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Interactions of human galectins with *Trypanosoma cruzi*

Binding profile correlate with genetic clustering of lineages

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Abstract

We report here the specific interaction between several members of the human galectin family with the three developmental stages of several genetic lineages of the protozoan parasite *Trypanosoma cruzi*. We provide data of specific and differential binding of human galectin (gal)-1, -3, -4, -7 and -8 to 14 strains of *T. cruzi* that belong to the six genetic lineages representing the genetic diversity of the parasite. It is shown that galectins preferentially bind forms present in the host, trypomastigotes and amastigotes, compared with the non-infective epimastigote present on the intestinal tract of the vector, reflecting the changes on glycosylation that occur during the metacyclogenesis and amastigogenesis process. Also, it is evidenced that galectin binding to the parasites promotes binding to the host cells and higher infection rates. In addition, evidence is provided indicating that the intracellular amastigotes may take over the cytosolic pool of some galectins when released to the extracellular medium. Finally, by applying unweighted pair group method analysis to the galectin-binding profile to either cell-derived trypomastigotes or amastigotes, we show that the differential-binding profile by the host galectins to the six lineages resembles the clustering based in genetic data. Therefore, the differential-binding profile for the six lineages could have implications in the immunopathology of Chagas' disease, affecting the complex network of immune responses on which galectins mediate, thus providing linkage clues to the notion that different lineages may be related to different clinical forms of the disease.

Key words: discrete typing units, galectin, host cell adhesion, *Trypanosoma cruzi*

Introduction

Trypanosoma cruzi is the causative agent of Chagas' disease, a chronic disorder endemic in Latin America (World 2010). The disease exhibits diverse clinical manifestations, starting with a short acute phase with variable parasitemia, followed by an indeterminate phase when no significant clinical symptoms (Coura and Borges-Pereira 2010; Rassi et al. 2010). After a few months, the chronic stage develops in ~30–35% of infected, causing mainly cardiac or digestive alterations. However, the host factors responsible of triggering immune mechanisms during disease progression still remain undetermined. *Trypanosoma cruzi* has a digenetic life cycle with at least three different life stages: epimastigotes and metacyclic trypomastigotes in the insect vectors and trypomastigotes and intracellular amastigotes in the mammalian hosts (de Souza 1984). The invasion of the human host mainly occurs by entrance through damaged skin or intact mucosa of metacyclic trypomastigotes released with the feces of infected insect vectors

after their blood meal. The trypomastigotes are able to infect a wide variety of host cells (de Souza 1984), where they transform into proliferating intracellular amastigotes. The amastigotes differentiate into bloodstream trypomastigotes that disseminate, before going through another intracellular cycle or taken up by the insect closing the life cycle. In order to adapt and survive into these different environments, the life forms present different plasma membrane composition, the main changes observed in the glycosylation profile of each biological form (de Lederkremer and Agusti 2009).

Trypanosoma cruzi is subdivided in six discrete typing units (DTUs) with differential geographical, transmission cycles, vector and host distribution (Macedo et al. 2004; Aquilino et al. 2012) and has been suggested that this polymorphism could be related to the clinical manifestations of the disease. However, little is known about the host molecules involved on the differential parasite recognition. Most of the pathogen-associated molecular patterns described so far in *T. cruzi* are glycosylated (such as GPI-linked, mucins and transsialidases) and control activation of the innate immune system and initiation of the acquired host immune response in the vertebrate host (Almeida et al. 2000; Procopio et al. 2002; Previato et al. 2004; Nogueira et al. 2007; Alcaide et al. 2010). Therefore, characterization of the interaction between parasite glycans and their host's putative receptors is of outstanding relevance. Among the wide array of host receptors, galectins have attracted attention over the last years due to the large number of immune functions ascribed to them. Galectins are a conserved family of animal lectins with preserved structure and calcium-independent affinity for β-galactosides (Vasta 2012), yet exhibiting different fine specificity and affinity. They are widely expressed on diverse cell types, from immune-privileged tissues to epithelial cells of intestinal tract. However, each one exhibits a restricted tissue distribution (Nio-Kobayashi et al. 2009) and shows a pleiotropic range of effects mainly related to inflammatory processes. Collectively, the final interplay of the galectin signaling network will be the cumulative result of each individual member interacting with its cognate ligands. It has become increasingly clear that different regulatory immune circuits can be associated to multiple activation of immune pathways triggered by PRRs (Dam and Brewer 2010a, 2010b; Amit et al. 2011; Chevrier et al. 2011). Hence, having several members of the galectin family to differentially scrutiny and mediate pathogen recognition constitutes a powerful mechanism to fine tune the subsequent immune response.

There have been reports suggesting involvement of galectins in *T. cruzi* infection, such as changes in expression of galectins and/or its ligands upon infection (Zuniga, Gruppi, et al. 2001; Zuniga, Rabinovich, et al. 2001; Vray et al. 2004; Silva-Monteiro et al. 2007), changes in subcellular location of gal-3 (Machado et al. 2014; Reignault et al. 2014). It has also been shown that gal-3 is able to promote trypomastigotes adhesion to extracellular matrix components (Moody et al. 2000; Kleshchenko et al. 2004). However, the relevance of galectins in *T. cruzi* infection is still far from being resolved.

Here we report the first systematic study of the binding of several members of the human galectin family towards three life forms of *T. cruzi*. Galectins present a higher affinity to the biological forms found in the vertebrate host, compared with the non-infective forms present in the insect vector. Secondly, by using unweighted pair-group method analysis of galectin-binding profile to epimastigotes and cell-derived trypomastigotes (CTT) of the six different DTUs, the 14 *T. cruzi* strains analyzed could be arranged in five clusters that closely resembled the grouping based solely on genomic data. In addition, several galectin ligands on the surface of the parasite had been identified.

Materials and Methods

Cells and parasites

Vero (green monkey kidney epithelial cells, ATCC CCL-81), LLC-MK2 (rhesus monkey, kidney epithelial cells, ATCC CCL-7), CaCo-2 (human colorectal adenocarcinoma cells, ATCC HTB-37) and THP-1 (human monocytes, ATCC TIB-202) cells were grown in RPMI complete medium containing 5% FCS, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL) (Gibco, Grand Island, NY) at 37°C in an atmosphere containing 5% CO_2. The mouse cardiac cell line HL-1 (Claycomb et al. 1998) was obtained and grown according to Dr. Claycomb (Department of Biochemistry & Molecular Biology, School of Medicine, Louisiana State University).

All parasite strains listed in Table I were genotyped as described in Zingales et al. (2012) and DTU assigned according to the new consensus on nomenclature on genetic lineages of *T. cruzi* (Zingales et al. 2009). Epimastigotes life forms were continuously cultured in liver infusion tryptose medium supplemented with 5% fetal calf serum, and 0.01% hemin as described previously (Alcina and Fresno 1988). CTT were obtained from the supernatant of infected Vero cells at 4–6 days post-infection and isolated by differential centrifugation, and recovered from the supernatant after 2 h incubation at 37°C, this procedure was repeated once to reduce the proportion of intermediate forms and amastigotes. The final population was never <95% highly motile trypomastigotes.

Amastigotes were obtained from 3 to 4 days infected Vero cells after lysis, either by Percoll discontinuous density gradient as described (Gamarro et al. 1985) or by anion-exchange chromatography (Marques et al. 2011). Microscopic examination indicated that 98 or 95%, respectively, of the population was homogeneous. No differences in the binding profile were detected. Given the highly consistent yield of the Percoll gradient method, it was the preferred method.

Galectins

Expression plasmid pQE60 containing the human gal-1 sequence was kindly provided by Dr. Elena Moisevaa (Leicester Warwick Medical School, UK). The protein was purified as described previously (Andersen et al. 2003). Expression plasmids for human gal-3 and -4 were provided by Dr. Hakon Leffler (Lund University, Sweden) (Patnaik et al. 2006). Expression plasmid pGEX containing the human gal-7 sequence was provided by Dr. Thierry Magnaldo (Institut Gustave Roussy, France) (Magnaldo et al. 1995). Four expression

