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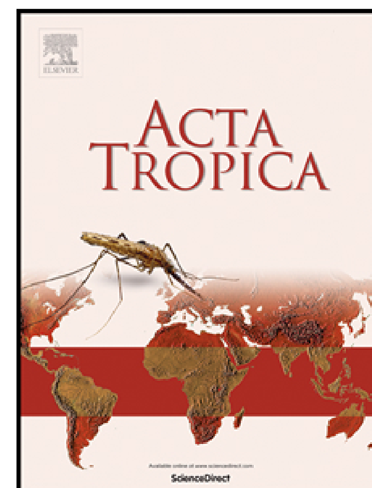

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Intranasal Trans-sialidase-based vaccine against Trypanosoma cruzi triggers a mixed cytokine profile in the nasopharynx-associated lymphoid tissue and confers local and systemic immunogenicity

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ABSTRACT

Trypanosoma cruzi, the agent of Chagas disease, can infect through conjunctive or oral mucosas. Therefore, the induction of mucosal immunity by vaccination is relevant not only to trigger local protection but also to stimulate both humoral and cell-mediated responses in systemic sites to control parasite dissemination. In a previous study, we demonstrated that a nasal vaccine based on a *Trans-sialidase* (TS) fragment plus the mucosal STING agonist c-di-AMP, was highly immunogenic and elicited prophylactic capacity. However, the immune profile induced by TS-based nasal vaccines at the nasopharyngeal-associated lymphoid tissue (NALT), the target site of nasal immunization, remains unknown. Hence, we analyzed the NALT cytokine expression generated by a TS-based vaccine plus c-di-AMP (TSdA+c-di-AMP) and their association with mucosal and systemic immunogenicity. The vaccine was administered intranasally, in 3 doses separated by 15 days each other. Control groups received TSdA, c-di-AMP, or the vehicle in a similar schedule. We demonstrated that female BALB/c mice immunized intranasally with TSdA+c-di-AMP boosted NALT expression of IFN-γ and IL-6, as well as of IFN-β and TGF-β. TSdA+c-di-AMP increased TSdA-specific IgA secretion in the nasal passages and also in the distal

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intestinal mucosa. Moreover, T and B-lymphocytes from NALT-draining cervical lymph nodes and spleen showed an intense proliferation after *ex-vivo* stimulation with TSdA. Intranasal administration of TSdA+c-di-AMP provokes an enhancement of TSdA-specific IgG2a and IgG1 plasma antibodies, accompanied by an increase IgG2a/IgG1 ratio, indicative of a Th1-biased profile. In addition, immune plasma derived from TSdA+c-di-AMP vaccinated mice exhibit *in-vivo* and *ex-vivo* protective capacity. Lastly, TSdA+c-di-AMP nasal vaccine also promotes intense footpad swelling after local TSdA challenge. Our data support that TSdA+c-di-AMP nasal vaccine triggers a NALT mixed pattern of cytokines that were clearly associated with an evident mucosal and systemic immunogenicity. These data are useful for further understanding the immune responses elicited by the NALT following intranasal immunization and the rational design of TS-based vaccination strategies for prophylaxis against *T. cruzi.*

1. INTRODUCTION

Chagas disease is a parasitic disease caused by *Trypanosoma cruzi* (Pérez-Molina & Molina, 2018). The disease affects 6-8 million people worldwide causing 14,000 deaths per year. Vector and congenital forms of natural transmission are frequent, however, the number of oral outbreaks caused by parasite-contaminated food ingestion is increasing in endemic countries (Coura, 2015). Independently of the route of infection, around 30% of infected individuals develop~~ed~~ after many years ~~of~~ cardiac and/or digestive manifestations of the disease (Rassi et al., 2010). The anti-parasitic treatment is based on Benznidazole or Nifurtimox, which cause~~s~~ several adverse effects leading to treatment interruption. Their efficacy is limited, justifying the identification of new alternatives to prophylaxis and therapy (Sales et al., 2017).

