A *Listeria monocytogenes*-Based Vaccine That Secretes Sand Fly Salivary Protein LJM11 Confers Long-Term Protection against Vector-Transmitted *Leishmania major*

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Cutaneous leishmaniasis is a sand fly-transmitted disease characterized by skin ulcers that carry significant scarring and social stigmatization. Over the past years, there has been cumulative evidence that immunity to specific sand fly salivary proteins confers a significant level of protection against leishmaniasis. In this study, we used an attenuated strain of *Listeria monocytogenes* as a vaccine expression system for LJM11, a sand fly salivary protein identified as a good vaccine candidate. We observed that mice were best protected against an intradermal needle challenge with *Leishmania major* and sand fly saliva when vaccinated intravenously. However, this protection was short-lived. Importantly, groups of vaccinated mice were protected long term when challenged with infected sand flies. Protection correlated with smaller lesion size, fewer scars, and better parasite control between 2 and 6 weeks postchallenge compared to the control group of mice vaccinated with the parent *L. monocytogenes* strain not expressing LJM11. Moreover, protection correlated with high numbers of CD4+ cells and low numbers of CD4+ IFN-γ+ IL-10+ T cells at 2 weeks postchallenge. Overall, our data indicate that delivery of LJM11 by *Listeria* is a promising vaccination strategy against cutaneous leishmaniasis inducing long-term protection against ulcer formation following a natural challenge with infected sand flies.

Leashmaniasis is a disease that is endemic in 98 countries, of which most are developing countries (1). There are approximately 0.9 to 1.6 million new cases annually and 350 million people at risk of getting infected. The most common form of the disease is cutaneous leishmaniasis, which is characterized by skin ulcers. The lesions commonly self-cure but can leave significant scarring and lead to social stigmatization. The large majority of cases of cutaneous leishmaniasis occur in Afghanistan, Iran, Syria, Algeria, Brazil, and Colombia (1).

*Leishmania* spp. are protozoans that are transmitted by phlebotomine sand flies. During feeding, the fly injects saliva into its host. Some of the saliva proteins induce vasodilation and prevent coagulation to facilitate feeding, while others have immunomodulatory effects (2). There is strong evidence that the fly saliva enhances the ability of *Leishmania* to establish an infection by about a thousandfold (3, 4). Conversely, the development of a delayed-type hypersensitivity (DTH) response to salivary components from uninfected sand flies compromises the ability of *Leishmania* to establish an infection (5, 6). The immunoprotective value of preexposure to the saliva of uninfected sand flies is further emphasized by the observation that the influx of refugees, humanitarian aid workers, tourists, and military personnel in locations with previously low levels of infection leads to dramatic outbreaks of leishmaniasis (7–15). Following these observations, specific salivary proteins have been identified as potent protective immunogens against the establishment of an infection with *Leishmania* and are being considered candidates for an anti-*Leishmania* vaccine (16–19).

Infected sand flies harbor the flagellated metacyclic promastigote form of *Leishmania*, which is transmitted into the dermis of a mammalian host during a blood meal. These parasites infect cells, predominantly professional phagocytes such as macrophages, in which they differentiate into nonflagellated amastigotes that divide and proliferate in parasitophorous vacuoles. Interestingly, the initial infection does not induce an inflammatory response, enabling the parasite to expand in numbers (20). However, it has been documented that resistance to *Leishmania* correlates with the development of a T-helper type 1 immune response driven primarily by gamma interferon (IFN-γ)-producing CD4+ T cells (21).

In this work, we used *Listeria monocytogenes* as a vector to vaccinate against a sand fly salivary protein. *L. monocytogenes* is an intracellular bacterial pathogen that has the ability to proliferate in the cytosol of infected cells and spread from cell to cell without exiting the intracellular milieu (22). Consequently, clearance of an infection is completely cell mediated (23) and is dependent on the...
development of a T_h1 immune response that is dominated by IFN-γ-secreting T cells (24). Over the past decade, there has been a strong interest in using *L. monocytogenes* as a vaccine vector because of its many useful characteristics (25–27). The bacterium can easily be attenuated without affecting its immunogenicity and can secrete large amounts of heterologous proteins in infected cells, leading to the generation of a specific cell-mediated immune response against that heterologous antigen. Therefore, we generated an attenuated *Listeria*-based vector that expresses a gene coding for the salivary protein LJ1M11 from the sand fly *Lutzomyia longipalpis* to assess the efficacy of a *Listeria*-based vaccine against cutaneous leishmaniasis. We observed a significant decrease in disease burden in mice vaccinated intravenously with *Listeria* secreting LJ1M11 and challenged by needle injection with purified parasites and sand fly saliva or with infected sand flies. Protection was associated with high numbers of CD4⁺, IFN-γ⁺, tumor necrosis factor alpha⁺/⁺ (TNF-α⁺/⁺), interleukin-10-negative (IL-10⁻) T cells and low numbers of CD4⁺, IFN-γ⁺/⁺, TNF-α⁻, IL-10⁺ T cells at 2 weeks postchallenge.