Table I. Strains of *T. cruzi* used in this work

Strain	DTU	Origin	Host/vector
Silvio/X10 c1	I	Belem, Brazil	*Homo sapiens*
Dm28c	I	Carabobo, Venezuela	*Didelphis marsupialis*
Esmeraldo c3	II	Bahia, Brazil	*Homo sapiens*
Y	II	Sao Paulo, Brazil	*Homo sapiens*
Tu18 c2	II	Tupiza, Bolivia	*Triatoma infestans*
Cm17	III	Carimaga, Colombia	*Dasyprocta fugilinosa*
M6241 c6	III	Para, Brazil	*Homo sapiens*
Can III c11	IV	Para, Brazil	*Homo sapiens*
10R26	IV	Santa Cruz, Bolivia	*Aotus* sp.
Bug2148 c11	V	Rio Grande do Sul, Brazil	*Triatoma infestans*
Sc43 c1	V	Santa Cruz, Bolivia	*Triatoma infestans*
Tula c2	VI	Tulahuen, Chile	*Homo sapiens*
VFRA c1	VI	Francia, Chile	*Triatoma infestans*
CL-Brener	VI	Rio Grande do Sul, Brazil	*Triatoma infestans*

plasmids pGEX4T2 containing the human gal-8 sequence and the mutant gal-8 R69H, gal-8 R233H and gal-8 R69H/R233H sequences were provided by Dr. Nozomu Nishi (Kagawa University, Japan) (Nishi et al. 2003). All human recombinant galectins were expressed and purified from bacterial pellets as described in the original manuscripts and freed of LPS (Lipopolysaccharide) by passage on a polymixin-Sepharose™ column as described by the manufacturer. Briefly, bacteria cultures were incubated with 1 mM isopropyl-1-thiogalactopyranoside for 3 h at 37°C to induce recombinant protein production. Bacteria were pelleted, suspended in phosphate-buffered saline 4 mM EDTA, 2 mM β-mercaptoethanol, 10 mM lactose (PBS-MELac) together with a protease inhibitor cocktail. After sonicating, bacteria were centrifuged to obtain the soluble fraction. Recombinant galectins present in this fraction were purified by affinity chromatograph on α-lactose-agarose and eluted with lactose 0.1 M and dialyzed against a buffer containing 0.1 mM β-mercaptoethanol and 10 μM lactose. Once purified, each galectin was kept at −20°C after freeze-drying solutions at concentrations >10 μM, in such way galectins were stable for a longer time avoiding proteolysis. Before each experiment, the integrity and purity of each galectin was assessed by SDS–PAGE and silver staining, and their sugar-binding capacity was periodically tested (Figure 2 shows a representative silver-stained gel of purified recombinant human galectins as used on this manuscript). Alexa-488-conjugated galectins were prepared by labelling with Alexa Fluor 488 Microscale Protein Labelling Kit (Life Technologies) following manufacturer guidelines.

Truncated gal-3 was obtained as described before (Kopitz et al. 2001).

Adhesion and infection assays of *T. cruzi* to host cells

To study parasite-cell adhesion, THP-1 and LLC-MK2 were used. *T. cruzi* CTT were labelled with the fluorescent dye CFSE according to manufacturer's instructions (carboxyfluorescein diacetate, succinimidyl ester, Life Technologies). THP-1 cells (10^6 cells/assay) were incubated with CFSE-labeled parasites in complete RPMI medium (cell : parasite ratio 1 : 3) for 15 min at 4°C to prevent parasite internalization with or without recombinant galectins. Then, cells were fixed in 1% paraformaldehyde in PBS. Samples were analyzed by flow cytometry using a FACScalibur flow cytometer (BD Biosciences). Fluorescence associated to mammalian cell gating was quantified as an indication of cell-attached parasites. To study adhesion of *T. cruzi* to LLC-MK2 cell line, cells were cultured in microtiter plate wells and CTT were added to each cell in a 1 : 10 cell : parasite ratio, in the presence or absence of recombinant galectins (0.2–2 μM) at 4°C for 15 min. After PBS washing, the number of parasites attached to the cells was directly counted in not <10 representative fields.

Vero or LLC-MK2 cells growing on glass coverslips at 25% confluency in 24-well plates were used for infection assays. Cell-derived trypomastigotes were added at an infection index of 10 for 4 h at 37°C, in the presence or absence of indicated galectins. The cells were washed three times to remove unattached parasites and kept at 37°C.

At indicated times, cells were washed twice with PBS, fixed with Bouiń's fixative solution and stained in Giemsa solution. The number of infected cells and number of intracellular amastigotes per cell were quantified counting at least 150 cells in three independent slides by two different observers.

Flow cytometry assays

Parasites were incubated with Alexa-488-labeled galectins (2 μM) for 15 min at 4°C. Unbound galectins were removed by washing three times with PBS, and parasites were then fixed in 1% paraformaldehyde for 20 min at 4°C. Relative fluorescence intensity was measured on a FACScalibur flow cytometer (BD Biosciences) and data were analyzed using FlowJo analysis software (Tree Star).

Identification of *T. cruzi* galectin ligands

To prepare galectin immobilized columns, 5–10 mg of gal-3 and -4 (purified as previously described) were covalently coupled to HiTrap NHS-activated columns according to manufacturer's instructions (Amersham Biosciences), including 5 mM lactose in the coupling buffer. Gal-7-GST and gal-8-GST were coupled to glutation-Sepharose columns.

Membrane proteins of CTT (Y strain) were obtained by labeling live parasites with Sulfo-NHS-SS-Biotin, lysed in buffer B (0.2 M NaCl, 20 mM Tris–HCl, 0.1% CHAPS and protease inhibitors cocktail). The solubilized cell surface biotinylated proteins were purified by chromatography on a Neutravidin column (Pierce) and eluted with DTT 50 mM. The eluted fraction was applied to columns containing the immobilized galectins. Columns were extensively washed with buffer B and buffer B plus 0.5 M NaCl, and then specifically eluted with buffer B plus 100 mM lactose. The eluted material was resolved by SDS–PAGE, transferred to nitrocellulose filters and probed with biotinylated galectins.

Identical nitrocellulose membrane lanes were cut into 10 slices, reduced with dithiothreitol, alkylated with iodoacetamide and digested overnight with trypsin in a 1 : 40 ratio. Peptides released were acidified with trifluoroacetic acid (TFA, final 0.1%), dried and redissolved in 5 μL TFA% and acetonitrile 33%. 2,5-Dihydroxybezoic acid 0.5 μL was used as matrix and mixed with 0.5 μL of peptide sample on an Anchor-chip (Bruker) and air dried. The spectra obtained were used for MASCOT in-house identification searches against the TryTrypDB *T. cruzi* protein database (Tcruzi_AnnotatedProteins-v7.0, http://TryTrypDB.org). The *T. cruzi* surface mucin AgC10 was identified among the galectin ligands by using a specific monoclonal antibody previously described by our laboratory (Alcaide and Fresno 2004a, 2004b).

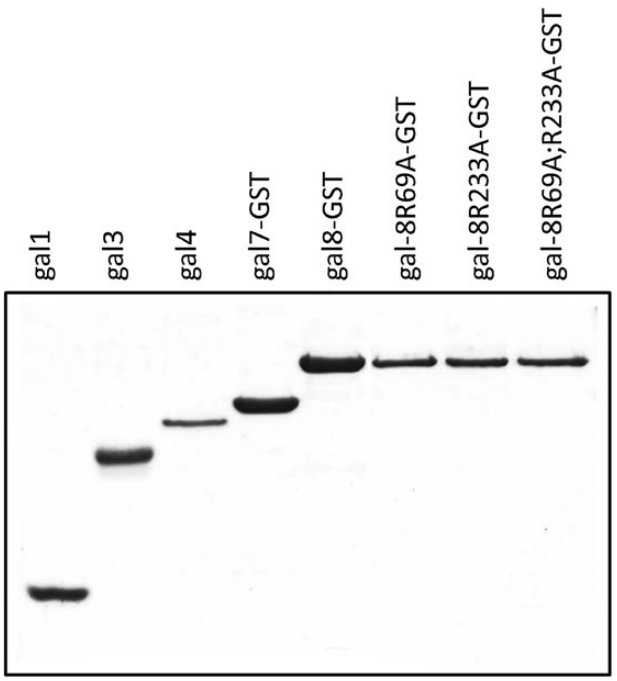

Fig. 1. Purified galectins are shown after SDS–PAGE. Samples of each purified recombinant galectin (between 1 and 3 μg) were separated by SDS–PAGE in a 12% gel and silver stained.

Confocal immunofluorescence microscopy

To study the subcellular location of endogenous galectins in infected cells, Vero cells were grown on cover slips to 25–30% confluence and then infected at an infection index (parasite : cell) of 10 with trypomastigotes, strain Y (DTU II), during 4 h at 37°C, after which free parasites were removed by washing three times with medium before incubate them at 37°C in an atmosphere containing 5% CO_2. After 3 days, the cells were fixed with 1% paraformaldehyde–0.5% glutaraldehyde in buffer sodium cacodylate 50 mM, pH 7.1, 70 mM NaCl, 0.1% saponin; washed three times with PBS–0.2% BSA, incubated with anti-galectin antibody and subsequently with Alexa-488-conjugated secondary antibody before images were acquired using Confocal LSM510 META microscope (Zeiss).

Unweighted pair-group method with arithmetic mean analysis

The clustering was done using Pearson coefficient comparing the median fluorescence index (MFI) of each DTU when tested against fluorescent conjugates of recombinant gal-1, -3, -4, -7 and -8 by using DendroUPGMA (Garcia-Vallve et al. 1999).

