response in *LdCen*$^{-/-}$-immunized group with regard to IL-2 and TNF-α. Although there were no significant differences in IL-10$^+$ cells in either CD4 or CD8 T cells among different groups, the ratios of IFN-γ/IL-10–producing cells were significantly high in both CD4 and CD8 T cells in both of the immunized groups (Fig. 6C). Overall, these results suggested a crucial role of both CD4 and CD8 T cells in protective immunity induced by the attenuated *L. donovani* parasite.

Adoptive transfer of total T cells/CD4 T cells or CD8 T cells from live-attenuated, parasite-immunized mice confers protection to recipient mice against L. mexicana *infection*

Induction of proinflammatory cytokine secretion from dLN-derived CD4 and CD8 T cells in immunized mice prompted us to characterize the specific T cells that have a role in cross-protection. Total T cells or CD4 or CD8 T cells were isolated from 6 wk postimmunized or nonimmunized mouse spleens by column-based purification as described in *Materials and Methods*; purity of total T cells is demonstrated in Supplemental Fig. 3. Mice that received total T cells from immunized mice (*Ldp27*$^{-/-}$ or *LdCen*$^{-/-}$) showed reduced lesion growth (Fig. 7A) and significant reduction in parasite burden (Fig. 7D) at 7 wk postchallenge, compared with recipients of T cells from nonimmunized mice. Mice that received CD4 or CD8 T cells from *Ldp27*$^{-/-}$-immunized mice showed significant reduction in lesion size and in parasite burden in lesions (Fig. 7B–D) at 7 wk postchallenge. Mice that received CD4 T cells from *LdCen*$^{-/-}$ showed controlled lesion growth as well as less parasite burden (Fig. 7B, 7D) compared with nonimmunized CD4 T cell recipient mice. However, mice that received CD8 T cells from *LdCen*$^{-/-}$ failed to control lesion growth as well as parasite burden (Fig. 7C, 7D).

Next we measured the proliferation capability of the different T cell subsets. We performed an adoptive transfer experiment with total T cells labeled with CFSE (Fig 7 E). Twenty-four hours after i.v. transfer, mice were challenged with *L. mexicana* in the ear. Four days after challenge dLNs from the infected ears were removed and stained with CD3/CD4 and CD8 Abs and analyzed by flow cytometry to measure the recruitment and proliferation of T cells in response to *L. mexicana* infection. We observed that there was a significantly higher percentage of CFSE$^+$ T cells/CD4 or CD8 T cells in mice that received T cells from immunized mice, compared with the mice that received T cells from nonimmunized mice (Fig. 7F, 7H, 7J). Moreover, we also gated those CFSE$^+$ donor T cells to measure proliferation of the subsets of T cells. Mice that received T cells from *Ldp27*$^{-/-}$-immunized mice showed significantly higher percentages of CD3, CD4, and CD8 T cell proliferation; however, mice that received T cells from *LdCen*$^{-/-}$-immunized mice showed a significantly higher percentage of proliferating CD3 and CD4 T cells, but not CD8 T cells (Fig. 7G, 7I, 7K). The proliferation capability of different subsets

