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ORIGINAL RESEARCH ARTICLE

Vesicles from different *Trypanosoma cruzi* strains trigger differential innate and chronic immune responses

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Trypomastigote forms of *Trypanosoma cruzi*, the causative agent of Chagas Disease, shed extracellular vesicles (EVs) enriched with glycoproteins of the gp85/*trans*-sialidase (TS) superfamily and other α-galactosyl (α-Gal)-containing glycoconjugates, such as mucins. Here, purified vesicles from *T. cruzi* strains (Y, Colombiana, CL-14 and YuYu) were quantified according to size, intensity and concentration. Qualitative analysis revealed differences in their protein and α-galactosyl contents. Later, those polymorphisms were evaluated in the modulation of immune responses (innate and in the chronic phase) in C57BL/6 mice. EVs isolated from YuYu and CL-14 strains induced in macrophages higher levels of proinflammatory cytokines (TNF-α and IL-6) and nitric oxide via TLR2. In general, no differences were observed in MAPKs activation (p38, JNK and ERK 1/2) after EVs stimulation. In splenic cells derived from chronically infected mice, a different modulation pattern was observed, where Colombiana (followed by Y strain) EVs were more proinflammatory. This modulation was independent of the *T. cruzi* strain used in the mice infection. To test the functional importance of this modulation, the expression of intracellular cytokines after in vitro exposure was evaluated using EVs from YuYu and Colombiana strains. Both EVs induced cytokine production with the appearance of IL-10 in the chronically infected mice. A high frequency of IL-10 in CD4+ and CD8+ T lymphocytes was observed. A mixed profile of cytokine induction was observed in B cells with the production of TNF-α and IL-10. Finally, dendritic cells produced TNF-α after stimulation with EVs. Polymorphisms in the vesicles surface may be determinant in the immunopathologic events not only in the early steps of infection but also in the chronic phase.

Keywords: *Trypanosoma cruzi*; extracellular vesicles; innate and chronic immunity; TLR2; α-galactosyl

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Trypanosoma cruzi is the causative agent of Chagas disease, one of the most important neglected infectious diseases in Latin America. Recent studies estimate that approximately 11 million people are infected, and about 100 million are at risk (1). This protozoan parasite is transmitted by insect vectors, orally, blood transfusion, organ transplantation and congenitally. During the life cycle, the parasites must face extremely adverse conditions both in the vertebrate and invertebrate hosts (2).

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In this context, *T. cruzi* employs a highly elaborated array of molecules and strategies to invade a wide range of host cells (3–6) and to escape from host immune responses (7–11). Invasion and immune resistance are vital processes required for survival, proliferation and establishment of *T. cruzi* infection. A number of parasite surface molecules have been implicated in host cell invasion and/or immunomodulation (12–16). Those include gp85/TS and mucin superfamilies of developmentally regulated glycosylphosphatidylinositol (GPI)-linked glycoproteins (14,17).

In addition to surface proteins, secreted factors also play an important role in parasite virulence. Some of those studies indicate that surface antigens can be released in soluble and/or membrane-bound forms (18–20). Secretion of virulence factors via extracellular vesicles (EVs) is well described in pathogens including protozoa, helminths, bacteria, fungi and virus (21–23). For example, Gram-negative bacteria secrete outer-membrane vesicles (OMVs) that are important for vaccination and delivery of a variety of virulence factors (23).

Recent secretome analyses of the trypanosomatids *Trypanosoma brucei* and *Leishmania donovani* have shown that a large proportion, if not most, of the secreted proteins are released as membrane-bound vesicles (24–27). Consistent with those observations, trypomastigote forms of *T. cruzi* also secrete proteins in EVs that are enriched with gp85/TS and mucins, crucial molecules for the host–parasite interaction (18). A distinguished feature of those EVs from Y strain is their ability to exacerbate parasite load and modulation of inflammation of the heart (19).

Studies comparing glycoconjugates from different *T. cruzi* strains and discrete typing units (DTUs) are not common (16,28). Purified GPI mucins isolated from Colombiana, Y and CL (DTUs I, II and VI, respectively) had the ability to differentially activate nitric oxide (NO) and cytokine production via TLR2 and modulate parasite invasion (8,16). However, those aspects remain unknown in EVs from different *T. cruzi* strains. In this work, we evaluated their role in the innate immune compartment and in the chronic phase.

Materials and methods

All experiments described in this session are summarized in the workflow (Fig. 1).

Ethics statement

All animals were handled in strict accordance with animal practice as defined by the Internal Ethics Committee in Animal Experimentation (CEUA) of Federal University of São Paulo (UNIFESP), Diadema, São Paulo, Brazil (protocol no. 3598). Knock-out mice handling protocol was approved by the National Commission on Biosafety (CTNBio) (protocol no. 01200.006193/2001-16).

Cell lines and culture

Tissue culture-derived trypomastigote forms from Colombiana (DTU I), YuYu (DTU I), Y (DTU II) and CL-14 (DTU VI) strains of *T. cruzi* were obtained after infection of green monkey (*Rhesus*) kidney LLC-MK_2 epithelial cells (ATCC, Manassas, VA) (29). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS; Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2. Murine macrophages (J774.1) were obtained from ATCC. Murine C57BL/6 macrophages were obtained after peritoneal washing. Both macrophage types were cultured in Roswell Park Memorial Institute (RPMI) supplemented with 10% FCS (37°C, 5% CO_2). Parasites and mammalian cells were regularly tested for the absence of *Mycoplasma* contamination (30).

Isolation, microscopy and characterization of EVs

Trypomastigotes from different *T. cruzi* strains (Y, Colombiana, CL-14 and YuYu) were obtained from the supernatant of LLC-MK_2 cells. Recovered parasites were washed 5 times in PBS (1,000g, 15 min) incubated for 2 h in RPMI with 5% glucose (37°C, 5% CO_2) for EVs release. Parasites were fixed and subjected to scanning electron microscopy as described (31). Trypomastigotes were removed by centrifugation (15 min, 1,000*g*), and EV-containing supernatants were filtered through 0.45-μm sterile cartridges (Fig. 1). For gel-exclusion chromatography, the filtered supernatant (1 mL) was concentrated 4 times and diluted to 100 mM ammonium acetate, pH 6.5. Then, it was loaded onto a Sepharose CL-4B column (1 × 40 cm, GE Healthcare, Piscataway, NJ) pre-equilibrated with 100 mM ammonium acetate, pH 6.5. The column was eluted with the same buffer at a flow rate of 0.2 mL/min. Fractions of 1 mL were collected, and then screened by chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) with chagasic anti-α-Gal antibodies, as previously described (19), and by a polyclonal antibody antiparasite membrane 460 (1:1,000), a kind gift from Dr Sérgio Schenkman (18,19). To identify the differential expression of α-galactosyl-containing glycoconjugates in the EVs, the fractions were pooled, concentrated in a speed-vac and its reactivity against anti-α-Gal antibodies (1:500) was tested by ELISA. Antirabbit or antihuman IgG-peroxidase conjugate (1:2,000) was used as secondary antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The reaction was developed using TMB substrate reagent set (BD Biosciences, San Diego, CA). Protein concentrations were determined using the Micro BCA protein assay kit (Thermo Scientific, Waltham, MA). To test for LPS contamination, CHO reporter cell lines TLR2 −/− TLR4 −/− (which do not express TLR2 or TLR4) and TLR4+ (expressing TLR4) were used as described elsewhere (8).

