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Intracellular Replication-Deficient Leishmania donovani Induces Long Lasting Protective Immunity against Visceral Leishmaniasis

Angamuthu Selvapandiyana, Ranadhir Dy, Susanne Nylen, Robert Duncan, David Sacks, and Hira L. Nakhasi

No vaccine is currently available for visceral leishmaniasis (VL) caused by Leishmania donovani. This study addresses whether a live attenuated centrin gene-deleted L. donovani (LdCen1^-/-) parasite can persist and be both safe and protective in animals. LdCen1^-/- has a defect in amastigote replication both in vitro and ex vivo in human macrophages. Safety was shown by the lack of parasites in spleen and liver in susceptible BALB/c mice, immune compromised SCID mice, and human VL model hamsters 10 wk after infection. Mice immunized with LdCen1^-/- showed early clearance of virulent parasite challenge not seen in mice immunized with heat killed parasites. Upon virulent challenge, the immunized mice displayed in the CD4+ T cell population a significant increase of single and multiple cytokine (IFN-γ, IL-2, and TNF) producing cells and IFN-γ/IL10 ratio. Immunized mice also showed increased IgG2a immunoglobulins and NO production in macrophages. These features indicated a protective Th1-type immune response. The Th1 response correlated with a significantly reduced parasite burden in the spleen and no parasites in the liver compared with naive mice 10 wk post challenge. Protection was observed, when challenged even after 16 wk post immunization, signifying a sustained immunity. Protection by immunization with attenuated parasites was also seen in hamsters. Immunization with LdCen1^-/- also cross-protected mice against infection with L. braziliensis that causes mucocutaneous leishmaniasis. Results indicate that LdCen1^-/- can be a safe and effective vaccine candidate against VL as well as mucocutaneous leishmaniasis causing parasites.


So far, several procedures have been used to develop live attenuated Leishmania parasites including long-term in vitro cultures, selection for temperature sensitivity, chemical mutagenesis, and irradiation (1, 2). Although such live attenuated lines have shown substantial protection against challenge in animal models, undefined genetic mutations and concerns arising from potential reversion to virulence make such vaccines unsuitable for human vaccination. Indeed, the persistence of asymptomatic infection especially in immunocompromised individuals raises the risk of reversion to clinical disease. Moreover, attenuations due to undefined genome alterations can reduce effective protective immunity, either they fail to persist long enough to elicit an immune response or lack critical epitopes to evoke the protective response (3). Alternatively, attenuation obtained through targeted genetic disruptions of essential growth regulating or virulence genes by homologous recombination is nonrevertible, hence can be safe (1). Moreover, sustained exposure of parasite Ag to the host eliminates the need of any adjuvants that are required in other nonliving vaccines (e.g., subunit vaccines).

There are several examples of targeted gene deletions that have been conducted for developing Leishmania-attenuated vaccine strains. Among the vaccine candidates studied for CL, L. major dihydrofolate reductase thymidylate synthase (dlhfr-ts) knockout parasites protect mice (4) but not Rhesus monkeys (5). L. major deficient in surface and secreted phosphoglycans (lpg^-), although unable to survive in sand flies and macrophages, retained the ability to persist indefinitely in mice and conferred protection against virulent challenge, even in the absence of a strong Th1 response (6, 7). Over time, however, lpg^- (a complete gene knockout) parasites unexpectedly regained virulence (8). L. major deleted for phosphomannomutase-protected mice, despite no increase in either effector or

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2 Abbreviations used in this paper: CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis; WI, wild type; FTAg, freeze-thaw Ag; WI, week immunized; WPC, week post challenge.

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memory response (9). In contrast, L. mexicana that also causes CL, deficient in cysteine proteinase genes ($\Delta$cpa and $\Delta$cpb), conferred protection in mice and hamsters against homologous challenge (10, 11). Among the vaccination studies in VL, mice immunized with a L. donovani strain deleted for biotin transporter (BT1) were similarly protected (12). Recent attempts using partial knockout parasites A2-A2rel gene cluster in L. donovani (13) and SIR2 gene in L. infantum (14) as immunogens induced protection against virulent challenge in BALB/c mice. However, such mutants cannot be used as vaccine candidates, because they still carry wild-type (Wt) allele/s and could cause disease.

