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Transcutaneous immunization using SLA or rLACK skews the immune response towards a Th1 profile but fails to protect BALB/c mice against a *Leishmania major* challenge

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ABSTRACT

Leishmaniasis is an expanding health threat worldwide complicated by the absence of an effective vaccine. We investigated transcutaneous immunization (TCI) as a needle-free immunization route which exploits the abundance of antigen presenting cells in the skin to induce both mucosal and systemic immunity. *Leishmania (L.) major* soluble antigens (SLA) or recombinant *Leishmania* homolog of receptors for activated C-kinase (rLACK) antigens were delivered transcutaneously together with cholera toxin (CT), to BALB/c mice. Mice were immunized at weeks 1, 4, and 7 with PBS, CT, SLA/CT or rLACK/CT. Two weeks after the final boost, antigen-specific IgG titers, IFN-γ ELISpot, and cytokine levels were assessed in half of the mice and the remainder were challenged with an intradermal (ear) injection of 5×10^4 *L. major* metacyclic parasites. Mice were monitored weekly and sacrificed after 7 weeks to assess the parasite burden and to study the ear lesion immunohistopathology. Our results show that TCI with SLA or rLACK yielded high levels of anti-SLA, anti-rLACK and anti-CT IgG antibodies. A Th1-type of immune response was demonstrated with a high frequency of IFN-γ secreting cells, high levels of IFN-γ production, and lower levels of IL-10 resulting in a high IFN-γ/IL-10 ratio in mice immunized with SLA/CT or rLACK/CT. After parasite challenge, rLACK immunization was not associated with protection. In addition, SLA/CT immunized mice had larger ear lesions and an increased parasite load in the ear. Immunohistochemistry of ear biopsies stained for nitric oxide synthase revealed that staining intensity was diminished in the SLA/CT group compared to the control group. This finding suggested that less parasite killing occurred at the site of the infection.

In conclusion, despite a strong Th1 type profile induced by TCI, exacerbation of infection occurred after challenge with *L. major*. This also correlated with low induction of nitric oxide.

1. Introduction

Leishmaniasis is a major public health problem that affects 98 countries. There are more than 350 million people at risk and an estimated incidence of 2 million new cases per year, with cutaneous leishmaniasis (CL) being the most common presentation [1]. There is no human vaccine to prevent CL, although leishmanization suggests that protective immunization is feasible [2,3]. Topical vaccines offer several advantages over injectable immunizations: recruitment of local skin immunity; ease of administration; less risk of transmitting blood borne infections; and simplified manufacturing processes [4].

Transcutaneous immunization (TCI) is a novel needle-free method that has great potential for increasing the adherence and effectiveness of global vaccination programs [5,6]. The demonstration of TCI feasibility in humans using an adjuvanted patch [7,8] and the observed protective immune responses when used for tetanus, diphtheria, malaria, and influenza vaccinations [9–11], as well as the resulting mucosal immunity, galvanized our interest in testing TCI as a *Leishmania* vaccine strategy.

TCI utilizes co-application of the antigen with an adjuvant such as cholera toxin (CT), an ADP-ribosylating bacterial exotoxin, and it

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elicits robust systemic and mucosal immune responses [12,13,6,12]. Studies in animal models have also indicated a requirement for an adjuvant in other effective *Leishmania* vaccines [14]. Antibody responses against both the CT and the coadministered antigens were induced in both rodents and humans, when CT was applied along with heterologous protein antigens. The systemic toxicities attributable to the use of CT by other application routes were not observed [6,12,15]. CT adjuvant promoted a balanced T helper (Th) Th1/Th2/Th17 response independent of interleukin (IL)-12 and IL-17 [16].

Apart from its ease of use, utilizing TCI for a *Leishmania* vaccine offered the distinctive advantage that the vaccine can be targeted to the skin where the *Leishmania* parasite establishes its presence and presumably shapes an initial host immune response. Dermal dendritic cells capture antigens and deliver them to the skin-draining lymph nodes to activate naïve or central memory T cells stimulating both local and systemic immune responses [17].

The protective immune response to *Leishmania* infection is predominantly cell-mediated immunity, and the induction of a Th1 pro-inflammatory immune response is critical to achieve protection [18]. The outcome of infection depends upon the type of the immune response generated. In resistant strains of mice (C57BL/6), *L. major* infection induced a Th1 type of response associated with a high level of interferon gamma (IFN-γ) which activates macrophages to kill parasites via effector nitric oxide (NO) production [19]. The lesions heal spontaneously and the animals are then protected against further infection. In susceptible BALB/c mice, *L. major* infection induced a Th2 response with high levels of IL-4, IL-5, and IL-13, resulting in disseminated infection [20,21]. In murine models, it has been previously shown that *Leishmania* soluble antigens (SLA) and *Leishmania* homolog of receptors for activated C-kinase (rLACK) antigens conferred protection to parasite challenge [22,23].