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Nowadays there are no prophylactic vaccines available against *T. cruzi*. Experimental vaccines against the parasite have been mainly exploring systemic routes of immunization, such as intramuscular or subcutaneous (Rodríguez-Morales et al., 2015). Mucosal vaccination may trigger the activation of the ~~common~~ mucosal immune system, leading to an effective local and systemic immune response, usually not achieved through parenteral administration. However, despite natural ways of *T. cruzi* transmission occurring through oral or conjunctive mucosa, this route of vaccination has been less assessed (Hoft & Eickhoff, 2002; Matos et al., 2014; Rodríguez-Morales et al., 2015; Sanchez Alberti et al., 2017).

Trans-sialidase (TS) family of proteins is encoded by at least 1430 genes in *T. cruzi*, which were subdivided into eight groups (GI-GVIII) based on sequence similarity and functional properties. The TS-GI includes genes of proteins with trans-sialidase activity. However, some genes of this group encode inactive forms since they have mutated amino acids in the catalytic domain or constitute pseudogenes (Campetella et al., 2020)). TS-GI are interesting as vaccine candidates because they are highly expressed on the parasite surface, can be released into circulation and they are among the best-characterized targets of the anti-parasite immune response (Buscaglia et al., 1999; Frasch, 2000). Furthermore, TS proteins are the main virulence factors of *T. cruzi* (Campetella et al., 2020). Additionally, TS-GI could bind glycans expressed on epithelial cells, suggesting that it could be involved in the invasion of mucosas (Butler et al., 2013). It is also noteworthy that TS are highly immunogenic in both mice and humans, while numerous experimental TS-GI-based vaccines have demonstrated protective efficacy (da Costa et al., 2021). In addition, a comparative study between different *T. cruzi*-derived antigens showed that TS-GI showed the best performance as vaccine immunogen (I. Bontempi et al., 2017). Therefore, the use of TS-GI in vaccine formulations has been justified based on these previous reasons. We recently demonstrated that an N-terminal fragment (TSnt) of 283 aminoacid residues, exerts protection when administered systemically

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(Prochetto et al., 2022). Moreover, intranasally administration of a C-terminal fragment (TSct) of 170 aminoacid residues plus c-di-AMP was highly immunogenic and elicited protection after oral challenge (Pacini et al., 2022). The c-di-AMP is a bacterial second messenger that acts as a potent mucosal adjuvant in mice and potentially in humans, activating the STING pathway (by "Stimulator of interferon genes"), resulting in the release of Type-I interferons (IFNs) and pro-inflammatory cytokines (Corrigan & Gründling, 2013; Dubensky et al., 2013; Ebensen et al., 2011a).

Although intranasal immunization with TS-derived vaccines shows promising results (Pacini et al., 2022), little is known about the response generated at the nasopharyngeal-associated lymphoid tissue (NALT), the target site of nasal immunization, and how it shapes the local and systemic response. Therefore, this study aimed to evaluate, for the first time, the cytokine profile induced at the NALT by the intranasal administration of a TSdA (dual-Antigen)-based vaccine, composed of both TSnt and TSct fragments and plus c-di-AMP. In addition, the associated mucosal and systemic immune profiles were investigated, since this knowledge may be crucial to facilitate the development of future *T. cruzi* nasal vaccines.

2. MATERIAL AND METHODS

2.1 Mice and parasites

BALB/c female mice (6-8 weeks old) and suckling mice were obtained and maintained at the animal facilities of the Centro de Investigación y Producción de Reactivos Biológicos (CIPReB-FCM-UNR). Protocols for animal studies were approved by the Institutional Animal Care & Use Committee (Res. Nro. 6698/2014). Recombinant Dm28c (DTU-I) metacyclic trypomastigotes of *T. cruzi* expressing β-galactosidase were used in the *ex vivo* assays. Tulahuén strain (DTU-VI) was used in the *in vivo* challenges.

