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A B S T R A C T

Development of a protective vaccine against Leishmania depends on antigen formulation and adjuvants that induce specific immunity and long-lasting immune responses. We previously demonstrated that BALB/c mice intranasally vaccinated with a plasmid DNA encoding the p36/LACK leishmanial antigen (LACK-DNA) develop a protective immunity for up to 3 months after vaccination, which was linked with the systemic expression of vaccine mRNA in peripheral organs. In this study, LACK-DNA vaccine was associated with biocompatible chitosan microparticles cross-linked with glyceraldehyde (CMC) to boost the long-lasting immunity against the late *Leishmania infantum* challenge. Infection at 7 days, 3 or 6 months after vaccination resulted in significantly lower parasite loads when compared with non-vaccinated controls. Besides, LACK-DNA-chitosan vaccinated mice showed long-time protection observed after the late time point challenge. The achieved protection was correlated with an enhanced spleen cell responsiveness to parasite antigens, marked by increased proliferation and IFN-γ as well as decreased IL-10 production. Moreover, we found diminished systemic levels of TNF-α that was compatible with the better health condition observed in LACK-DNA/CMC vaccinated-infected mice. Together, our data indicate the feasibility of chitosan microparticles as a delivery system tool to extend the protective immunity conferred by LACK-DNA vaccine, which may be explored in vaccine formulations against *Leishmania* parasite infections.

Leishmaniasis is a neglected tropical disease affecting over 12 million individuals worldwide with approximately 2 million new cases reported yearly. In humans, the disease manifestation ranges from self-healing cutaneous leishmaniasis (CL) to severe visceral leishmaniasis (VL), which is a fatal and systemic disease, if left untreated [1].

Disease control depends exclusively on chemotherapy, based on pentavalent antimonials, oral miltefosine, liposomal amphotericin B and paromomycin. The use of these drugs has significantly reduced mortality caused by VL, however, they are very toxic, expensive and have frequently been associated with induction of drug-resistant strains [2–4]. Thus, the development of a prophylactic or therapeutic vaccine is the most cost-effective way of controlling this infectious disease [3,5,6], but currently no vaccine against human VL exists.

Non-invasive immunizations including mucosal administration of vaccines have emerged in order to reduce or eliminate

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disadvantages observed with parenteral route delivery, which includes cross-contamination, needlestick injury, under-or overdosing, increased cost as well as low acceptance [6,7]. By using this strategy, several vaccine candidates based on whole parasite antigens, purified proteins and DNA have been tested in order to promote specific protection against pathogens [8–11]. DNA represents a promising technology that has shown advantages over traditional, attenuated and subunit vaccines, especially related to its low cost of production, stability and ability to induce both cellular and humoral immunity [12–14].

We have successfully used for many years the intranasal route to deliver the LACK-DNA vaccine candidate, a plasmid encoding the cytoplasmic LACK protein from *Leishmania infantum* that have provided protective immune responses in hamsters and mice against both cutaneous and visceral leishmaniasis [12,14–16]. Nevertheless, we have also shown that vaccinated mice with LACK-DNA alone did not have protective immunity against the late parasite challenge (6 months post-vaccination), suggesting the need for formulation adjustment in order to mediate a long-lasting immunity [12].

Particles based techniques as a delivery system to antigens and DNA have emerged as one of the most promising strategies to induce strong immune responses [3,17,18]. Besides, this approach can protect the antigen from premature degradation by proteolytic enzymes, promoting an efficient antigen uptake by APCs or M cells [19]. In this regard, chitosan microparticles have been widely used offering several advantages compared to other biodegradable polymers such as mucoadhesive properties and low toxicity [20]. Moreover, they are highly biodegradable and biocompatible as well as efficient to increase residual time at the site of absorption, prolonging the release of antigens and promoting long last immunity [17,18,21].