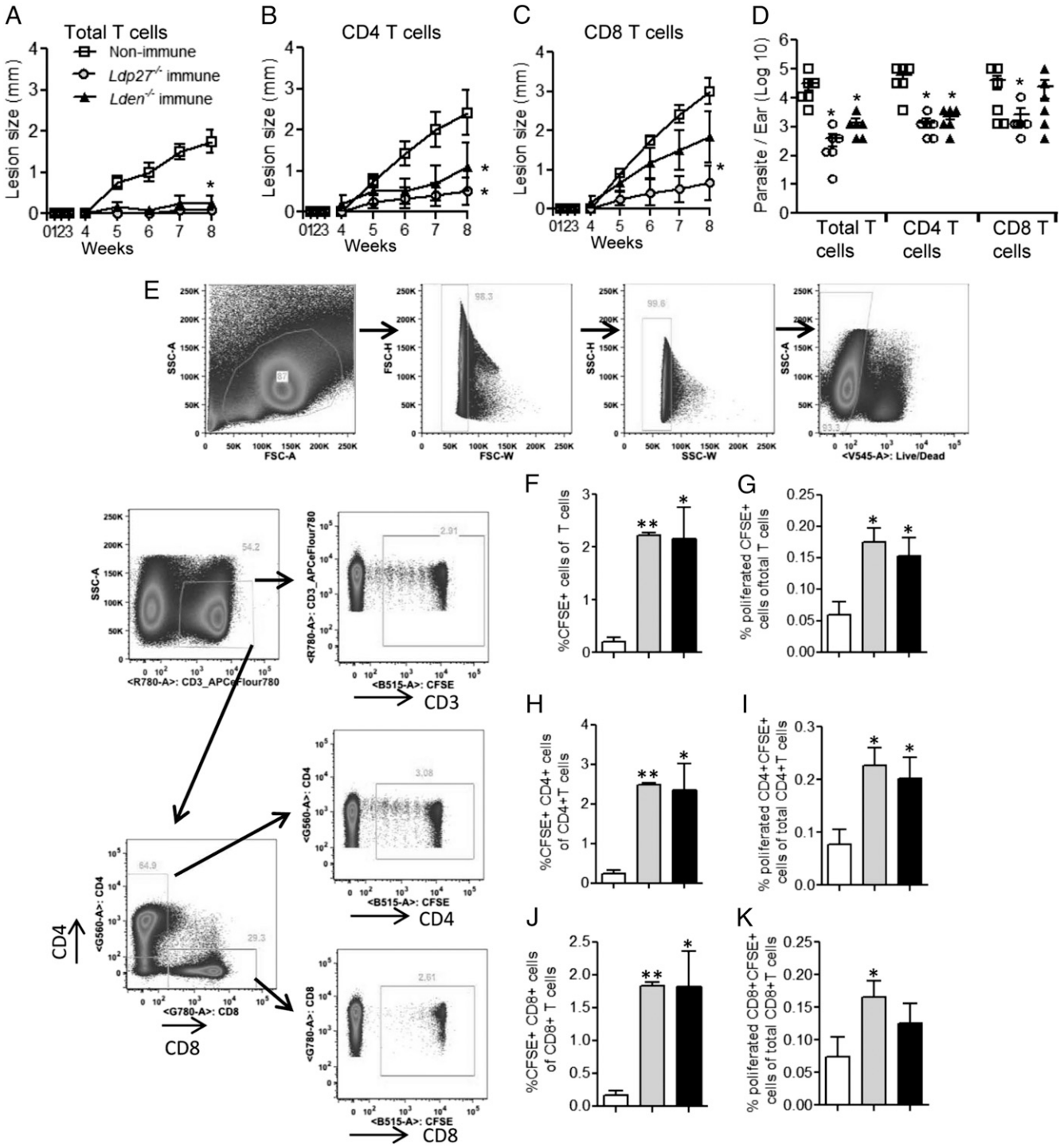

FIGURE 7. Live-attenuated *Leishmania* vaccine-induced host-protective immunity is dependent on both CD4 and CD8 T cells. Lesion development was measured in recipient mice. (**A**) Pan T cells, (**B**) CD4 T cells, (**C**) CD8 cells isolated from splenocytes of 6 wk postimmunized mice, and (**D**) parasite burden measured from the infected ear of these recipient mice groups ($n = 6$) are shown. □, Nonimmune; ○, *Ldp27*$^{-/-}$ immune; ▲, *LdCen*$^{-/-}$ immune. Proliferation capability of T cells of immunized mice was measured by adoptive transfer of CFSE-stained pan T cells in naive mice and challenged with *L. mexicana*; CFSE-stained cells from ear dLNs were studied by flow cytometry. (**E**) Representative dot plots showing the gates used for identifying the individual cell type and their proliferation capacity. (**F**, **H**, and **J**) Bar diagram shows recruitment of CFSE$^+$/CD3$^+$ or CFSE$^+$/CD4$^+$ or CFSE$^+$/CD8$^+$ T cells in the LNs of recipient mice as well as their proliferation capacity (**G**, **I**, and **K**). (G), (I), and (K) represent percentage of proliferated T cells in recruited CFSE$^+$ cells. $*p < 0.05$, $**p < 0.01$.

of T cells correlated with the lesion growth and parasite burden in respective groups. Taken together, these results suggest that *L. donovani*–attenuated, parasite-induced strong T cell immunity and both CD4 and CD8 T cells are crucial in controlling the parasite burden against wild-type *L. mexicana* infection.

