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RESEARCH

Immunomodulatory activity of *Trypanosoma cruzi* recombinant antigen combination TSA-1-C4 and Tc24-C4 induce activation of macrophages and CD8$^+$ T cells

Victor Manuel Dzul-Huchim[1] · Miguel Rosado-Vallado[1] · Antonio Euan-Canto[2] · Julio Torres-Romero[2] · Jaime Ortega-Lopez[3] · Julio Vladimir Cruz-Chan[1] · Liliana Estefania Villanueva-Lizama[1] · Victor Arana-Argáez[2]

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Abstract

Chagas disease is a chronic infection caused by the protozoan parasite, *Trypanosoma cruzi*, with limited benefits of the currently available anti-parasitic chemotherapeutic approaches to halt the progression of heart disease. Recombinant TSA-1-C4 and Tc24-C4 proteins have been developed as promising antigen candidates for therapeutic vaccines, leading to propose them in combination as a bivalent recombinant protein strategy. In this study, we evaluated the immunomodulatory effect of the combined TSA-1-C4 and Tc24-C4 recombinant proteins by in vitro assays using murine macrophages. Macrophages from naïve Balb/c mice were isolated and stimulated with TSA-1-C4 plus Tc24-C4 recombinant proteins, hence, supernatants were recovered to measure host NO, H_2O_2, as well as, TNF-α, IL-1β, IL-6 and IL-10 cytokine responses. Later, stimulated macrophages were co-cultured with CD8$^+$ T cells from naïve mice, and inflammatory cytokine-profiles were measured from supernatants. We observed that combining both antigens promotes the activation of host macrophages by NO and H_2O_2 release; moreover, these macrophages also induced considerable pro-inflammatory immune-responses mediated by TNF-, IL-1β and IL-6 cytokines compared to either TSA-1-C4 or Tc24-C4 stimulated macrophages. In addition, naïve CD8$^+$ T cells in presence of TSA-1-C4 plus Tc24-C4 stimulated-macrophages similarly boosted the pro-inflammatory immune profile by significant production of IFN-γ and TNF-α cytokines. These results support immunological advantages for the use of TSA-1-C4 and Tc24-C4 combination as vaccine candidates against *T. cruzi*.

Keywords Chagas disease · *Trypanosoma cruzi* · TSA-1-C4 · Tc24-C4 · Immunomodulatory · Macrophages · CD8$^+$ T cell

Introduction

Chagas disease (CD) is a neglected tropical disease caused by the intracellular parasite *Trypanosoma cruzi* and transmitted through contact with infected excretes of triatomine bugs. The illness affects approximately 6–7 million people worldwide and is a major public health problem in the Americas (WHO 2018). The acute phase of CD is distinguished by parasites in peripheral blood and consequent non-specific febrile illness that typically resolves within 4–8 weeks (Dias et al. 1956; Rassi et al. 2010). Once resolved, infection is followed by a chronic phase, in which most patients remain asymptomatic throughout life. However, 20–30% of infected individuals progress to chronic chagasic cardiomyopathy (CCC), leading to severe heart disease or premature death (Teixeira et al. 2002; Rassi et al. 2010; Ribeiro et al. 2012). Benznidazole (BNZ), the first-line pharmacological treatment for CD is associated with drug toxicity and

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✉ Victor Arana-Argáez
victor.arana@correo.uady.mx

[1] Centro de Investigaciones Regionales (C.I.R.) Dr. Hideyo Noguchi, Universidad Autónoma de Yucatán (UADY), Calle 43 S/N entre calle 96 y calle 40 Colonia Inalámbrica, Mérida, Yucatán C.P. 97069, Mexico

[2] Facultad de Química, Universidad Autónoma de Yucatán (UADY), Calle 43 S/N entre calle 96 y calle 40 Colonia Inalámbrica, Mérida, Yucatán C.P. 97069, Mexico

[3] Centro de Investigación y Estudios Avanzados (CINVESTAV), del Instituto Politécnico Nacional (I.P.N.), San Pedro Zacatenco Gustavo A. Madero, Ciudad de México C.P. 07360, Mexico

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long regimens. In patients with CCC, BNZ often fails to significantly reduce the parasite burden in heart, or ameliorate host tissue damage (Viotti et al. 2009; Jackson et al. 2010; Urbina 2010). Therefore, several studies have been consolidated on evaluating alternatives for the treatment and prevention of *T. cruzi*-induced CCC. This includes new immunotherapies or therapeutic vaccines in patients living with CD, and examining the specific impact of these novel biologics on both innate and adaptive immune responses. In the last years, there has been an increased interest in vaccine candidate development, exploring a variety of antigens, adjuvants, and delivery systems (plasmids, adenoviruses, peptides, and recombinant proteins) for the immunotherapy of CD (Martinez-Campos et al. 2015; Pereira et al. 2015; Teh-Poot et al. 2015; Barry et al. 2016; Jones et al. 2018; de la Cruz et al.2019; González-López et al. 2022; Prochetto et al. 2022). The urgency is to find candidate antigens that could delay the progression of CCC in patients who fail antiparasitic chemotherapy. The mutated recombinant proteins: trypomastigote surface antigen known as TSA-1-C4 and mutated flagellar calcium-binding protein, Tc24-C4, administered individually in conjunction with Toll like receptors-4 (TLR-4) agonist adjuvants have demonstrated immunogenicity in murine models (Gunter et al. 2017; Seid et al. 2017; Jones et al. 2018; Cruz-Chan et al. 2021; Jones et al. 2023). In recent years, strategies based on the use of two or more antigens as part of formulation of therapeutic vaccines have been proposed in pathologies such as yellow fever, leishmaniasis, malaria, and cancer eliciting multiple antigen-specific immune responses driving host-protection (Maciel et al. 2015; Si et al. 2017; Salehi-Sangani et al. 2019; Sklar et al. 2021). All these promising results have allowed proposing the recombinant proteins TSA-1-C4 and Tc24-C4 in combination as a bivalent recombinant protein strategy. However, little has been investigated about the immunomodulatory activity of both recombinant proteins to induce innate as well as adaptive immune response.