### MATERIALS AND METHODS

#### Vaccine strain.

*Listeria monocytogenes* strain DP-L1942, which carries a deletion in the *actA* gene, was used to alleviate the safety concerns regarding a *Listeria*-based vaccine (28). *ActA* is a surface protein that mediates actin polymerization, enabling *L. monocytogenes* to move in the cytosol of host cells and to spread from cell to cell without escaping the intracellular milieu (29). The *actA* deletion mutant is as efficient as the wild-type strain at escaping vacuoles formed upon initial entry into cells and at multiplying in the cytosol of infected cells. However, it is incapable of spreading from cell to cell without escaping from the intracellular milieu, resulting in a 3-log attenuation defect in a mouse model of infection (30). The *actA* deletion strain confers protective immunity at the same level as the wild-type strain.

In an effort to improve vaccine safety and efficacy, the *mdrM* gene was deleted. This gene codes for a multiple-drug resistance transporter that contributes to the activation of type I interferon in infected cells (31). This innate immune response to a cytotoxic infection by *L. monocytogenes* decreases the ability of the host to control the infection (32, 33). An internal in-frame deletion of the *mdrM* gene was introduced in strain DP-L1942 by allelic exchange as described previously (34), generating the control strain HEL-1207. The vaccine strain (HEL-1325) was created by introducing in HEL-1207 a construct comprised of the sequences coding for the *ActA* signal sequence and N terminus (100 amino acids), followed by a FLAG tag and the gene coding for LJ1M11 minus its signal sequence (Fig. 1A).

Secretion of LJ1M11 by intracellular bacteria was verified by pulse labeling infected J774 mouse macrophage-like cells as described previously (35). Fitness of the recombinant strains was verified by performing intra- cellular growth curves in J774 cells as previously described (36).

#### Mice and sand flies.

Six- to 8-week-old C57BL/6 mice were used for this study. *Lutzomyia longipalpis* sand flies were reared at the Laboratory of Malaria and Vector Research (LMVR), NIAID, NIH. Four- to seven-day-old adult flies were dissected to obtain salivary glands, which were stored in phosphate-buffered saline (PBS) at −80°C. Salivary gland homogenates (SGH) were prepared by sonication. Lysates were cleared by centrifugation. When used for animal infections, sand flies were preinfected with *L. major* promastigotes as described previously (6).

#### Parasites.

*Leishmania major* WR 2885, a recent isolate originating from Iraq and typed at the Walter Reed Army Institute of Research, was used for this vaccine study (16). Frozen aliquots of *L. major* promastigotes were thawed, passed in *vitro* once or twice, and allowed to grow to stationary phase. Parasites were pelleted, washed once, and resuspended in Dulbecco’s modified Eagle medium (DMEM). A discontinuous Ficoll gradient was prepared with 20% and 10% Ficoll type 400 in MilliQ water and overlaid with the parasite suspension. After centrifugation at 2,000 rpm for 10 min with slow deceleration, the metacyclic promastigotes were recovered above the 10 to 20% interface. The parasites were washed in DMEM, and counts were determined using a hemacytometer. A final suspension was prepared in PBS.

#### CLA.

Parasites grown in flask, as described above, were pelleted by centrifugation, washed three times in PBS, and freeze-thawed multiple times to generate a crude lysate. Protein content was measured using a bichinonic acid (BCA) assay, and aliquots of crude *Leishmania* antigen (CLA) in PBS were stored at −80°C.