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2.2 Immunization schedule and samples collection

TS dual-Antigen (TSdA) results from the combination of TSnt+TSct fragments (Prochetto et al., 2022; Quintana et al., 2018). Details of fragments and the identity percentage among strain used in these study are given in the Supplementary data (S1). BALB/c female mice (n=4-7/group) were immunized intranasally with 3 doses (one every two weeks) of formulations containing: Saline solution alone (Co group), 5µg of each TS fragment (TSdA group), TSdA plus 5µg of c-di-AMP by Sigma-Aldrich, USA (TSdA+c-di-AMP group), and 5µg of c-di-AMP (c-di-AMP group). Blood and feces were collected on day 0, and 15 days after the second and third doses. Fecal samples were pooled (2-3 animals/sample) and processed as previously published (Pacini et al., 2022). Mice were sacrificed 15 days after the last dose of immunization and NALT, cervical lymph nodes (CLN), and spleens were obtained for qPCR and *ex vivo* assays. Nasal lavages were also performed by retrograde infusion through the trachea with 0.3 mL Protease Inhibitor (SIGMA).

2.3 *Determination of the TSdA-specific antibody levels in plasma, nasal lavage, and fecal samples*

Evaluation of the TSdA-specific antibody levels in plasma, nasal lavage, and fecal samples were performed as previously published (I. A. Bontempi et al., 2015; Pacini et al., 2022). Details of ELISA are given in Supplementary data (S1).

2.4 TSdA-specific cellular assays.

A delayed-type hypersensitivity test was performed by intradermal challenge with TSdA (2.5 µg of each fragment) in the hind footpad of mice 15 days after the last immunization. Footpad swelling was measured until 72h post-challenge, as informed previously (Pacini et al., 2022). Spleens and CLN were homogenized and $1{\times}10^6$ cells/well were cultured in 48-well plates (GBO), in complete DMEM medium alone or stimulated with TSdA (10µg/well). After 60h of culture, cells were incubated with anti-FcγII/III-R

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antibodies and stained with anti-CD4/Percp and anti-CD8/PE and anti-B220/APC-Cy7 and anti-Ki67/FITC (all from BD Pharmingen) to determine lymphocyte proliferation. The samples were acquired in a BD FACSAriaII cytometer. A minimum of 1×10^5 events were acquired for each sample. FlowJo Software (Beckton-Dickinson, USA) was used for sample analysis.

2.5 NALT isolation and cytokine pattern profile by RT-qPCR

Total RNA was isolated from palates using TRI-Reagent® and reverse-transcribed to cDNA using the RevertAid Reverse Transcriptase (Thermo-Fisher Scientific, USA). Real-time PCR was performed with the StepOnePlus Real-Time PCR System (Applied-Biosystems, USA) using the Mix-5x-HOT-FIREPol®EvaGreen® qPCR-Mix Plus with ROX (Solis-BioDyne, Estonia). Details of amplification are included in the Supplementary data (S1). Primers (Table 1) were designed in our laboratory and purchased from Eurofins-Genomics, USA or Invitrogen, USA.

2.6 Ex-vivo and in-vivo evaluation of the activity of immune plasma

Dm28c/pLac *T. cruzi* cell line was obtained by transfection of *T. cruzi* Dm28c strain from DTU I with pLacZ plasmid (Dm28c/pLacZ). Vero cells ($5x10^3$) were seeded onto 96-well plates and, after o.n. incubation at 37°C, were infected with $5x10^4$ Dm28c/pLac parasites, previously incubated in 1/10 diluted plasma from immunized mice (5 mice/pool) during 1h at 37°C in DMEM, or without plasma. After o.n. incubation, Vero cells were washed to eliminate non-penetrating parasites and incubated for additional 72h. The β-galactosidase activity expressed by the recombinant amastigotes hydrolyzes the chromogenic substrate, chlorophenol red β-D-galactopyranoside (CPRG), to chlorophenol red, which can be easily measured colorimetrically at 595nm as an indirect way of determining parasite numbers and cell invasion (Alonso et al., 2021). Uninfected cells were used as blanks, while cells coincubated with parasites in absence of

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immune plasma were used to estimate the 100% of invasion. Inhibition percentage was calculated as follows: {1-[(ABS-595nm of infected cells in presence of immune plasma)/(ABS-595nm of infected cells in absence of immune plasma]}x100.

Suckling mice (5 days old) were intraperitoneally challenged with a lethal dose of 500 bloodstream trypomastigotes of the Tulahuen strain. Parasites were previously incubated for 1h with immune plasma pools (5 animals/pool) derived from immunized animals. Survival was monitored daily.