Results

Galectins bind to *T. cruzi*

First, we screened the binding of highly purified recombinant human galectins conjugated to Alexa-488 by flow cytometry to live epimastigotes, amastigotes and CTT. The purity of the recombinant galectins employed in the study was assessed by silver-stained SDS–PAGE gel (Figure 1). Individual life forms of *T. cruzi* were analyzed separately. Figure 2A shows a representative flow cytometry histogram obtained for the Y strain (DTU TcII) in which a differential-binding profile is observed. There was a higher affinity (in terms of higher MFI) to the forms present in the host, i.e., CTT and amastigotes, than to the non-infective in vitro cultured epimastigotes to which only gal-7 showed a binding clearly distinctive from negative controls. Gal-7 and -8 showed the highest affinity when trypomastigotes were tested, whereas gal-1 and -4 were shown to bind amastigotes (Figure 2B) in a clear indication of different glycan exposition on the different life forms, evidencing substantial differences in the specificity for each galectin. We did not find any quantitative difference between trypomastigotes obtained by metacyclogenesis or CTT (not shown), suggesting that the change in the surface exposed glycans is inherent to the life form and not to the differentiation strategy used to generate them. The binding to CTT was saturable, reaching a plateau at galectin concentrations close to 20 μM (Figure 2C) similar feature was found for amastigotes and epimastigotes (not shown).

As galectins are soluble proteins located in the cytosol, it is reasonable to propose that the intracellular pool might bind to amastigotes exposing galactosides on their surface. In order to test this, purified extracellular amastigotes from disrupted-cell supernatants were galactose washed in order to remove any host galectin bound. Under those conditions, the binding of galectins to parasites was significantly

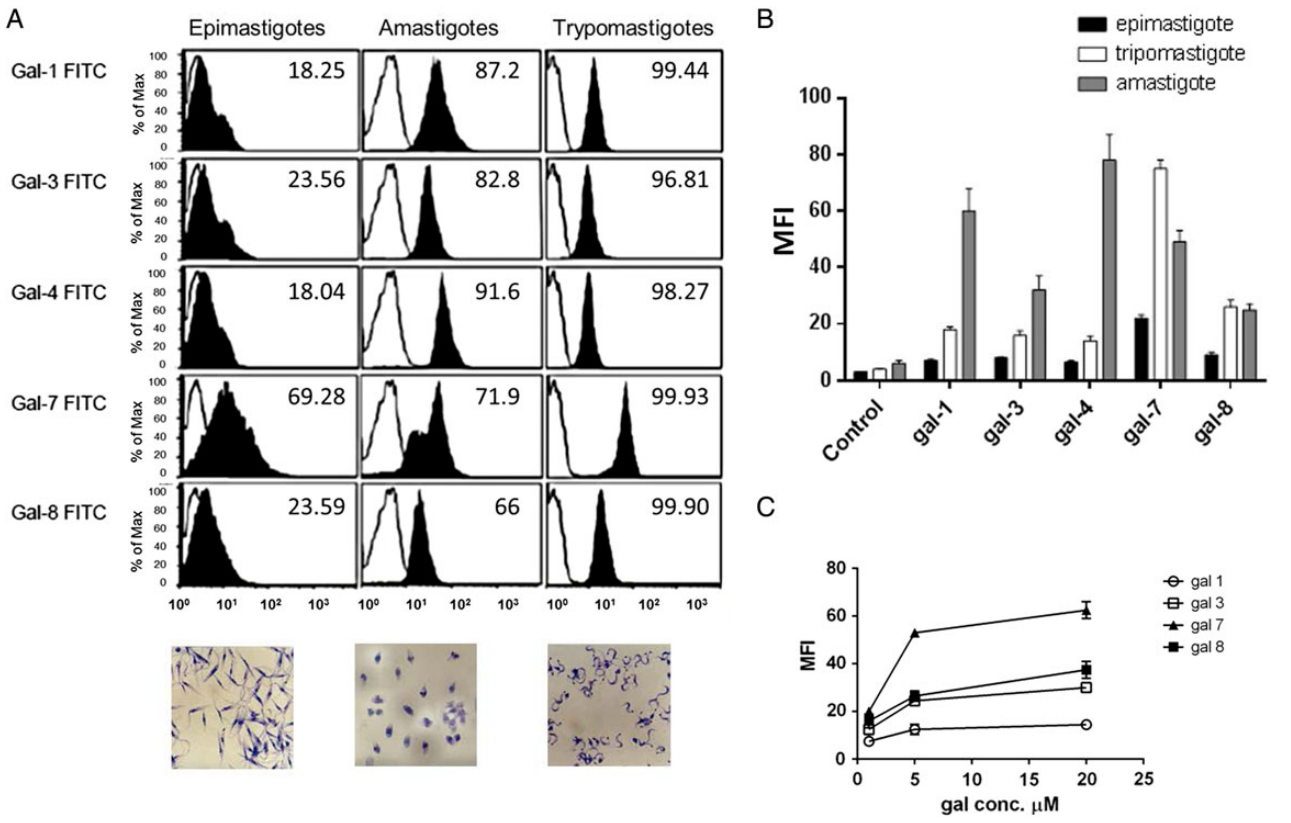

Fig. 2. Human recombinant galectin binding to *T. cruzi* (strain Y, DTU II) is specific for different biological stages. Alexa488-labeled recombinant human galectins were incubated with three parasite life cycle stages at 4°C and galectin binding was quantified by flow cytometry. (**A**) Alexa-488-galectins were able to bind *T. cruzi*, and the affinity of galectins was higher for the infective forms of the parasites under the same experimental conditions. Parasites incubated with galectins are shown in solid black, unlabeled negative controls in white. The insets show a representative micrograph of the parasite population employed for each analysis. (**B**) Mean of fluorescence index (MFI) of galectins (5 μM) binding to the three life forms. Median ± SD of five independent experiments is shown. (**C**) MFI of Alexa-488-labeled recombinant human gal-1, -3, -7 and -8 bound to CTT vs. galectin concentration. The median ± SD of five different experiments is shown.

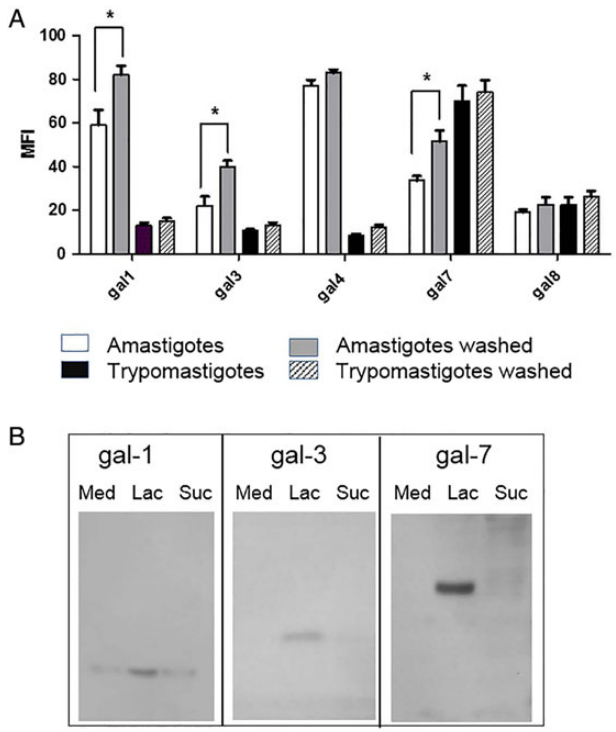

Fig. 3. Amastigotes and CTT bind host galectins. (**A**) Amastigotes or cell-derived trypomastigotes were washed with lactose or sucrose as described in Materials and Methods before being submitted to flow cytometry analysis of galectin binding. The median ± SD of three different experiments is shown. Asterisk indicates significant at $P < 0.05$. (**B**) Wash media from amastigotes recovered by centrifugation and filtration on 0.22 μM was resolved by SDS–PAGE and WB with specific anti-galectin antibodies (for gal-1 and -3) or streptavidin–HRP for biotinylated gal-7.

increased for gal-1, -3 and -7 ($P < 0.01$, Figure 3A). This is a clear indication that amastigotes are recruiting intracellular galectin (or any other galactoside-binding protein) from the infected host cells cytosolic pool. No significant difference was observed regarding gal-4 and -8. In contrast, when CTT were subjected to the galactose wash; no increase in the MFI was found when compared with control non-washed trypomastigotes (Figure 3A), suggesting either they are not recruiting intracellular galectin or the bound proteins are released/processed shortly after being exposed to the extracellular milieu. Another alternative is that trans-sialidase action makes any exposed galactose residue, cryptic for the galectins.

The protein content obtained after galactose washing of isolated intracellular amastigotes was subjected to SDS separation and western blot to detect host galectins by using specific antibodies or streptavidin–HRP to detect biotinylated-gal-7 (Figure 3B). As expected, when sucrose or mannose was used instead of galactose, galectins were not released from amastigotes, and it did not affect galectin binding observed by FACs (not shown).

