DELEÇÃO DIRECIONADA DE CENTRINA EM *LEISHMANIA BRAZILIENSIS* UTILIZANDO CRISPR-CAS9: GERAÇÃO DE UMA LINHAGEM ATENUADA PARA IMUNOPROFILAXIA

RESUMO

As Leishmanioses são doenças tropicais negligenciadas e não existem vacinas para uso humano. Na América Latina, a *Leishmania braziliensis* é o principal agente causador da leishmaniose tegumentar. Aqui, visando o desenvolvimento de uma cepa atenuada de *L. braziliensis* com fins de imunoprofilaxia, nós empregamos a tecnologia CRISPR/Cas9 com sucesso. Geramos uma linhagem de parasitas transgênicos expressando Cas9 e T7 RNA polimerase, a qual foi empregada para a deleção direcionada de centrina, uma proteína do citoesqueleto envolvida na duplicação do centrossoma em eucariotos. O sequenciamento do genoma completo de *L. braziliensis* deficiente em centrina (*LbCen*$^{-/-}$) não indicou a presença de mutações fora do alvo. *In vitro*, as taxas de crescimento de promastigotas de *LbCen*$^{-/-}$ e de tipo selvagem foram semelhantes, mas os amastigotas axênicos e intracelulares de *LbCen*$^{-/-}$ mostraram um fenótipo multinucleado, com sobrevivência diminuída em macrófagos infectados. Após a inoculação experimental em camundongos BALB/c, a *LbCen*$^{-/-}$ não induziu o desenvolvimento de lesões e os parasitas foram eliminados rapidamente. Porém, a imunização com *LbCen*$^{-/-}$ não conferiu proteção contra um desafio subsequente, diferentemente de outras linhagens com esta mesma deficiência. A resposta imune induzida pela inoculação com *LbCen*$^{-/-}$ foi caracterizada por níveis significativamente mais elevados de IL-4 e IL-10, não observados em camundongos leishmanizados com *LbWT*. Portanto, a imunização com *LbCen*$^{-/-}$ não é capaz de induzir uma resposta imune do tipo Th1, tornando os camundongos suscetíveis a desafios e a *LbCen*$^{-/-}$ não pode reproduzir a imunidade observada na leishmanização. Em conclusão, a deficiência da centrina em *L. braziliensis* causa atenuação do parasita, interrompe a capacidade de causar doença, mas não é capaz de estimular uma resposta imune protetora.

Palavras-chave: leishmaniose cutânea, CRISPR-Cas9, vacina viva atenuada, imunização, proteção.

TARGETED CENTRINA DELETION IN LEISHMANIA BRAZILIENSIS USING CRISPR-CAS9: GENERATION OF AN ATTENUATED CELL LINE FOR IMMUNOPROPHYLAXIS

SUMMARY

Leishmaniases are neglected tropical diseases and there are no vaccines for human use. In Latin America, *Leishmania braziliensis* is the main causative agent of tegumentary leishmaniasis. Here, aiming at the development of an attenuated strain of *L. braziliensis* for immunoprophylaxis purposes, we successfully employed the CRISPR/Cas9 technology. We generated a line of transgenic parasites expressing Cas9 and T7 RNA polymerase, which was employed for the targeted deletion of centrin, a cytoskeletal calcium-binding protein involved in centrosome duplication in eukaryotes. Whole genome sequencing of centrin-deficient *L. braziliensis* (*LbCen*$^{-/-}$) did not indicate the presence of off-target mutations. *In vitro*, growth rates of *LbCen*$^{-/-}$ and wild-type promastigotes were similar, but axenic and intracellular *LbCen*$^{-/-}$ amastigotes showed a multinucleated phenotype with impaired survival in infected macrophages. After experimental inoculation in BALB/c mice, *LbCen*$^{-/-}$ failed to induce lesion development and the parasites were rapidly eliminated. Furthermore, immunization with *LbCen*$^{-/-}$ did not confer protection against subsequent challenges, unlike other centrin-deficient leishmanias. The immune response induced by inoculation with *LbCen*$^{-/-}$ was characterized by significantly higher levels of IL-4 and IL-10, not observed in mice leishmanized with *LbWT*. Therefore, immunization with *LbCen*$^{-/-}$ is not capable of inducing a Th1-type immune response, making mice susceptible to challenges, and *LbCen*$^{-/-}$ cannot reproduce the immunity observed in leishmanization. In conclusion, centrin deficiency in *L. braziliensis* causes parasite attenuation, interrupts the ability to cause disease, but otherwise cannot stimulate a protective immune response.

Key words: cutaneous leishmaniasis, CRISPR-Cas9, live-attenuated vaccine, immunization, protection.