The principal control for *T. cruzi*-infection is dependent on MHC class I presentation of cytoplasmic antigens, which activate $CD8^+$ cytotoxic T cells and release cytotoxic granules (perforin and granzymes) (Padilla et al. 2009; Parodi et al. 2009). Besides, pre-clinical studies in murine models, have also found that a T helper (Th) 1 type immune response with evidence for IFN-γ and TNF is required to reduce parasite dissemination from acute *T. cruzi*-infection (Martin and Tarleton 2004; Dumonteil et al. 2012; Tarleton 2015). However, adaptive immune response are characterized by being delayed; therefore, innate immune responses are critical during *T. cruzi* early infection (Sathler-Avelar et al. 2009; Rodrigues et al. 2012; Arango-Duque and Descoteaux 2014). Phagocytic cells and complement system during innate immunity are the first lines of defense against pathogens and drives adaptive immune responses. Since studies suggest that *T. cruzi*-infection may cause morbidity when innate effector functions are lacking or down-regulated, there is a necessity to evaluate the ability of TSA-1-C4 and Tc24-C4 to triggered immune innate and adaptative responses, which means to evaluate the immunomodulatory activity of the recombinant proteins.

Macrophages are recognized as one of the major cellular components in the inflammatory responses during early stages of pathogen infections, as they rapidly recognize, engulf, and destroy pathogens or apoptotic cells (Arango-Duque and Descoteaux 2014; Oishi and Manabe 2016). Depending on the type of activation manifested by cytokine production and expression of surface markers, macrophages are classified as M1 or M2 subgroups. The M1 macrophages, classically activated (e.g. by lipopolysaccharides (LPS) or IFN-γ) operate by pro-inflammatory signaling pathways such as TNF-α, IL-1β, and IL-6, as well as, reactive oxygen species (ROS), and reactive nitrogen species (RNS), which result in parasite elimination or growth inhibition (Kumar and Tarleton 2001; Boscá et al. 2005; Cuervo et al. 2008). Therefore, nitric oxide (NO) and hydrogen peroxide (H2O2) molecules are regarded as potential indicators of functionality and activation of M1 macrophages (MacMicking et al. 1997; Nathan and Cunningham 2013; Canton et al. 2021). In contrast, anti-inflammatory M2 macrophages are induced by IL-4 or IL-10, and characterized by regulatory activity (Stout and Suttles 2004). Besides, macrophages usually are also recognized by their ability to perform several adaptive functions including "antigen presenting cell" (APC) roles. Therefore, examining macrophage responses after exposition to recombinant TSA-1-C4 and Tc24-C4 antigens allows for further exploration of both innate and adaptive immunity as well as its effect as a bivalent recombinant protein strategy. Here, we examine the immunomodulatory role of the two vaccine antigens TSA-1-C4 and Tc24-C4 in terms of activate macrophages and provide new information about the activation of the principal cytotoxic T cell subpopulation, $CD8^+$ cells, by in vitro assays. Among our results, we find that there are benefits and synergies in combining TSA-1-C4 and Tc24-C4 relative to either antigen alone, these data support immunological advantages for the use of recombinant protein combination as vaccine candidates against *T. cruzi*.

Materials and methods

Mice

For this study, a total of five Balb/c mice (female and male) were obtained at 4–6 weeks old from Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatán (UADY). All animals received

ad libitum food and water and were maintaining with 12-hrs light/dark cycle.

Recombinant antigens

The recombinant TSA-1-C4 and Tc24-C4 antigens were obtained from the Departamento de Biotecnología y Bioingeniería from the Centro de Investigación y Estudios Avanzados (CINVESTAV) of the Instituto Politécnico Nacional (IPN), México. Each TSA-1-C4 or Tc24-C4 coding sequence was cloned into a pET41a+ E. coli expression vector (Seid et al. 2017; de la Cruz et al. 2019). The resulting plasmid DNA was transformed into BL21 (DE3) cells induced with isopropyl-beta-D-1-thiogalactoside (IPTG) for protein expression. Recombinant proteins were purified by ion exchange (IEX) and size exclusion chromatography (SEC) and diluted in PBS 1X pH 7.4 (Seid et al. 2017). The integrity and size of each recombinant protein was analysed by SDS-PAGE electrophoresis (Supplementary Fig. 1) and concentration was determined by Bradford assay.