Antileishmanial Ab response in the serum of immunized and nonimmunized/challenged mice

It has been previously reported that *L. mexicana* hijacks host IgG to induce a suppressive IL-10 response through FcγRs, the cell surface receptor for IgG (44). Particularly, the host IgG1 subclass

is crucial and pathogenic in *L. mexicana* infection, because it has been shown that IgG1 knockout mice control the progression of infection (45, 46). In this study, we analyzed sera from immunized/challenged and nonimmunized/challenged mice as described in Fig. 1 for determination of *L. mexicana*–specific IgG, IgG1, and IgG2a titers. In *Ldp27*$^{-/-}$-immunized/challenged mice there were significantly reduced titers of IgG and IgG1, as well as a concomitant increase in IgG2a titers compared with nonimmunized/challenged mice (Supplemental Fig. 4A–C). However, there was no significant difference in IgG and IgG1 titers between the *LdCen*$^{-/-}$-immunized/challenged mice compared with nonimmunized/challenged mice. Interestingly, there was an increase in IgG2a titers in *LdCen*$^{-/-}$-immunized mice compared with both nonimmunized as well as *Ld27*$^{-/-}$-immunized/challenged mice. The ratio of IG2a/IgG1 is significantly high in both the *Ldp27*$^{-/-}$- or *LdCen*$^{-/-}$-immunized/challenged mice compared with the naive challenged mice, with some difference between the two live-attenuated parasites, suggesting that the increase in IgG2a and decrease in IgG1 levels may have an independent role in protection by each of these attenuated parasite strains.

BMDCs infected with Ldp27$^{-/-}$ *and* LdCen$^{-/-}$ *parasites exhibit protective immune responses*

We have previously shown that *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ parasites induced long-term protective responses in murine immunization models (12, 14). Because DCs play a critical role in naive T cell activation and differentiation during *L. donovani* infection (47), we assessed the ability of *Ldp27*$^{-/-}$ or *LdCen*$^{-/-}$ parasites to modulate DC responses using an in vitro model of BMDCs. Early parasite infection was estimated by infecting BMDCs with CFSE-stained parasites and measuring phagocytosis by flow cytometry. We observed that *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ parasites have similar rates of infection compared with *LdWT* parasites at 3 and 7 h postinfection (Fig. 8A). However, BMDCs infected with *Ldp27*$^{-/-}$ or *LdCen*$^{-/-}$ displayed significantly lower percentages of infected cells and parasites per infected cell compared with those infected with *LdWT* at 24 and 72 h postinfection (Fig. 8B, 8C), thus indicating that *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ are unable to persist in BMDCs for prolonged periods. IL-12 produced by DCs plays an important role in the development of Th1 cells (48, 49). *Ldp27*$^{-/-}$ or *LdCen*$^{-/-}$ parasite-infected DCs produced significantly more IL-12 (1.6-fold and 1.3-fold, respectively) than did those infected with WT parasites (Fig. 8D).