RESULTADOS

CAPÍTULO I. Deleção direcionada de Centrina em *Leishmania braziliensis* usando edição baseada em CRISPR-Cas9

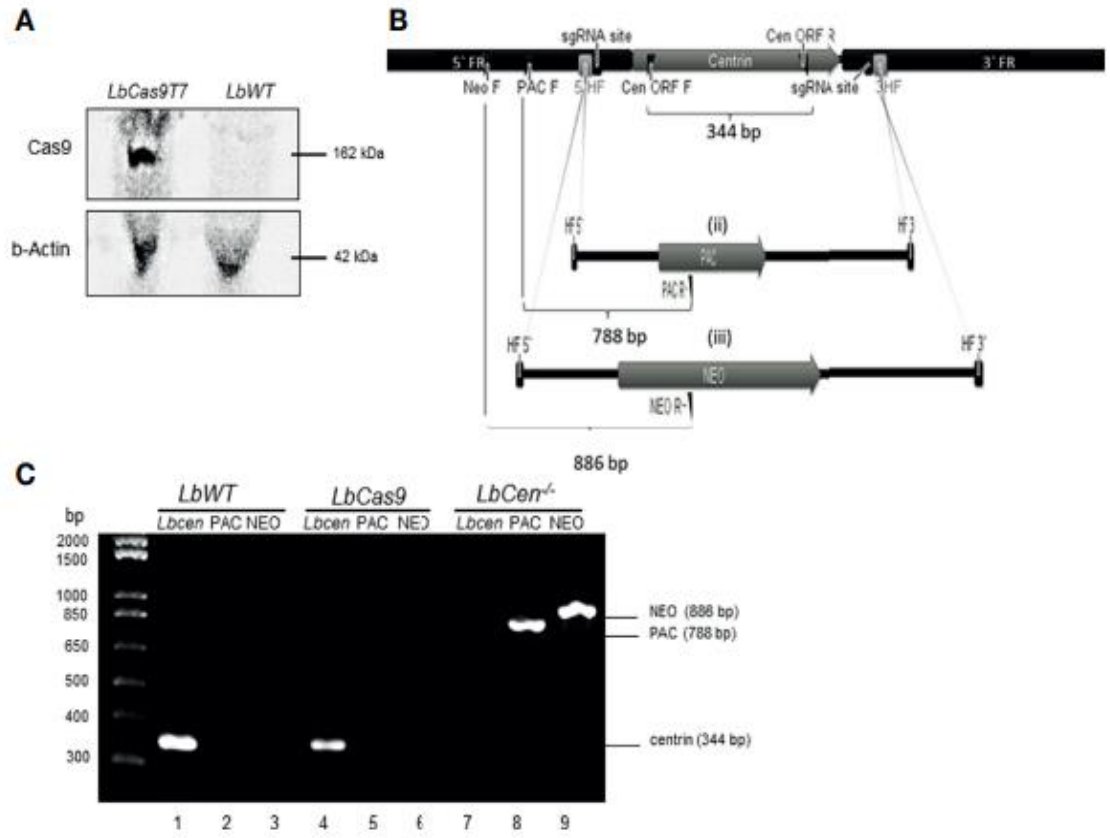

FIGURE 2 | Generation of centrin−/− *L. braziliensis*. (A) Western blot of whole cell lysates probed with anti-Cas9 antibody and anti-b-Actin. *LbWT*, parental cell line, *LbCas9T7*, *L. braziliensis* expressing Cas9 and T7. (B) In silico representation of the CRISPR-Cas9 based deletion of the *L. braziliensis* putative centrin gene (i) centrin genomic locus indicating sgRNA (guide RNA) binding sites at both 5` and 3` UTRs, 3`& 5` HF (homology flank) or flanking regions (30 bp) and primers for the correct integration (flag). PAC and NEO forward primers (PAC F and NEO F), Cen ORF forward and reverse (CEN ORF F and CEN ORF R) diagnostic primers for detection of centrin gene (amplification of 344 bp fragment). (ii) & (iii) Donor cassettes containing PAC and NEO antibiotics markers, indicating diagnostic reverse primers (PAC R and NEO R and size of expected amplicons for the confirmation of correct integration of the cassettes (788 and 886 bp, respectively). (C) PCR analysis of generated cell lines: test for the presence of the Centrin in *LbWT* parental line and in LbCas9T7; test for the integration of the PAC and NEO-resistance genes in *LbCen−/−*. PCR products were analyzed on a 1% agarose gel.