Herein, we present a novel strategy to combine the highly successful LACK-DNA intranasal delivery with chitosan microparticles to optimize a vaccine formulation against *L. infantum*. We found that this association can significantly boost the antigen-immunogenicity and provide a better protective and long-lasting host immune response.

1. Methods

1.1. Animals

BALB/c mice were originally purchased from Jackson Laboratory (Bar Harbor, Maine). They were bred and maintained at our own facilities, using sterilized bedding, filtered water and pelleted food. Female animals were used at 6–8 weeks of age. The experimental protocols were approved by the Ethical Committee for Experimental Animal Use established at the Federal University of Rio de Janeiro under registration number IBCCF 118.

1.2. Parasites and antigens

L. infantum strain MHOM/BR/1974/M2682 amastigotes were routinely isolated from the spleens of infected mice and cultured at 25 °C as promastigotes in DMEM medium pH 6,8 supplemented with 20% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, and 20 μg/ml of gentamicin (herein named DMEM 20% HIFCS). For L. *infantum* antigen (LiAg), late-log-phase culture promastigotes were centrifuged, washed three times in phosphate buffer saline (PBS) and disrupted by three rounds of freezing and thawing. Protein content was determined by the Lowry method. The recombinant LACK antigen was kindly provided by Dr. Vicente Larraga (Centro de Investigaciones Biologicas, Madrid, Spain). For pCI-neo-LACK (LACK-DNA), the gene encoding the p36 *L. infantum* LACK protein was inserted downstream of the cytomegalovirus promoter in the EcoRI/XbaI site of the pCI-neo expression vector (Promega), as described previously [26]. Endotoxin-free control and LACK-encoding plasmids were isolated using EndoFreePlasmid Mega kit (Qiagen) according to the manufacturer's instruction.

1.3. Microparticles of chitosan cross-linked with glyceraldehyde (CMC)

Chitosan microparticles were prepared by spray drying technique as described by Oliveira (2005) [27]. For cross-linking, chitosan microparticles were suspended in acetone: water solution (2:1) containing 1.5% of glyceraldehyde and maintained under agitation at 500 rpm for 30 min at room temperature, followed by filtration in 0.22 μm membrane and dried in vacuum at room temperature for 24 h. The particles obtained presented an average size of 5 μm (Malvern MasterSizer, model E, UK), span index of 2.21 (Malvern MasterSizer, model E, UK) and zeta potential of +55.3 ± 0.6 mV (Zetamaster Malvern, UK).

1.4. LACK DNA adsorption in CMC

LACK-DNA plasmid was extracted by alkaline lysis method using DNA LPS-free extraction kit, according to the manufacturer's instructions (Quiagen Giga-Prep - USA). 50 mg of DNA was mixed with 50 mg of CMC and added to 25 ml of citrate-phosphate buffer and ethanol (2:1) at pH 5.5, for 2 h at 37 °C. The adsorption rate was determined by free DNA amount in the supernatant at 260 nm (NanoDrop 2000, Thermo Scientific). pCi-neo empty plasmid were used as a negative control.

1.5. Vaccination and infection

Mice were vaccinated by intranasal route (i.n.) with LACK-DNA as previously described in Ref. [28]. Briefly, animals held upright received 10 μL of PBS containing 30 μg of LACK-DNA adsorbed with CMC-Gly in each nostril (LACK-DNA/CMC). Controls received phosphate buffer saline (PBS) or CMC-Gly alone (CMC). A booster dose was given 7 days later. The infection was done at one week, three months or six post second vaccine dose by the *i.v.* route with 10^7 *L. infantum* promastigotes at the stationary phase of growth.

1.6. Determination of the parasite burden

On day 30 post infection, the parasite burden in each liver and spleen was determined by Limiting Dilution Assay. Briefly, each organ was weighted and homogenized in DMEM 20% HIFCS. Serial dilutions of single-cell suspensions were cultured for 12 days at 25 °C. The original numbers of parasites in each organ was calculated from the reciprocal of the highest dilution containing promastigotes.