In this study, we show that delivering *Leishmania* antigens, SLA and rLACK by TCI along with CT induced humoral and cellular immune responses in BALB/c mice. However, despite the generation of a strong Th1 response, these antigens failed to induce protection against *Leishmania* parasite challenge. Furthermore, SLA/CT exacerbated *Leishmania* infection.

2. Materials and methods

2.1. Mice

Six- to eight-week-old female BALB/c mice (Jackson Laboratory) were used in the present study. The study was conducted in compliance with the Animal Welfare Act, complied with the ARRIVE guidelines and adhered to the principles in the guide for care and use of laboratory animals. The investigators used facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The Institutional Animal Care and Use Committee approved all animal experiments.

2.2. Parasite culture

Leishmania major strain expressing luciferase used in this study was a gift from Dr. M. Wilson, (University of Iowa, Iowa City, IA) [24]. Parasites were cultured at 26 °C in Schneider's medium containing 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% heat-inactivated fetal calf serum (Hyclone). The stationary phase was reached after 6 days of culture, and metacyclic parasites were then purified by using a Ficoll density gradient (Sigma) as previously described [25].

2.3. Antigen preparation

SLA were prepared from the stationary phase culture as described previously [26]. Briefly, parasites were lysed, sonicated, and subjected to ultracentrifugation.

2.4. Expression and purification of recombinant LACK protein in Escherichia coli

Recombinant LACK protein was produced and purified as previously described [27] using *E. coli* M15 cells harboring the recombinant plasmid PQE-30-LACK (gift from Dr. J. Blackwell).

2.5. Immunization of mice and parasite challenge

Female BALB/c mice (five animals per group) were immunized by TCI by directly placing the antigens onto their shaved back [6]. The antigen doses determined separately in a dose ranging experiment were 50 μg of SLA or rLACK mixed with 5 μg of CT (List Biological Laboratories) adjuvant in a total volume of 50 μl. Control groups received only CT or PBS. Each mouse received three TCI treatments administered at 3-week intervals on weeks 1, 4, and 7. Mice were bled on weeks 0, 3, 6, and 9. The sera were stored at −20 °C until analyzed. Two weeks after the last immunization, 5×10^4 metacyclic *L. major* promastigotes diluted in 10 μl PBS were inoculated in the right ear of mice. After challenge, lesion development was monitored at weekly intervals by measuring ear thickness with a metric caliper (Mitutoyo), and the lesion size was calculated by subtracting the size of the contralateral uninfected ear. Lesion development was also monitored by *in vivo* imaging using a Carestream system (Bruker Corporation) [24]. The net intensity of bioluminescence was determined using the Carestream Molecular Imaging Software by drawing regions of interest (ROI) and computation. The entire experiment was repeated 3 times for SLA and 2 times for rLACK.

2.6. Antibody measurement by enzyme-linked immunosorbent assay (ELISA)

Serum samples collected from immunized mice were analyzed by ELISA for the presence of IgG antibodies specific to rLACK and SLA, as previously described [27]. Individual samples were analyzed in triplicate at each time point and results plotted as an absorbance at 450 nm (OD at 450 nm) for SLA and rLACK. The CT titer was determined as previously described with some modifications [28]. Briefly, plates were coated with CT, washed and blocked in phosphate buffered saline (PBS) containing 0.5% gelatin (Sigma Aldrich) for 1 h at 37 °C. After incubation with diluted sera for 2 h, plates were washed and incubated in the presence of horseradish peroxidase (HRP) conjugated goat anti-mouse total immunoglobulin G (IgG) at a 1:5000 dilution. Plates were then washed and incubated in the presence of the substrate, 3,3′,5,5′-Tetramethylbenzidine (TMB) for 30 min. The reaction was stopped by the addition of stop solution and OD was read at 450 nm with a correction at 630 nm. The results are plotted as OD at 450 nm (for SLA and rLACK) or as end point titers (for CT) and represents the inverse of the highest serum dilution that was greater than twice the background.

2.7. Enzyme-Linked ImmunoSpot (ELISpot) and cytokine quantification

Two weeks after the second TCI boost (week 9), groups of mice were sacrificed and the spleen and popliteal lymph nodes (LN)

were harvested. Single-cell suspensions from spleen and LN were made using a 70 μm cell strainer. For the ELISpot assay, cells (5×10^6/ml) were plated in 96-well plates (50 μl per well) coated with anti-IFN-γ antibody (BD Biosciences) in complete tissue culture medium (RPMI supplemented with 10% FBS, 5×10^{-5} μM 2-ME, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) and stimulated with 20 μg/ml SLA or rLACK for 72 h at 37 °C, 5% CO_2. The ELISpot was performed following the instructions provided in the kit (Mouse IFN-γ ELISpot set, BD Biosciences). Spots were enumerated using the Immunospot Microanalyzer (Cellular Technology Limited) and plotted as number of IFN-γ secreting cells in the presence of SLA or rLACK minus the number of spots without the stimulus. For cytokine induction measurements, cells (2×10^6/ml) were plated in 24-well plates (500 μl per well), stimulated with the same antigens as described above and incubated at 37 °C for 48 h (for IL-10, TNF-α and IL-4) or 72 h for IFN-γ [29]. Culture supernatants were assayed for cytokines using ELISA (Ready Set Go kits, E-Biosciences).