#### Mouse experiments.

Mice were anesthetized with isoflurane and vaccinated intradermally in the pinna of the ear (10 µl), intramuscularly (i.m.) in the hamstring (10 µl), subcutaneously at the base of the neck (100 µl), or intravenously in the retro-orbital sinus (100 µl). The vaccination dose was 5 × 10⁶ CFU three times at 2-week intervals.

Mice were challenged either 3 or 12 weeks after the last boost to assess short- and long-term protection, respectively. Anesthetized mice were challenged intradermally in the pinna of the ear with 500 metacyclic promastigotes and SGH (equivalent to half a pair of salivary glands) in a total volume of 10 µl. Sand fly challenges were performed at the NIAID LMVR animal facility as described previously (6). Ear lesions were measured on a weekly basis starting at day 7 postchallenge. Needle challenges generate single-ear lesions that were measured for thickness and diameter. These measurements were transformed into lesion area. Sand fly challenges generate multiple lesions that sometimes coalesce. For this reason, we measured the thickness of the biggest ear lesion.

#### Processing of infected ears and flow cytometry experiments.

Ear pinna dermal sheets were collected, treated with ethanol, separated, and digested in 500 µl of DMEM containing Liberase TL (250 µg/ml; Roche) at 37°C for 1.5 h. Digested tissues were minced and homogenized. Ear cells were washed with complete tissue culture medium (CTCM; containing DMEM, 10% fetal bovine serum [FBS], 1 mM Na pyruvate, 0.1 mM non-essential amino acids, 2 mM l-glutamine, 30 mM HEPES, and 50 µM β-mercaptoethanol). The resulting suspension was filtered, spun down, and resuspended in CTCM.

Parasite loads in the challenged ears were determined by limiting-dilution assays as described previously (37). Parasite titers were determined from the highest dilution at which growth was visible.

Ear cells were mixed with CLA-prepulsed bone marrow-derived dendritic cells (DC) to stimulate cytokine production by immune cells, improving the sensitivity of fluorescence-activated cell sorter (FACS) analysis. Briefly, DC were prepared as previously described (38) and incubated with or without CLA (25 µg/ml) for 6 to 7 h before adding ear cells at a ratio of 1 DC per 5 ear cells. After an overnight incubation, cells were treated for 4 h with brefeldin A (10 µg/ml) and phorbol myristate acetate (PMA; 5 ng/ml), collected in FACS buffer (10% normal mouse serum, 0.5% bovine serum albumin [BSA], 0.02% NaN₃ in PBS), and incubated with the following antibodies against surface and intracellular markers: CD4-allophycocyanin (APC)-H7, CD8a-APC, TNF-peridinin chlorophyll protein (PerCP)-Cy5.5, IFN-phiocerthrin (PE)-Cy7, and IL-10-PE. Cells were collected on a BD Canto flow cytometer and analyzed using FlowJo software. Isotype controls and representative plots of CD4⁺ T cells stained for IFN-γ, TNF-α, and IL-10 are shown in Fig. S1 in the supplemental material. Integrated median fluorescence intensities (iMFHI) were calculated for the cells by multiplying the MFI by the frequency of cells producing the particular cytokine, and the data are reported in Table S1 in the supplemental material.

#### Data and statistical analyses.

Ratios were obtained by comparing values from each individual mouse from a vaccine group to the calculated means from the corresponding control group. Statistical analyses were performed using a two-tailed, unpaired *t* test or a one-way analysis of variance (ANOVA) test with Bonferroni posttest analysis.

#### Ethics statement.

All animal experimental procedures were reviewed and approved by the Cornell University Institutional Animal Care and Use Committee, NIAID Animal Care and Use Committee, and U.S. Army Medical Research and Material Command Animal Care and Use Review
Office. All animal experiments were carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (39).

**RESULTS**

*L. monocytogenes* vaccine vector confers the greatest protection against cutaneous leishmaniasis when administered intravenously. We generated an attenuated *Listeria* vaccine strain (HEL-1325) in which LJM11 was fused to the N terminus of the *Listeria* ActA protein (Fig. 1A). This strain secreted large amounts of the fusion protein (55.4 kDa) in infected cells (Fig. 1B) and had an intracellular growth rate that was similar to that of the parent strain (Fig. 1C). This attenuated *Listeria* vaccine strain persisted for several days in tissues of infected mice, inducing a specific T cell immune response against LJM11 (Fig. 1D; also see Fig. S2 and S3 in the supplemental material).