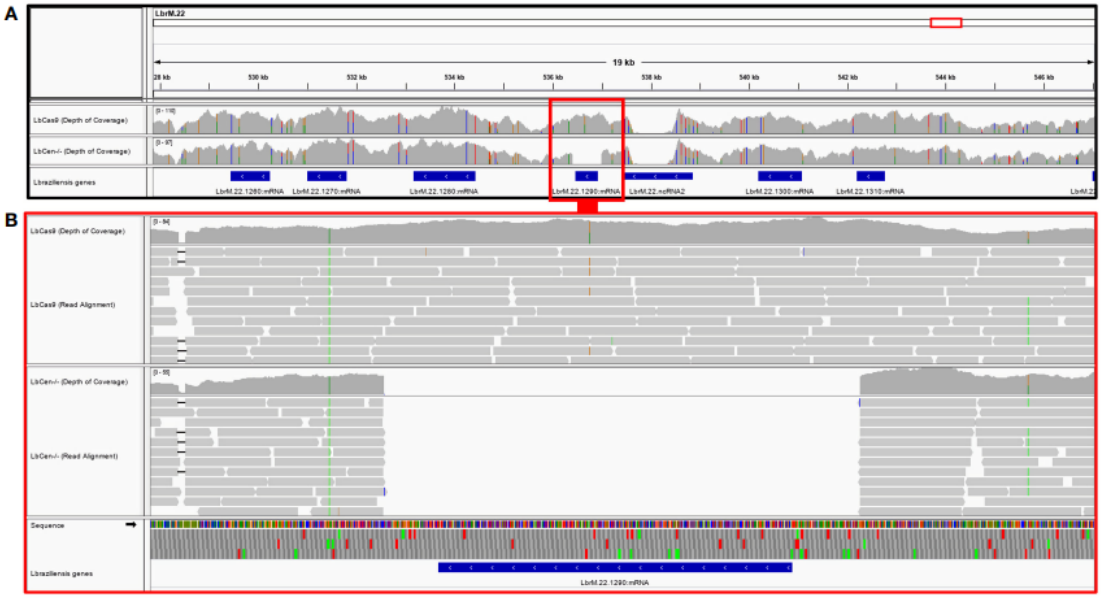

FIGURE 3 | Deletion of the LbrM.22.1290 centrin gene as confirmed by whole genome sequencing. (A) MHOM/BR/75/M2904 *L. braziliensis* chromosome 22 encompassing the 527,855–547,147 region covered by LbCas9T7 and lbCen−/− genomic read libraries. Blue boxes represent *L. braziliensis* genes, drawn in scale; LbrM.22.1290 centrin gene region is highlighted by red box. Gray histograms above the genes represent the read depth in each genomic position for each genomic library, where colored markings denote SNPs in the reads when compared to the reference genome. (B) Read mapping in the genomic region encompassing the LbrM.22.1290 gene. Mapping of individual reads is represented by gray boxes. An expected number of reads mapped into the LbrM.22.1290 centrin gene region in the LbCas9T7 isolate, whereas no read from *LbCen−/−* library mapped into this gene.