2.8. Parasite quantification

Parasite load was quantified by a limiting-dilution technique adapted from the work of Titus et al. [30]. The excised ear or ipsilateral cervical lymph nodes were homogenized, and serial 10-fold dilutions were plated in triplicate in 96-well flat-bottom microtiter plates containing Schneider's medium supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum. Plates were incubated for 10 days at 26 °C. Then the number of viable parasites (motile) was determined microscopically, calculated from the reciprocal of the highest dilution at which promastigotes could be detected.

2.9. Histopathological analyses and immunohistochemistry

Mice were euthanized 7 weeks post-*Leishmania* infection; their ears were further evaluated. A subset of the ear tissues was formalin-fixed and processed as previously described [31]. Hematoxylin and eosin (H&E) staining as well as immunostaining for iNOS (Inducible Nitric Oxide Synthase), MHC-II (Major Histocompatibility Complex 2), macrophage (mph) and IL-10 were performed by Histoserv (Gaithersburg, MD). The slides were analyzed by a dermatopathologist at the Walter Reed National Military Medical Center.

2.10. Statistical analysis

Previous data indicated that the coefficient of variation for the IFN-γ/IL-10 ratio is about 0.35. ANOVA has an 80% power to detect a 2-fold increase with 5% two-sided significance level if the sample size is 5 animals per group. At a minimum, challenge experiments were performed twice in our study. Statistical significance was analyzed using PRISM7 (GraphPad Software, CA). The data are expressed as the mean ± SD. Differences were tested using an unpaired Student's t-test or the Mann-Whitney test for samples with non-parametric distributions, $p < 0.05$ was regarded as significant.

3. Results

3.1. Administration of SLA and rLACK by the transcutaneous route resulted in high antibody levels

SLA and rLACK were prepared (Supplemental Fig. S1) and evaluated for immunogenicity after TCI. Serum samples were collected before immunization (pre-bleed) and every 2 weeks after each immunization. Individual serum samples from each group were analyzed for the presence of IgG antibodies specific to SLA, rLACK, and CT by ELISA. The data from two or three independent experiments are combined in Fig. 1. The antibody responses to SLA (Fig. 1A) and rLACK (Fig. 1B) increased over time and were highest at the terminal bleed (week 9). At the final time point, the antibody response to SLA was significantly stronger compared to the rLACK antibody response ($p = 0.0007$, further elucidated with endpoint titers (Supplemental Fig. S2) High levels of anti-CT titers were seen after the first immunization and the titers peaked two weeks after the second boost (Fig. 1C). No differences in immunoglobulin subclasses IgG1 and IgG2a were found between the groups (Supplemental Fig. S3).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.11.052.

3.2. Association of SLA and rLACK immune response with a Th1 profile

A high frequency of IFN-γ secreting cells was seen after TCI immunization with CT-adjuvanted SLA or rLACK antigens (median of 224 and 109.3 spot forming cells/million splenocytes, respectively) (Fig. 2A). SLA/CT or rLACK/CT immunization resulted in high levels of IFN-γ (6224 pg/ml and 7252 pg/ml, respectively) compared to control mice (5 pg/ml for PBS and 1 pg/ml for CT) in the supernatants of antigen-stimulated splenocytes, as measured by ELISA (Fig. 2B). Cells from SLA or rLACK immunized mice produced approximately 10-fold lower amounts of IL-10 compared to IFN-γ (Fig. 2C). This resulted in a high IFN-γ/IL-10 ratio of 15.3 and 55, respectively for SLA and rLACK antigens (Fig. 2D). Very low levels of IL-4 (Supplemental Fig. S4) and TNF-α (data not shown) were observed.

3.3. Durability of Th1 immune response

To assess if the above profile was maintained over time, some mice were not sacrificed until 2 months after the third immunization. An elevated antibody response to SLA (Fig. 3A), a high frequency of IFN-γ secreting cells (266 spot forming cells/ million splenocytes) (Fig. 3B), and production of high levels of IFN-γ (6200 pg/ml) from the supernatants of antigen-stimulated splenocytes (Fig. 3C) were seen at 8 weeks after the last boost. Thus, the IFN-γ response persisted for over 2 months. The long term response to rLACK delivered through TCI was not assessed in our study.