Initially, various routes of vaccine delivery were tested to identify parameters conferring the best level of protection in a mouse model of cutaneous leishmaniasis (3). Groups of mice were vaccinated intravenously, intradermally, subcutaneously, or intramuscularly and challenged intradermally in the ear pinna with purified promastigotes and sand fly SGH. An age-matched unvaccinated group of mice was included in the challenge. Levels of secreted IFN-γ and IL-10 were measured by ELISA (the limit of detection was 15 and 70 pg/ml for IFN-γ and IL-10, respectively). Data were analyzed using one-way ANOVA with Bonferroni posttest analysis. *n* = 3 mice per group.

![Fig 1](https://iai.asm.org/content/fig1.png)

**FIG 1** *Listeria* vaccine vector. (A) Schematic representation of the *L. monocytogenes* vaccine construct. The various sequence parts code for the *actA* promoter (*actA* P), ActA signal sequence (ActA SS), 100 amino acids of the ActA N terminus, Flag tag (FLAG), and *Lutzomyia longipalpis* salivary gland protein LJM11 (LJM11). (B) Infected J774 cells were pulse labeled with [35S]methionine in the presence of host protein synthesis inhibitors. Host cells were lysed, and secreted bacterial proteins were resolved by SDS-PAGE and detected by autoradiography. Molecular mass markers, in kDa, are indicated on the far left; the location of the ActA/LJM11 chimera and a degradation product (*) are indicated on the far right. HEL-1207, *L. monocytogenes* Δ*actA ΔmdrM*; HEL-1325, HEL-1207 *actA-LJM11*. (C) Intracellular growth kinetics of *L. monocytogenes* in J774 cells. (D) Immune response to LJM11 in splenocytes from vaccinated mice. Mice were vaccinated intravenously as described in Materials and Methods with the control vector (HEL-1207) or the vaccine vector (HEL-1325). Three weeks later, spleens were collected and splenocytes were incubated overnight with dendritic cells that had been pulsed with LJM11 or left untreated. Levels of secreted IFN-γ and IL-10 were measured by ELISA (the limit of detection was 15 and 70 pg/ml for IFN-γ and IL-10, respectively). Data were analyzed using one-way ANOVA with Bonferroni posttest analysis. *n* = 3 mice per group.
group vaccinated intravenously suffered a disease burden that was about threefold less than that of the unvaccinated and subcutaneously vaccinated groups and about twofold less than the intradermally and intramuscularly vaccinated groups. The overall protection was highly significant for the group vaccinated intravenously (\(P < 0.001\)) compared to the group vaccinated subcutaneously and to the control group. Protection was also significant for the group vaccinated intradermally (\(P < 0.05\)) compared to the control group.

The experiment described above did not include a vaccine vector control for any of the four routes of vaccination tested. To test the possibility that protection conferred by intravenous vaccination was due to a nonspecific immune response to the vaccine vector, we performed an experiment in which we vaccinated mice intravenously with the vaccine vector (HEL-1207) or with PBS. Mice were challenged as described 3 weeks postvaccination with \(L.\) major plus half a pair of sand fly salivary glands. (A) Ear lesion areas (diameter times thickness) were measured weekly and plotted. (B) Disease burden of mice vaccinated and challenged. The area under the curve was measured for each individual mouse and plotted. The experiment was repeated twice with \(n = 6\) mice per group. (C) Mice were primed and boosted intravenously twice at 2-week intervals with the vaccine vector control (HEL-1207) or PBS and challenged 3 weeks later as described above. Ear lesion areas are reported. Data were analyzed using the one-way ANOVA statistical test with Bonferroni posttest analysis. ***, \(P < 0.001\); *, \(P < 0.05\).