Isolation, culture and stimulation of murine macrophages

A total of five naïve mice were euthanized intramuscular via using xylazine/ketamine (10mg/kg, 100mg/kg)-induced deep anesthesia followed by cervical dislocation. Under sterile conditions, the abdomen was disinfected with ethanol 70% and the peritoneal cavity was exposed, resident macrophages were harvested by injection of cold phosphate-buffered saline (PBS) in the peritoneal cavity. Consecutively, macrophages were pelleted by centrifugation for 10 minutes at 600 x g and 4°C and were resuspended in Dulbecco´s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco) (Villa de la Torre et al. 2016; Arana-Argáez et al. 2020). Cell viability was assessed by trypan blue exclusion and cell quantification was determined by Neubauer chamber. A total of $1x10^5$ cells were cultured in a 24-well plate under 5% CO_2 and 37 °C for 48 hrs, afterwards, non-adherent cells were removed by DMEM washing. In order to stimulate cells, we added recombinant TSA-1-C4 and Tc24-C4 antigens mixed in equal proportion to reach final concentrations per well to 100, 50, 25, 12.5, and 6 µg/mL diluted in DMEM. Stimulated cells remained on incubation under 5% CO_2 and 37 °C for 48 hrs , later, supernatants were collected and stored at −80ºC until further analysis. The maximum concentration tested here was based on the previous reports that used recombinant proteins for immunization assays (Jones et al. 2018). Individual stimulation with recombinant TSA-1-C4 or Tc24-C4 antigens were also included.

Measurement of NO release

Supernatants of stimulated peritoneal murine macrophages cultures were collected and nitrite releasing, as an indicator of NO synthesis, was analysed by Griess reagent (Zamani et al. 2014; Arana-Argáez et al. 2021). Briefly, 50 µL of supernatants from each culture were placed into a 96-well plate and mixed with an equal volume of Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 5% H_3PO_4), followed by incubation at room temperature and protected from light for 15 minutes. Consecutively, the absorbance was measured at 490 nm using a microplate absorbance reader (Thermo Scientific, Multiskan FC). Nitrite concentration was determined based on a sodium nitrite ($NaNO_2$) standard curve ranging from 0 to 50 µM. Non-stimulated cells were included as control to display basal levels of $NaNO_2$ produced by macrophages. All experimental variants were performed in triplicate.

Measurement of H2O2 release

The supernatants of stimulated peritoneal murine macrophages cultures were collected and analyzed for H_2O_2 concentrations by using a colorimetric assay (HANNApro) adapted to the laboratory conditions (Pick and Mizel 1981; Arana-Argáez et al. 2021). Briefly, 50 µL supernatants from each culture were placed into a 96-well plate and were diluted in 10 µL of sulfuric acid (H_2SO_4) 15% and 10 µL of potassium iodide (KI) 0.03M, followed by incubation at room temperature and protected from light for 20 minutes. Afterwards, the absorbance was measured at 490 nm using a microplate absorbance reader (Thermo Scientific, Multiskan FC). Hydrogen peroxide concentration was determined based on a H_2O_2 standard curve ranging from 0 to 50 µM. Non-stimulated cells were included as control in order to show basal levels of H_2O_2 produced by macrophages. All experimental variants were performed in triplicate.

$CD8^+$ T cell purification and co-culture with stimulated macrophages

$CD8^+$ T cells were purified by magnetic negative selection using a $CD4^+$ T cells isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany) from spleen cells according to the manufacturer's instructions. Briefly, spleens were removed from euthanized mice, and mechanically perfused by injection of cold PBS. Splenocytes were rinsed with supplemented DMEM pH 7.4, and pelleted by centrifugation for 10 minutes at 160 x g, 4ºC. Cells were resuspended in lysis solution (Tris base 0.17 M pH 7.2 and NH_4Cl 0.16 M) for 15 minutes at 37º C to remove erythrocytes (de la Cruz et al. 2019). Consecutively, cells were completed with PBS and pelleted by centrifugation at the same conditions

mentioned. Supernatant was decanted, and the splenocyte pellet was resuspended in supplemented DMEM, cell viability was assessed by trypan blue exclusion and cell numbers were determined by Neubauer chamber. Supernatants from a culture of peritoneal macrophages stimulated with 100, 50, 25, 12.5 or 6.25 µg/mL of recombinant TSA-1-C4 plus Tc24-C4 combination were removed through DMEM washing after 48 hrs, hence, $2x10^5$ of $CD8^+$ T cells were added at 1:2 proportion (lymphocyte:macrophages). Co-culture was performed using a 24-well plate and incubated under 5% CO_2 and 37 °C for 48 hrs, afterwards, supernatants were collected to measure cytokine production.

Determination of cytokines

Concentration of IFN-γ, TNF-α, IL-1β, IL-4, IL-6 and IL-10 cytokines from the supernatants was determined by commercial Enzyme-linked immunosorbent assay kits (ELISA) (Peprotech, Inc.) according to the manufacturer´s instructions. Briefly, plates were coated with either IFN-γ, TNF-α, IL-1β, IL-4, IL-6, or IL-10 antibodies. Later, plates were blocked with PBS 1x + BSA 1% for 2 hrs at 37 ºC. Supernatant samples were added by triplicate. After 1 h, avidin-HRP conjugated antibody diluted 1:2000 in PBS 1x was added per well. Plates were incubated for 30 min under dark conditions and subsequently ABTS solution with hydrogen peroxide was added. Absorbance was measured at 490 nm using a microplate absorbance reader (Thermo Scientific, Multiskan FC). Cytokine concentration in supernatants was calculated by standard curves (0–2000 pg/mL). Macrophages stimulated with LPS (1 µg/mL) for TNF-α, IFN-γ, IL-1β and IL-6 detection or methotrexate (MTX, 10 µg/mL) for IL-4 or IL-10 detection were used as positive controls. Non-stimulated macrophages (only supplemented DMEM) were used as basal control. All experimental variants were performed in triplicate.