Despite limitations with most of the mutant strains, these experiments clearly demonstrate the potential as well as the pitfalls of generating live attenuated Leishmania vaccines by targeted gene deletions. Hence it is critical to develop attenuated lines through complete gene knockouts that generate avirulent organisms that persist for a brief duration but eventually are completely eliminated, thereby inducing effective immunity without clinical disease or the risk of reactivation. Recently, we have developed a L. donovani strain completely deleted for the $\text{centr}1$ gene (15). $\text{centr}1$ is a calcium-binding basal body-associated protein involved in cell division in protozoan parasites like Leishmania, Trypanosoma, and Plasmodium (16–18) and centrosome associated in higher eukaryotes (19). We demonstrated that deletion of Ld$\text{Centr}1$ (Ld$\text{Centr}1^{-/-}$) did not affect the growth of the promastigote in vitro, however, the growth of the mammalian-infecting, amastigote form of the parasite, was blocked. These amastigotes showed failure of basal body duplication and cytokinesis, resulting in large multinucleate cells in culture and ex vivo in human macrophages (15). We tested this unique parasite line for its safety and protective efficacy against homologous and heterologous virulent Leishmania challenge and the immune response correlating with protection in rodent animal models.

Materials and Methods

Animals and parasites

Five- to six-week-old female BALB/c mice from National Cancer Institute, SCID mice (BALB/c background) from The Jackson Laboratory, and 40–50 gm male Syrian golden hamsters from Charles River Laboratories were used in the experiments. Procedures used were reviewed and approved by the Animal Care and Use Committee, Center for Biologics Evaluation and Research, Food and Drug Administration. Among parasites, the Wt, $\text{centr}1$-deleted (Ld$\text{Centr}1^{-/-}$) and $\text{centr}1$ deleted but episomally $\text{centr}1$ protein expressing line (Ld$\text{Centr}1^{-/-}$ AB) of L. donovani (15) were used. The parasite culture procedure and the routine molecular biology practices were as previously described (16).

Vaccinations and challenge studies

In independent experiments, the mice were inoculated/vaccinated via tail vein with 3 million metacyclic parasites of either L. donovani Wt, or Ld$\text{Centr}1^{-/-}$ parasites. Infective-stage metacyclic promastigotes of L. donovani were used (in stationary cultures by density gradient centrifugation as described (20)). Control groups (naive) corresponded to mice that were isolated from stationary cultures by density gradient centrifugation (21). As an additional confirmation of the presence of parasites in tissues, total DNA samples obtained from infected mouse and hamster spleens were used as templates in a Taqman-based real-time PCR. The amplification target was on the kinetoplast minicircle DNA of the parasite. The primers and methods were as previously described (22), with the addition of a fluorescent probe for detection. The probe had the sequence 5'-RAAARKKVRTCRCA GAAAYCCCGT-3'. A Black Hole Quencher moiety is coupled to the 3' end and Calfluor Red is coupled to a C6 linker at the 5' end. The degenerate linker is locked according to the Nomenclature Committee of the International Union of Biochemistry (http://www.chem.qmul.ac.uk/subnb/misc/naseq.html). The degenerate probe allows detection of sequence variants of the minicircle found in Leishmania and is added to the reaction mixture at a final concentration of 1.5 pmol/$\mu$l. To evaluate the number of Leishmania cells that were represented by a given Ct value, a standard curve was constructed by infecting macrophages (isolated, differentiated primary human monocytes) with stationary phase Leishmania. After 24 h, macrophages were trypsinized, scraped into PBS, the macrophage cells/$\mu$l were determined by hemocytometer, and the Leishmania amastigotes per macrophage were determined microscopically from a smear of Diff-Quick (Baxter Healthcare Corporation) stained cells. A calculated volume of the cell suspension was added to DirectPCR Lysis Reagent (Viagen Biotech) to produce lysates of 10 parasites/$\mu$l, 1 parasite/$\mu$l, and 0.1 parasite/$\mu$l. One microliter of each concentration was used as template in real-time minicircle PCR with four replicates to determine the mean Ct value.