Vaccinated mice are protected short term against a needle challenge. In the next set of experiments, mice were vaccinated intravenously with either \(L.\) monocytogenes expressing LJM11 (HEL-1325; the vaccine group) or the isogenic \(L.\) monocytogenes strain not expressing LJM11 (HEL-1207; the control group) to assess the specificity of the protective immune response. Short-term immune protection was assessed by challenging the two groups of mice intradermally 3 weeks after the last boost either with purified parasites mixed with sand fly SGH (needle challenge) or infected \(Lutzomyia\) longipalpis sand flies (sand fly challenge). Ear lesion sizes were recorded weekly. Because a needle challenge generates a single ear lesion, we measured lesion thickness and diameter and transformed these measurements into lesion area. In contrast, a sand fly challenge generates multiple lesions that sometimes coalesce. For this reason, we measured the thickness of the biggest ear lesion. In addition to measuring lesion sizes, a subset of mice from each group was sacrificed at 2, 6, and 11 weeks postchallenge to determine parasite counts and characterize the immune response in the infected ears.

Results from the short-term needle (STN) challenge groups indicated that mice in the vaccine group developed smaller lesions than those in the control group between 3 and 10 weeks postchallenge (\(P\) values between 0.0122 and \(<0.0001\)) (Fig. 3A), concurrent exhibiting a significantly smaller disease burden (\(P < 0.0001\)) (Fig. 3B). Ear lesions were monitored carefully for the development of scars or ulcers, and those that developed either over the duration of the experiment were scored. Five percent of mice in the vaccine group and 55% of mice in the control group developed scars or ulcers (\(P = 0.010\)) (Fig. 3C). Ear parasite num-
bers were about the same in both groups of mice at 2 and 11 weeks postchallenge (Fig. 3D). However, between 2 and 6 weeks postchallenge, ear parasite numbers increased by three logs in the control group as opposed to one log in the vaccine group. The difference in ear parasite numbers between vaccine and control groups at 6 weeks postchallenge was statistically significant (P < 0.001). Overall, results from the STN challenge indicated that the vaccine is effective in decreasing overall disease burden, preventing the formation of scars and decreasing parasite replication between 2 and 6 weeks postchallenge.

Results from the short-term sand fly (STSF) challenge groups indicated that mice developed similar-sized lesions in vaccine and control groups (Fig. 3E) with no significant difference in disease burden (Fig. 3F). Scars developed in 65 and 40% of the mice in control and vaccine groups, respectively (Fig. 3G), but this difference was not statistically significant. Ear parasite numbers were approximately 1.5 logs higher in the vaccine group than in the control group at 2 weeks postchallenge (Fig. 3H). However, between 2 and 6 weeks postchallenge, ear parasite numbers increased by nearly three logs in the control group as opposed to one log in the vaccine group. By 11 weeks postchallenge, ear parasite numbers were similar in both groups of mice. Overall, results from the STSF challenge indicated that the vaccine did not confer long-term protection in mice.

Vaccinated mice are protected long term against a sand fly challenge. Long-term protection assays were performed by challenging mice 12 weeks postvaccination. Results from the long-term needle (LTN) challenge indicated that the vaccine group (HEL-1325) of mice had significantly smaller ear lesions than the control group (HEL-1207) at 6 and 7 weeks postchallenge (P values of 0.012 and 0.004, respectively) (Fig. 4A). However, the overall burden of disease was not significantly different between the two groups (Fig. 4B). Scars developed in 62 and 45% of the mice in control and vaccine groups, respectively (Fig. 4C). Ear parasite numbers were similar between the two groups at all time points (Fig. 4D). Overall, results from the LTN challenge indicated that the vaccine did not confer long-term protection in mice.

Results from the long-term sand fly (LTSF) challenge indicated that the vaccine group (HEL-1325) developed significantly smaller ear lesions between 4 and 8 weeks postchallenge than the control group (HEL-1207) (P values between 0.027 and 0.0015) (Fig. 4E), concurrently exhibiting a significantly smaller disease burden (P = 0.019) (Fig. 4F). Scars developed in 52 and 15% of the mice in control and vaccine groups, respectively, and this difference was statistically significant (P = 0.017) (Fig. 4G). Similar to the STSF challenge, ear parasite numbers started 1.5 logs higher in the vaccine group than in the control group at 2 and 6 weeks postchallenge. Parasite numbers did not change in either group between 6 and 11 weeks postchallenge. Overall, results from the LTSF challenge indicated that although parasite numbers were not significantly different between the vaccine and control groups by the end of the experiment, the vaccine is effective in decreasing overall disease burden and preventing the formation of scars.