Statistical analysis

All tests were analyzed in GraphPad Prism software version 9.0.c. Normality was verified using Shapiro-Wilk test. Data were analyzed by one-way ANOVA or Kruskal–Wallis tests for multiple groups, depending on its distribution followed by Tukey or Dunn's post hoc test. Differences between groups were considered statistically significant when P-value was less than 0.05.

Results

Stimulation with recombinant proteins TSA-1-C4 and Tc24-C4 activates peritoneal murine macrophages

In this study, we evaluated the immunomodulatory effect of the recombinant TSA-1-C4 plus Tc24-C4 antigen combination at different concentrations through activation of peritoneal macrophages and $CD8^+$ T cells from naïve mice by in vitro assays.

According to our data, peritoneal murine macrophages stimulated with 100, 50, 25, and 12.5 µg/mL of TSA-1-C4 plus Tc24-C4 combination or either antigen alone, exhibited significant NO and H_2O_2 levels compared with

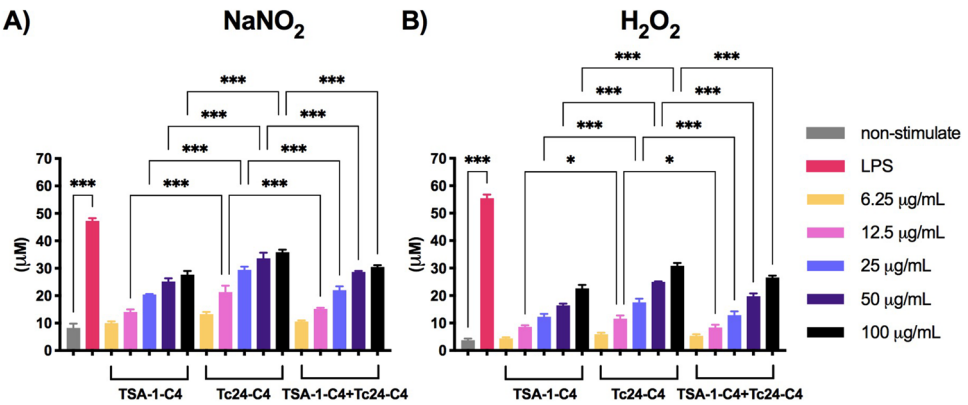

Fig. 1 Measurement of NO and H_2O_2released from supernatants of peritoneal macrophages after TSA-1-C4 plus Tc24-C4 stimulation. Peritoneal cells isolated from five naïve mice were cultured in supplemented DMEM for 48 hrs, non-adherent cells were removed through DMEM washing. Cells were stimulated with TSA-1-C4 and Tc24-C4 antigens mixed in equal proportion to reach final concentrations per well to 100, 50, 25, 12.5, and 6 µg/mL for 48 hrs. Supernatants were recovered to measure $NaNO_2$ (as indicator of NO concentration) (**A**) and H_2O_2(**B**) release. Data are represented by the mean ± S.D. Significance was calculated by one-way ANOVA and Tukey's multiple-comparison test. One, two, and three symbol characters were used to annotate the *P* values of <0.05, <0.01, and <0.001, respectively. Range of detection: 5–1400 and 5–1000 µM for $NaNO_2$ and H_2O_2 respectively.

non-stimulated macrophages (Supplementary Table 1). A non-significant increment was observed in peritoneal macrophages stimulated with 6.25 µg/mL of TSA-1-C4, Tc24-C4 or TSA-1-C4+Tc24-C4 compared with non-stimulated macrophages (Fig. 1,A-B). As we expected, LPS stimulation induced the highest levels of NO and H_2O_2 molecules for both assays, moreover; we observed a dose-dependent effect, while higher is the concentration of antigen-stimulation, higher are the levels of NO or H_2O_2 molecules in supernatants from peritoneal macrophages (Fig. 1,A-B). We also observed that releasing of NO and H_2O_2 molecules from Tc24-C4 stimulated macrophages (100, 50, 25, and 12.5 µg/mL) was significantly higher compared to TSA-1-C4 plus Tc24-C4 or individual TSA-1-C4 stimulated cells (Fig. 1,A-B).

Peritoneal murine macrophages stimulated with recombinant TSA-1-C4 plus Tc24-C4 display an inflammatory immune profile

We were also interested in evaluating the inflammatory immune profile of peritoneal murine macrophages activated by TSA-1-C4 plus Tc24-C4 recombinant antigen combination, hence, we measured levels of the principal