1.7. Splenocyte proliferation assay

Mice splenocytes were suspended in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (Sigma–Aldrich, USA). The concentration of splenocytes was adjusted to 5×10^5 cells/well in a 96-well culture plate. Cells were stimulated in the presence of Con A (10 μg/ml) [Sigma–Aldrich, USA], *L. infantum* antigen (50 μg/ml) or recombinant LACK (5 μg/ml). Cultures were incubated at 37 °C in a CO_2 incubator with 5% CO_2 for 3 days followed by 3H-thymidine addition (1 μCi) for 18 h. Cells were counted in a liquid scintillation counter (Beckman, USA) and the results expressed as stimulation index.

1.8. Cutaneous hypersensitivity reaction

On day 1 of i.v. infection, vaccinated and non-vaccinated mice were injected in the hind footpad with 20 μg of LiAg in 20 μl of PBS. Footpad swelling was measured with a dial caliper and the results were expressed as the difference between the thickness of the injected and pre-injected footpads.

1.9. Cytokines

Thirty days after infection, single cell suspensions were prepared from spleens at 5×10^6 cells/ml in DMEM 10% HIFCS supplemented with 50 μM 2-mercaptoetanol. Cells were incubated at 37 °C in 24-well flat-bottom plates in the presence or absence of LiAg (50 μg/ml), rLACK (5 μg/ml) or medium alone for 72 h. The cytokine production was determined in the culture supernatants. TNF-α was assessed in sera by ELISA assay following the manufacturer's instructions (R&D Systems, Minneapolis, USA).

1.10. Statistics

Data were statistically analysed using Prisma software. Means of normally distributed variables were compared by ANOVA analysis simple factorial test and by one way ANOVA-Tukey's honestly significant difference (Tukey's HSD) post-hoc method and were considered significantly different when $p < 0.05$.

2. Results

2.1. Long lasting immunity is conferred by intranasal vaccination with LACK-DNA/chitosan microparticles

We previously demonstrated that intranasal immunization of mice with LACK-DNA was able to confer a protective immune response for up to 3 months after vaccination [12]. Now, we investigate whether intranasal vaccination with LACK-DNA in association with chitosan microparticles (CMC) may extend the specific cell-mediated immune responses and protection. Thus, vaccinated-mice were challenged 1 week, 3 months and 6 months after the booster dose and the parasite burden was accessed at the parasitic peak, thirty days after infection. Our data demonstrate that both LACK-DNA- and LACK-DNA/CMC- vaccinated mice had a significant reduction of liver and spleen parasite burden compared to control groups. This was observed at 1 week and 3 months after vaccination (Fig. 1A and B). Interestingly, only LACK-DNA/CMC vaccination was able to induce parasite control in both liver and spleen at the late challenge (6 months) (Fig. 1C), suggesting its capacity to confers a protective long lasting immunity.