2.7.Statistical analyses

Results correspond to two independent experimental rounds. Data were analyzed using nonparametric tests (Kruskal-Wallis followed by Mann-Whitney U-test). Analyses were performed using GraphPad Instat-4.0 software (USA). Differences between groups were considered significant when $p < 0.05$.

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3.RESULTS

3.1.TSdA-specific sIgA common mucosal responses are linked to a mixed cytokine pattern triggered in the NALT after intranasal vaccination with TSdA+c-di-AMP

To evaluate the immunogenicity of TSdA+c-di-AMP formulation after intranasal vaccination, we focused on the early immune responses involving TSdA-specific IgA secretion at the common mucosa system, the expression profile of cytokines induced at the NALT, ex-vivo lymphocyte proliferation from draining lymph nodes (Fig.1A). A rise in fecal specific-IgA antibodies became evident after the second dose of TSdA+c-di-AMP and TSdA mice (Fig.1B). After the third dose, augmented contents of specific-sIgA in nasal lavages (Fig.1C) and feces (Fig.1D) were detected in TSdA+c-di-AMP compared with Co, TSdA, or c-di-AMP groups.

TSdA-vaccinated animals also showed an increase in fecal sIgA, but greater only than the Co group (Fig.1D).

NALT cytokine expression after intranasal immunization with TS-derived immunogens plus c-di-AMP was unexplored. Thus, to determine whether a TSdA+c-di-AMP triggers a particular cytokine profile linked to a better immunogenic response, we evaluated by RT-qPCR the expression of a wide range of cytokines associated with diverse immune patterns of response. As can be observed in Fig.1E-J, a significant increase of IFN-γ, IFN-β, IL-6, and TGF-β gene expression was observed in the NALT of TSdA+c-di-AMP immunized mice compared to other groups (except for IL-6, where the difference versus c-di-AMP is not significative). Interestingly, TSdA+c-di-AMP prompted to increase IL-17A and IL-4 expression (detectable in ≈50% of mice/group). These results indicated that TSdA+c-di-AMP promoted a marked mixed cytokine environment in the NALT which differed significantly for the other vaccinated groups and result favorably for the TSdA-specific IgA induction in both nasal and digestive mucosa (Fig.1K).

Antigens that drain from nasal passages and palate regions via pharyngeal lymphatics, reach the CLN (Fig.1A), acting as an effector site for NALT (Lohrberg et al., 2018; Lohrberg & Wilting, 2016). *Ex-vivo* TSdA-restimulation of cervical lymphocytes from the TSdA+c-di-AMP animals induces a clear enhancement of T and B-cell proliferation (Fig.1L-M).

3.2.TSdA+c-di-AMP conferred a strong systemic immune response and protects against parasite invasion

Here, we evaluated whether intranasal vaccination with TSdA+c-di-AMP was also effective in priming systemic immune responses and also whether this effect is reflected on the cross-talk between the mucosal and systemic immune systems. TSdA+c-di-AMP provokes a robust response of specific-IgG2a (Th-

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1-biased) plasma antibodies. A similar situation occurs regarding specific-IgG1 antibodies (Th-2-biased), although it only reaches statistical significance in the TSdA+c-di-AMP group when contrasted with the Co or c-di-AMP groups, but not against TSdA alone. Additionally, the relative IgG2a/IgG1 ratio is increased in the TSdA+c-di-AMP mice, indicating that TSdA+c-di-AMP vaccine prompted a systemic Th1-biased humoral response compared to the rest of the groups (Fig.2C). At difference that was observed with the specific-IgA, the systemic response became evident after the third dose (Fig.2D-E). Moreover, parasites exposed to immune plasma from TSdA+c-di-AMP vaccinated animals showed a diminished invasion capacity (≈50%), and caused delayed mortality of highly susceptible mice, as seen in Fig.2F-G.

As shown in Fig.2H, TSdA+c-di-AMP vaccinated mice showed pronounced footpad swelling after TSdA challenge (Fig.2H). Likewise, the global response between 0-72h is also evident when observing the area under the curve (Fig.2I). Moreover, after *ex-vivo* restimulation with the TSdA, the absolute number of splenic T-lymphocytes and B-lymphocytes expressing Ki67 were markedly increased in the TSdA+c-di-AMP group (Fig.2J-L). Collectively, these results showed that intranasal administration of TSdA+c-di-AMP elicited a robust systemic humoral and cellular response against TSdA.