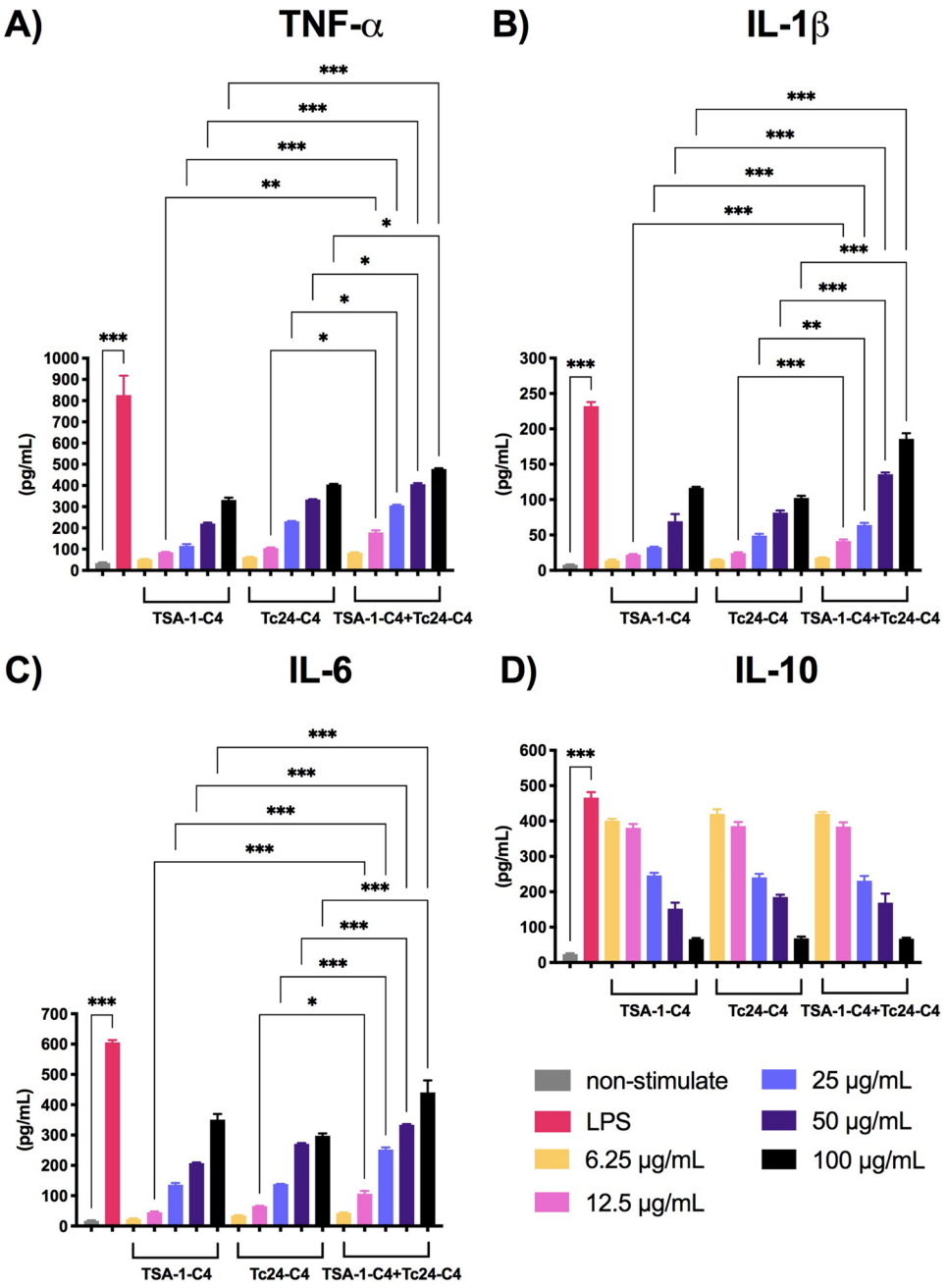

Fig. 2 Effect of recombinant TSA-1-C4 plus Tc24-C4 combination on cytokine production by peritoneal macrophages. Peritoneal cells isolated from five naïve mice were cultured in supplemented DMEM for 48 hrs, adherent cells were stimulated with TSA-1-C4 and Tc24-C4 antigens mixed in equal proportion to reach final concentrations per well to 100, 50, 25, 12.5, and 6 µg/mL for 48 hrs. Supernatants were recovered to measure TNF-α (**A**)**,** IL-1β (**B**)**,** IL-6 (**C**) and IL-10 (**D**) production. Data are represented by the mean ± S.D. Significance was calculated by one-way ANOVA and Tukey's multiple-comparison test. One, two, and three symbol characters were used to annotate the *P* values of <0.05, <0.01, and <0.001, respectively. Range of detection: 23–1500, 32–4000, 32–2000 and 32–2000 pg/ml for TNF-a, IL-1β, IL-6, and IL-10 respectively.

pro-inflammatory signaling pathways (TNF-α, IL-1β, IL-6) and the regulatory cytokine IL-10 by ELISA kit-test as we described previously.

When we measured TNF-α, IL-1β and IL-6 production by macrophages after the stimulation with TSA-1-C4 plus Tc24-C4 combination, we observed significant cytokine-production for all concentrations evaluated, except for 6.25 µg/mL compared to non-stimulated macrophages (Supplementary Table 2). Moreover, peritoneal murine macrophages stimulated with LPS induced the highest production of TNF-α, IL-1β, and IL-6 cytokines as we expected (Fig. 2,A-C). According to our assay, we observed a dose-dependent-effect, while higher is the stimulation, higher is the production of each cytokine, besides, TSA-1-C4 plus Tc24-C4 stimulated macrophages induced a significant increase in the pro-inflammatory cytokine-production compared to individuals TSA-1-C4 or Tc24-C4 stimulated cells for all concentrations evaluated, except for 6.25 µg/mL (Fig. 2,A-C).