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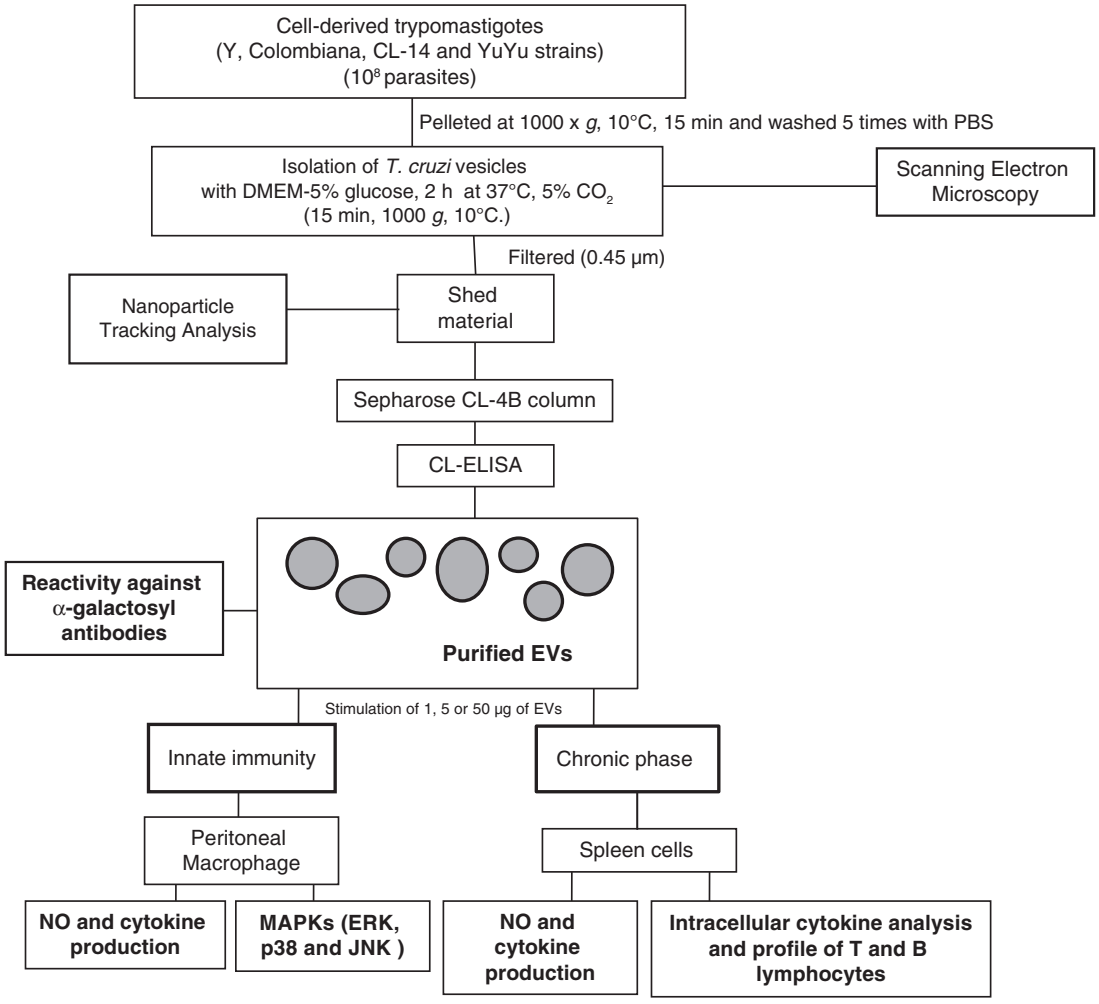

Fig. 1. Procedures employed for the production, fractionation and characterization of *T. cruzi* EVs from different strains.

Nanoparticle tracking analysis

Size, distribution and concentration of isolated vesicles were measured in a Nanosight NS300 instrument (Malvern Instruments Ltd, Malvern, UK) equipped with a 405-nm laser and coupled to a CCD camera (the laser emitting a 60-mW beam at 405-nm wavelength), and data were analysed using the nanoparticle tracking analysis (NTA) software (version 2.3 build 0017). The detection threshold was set to 10. Blur, Min track Length and Min Expected Particle Size were set to auto. To perform the measurements, samples were diluted 100 times with PBS. Readings were taken in triplicates during 30 s at 20 frames per second, at camera level set to 14 and manual monitoring of temperature (19°C).

Purification of murine peritoneal macrophages

For the innate immune experiments, thioglycollate-elicited macrophages were extracted from wild-type C57BL/6 and its respective knockouts (TLR2 −/− and TLR4 −/−) by peritoneal washing with ice-cold serum-free RPMI and enriched by plastic adherence for 1h (37°C, 5% CO_2). Cells (3×10^5 cells/well) were washed and cultured in RPMI, 2 mM glutamine, 50 U/mL of penicillin and 50 µg/mL streptomycin supplemented with 10% FBS in 96-well culture plates (37°C, 5% CO_2). Cells were primed with interferon-γ (IFN-γ) (25 IU/mL) for 18 h prior to incubation with vesicles (1, 5 and 50 µg/mL) from Colombiana, YuYu, Y or CL-14 strains. A positive control included live parasites from Y strain (MOI 10:1). Control glycoconjugates included lipophosphoglycan (LPG) from *Leishmania braziliensis* (10 µg/mL) and lipopolysaccharide from *Escherichia coli* (LPS; 100 ng/mL) (32). Negative controls included medium and medium-containing IFN-γ (25 IU/mL). Cells were incubated for 48 h and the supernatants collected for cytokine (IL-1β, IL-6, IL-10, IL-12/IL-23p40, IL-12p70 and TNF-α) and NO measurements. Results are representative of 2 experiments performed in duplicate.

Preparation of cell lysates and MAPKs

In order to evaluate the signalling events prior to EVs stimulation, J774.1 macrophages were placed on 24-well

tissue culture plates (3×10^6/well) for 18 h. The cells were washed with warm RPMI and incubated with vesicles isolated from Colombiana, YuYu, Y or CL-14 (5 μg/mL) strains. Four time points (5, 15, 30 and 45 min) were assayed. Controls with medium (negative control) or LPS (100 ng/mL, positive control only for 45 min incubation) were included. After each time point, cells were washed with ice-cold PBS and lysed in RIPA lysis buffer (Sigma) in the presence of protease inhibitor cocktail (Thermo Fisher Scientific). Cells were harvested with a plastic scraper and centrifuged at 13,000*g* (4°C, 10 min). Supernatants were transferred to new tubes and stored at −20°C. Cell lysates were resolved by SDS–PAGE, transferred to a nitrocellulose membrane and blocked (5% non-fat milk in TBS–0.1% Tween 20) for 1 h. Primary Abs (1:1,000) [anti-dually phosphorylated ERK (Sigma), anti-dually phosphorylated p38 (Santa Cruz Biotechnology, Dallas, TX) and JNK (Sigma)] were added. Total p38 primary antibody (Sigma) was used as the normalizer. All mAbs were incubated for 16 h at 4°C. Membranes were washed (3×10 min) with TBS–0.1% Tween 20 and incubated 1 h with antimouse IgG conjugated with peroxidase (1:10,000). The reaction was visualized using luminol (33).

Splenocyte cultures derived from chronic infections

For the chronic assays, the 4 strains of *T. cruzi* (Y, Colombiana, CL or YuYu) were intraperitoneally injected (50 parasites/animal, 15 per group) in C57BL/6 mice. Parasitaemia was detected daily up to 15 days to confirm infection. Only the animals whose infection was parasitologically diagnosed were selected. Those mice were maintained for 6 months (∼180 days). After this period, the animals were anaesthetized and the spleens removed (34). Spleen cells (1×10^6/well) from mice infected with each *T. cruzi* strain were incubated for 48 h with EVs (5 μg/mL) isolated from the 4 strains. Supernatants were collected for NO and cytokine assays. Total *T. cruzi* antigen (10 μg/mL) and culture medium were added as positive and negative controls, respectively. Results are representative of 2 experiments in duplicate.

Cytokines and nitrite measurements

For CBA multiplex cytokine detection, supernatants were collected and cytokines were determined using the BD CBA Mouse Cytokine assay kits according to the manufacturer's specifications (BD Biosciences). IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12/IL-23p40, IL-17, IFN-γ and TNF-α were measured. Flow cytometry measurements were performed on a FACSCalibur flow cytometer (BD Biosciences). Cell-Quest™ software package provided by the manufacturer was used for data acquisition, and the FlowJO software 7.6.4 (Tree Star, Inc., Ashland, OR) was used for data analysis. A total of 2,400 events were acquired for each preparation. Nitrite concentrations were determined by Griess reaction (Griess Reagent System, 2009).

Immunostaining for cell surface markers and intracellular cytokines

To identify the sources of the cytokines, splenocytes (1×10^6 cells) were cultured with RPMI supplemented with 10% FCS and incubated with vesicles (5 μg/mL) for 24 h (37°C, 5% CO_2). Three positive controls were performed to evaluate the sample viability. Lymphocytes were treated with phorbol 12-myristate 13-acetate (25 ng/mL) (Sigma) and ionomycin (1 μg/mL) (Sigma), whereas monocytes and DCs were treated with LPS (100 ng/mL). The viability of these controls was confirmed by the high levels of cytokine production (data not shown). Afterward, all cultures were incubated in the presence of Brefeldin (10 μg/mL) (4 h, 37°C, 5% CO_2) and treated with 100 μL of 2 mM EDTA in PBS for 15 min to block the reaction (35).

After incubation, spleen cells were washed once (800g, 5 min at 4°C) with FACS buffer prepared in PBS (0.15 M) supplemented with 0.5% of bovine serum albumin and 0.1% sodium azide. Cells were resuspended in 100 μL of FACS buffer and immunostained with antimouse monoclonal antibodies including: F4/80 (clone BM8/PE-Cy5), CD11b (clone M1/70/ PE-Cy7), CD11c (clone N418/ FITC) and CD19 (clone 1D3/PE-Cy7) from eBioscience (San Diego, CA); and MHCII (clone M5/114.15.2/ APC), CD3 (clone 145-2C11/PerCP), CD4 (clone GK1.5/Alexa fluor 700) and CD8 (clone 53–6.7/Alexa fluor 647) from BioLegend (San Diego, CA) in the dark (30 min, 4°C). The lysing/fixation occurred in the presence of 150 μL FACS brand Lysing solution (BD Biosciences) for 10 min. Cells were washed once (800 g, 10 min, 4°C) with FACS buffer. Membrane-stained cells were permeabilized by incubation for 15 min with 200 μL of FACS permeabilization buffer (FACS buffer supplemented with 0.5% saponin) and washed again (800*g*, 10 min at 4°C) with FACS buffer. Finally, cells were incubated in the dark (30 min) at room temperature, in the presence of 25 μL (1:50) of PE-labelled antimouse cytokines (IL-10, IFN-γ and TNF-α) (eBioscience). The material was fixed with 200 μL of FACS FIX Solution (10 g/L of paraformaldehyde, 10.2 g/L of sodium cacodilate and 6.63 g/L of sodium chloride, pH 7.2) (Sigma) and stored at 4°C prior to flow cytometry acquisition and analysis.