Intracellular staining and flow cytometry

Splenocytes were plated in 24-well plates and stimulated with freeze-thaw Ag (FTAg) (23) or no Ag (control) in complete RPMI 1640 medium. After 5 h at 37°C brefeldin A was added to the wells. After 7 h at 37°C, cells were fixed with 1% paraformaldehyde for 15 min. The cells were washed twice in PBS and fixed with cytofix/Cytoperm kit for 20 min (room temperature), intracellular staining with Alexa Flour 700 anti-IFN-γ, FITC anti-CD4, Human Adsorbed, Rabbit anti-mouse IgG (H + L)-HRP; Rabbit anti-mouse IgG1-HRP, FITC anti-CD8 Abs for 30 min (each with 1/1000 dilutions). SureBlue (KPL) was used as a peroxidase substrate. After 15 min, the reaction was stopped by the addition of 100 $\mu$l of 1 M H$_2$SO$_4$, and the absorbance was read at 450 nm.

NO quantifications

Splenocytes or macrophages obtained from peritoneal fluid (24) were cultured in complete, endotoxin-free RPMI 1640 medium (certified LPS free by the manufacturer). NO (nitrite/nitrate) production was determined for the supernatants from unstimulated cultures and cultures stimulated with FTA g for 48 h at 37°C by the Griess reaction kit (Sigma-Aldrich).

Statistical analysis

Statistical analysis of differences between means of groups was determined by two-sample t test assuming unequal variance. A p value < 0.05 was considered as highly significant.

Results

Growth attenuation and limited persistence of Ld$\text{Centr}1^{-/-}$ parasites in mice

Centr1-deleted L. donovani metacyclics when used to infect human macrophages in vitro or injected in susceptible BALB/c mice, displayed an attenuated growth (i.e., reduction in the number of
The data presented are representative of two or more experiments with similar results. Mean and SEM of four or more mice in each group are from the organs were measured after different time points post challenge. Similarly, we did not observe any LdCen1−/− parasites at 12 wk postinfection in SCID mice, indicating that their clearance is not dependent on functional T and B cells (Fig. 1b).

**FIGURE 1.** Avirulence and immunoprotective properties of centrin (LdCen1) knockout L. donovani (LdCen1−/−) parasites in mice. a, Survival of parasites of LdCen1−/−, LdCen1+/− epimorphically expressing LdCen1 protein (add back; LdCen1+/− AB), and Wt in BALB/c mice. Parasite burdens from the organs of mice infected with metacyclic parasites at 5 wk post infection are shown. b, Centrin knockout L. donovani does not survive for long in BALB/c and SCID (BALB/c) mice. Parasite load in organs of mice infected with metacyclics of either Wt or LdCen1−/− parasites were measured at 5 or 12 wk after infection. c, Protection of LdCen1−/− vaccinated BALB/c mice against virulent challenge. Mice vaccinated with LdCen1−/− parasites were challenged after different time periods with virulent Wt L. donovani and the challenge parasite burdens from the organs were measured after different time points post challenge. The data presented are representative of two or more experiments with similar results. Mean and SEM of four or more mice in each group are shown. Cha, challenged; Imm, immunized; ND, not detected; WI, wk after infection; WC, wk after challenge; *, p < 0.01.

infected macrophages over time; supplementary Fig. 1a), and the number of parasites per spleen or liver 5 wk postinfection compared with control (Fig. 1a). This is the direct consequence of centrin1 deficiency, because LdCen1−/− cells expressing centrin1 protein from a transfected plasmid were rescued for growth both in macrophages and in mice (supplementary Fig. 1a and Fig. 1a). To analyze the persistence of LdCen1−/− in mice, BALB/c mice were injected with LdCen1−/− or Wt parasites and were monitored up to 12 wk post infection. Few LdCen1−/− parasites were seen at 5 wk post infection in spleen and liver, measured by limiting dilution, and by 12 wk, the parasites were completely cleared from both organs (Fig. 1b). The Wt-infected mice had significant parasite burden in these organs at both the time points (Fig. 1b). Similar results were obtained in a confirmatory real-time PCR study using DNA from spleens of mice infected either with Wt or LdCen1−/− parasites (supplementary Table 1). Detection of the parasite minicircle DNA target indicated the presence of a substantial number of parasites in both Wt-infected (6 wk) and LdCen1−/−-infected (5 wk) mice. However, the mice infected with LdCen1−/− for 12 wk showed a level equivalent to naïve mice, which can be considered as a background, signifying either no LdCen1−/− parasites or significantly reduced parasite burden in mice at this time point. Hence, it appears that the parasites are present for at least 5 wk in the viscera as opposed to disappearing within just a few days. Similarly, we did not observe any LdCen1−/− parasites at 12 wk postinfection in SCID mice, indicating that their clearance is not dependent on functional T and B cells (Fig. 1b).