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4.DISCUSSION

Nowadays, TS-GI are considered promising candidates for a *T. cruzi* vaccine (da Costa et al., 2021). Most of these studies have been performed through systemic routes of immunizations, and to a lesser extent by mucosal routes. Previously, we demonstrated that intranasal administration of a TS-fragment plus c-di-AMP was highly immunogenic and elicited prophylactic capacity (Pacini et al., 2022). However, the immune profile induced locally by TS-based nasal vaccines and how they can shape the systemic response

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remained unexplored. Here, we showed that a TSdA-based vaccine administered intranasally induces a mixed pattern of cytokine in the NALT, that is clearly associated with local and systemic immunogenicity. The TSdA-specific IgA secretion is crucial to develop a prophylactic vaccine against *T. cruzi* conjunctive or oral infection. Coincidently, fecal TSct-specific IgA production was observed when TSct was administered intranasally (Pacini et al., 2022). Other authors have also advertised IgA secretion against other antigens when administered by oral or nasal route (Ebensen et al., 2011b; Matos et al., 2014), reinforcing the idea that it is possible to artificially provoke a mucosal *T. cruzi*-specific response. Here, increased TSdA-specific IgA in nasal lavages of TSdA+c-di-AMP vaccinated animals was associated with enhanced mRNA expression of NALT IL-6 and TGF-β, two cytokines involved in IgA class-switching (Ramsay et al., 1994; Takaki et al., 2017; Tezuka & Ohteki, 2019). Moreover, IgA secretion was associated with IFN-γ upregulated expression. Despite IL-17A raise in NALT did not reach statistical significance, it is known that systemic protective cell immunity in mice and humans with Chagas disease requires in addition to Th-1, the involvement of Th-17 cell B(Gutierrez et al., 2009; Magalhães et al., 2013; Miyazaki et al., 2010). However, Th1/Th17 combined role at the inductive mucosal site is less known. Recently, it was noticed using IL-17 KO mice that Th-17 T cells and IL-17A are key players in providing mucosal protection against gastric *T. cruzi* infection (Cai et al., 2021). TSdA+c-di-AMP induces in NALT a Th-1/Th-17-biased pattern accompanied by IL-4(Th-2) expression in ≈50% of animals. Moreover, a STING-driven IFN-β expression in the NALT of TSdA+c-di-AMP and c-di-AMP vaccinated animals suggested that other direct and indirect protective mechanisms could be also induced. Accordingly, c-di-AMP can activate NALT dendritic cells, increasing the expression of CD80/CD86 costimulatory markers and also MHC-II molecules, favoring the priming of different Th-cells (Škrnjug et al., 2014b). This effect may be induced by the expression of IFN-β

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by dendritic cells localized in the NALT since c-di-AMP administered intranasally can modulate in-situ both innate and adaptive T and B-cell response through STING pathways in other models (Škrnjug et al., 2014a). Moreover, type-I IFN nasal administration enhances specific-IgA, but also plasma IgG2a secretion during viral respiratory infections (Staats & Thompson, 2011). Hence, it is conceivable that NALT c-di-AMP/STING/IFN-β axis also contributed to the global humoral immunogenicity elicited by TSdA+c-di-AMP vaccine.

Of note, TSdA+c-di-AMP vaccinated mice showed an earlier humoral mucosal response if compared to systemic, similar to what was evidenced in viral infections of the air tract (Alu et al., 2022). Despite that, we highlight that the specific-IgA secretion induced by TSdA+c-di-AMP is not limited to the nasal cavity, but also at least at one distal mucosal site, the intestinal tract. These results indicate that B-lymphocytes activated at the NALT-inductive site may instruct to home back to the lymphoid structures of the nasal cavity, but some of them also seed the distal intestinal mucosa. This fact is relevant in the context of a mucosal vaccine designed to prevent oral infection, because seeding TSdA-specific effector or memory B-lymphocytes in the intestinal tract may help to protect against parasite epithelial invasion.