Flow cytometry acquisition and analysis

Data were collected using FACSFortessa (BD Biosciences – Immunocytometry Systems) with Diva software (BD Biosciences) and analysed by FlowJo software (Tree Star). Flow cytometry acquisitions of 100,000 and 200,000 immunostained cells/samples were performed for lymphocytes and monocytes, respectively. Distinct gating strategies were used to analyse the cytokine-expressing leucocyte subpopulations from innate and adaptive immunity, including monocytes, DCs, T and B lymphocytes. The singlets (single cells) were selected excluding the doublets (FSC-A/FSC-H), and the cells were gated by forward and side scatter to

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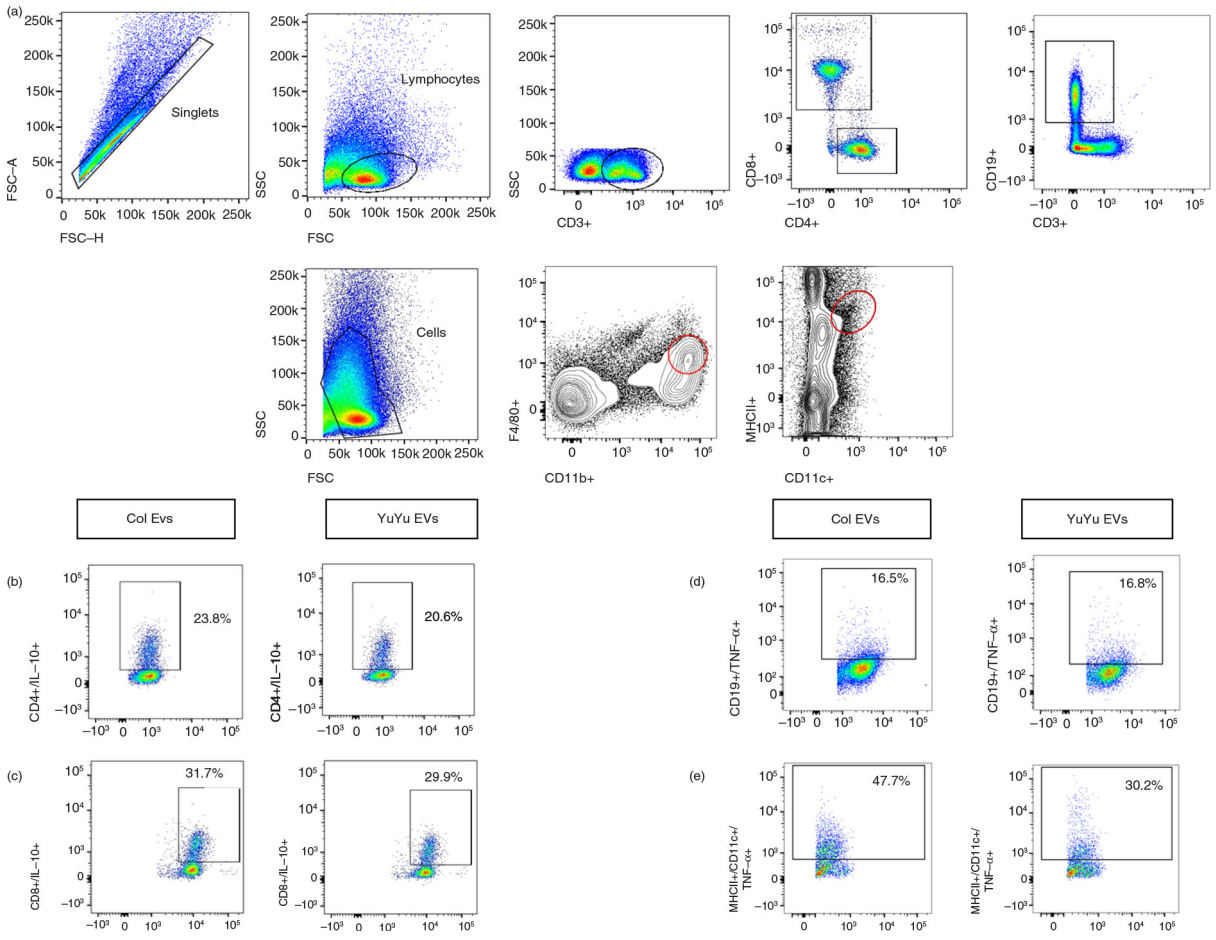

Fig. 2. Flow cytometry strategy for intracellular cytokine production by spleen cells from infected mice. (a) Dot plots of CD4, CD8, CD19, F4/80/CD11b and MHCII/CD11c expression. The representative dot plots illustrating the frequency (%) of cytokines from spleen cells are shown for Colombiana and YuYu strains. (b) CD4+IL-10+ cells. (c) CD8+IL-10+. (d) CD19+TNF-α+. (e) MHC/CD11c+TNF-α+.

separate lymphocytes from monocytes (Fig. 2a). Following the initial gate selection, the frequencies of cytokine-positive cells were quantified by statistics applied on T lymphocytes (CD3+/CD4+ or CD3+/CD8+), B lymphocytes (CD19+), DCs (CD11c+/MHC-II+) and monocytes (CD11b+/F4/80+) vs. anticytokine-PE dot plots (Fig. 2b–e).

Statistical analysis

For nitrite and cytokine measurements, the Shapiro–Wilk test was conducted to test the null hypothesis that data were sampled from a Gaussian distribution. The p-value ($p > 0.05$) showed that data did not deviate from Gaussian distribution. For this reason, Student's t-test and ANOVA were performed to test equality of population medians among groups and independent samples. The comparative analysis between control and stimulated culture was performed by Mann-Whitney pair test. Data were analysed using GraphPad Prism 5.0 software (Graph Prism, San Diego, CA) and $p < 0.05$ was considered significant.

Results

Purification and α-galactosylation in T. cruzi-derived EVs

Trypomastigotes from *T. cruzi* constantly shed EVs into the culture medium [18–20] throughout the whole body, as shown for Y strain (Fig. 3a–d). EVs were collected from the supernatant of 1×10^8 trypomastigotes from Y, Colombiana, CL-14 and YuYu strains after incubation of the parasites for 2 h at 37°C in culture medium. NTA analysis showed a heterogeneous profile for the different strains (Fig. 4a–f). The average size was very similar for all strains (Fig. 4e), whereas the particles' concentrations were lower for strains Y, Colombiana and CL-14 (Fig. 4f), in contrast with YuYu strain. Size distribution according to the D10, D50 and D90 did not vary significantly among the 4 strains analysed (Supplementary Fig. 1).

EVs released into the medium were then purified by gel-exclusion chromatography [18–19] for further experiments (Fig. 1). Polyclonal antibodies raised against total

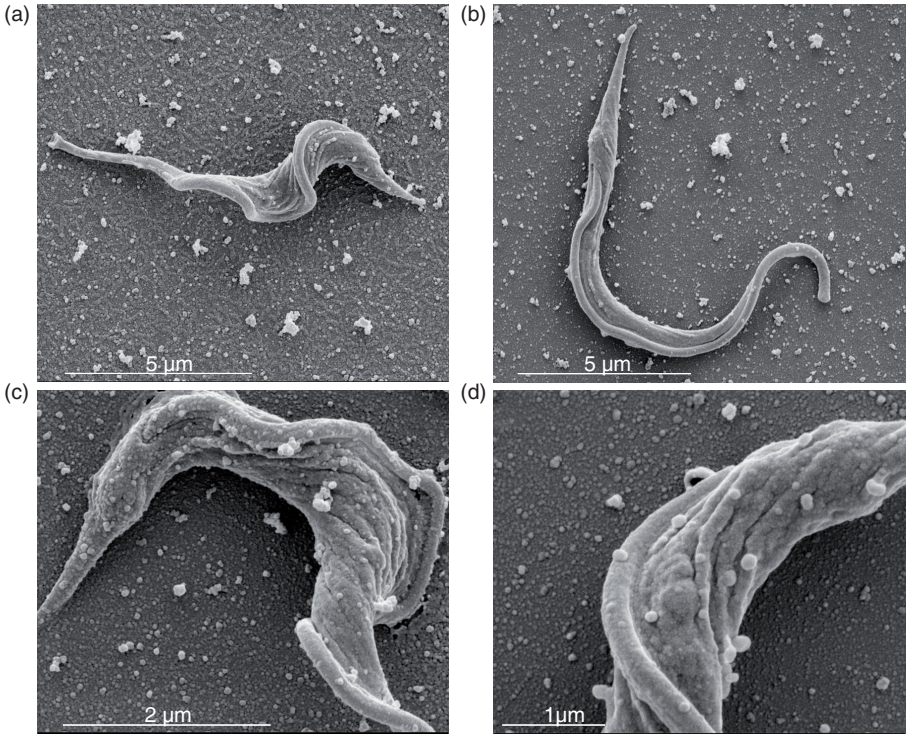

Fig. 3. *Trypanosoma cruzi* (Y strain) trypomastigotes spontaneously shed vesicles from their entire membrane surface. Scanning electron microscopy (SEM) of parasite membrane shedding after incubation in culture medium (a–d, bars: 1–5 µm). Magnification: (a) 27,383 ×, (b) 25,242 ×, (c) 60,470 × and (d) 92,084 ×.