An alternative explanation to the "coating" with endogenous galectins as responsible for the diminished binding of exogenous galectins would be that the recently released amastigotes do not expose the galectin ligands and are unmasked or exposed immediately after being released in the media.

In order to test this hypothesis, we set up in vitro infections of LLC-MK2 or Vero cells and after 4/5 days the cells were processed for immunofluorescence to examine the pattern of endogenous galectin distribution. Interestingly, non-infected cells expressed a uniform cytosolic staining pattern for gal-1 and -3 (Figure 4D and E, arrow head), in clear contrast to infected cells where galectin expression was accumulated on the cell surface of intracellular amastigotes (Figure 4A and B arrows), confirming that intracellular amastigotes are coated by host endogenous galectins. The possibility of a cross-reacting endogenous parasite protein homologous to host galectin was ruled out by flow cytometry (as shown in the controls in Figure 3A) and reports in the literature showing no specific cross-reactivity of anti-gal antibodies against *T. cruzi* antigens (Giordanengo et al. 2001).

Galectin binding to parasites is carbohydrate recognition domain dependent

Our data showed that the binding of galectins to *T. cruzi* was inhibited by lactose, but not sucrose, suggesting that the lactose-binding domain is involved in the parasite–protein interaction. To confirm that the binding of the human galectins to the parasites was carbohydrate recognition domain (CRD) mediated, we next carried out the binding assays in the presence and absence of specific inhibitory ligands of galectins. Galectin binding was completely inhibited by lactose at 50 mM (Figure 5A) or thiodigalactoside at 10 mM, indicating that the CRD was involved in the recognition to the sites exposed on the surface of the parasites. However, we failed to inhibit galectin binding when β-galactosidase-treated parasites were used, perhaps as a result of an incomplete access of β-galactosidase to the ligands due to steric hindrance from the dense mucin layers or/and to high protein turnover rate found in *T. cruzi*.

Unambiguous evidence supporting the implication of the CRD for the tandem-repeat gal-8 was obtained by using the recombinant human protein carrying punctual mutations (gal-8R69, gal-8R233 and gal-8R69R233, resulting in a non-functional N-, C terminal and both N- and C-terminal CRDs, respectively) that abolished the binding to their natural ligands (Nishi et al. 2006). Gal-8R69 and gal-8R233 presented reduced binding to CTT and the null double mutant showed an absolute lack of binding, confirming that the CRD of gal-8 is directly involved in the binding to parasite ligands.

Gal-3 is the only one member of the chimera-type galectin group, constituted by a CRD at the C-terminus and a collagen-like domain at the N terminus that could bind *T. cruzi* in a CRD-independent manner. In order to determine which domain of gal-3 is responsible for the interaction with *T. cruzi*, we studied the binding properties of a truncated gal-3 constituted only by the C-terminus domain including the CRD after releasing the collagenous domain by collagenase treatment (Kopitz et al. 2001). As shown in Figure 5C, removal of the collagen-like N-terminal domain does not affect the binding to trypomastigotes and amastigotes, indicating that the recognition and binding of gal-3 to the parasite is also CRD mediated.

Galectin binding promotes adhesion to host cells

We next tried to address the functional relevance of the galectin-trypomastigote interaction on parasite adhesion and/or infection to host cells. To evaluate this, we next performed in vitro adhesion and infection assays using several cell lines as models.

Parasite adhesion to THP-1 cells in the presence of recombinant galectins was quantified by using a cell cytometry approach in which the gating of free CFSE-labeled live CTT (strain Y) can be easily separated from unlabeled THP-1 cells according to their respective forward and size scatter values. Therefore, any increase in fluorescence in the cell's gate is proportional to the number of CFSE-labeled

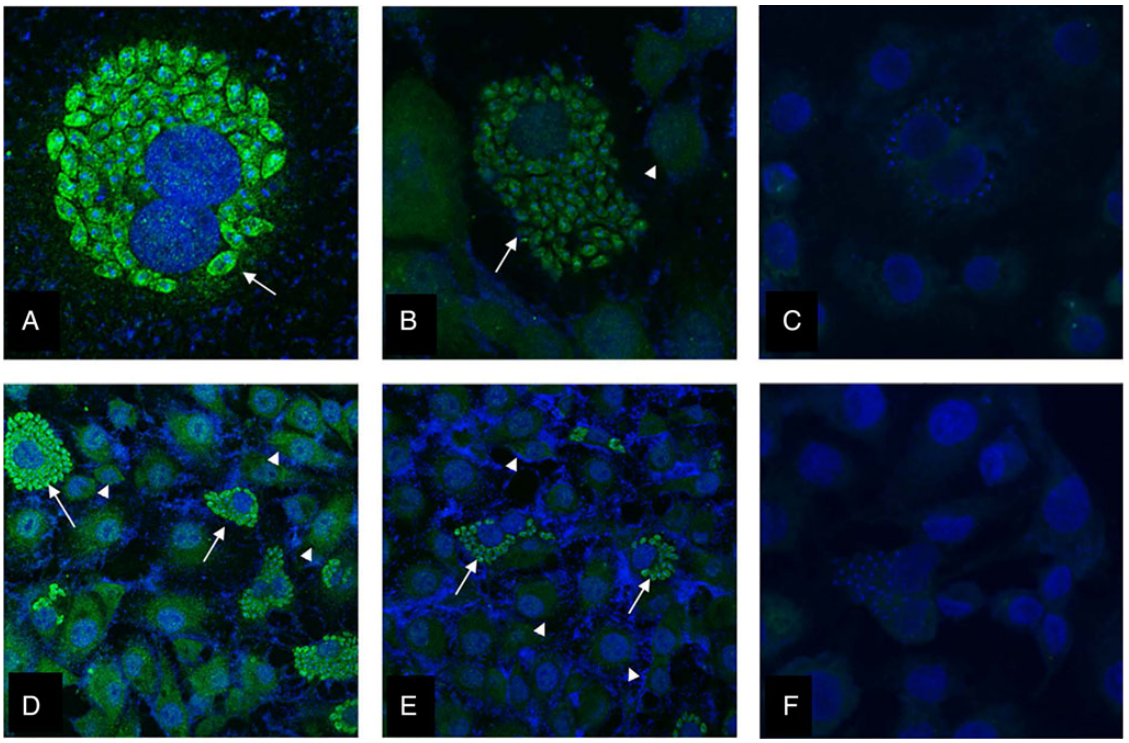

Fig. 4. Intracellular amastigotes bind intracellular pool of galectins. LLC-MK2 cells (**A**, **C** and **D**) or Vero (**B**, **E** and **F**) growing onto glass coverslips were infected as described. After 4 days p.i., the cells were processed for immunofluorescence and stained with anti-gal-1, -3 and -7 or just secondary antibody.

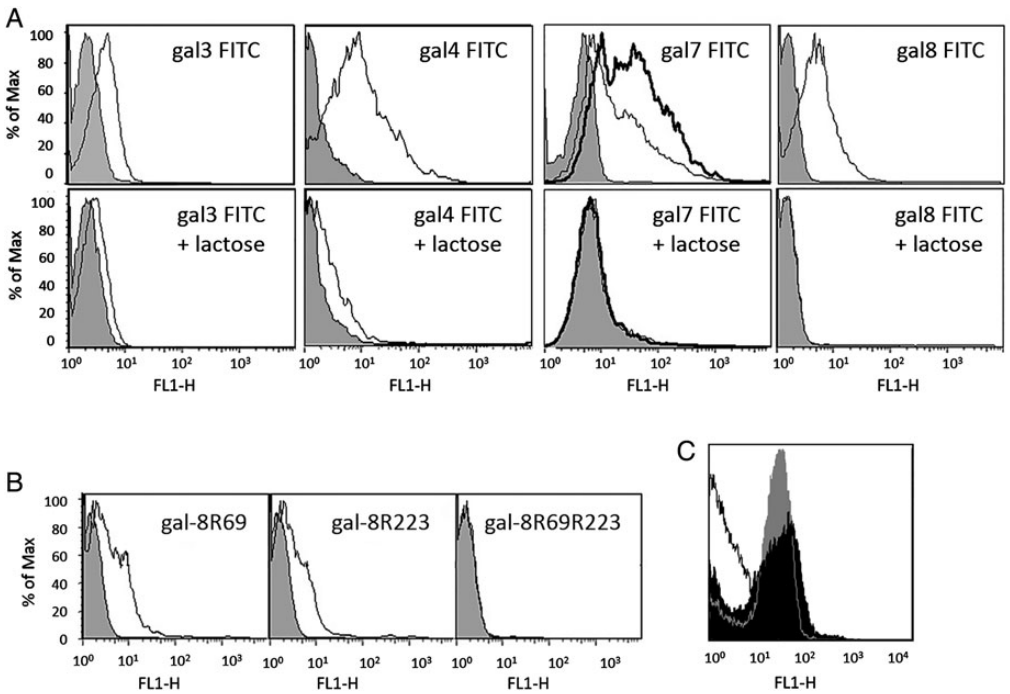

Fig. 5. Galectin binding to trypomastigotes is mediated by the CRD. (**A**) Negative controls are in solid gray, thin black line refers to parasites after incubation with FITC galectins, 2 μM, for 15 min at 4°C. Parasite-associated fluorescence was quantified by flow cytometry. In the case of gal-7, the thicker black line (—) corresponds to a galectin concentration of 20 μM, and the thin line (–) to 2 μM. (**B**) Metacyclic trypomastigotes were incubated with FITC-labeled gal-8, and with the FITC-labeled mutants R69, R233 and R69R233 for 15 min at 16°C. The mean of fluoresce intensity was measured by flow cytometry. All the mutants showed a reduced binding capacity to *T. cruzi*. (**C**) Flow cytometry analysis of truncated gal-3 binding to trypomastigotes of *T. cruzi* strain Y. Negative control (no gal-3, white), full-length gal-3 (gray trace), truncated C-terminal CRD of gal-3 (black trace).