The enhanced *ex-vivo* proliferation of CLN and splenic B-cells in addition to specific and protective plasma antibodies detected in TSdA+c-di-AMP immunized animals point out the clear establishment of a systemic response triggered by intranasal immunization. In addition, TSdA+c-di-AMP immunization also provokes a TSdA-specific cellular response at mucosal and systemic compartments, assessed by the NALT IFN-γ expression accompanied by IL-17/IL-4 expression in ≈50% of animals, the marked footpad swelling and the increased *ex-vivo* proliferation of CLN and splenic T-lymphocytes after TSdA restimulation. Overall, the results shown here indicate that TSdA+c-di-AMP administered by the nasal route triggers a NALT

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mixed pattern of cytokines that are associated with a clear immunogenic profile, being a promising vaccine candidate against *T. cruzi.* Given that one of the natural routes of parasite infection is the oral mucosa, which is in close contact with the NALT, this vaccine would provide both optimal local and systemic protection against oral parasite dissemination.

CONFLICT OF INTEREST STATEMENT

None of the authors have a conflict of interest in relation to the content of the present work.

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AUTHOR CONTRIBUTIONS

Conception and design: PAR/MI. Obtention of TSct from *L.lactis*: MC/BVS/EM/PMF. Obtention of TSnt from *E.coli*: PMF/PE/IM. Database: PMF/DB/BBC. *In-vivo* experiments: PMF/BCC/DB/GFB/FC/PAR. *Ex-vivo* experiments: PMF/CP/dHMA. Statistical analysis: PMF/PAR. PAR wrote the first draft of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Table 1: Primer sequences used for RT-qPCR

GENE	PRIMER SEQUENCE (5'-3')
IFN-γ	Fwd: AGACAATCAGGCCATCAGCAAC Rev: CTCATTGAATGCTTGGCGCTG
IL-17A	Fwd: CAAAGCTCAGCGTGTCCAAA Rev: CTTCCCAGATCACAGAGGGATA
IL-4	Fwd: GCCAAACGTCCTCACAGCAA Rev: TCTGCAGCTCCATGAGAACACT
IFN-β	Fwd: TGGGAGATGTCCTCAACTGC Rev: CCAGGCGTAGCTGTTGTACT
TFG-β	Fwd: TGACGTCACTGGAGTTGTACGG Rev: GTTCATGTCATGGATGGTGC
IL-6	Fwd: GAGGATACCACTCCCAACAGACC Rev: AAGTGCATCATCGTTGTTCATACA
GAPDH	Fwd: AGCAATGCATCCTGCACCACCA Rev: ATGCCAGTGAGCTTCCCGTTCA

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FIGURE LEGENDS

Figure 1: Mucosal and drain lymph node response after intranasal nasal immunization with TSdA+c-di-AMP

BALB/c female mice were immunized intranasally (3 doses, one every 15 days). Feces and nasal lavage were assessed by ELISA for TSdA-specific IgA antibodies. **A)** Schematic of mouse nasopharynx-associated lymphoid tissue (NALT), the posterior palate, nasal passages (NP), and the drain cervical lymph node (CLN). Arrows indicate the sequence of immune response developed locally. **B)** Kinetics of fecal TSdA-specific IgA secretion before immunization and after second and third doses in all immunized groups. **C)**

TSdA-specific IgA in nasal lavages after immunization completion, *=p < 0.05. **D)** TSdA-specific IgA in fecal extracts after immunization completion, *=p < 0.05. Fifteen days after immunization, palates were obtained for measured NALT cytokine expression through RT-qPCR for: **E)** IFN-γ; **F)** IL-17A; **G)** IL-4; **H)** IFN-β; **I)** TFG-β; and **J)** IL-6; *=p < 0.05. **K)** Radial chart showing the association between immune parameters (antibodies and gene cytokine expression) triggered by each vaccine formulation with respect to the Co group. The magnitude of each axis represents the delta value obtained between each vaccinated group and the Co group. Thus, increasing or decreasing the central polygon areas reflects either the higher or lower contribution of each immune parameter. Cervical lymphocytes were obtained 15 days after immunization completion and restimulated *ex vivo* with TSdA. **L)** Gating strategy to evaluate cervical lymphocyte proliferation by Ki67 staining. **M)** TSdA-specific proliferation of CD4+ and CD8+ T-cells and B220+ B-cells. α=p < 0.05 with respect TSdA-stimulated group, *=p < 0.05 vs Co group, # =p < 0.05 vs TSdA group, ϕ=p < 0.05 vs c-di-AMP group. (One-way-ANOVA Kruskal-Wallis test followed by the U de Mann-Whitney test).