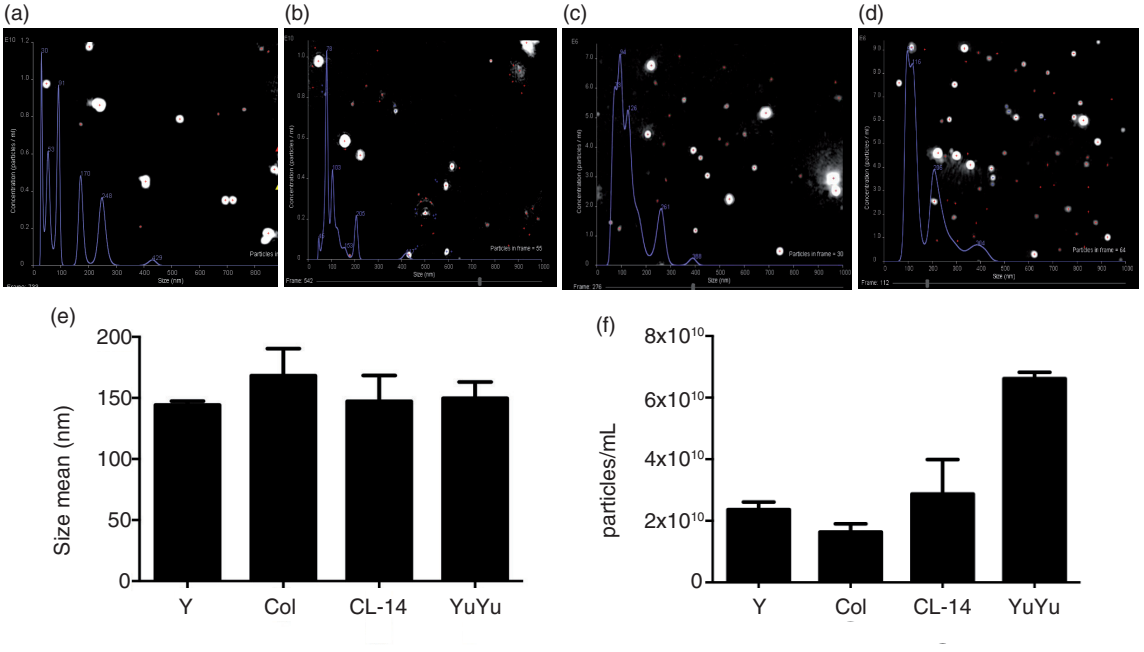

Fig. 4. Nanoparticle tracking analysis of the EVs isolated from different strains of *T. cruzi*. Graphic demonstration of size distribution and concentration for Y (a), Colombiana (b), CL-14 (c) and YuYu (d) strains. The average size (nm) (e) and concentration (particles/mL) (f) of the vesicles for all strains are represented. Data are representative of 3 independent experiments.

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trypomastigote membrane (antibody named 460) were employed for the selection of positive fractions by ELISA. Those antigen-containing fractions were pooled and the protein content was estimated. Lower protein content was detected in EVs derived from Y, Colombiana and CL-14 strains. EVs from the YuYu strain exhibited the highest protein level of liberated proteins (Fig. 5a), in contrast to EVs from Colombiana strain. Analysis of α-galatosyl-containing glycoconjugates expression in EVs has shown that those obtained from Y, CL-14 and YuYu strains were richer in α-galactosyl residues (Fig. 5b). EVs derived from the Y strain showed the lowest protein and the highest carbohydrate contents. Altogether, those results suggest that EVs from different strains have protein and α-galactosyl contents that are not directly correlated with each other.

Trypanosoma cruzi *EVs differentially activate TLR2*

Since EVs from different strains of *T. cruzi* are polymorphic when expression of α-galactosyl residues and protein content are considered, their ability to induce NO and cytokines in murine macrophages was investigated. A pilot experiment was designed to test a panel of pro- and anti-inflammatory cytokines (IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12/IL-23p40, IL-17, IFN-γ and TNF-α). No production of the cytokines IL-1β, IL-2, IL-3, IL-4, IL-5, IL-10, IL-17 and IL-12/IL-23p40 was detected when macrophages were primed with EVs from each of the strains (data not shown). For this reason, only the remaining cytokines were used in the next experiments. In comparison to EVs from YuYu and CL-14, no significant levels of cytokines were detected after incubation with EVs from Y and Colombiana strains in all concentrations tested, especially for TNF-α and NO (Figs. 6 and 7). A similar profile was observed for IL-6 (Supplementary Fig. 2). Consistent with those observations, no activation of the CHO cells transfected with CD25 reporter coupled to NF-κB was detected in TLR2- TLR4- and TLR4+ subsets confirming that the EVs preparations were free of LPS (Supplementary Fig. 3). Since the results were similar when 1 or 5 μg/mL was employed, the concentration 5 μg/mL was chosen for the next experiments.

EVs from T. cruzi *strains activate MAPKs in J774.1 macrophages*

To better characterize the signalling events after EVs exposure, J774.1 macrophages were incubated with vesicles (5 μg/mL) from each of the strains. MAPK activation was assessed as a function of time and analysed by densitometry. Although all EVs activated ERK 1/2, JNK and p38, no visible differences were detected among the strains (Fig. 8a–c).

T. cruzi *EVs differentially activate chronic-infected mice splenocytes*

Previous studies demonstrated that EVs are very important for heart tissues invasion and pathology during infection (19). The impact of *T. cruzi* EVs isolated from different strains during the chronic phase was assessed in splenocyte cells after 180 days post-infection of the animals with *T. cruzi*. No production of IL-2, IL-4 and IL-5 was detected (data not shown). Regardless of the type of strain employed for mice infection, all strain-derived vesicles were able to induce expression of TNF-α, IFN-γ, IL-6 and IL-10 in all groups. Similar data were observed for NO (Supplementary Fig. 4). Interestingly, Colombiana and Y EVs were more pro-inflammatory than those from YuYu and CL-14 strains ($p > 0.05$; Figs. 9 and 10).

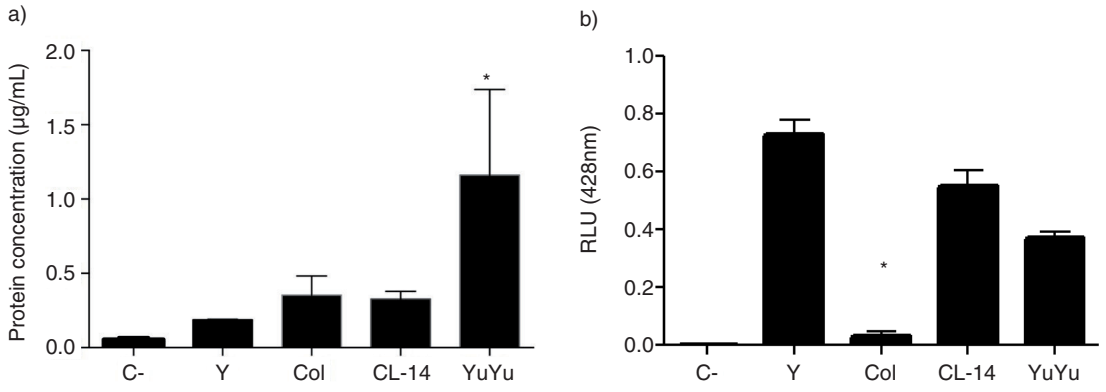

Fig. 5. Protein and terminal α-galactosyl residues measurement in EVs isolated from 4 *T. cruzi* strains. (a) Protein concentration in EV-pooled (b) EVs reactivity with anti-α-Gal antibodies (1:500) determined by ELISA. Negative control corresponds to the medium with 5% glucose without EVs. Bars express the mean value ± SD of 4 separate studies (*$p < 0.05$).