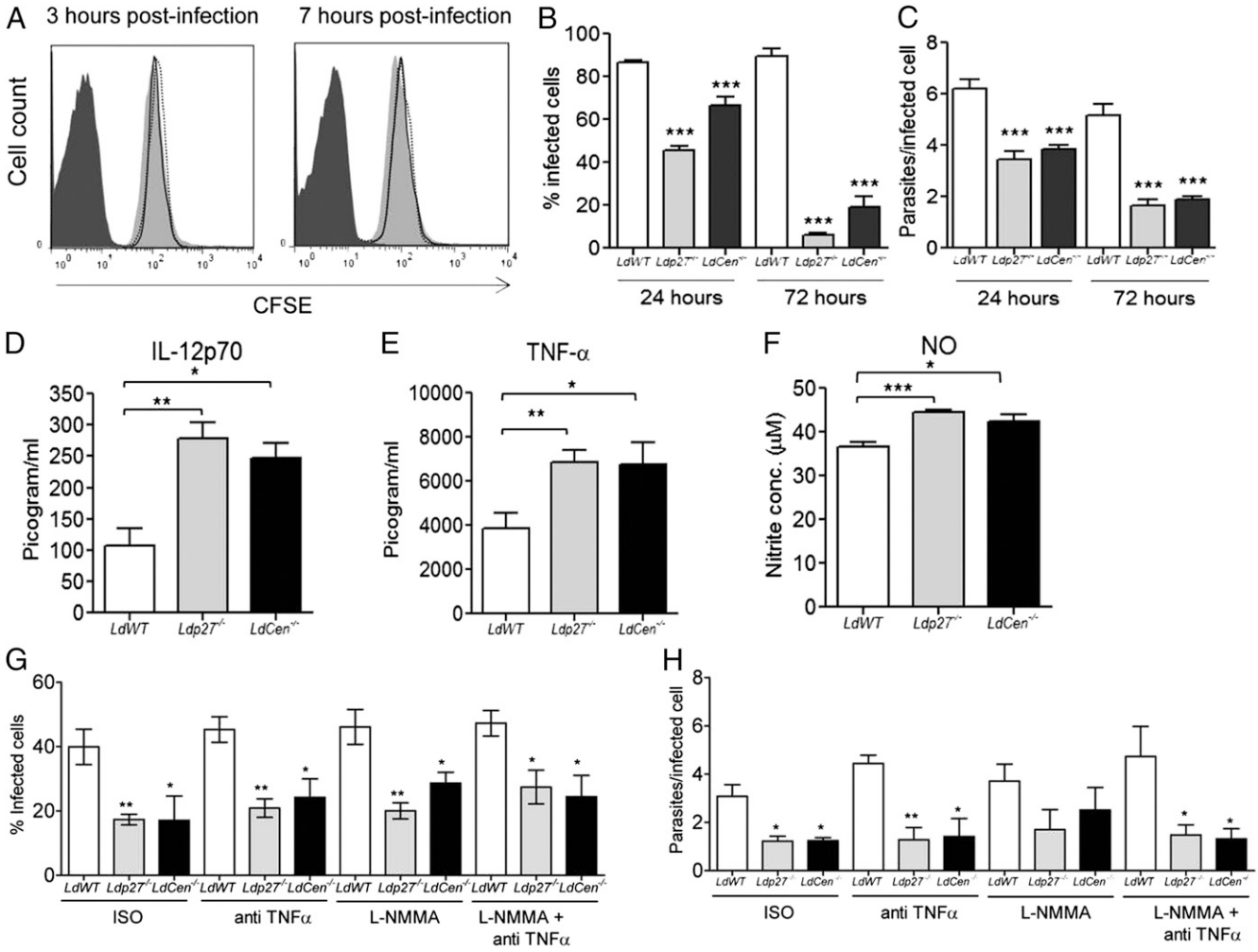

FIGURE 8. *LdWT*, *Ldp27*$^{-/-}$, and *LdCen*$^{-/-}$ parasite uptake, persistence, and cytokine responses in BMDCs. (**A**) BMDCs cultured from BALB/c mice were infected with CFSE-stained parasites for 3 and 7 h. *LdWT* (indicated as black-dotted histogram), *Ldp27*$^{-/-}$ (indicated as black-filled histogram), and *LdCen*$^{-/-}$ (indicated as gray-filled histogram) parasite uptake was measured at the indicated time points by flow cytometry. (**B** and **C**) BMDCs were infected with parasites (1:4) and intracellular parasite numbers were visualized by Giemsa staining and estimated microscopically at 24 and 72 h postinfection. BMDCs were infected with parasites (1:4) and stimulated with LPS (1 μg/ml) for 24 h. Culture supernatants were collected to analyze IL-12 (**D**) and TNF-α (**E**) production by ELISA and NO (**F**) production by the Griess assay. The data presented are representative of two independent experiments. Percentage of infected DCs (**G**) and parasites per infected cell (**H**) were measured in DCs treated with the blocking Ab anti–TNF-α or L-NMMA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Furthermore, we measured TNF-α and NO levels, as both molecules are directly involved in the killing of intracellular parasites (50). Interestingly, *Ldp27*$^{-/-}$- or *LdCen*$^{-/-}$-infected BMDCs secrete significantly more TNF-α (Fig. 8E) and NO (Fig. 8F) compared with *LdWT*-infected cells. The higher rates of *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ clearance in DCs (Fig. 8B, 8C) may be attributed to differences in cytokine and NO production in *Ldp27*$^{-/-}$- and *LdCen*$^{-/-}$-infected BMDCs compared with *LdWT*-infected BMDCs. To further confirm whether enhanced TNF-α or NO has any role in clearing the attenuated parasites, we treated DCs with anti–TNF-α Ab or L-NMMA to inhibit TNF-α secretion or NO production, respectively, and measured parasite burden (Fig. 8G, 8H). We observed that both anti-TNF-α– and L-NMMA–treated DCs that were either infected with *Ldp27*$^{-/-}$ or *LdCen*$^{-/-}$ parasites had significantly fewer parasites compared with *LdWT*-infected DCs, similar to isotype Ab–treated BMDCs. These observations clearly suggest that both the live-attenuated parasites have inherent growth defects, that is, they are unable to survive in host cells and are not controlled by the enhanced proinflammatory response.