**Protective efficacy of LdCen1−/− parasites against virulent infection**

The ability of LdCen1−/− parasite to protect against Leishmania infection was determined in 5-wk immunized mice (WI) followed by challenge with virulent Wt L. donovani. Spleen and liver were analyzed for parasite burden 4, 8, and 10 wk post challenge (WPC), measured by limiting dilution. The results showed that the immunized mice had ~2-log-fold reduced (p < 0.01) parasite burden in spleen and undetectable parasite in liver compared with naïve-challenged mice at 10 WPC (Fig. 1c). However, there was no significant change in the weight of liver or spleen of immunized or unimmunized mice. To evaluate the ability of LdCen1−/− immunization to confer lasting protection, mice were challenged with virulent Wt L. donovani 12 or 16 wk following LdCen1−/− immunization, and evaluated after 10 wk. The parasite burden in the spleen and liver indicated that the level of protection in both immunized groups (12 WI and 16 WI) was similar to the level observed for the 5 WI group, indicating a sustained protective response after immunization (Fig. 1c). A similar result was observed in a separate experiment where mice were challenged after 24 wk of immunization (data not shown). No protection was observed 8 WPC in mice immunized with heat killed 3 million LdCen1−/− metacyclics challenged 5 wk after immunization (supplementary Fig. 1b), suggesting that live attenuated parasites were required to induce protective immunity. Overall, the results suggest that mice immunized with LdCen1−/− elicit a strong and sustained protection against the virulent challenge.

**Induction of multifunctional Th1 effector cells correlates with LdCen1−/−-induced protection**

The involvement of IFN-γ-producing Th1 cells in immunity against Leishmania is described (25). We also observed an absolute requirement for IFN-γ in LdCen1−/−-induced immunity. IFN-γ knockout mice immunized with LdCen1−/− for 5 wk followed by challenge were not protected. Parasite burden from these mice was similar to naïve-challenged control observed at 10 wk post challenge (Fig. 2). To further investigate the cell-mediated responses induced by LdCen1−/− parasites and find a correlate of protective immunity, we measured the Th1 cytokines IFN-γ, TNF, and IL-2 produced by splenic CD4+ and CD8+ T cells from the BALB/c mice using multiparameter flow cytometry. Spleen cells grown in vitro with or without freeze thaw Ags followed by multicolor staining were gated based on forward and side scatter in FlowJo to select only the lymphocyte population devoid of dead cells and other larger leukocytes (Fig. 3a, left) and further gating of the lymphocytes for the CD3+ (Fig. 3a, middle) followed by gating of CD3+ in to CD4+ and CD8+ T cells (Fig. 3a, right). Seven distinct populations of cytokine-producing cells were defined from the CD4+ and CD8+ T cells based on different combinations of IFN-γ,
TNF, and IL-2 (a representative analysis is shown in Fig. 3b). The quality of the Th1 response is based on the relative frequency of these distinct populations (26). Five weeks after immunization with LdCen1\(^{-/-}\), single cytokine-producing CD4\(^+\) and CD8\(^+\) T cells, making either IFN-\(\gamma\) or IL-2, were more predominant than multicytokine-producing subpopulations post immunization (Fig. 3, c and d). Similar analysis conducted with spleens of immunized mice analyzed 10 WPC showed a significantly higher percent (2- to 10-fold; \(p < 0.01\)) of the cytokine-producing subpopulations than in the naive challenged mice (Fig. 3, e and f). Importantly, the expansion of multifunctional cells was clearly evident in the immunized mice 10 WPC. Interestingly, the percent of both CD4\(^+\) and CD8\(^+\) T cells that make TNF alone increased significantly (up to 2-fold; \(p < 0.01\)) after challenge in LdCen1\(^{-/-}\) immunized mice. The percentage of T cells that produce Th1 cytokines increased after immunization and the increased frequency of multifunctional cells after challenge strongly correlated with protection.