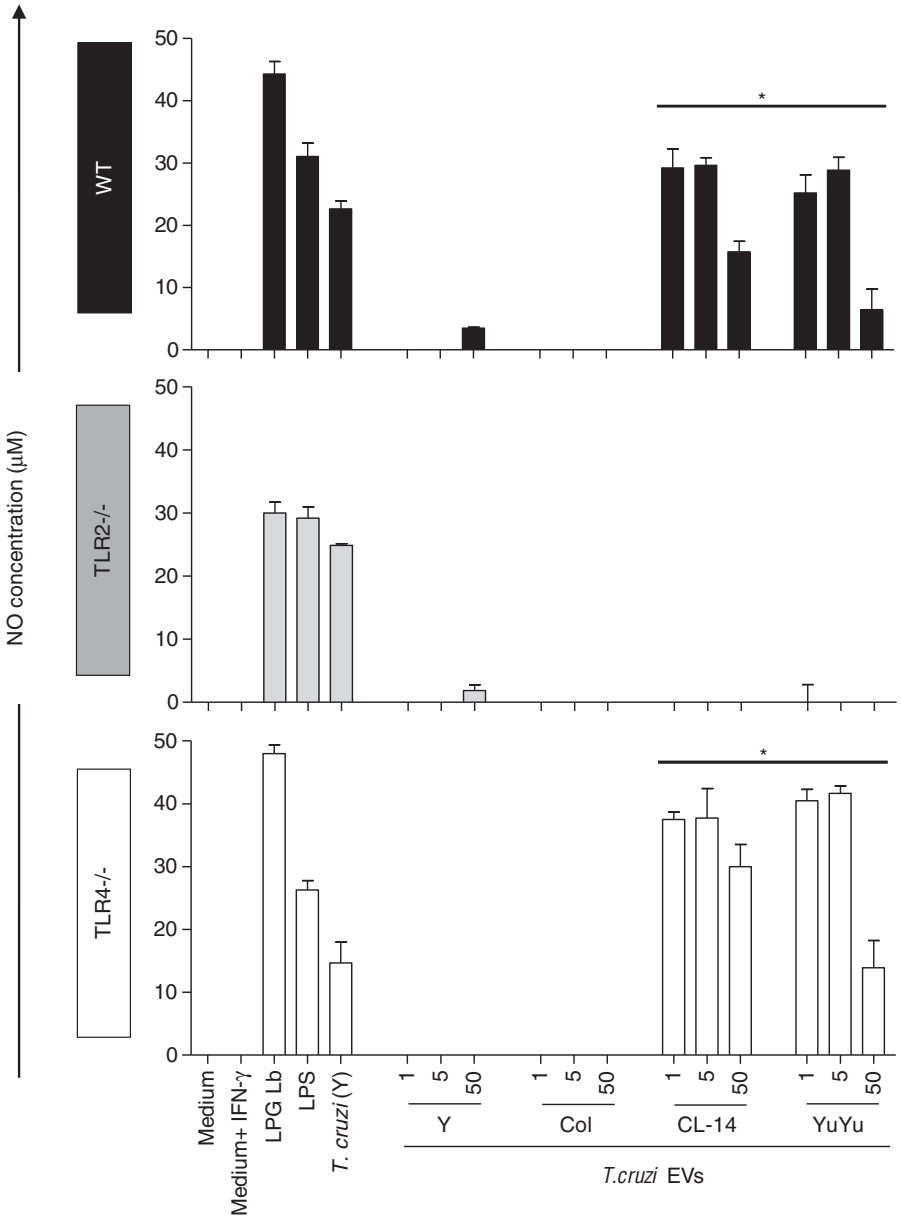

Fig. 6. Nitric oxide (NO) production by murine macrophages stimulated by EVs from CL-14 and YuYu strains is dependent on TLR2. Murine macrophages (C57BL/6, TLR2 −/− and TLR4 −/−) were stimulated with different concentrations of *T. cruzi* EVs (1, 5 and 50 μg). Cells were pre-incubated with IFN-γ (100 U/mL) for 18 h prior to addition of EVs or LPS, LPG and live parasites MOI 10:1 (positive controls). Negative controls included medium and medium + INF-γ. LPG Lb, *L. braziliensis* LPG; LPS, lipopolysaccharide (LPS) from *E. coli*; *T. cruzi* (Y), live parasites of *T. cruzi* (Y strain). Bars express the mean value ± SD of 2 separate studies (*$p < 0.05$).

EVs from Colombiana and YuYu strains induce similar levels of intracellular cytokines in leucocyte subpopulations

The previous cytokine analyses did not show major differences after EVs stimulation in mice infected with each of the 4 strains. For this reason, only 2 strains (Colombiana and YuYu) were chosen for intracellular cytokine detection in spleen cells (monocytes, DCs, T and B lymphocytes). In general, no quantitative cytokine differences were detected between splenocytes from YuYu and Colombiana-infected mice (Figs. 11 and 12). In CD4+ and CD8+ T lymphocytes, a higher frequency of IL-10+ than INF-γ+ or TNF-α+ cells (Fig. 11) was observed. A mixed profile of cytokine induction was observed in B cells with the production of TNF-α and IL-10 (Fig. 12a–c). Dendritic cells also produced TNF-α after stimulation with EVs from both strains (Fig. 11d). No relevant production of those cytokines was detected in monocytes (data not shown).

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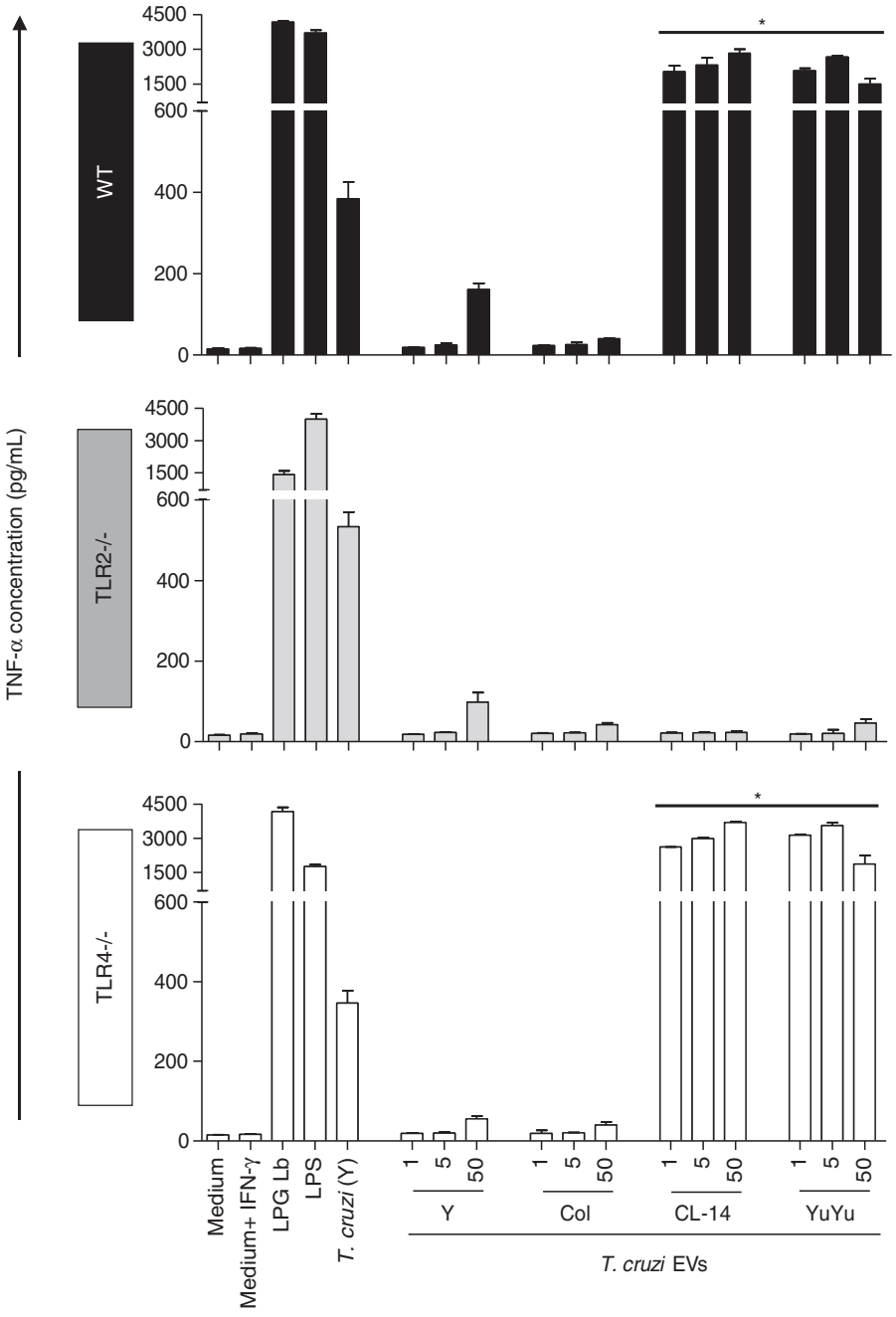

Fig. 7. TNF-α production by murine macrophages stimulated by EVs from CL-14 and YuYu strains is dependent on TLR2. Murine macrophages (C57BL/6, TLR2 −/− and TLR4 −/−) were stimulated with different concentrations of *T. cruzi* EVs (1, 5 and 50 μg). Cells were pre-incubated with IFN-γ (100 U/mL) for 18 h prior to addition of the EVs, and controls (LPS, LPG and live parasites MOI 10:1) (positive controls). Negative controls included medium and medium + INF-γ. IFN-γ, gamma-interferon; LPG Lb, *L. braziliensis* LPG; LPS, lipopolysaccharide from *E. coli*; *T. cruzi* (Y), live parasites of *T. cruzi* (Y strain). Bars express the mean value ± SD of 2 separate studies (*$p < 0.05$).