To determine whether *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ infection in DCs is able to promote protective T cell responses in vitro, we pulsed BMDCs infected with *LdWT*, *Ldp27*$^{-/-}$, or *LdCen*$^{-/-}$ parasites with OVA peptide and cocultured them with OVA-specific (DO11.10), CFSE-labeled CD4$^+$ T cells and evaluated T cell proliferation after 7 d by flow cytometry. As shown in Fig. 9A, the percentage of proliferating T cells is significantly enhanced upon coculture with BMDCs infected with *Ldp27*$^{-/-}$- and *LdCen*$^{-/-}$-infected BMDCs compared with those cocultured with *LdWT*-infected BMDCs. In particular, *LdCen*$^{-/-}$-infected DCs induced proliferative responses that were comparable to uninfected DCs. Cytokine production was measured in culture supernatants after 7 d of coculture. IFN-γ levels in *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ T cell cocultures were slightly higher than in *LdWT*-infected BMDC cocultures, although the difference was not statistically significant (Fig. 9B). Interestingly, whereas *LdWT*-infected BMDCs induced high IL-10 production compared with OVA-pulsed uninfected DCs, IL-10 production upon coculture with *Ldp27*$^{-/-}$- and *LdCen*$^{-/-}$-infected BMDCs was significantly lower compared with *LdWT*-infected BMDCs and similar to the uninfected controls (Fig. 9C). Overall, the IFN-γ/IL-10 ratio was significantly higher in *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ T cell cultures compared with *LdWT* cocultures (Fig. 9D). Taken together, these data indicate that *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ infection in DCs is capable of inducing typically protective responses in an in vitro system and is consistent with our in vivo studies using these parasite cell lines as immunogens (12, 14).

Discussion

Previous studies from our laboratory showed that genetically modified *L. donovani* parasites (i.e., *Ldp27*$^{-/-}$ or *LdCen*$^{-/-}$) induced host-protective response in susceptible BALB/c mice against wild-type *L. donovani* parasite infection (homologous infection) and also conferred significant protection against heterologous infections (*L. major* and *L. braziliensis*, respectively) (12, 14). However, the mechanisms of cross-protection against such heterologous *Leishmania* infections remained unexplored. Furthermore, there are differences in the mechanism of pathogenesis among various Old and New World *Leishmania* species causing CL (51). Hence, in this study we characterized the *Ldp27*$^{-/-}$- or *LdCen*$^{-/-}$-induced cross-protection against *L. mexicana* infection by analyzing specific aspects of the host immune response.

Both *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ immunization confer significant protection in BALB/c mice against wild-type *L. mexicana* infection. Although the lesion parasite load was significantly lower in *Ldp27*$^{-/-}$- and *LdCen*$^{-/-}$-immunized mice compared with non-immunized mice, *Ldp27*$^{-/-}$-immunized mice displayed the lowest parasite burdens, and no parasites were detectable in the lesions