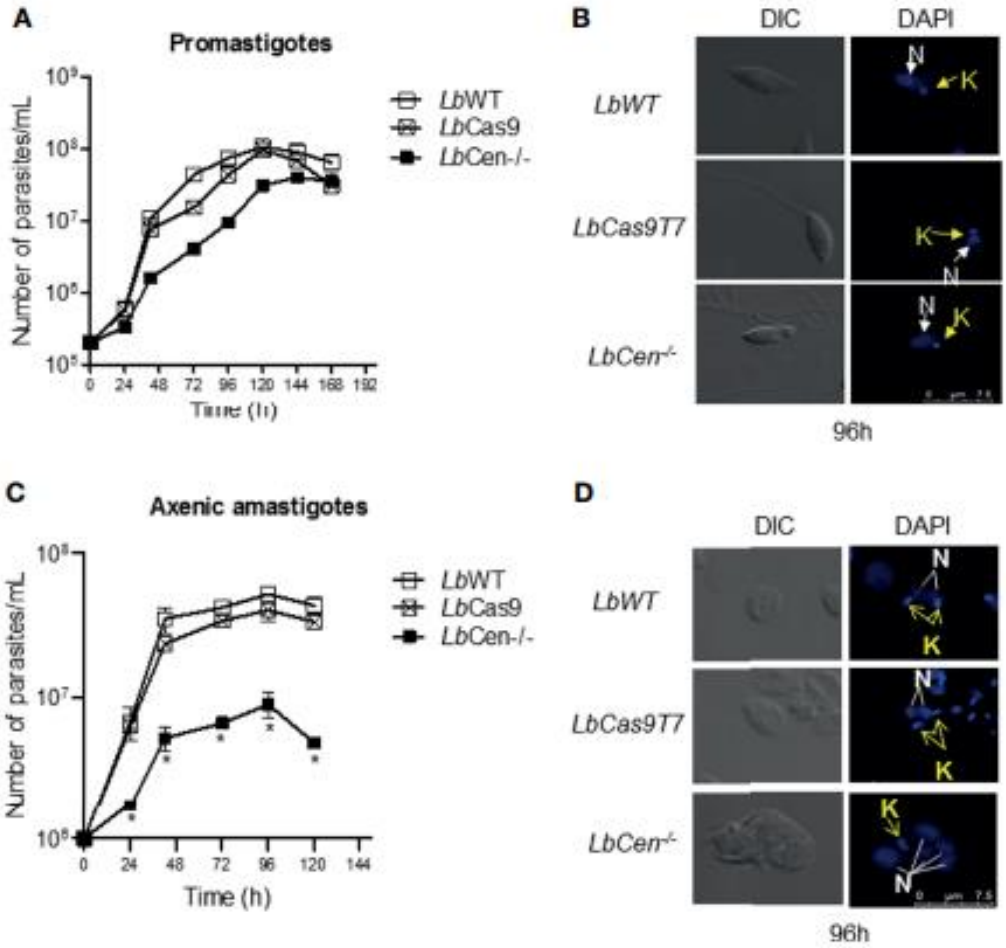

FIGURE 5 | Kinetics of *LbCas9T7* and *LbCen−/−* promastigote growth in vitro. (A) Promastigote cultures were started at 5 × 105 parasites/ml and were maintained at 26 °C for 7 days in supplemented Schneider media. Parasite numbers were determined daily by counting using a hemocytometer. Data is plotted as mean ± SEM and is from a representative experiment, performed in triplicate. (B) DIC and fluorescent (DAPI) representative micrographs of *LbWT*, *LbCas9T7*, and *LbCen−/−* promastigotes after 96 h of culture. (C) Axenic amastigote cultures were started at 1 × 106 parasites/ml and were maintained at 34°C for 5 days in supplemented Schneider media, pH 5.5. Parasite numbers were determined daily by counting using a hemocytometer. Data is plotted as mean ± SEM, and is from a representative experiment, performed in quadruplicate, *$p < 0.05$. (D) DIC and fluorescent (DAPI) representative micrographs of *LbWT, LbCas9T7,* and *LbCen−/−* amastigotes after 96 h of culture.

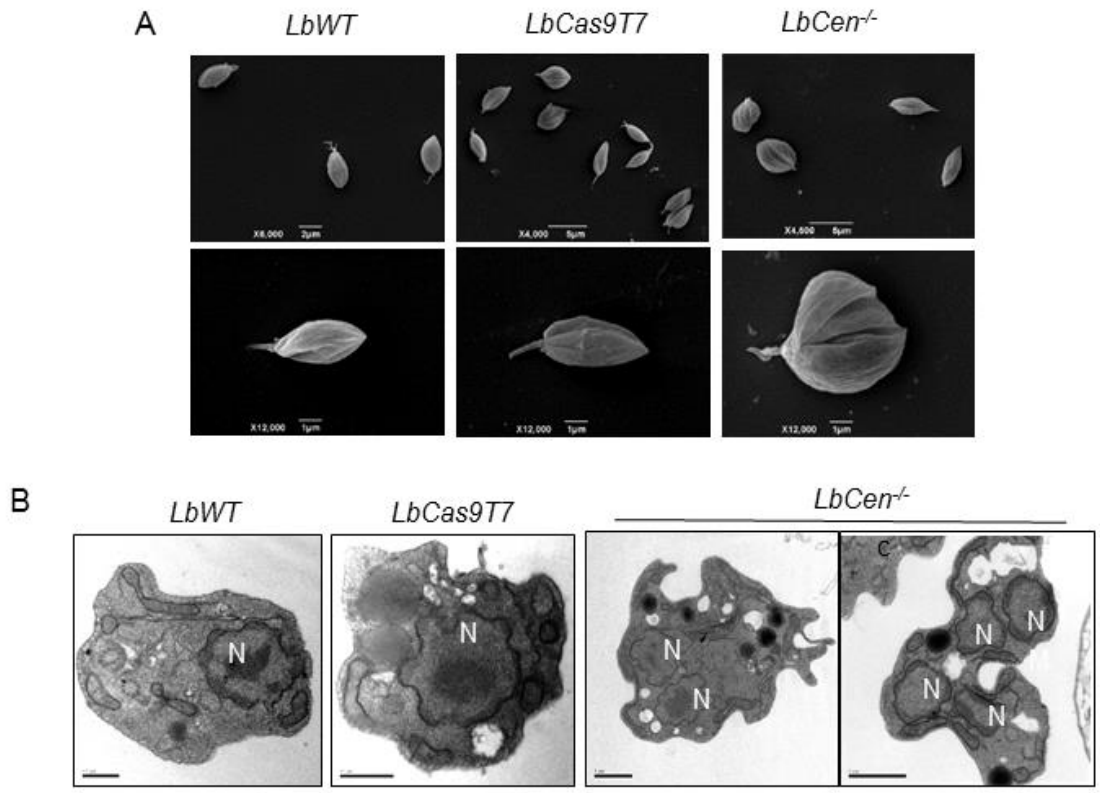

FIGURE 6 | Ultrastructural analysis of *LbCen−/−* axenic amastigotes. *LbWT, LbCas9T7*, and *LbCen−/−* axenic amastigotes were harvested, fixed and prepared for scanning (A) or transmission electron microscopy (B). Transmission electron micrographs of axenic amastigotes showing the presence of a single nucleus (N) in *LbWT* and in *LbCas9T7* and the presence of multi nuclei in *LbCen−/−*. Scale bars, 0.5 µm (*LbWT* and *LbCas9T7*); 1 µm (*LbCen−/−*).