3.4. Lack of protection of TCI-immunized BALB/c mice against L. major challenge

Groups of five age-matched female mice were immunized three times with CT-adjuvanted SLA or rLACK antigens. Control mice received PBS or CT. Mice were challenged 2 weeks after the last TCI boost with an intradermal injection into the right ear (5×10^4 metacyclic stage *L. major* parasites). Lesion development was monitored for up to seven weeks after challenge. The experiment was repeated three times for SLA-CT and two times for rLACK-CT with reproducible findings. We observed no difference in the disease course between mice in the control groups and mice that received rLACK/CT (Fig. 4A). However, there was a significant increase in lesion size and ulceration (documented also in photographs, Supplemental Fig. S5) as well as ear thickness measurements in the group immunized with SLA/CT, starting from week three. This suggested that not only did CT-adjuvanted SLA administered by TCI fail to protect against a *Leishmania* challenge, but it led to disease exacerbation (Fig. 4A). Infection was also monitored over time by detecting the parasite bioluminescence in the whole

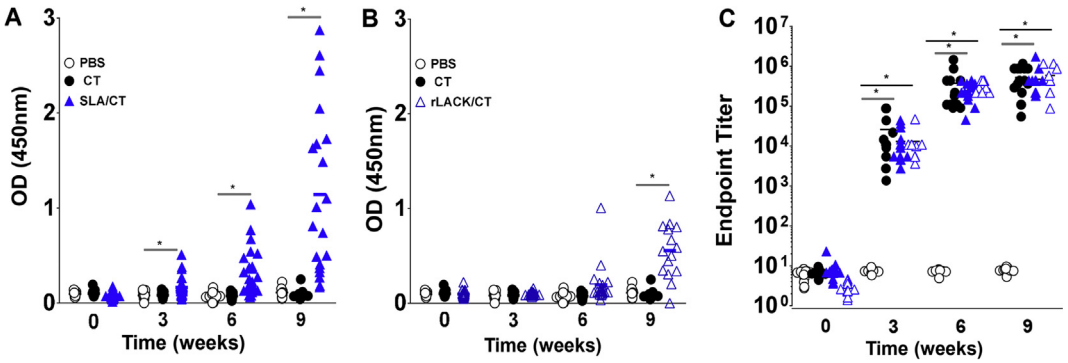

Fig. 1. Kinetics of antibody response. (A, B) Mice (n = 15 for PBS and CT, n = 20 for SLA/CT and rLACK/CT) were immunized three times at 3-week intervals and bled 15 days after each immunization. The levels of (A) anti-SLA and (B) anti-rLACK specific antibodies in individual mouse serum at a dilution of 1:100, over time following TCI were analyzed by ELISA, and the values at OD 450 nm are plotted. (C) Cholera toxin specific IgG antibody titers. Serum was analyzed for CT specific IgG by ELISA as described in the Materials and Methods. The end point titer is defined as the reciprocal of the highest dilution, which is twice the background value. The results obtained from three independent experiments for PBS, CT, and SLA/CT groups and from two experiments performed with rLACK/CT are plotted. The star indicates statistical significance ($p < 0.05$).