Figure 2: Systemic response elicited after intranasal nasal immunization with TSdA+c-di-AMP

The humoral and cellular systemic responses triggered by intranasal immunization were evaluated during and after immunization schedule completion. Specific plasma antibodies were evaluated by ELISA: **A)** TSdA-specific IgG2a (15 days after 3rd doses); **B)** TSdA-specific IgG1 (15 days after 3rd doses); **C)** IgG2a/IgG1 ratio (15 days after 3rd doses); **D-E)** Kinetics of TSdA-specific IgG2a e IgG1 respectively before immunization and after second and third doses. **F)** *In vitro* inhibition assay: Recombinant Dm28cβ-gal parasites were preincubated with immune plasma from vaccinated animals previous to infection of non-phagocytic Vero cells. The graph shows the percentage of inhibition of invasion after 72 h post-exposition. **G)** Mortality of suckling mice intraperitoneally infected with Tulahuen parasites that

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were previously exposed to immune plasma (IP) derived from TSdA, TSdA+c-di-AMP, or vehicle-treated animals (Log-rank test, $p<0.05$). **H)** A delayed-type hypersensitivity test (DHT) was performed 15 days after the last immunization. The thickness of the footpad was measured 24, 48, and 72h after the intradermal injection of 5µg of TSdA. The opposite footpad served as a control. Results are expressed as the difference between the value of the footpad control and the TSdA-inoculated one (delta, Δmm). Representative images showed footpad swelling after 48h; **I)** The global response after DHT was calculated as the area under the curve (AUC) from 0 to 78 hours after the TSdA challenge. Splenocytes were obtained 15 days after immunization completion and restimulated *ex vivo* with TSdA during 60h and stained with Ki67 to estimate TSdA-specific proliferation: **J)** CD4+Ki67 T-cell; **K)** CD8+ki67 T-cells; and **L)** B220+ki67 B-cells. The levels of significance are defined as follows: $*p < 0.05$ and $**p < 0.01$. (One-way-ANOVA Kruskal-Wallis test followed by the U de Mann-Whitney test).

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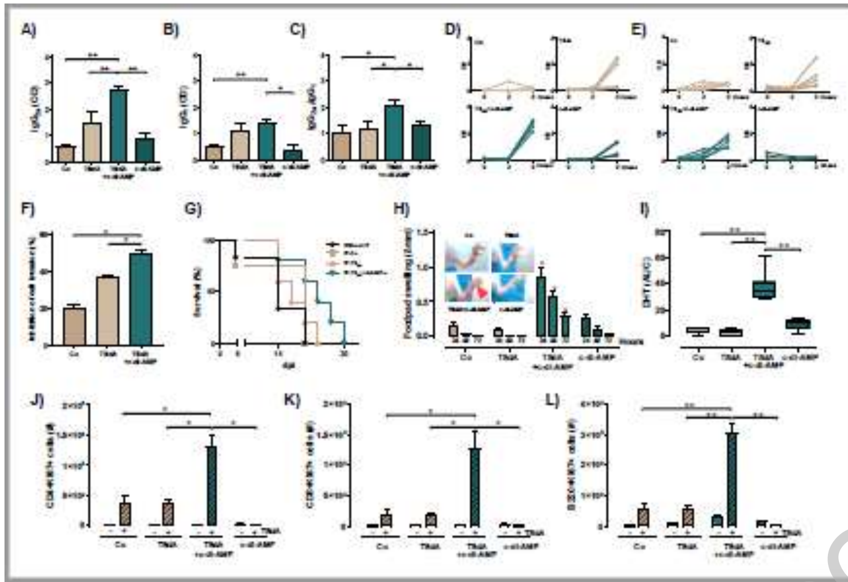

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CONFLICT OF INTEREST STATEMENT

None of the authors have a conflict of interest in relation to the content of the present work.

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