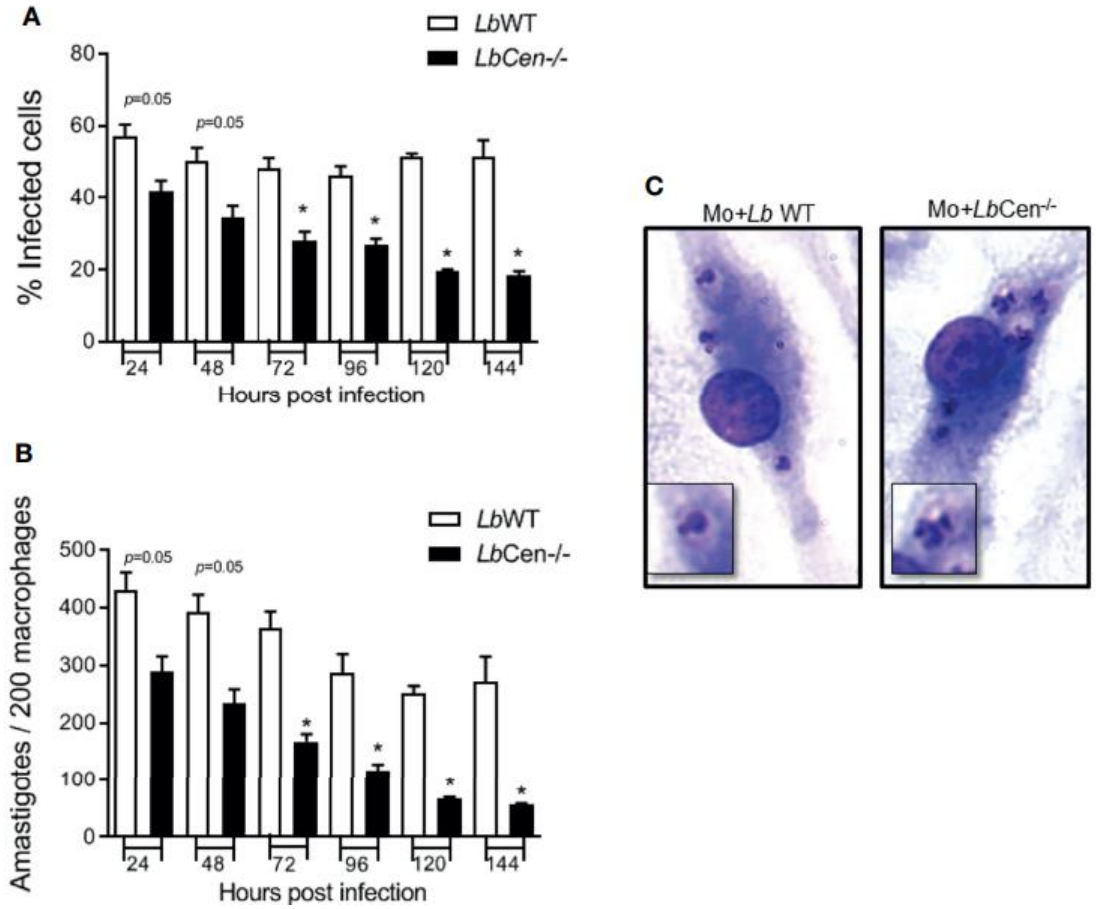

FIGURE 7 | Macrophage infection with *LbCen−/−*. BMDM were infected with *LbWT* or *LbCen−/−* (10:1, parasite/macrophage ratio) promastigotes for 24 h. Cells were extensively washed and further cultured for 48, 72, 96, 120 or 144 h. Cells were stained with H&E and evaluated for the percentage of infection (A) and the number of amastigotes per 200 macrophages (B) by optical microscopy. (C) Photomicrographs showing infected macrophages at 96 h. Data (mean ± SEM) are pooled from four independent experiments, each performed in quadruplicate. *p < 0.05.