Discussion

T. cruzi infection induces important changes in the host's cellular immune response not only in the innate compartment but also in the adaptive immunity context. Parasite and host-related factors may affect the onset of clinical forms. In the host, T cells especially are critical for pathology modulation (36). Many studies have evaluated the cytokine production in different clinical forms (cardiac/intestinal and or indeterminate) providing insights into the understanding of the immunopathological events in Chagas disease (9). Consistent with those observations, a recent report has associated clinical forms

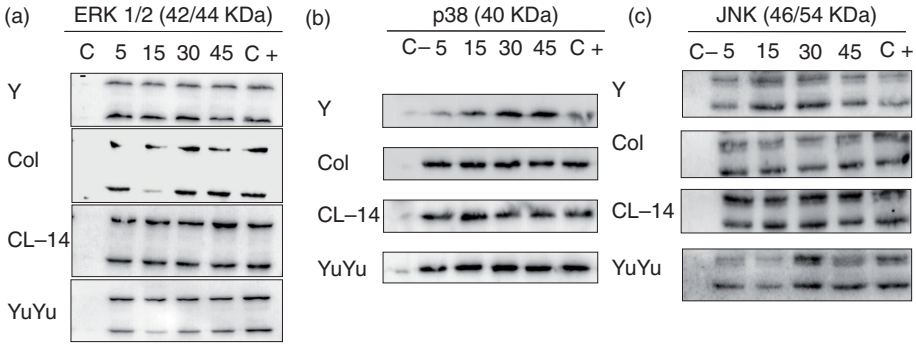

Fig. 8. *Trypanosoma cruzi* EVs equally activate MAPKs (ERK 1/2, p38 and JNK) from J774.1 macrophages. Cells were stimulated with EVs (5 μg/mL) at different time points (5, 15, 30 and 45 min). Dually phosphorylated MAPKs were detected by western blot: (a) ERK 1/2, (b) p38 and (c) JNK. C-, negative control (medium); C+, positive control (LPS from *E. coli*) (100 ng/mL).

with different DTUs. Here, similar to the tGPIs (16), a clear association between DTU and the parameters studied was not demonstrated, suggesting that those variations are probably strain-specific. For example, strains YuYu and Colombiana (DTU I) exhibited a different pro-inflammatory profile in the previous experiments.

EVs have been isolated and characterized in a number of pathogens including viruses, bacteria, fungi and parasites, and were described as having a pivotal role during the modulation of the host immune system. Those vesicles exhibit a complex array of molecules and may be considered a packet of pathogen-associated molecular patterns (PAMPs) reviewed in (22,23). In this sense, trypomastigote forms of *T. cruzi* continuously shed vesicles that are enriched with gp85/TS, mucin-associated surface proteins and other α-Gal-containing glycoconjugates,

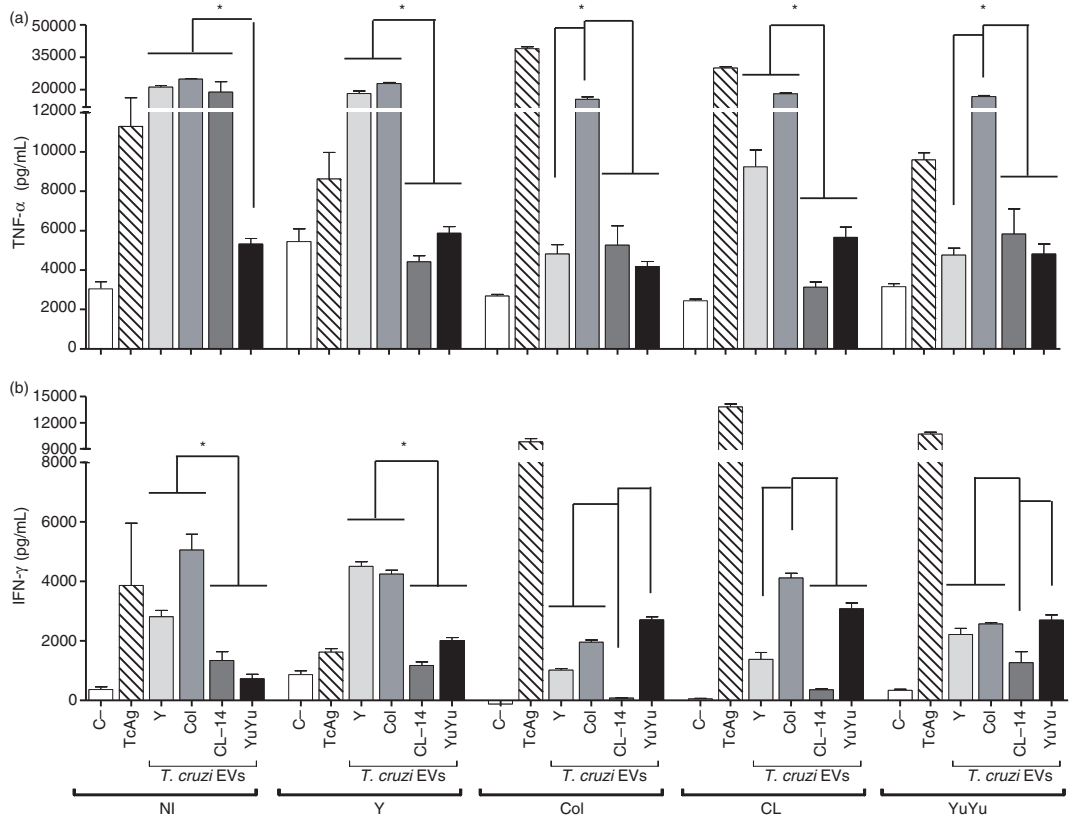

Fig. 9. *Trypanosoma cruzi* EVs from Colombiana and Y strains display a higher pro-inflammatory activity in splenocytes derived from chronically infected mice. Cells were incubated with EVs (5 μg/mL) or total *T. cruzi* antigen (10 μg/mL) (positive control). Spleen cells from non-infected mice with and without the addition of a new stimulus were also used as controls. TNF-α (a) and IFN-γ (b) concentrations (pg/mL) were determined by CBA. Medium, negative control; TcAg, soluble trypomastigote antigen of *T. cruzi*; Bars express the mean value ± SD of 2 separate studies (*$p < 0.05$).

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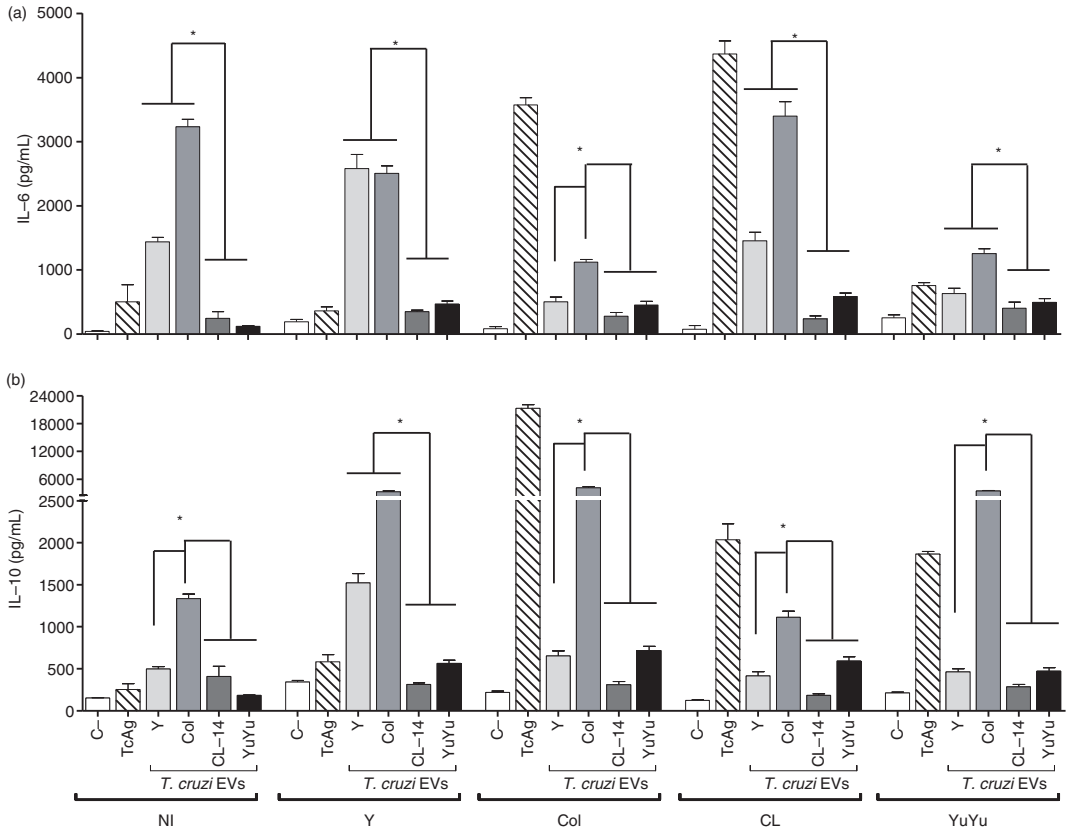