To have a perspective of the regulatory immune response, we measured levels of IL-10 cytokine-production by TSA-1-C4 plus Tc24-C4 stimulated macrophages. According to Supplementary Table 2, we observed that, peritoneal macrophages stimulated with either 100, 50, 25, 12.5 and 6.25 µg/mL of TSA-1-C4 plus Tc24-C4 recombinant antigen combination showed significant IL-10 production compared to non-stimulated macrophages. As we expected MTX stimulation induced the highest levels of the cytokine (Fig. 2,D). By the other hand, we observed an inverse dose-dependent-effect, while higher is the concentration, lower is the IL-10 production (Fig. 2,D). No significant differences were found comparing TSA-1-C4 plus Tc24-C4 stimulated macrophages with either TSA-1-C4 or Tc24-C4 stimulated cells for all concentrations evaluated.

Peritoneal murine macrophages stimulated with recombinant TSA-1-C4 plus Tc24-C4 activates CD8$^+$ T cells

With the purpose to evaluate whether the combination of TSA-1-C4 plus Tc24-C4 stimulated macrophages activates CD8$^+$ T cells, peritoneal murine macrophages stimulated with 100, 50, 25, 12.5 and 6.25 µg/mL of TSA-1-C4 plus Tc24-C4 recombinant antigen combination were co-cultured with CD8$^+$ T cells from naïve mice. IFN-γ, TNF-α, IL-4 and IL-10 were measured from supernatants by ELISA kit-test as we mentioned previously.

As we describe in Supplementary Table 3, supernatants from co-cultures of CD8$^+$ T cells and peritoneal macrophages stimulated with either 100, 50, 25 and 12.5 µg/mL of TSA-1-C4 plus Tc24-C4 recombinant antigen combination exhibited significant IFN-γ, TNF-α, IL-4 and IL-10 levels compared to CD8$^+$ T cells co-cultured with

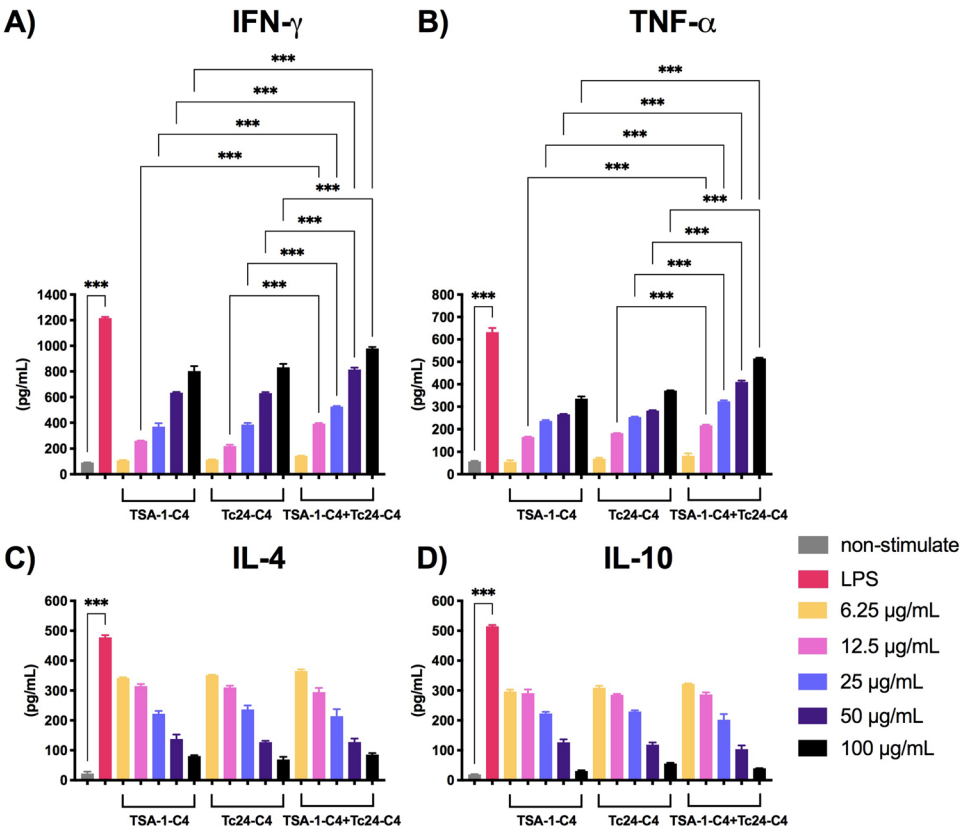

Fig. 3 Effect of recombinant TSA-1-C4 plus Tc24-C4 combination on cytokineproduction by CD8$^+$ T cells. Peritoneal cells isolated from naïve mice were stimulated with TSA-1-C4 and Tc24-C4 antigens mixed in equal proportion to reach final concentrations per well to 100, 50, 25, 12.5, and 6 µg/mL; after 48 hrs, supernatant was removed and cells were co-cultivated with CD8$^+$ T cells from naïve mice. Supernatants were recovered to measure IFN-γ (**A**), TNF-α (**B**), IL-4 (**C**) and IL-10 (**D**) production. Data are represented by the mean ± S.D. Significance was calculated by one-way ANOVA and Tukey's multiple-comparison test. One, two, and three symbol characters were used to annotate the *P* values of <0.05, <0.01, and <0.001, respectively. Range of detection: 63–4000, 23–1500, 8–1000 and 32–2000 pg/ml for IFN-γ, TNF-α, IL-4, and IL-10 respectively.