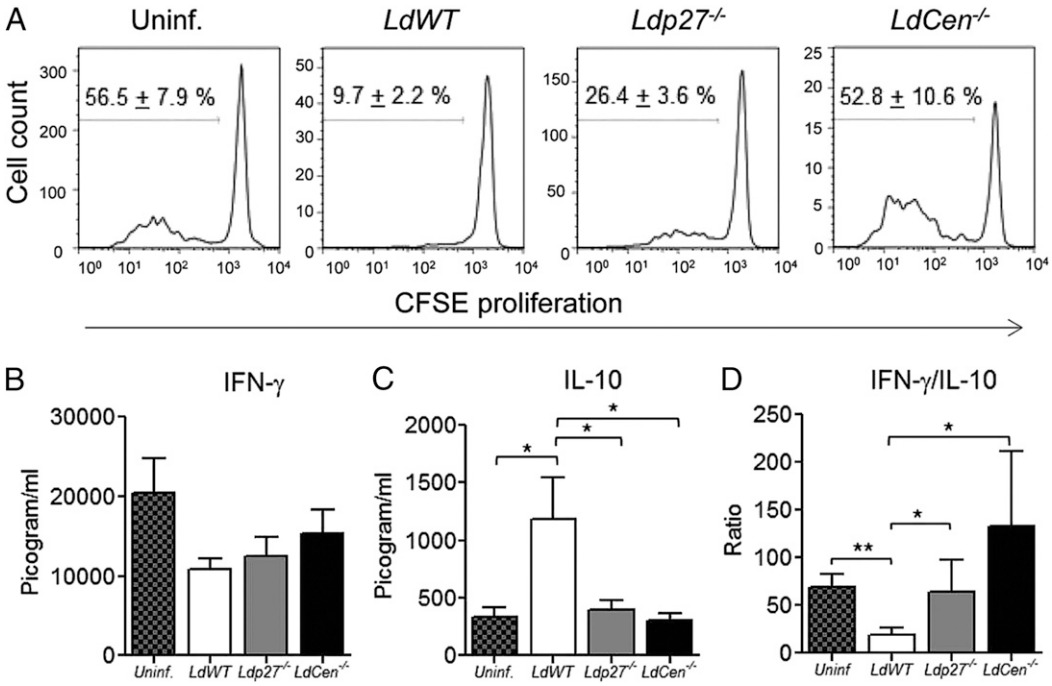

FIGURE 9. T cell proliferation and cytokine production upon coculture of parasite-infected DCs with OVA-specific transgenic T cells. (**A**) BMDCs cultured from BALB/c mice were pulsed with OVA peptide (2 μg/ml) for 2 h and infected with *LdWT*, *Ldp27*$^{-/-}$, or *LdCen*$^{-/-}$ parasites for 18 h. CD4$^+$ T cells were purified from DO11.10 OVA transgenic mice, stained with CFSE, and cocultured with parasite-infected DCs for 7 d. T cell proliferation was estimated by flow cytometry by gating on CD4$^+$ cells. (**B**–**D**) Culture supernatants were collected at 7 d of coculture to assay IFN-γ and IL-10 by ELISA. The data presented are representative of two independent experiments. *$p < 0.05$, **$p < 0.01$.

of many mice in this group. Furthermore, *Ldp27*$^{-/-}$-immunized mice also showed significantly higher DTH response and T cell proliferation against *L. mexicana* Ag compared with *LdCen*$^{-/-}$-immunized mice. This difference in response could be due to the persistence of parasites in animals allowing for longer exposure to parasite Ags (14). For example, we have previously observed that *Ldp27*$^{-/-}$ parasites persist for at least 10–12 wk, allowing longer exposure to parasite Ags as compared with *LdCen*$^{-/-}$ parasites, which persist for only 4–6 wk in mice (12, 14). Therefore, it is likely that the abundance of *Ldp27*$^{-/-}$ parasites in 6 wk post-immunized mice induce more effector T cells at the time of challenge compared with *LdCen*$^{-/-}$-immunized mice.

CL causes robust tissue inflammation at the site of parasite infection involving all of the immunomodulatory cells, including mononuclear cells, polymorphonuclear cells, eosinophils, and mast cells (40, 41, 50). In the present study, H&E-stained ear sections of nonimmunized/challenged or immunized/challenged mice revealed an abundance of polymorphonuclear cells as well as mononuclear cells, fibroblasts, and eosinophils in the lesion. However, immune-stained sections revealed more mature macrophages as well as MHC-II–expressing APCs in the lesions of immunized mice as compared with those from nonimmunized/challenged mice. This observation is consistent with earlier studies describing the importance of macrophages in host-protective immune responses in *L. mexicana* infection (40). Mast cells also play an important role in CL, and the presence of degranulated mast cells at the site of *L. major* infection correlates with the development of severe pathology (42, 52). Mast cells produce IL-4 and IL-13, which appear to be required for the establishment of the Th2 response in CL (42, 53, 54). Mast cells also release histamine, which regulates the immune response by inhibiting IL-12 production while simultaneously stimulating the generation of IL-10, IL-5, and IL-6 by monocytes and DCs (55, 56). Indeed, it was reported earlier that *L. mexicana* lipophosphoglycan induces degranulation of mast cells in susceptible BALB/c mice and thus promotes an inflammatory response associated with increased parasite numbers (42). Similarly, we also observed significant numbers of degranulated mast cells in the lesions of nonimmunized/challenged mice that correlated with more parasites in the lesion. In contrast, the lesions of immunized/challenged mice harbored fewer mast cells and most of them were not degranulated; this correlated with either significantly lower parasite burdens or complete absence of parasite altogether. The analysis of lesion infiltration of mast cells indicated a more controlled inflammatory response in immunized mice that is evidenced by reduced tissue damage. The role of eosinophils is not very clear in the pathogenesis of CL because eosinophils persist even in chronic cutaneous lesions of resistant or susceptible mice (57, 58). It was reported earlier that eosinophils are involved in the control of infection through their phagocytic capability as well by releasing cytotoxic granules (57, 58). Alternatively, the presence of eosinophils is characteristic of a Th2-type immune response that favors parasite survival and proliferation (59). The significant infiltration of eosinophils in the lesion of nonimmunized mice reflects the Th2 environment, and reduced infiltration of eosinophils in immunized mice reflects the Th1 type of immune environment. The analysis of lesion infiltration of mast cells indicates a more controlled inflammatory response in immunized mice that would suggest reduced tissue damage.