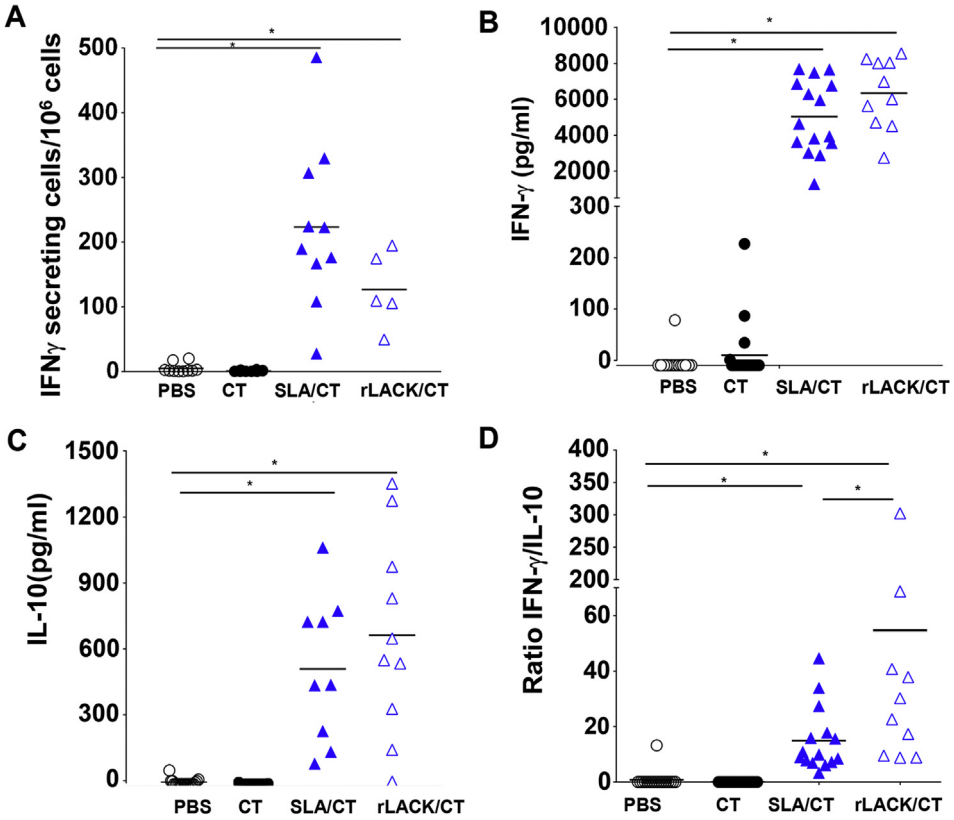

Fig. 2. Cellular responses to SLA and rLACK vaccination. (A) Determination of the frequency of IFNγ-secreting cells two weeks after the third TCI boost (before infection). Splenocytes were prepared from each group of mice, plated in triplicate on IFN-γ antibody coated plates and stimulated with *L. major* SLA antigens for 72 h. IFN-γ-secreting spots were enumerated and plotted after subtracting the non-specific spots from unstimulated wells. (B–D) Cytokine analysis of supernatants from splenocytes of mice. Splenocytes were prepared from the each group of mice and stimulated with *L. major* SLA antigens. Culture supernatants were collected after 48 h (for IL-10) or 72 h (for IFN-γ) of stimulation, and cytokines were measured by ELISA. (B) IFN-γ, (C) IL-10, and (D) ratio of IFN-γ/IL-10. Data are representative of 2 independent experiments (n = 10 for PBS, CT and SLA/CT, n = 5 for rLACK/CT). The star indicates statistical significance ($p < 0.001$).

animal using the Carestream *in vivo* Imaging System. This technique allows the estimation of parasite loads longitudinally and in contrast to ear thickness measurements, is unaffected by inflammation at the site of infection. The net intensity of the parasite burden was graphed at various time points post-infection (Fig. 4B). Both ear thickness measurement and Carestream *in vivo* imaging yielded similar results; confirming that SLA TCI led to disease exacerbation.

Parasites infiltrating the ear at the inoculation site and in the draining ipsilateral cervical lymph nodes were enumerated by limiting dilution assay at the end of the observation period (week 7 post-infection) (Fig. 5A). The increase in parasite load measured in the ears of mice immunized with SLA/CT and rLACK paralleled the increase in the ear thickness and *in vivo* imaging bioluminescence (Fig. 4A-B). The parasite load in the cervical lymph nodes did not correlate with lesion size (Fig. 5B). Quantitative PCR with

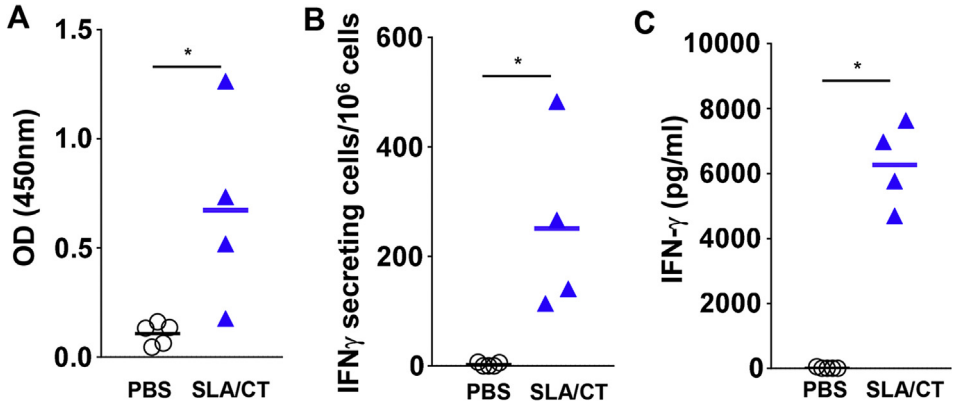

Fig. 3. Immune responses at 2 months post last TCI boost. (A) Anti-SLA IgG antibodies were determined by ELISA in the sera of PBS (n = 5) and SLA/CT immunized (n = 4) mice. (B) The frequency of IFN-γ secreting splenocytes was determined by ELISPOT. (C) IFN-γ secretion in splenocytes cultured with SLA for 72 hrs. The star indicates statistical significance ($p < 0.05$).