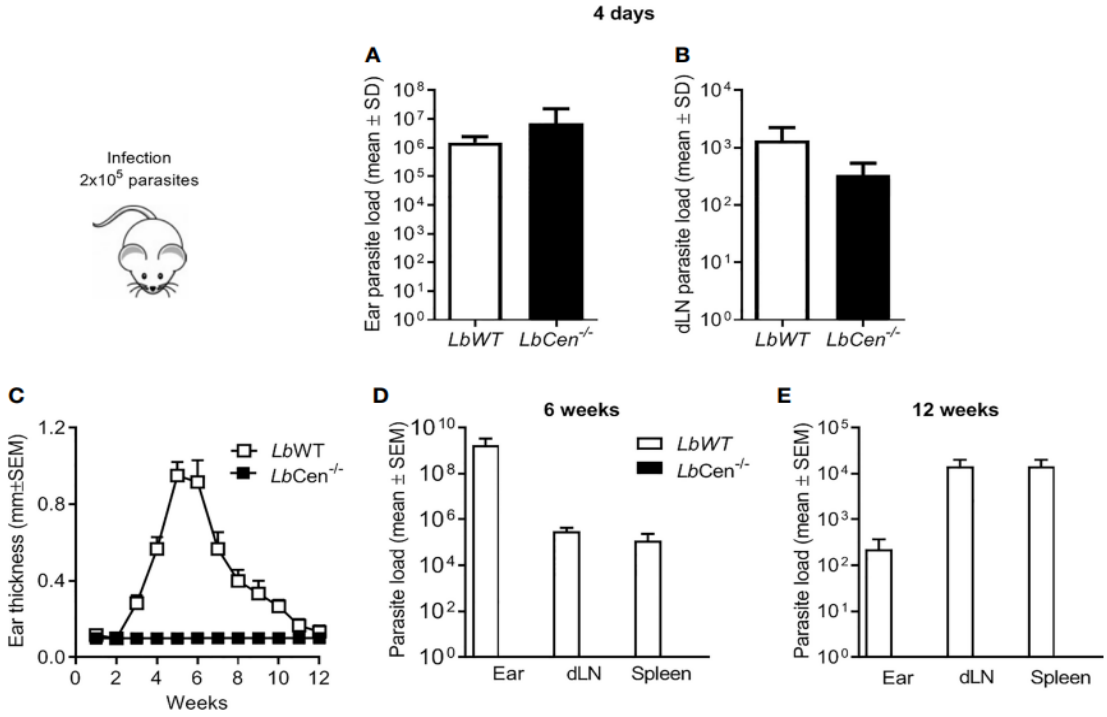

FIGURE 8 | Parasite load in mice inoculated with *LbCen−/−*. BALB/c mice (10 per group) were infected with 2 × 105 *LbWT or LbCen−/−* promastigotes, in the ear dermis and parasite load was determined, four days later, at the inoculation site (ear) (A) and in draining lymph nodes (B) by Limiting Dilution Analysis. Data (mean ± SEM) are from one representative experiment. BALB/c mice (10 per group) were infected as described and lesion development was measured weekly (C). Six (D) and twelve (E) weeks post infection, parasite load was evaluated by Limiting Dilution Analysis. Data (mean ± SEM) are from one representative experiment.

CAPÍTULO II. Imunização com *Leishmania braziliensis* deficiente em centrina não confere proteção contra infecção subsequente

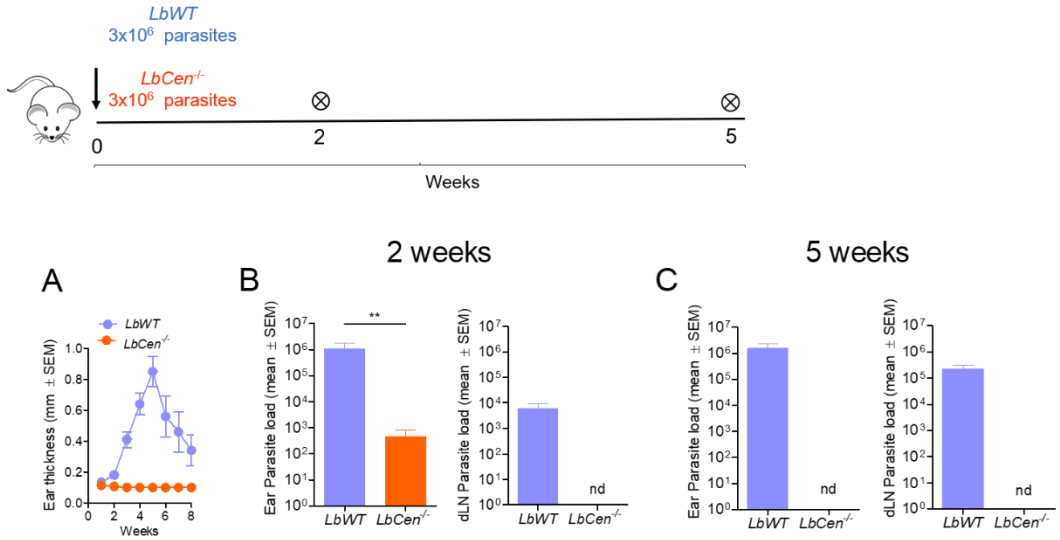

Figure 3. *LbCen-/-* parasites are safe and do not cause lesion development in BALB/c mice. BALB/c mice (10 per group) were inoculated with 3x10^6 *LbWT* or *LbCen*$^{-/-}$ stationary promastigotes, in the ear dermis, and lesion development was measured weekly (A). Parasite load was determined, at 2 (B) and 5 (C) weeks post infection, at the inoculation site (ear) and in draining lymph nodes by Limiting Dilution Analysis. Results are expressed as means ± SEM and are from one representative experiment.