L. mexicana causes a progressive disease in susceptible BALB/c mice inducing a Th2-type immune response and developing significant pathology, whereas induction of Th1 cytokines leads to disease resistance (50). Th1-mediated protection is driven by IFN-γ–induced production of NO in infected cells, leading to parasite killing, whereas Th2-associated cytokines IL-4 and IL-10 are known to result in exacerbation of disease (50). It has been shown that both IL-4 and IL-10 are crucial for *L. mexicana* disease progression and that genetically susceptible mice lacking IL-4 or IL-10 resolve infection with the development of a Th1-like response and production of an IFN-γ response (44, 60). IL-10 immunosuppresses DCs by inhibiting Ag presentation to T cells, thus decreasing IL-12 production and inhibition of iNOS (39). Interestingly, *L. mexicana* inhibits recruitment of monocytes and monocyte-derived DCs to the site of infection and treatment with anti–IL-10R Ab results in increased recruitment of monocytes to the lesion along with increased production of IFN-γ and iNOS (40). Moreover, in *L. mexicana* infection, IL-10–mediated suppression of protective immune responses is a crucial phenomenon for chronic infection, as IL-10–deficient mice control parasite numbers and resolve infection via an enhanced Th1 response (44). In the present study, we observed that lesions of immunized mice produced less IL-10 compared with nonimmunized mice with a concomitant increase in the amounts of iNOS. It has also been reported that *L. mexicana* induces host IgG, which leads to increased IL-10 secretion, promoting the establishment of a chronic nonhealing infection (46, 61, 62). In this study, we found that immunization with live-attenuated *L. donovani* parasites induces more IgG2a, which reflects induction of a protective Th1 response, and thus alters *L. mexicana*–induced pathogenesis. These observations clearly suggest that live-attenuated *L. donovani* parasite immunization could diminish IL-10 induction by *L. mexicana* infection, resulting in enhanced iNOS expression and clearance of parasites from the lesion.

The Th1/Th2 paradigm is well established in the case of *L. major* infection; however, it has been reported that *L. mexicana*–induced immune responses do not follow the same paradigm as *L. major*. For example, IL-4 gene–deficient mice are significantly resistant to *L. mexicana* infection (60), whereas *L. major* infections can lead to development of chronic lesions in IL-4 knockout mice (63). Apart from IL-4, IL-13, another cytokine, contributes to the chronicity of *L. mexicana* infection (53). However, in our study we observed a very distinct Th1/Th2 dichotomy in *L. mexicana*–infected mice. We found that cells cultured from LNs of immunized/challenged mice produce significantly more *L. mexicana* Ag–specific IFN-γ secretion but lower Th2 cytokines IL-4, IL-5, IL-6, IL-10, and IL-13. It is well established that other than CD4 T cells, CD8 T cells are crucial for controlling the *Leishmania* parasite growth by changing the immune response from Th2 toward a Th1 response (43, 64). In particular, it has been observed that in the lesions of *L. mexicana* infection there were a significant number of CD8 T cells during the healing phase (65). We also have observed in immunized mice dLNs that there is a higher percentage of CD4 and CD8 T cells secreting IFN-γ, IL-2, and TNF-α, confirming a crucial role of both CD4 and CD8 T cells in immunized mice. Particularly in the *Ldp27*$^{-/-}$-immunized mice where we observed robust protection, there was a potent Th1 immune response as well as a strong CD8 T cell response against *L. mexicana* infection. Additionally, adoptive transfer of total T cells from immunized mice conferred better protection in terms of controlling lesion and parasite growth than did the CD4 or CD8 T cells alone against challenge with *L. mexicana* in the recipient mice. These results further confirm that protection in immunized mice is due to effective induction of Th1 response along with potent CD8 T cell activation, as has been observed by others (19, 40, 66). Moreover, splenocytes of *L. donovani*–immunized and *L. mexicana*–challenged mice showed a mixed Th1/Th2 (IFN-γ and IL-4) response against *L. donovani* Ags. Interestingly, the ratio of IFN-γ/IL-4 was significantly high only

in *LdCen*$^{-/-}$-immunized mice, suggesting less abundance of *LdCen*$^{-/-}$ parasites in the spleen at that time point. Furthermore, enhanced IgG2a titers in the serum of *LdCen*$^{-/-}$-immunized mice might be the result of enhanced IFN-γ production. Overall, these observations are consistent with our previous studies using both *Ldp27*$^{-/-}$ and *LdCen*$^{-/-}$ as immunogens against *L. donovani* infection suggesting the presence of a systemic immune response against immunizing agents (14).