parasites that have been attached to those cells. Mixtures of parasites and cells in the presence or absence of galectins were submitted to analysis. We limited the analysis time to 15 min in order to look just for adhesion, and the temperature to <16°C to keep parasites live but blocking endocytosis of galectins, limiting protease activity. A positive correlation between the affinity shown by individual galectins and their ability to promote parasite adhesion to host cells was observed (Figure 6A). Thus, gal-3, -7 and -8, the galectins that showed a higher affinity towards parasite infective forms, were those that promoted parasite adhesion to THP-1 cells, whereas, gal-1 and -4, with lower affinity towards trypomastigotes (strain Y), did not increase parasite adhesion to THP-1 cells compared with control (no exogenous galectin). Similar results were obtained with other different strains tested (data not shown).

Like the galectin binding to isolated cells, the galectin-mediated adhesion of parasite to host cells was shown to be dependent on the CRD as it was abolished by specific haptens, such as lactose, or by mutation of both CRDs found in gal-8 (Figure 6B).

As monomeric gal-1 and -7 exhibited concentration-dependent oligomerization affecting their valency, we decided to test the effect of such functional aggregation concentration dependent on the binding. Thus, two concentrations of gal-1 and -7 were tested; 0.2 μM (mainly monomeric) and 2 μM (mainly dimeric and/or higher oligomers). The data shown in Figure 6C suggest that cell-binding promoting activity of this galectin relies on its multivalent properties acting as a molecular bridge between parasites and host cells.

Similar assays were carried out with several adherent cell lines to mimic the physiological environment found in vivo, where *T. cruzi* infects epithelial cells, showing a broadly similar pattern of galectin-induced adhesion (Figure 6D).

To further investigate the relevance of galectin–*T. cruzi* interaction, a new set of experiments was conducted to define whether the observed galectin-mediated adhesion to host cells translates into higher infection rates. Data shown in Figure 7 support that idea, as in the presence of galectins, concomitant with an increased binding to the host cells, there is an increased infective ability evidenced by both

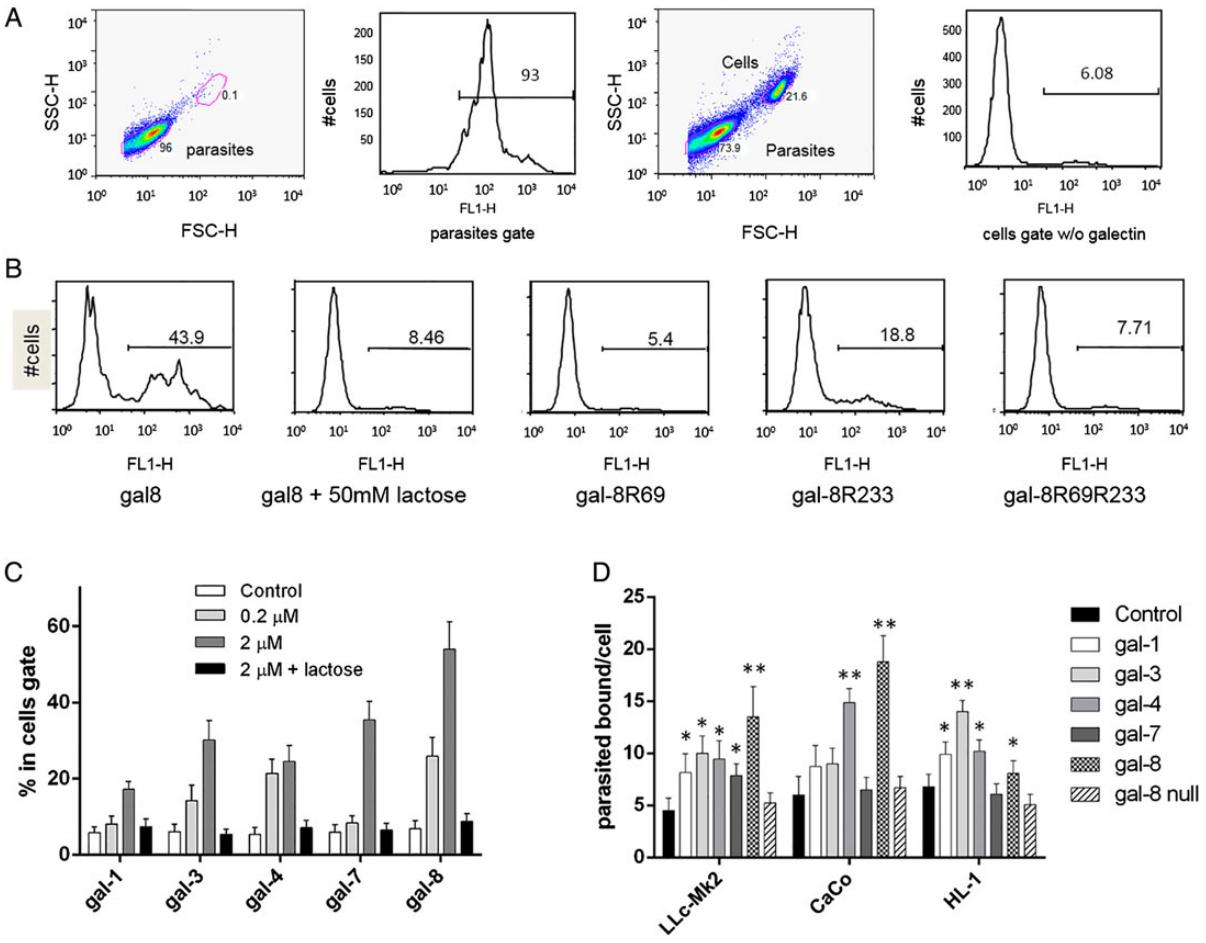

Fig. 6. Recombinant human galectins promote parasite adhesion to host cells. (**A**) CTT (strain Y) were labeled with CFSE, and incubated with non-labeled cells. Parasites and cells were resolved by flow cytometry due to their different size, and the fluorescence intensity associated to the cell gate (THP-1) was quantified. Almost 100% of the parasites presented high fluorescence intensity, and only 6% of the cells showed fluorescence intensity when incubated with parasites in the absence of rGals. (**B**) Aliquots of gal-8 was added to the mixture of cells and parasites for 10 min at 16°C, and the rate of positive cells increased to 43.9% of the total. This binding was abolished in the presence of lactose 50 mM, or when gal-8 R69, R233 and R69R233 were used instead of the wild-type galectin. (**C**) Mean fluorescence intensity is shown using the same approach with gal-1, -3, -4, -7 and -8, at 0.2 and 2 μM, in the presence and absence of lactose. Representative results from two independent experiments performed is shown. (**D**) CTT (strain Y, DTU II) were incubated with an LLC-MK2, CaCo and HL-1 cells monolayer for 15 min at 16°C in the presence or absence of 2 μM recombinant galectin, after extensive washing, the attached parasites were counted.