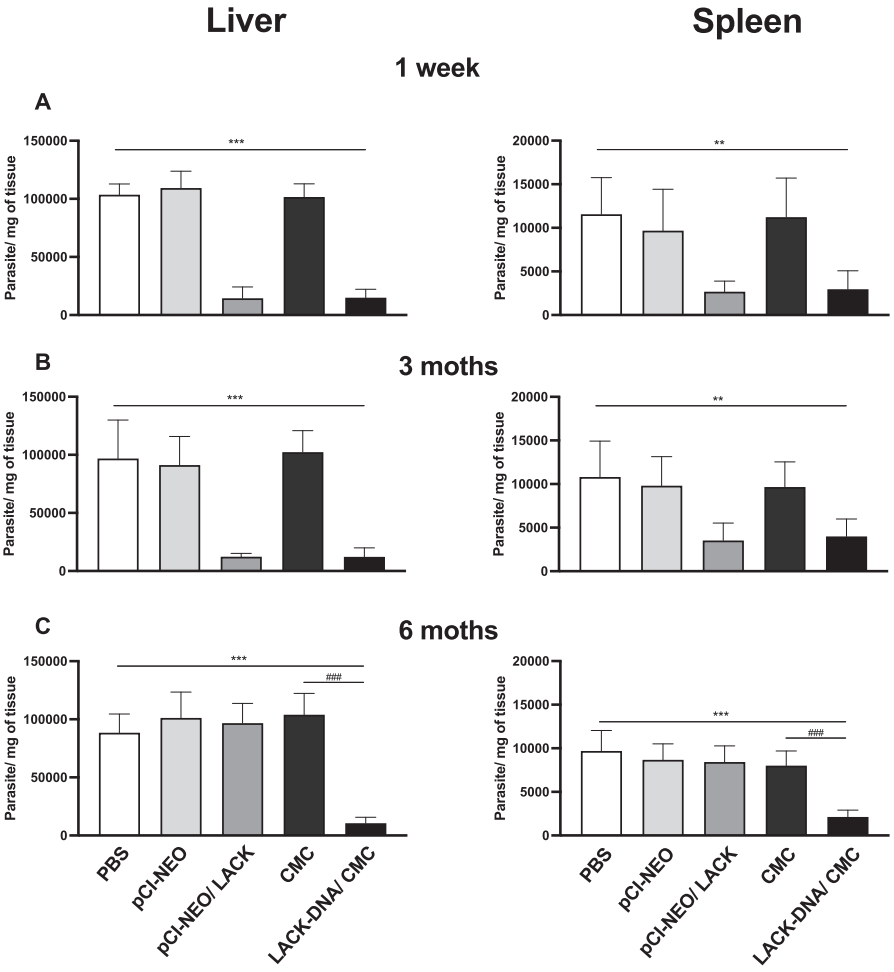

Fig. 1. Parasite burden in infected mice after different times of vaccination. Mice received two *i.n.* doses of 30 μg of naked LACK-DNA; 30 μg of pCI-neo plasmid alone; 30 μg of LACK-DNA adsorbed to CMC (LACK-DNA/CMC); 1,5 mg of naked CMC or 20 μl of PBS alone with one week interval. After 1 week, 3 months or 6 months of vaccination, the animals were i.v.-challenged with *L. infantum*. The parasite burden in individual organs was measured.

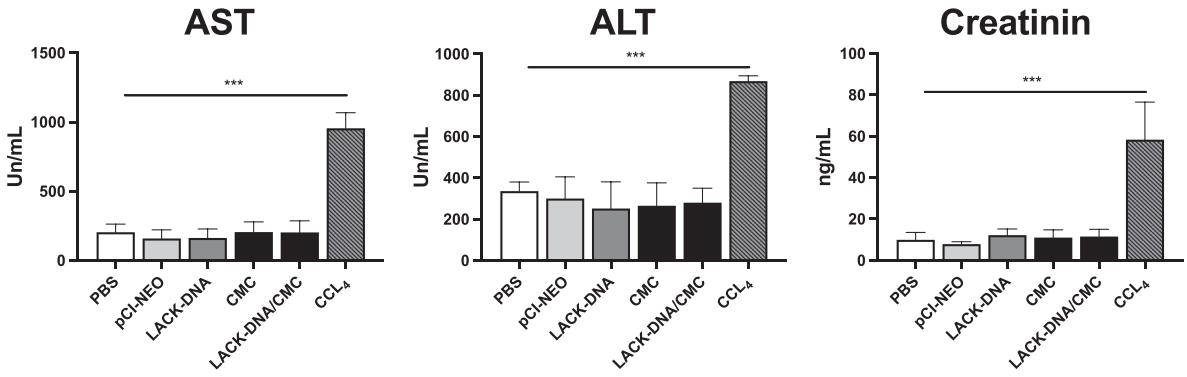

Fig. 2. Biocompatibility effect of vaccination and parasite-specific cytokines production. Mice were vaccinated as described in (Fig. 1). Twenty-four hours post booster the levels of transaminase AST, ALT and Creatinine in the serum were evaluated by colorimetric assay. The results are represented as arithmetic means ± S.D of three independent experiments (n = 12/group). ****p < 0.0001.