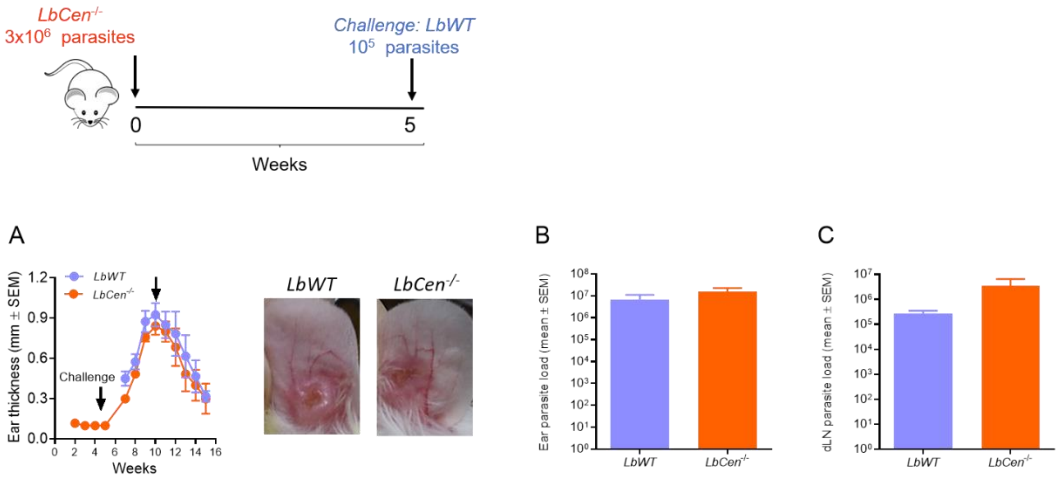

Figure 4. Immunization with *LbCen*$^{-/-}$ does not confer protection against a challenge with *LbWT.* BALB/c mice (n=10) were inoculated with 3x10^6 *LbCen*$^{-/-}$ stationary promastigotes, in the ear dermis. After 5 weeks mice were challenged in the opposite ear with 2x10^5 *LbWT* stationary promastigotes and, lesion development was measured weekly (A). Parasite load was determined 5 weeks post infection, at the inoculation site (ear) (B) and in draining lymph nodes (C) by Limiting Dilution Analysis. Results are expressed as means ± SEM and are from one representative experiment.

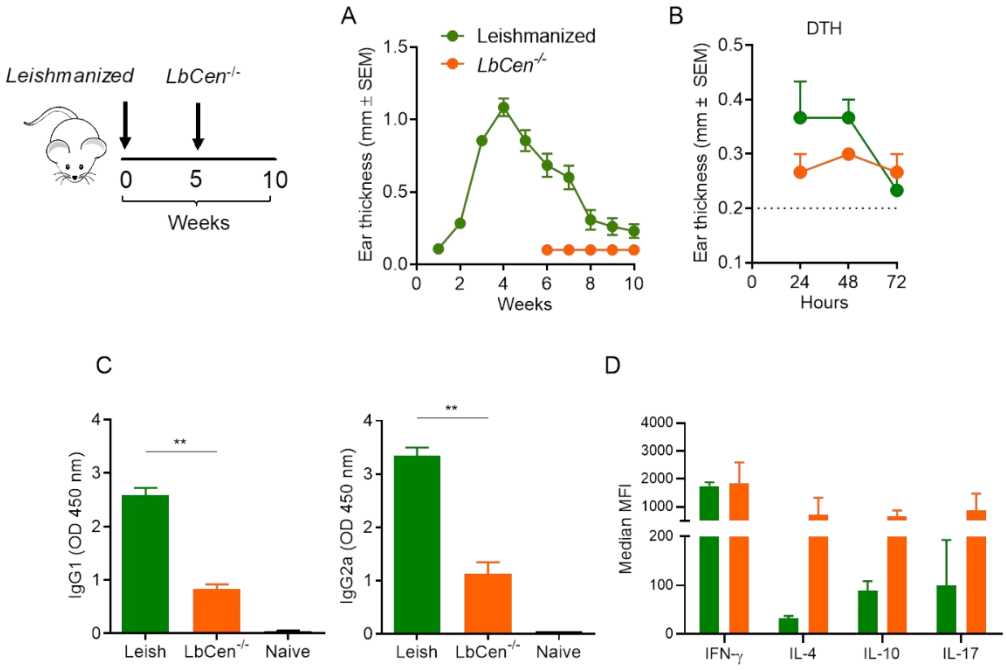

Figure 6. Mice immunized with *LbCen*$^{-/-}$ do not recapitulate the immune response observed in leishmanized mice. Mice were inoculated with *LbWT* or *LbCen*$^{-/-}$. (A) Lesion development after parasite inoculation. (B) DTH response. (C) IgG1 and (D) IgG2a presence was determined by ELISA. (E) Recall responses in draining lymph nodes after re-stimulation with SLA was determined using Luminex assay. Results are expressed as means ± SEM and are from one representative experiment.