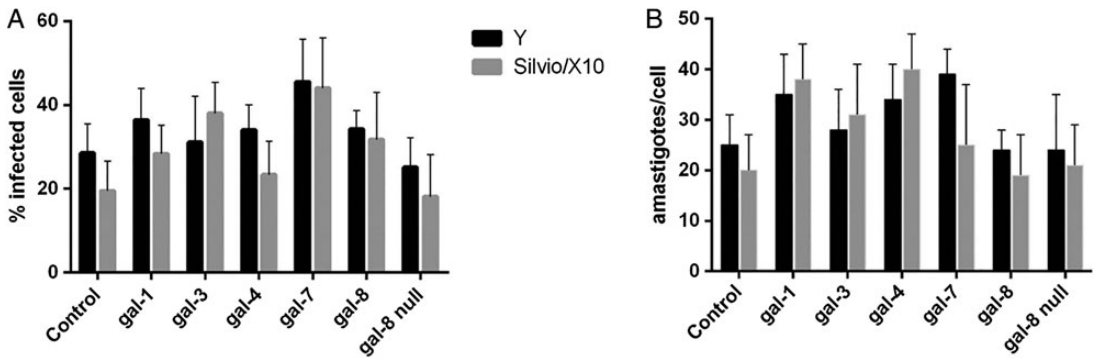

Fig. 7. Galectin binding promotes productive infections. CTT (strain Y, DTU II) were incubated with LLC-MK2 cells for 15 min at 16°C in the presence or absence of 2 μM recombinant galectin, after extensive washing, the cells were incubated at 37°C for up to 5 days. At indicated times, cells were washed twice with PBS, fixed with Bouińs fixative solution and stained in Giemsa solution. The intracellular amastigotes were quantified by counting randomly at least 300 cells.

the number of infected cells (Figure 7A) and the number of amastigotes per cell (Figure 7B).

Identification of surface mucins as galectin ligands on *T. cruzi*

Once it was shown that galectins bind to *T. cruzi*, attempts to identify the galectin ligand on the parasite cell surface were carried out by using biotin-labeled recombinant galectins. It was detected a diverse array of protein bands ranging from 10 to 100 kDa, specific and variable for individuals galectins. A discrete band ~80–70 kDa was common for all galectins tested; however, the total number of proteins detected was qualitatively different for each galectin (Figure 8A). Gal-4 seemed to be the more restricted ligand galectin, with just the band ~80–70 kDa detected in the eluate, in contrast to gal-1 that showed the most complex pattern of ligands. Interestingly, gal-7 and -8 showed an almost identical pattern of ligands. To identify the parasite proteins contained in the samples, identical membranes were sliced and subjected to peptide mass fingerprinting. The identified proteins are shown in Table II. By using specific antibodies again parasite mucins, the presence of the mucin AgC10 among the human gal-7 and -8 ligands was demonstrated (Figure 8B).

Unweighted pair-group median arithmetic analysis

As galectins are relevant factors in the outcome of immune responses, we decided to test whether a differential galectin binding could be associated to different DTUs described for *T. cruzi*, since DTUs are also associated to clinical manifestations. A similar binding pattern to different *T. cruzi* strains would indicate that inter-strain differences on exposed glycans are not significant and hence not being a discriminatory element, or in contrast, showing strain specific galectin binding (distinguishing specific traits like type of galactose containing glycans exposed on the cell surface). In a similar fashion as the referenced strain Y, intracellular amastigotes and CTT from the strains tested (Table I) were the forms that showed the highest binding for all galectins tested. Furthermore, some differences were found when the MFI for each galectin-binding profile was analyzed (shown in Tables III and IV). Based on these differences, we decided to carry out an unweighted pair-group median arithmetic (UPGMA) analysis of MFI values vs. DTUs to identify whether there was a common pattern for strains belonging to the same lineage. The resulting dendrogram shows that the 14 strains were grouped in six clearly discrete clusters when CTT were analyzed (Figure 9A) and in five clusters when amastigotes were utilized (Figure 9B) where the last cluster included strains from DTUs IV and V.

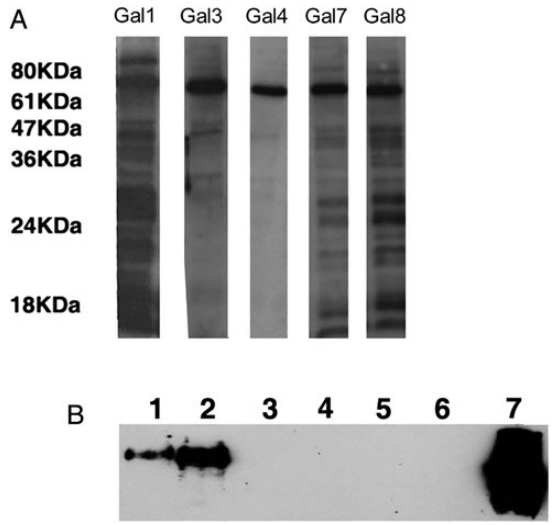

Fig. 8. Identification of parasite galectin ligands. (**A**) CTT protein extracts were applied to immobilized galectin columns, washed extensively and eluted with 100 mM lactose, resolved by SDS–PAGE, transferred to nitrocellulose membranes and blotted with biotin-labeled recombinant galectins, and revealed with streptavidin–HRP. (**B**) Either Gal-7-GST or Gal-8-GST was immobilized to Glutation-Sepharose® and CTT extracts were applied. GST immobilized was used as a control. After extensive washing, the column was eluted with 100 mM lactose. Eluted fractions were subjected to western blot using specific antibodies against *T. cruzi* mucin AgC10. 1: Gal-7-GST column lactose elution, 2: Gal-8-GST column lactose elution, 3: GST column lactose elution, 4: blank Sepharose lactose elution, 5, 6: washing elutions, 7: purified AgC10.

Discussion

Specific interactions between galectins and glycoconjugates are considered to be critical determinants in pathogen recognition (Rabinovich and Gruppi 2005; Vasta 2009; Paz et al. 2010). There is no direct evidence whatsoever regarding different galectins binding to the three life forms of this pathogen, and more important yet, there is no data on differential interaction of innate immune components (c-type lectins, galectins, etc.) to different parasite genetic lineages. Under this premise, we

Table II. Proteins from *T. cruzi* cell-derived trypomastigotes (strain Y) bound to galectins

	Protein (MW)	Sequence of identified peptide	Accession number
Gal-1	Surface protease GP63 putative	R.GRPVVGVINPR.H	Q4CM87
		R.NVGEVTGGEEPASPVTVSVGSDWAPLR.I	
		R.LLVRPLDGPLVVPR.F	
		R.SSVHVVNSR.N	
		R.FREGSVCGK.F	
	GP90	K.AYTVLGPTDGTDNRVGFFYHPTTTTK.L	AAM47176
		K.QSTIDAHEVK.L	
		K.LTESDSEVMWPVNTR.V	
		K.VFLLVGSLGELK.E	
		R.EPTDSEPTGGITWGEIK.S	
	GP82	R.GEIDAQYAVDGK.L	ABR19835
		K.GNLDVVLSPTTTMK.G	
		R.KVMLYTQR.G	
	Mucin TcMUCII, putative	K.MNVNSEGSNTQEDEEGGRNK.A	EAN93978.1
	Calcium-binding protein	K.VEDPAALFK.E	BAA13411.1
		K.LDEFTPR.V	
	Surface protein-1	K.APSESTPLLGAGLGDNDGTK.F	AAB18265.1
	Trans-sialidase, putative	K.SLLGQIAPQAQGDSK.V	EAN98599.1
		K.NFFLYNRPLSADELK.M	
Gal-3	Surface protease GP63 putative	R.LLVRPLDGPLVVPR.F	Q4CM87
		K.VDILENVILSEAAK.M	
	GP90	K.AYTVLGPTDGTDNRVGFFYHPTTTTK.G	AAM47176
		K.LTESDSEVMWPVNTR.V	
		K.TTESGTWEPGKEYQVAL.M	
		R.KVMLYTQR.G	
		R.EPTDSEPTGGITWGEIK.S	
	GP82	K.LVVGEVTKPSAGGEPSG.W	ABR19835
		K.FTGFGSGAIWPVNNR.E	
	Trans-sialidase, putative	K.SLLGQIAPQAQGDSK.V	EAN98599.1
Gal-4	Surface protease GP63 putative	R.LLVRPLDGPLVVPR.F	Q4CM87
		R.SSVHVVNSR.N	
		R.FREGSVCGK.F	
		R.NVGEVTGGEEPASPVTVSVGSDWAPLR.I	
		R.VAVHEM*AHALGFIVTDM*EGQALVK.R	
		K.VDILENVILSEAAK.M	
	GP90	K.GELSSSLLYSDGNLQLLQQR.G	AAM47176
		R.VGFFYHPTTTTK.G	
		K.SQSFFSDLK.L	
		R.EPTDSEPTGGITWGEIK.S	
		K.EDGENCLLSTGVSPAK.C	
Gal-7	Surface protease GP63 putative	K.VDILENVILSEAAK.M	Q4CM87
		R.LLVRPLDGPLVVPR.F	
		R.FREGSVCGK.F	
	Mucin TcMUCII, putative	K.MNVNSEGSNTQEDEEGGRNK.A	EAN93978.1
Gal-8	Surface protease GP63 putative	R.FREGSVCGK.F	Q4CM87
		R.LLVRPLDGPLVVPR.F	
	GP90	R.KVMLYTQR.G	AAM47176
		K.LTESDSEVMWPVNTR.V	
		K.AYTVLGPTDGTDNRVGFFYHPTTTtK.G	
		R.EPTDSEPTGGITWGEIK.S	
	GP82	K.NVFLYNPRPLGADELR.M	ABR19835
		K.FTGFGSGAIWPVNNR.E	
		K.GNLDVVLSPTTTMK.G	
	Mucin TcMUCII, putative	K.MNVNSEGSNTQEDEEGGRNK.A	EAN93978.1

decided to ascertain whether different human galectins are able to specifically recognize glycans exposed on the cell surface of the three life forms of the pathogenic parasite *T. cruzi*.