2.2. Intranasal vaccination with LACK-DNA/CMC does not promote acute systemic toxicity and enhances specific protective immune responses

To investigate whether intranasal vaccination with chitosan microparticles could induce acute systemic toxicity, we accessed the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine 24 h after the booster dose. No toxicity or change in biochemical parameters of AST, ALT and creatinine were found after intranasal delivery of CMC or its association with LACK-DNA (Fig. 2). In contrast, positive control mice that were injected with carbon tetrachloride (CCL_4) exhibited a significant increase of all biochemical parameters when compared with negative control or vaccinated groups (Fig. 2).

The cutaneous hypersensitivity reaction (DTH) to locally injected *L. infantum* antigen was assessed as an indication of cell-mediated immune response. Previously, LACK-DNA immunized mice demonstrated increased DTH responses up to 3 months after vaccination. To establish the critical effect of association with CMC in the prolongation of LACK DNA immunity, we accessed the DTH 6 months after

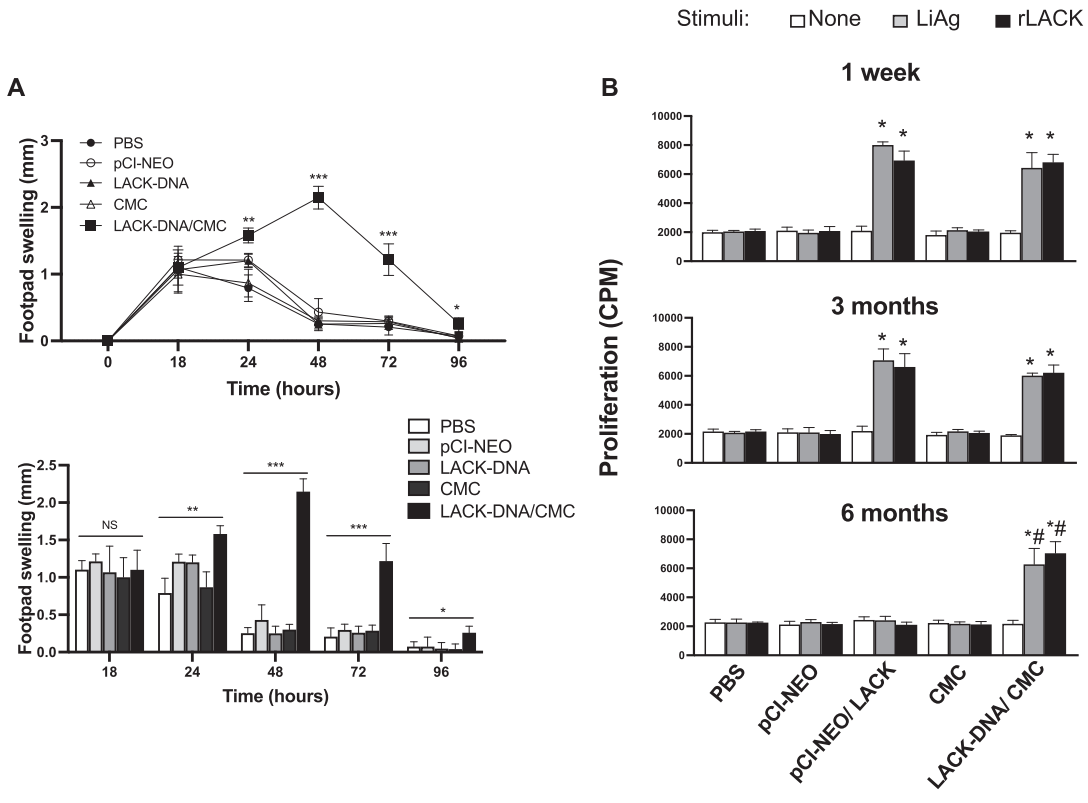

Fig. 3. Parasite-specific lymphoproliferative response in infected mice after different times of vaccination. Mice were vaccinated and infected after the indicated times. On day 30 of infection, their spleen cells were harvested and stimulated with LiAg (50 μg/ml); recombinant LACK protein (5 μg/ml); or medium alone. The lymphoproliferative response was determined by 3H-thymidine incorporation after 3 days of culture. The results are represented as arithmetic means ± S.D of three independent experiments (n = 21/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared with the respective PBS controls. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ as compared with the LACK-DNA group.