We also quantitated T cells that produce IL-10, a cytokine involved in the pathogenesis of VL (27). The ratio of IFN-\(\gamma\) to IL-10 serves as an additional correlate of immune protection (14). In the restimulated CD4\(^+\) T cells from the spleen, the IFN-\(\gamma\)/IL-10 ratio was significantly higher in the immunized mice both at the time of challenge (5 WI) and after challenge (5 WI plus 10 WC) compared with either naive or naive-challenged controls (Fig. 3g). The results thus indicate an increased IFN-\(\gamma\) secretion coinciding with reduced IL-10 production among the immunized mice, indicating a strong Th1 response that could be accountable for protective immunity.

Recent studies in CL infection in mice indicate a role for nonregulatory T cells, which simultaneously produce IFN-\(\gamma\) and IL-10, for immune suppression during pathogenesis of a non-healing lesion (28). Hence, we also looked at IFN-\(\gamma\)-T cells that coproduce IL-10 cytokine after immunization with LdCen1\(^{-/-}\). The results indicated a significantly higher percentage of CD4\(^+\) T cells positive for both IFN-\(\gamma\) and IL-10 in the 5-wk-immunized mice than in controls (Fig. 3h). No increase of such cells in the CD4\(^+\) population was observed either 12 or 16 wk after immunization (data not shown). The presence of IFN-\(\gamma\) and IL-10 coproducing CD4\(^+\) cells early after immunization (5W, a time point when LdCen1\(^{-/-}\) parasites are still detectable), might reflect immune modulation to reduce inflammation-mediated host damage.

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

We evaluated the humoral response in the immune-challenged mice. Sera from BALB/c mice taken 10 wk post challenge after 5, 12, or 16 immunization weeks were measured for Leishmania-specific IgG, IgG1, and IgG2a responses. Results indicated a higher level of all three Ab populations in the immune-challenged groups compared with the naive challenged groups (Fig. 4a). Importantly, the immunization led to a selective increase in Th1-driven IgG2a Ab levels during infection (\(p < 0.01\)). The results indicate a higher Leishmania specific humoral immune response generated by immunization with LdCen1\(^{-/-}\) that correlates with the increase in the Th1 response.

**Increased NO production in spleen cells in the immune-challenged mice**

Because the production of NO by macrophages is a key factor in killing Leishmania, we determined the level of NO produced by in vitro Ag restimulated splenocytes that included macrophages derived from the immune-challenged mice by ELISA. Interestingly, significantly higher (\(p < 0.01\)) levels of the reactive NO radical (nitrite) were demonstrated in the supernatants of splenocytes of the three immune-challenged groups (Fig. 4b). We also analyzed Ag-specific release of nitrite from the cultured macrophages derived from the peritoneal fluids of mice. There is a small increase in NO production in macrophages of the naive mice, suggesting a background level in this assay, as well as in the naive challenged mice with the addition of FTAg. However, in the immunized, challenged mice the production of NO in response to FTAg is significantly higher (\(p < 0.01\)) than the naive challenged mice, which clearly correlates with protection (supplemental Fig. 2).

**Safety and efficacy of LdCen1\(^{-/-}\) in the hamster model**

Because golden Syrian hamsters are considered a more appropriate model for VL (29), we evaluated the safety and protection of LdCen1\(^{-/-}\) against L. donovani challenge in this species. The LdCen1\(^{-/-}\) parasite burden in spleens, measured by limiting dilution, was 3-log-fold less than Wt L. donovani 5 wk after inoculation in hamsters and no LdCen1\(^{-/-}\) parasites were found in the liver, whereas at 10 wk post immunization, no LdCen1\(^{-/-}\) parasites were observed in any of the organs in contrast to hamsters inoculated with virulent parasites, which had significantly higher number of parasites in both spleen and liver (Fig. 5a). Further persistence of the parasites in the hamster model was evaluated by performing real-time minicircle PCR on spleen DNA samples collected more than 3 mo postinfection. Samples from Wt-infected hamsters gave a Ct value indicating a substantial number of parasites, while at the same time point, LdCen1\(^{-/-}\)-infected samples gave a Ct value equivalent to the uninfected hamsters. After 5 wk of immunization with LdCen1\(^{-/-}\) and challenge with virulent L. donovani, the immunized hamsters had significantly lower parasite burden in both the organs, measured by limiting dilution, compared with the naive-challenged animals, with a reduction of 99.9% in spleen and 99.7% in liver (\(p < 0.01\)) (Fig. 5b).