Fig. 10. *Trypanosoma cruzi* EVs from Colombiana and Y strains display differential immunomodulatory activity in splenocytes derived from chronically infected mice. Cells were incubated with EVs (5 μg/mL) or total *T. cruzi* antigen (10 μg/mL) (positive control). Spleen cells from non-infected mice with and without the addition of a new stimulus were also used as controls. IL-6 (a) and IL-10 (b) concentrations (pg/mL) were determined by CBA. Medium, negative control; TcAg, soluble trypomastigote antigen of *T. cruzi*; bars express the mean value ± SD of 2 separate studies (*$p < 0.05$).

such as mucins (16,37). BALB/c mice injected with *T. cruzi* EVs and challenged with tripomastigote forms developed severe heart pathology with an intense inflammatory reaction, an increased number of intracellular amastigotes and mortality (19). These results indicate that molecules present in vesicles are important during the immunopathological events in Chagas disease. However, no information about the role of EVs from different *T. cruzi* strains in those processes is available. In this paper, purified EVS, free of LPS from 4 strains belonging to DTUs I (Colombiana and Yu-Yu), II (Y) and VI (CL-14) (28), were chosen to evaluate their role in the innate immune compartment and in spleen cells from chronically infected mice.

A clear polymorphism was detected early in the EVs. Vesicles from Y, CL-14 and YuYu strains were richer in α-Galactosyl epitopes (Galα1-3Galβ1-(3)4GlcNAc-R) (38). Those glycoconjugates are extremely immunogenic resulting in the production of high levels of lytic anti-α-Gal antibodies in chagasic patients and are important for infection control (39–41). A distinguished feature was observed for Colombiana strain, which is extremely virulent and often leads to mortality in mice (42). Since EVs from that strain express much less α-Gal epitopes, one can hypothesize that this fact would make Colombiana strain less susceptible to the action of lytic antibodies. Consistent with those observations, purified tGPI-mucins from Y, CL and Colombiana strains were also differentially recognized by anti-α-Gal antibodies from human patients (16). Those data indicate the presence of not only variable amounts of terminal α-Gal residues but also other changes in the structure of this epitope. No substantial differences in protein content were observed for the Y, CL-14 and Colombiana EVs. However, EVS isolated from YuYu strain have the highest protein amount. To further characterize and understand the role of EVs, proteomic analyses are under way to determine EVs composition from *T. cruzi*, as performed for other pathogens, such as *Plasmodium yoelii* (43), *Leishmania mexicana* (44) and the trematodes *Echinostoma caproni* and *Fasciola hepatica* (45). To evaluate the implications of such polymorphisms in antigenicity, pathogenesis and virulence among different strains, purified EVs from *T. cruzi*, were incubated with host cells.

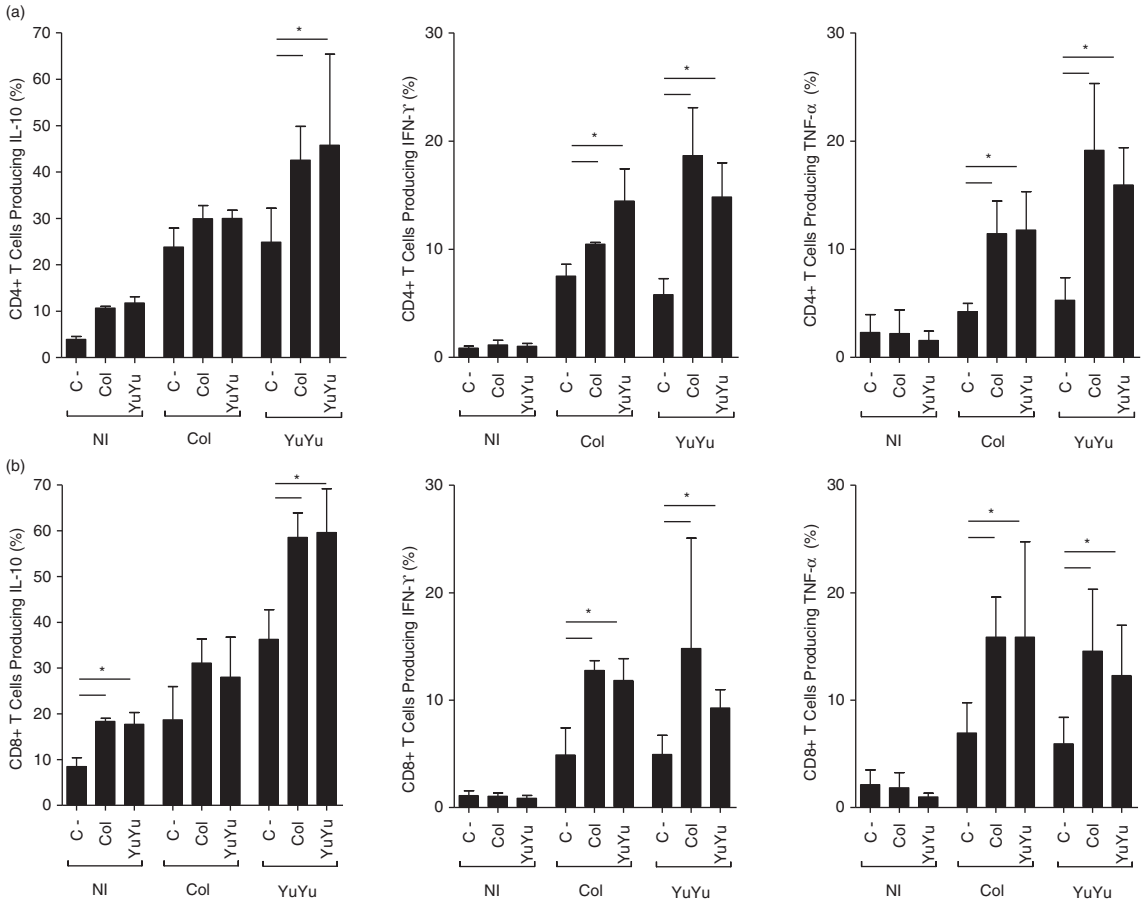

Fig. 11. Intracellular cytokine production by T lymphocytes is similar after stimulation with EVs from Colombiana or YuYu strains. (a) CD4+ production of intracytoplasmic cytokines (IL-10 with IFN-γ and TNF-α). (b) CD8+ production of intracytoplasmic cytokines (IL-10 with IFN-γ and TNF-α). Immunophenotypic staining was performed as described in the Material and Methods section. Bars express the mean value ± SD of 2 separate studies (*$p < 0.05$).

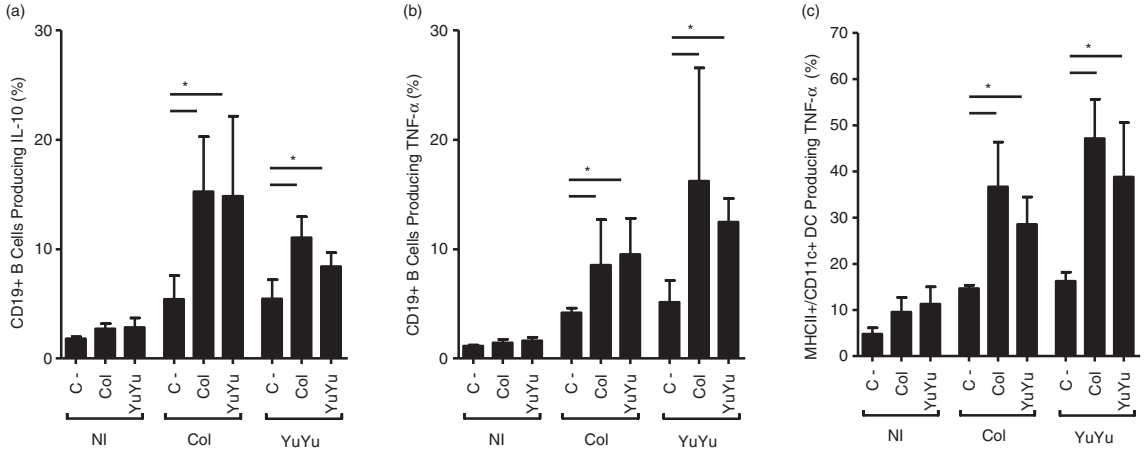

Fig. 12. Intracellular cytokine production by B and DC is similar after stimulation with EVs from Colombiana or YuYu strains. (a) CD19+IL-10+ production. (b) CD19+TNF-α+production. (c) MHCII/CD11c+TNF-α+ production. Immunophenotypic staining was performed as described in the Material and Methods section. Bars express the mean value ± SD of 2 separate studies (*$p < 0.05$).