Still, it is not clear whether parasite persistence or central memory T cells are essential for host protection against reinfection with *Leishmania*. In our previous study, we demonstrated induction of effector memory T cells in live-attenuated *L. donovani* parasite-immunized mice against homologous infection (14). In the present study, we have confirmed that adoptively transferred CD4 and CD8 T cells from immunized mice proliferated significantly in naive mice after heterologous parasite *L. mexicana* infection as well. These results thus indicate that immunized mice might have more *Leishmania*-specific effector T cells residing in their lymphoid organs, which may help to clear wild-type *L. mexicana* parasites. Collectively, these findings indicate that the persistence of parasites that induce effector T cell generation in addition to central memory T cells might be necessary for better protection against virulent challenge (67, 68).

The DCs play a critical role in initiating and shaping Th1-protective responses in *Leishmania* infection (69, 70). Several studies have reported that *Leishmania* parasites prevent development of protective Th1 immunity by dysregulating DC function (69–71). We therefore examined the effect of attenuated *LdCen*$^{-/-}$ and *Ldp27*$^{-/-}$ parasites on LPS-induced DC maturation and cytokine production. We had previously found that centrin and p27 genes are required for survival of *L. donovani* in macrophages, because both *LdCen*$^{-/-}$ and *Ldp27*$^{-/-}$parasites, which are phagocytosed by both mouse and human macrophages, do not survive after a certain period of infection (13, 72). Similarly, in this study, we found that both attenuated parasite (*LdCen*$^{-/-}$ and *Ldp27*$^{-/-}$) strains were phagocytosed by DCs at similar rates to wild-type parasites (*LdWT*); however, unlike wild-type parasites, both of the attenuated strains fail to survive inside DCs. Interestingly, DCs infected with both attenuated strains, as well as *LdWT* parasites, showed comparable expression of MHC-II and costimulatory molecules, including CD80 and CD86 upon activation with LPS (data not shown), as was also observed in other studies (73). In addition to Ag presentation, DCs also modulate the host immune response by producing cytokines, including IL-12 (48, 49). Interestingly, DCs infected with *Ldp27*$^{-/-}$ or *LdCen*$^{-/-}$ parasites produced significantly more IL-12 compared with those infected with *LdWT* parasite-infected DCs. Both TNF-α and NO have been shown to be directly involved in the killing of intracellular parasites (50). Enhanced secretion of both the molecules in the culture supernatants of attenuated parasite-infected DCs compared with *LdWT* infection clearly indicated that attenuated parasites not only have impaired growth inside DCs, but they also induce a proinflammatory response. The impairment of growth of the attenuated parasites in DCs does not require the proinflammatory response in such cells, because we observed that both of the live-attenuated parasites are cleared from DCs independent of the higher TNF-α or NO generation. Although they are cleared by host cells, they also induce a strong proinflammatory response, suggesting that such parasites can be safely used as vaccine candidates. These observations further confirm our earlier studies that both the live-attenuated parasites are safe because they do not survive either in IFN-γ$^{-/-}$ mice or immune-suppressed mice (12, 14). Functional Ag presentation studies demonstrate that OVA-pulsed DCs infected with *LdCen*$^{-/-}$ and *Ldp27*$^{-/-}$ parasites were significantly better activators of OVA-specific T cells compared with those infected with *LdWT*, as indicated by OVA-specific proliferation of T cells. Furthermore, such an interaction between DCs and T cells in vitro also resulted in suppression of IL-10 release compared with *LdWT* parasite-infected DCs. These observations clearly indicate that unlike wild-type *L. donovani* parasites, both the attenuated parasite strains can activate DCs to promote their interaction with T cells and stimulate their ability to regulate parasite growth. Furthermore, in vitro studies confirm our earlier observation that the protection elicited by *L. donovani* live-attenuated parasites in vivo in animal models is mediated by T cells by shifting toward a Th1 response (12, 14).

In conclusion, we demonstrate that both strains of attenuated *L. donovani* parasites are capable of inducing a host-protective immune response against *L. mexicana* parasites through the induction of a strong DTH induration response, which correlates with a significantly higher percentage of *L. mexicana* Ag–specific proliferating T cells. Furthermore, the cross-protection was associated with an induction of a Th1 immune response involving both CD4 and CD8 T cells and concomitant suppression of a Th2 cytokine response, suggesting the role of *Leishmania*-specific effector memory T cells residing in the LNs that clear the parasites. Additionally, we demonstrate that initial infection in DCs with live-attenuated parasites activates DCs to influence the induction of a Th1-protective response. Interestingly, in a head-to-head comparison, we observed that there are differences in the immune response between the two live-attenuated *L. donovani* parasites against *L. mexicana* infection. Finally, we observed a cross-protection against *L. mexicana* that is long-lasting. These studies thus suggest that live-attenuated *L. donovani* parasites can serve as pan-*Leishmania* vaccine candidates.

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Disclosures

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