**Centrin1 KO L. donovani cross protects mice against challenge with L. braziliensis**

We wanted to determine whether immunization with LdCen1\(^{-/-}\) could provide heterologous protection against infection involving other Leishmania species. For this purpose, 5 wk LdCen1\(^{-/-}\) immunized as well as naive BALB/c mice were challenged by s.c. inoculation in the footpad with 2 million metacyclics purified from L. braziliensis, the causative agent of...
The naive, challenged mice developed progressive lesions during the 7 wk of observation, whereas the immunized mice developed significantly smaller lesions (3-fold; p < 0.01) throughout the 7 wk (Fig. 5c). The immune-challenged mice showed significantly lower parasite burdens both in the footpads and lymph nodes compared with the controls (Fig. 5d), with a 99.9% reduction in both the tissues (p < 0.02; p < 0.01). A similar type of experiment conducted to examine cross

**FIGURE 3.** Multiparameter flow cytometry based analysis for distinct Th1/Th2 cytokine populations. A seven color cytometry panel was used to measure the four cytokines, IFN-γ, TNF, IL2, and IL10, with the help of three T cell membrane markers CD3, CD4, and CD8. a. The common gating steps shown in this study through FlowJo analysis are as explained in the Results section. b. Representative fluorescence intensity distributions of single, double, or triple positive cytokines (IFN-γ, TNF, and IL-2) expressing T cells in spleens of vaccinated (5 wk post vaccination) and naive mice. CD3+CD4+ or CD3+CD8+ lymphocytes from spleens showing either IFN-γ+ or IFN-γ− are each further separated into cells that also make either TNF, IL-2, both TNF and IL-2, or none. c–f. Flow cytometric analysis of Leishmania Ag-specific multifunctional T cells. Leishmania Ag-specific multifunctional effector cells that were measured from splenocytes of LdCen+/− vaccinated mice at the time of challenge (5WI, 5 wk post immunization) and after challenge (5WI plus 10WPC: 10 wk post challenge) (c and d). The percentages of CD4+ (c and e) and CD8+ (d and f) T cells producing the cytokines IFN-γ, TNF, and IL-2 and combinations of these. The analysis was conducted using multiparameter flow cytometry (as in Methods). g. Ratio of IFN-γ to IL-10 producing CD4+ T cells from spleens at the time of challenge (naive and immunized (5W)) and after challenge (naive-challenged and immune-challenged (5WI plus 10WPC)). h. CD4+ T cells from spleens that simultaneously produce IFN-γ and IL-10 at 5W post immunization with LdCen+/−. The data presented are representative of two or more experiments with similar results. Mean and SEM of four or more mice in each group with error bar indicating SEM are shown. Cha, challenged; Imm, immunized; W, wk infected; WI, wk after immunization; WPC, wk after challenge; *, p < 0.01.
protection of \( \text{LdCen}^{-/-} \) against \( L.~\text{major} \) showed a moderate but delayed protection (data not shown).

**Discussion**

In pursuit of a candidate live attenuated vaccine, we generated a \( L.~\text{donovani} \) parasite that had a complete deletion for a cell division gene, centrin1 (\( \text{LdCen}^{-/-} \)) (15). We have demonstrated that \( \text{LdCen}^{-/-} \) attenuation is due to the failure of cytokinesis, resulting in multiplication of cellular organelles, cell enlargement, and eventual programmed cell death. We think that the genetically targeted and defined attenuation reduces the risk of reversal to virulence, a concern generally raised for attenuated organisms that are created by random genomic mutations. \( \text{LdCen} \) deletion specifically attenuated the amastigote stage of the parasite that replicates inside macrophages, and has no effect on the growth of the promastigote form. This could be advantageous to grow large quantities of parasites for vaccine trials.

To evaluate it as a vaccine candidate, the \( \text{LdCen}^{-/-} \) parasite was tested both in susceptible BALB/c mice and Syrian hamsters. \( \text{LdCen}^{-/-} \) parasites showed persistence of low numbers of parasites both in mice and hamsters for at least 5 wk. Ten (both in mice and hamsters) or 16 wk postinfection and in some cases even longer periods (24 wk; data not shown), \( \text{LdCen}^{-/-} \) parasites were not detected in liver or spleen of mice, suggesting the complete clearance demanded of a safe vaccine candidate. Similar observations in SCID mice lacking both T and B cells reinforce its safety even in the immune compromised host.