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Extensive studies have reported on the biological activities of tGPI-mucins, which corresponds to 60–80% of the parasite *T. cruzi* surface molecules (37,46) and may be one of the main components of EVs. Those are potent agonists of TLR1/TLR2 and TLR2/TLR6 (47). Interestingly, tGPIs from Colombiana, Y and CL strains differentially induced NO and proinflammatory cytokines (IL-12 and TNF-α) in murine macrophages (16,37).

EVs from all strains induced different levels of NO, IL-6 and TNF-α in macrophages. An interesting aspect of this activation is that although they have the expression of many molecules, EVs were very specific for TLR2, particularly in the case of CL-14 and YuYu strains. This ability was lower for Y and Colombiana strain, showing that differential stimulation by EVs varies in the innate immune compartment. This result may be due to the content of other components, such as GIPL, a TLR4 agonist (48), did not seem to be important in this activation. On the other hand, membrane molecules like cruzipain could also be involved. Those were able to decrease NO production by down-regulating the iNOS expression in macrophages (49).

Those data are in accordance with previous in vivo results showing that EVs from the Y strain induced a local reduction of iNOS expression resulting in higher tissue parasitism (19). Recently, it was demonstrated that strains belonging to different DTUs vary in the ability to bind to human galectins, suggesting the existence of glycoconjugate polymorphisms in *T. cruzi* (13,14,50). In other tripanosomatids, such as *L. donovani*, a more immunomodulatory profile was observed by promoting IL-10 production and consequent inhibition of TNF-α by the parasite EVs (27).

The MAPKs transduce a variety of extracellular stimuli through a cascade of protein phosphorylations like ERK1/2, JNK and p38, leading to the activation of transcription factors (51). Previous studies have correlated that tGPI mucins from Y strain were able to trigger phosphorylation of ERK-1/2 and p38 in murine macrophages resulting in the production of IL-12 and TNF-α (52,53). Since tGPI mucins are major glycoconjugates on the membrane surface and may be also found in EVs, the next step was to evaluate their ability to activate MAPKs. All EVs were equally able to induce MAPKs (ERK 1/2, p38 and JNK) signalling in J774.1 macrophages during the very initial steps of innate immune response. However, this did not result in NO and cytokine production for the EVs from Y and Colombiana strains, reinforcing that inhibition of MAPKs is likely to occur downstream in the pathway. This is in accordance with the fact that EVs are internalized by the cells (20), thus modulating the further intracellular events such as NO and cytokine production after 48 h. This may suggest 2 possible mechanisms: (a) EVs can activate MAPKs via TLR2 and/or via internalization (CL-14 and YuYu strains) and (b) EVs can be internalized and activate MAPKs (Colombiana and Y strains). Glycoconjugates (LPGs and GIPLs) from other trypanosomatids including *Leishmania braziliensis*, *Leishmania infantum* and different strains of *Leishmania enriettii* were also able to differentially activate MAPKs, thus reinforcing that intra and interspecies variation are important in the early events of immune response (32,33,54). In summary, those data indicate that regardless of the type of *T. cruzi* EVs, they were all sensed early by the cells from the innate immune compartment. Therefore, a differential expression of NO and pro-inflammatory cytokines were observed later on.

The transition from the acute to the chronic phase is a well-known mechanism in the natural history of Chagas Disease. It is characterized by the establishment of an effective immune response and a decrease in blood parasitaemia (55). The initial events in the immune compartment are crucial for the development of a successfully acquired immune response (56). Based on these observations, our next step was to evaluate the role of EVs from all *T. cruzi* strains in splenocytes from chronically infected C57BL/6 mice. Mice were infected with a low number of parasites from all strains and those that survived the acute phase were kept for 6 months (~180 days) prior to splenocyte recovery. Since CL-14 strain is not infective to mice (57), its parental isolate (CL) has been employed, resulting in detectable parasitaemia. An interesting aspect was observed in these experiments, in which the strains that did not activate the innate immune compartment (Y and Colombiana) were the most pro-inflammatory in the chronic phase. Regardless of the type of strain used for in vivo infection, Colombiana and Y strains EVs exhibited a more pro-inflammatory role than those from YuYu and CL-14 strains. Similar to macrophages, splenocytes also produced NO, TNF-α and IL-6 in addition to IFN-γ. As expected, the appearance of IL-10 in detectable levels occurred in those cells confirming its role modulating the inflammatory events in the chronic phase (58–60). Depending on the strain circulating in the host and considering the fact that blood tripomastigotes may shed vesicles during the acute and chronic phase, those data indicate that EVs from *T. cruzi* may be crucial for the immunopathological mechanisms occurring in the tissues and spleen. Altogether, differential expression of antigenic molecules in all EVs was able to activate either the cells from innate compartment or the splenocytes from infected and non-infected mice. This stimulation was independent on the strain used for the mice infection reinforcing the role of EVs as antigenic stimuli.

Interestingly, for the intracellular cytokine production, they exhibited a similar profile, especially in their ability to activate spleen cells from infected mice with them. Those responses were in general higher than those seen for spleen-derived cells from mice infected with the Y and CL strains. CD4+ T cells and monocyte/macrophages

act as key orchestrators of the cellular response during chronic Chagas disease. These cells produce pro- and anti-inflammatory cytokines after stimulation with total crude parasite extract of *T. cruzi* (60,61). Analysis of the inflammatory infiltrates in infected mice primed with vesicles from Y strain also revealed a dominance of CD4+ and CD8+ T lymphocytes and macrophages (19). Our data demonstrated that, regardless the strain, in vitro stimulation with EVs increased the frequency of IL-10+ CD4+ and CD8+ T cells in mice infected with YuYu and Colombiana strains. This is consistent with the previous data showing that IL-10 was detected in splenocytes derived from mice in the chronic phase. IL-10 is a very important cytokine involved in the immunomodulatory mechanisms during chronic Chagas disease (62). The ability to produce IL-10 in chronic phase may be important to modulate the fine balance between inflammatory and anti-inflammatory cytokines avoiding tissue damage (58).

The specific role of B cells in Chagas disease is still unclear. In general, these cells can contribute to immunity in multiple ways including producing antibodies, antigen presentation or cytokine secretion (41,63,64). A mixed profile of cytokine induction was observed after EVs stimulation with the production of IL-10 and TNF-α by CD19+ B cells, key cytokines often involved in the immunomodulatory processes triggered by pathogens. Additionally, B-cell activation may account for some of the observed increases in cytokine levels post-infection with *T. cruzi* (65). Recently, it was observed that an increase in the expression of regulatory B cells in patients from Chagas disease is associated with higher levels of IL-10 (66). These observations reinforce the role of B cells to produce a variety of cytokines, and there is growing interest in the potential of B cells to modulate immune responses by regulating their profiles of effector cytokine secretion (67). In summary, B and T cells from chronically infected mice were readily able to trigger cytokine production after EV exposure. However, their role in antigen presentation remains unclear for EVs.

Finally, monocytes and DCs are innate immune cells that are very important in the early events of immune responses against *T. cruzi* (68,69). Here, their role in the chronic phase was also determined. No production of IL-10 was detected after stimulation with EVs by either monocytes and or DC, suggesting that those cells are not related to its production in the later phases of the disease. On the other hand, DCs were still an important source of TNF-α. However, in *L. donovani*, treatment with vesicles prior to challenge with parasites exacerbated infection and also promoted IL-10 production in the spleen by DCs (27). Those experiments suggest that during the chronic phase, the EVs from *T. cruzi* seem to be more pro-inflammatory for DCs than those from *L. donovani*, which were immunosuppressive.

Conclusions

We have demonstrated that EVs from *T. cruzi* strains were able to differentially modulate the early and later events of immune responses. Although a clear association with DTUs was not observed, those EVs were very potent agonists of TLR2, especially for CL-14 and YuYu strains. On the other hand, EVs from Y and Colombiana strains were weak activators of the innate immune compartment but were very pro-inflammatory in chronic phase-derived splenocytes. An important aspect of EVs is their ability to modulate the events in the chronic phase due to the production of IL-10 by T and B cells. Additional studies are still needed to understand better the impact of EVs on the immune system of patients and correlate with distinct clinical forms of Chagas disease. Understanding the mechanisms involved in the immune responses to infection by parasites is important to prevent and control the immunopathological events during Chagas disease.

Authors' contributions

Conceived and designed the experiments: PMN, OAM-F, ACOS, SRB, RPS, MAC and ACTT. Performed the experiments: PMN, SRB, KR, JHC, ACOS, NLP, RPS and ACTT. Analysed the data: PMN, ACOS, OAM-F, MAC, NLP, MJMA, RPS and ACTT. Wrote the paper: PMN, MJMA, WC, RPS and ACTT.

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Conflict of interest and funding

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