**FIG 3** Short-term needle and sand fly challenges. Mice were vaccinated intravenously three times at 2-week intervals and challenged intradermally 3 weeks after the last boost. Control group, HEL-1207; vaccine group, HEL-1325. (A to D) Mice were challenged by needle injection with *L. major* plus half a pair of salivary gland homogenate. (E to H) Mice were challenged with infected sand flies. (A and E) Lesion area measurements for 10 weeks postchallenge. (B and F) Disease burden calculated by measuring the area under the curve for each individual mouse. (C and G) Percentage of mice that developed scars over the duration of 11 weeks. (D and H) Parasite numbers per ear at 2, 6, and 11 weeks postchallenge. Experiments were repeated twice, and data shown are pooled from both experiments; n = 30 mice per group (10 processed at each time point). Data were analyzed using a *t* test comparing vaccine and control groups. ***, P < 0.001; **, P < 0.01; *, P < 0.05. The control group in panel A is the same as the vector control group in Fig. 2C.
A combination of high CD4+ IFN-γ+ TNF-α+/− IL-10− and low CD4+ IFN-γ+/− TNF-α− IL-10+ T cell populations at 2 weeks postchallenge is predictive of protection. Analysis of CD4+ and CD8+ T cells recruited at the site of challenge indicated that, on average, more than 97% of the specific immune response to Leishmania was mediated by CD4+ T cells (Fig. 5A). At 2 weeks postchallenge, there were significantly higher numbers of CD4+ IFN-γ+ TNF-α+/− IL-10− T cells in every vaccine group than in the respective control groups (Fig. 5B to E). The share occupied by CD4+ IFN-γ− TNF-α+/− IL-10− T cells was 68% and 64% in the protected groups (STN and LTSF vaccine groups) and between 36% and 57% in the nonprotected groups (LTN and STSF vaccine groups and all control groups) (Fig. 5F to I). There were no significant differences between control and vaccine groups in the number of cells producing TNF-α for any of the experimental challenges, although the share occupied by CD4+ IFN-γ+/− TNF-α+ IL-10− T cells was lower, 42% and 38%, in the protected groups (STN and LTSF vaccine groups) and between 43% and 74% in the nonprotected groups (LTN and STSF vaccine groups and all control groups) (Fig. 5F to I). The data also indicate a significantly smaller number of CD4+ IFN-γ− TNF-α− IL-10+ T cells in the protected LTSF vaccine group (Fig. 5E) and a significantly larger number of CD4+ IFN-γ− TNF-α+ IL-10+ T cells in the nonprotected STSF vaccine group compared to their respective control groups (Fig. 5C). Although the share occupied by IL-10+ (with or without IFN-γ) in the protected groups was not always lower than that in the nonprotected groups as a whole, the protected vaccine groups (STN and LTSF) had 3.5- to 4-fold fewer CD4+ IFN-γ+/− TNF-α− IL-10− T cells than their respective control groups, whereas this difference was 0.3- and 1.6-fold between the nonprotected vaccine groups (LTN and STSF) and their respective control groups (Fig. 5F to I).