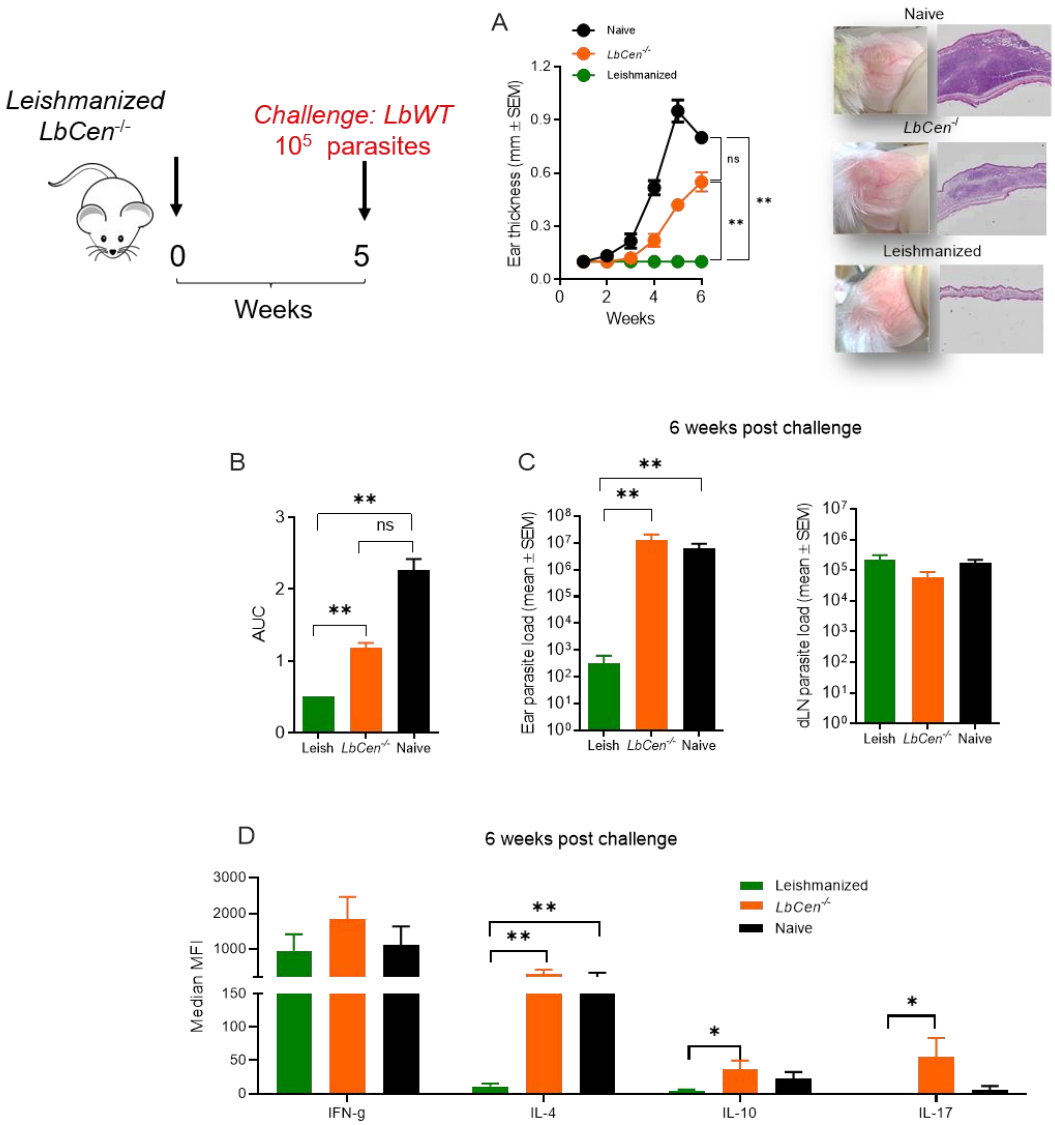

Figure 7. Lack of protection against a live challenge in mice immunized with *LbCen*$^{-/-}$. Mice were inoculated with *LbWT* or *LbCen*$^{-/-}$. Then, mice were challenge with live *LbWT.* (A) Lesion development following challenge was measured and histopathological analysis of ears was performed. (B) Area under the curve (AUC) of ear thickness was determined. (C) Parasite load was evaluated in ears and draining lymph nodes by limiting dilution analysis. (D) Cytokine response in draining lymph nodes following re-stimulation with SLA was determined by Luminex Assay. Results are expressed as means ± SEM and are from one representative experiment. *p<0,05; **p<0,01.