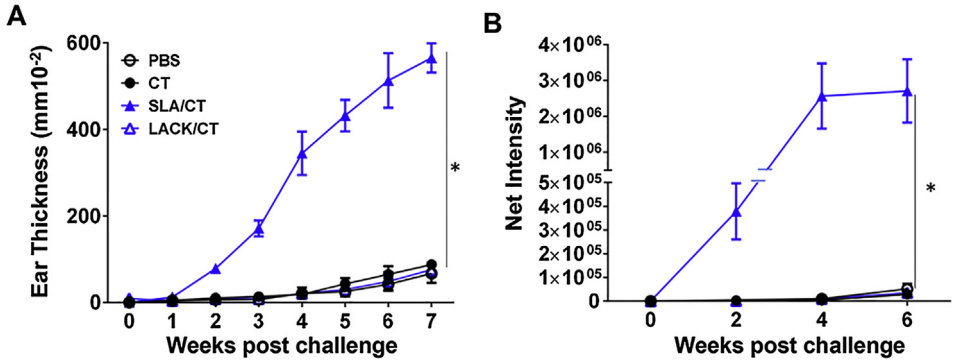

Fig. 4. Progression of leishmaniasis following TCI. (A) Measurement of ear swelling in mice immunized with PBS, CT, SLA/CT or rLACK/CT. Mice (five animals per group) were vaccinated on their shaved backs three times at 3-week intervals by the TCI route. Two weeks after the final immunization, the animals were challenged intradermally in the right ear with an inoculation of 5×10^4 metacyclic *L. major* parasites, and the ear thickness was measured weekly thereafter with a set of calipers. Control mice were vaccinated with CT or PBS alone. The experiment was performed three times for SLA/CT and twice for rLACK/CT with similar outcomes. The data are shown from a representative experiment (B) Evaluation of parasitic burden by bioluminescence. Carestream *in vivo* imaging data were collected from infected mice (n = 5) at 2, 4, and 6 weeks post-infection. The net intensity was determined by measuring the luminescence in the infected ear after subtracting the background intensity. The graph represents the average bioluminescence net intensity at various time points post-infection. The star indicates statistical significance ($p < 0.05$).

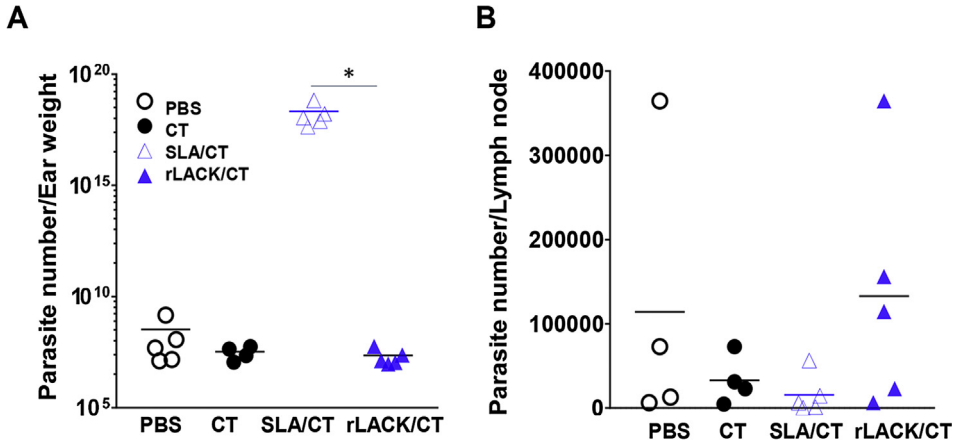

Fig. 5. Determination of parasite burden. (A) Parasite burden in the infected ear and (B) draining cervical lymph node of mice TCI vaccinated with PBS, CT, rLACK/CT or SLA/CT antigens and then challenged with *L. major* parasites. The parasite burden was determined by limiting-dilution assay at week 7 post-challenge. The results are expressed as the mean of the $\log_{10}$ dilutions positive for *L. major* promastigotes (divided by the ear weight). The results represent triplicate measurements from five mice in each group. The results shown are from a representative experiment out of three performed with SLA/CT and two with rLACK/CT. The star indicates statistical significance.

a kinetoplast target confirmed a higher parasite load in the ears of mice immunized with SLA/CT (data not shown).

3.5. CL exacerbation was associated with decreased iNOS expression

Infected ear lesions from mice in the PBS and SLA/CT immunized group (2 mice/group) were subjected to immunohistochemistry analysis. The cellular distribution of antigen presenting cells (macrophages and MHC-II expressing cells) and cells secreting IL-10 and iNOS (inducible nitric oxide synthase) were analyzed. Infected ears from immunized mice contained similar number of macrophages and cells expressing MHC-II and IL-10 as PBS non-immunized mice (compare Fig. 6E, D and C with Fig. 6J, I and H). Interestingly, expression of iNOS was much lower in the lesions of mice immunized with SLA/CT compared to PBS non-immunized mice (compare Fig. 6B with Fig. 6G).

4. Discussion

To date, no vaccine against leishmaniasis is available for use in humans. Development of an effective *Leishmania* vaccine for CL depends significantly on a variety of factors. To achieve better protection, the selection of effective antigen and adjuvant combinations and the choice of delivery system must be considered [32]. The topical application of antigen-adjuvant to the abraded skin surface by TCI offered an attractive needle-free vaccination route as compared to the commonly used injection route because of the skin's ease of administration, accessibility and immunocompetence [33,34].