The balance between CD4+ IFN-γ+ IL-10− and CD4+ IFN-γ− IL-10+ T cells has been shown to be an indicator of protection in the mouse model of cutaneous leishmaniasis (40–42). Therefore, to assess this balance, we calculated the vaccine/control ratios for CD4+ T cells expressing these cytokines with or without TNF-α at 2 weeks postchallenge and for disease burden. The ratios eliminate the inherent cytokine staining variability between samples processed on different days and lesion size variability for challenges performed with different preparations of metacyclic promastigotes or infected sand flies. For example, control and vaccine groups from an STN challenge were challenged together and processed together but on a different day than control and vaccine groups from an STSF challenge. The protected STN and LTSF challenge groups had a nearly 6-fold difference in ratios of CD4+ IFN-γ+ TNF-α+/− IL-10− (ratio of 2.3) and CD4+ IFN-γ+/− TNF-α− IL-10− (ratio of 0.4), respectively, with low disease burden ratios of 0.2 and 0.4, respectively (Fig. 6). The unprotected LTN challenge group had ratios of 1.3 and 0.7 for CD4+ IFN-γ+/− TNF-α− IL-10− and CD4+ IFN-γ+/− TNF-α− IL-10−, respectively, with a disease burden ratio of 0.6. The other unprotected STSF challenge group had ratios of 1.9 and 3.5 for CD4+ IFN-γ+/− TNF-α+/− IL-10− and CD4+ IFN-γ+/− TNF-α− IL-10−, respectively, with a disease burden ratio of 0.8. Overall, the results reveal a pattern in which a combination of high CD4+ IFN-γ+ TNF-α+/− IL-10− and low CD4+ IFN-γ+ TNF-α− IL-10+ T cell numbers at 2 weeks postchallenge is predictive of lower disease burden.
FIG 5 Recall response to vaccine at 2 weeks postchallenge. Cells collected from ears were incubated overnight with dendritic cells prepulsed with CLA and then treated for 4 h with brefeldin A and PMA. Cells were stained for surface markers and intracellular cytokines and analyzed by flow cytometry. Results were analyzed by gating for CD4 surface expression and intracellular cytokine expression (IFN-γ and TNF-α, IFN-γ, IL-10, TNF-α, or IFN-γ and IL-10). The total number of *Leishmania*-specific cytokine-producing CD4+ T cells in both control (HEL-1207; black bars) and vaccine (HEL-1325; gray bars) groups of mice is shown for short-term needle challenge (STN) (A), long-term needle challenge (LTN) (B), short-term sand fly challenge (STSF) (C), and long-term sand fly challenge (LTSF) (D). SSC, side scatter; FSC, forward scatter. (F to I) Data were converted into pie charts showing the contribution of each cytokine combination to the total number of cytokine-producing CD4+ T cells. Experiments were repeated twice, and data shown are pooled from both experiments. n = 10 (n = 5 mice per group per experimental repeat). Data were analyzed using the t test comparing vaccine and control groups. **, P < 0.01; *, P < 0.05.
DISCUSSION

In this study, we used *Listeria monocytogenes* as a vaccine delivery vector for LJM11, a sand fly salivary protein that has been shown to be a potent vaccine antigen (16, 43). Our results indicate that delivery of LJM11 by *Listeria* is a promising vaccination strategy against cutaneous leishmaniasis inducing long-term protection against the formation of ulcers and scars and resulting in an overall decrease in disease burden following a natural challenge with infected sand flies. In addition, these studies reinforce the value of LJM11 as a protective immunogen and validate the use of *Listeria monocytogenes* as a delivery vaccine vector.

Given that the formulation for a vaccine can have great effects on its efficacy, we set out to determine whether *Listeria* could be used as an effective vaccine delivery model. Among the advantages of using this system for vaccination are that *Listeria monocytogenes* can be attenuated without affecting its ability to induce a strong cell-mediated T<sub>1,1</sub> immune response (30), and the antigen of interest is secreted directly in the cytosol of host cells, bypassing the need for DNA or protein purification. Our *Listeria* vaccine vector can express an abundant amount of LJM11 in infected cells, and vaccinated mice developed a robust immune response to this protein.

Cutaneous leishmaniasis is, by definition, an infection of the skin, an organ that is populated by specialized sets of dendritic cells that contribute to homing receptor imprinting on T cells (44–46). Accordingly, mice vaccinated intradermally were protected against disease, whereas those vaccinated intramuscularly or subcutaneously were not, presumably because intradermal delivery of the antigen led to the imprinting of a larger number of T cells that readily migrated to the skin upon challenge. However, the best level of protection was observed in mice vaccinated intravenously. Upon intravenous injection, bacteria are delivered systematically to a large number of secondary lymphatic tissues within minutes of injection. Seemingly, this would lead to production of a larger quantity of antigen, exposure of the antigen to different subsets of dendritic cells, and a more robust induction of T cell proliferation at central and peripheral sites. However, an intravenous vaccine would not be practical, as it would require specialized technical personnel to administer, creating an increased level of complexity in areas where cutaneous leishmaniasis is endemic. A future challenge will be to find ways to improve protection conferred by an intradermal vaccine. An initial approach could be to combine the vaccine with an inhibitor of IL-10, as this cytokine activates regulatory T cells, compromising clearance of the infection (41).