While searching for correlates of protection against homologous challenge, we found that \( \text{LdCen}^{-/-} \) immunized mice developed an increased percent of \( \text{Leishmania} \) specific CD4\(^+\) T cells that coproduce IFN-\( \gamma \) and IL10 during postchallenge with \( L.~\text{braziliensis} \). We also observed an increased IFN-\( \gamma \)/IL-10 ratio in the splenocyte supernatant measured by ELISA after 4 wk. Cross-protection of BALB/c mice vaccinated with \( \text{LdCen}^{-/-} \) against heterologous challenge with \( L.~\text{braziliensis} \). Five wk post vaccinated mice were infected with metacyclics of \( L.~\text{braziliensis} \) injecting i.m. in the left hind footpad of each mouse. Graph shows footpad swelling due to infection in the injected footpad calculated by measuring the difference in the footpad size between the two hind footpads. Parasite numbers per organ of infected footpad or draining lymph nodes from 7 wk postchallenge with \( L.~\text{braziliensis} \) are shown. The data presented are representative of two experiments with similar results. Mean and SEM of four animals in each group are shown. Cha, challenged; Imm, immunized; ND, not detected; W, wk after infection; *, \( p<0.02 \) and **, \( p<0.01 \).

**FIGURE 5.** Avirulence and immuno-protective properties of \( \text{LdCen}^{-/-} \) parasites in hamsters. a, Parasite load in organs of hamsters infected with either \( \text{Wt} \) or \( \text{LdCen}^{-/-} \) metacyclic parasites was measured at 5 and 10 wk after infection. b, Protection of the \( \text{LdCen}^{-/-} \) vaccinated hamsters against virulent challenge. Hamsters vaccinated with \( \text{LdCen}^{-/-} \) parasites were challenged after 5 wk of immunization with virulent Wt \( L.~\text{donovani} \) and the challenge parasite burdens from the organs were measured after 4 wk. c, Cross-protection of BALB/c mice vaccinated with \( \text{LdCen}^{-/-} \) against heterologous challenge with \( L.~\text{braziliensis} \). Five wk post vaccinated mice were infected with metacyclics of \( L.~\text{braziliensis} \) injecting i.m. in the left hind footpad of each mouse. Graph shows footpad swelling due to infection in the injected footpad calculated by measuring the difference in the footpad size between the two hind footpads. d, Parasite numbers per organ of infected footpad or draining lymph nodes from 7 wk postchallenge with \( L.~\text{braziliensis} \) are shown. The data presented are representative of two experiments with similar results. Mean and SEM of four animals in each group are shown. Cha, challenged; Imm, immunized; ND, not detected; W, wk after infection; *, \( p<0.02 \) and **, \( p<0.01 \).

**FIGURE 4.** a, Humoral response in the virulent challenged mice vaccinated with \( \text{LdCen}^{-/-} \) parasites. ELISA measurement of IgG, IgG1, and IgG2a Abs in sera from the mice immunized with \( \text{LdCen}^{-/-} \) parasites for 5, 12, or 16 wk or naive before challenge with virulent \( L.~\text{donovani} \). Sera were collected 10 wk post challenge. b, \( \text{Leishmania} \) Ag specific stimulation of NO synthase (NOS2) in the splenocytes of naive, naive challenged and \( \text{LdCen}^{-/-} \) immunized and Wt challenged mice. The activity of NOS2, indicated by the amount of released nitrite (NO) in the splenocyte supernatants was measured by the Griess reaction. The data presented are representative of two experiments with similar results. Mean and SEM of three or more mice in each group are shown. Cha, challenged; Imm, immunized; FTAg, Freeze-thaw Ag; WI, wk after immunization; WPC, wk after challenge; *, \( p<0.01 \).
possible immune modulation at this time as also observed previ-
ously, during Leishmania infection in mice (28). In the Leishmania case, the nonregulatory T cells (CD4+CD25+Foxp3+) that were the source of IL-10 (most of them also produced IFN-γ) were immu
nosuppressive during protozoan infection for the reduction of in-
fec tion mediated damage to host cells. Hence, the LdCen1−/− im-
munization may be facilitating a desirable balance in the immune re-
response allowing brief parasite persistence that facilitates protec-
tion without causing host damage.