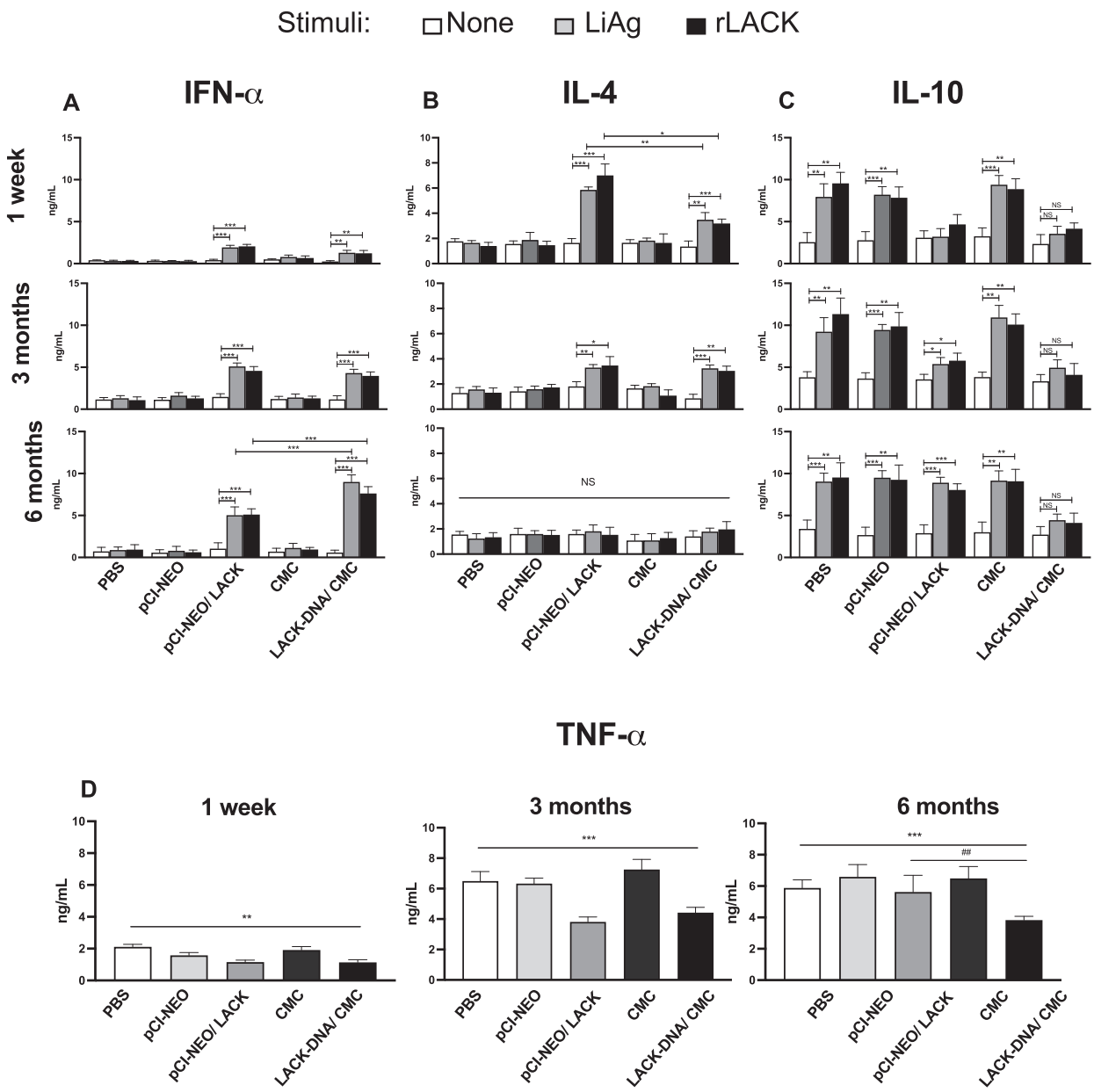

Fig. 4. Parasite-specific cytokine response in mice infected after different times of vaccination. Mice (n = 8) were vaccinated and infected after the indicated times. On day 30 of infection, their spleen cells were harvested and stimulated in vitro with LiAg (50 μg/ml); recombinant LACK protein (5 μg/ml); or medium alone. Determination of vaccine-induced IFN-γ, IL-4 and IL-10 cytokines were measured in the supernatants by ELISA. TNF-α was accessed by ELISA in the individual sera. The results are represented as arithmetic means ± S.D of three independent experiments (n = 21/group). *$p < 0.05$, **$p < 0.01$ as compared with the respective PBS controls. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ as compared with the LACK-DNA group.

vaccination. Interestingly, mice that were pre-immunized with LACK-DNA/CMC exhibited significant swelling as compared to non-vaccinated controls as well as LACK-DNA alone (Fig. 3A), observed at 24, 48 and 72 h after skin challenge with antigen. Moreover, compared to control groups, splenocytes from LACK-DNA-vaccinated mice 1 week or 3 months prior infection strongly proliferated after LiAg or rLACK recall (Fig. 3B). Similarly, LACK-DNA/CMC vaccinated mice showed significant lymphoproliferative response compared to negative controls and LACK-DNA group (Fig. 3B).

Cytokines analyses showed an increased IFN-γ production by LACK-DNA or LACK-DNA/CMC vaccinated mice when compared to control groups after all evaluated times (Fig. 4A). However, higher IFN-γ production was observed by LACK-DNA/CMC as compared to LACK-DNA vaccinated mice at the late challenge (6 months) (Fig. 4A). LACK-DNA and LACK-DNA/CMC vaccination induced increased IL-4 production in response to LiAg or rLACK in vitro