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

Angamuthu Selvapandian,* Ranadhir Dey,* Susanne Nylen,† Robert Duncan,* David Sacks,† and Hira L. Nakhasi1*  

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 naïve 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.  


The obligate kinetoplastid protozoan parasites of the genus *Leishmania* are spread by sand fly vectors and cause a spectrum of diseases collectively known as leishmaniasis that ranges from self-healing cutaneous leishmaniasis (CL),2 mucocutaneous leishmaniasis (MCL), and to fatal visceral leishmaniasis (VL). Leishmaniasis is endemic to 88 countries in the tropical and subtropical world, affecting 12 million people and threatening 350 million more. Drug treatment requires long-term medication, which is expensive and highly toxic. A vaccine for leishmaniasis has been a goal for a century, but there are still no effective vaccines (1). The knowledge that a cured disease either due to a natural infection or cutaneous leishmanization (1) protects the individual from reinfection and the persistence of a few parasites in the body can impart life-long protection against leishmaniasis, has encouraged researchers to develop live attenuated *Leishmania* vaccine candidates.

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 random 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 (*dlftrs−*) knockout parasites protect mice (4) but not Rhesus monkeys (5). *L. major* deficient in surface and secreted phosphoglycans (*lpgL−*), 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, *lpgL−* (a complete gene knockout) parasites unexpectedly regained virulence (8). *L. major* deleted for phosphomannomutase-protected mice, despite no increase in either effector or
memory response (9). In contrast, L. mexicana that also causes CL, deficient in cysteine proteinase genes (Δpda and Δpbl), 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 biopterin 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 centr1 gene (15). Centr1 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 LdCent1 (LdCent1Δ–Δ) 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 multinucleated parasites. Infective-stage metacyclic promastigotes of L. donovani (LdCent1Δ–Δ) and centr1 deleted but episomally expressing the centr1 protein expressing line (LdCent1Δ–Δ 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 cells of either Ldonovani Wt, or LdCent1Δ–Δ parasites. Infective-stage metacyclic promastigotes of L. donovani were used in stationary cultures by density gradient centrifugation as described (20). Control groups (naïve) corresponded to mice that received a saline solution (PBS). Mice vaccinated with LdCent1Δ–Δ parasites after different time periods were challenged via tail vein with 3 million virulent Wt L. donovani metacyclic parasites. Age-matched naïve mice as control groups were also similarly challenged with 3 million virulent metacyclic L. donovani parasites. In separate experiments, mice vaccinated (with LdCent1Δ–Δ) or not (naïve with saline) were also challenged by infecting i.m. on left hind footpad with 3 million metacyclic L. braziliensis parasites. Hamsters were inoculated intracardially with 10 million metacyclics of Wt or LdCent1Δ–Δ parasites. The hamsters vaccinated with LdCent1Δ–Δ or not (naïve with saline) were challenged after 5 wk intracardially with 10 million metacyclic virulent L. donovani parasites. After post-challenge periods, parasite load was recorded from spleens and livers from the L. donovani-challenged mice and hamsters and footpads and lymph nodes from the L. braziliensis-challenged mice by culturing the separated host cell preparations by limiting dilutions as previously described (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′-RAAARKVRTRCA GAAAAYCCTG–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 letter code is according to the Nomenclature Committee of the International Union of Biochemistry (http://www.chem.qmul.ac.uk/symbm/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/μl. To evaluate the number of Leishmania cells that were represented by a given Ct value, a standard curve was constructed by infecting macrophages (differentiated primary human monocytes) with stationary phase Leishmania. After 24 h, macrophages were trypsinized, scraped into PBS, the macrophage cells/μl 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/μl, 1 parasite/μl, and 0.1 parasite/μl. 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 36 h at 37°C brefeldin A was added to the wells. After 7 h at 37°C, cells were fixed with anti-CD 16/32 (5 μg/ml) at 4°C, stained with PerCP-Cy5.5 anti-CD4, PE-Cy7 anti-CD8 Abs for 30 min (each with 1/300 dilution; 4°C), fixed with Cytofix/Cytoperm kit for 20 min (room temperature), intracellular staining with Anti-IL-2, which was from eBioscience. Electronic compensation was performed with single-stained cells with individual mAbs used in the test. All Abs and reagents were purchased from BD Biosciences except Pacific blue anti-IL-2, which was from eBioscience. Electronic compensation was performed with single-stained cells with individual mAbs used in the test samples. Cells were acquired on LSRII with DIVA software (BD Biosciences) and analyzed by FlowJo software (Tree Star).