non-stimulated macrophages. As we expected, LPS and MTX controls induced the highest production of IFN-γ and TNF-α, or, IL-4 and IL-10 cytokines (Fig. 3,A-D). Furthermore, we observed a proportional dose-dependent effect when we evaluated pro-inflammatory cytokines (IFN-γ and TNF-α), while higher is the antigen-stimulation, higher is the cytokine production, meanwhile, we observed an inverse dose-dependent effect to evaluate the anti-inflammatory profile by IL-4 and IL-10 measurement, while higher is the antigen-stimulation, lower is the cytokine-production (Fig. 3,A-D). Our data revealed a high production of IFN-γ and TNF-α by $CD8^+$ T cells co-cultured with TSA-1-C4 plus Tc24-C4 stimulated cells compared to individuals TSA-1-C4 or Tc24-C4 stimulated cells for all concentration evaluated except for 6.25 µg/mL. For last, we did not observe statistical differences when comparing IL-4 or IL-10 production in CD8+ T cells co-cultured with TSA-1-C4 plus Tc24-C4 stimulated cells compared to individual TSA-1-C4 or Tc24-C4 stimulated cells for all concentration evaluated (Fig. 3,C 3,D).

Discussion

The recombinant proteins TSA-1-C4 and Tc24-C4 have been proposed as potential antigens for the development of vaccines candidates, with previous studies demonstrating their therapeutic effect. However additional work is needed to understand their immunologic mechanisms and evaluate their immunomodulatory effects through specific assays.

In this study, we found that peritoneal murine macrophages stimulated with either TSA-1-C4, Tc24-C4 or the combination of recombinant antigens induces releasing of host NO, H_2O_2 molecules, as well as, TNF-α, IL-1β, IL-6 and IL-10 cytokines, which are indicator of activation and functionality. This is the first report showing releasing levels of NO and H_2O_2 molecules using in vitro cultures of macrophages expose to recombinant TSA-1-C4 and Tc24-C4 proteins. Particularly, we observed that Tc24-C4 alone is a good inducer of NO and H_2O_2 molecules, even compared with the combination of TSA-1-C4 plus Tc24-C4 proteins, which induction was slightly lower. We assume that TSA-1-C4 antigen could have a downregulating effect on cytotoxic molecules releasing by macrophages. Previous studies suggest that, ROS and NO molecules are essential for parasite proliferation and growth, due to provide ideal conditions (e.g., iron availability in macrophages) for parasite-replication inducing a toxic effect in the host (Goes et al. 2016; Paiva et al. 2018).

We also evaluated the inflammatory cytokine profile from supernatants of TSA-1-C4 plus Tc24-C4 stimulated macrophages. Here, we observed that when we increase the concentration of either, TSA-1-C4, Tc24-C4 or their combination, the production of TNF-α, IL-1β and IL-6 increase, while the regulatory cytokine IL-10 tends to reduce, therefore, we suggest that macrophages stimulated with the recombinant antigens can induce an inflammatory profile which is it a dose-dependent effect. Moreover, we demonstrated that the combination TSA-1-C4 plus Tc24-C4 increase significatively the production of TNF-α, IL-1β and IL-6 compared to individual TSA-1-C4 or Tc24-C4 stimulated-macrophages from naïve mice evidencing the capacity to induce a strong inflammatory response. It has been reported during early stages of *T. cruzi* infection, macrophages release cytokines, such as TNF-α, IL-1β, and IL-6, which induce the expression of adhesion molecules, causing chemotaxis and migration of T cells, hence are critical for control of parasite-replication and protective immunity (Kumar and Tarleton 2001; Barton 2008; Arango-Duque and Descoteaux 2014). Other studies have reported similar data to our findings. After stimulation with Tc24-C4 recombinant protein, production of cytokines as TNF-α or IL-6 was reported in supernatants of spleen cells from *T. cruzi*-infected Balb/c mice immunized with a vaccine formulated with the recombinant Tc24-C4 antigen in addition with TLR-4 agonist adjuvants (Jones et al. 2018; Cruz-Chan et al. 2021; Jones et al. 2023; Poveda et al. 2023). On the other hand, limited information is currently published related to TSA-1-C4, however, pro-inflammatory profile was observed in supernatants of spleen mononuclear cells from *T. cruzi*-infected and TSA-1-C4+E6020-SE vaccinated mice after TSA-1-C4 stimulation (data in publication process).

Protective immunity against intracellular parasites as *T. cruzi* is mediated by $CD8^+$ T cells, which release cytotoxic molecules such as, perforin and granzymes from cytotoxic granules (Kumar and Tarleton 1998; de Alencar et al. 2009). Moreover, $CD8^+$ T cells modulate the immune response through secretion of cytokines, which are required for activation of APCs, macrophages and T cells or downregulate the extensive inflammatory response by production of regulatory cytokines (Martin and Tarleton 2004; Padilla et al. 2009). In this study, we measured the major cytokines representing for pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory (IL-10 and IL-4) immune responses in supernatants from co-cultures of TSA-1-C4 and Tc24-C4 stimulated macrophages and $CD8^+$ T cells from naïve mice. All stimulus conditions (regardless of if the recombinant proteins were used in combination or not) induced pro-inflammatory and anti-inflammatory cytokines responses, through a dose-dependent effect. As we hypothesized, we also observed that there is a benefit to use the bivalent recombinant protein combination, our data reveals that the macrophages stimulated with recombinant TSA-1-C4 and Tc24-C4 combination can activate $CD8^+$ T cells which are characterized by inducing a