recall and that was not observed after the late challenge (6 months) post vaccination (Fig. 4B). At 6 months post vaccination, infected controls or LACK-DNA vaccinated mice were unable to suppress the IL-10 production (Fig. 4C). Complementary, both LACK-DNA or LACK-DNA/CMC vaccinated 1 week or 3 months prior infection presented lower TNF-α levels compared to non-vaccinated groups (Fig. 4D). Interestingly, only LACK-DNA/CMC vaccinated mice showed a significant reduction of TNF-α levels when challenged 6

months after booster (Fig. 4D). This was compatible with their healthy appearance in contrast to non-vaccinated controls, and to a lesser extent LACK-DNA vaccinated mice, that showed unhealthy appearance and prostrate behaviour.

3. Discussion

In the present study, LACK-DNA plasmid was associated with chitosan nanoparticles as a vaccine delivery system to increase its protective immune response against visceral leishmaniasis in mice. This approach has been used to enhance both vaccine efficacy and to protect the DNA from nasal mucosa degradation. Moreover, complexation in microparticles has shown to improve antigen uptake by professional antigen-presenting cells (APCs) and promote a slow antigen release at the targeting site of vaccination [17,18,22]. CMC also offers advantages such as favourable size, stability in the target site, polycationic activity as well as immunomodulatory properties [18]. Here, spherical-shaped CMC microparticles given by intranasal route to BALB/c-vaccinated mice averaged 5 μm (data not shown). It is known that particles smaller than 10 μm are phagocytized by antigen-presenting cells at mucosal surfaces, leading to immune response enhancement. Thus, CMC presented required size for APCs uptake, necessary for immune response induction.