In the present study, we observed a robust Th1-specific serum Ab (IgG2a) response in the immune-challenged mice, further sup-
porting the observation that LdCen1−/− induces a generalized Th1 type response. In addition, the increased release of NO observed in the splenocyte culture derived from the immune-challenged mice coincided with the increase of Leishmania specific T cells producing IFN-γ, TNF, and IL-2 cytokines. NO production induced by Th1 cytokines is a main leishmanicidal mechanism of murine mac-
rophages (31, 32). Thus, in the LdCen1−/−-immunized mice, NO production by macrophages that are stimulated by Th1 cytokine producing T cells correlates with the control of infection.

Importantly, immunization with LdCen1−/− protected both mice and hamsters against virulent homologous challenge, as in-
dicated by significantly reduced parasite burdens in the organs of the immune-challenged mice and hamsters compared with naive controls. The protective response was sustained for up to 24 wk after immunization. Complete elimination of wild-type parasites from the livers of LdCen1−/−-immunized mice after virulent chal-
lenge, is superior to the protection induced by L. donovani BT1−/−, the only other complete gene knockout strain reported to target VL, which reduced parasite level in liver by 75% of the level in naïve challenged mice (12). The dhfr-ts auxotropic L. major line was safe in both mice and rhesus monkeys but protected only the mice (4, 5). Because the immune responses due to dhfr-ts were not studied in those animals, it would be difficult to know if there were weakness in the mouse immune response that could have predicted failure in the monkey. However, the quality of LdCen1−/−-induced immune response in mice and protection in mice and hamsters encourage us to proceed with immune studies in higher animal models. The level of protection achieved by LdCen1−/− was similar to the protection due to L. infantum SIR2−/− in mice (14). Because L. infantum SIR2−/− is a partial knockout, it may be at a greater risk of reversion and therefore is unlikely to be pursued as a vaccine candidate. Lpg2−/−, the only complete gene knockout that has reverted to virulence in mice, is also known to establish prolonged persistence in the host (7), which may have permitted the acquisition of compensating muta-
tions. Because LdCen1−/− does not persist in mice and hamsters beyond 2 mo, such a reversal to virulence is less likely.

There are several possible explanations for the protection by LdCen1−/− parasite vaccination. First, there could be residual par-
site burden (beyond the detection sensitivity of our methods, which would allow continued presence of Leishmania-specific ef-
fector cells and maintain anti-Leishmania immunity (21, 33). Sec-
cond, vaccination with LdCen1−/− parasites could lead to generation of a central memory T cell response after the KO parasites are cleared that could develop into an effector memory T cell response upon challenge, and provide protection (34). Third, there could be Ag persistence in peripheral tissues long after the parasites are cleared. These persistent Ag “deposits” may contribute to specific memory T cells through the activation of naive T cells as has been reported for some viral infections (35, 36). However, these hypo-
theses need to be tested and are the subject of future studies.

Finally, heterologous protection induced by infection with one virulent species of Leishmania against infection with another spe-
cies of Leishmania has been documented in animal model studies (37–39). In mice, experimental vaccination using dp72 protein iso-
lated from L. donovani cross-protected against Leishmania infection (40) and immunization with exogenous Ags (LmSEAGs) of L. ma-
jor cross-protected against L. donovani (41). Our results show that LdCen1−/−-immunized mice were cross-protected to a high de-
gree against a heterologous challenge with L. braziliensis, that causes MCL, and to some degree against L. major that causes CL. Therefore, our results confirm that the live attenuated parasites also confer resistance to infection with other species.

In the literature, several routes of administration of Leishmania vaccine candidates have been studied with varied degree of pro-
tection (reviewed in Ref. 42). In the present study, we have used the i.v. route in mice and intracardial route in hamster, however we recognize that in general a recommended route of administration of a vaccine in humans is either intradermal or i.m. Future studies in our laboratory will focus on defining the ideal route of admin-
istration for optimal protection for LdCen1−/− parasites.

In summary, this study demonstrates that in mice and hamsters, the live attenuated LdCen1−/− is highly immunogenic and confers a significant degree of protection against L. donovani and also is capable of inducing cross protection against L. braziliensis. The vaccine-elicited parasite Ag-specific Th1 responses coinciding with a robust Ab response and NO production, all strongly corre-
lated to a sustained protection. This report thus presents a well characterized amastigote specific, live attenuated L. donovani can-
didate vaccine that has been evaluated for its in vivo persistence, immunological response, and protective (against homologous and heterologous challenges) efficacy.

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