Skin is an important part of the body's defense against invasion by infectious organisms [35] and may also be a target of chronic infections such as vector bite-inoculated *Leishmania* where the parasites establish their presence through avoidance of the immune system [36]. The skin has professional antigen presenting cells (APC) such as dendritic cells (DC) in the dermis, and Langerhans cells, macrophages, and sentinel T cells [37] in the epidermis, which can activate both *in vitro* and *in vivo* primary immune responses [38]. As for local immune responses in the skin following *Leishmania* infection, cure was associated with the production of IFN-γ only, while IL-10 was present in persisting lesions [20].

TCI requires the co-application of an adjuvant with an antigen to elicit robust systemic and mucosal immune responses [6,12,13,15]. TCI also yields potent cellular immunity, such as antigen-specific $CD4^+$ and $CD8^+$ T cell responses [6,39]. This is critical in leishmaniasis vaccine development as studies in mice showed that protective immunity to *Leishmania* required the development of parasite-specific Th1 responses, as characterized by IFN-γ and TNF-α production [40]. These inflammatory cytokines were required for the generation of reactive oxygen and nitrogen species by infected macrophages; these species (such as NO) enabled the killing of intracellular parasites. Conversely, a Th2 immune response was associated with susceptibility to leishmaniasis [41].

In this study, we used TCI as a route for vaccine delivery of two *Leishmania* antigens, namely SLA and rLACK, in combination with CT as an adjuvant, and assessed the degree of protection against parasite challenge in a murine CL model. As a proof of concept we tested SLA [42], a total parasite antigen, and rLACK, a recombinant protective antigen in BALB/c mice [23]. When used with appropriate adjuvants, SLA has been shown to induce protection [22]. Vaccination with rLACK antigen in the presence of recombinant IL-12 evoked $CD4^+$ T cell-induced protection in mice against *L. major* infection. This protection correlated with an increase in IFN-γ and a reduction of IL-4 [23]. We demonstrated that TCI induced a strong humoral response to CT as well as to SLA and rLACK, with the antibody response to SLA being higher that the antibody response to rLACK (Fig. 1A), suggesting that TCI was a suitable route to deliver *Leishmania* antigens. TCI also elicited strong Th1-like responses characterized by high levels of IFN-γ, low levels of IL-10, and negligible TNF-α and IL-4 production. These results suggested that our vaccine preparations might have the potential to achieve protection against murine CL because a high ratio of IFN-γ/IL-10 is considered a strong pre-challenge indicator of leishmaniasis vaccine success [43]. However, after parasite challenge TCI was not associated with protection and was even associated with exacerbation of the infection. SLA-TCI vaccinated mice had large skin lesions with severe local tissue injury, and a high number of parasites, which persisted at least 7 weeks post-infection. The observed absence of protection (with rLACK especially) could be explained by the fact that the immune responses to the same antigen induced by various immunization strategies could be different and sometimes even contrasting [44].

Additionally, we had considered whether TCI might change the local skin microbiome thus potentiating CL. It was recently shown that *Leishmania* infection induced a change in the skin microbiota and a pre-existing skin microbiome can influence the outcome of the infection or the disease [45]. Interestingly, intradermal immunization of BALB/c with SLA/CT led to the same exacerbation (results not shown), which suggests that the route of administration was not the determining factor. Exacerbation of disease was demonstrated in other studies, but the etiology of exacerbation was not clearly understood [46]. Two *Leishmania* antigens, lmd29 and 584C, lost their exacerbatory phenotype in BALB/c mice

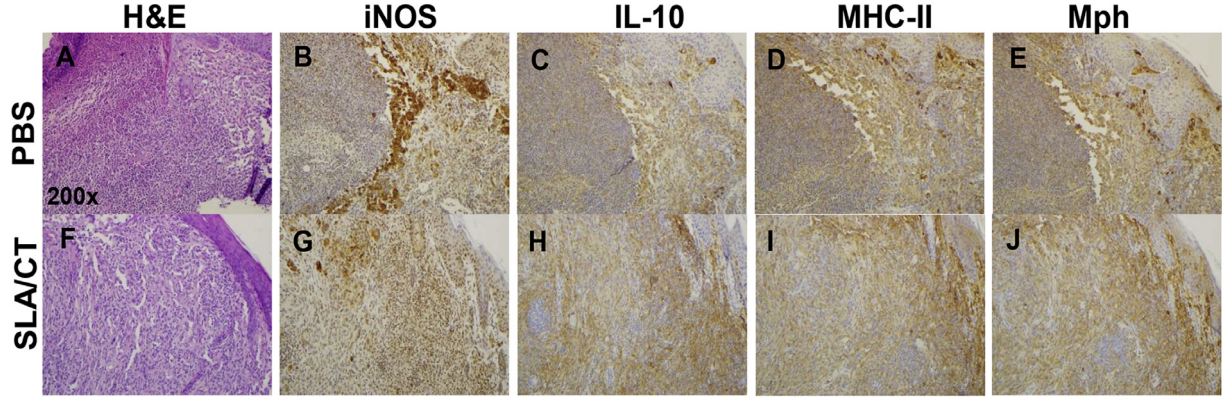

Fig. 6. Immunohistochemical analysis of the ear lesions 7 weeks post *L. major* infection. Mice were injected with SLA/CT (lower panels) or PBS (upper panels) by TCI. Seven weeks later, ear samples were fixed in 10% neutral formalin and paraffin embedded. Four- to 5-mm sections were cut and stained (A, F) with H&E; or with antibodies specific to (B, G) iNOS; (C, H), IL-10; (D, I) MHC-II; and (E, J) macrophages. Brown color demonstrates respective Ab-stained cells. Original magnification: 200×.