Ab responses

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

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 LdCent1Δ–Δ 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
infection; WC, wk after challenge; shown. Cha, challenged; Imm, immunized; ND, not detected; WI, wk after infection. 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 mini-circle 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 naive 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 naive-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 naive-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, IL-2, and 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 LdCen1Δ−/−, single cytokine-producing CD4+ and CD8+ T cells, making either IFN-γ 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 LdCen1Δ−/− 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-γ to IL-10 serves as an additional correlate of immune protection (14). In the restimulated CD4+ T cells from the spleen, the IFN-γ/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-γ 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-γ and IL-10, for immune suppression during pathogenesis of a non-healing lesion (28). Hence, we also looked at IFN-γ+ T cells that coproduce IL-10 cytokine after immunization with LdCen1Δ−/−. The results indicated a significantly higher percentage of CD4+ T cells positive for both IFN-γ 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-γ and IL-10 coproducing CD4+ cells early after immunization (5W, a time point when LdCen1Δ−/− 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 LdCen1Δ−/− 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 LdCen1Δ−/− in the hamster model**

Because golden Syrian hamsters are considered a more appropriate model for VL (29), we evaluated the safety and protection of LdCen1Δ−/− against L. donovani challenge in this species. The LdCen1Δ−/− parasite burden in spleens, measured by limiting dilution, was 3-fold less than that of L. donovani 5 wk after inoculation in hamsters and no LdCen1Δ−/− parasites were found in the liver, whereas at 10 wk post immunization, no LdCen1Δ−/− 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, LdCen1Δ−/−-infected samples gave a Ct value equivalent to the uninfected hamsters. After 5 wk of immunization with LdCen1Δ−/− 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 LdCen1Δ−/− could provide heterologous protection against infection involving other Leishmania species. For this purpose, 5 wk LdCen1Δ−/− 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...
MCL. 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
protection of \textit{LdCen}^{-/—} against \textit{L. major} showed a moderate but delayed protection (data not shown).

Discussion

In pursuit of a candidate live attenuated vaccine, we generated a \textit{L. donovani} parasite that had a complete deletion for a cell division gene, centrin1 (\textit{LdCen}^{-/—}) (15). We have demonstrated that \textit{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. \textit{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 \textit{LdCen}^{-/—} parasite was tested both in susceptible BALB/c mice and Syrian hamsters. \textit{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), \textit{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 \textit{LdCen}^{-/—} immunized mice developed an increased percent of \textit{Leishmania} specific CD4+ and/or CD8+ T cells expressing Th1 cytokines (IFN-γ, TNF, and IL-2) either singly or in multiple combinations. Multifunctional effector cells associated with protection have been described by others in mice vaccinated with Leish-111f recombinant polyproteins plus adjuvant (30) and with \textit{Leishmania} MML protein expressed in a replication-defective adenovirus (26). We found an especially strong increase of TNF-producing T cells, reinforcing prior data suggesting that TNF along with IFN-γ secretion are important cytokines for protection in vivo (26). We also observed an increased IFN-γ/IL-10 ratio among the CD4+ T cells in the \textit{LdCen}^{-/—} vaccinated mice both at the time of and after challenge, revealing another correlate of protection. A similar polarization to an increased IFN-γ to IL-10 ratio in the splenocyte supernatant measured by ELISA after \textit{L. infantum} challenge in mice immunized with SIR2^{+/-} \textit{L. infantum} was observed (14). We also observed an increase of CD4+ T cells that coproduce IFN-γ and IL10 during the initial period (5W) of infection with \textit{LdCen}^{-/—}, suggesting
possible immune modulation at this time as also observed previ-
ously, during *L. major* infection in mice (28). In the *L. major* 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
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 produc-
ing 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* auxotrophic 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-
asite 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-
ond, vaccination with *LdCen1*−/− parasites could lead to genera-
tion 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 hy-
potheses need to be tested and are the subject of future studies.