Two different types of challenges were used to determine the short- and long-term protection effects of the vaccine: a needle challenge in which mice were injected with purified metacyclic promastigotes mixed with sand fly saliva, and a natural challenge in which mice were exposed to infected sand flies. The needle challenge is more practical, less time-consuming, more economical, less traumatic for the mice, and more precise than a natural challenge. For these reasons, most experiments were performed using the needle challenge. Ultimately, when optimal vaccine parameters were determined, we aimed at testing vaccine efficacy against a natural challenge using infected sand flies. During a natural challenge, sand flies feed multiple times on the mouse ear, causing more inflammation than a needle challenge. In addition, the challenge dose cannot be controlled during a natural challenge, adding a variable that is absent from the needle challenge. Nevertheless, challenging mice with infected sand flies is imperative to the assessment of vaccine efficacy, as this type of challenge reproduces more closely what happens when an individual becomes infected.

Conferring protection long after the last boost and preferably for the life of the host is of primary concern when developing a vaccine. Vaccinated mice were protected short term against a needle challenge but not long term. On the contrary, vaccinated mice were protected long term against a sand fly challenge but not short term. There are obvious differences in these two types of challenges, as described above. Needle injections are administered quickly and at a single site, while sand flies bite repeatedly, creating more trauma and inflammation at the site of infection. Furthermore, sand flies secrete a mucin-like gel that has been reported to exacerbate infection (47). It would be reasonable to speculate that the extra inflammatory reaction caused by sand fly bites combined with a strong recall response in the short-term challenge hastened the development of a regulatory T cell response, abrogating the protective immune response to infection (40). However, vaccination with the purified protein was shown to confer short- and long-term protection against a sand fly challenge (16). Perhaps the structure of LJM11 is modified when made as a chimeric protein by *Listeria*, potentially affecting the presentation of important amino acids. These results reinforce the need to use a challenge method that is as close as possible to a natural challenge for truly testing vaccine efficacy.

At 11 weeks postchallenge, parasite numbers were equivalent in all of the groups, despite the fact that the overall disease burden and the numbers of mice with scars were significantly lower in the protected groups (STN and LT5F). It was not uncommon to find...
mice with equal parasite numbers but very different pathology at 11 weeks postchallenge (see Fig. S4 in the supplemental material). Nonetheless, parasite numbers were significantly lower at 6 weeks postchallenge in the protected vaccinated groups than in their respective control groups and did not significantly increase between 6 and 11 weeks. These results suggest that early control of parasite replication is most important to prevent lesion development and that parasite clearance is not essential to prevent the pathology.

Vaccinated mice challenged with infected sand flies had higher parasite numbers than control mice at 2 weeks postchallenge. Control and vaccinated groups were challenged simultaneously, and analysis of flies postinfection indicated no differences in the percentages of fed flies between mice. Therefore, it appears that the recall response to LJM11 was favorable to initial parasite survival and/or growth following a sand fly challenge. The immune response in the hours and days following challenge ought to be characterized to address these differences.

Crucial to the control of an infection with Leishmania is the development of a Th1 immune response, characterized mainly by the production of IFN-γ by CD4⁺ T cells (16, 48). Although CD8⁺ T cells can also serve as a source of IFN-γ, their cytolytic effect exacerbates immunopathology and mediates the development of metastatic lesions (49). However, we focused our analysis on CD4⁺ T cells, because they consistently accounted for more than 97% of the total numbers of T cells specific to L. major at 2 weeks postinfection. Induction of multifunctional CD4⁺ T cells producing IFN-γ with or without TNF-α and few CD4⁺ T cells producing IL-10 with or without IFN-γ is also very important to disease control (41, 50). Accordingly, our results indicate that protected vaccinated groups generated high CD4⁺ IFN-γ⁺ TNF-α⁺/− IL-10⁻ and low CD4⁺ IFN-γ⁺/− TNF-α⁺ IL-10⁺ T cell numbers. This immune profile at 2 weeks postchallenge was predictive of the outcome disease burden for these 11-week-long experiments.

We conclude that the Listeria-based vaccine secreting the sand fly salivary protein LJM11 during intracellular infection generates an immune response that contributes to curbing disease burden and reducing the number of scars and ulcers in mice challenged with infected sand flies 3 months postvaccination. Protection correlates with a high ratio of CD4⁺ IFN-γ⁺ TNF-α⁺/− IL-10⁻ to CD4⁺ IFN-γ⁺/− TNF-α⁺ IL-10⁺ at 2 weeks postchallenge and lower parasite numbers at 6 weeks postchallenge.

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