rendered resistant by disruption of genes encoding classical Th2 cytokines, indicating a role for IL-4 and IL-10 in vaccine-induced exacerbation [47]. Another factor contributing to disease exacerbation could be the presence of a high anti-SLA IgG before parasite challenge. It was demonstrated that IgG antibody not only failed to provide protection against *Leishmania*, but contributed to disease progression [48].

It has been shown that direct antigen presentation by specific B cells was involved in susceptibility of BALB/c mice to infection with *L. major* LV39 and B cell function led to disease exacerbation [49]. In murine visceral leishmaniasis, disease exacerbation was a direct result of circulating immunoglobulins (IgM and/or IgG) [50] and mice lacking B cells were found to be less susceptible to *L. donovani* infection [51]. Additionally, IgG immune complexes exacerbated infection by inducing IL-10 production in macrophages [48]. IL-10 promoted B cell survival, and plasma cell proliferation, and also an IgG isotype switch toward IgG1 and IgG3 [52]. In the mouse, antibodies of the IgG1 isotype were produced mainly during a Th2 immune response, and IgG2a antibodies were produced mainly during Th1 immune responses [53]. In our study, despite no difference seen in Ig isotypes and induction of low levels of IL-10 before challenge, an exacerbation of the disease was observed. We speculated that antibody-antigen complexes were probably detrimental to the host.

In *Leishmania* vaccine trials, as in human leishmaniasis, the immune response is complex and either Th1 or Th2 phenotypic cells can develop. The biological phenotype of the immune response is determined by the predominance of one cell type over the other, not merely by the presence or absence of Th1 or Th2 type immune cells [54]. Our results confirmed the observations made by Campos-Neto that antigens stimulating a Th1 response during the disease showed no protective effect as a vaccine, while antigens associated with an early Th2 response were protective if a Th1 response to them was generated before infection [46]. This lack of correlation with protection was also observed with eight other leishmanial antigens that have been discovered and selected on the basis of the Th1/Th2 paradigm using peripheral blood mononuclear cells from cured leishmaniasis patients as immunological readouts [55,56].

Based on the observed exacerbation with SLA/CT delivered through TCI, we investigated the role of inflammatory cells after infection. Inflammatory cells and soluble mediators recruited to the site of infection are important in the lesion healing response versus disease progression [31]. No differences in macrophages, APC, or cells secreting IL-10 were observed between the SLA-vaccinated or control mice. However, lower levels of iNOS were expressed at the site of infection in the SLA group. Nitric oxide (NO) production by the inducible NO synthase (iNOS or NOS2) represents one of the major microbicidal mechanisms of murine macrophages [57]. In the presence of NO, parasites were less metabolically active, resulting in reduced proliferation [58]. *L. major* infection can be reactivated in chronically infected healthy C57BL/6 mice following iNOS inhibitor treatment [59]. The expression of iNOS prevented uncontrolled parasite replication and reduced the level of T cell activity [59], suggesting an association between iNOS expression and leishmaniasis outcome. The lower expression of iNOS in SLA/CT vaccinated mice could explain the lesion exacerbation seen, resulting in the host's inability to contain the parasites. We also examined the arginase activity as a mechanism of local T cell hyporesponsiveness in the ear. High arginase activity, a hallmark of nonhealing leishmaniasis, has shown to be primarily expressed locally at the site of pathology. It causes local depletion of L-arginine, which impairs the capacity of T cells in the lesion to proliferate [60]. Arginase-mediated L-arginine depletion induces down-regulation of CD3 zeta, the main signaling chain of the TCR, and functional T cell hyporesponsiveness [61]. In our study, no differences were seen in the ears immunostained with anti arginase antibody (data not shown) between the PBS and SLA/CT groups. In conclusion, we demonstrated that despite the presence of a strong Th1 response before challenge, TCI with either SLA or rLACK was not associated with protection. We suggest that the high humoral response seen before challenge, especially in the SLA TCI treated group, as well as the low iNOS expression impacted the disease outcome. In mice, NO is considered essential for controlling *Leishmania*, as iNOS-deficient mice are susceptible to *L. major* infection even though they develop a greater Th1–type response compared with wild-type mice [62].

5. Conflict of interest

No competing interests to declare

6. Disclaimer

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Army, Department of Defense, nor the U.S. Government. All authors have approved the final article.

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