It is known that the parasite surface glycoconjugates are different in every biological stage of the parasite (de_Lederkremer and Colli 1995; Ferguson 1997; Bourguignon et al. 1998; Colli and Alves 1999; de Lederkremer and Agusti 2009). Our results show that the life forms present in the host (amastigotes and trypomastigotes) are recognized to a greater extent than the non-infective epimastigotes, reflecting the changes that take place in membrane composition, accessibility and exposure of glycans on the infective forms, probably related to the infective capacity.

Table III. Median fluorescence index (MFI) for each galectin-FITC against CTT from the indicated strains

Strain	DTU	MFI gal-1	MFI gal-3	MFI gal-4	MFI gal-7	MFI gal-8
Silvio/X10 c1	TcI	15 ± 3	48 ± 5	14 ± 4	48 ± 4	19 ± 4
Dm28c	TcI	17 ± 4	47 ± 7	15 ± 5	51 ± 9	22 ± 5
Esmeraldo c3	TcII	15 ± 3	19 ± 4	16 ± 7	80 ± 6	30 ± 8
Y	TcII	18 ± 4	15 ± 6	19 ± 5	77 ± 8	26 ± 6
Tu18 c2	TcII	17 ± 5	18 ± 4	21 ± 6	75 ± 8	28 ± 9
Cm17	TcIII	19 ± 3	38 ± 5	20 ± 5	55 ± 7	39 ± 7
M6241 c6	TcIII	22 ± 5	39 ± 7	24 ± 6	60 ± 10	41 ± 6
Can III	TcIV	28 ± 7	38 ± 5	19 ± 7	65 ± 8	46 ± 7
10R26	TcIV	35 ± 7	37 ± 6	18 ± 8	57 ± 10	31 ± 8
Bug2148 c11	TcV	18 ± 5	40 ± 5	16 ± 7	68 ± 6	31 ± 3
Sc43 c1	TcV	19 ± 5	42 ± 3	19 ± 4	78 ± 7	32 ± 2
Tula c2	TcVI	20 ± 5	26 ± 6	21 ± 5	57 ± 5	21 ± 3
VFRA c1	TcVI	21 ± 4	28 ± 2	22 ± 8	49 ± 4	20 ± 3
CL-Brener	TcVI	19 ± 3	27 ± 4	25 ± 4	55 ± 4	24 ± 3

Each flow cytometry analysis was carried out three times and the median ± SD is shown.

Table IV. Median fluorescence index (MFI) for each galectin-FITC against amastigotes from the indicated strains

Strain	DTU	MFI gal-1	MFI gal-3	MFI gal-4	MFI gal-7	MFI gal-8
Silvio/X10 c1	TcI	19 ± 7	25 ± 4	91 ± 10	78 ± 8	47 ± 8
Dm28c	TcI	27 ± 5	27 ± 6	87 ± 8	71 ± 7	41 ± 9
Esmeraldo c3	TcII	87 ± 5	29 ± 8	125 ± 10	79 ± 3	30 ± 8
Y	TcII	78 ± 8	31 ± 5	94 ± 8	58 ± 9	21 ± 4
Tu18 c2	TcII	97 ± 7	35 ± 9	130 ± 12	75 ± 8	28 ± 9
Cm17	TcIII	45 ± 6	88 ± 43	150 ± 11	98 ± 8	61 ± 7
M6241 c6	TcIII	41 ± 8	97 ± 6	112 ± 9	87 ± 11	71 ± 8
Can III	TcIV	39 ± 6	65 ± 7	75 ± 8	99 ± 7	44 ± 8
10R26	TcIV	45 ± 4	59 ± 8	85 ± 7	125 ± 15	43 ± 3
Bug2148 c11	TcV	54 ± 7	71 ± 2	67 ± 4	98 ± 3	41 ± 8
Sc43 c1	TcV	61 ± 3	81 ± 3	57 ± 9	108 ± 11	29 ± 7
Tula c2	TcVI	27 ± 4	52 ± 8	90 ± 4	57 ± 5	31 ± 5
VFRA c1	TcVI	32 ± 8	68 ± 3	81 ± 9	65 ± 3	40 ± 6
CL-Brener	TcVI	39 ± 7	77 ± 9	78 ± 10	70 ± 7	38 ± 7

Each flow cytometry analysis was carried out four times and the median ± SD is shown.

The major glycoconjugates of *T. cruzi*, the mucins, are longer in the trypomastigotes and contain additional α-galactopyranosyl residues (Almeida et al. 1999; Buscaglia et al. 2006). Those changes are supposed to allow adaptation and survival in a new environment, where the parasite will encounter immune mechanisms controlled by immune regulators, such as galectins. Whether the parasite evolved to display galectin ligands in the infective forms, or the host developed galectins with greater affinity towards infective form glycans is difficult to predict over the common evaluative pressure of both parts. In that context is worth mentioning that mannose receptor and mannose-binding proteins have been described to favor binding of amastigotes to macrophages that had not been activated by IFN-γ (Kahn et al. 1995).

We observe that intracellular amastigotes are the parasite form that binds galectins with the highest affinity. In that sense, it results interesting that intracellular amastigotes are "coated" with the cytosolic soluble galectin pool from infected cells. It has been described an alternative infection cycle inside the host, where intracellular amastigotes released from lysate cells may initiate a new round of infection by attaching and then infecting neighbor cells. Besides altering the normal functions of cytosolic galectins (by altering the intracellular pool) that galectin coating of amastigotes could favor a fast entry into neighbor cells by promoting adhesion and facilitating invasion with galectins acting as bridge between cells and parasites. Another possibility for the "decoration" with galectins would be to induce a receptor rearrangement of the galectin lattice at the host cell surface modification in that way the signaling pathways that allow the amastigote to survive in the extracellular milieu before obligate transformation to trypomastigotes or initiate a new invasion cycle or simply tagging the amastigotes with a danger signal that favors the engulfment by macrophages. Whether this "coating" or "recruiting" of intracellular galectins confers to the parasite any advantage is just a matter of speculation at the moment. Remarkably, recent findings showed a recruitment of structures expressing gal-3 to vacuoles containing *T. cruzi* amastigotes (Machado et al. 2014; Reignault et al. 2014), and some reports showed that gal-3 covers intracellular Mycobacterium-containing phagosomes promoting pathogen killing (Beatty et al. 2002). An intriguing alternative is that sequestering cytosolic gal-3 may impair the phagocytic capacity of already infected cells by reducing actin cytoskeletal rearrangement (Sano et al. 2003). There are several reports showing the ability of galectins to bind to pathogens, such as viruses (Levroney et al. 2005; Ouellet et al. 2005), bacteria (Barboni et al. 2005; Fowler et al. 2006), (Mey et al. 1996), *Candida* sp. (Kohatsu et al. 2006) and the helminth *Schistosoma mansoni* (van den Berg et al. 2004). In addition, gal-3 and -9 bind *Leishmania major* (Pelletier and Sato 2002; Pelletier et al. 2003; van den Berg et al. 2004). Regarding *T. cruzi*, only gal-3 has been described to interact with the parasite (Moody et al. 2000; Kleshchenko et al. 2004) to promote adhesion to coronary artery smooth muscle cells and to extracellular matrix via a laminin bridge. Our data provide evidence that gal-3 binds *T. cruzi* amastigote surface, and for the first time we provide evidence that also other galectin members like gal-1, -4, -7 and -8 can bind the parasite, preferentially amastigotes and CTT of all genetic lineages of *T. cruzi*. Of note, galectin binding may render more productive infections for the parasite protozoan. The relevance of *T. cruzi*–galectin interaction might vary depending on the individual galectin and on the cell type or matrix component that the parasite interacts with. If that is the case, our data suggest that *T. cruzi* could use different combinations of galectins to adhere and to infect cells in different tissues, perhaps reminiscent of the differential tropism exhibited by different lineages.

Gal-7, located mainly on skin and stratified epithelia (Magnaldo et al. 1995, 1998), is the only galectin that showed significant binding to the epimastigotes form. Epimastigotes are present on the vector and despite some reports showing infectivity (Burger et al. 1982), they are generally considered to be non-infective. The significance of this might be related to the presence of epimastigotes or metacyclic trypomastigotes excreted in the feces (Schaub and Losch 1988). That opens the possibility that gal-7, located at the point of parasite entry, such as the skin, could have evolved to interact with parasite forms found there at the moment of infection, before new infective forms arise in the host.