The successful use of chitosan-DNA association given intranasally has been demonstrated in viral respiratory infections models, hepatitis B virus and parasites [21,23–26]. In previous work, we demonstrated the correlation between systemic LACK-mRNA expression after LACK-DNA intranasal immunization and the protective immunity duration against visceral leishmaniasis. BALB/c mice infected with *L. infantum* at 7 days or 3 months after vaccination presented significantly lower parasite loads than non-vaccinated controls. However, when challenged 6 months after vaccination, they responded similarly to non-vaccinated controls [12].

The ability to confer long protection, preferably for the host's lifetime, is the primary aim for vaccine development. Due to their cationic nature, chitosan microparticles are useful materials to interact with negatively charged substances such as mucosa surfaces and DNA molecules [27], increasing antigen half-life and resulting in improved vaccine efficacy [22,28]. In addition, previous studies have suggested that chitosan microparticles can permeate mucosal epithelium by stretching GAP junctions thus allowing vaccines a better access to the underlying lymphoid tissue [29,30]. Taken together, all these characteristics seem to support the adequacy of CMC as an efficient delivery vector to enhance the duration of LACK DNA intranasal vaccination.

The use of chitosan particles as vaccine adjuvants has shown to induce both cellular and humoral immune responses, which has been often related to vaccine success in humans and animal models [18,31]. Although humoral responses do not always correspond to protection [39], a strong antigen-specific cellular immune response can be related to VL healing [32]. In the present study, splenocytes from LACK-DNA/CMC vaccinated mice exhibited enhanced lymphoproliferative responses after LiAg and rLACK in vitro antigen recall, demonstrating the correlation between cellular immune response and resistance to L. infantum infection, as indicated by the lower parasite burden. Besides blastogenesis, the ability to develop a robust Th1 immune response associated with IFN-γ production is crucial to visceral leishmaniasis control [12] whereas IL-13/IL-4 and IL-10 are key cytokines associated with the disease progression. Notably, LACK-DNA vaccination led to increased memory production of IFN-ɣ when given in association with CMC. Previous studies also demonstrated the capacity of chitosannanoparticles-DNA to induce a potent IFN-ɣ production [12,23] and the superior capacity of chitosan to enhance cell-mediated immune responses [33]. Moreover, only LACK-DNA/CMC vaccination led to controlled production of IL-10 and TNF-α in mice infected 6 months after vaccination, suggesting that this formulation provided strong and long-lasting protection against the intracellular parasites.

We have previously demonstrated that after intranasal instillation with naked LACK DNA in physiological solution, LACK-DNA is absorbed by the nasal mucosa and LACK mRNA expressed in different organs including the spleen and lymph nodes [12]. In this way, LACK protein may be synthesized and presented by APCs directly in primary lymphoid organs. That may explain why the outcome of *L. infantum* infection in mice is different when LACK-DNA is given by intranasal and subcutaneous routes [34]. The deleterious effect of s.c. vaccination is possibly due to the rapid induction of IL-4- producing LACK-specific CD4+ Vβ4-Vα8 T cell repertoire that naturally circulates in susceptible BALB/c mice due to cross-reaction with LACK-like proteins produced by the gut microflora [35,36]. Intranasal vaccination with LACK DNA, on the other hand, may somehow prevent the expansion of that T cell repertoire through mucosal tolerance. The protective T cell repertoire responsible for the protective immunity remains to be determined. Whether or not CMC acts only as a mucoadhesive and transepithelial carrier delivering the LACK DNA more effectively to the lamina propria dendritic cells, or also as an immunostimulatory agent affecting systemic LACK expression is another interesting point for further investigation. Taken together, our results show that CMC is a good adjuvant to enhance systemic immune responses induced by LACK-DNA vaccine in visceral leishmaniasis, and a general potential strategy to improve intranasal vaccination with DNA.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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