Finally, heterologous protection induced by infection with one
vivulnt 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 *L. major* 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
confers 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 intraderal 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*
candidate vaccine that has been evaluated for its in vivo persistence,
immunological response, and protective (against homologous and
heterologous challenges) efficacy.

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**Disclosures**

The authors have no financial conflict of interest.

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REPLICATION DEFICIENT Leishmania INDUCES PROTECTIVE IMMUNITY


Legends to Supplementary Figures:

**Supplementary Figure 1:** (a) Episomal expression of centrin1 protein in the *LdCen1*<sup>−/−</sup> parasites restores positive growth in cultured macrophages. Survival of parasites of *LdCen1*<sup>−/−</sup> and *LdCen1*<sup>−/−</sup> episomally expressing LdCen1 protein (add back; *LdCen1*<sup>−/−</sup> AB) and wild type (Wt) in macrophages is shown. Newly differentiated macrophages *in vitro* from freshly elutriated human monocytes in the presence of Macrophage colony-stimulating factor (M-CSF) in culture for 7 days were infected with metacyclic parasites and the percent of infected macrophages at different time periods post infection up to day 10 were measured. The data presented are the mean and S.E.M. of three independent experiments. At each time point in an experiment at least 300 total macrophages were counted. (b) Heat-killed (HK) *LdCen1*<sup>−/−</sup> parasites do not protect mice against virulent challenge. BALB/c mice vaccinated with either *LdCen1*<sup>−/−</sup> parasites, heat killed *LdCen1*<sup>−/−</sup> or PBS alone were challenged 5 wk post immunization with virulent *L. donovani*. Graph shows the number of challenge parasites in spleen and liver measured 8 wk post challenge. The data presented are representative of two experiments with similar results. Mean and S.E.M. of four animals in each group are shown. *, p<0.01.

**Supplementary Figure 2:** *Leishmania* antigen specific stimulation of nitric oxide synthase (NOS<sub>2</sub>) in the *in vitro* cultured peritoneal macrophages from naïve mice, mice immunized with *LdCen1*<sup>−/−</sup> for 5 wk followed by 10 wk challenge or naïve challenged mice. The activity of NOS<sub>2</sub>, indicated by the amount of released nitrite (NO) in the splenocyte supernatant, was measured by the Griess reaction. The data presented are representative of two experiments with similar results. Mean and S.E.M. of three or more mice in each group are shown. Cha’: challenged; FTAg: Freeze-thaw antigen; Imm’: immunized; WI: wk after immunization; WC: wk after challenge *, p<0.01.
Fig S1

(a) Percent infected macrophages over time for different genotypes: Wt, LdCen1/−, and LdCen1/− AB. Graph shows a decrease in infected macrophages over time for all genotypes, with significant differences marked by asterisks.

(b) Parasites per organ comparison between Naive, HK, and LdCen1/− genotypes for Spleen and Liver. Spleen shows a significant decrease in parasites for the LdCen1/− genotype compared to the Naive and HK groups. Liver shows a trend similar to Spleen but is not statistically significant.
Fig S2

Nitrite Conc' (µM)

-FTAg
+FTAg

Naive
Naïve Cha'
Imm' Cha'

5WI + 10WC
Supplemental Table 1. Real-time PCR confirmation of parasite presence in spleens of mice infected with either the virulent or \textit{LdCen1}^{-/} parasites

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ct   Values$^a$</th>
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<tbody>
<tr>
<td>Naïve</td>
<td>39.6 ± 0.6</td>
</tr>
<tr>
<td>6 wk infection with wild type parasites</td>
<td>31.2 ± 4.9</td>
</tr>
<tr>
<td>5 wk infection with \textit{LdCen1}^{-/} parasites</td>
<td>31.9 ± 1.8</td>
</tr>
<tr>
<td>12 wk infection with \textit{LdCen1}^{-/} parasites</td>
<td>37.7 ± 2.3</td>
</tr>
<tr>
<td>Intracellular Amastigotes$^b$ (cell count per reaction)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>28.5 ± 0.4</td>
</tr>
<tr>
<td>1</td>
<td>30.1 ± 0.4</td>
</tr>
<tr>
<td>0.1</td>
<td>33.5 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$ Mean and S.E.M. of three mice in each group are shown.

$^b$ Standard curve of ex vivo infected macrophages, 4 replicates at each concentration (see Materials and Methods for details)