significant Th1-type response mediated by a strong production of IFN-γ and TNF-α compared with individual TSA-1-C4 or Tc24-C4 antigen stimulation. It is not known what are the receptor for these antigens, and they are not contaminated with LPS. Most of the Pattern Recognition Receptors (PRRs) are not triggered by proteins, but by lipids, carbohydrates and nucleic acids. Due to the limitations of the study, we could not clarify how the recombinant proteins interact with these PRRs on macrophages. In accordance with our data, some studies have reported immunological profiles mediated by Tc24 antigen-specific $CD8^+$ T cells in in vitro spleen cells culture from T. cruzi-infected Balb/c mice and immunized with Tc24-C4 recombinant vaccines (Jones et al. 2018; Cruz-Chan et al. 2021), while in a recent study it was demonstrated that re-stimulation with recombinant Tc24-C4 protein in spleen cells from Tc24-C4 + GLA-SE vaccinated mice induces an antigen-specific $CD8^+IFN\text{-}\gamma^+$ immune profile (Poveda et al., 2023). Similarly, TSA-1 antigen-specific $CD8^+IFN\text{-}\gamma^+$ T cells was observed in spleen cells cultures from Balb/c mice immunized with TSA-1 in conjunction with Monophosphoryl-Lipid A (MPLA) adjuvant (de la Cruz et al. 2019).

This study supports the immunomodulatory effect of the bivalent recombinant protein strategy; and it is accordance with a previous study which evaluated the therapeutic efficacy of a vaccine-linked chemotherapy formulated by the recombinants TSA-1-C4 and Tc24-C4 proteins given during *T. cruzi*-chronic infection using a pre-clinical model (Dzul-Huchim et al. 2022). Moreover, the bivalent vaccine, comprised with the two recombinant T. cruzi antigens, TSA-1-C4 and Tc24-C4, has confirmed its immunogenicity in non-human primate trials (Dumonteil et al. 2020). Other studies have also evidenced the beneficial effect of recombinant vaccines formulates with TSA-1-C4 or Tc24-C4 reporting reduction in parasitemia, cardiac inflammation, parasite load in heart as well as, increase in survival using murine models, supporting the data showed in this study (Seid et al. 2017; Jones et al. 2018; Cruz-Chan et al. 2021; Dzul-Huchim et al. 2022; Jones et al. 2023). Furthermore, a recent study it was showed that the combination of BZN with a vaccine formulated with Tc24-C4 improves cardiac structure and function in a murine model of chronic Chagas disease delaying the development of CCC (Jones et al. 2023).

Some limitations were observed in this study. Despite we recovered peritoneal macrophages from cavity peritoneal of mice, is possible that there were also other cells lines in our environment, such as dendritic cells. Regarding the co-culture of macrophages and $CD8^+$ T cells, we are aware that there is a bias in the origin of cytokine production, since, although we removed the cytokines in supernatant from the macrophage culture prior to the addition of $CD8^+$ T cells, there may be a percentage of cytokines produced by the macrophages and not by the $CD8^+$ T cells. Measurement of cytokines by other techniques highly sensitives as cytometric-bead-array (CBA) assay also could support our findings. Furthermore, it has been demonstrated that IL-17 family of cytokines plays a critical role in host survival by regulating exuberant inflammation during *T. cruzi* infection, suggesting that IL-17 cytokine have protective roles during adaptive immunity (Tosello-Boari et al. 2012, 2018; Cruz-Chan et al. 2021). For these reasons, we believe that further studies, would be relevant to evaluate the Th17 profile induced by the recombinant TSA-1-C4 plus Tc24-C4 antigen combination. As perspective in vitro assays including macrophages previously stimulated with the recombinant proteins and *T. cruzi* parasites will be necessary in order to know the cytotoxicity activity. With the data showed in the present study in addition to the results in previous studies using murine models infected with *T. cruzi* and treated with TSA-1-C4 and Tc24-C4 vaccine candidates (Seid et al. 2017; Jones et al. 2018; Cruz-Chan et al. 2021; Dzul-Huchim et al. 2022 Jones et al. 2023), we assume that macrophages will perform both their phagocytic and APC functions supporting the cellular immune response to combat the infection.

Conclusion

In sum, we demonstrate by in vitro assays the beneficial effect of the TSA-1-C4 plus Tc24-C4 recombinant protein combination, which drive a strong immunomodulatory activity evidenced by the activation of macrophages and CD8+ T cells associated with an inflammatory immune profile.

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Data availability No datasets were generated or analyzsed during the current study.

Declarations

Ethics approval All studies were approved by the institutional bioethics committee of the "Centro de Investigaciones Regionales Dr. Hideyo Noguchi,", Universidad Autónoma de Yucatán (Reference #CEI-08-2019) and were maintained in accordance with the principles and guidelines of National Institutes of Health Guide for Treatment and Care for Laboratory Animals and by the Mexican Official Norm for Animal Care and Handing,NOM-062-ZOO-1999.

Consent to participate Not applicable.

Conflict of interests The authors declare no competing interests.

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