Using a classical approach of affinity chromatography allowed us to partially identify, by peptide mass fingerprinting, a very limited and restricted group of membrane proteins from CTT as ligands. Two proteins were found to be recognized by all the human galectins tested, the putative protease GP63, GP90. The GP82, a putative mucin (TcMUCII) and a putative transialidase were also common ligand partners for the human galectins tested in this study. Using an alternative approach

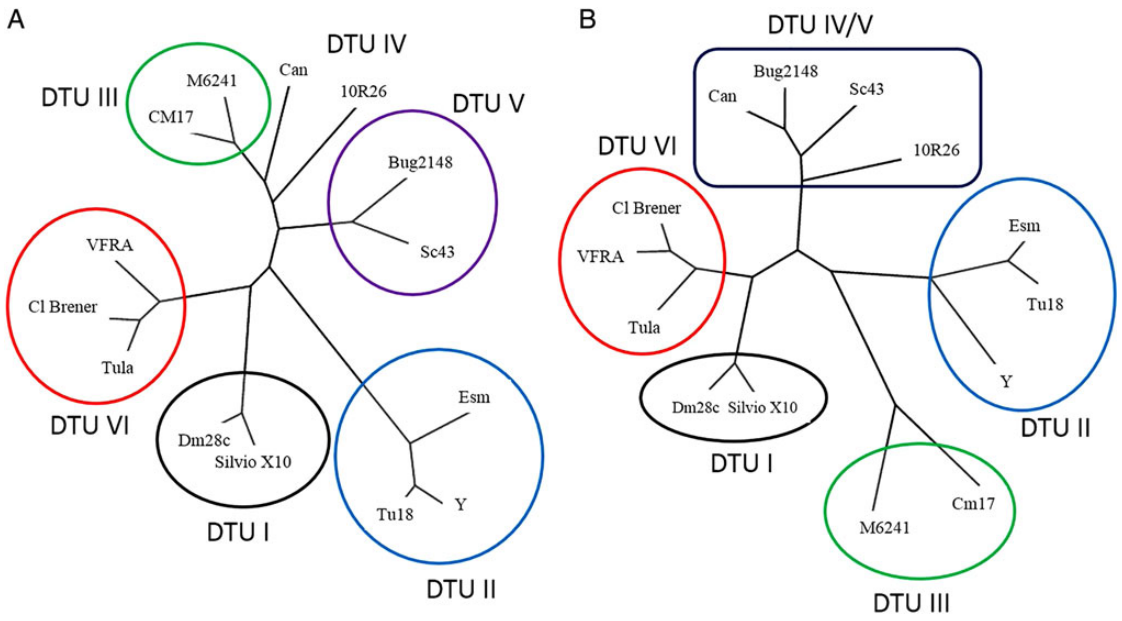

Fig. 9. UPGMA analysis of galectin-binding profiles. A matrix was built with the data shown in Tables III and IV, presenting the Median Fluorescence Index (MFI) for the binding of each galectin to CTT (**A**) or amastigotes (**B**) of each parasite strain in order to construct a dendrogram reflecting relationships between the different *T. cruzi* DTUs represented by the reference strains used in this study.

like western blot using soluble streptavidin-labeled galectins, in which the multivalency of galectins is preserved, we could observe many more unidentified ligands. A band ~70–80 kDa was the main *T. cruzi* trypomastigote ligand of all the galectins tested. We could identify just one potential ligand of gal-3, -7 and -8 as the mucin AgC10 (Kierszenbaum et al. 2002; Alcaide and Fresno 2004a, 2004b). Moody et al. (2000) showed a 45 kDa parasite mucin that was detected as a gal-3 ligand that could be AgC10. Gal-3 could be implicated in the immunosuppressor activities attributed to the mucin, as is known that gal-3 modulates T cell responses by control of TCR clustering at the immune synapse (Demetriou et al. 2001). Other galectins might be concerned as it could bind to common receptors (Patnaik et al. 2006), although the biological functions derived from different transduction signals must not be necessarily the same.

As galectins are multivalent proteins, they can cross-link receptors mediating cell–cell adhesion and/or cell–extracellular matrix. Therefore, experiments were conducted to evaluate the ability of galectins to promote parasite adhesion to host cells. The galectin-induced adhesion was both concentration- and CRD-dependent. When gal-8 null mutant was used, adhesion was abrogated, suggesting that an active CRD is required to favor parasite adhesion to host cells. Nevertheless, the gal-8 mutant that has a non-functional N-terminal domain, gal-8R233, is still able to promote parasite adhesion to host cells, although in a lesser extent than the wild type. Gal-8R233 is supposed not to cross-link glycosylated receptors, as only one-terminal domain of the protein keeps CRD. It could be argued that the mutated CRD interacts with some host molecules in a sugar independent way. However, that is not occurring in the parasite binding because lactose inhibits wild-type gal-8 binding to the parasite. Another explanation, although gal-8 is a tandem repeat, is that the protein can form homodimers through the N-terminal CRD, as it has been published before (Stowell et al. 2008). In that way, gal-8 could still bind with relative high affinity to cognate ligands.

A way in which the galectin binding to the parasites could be modified by altering the galectin ligands exposure, for example by β-galactosidases or the sialyltransferase ST6 (Zhuo and Bellis 2011) acting as negative regulators. It could be argued that the transialidase (TS) from *T. cruzi*, considered a virulence factor, could act as such modulator, but that is not the case as the TS does not transfer sialic acids linked α2–6 (Vandekerckhove et al. 1992) and hence could not modify the galectin binding to host self-ligands; however, there are no reports of *T. cruzi* β-galactosidase as virulence factor.

By evaluating whether different reference strains with clearly distinct biological behavior exhibit differential binding to host immune proteins like galectins, support to the notion that specific traits in the different genetic lineage or DTUs of *T. cruzi* could be associated to differential pathology is provided. If there is a differential galectin-binding profile for each genetic lineage that could mean that different immune responses networks would be triggered at different tissues and eventually be correlated to the differential course of the Chagas' disease pathology. This analysis allowed to build a tree with six groups clearly defined that closely resembles the one built solely on genetic data.

A parallel analysis taking the similarity matrix build on amastigote-galectin-binding profile data arranged the different lineages in five clusters containing each one a single DTU and a mixed cluster containing DTUs IV and V together.

Our findings are compatible with the idea that DTUs II (arranged in the tree far apart from the rest) and I are ancient parental lineages and that DTUs IV and V are recent ones derived from at least one recombination event (one cluster together or very close). This is to our best knowledge the first report of clustering the DTUs based on biological properties.

A great limitation of UPGMA is that it assumes the same evolutionary speed on all lineages, i.e., the rate of mutations is constant over time for all lineages in the tree, and so we must be very cautious implying that the rate of changes in glycan modifications on each lineage is constant and that the distances reflect true evolutionary distance. It is clear that further studies including more strains and isolates is necessary before any conclusions can be raised.

The results also reflect changes in the composition and quantity (ratio of enzymes involved in synthesis/modification of glycans) of "glycogenes" in the lineages genomes. It must be considered here that glycoconjugates are not direct products of the genome, but the result of a series of variable sequences of "glycogenes" actions, and subtle changes on them may result in a great diversity of structures, and for sure such variations may have profound implications in virulence, antigenicity and immune response deriving in variable pathogenesis (Varki 2011). It is known that *T. cruzi* lineages (Araujo et al. 2002), or different strains showing dissimilar infectivity/virulence (Piazza et al. 1996) can be distinguished in vitro by their different affinity towards PNA, suggesting that different lineages exhibit different glycans on their surface. This has been recently confirmed by Soares et al. (2012) showing differential expression of α-galactosyl residues in *T. cruzi* GPI-mucins, and by our group showing differential glycan profile by different strains of *T. cruzi* (Bonay and Staudacher, in preparation).

The composition of lineages in a natural infecting population may play a role in determining the outcome of the infection and may reflect the differential interaction with host proteins regulating the immune response. Providing evidence of discriminant reactivity of a family of immune relevant proteins against different lineages imposes new views on the causes and progression of the wide spectra of disorders associated to the disease. In addition, it reinforces the notion that differential outcomes of *T. cruzi* infection could be influenced by the complexity of the infecting *T. cruzi* population that interferes with host factors related to regulation of acute inflammatory response essential for protection against infection, but may also contribute to pathology. This represents the first step to extend this analysis to another relevant protein in order to increase the view on how the innate immune system perceives the different parasites/strains and thus provides a clue to elaborate complex networks of interactions and deciphers the fine-tuning events occurring with complex pathogens.

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Conflict of interest statement

None declared.

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Abbreviations

CRD, carbohydrate recognition domain; CTT, cell-derived trypomastigotes; DTUs, discrete typing units; TFA, trifluoroacetic acid; UPGMA, unweighted pair-group median arithmetic

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