



Giardia lamblia: identification of molecules that contribute to direct mast cell activation

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Abstract

Mast cells play a central role in the early clearance of the intestinal parasite *Giardia lamblia*. In a previous study, we reported that *G. lamblia* live trophozoites or trophozoite-derived total soluble extract induced direct activation (IgE-independent) of mast cells and release of IL-6 and TNF- α . To identify the *Giardia* molecules and the mast cell receptors involved in this activation, trophozoite-derived total soluble proteins separated into three fractions (F1–F3) were evaluated for its ability to activate mast cells in vitro. F2 activated mast cells in a greater extent than F1 and F3. Furthermore, F2 induced the release of IL-6 and TNF- α by mast cells. TLR2 and TLR4 expression increased slightly after mast cell stimulation with either F2 or total soluble extract; however, these receptors were not involved in F2 or total soluble extract-induced proinflammatory cytokine production. Proteins present in F2 as unique and high-intensity bands identified by liquid chromatography coupled with tandem mass spectrometry, include molecules with important biological activities such as enolase and arginine deiminase (ADI). Recombinant ADI and enolase were tested for their ability to activate mast cells, but only ADI induced a significant release of IL-6 and TNF- α . ADI product, citrulline but not ammonium, also induced mast cell release of TNF- α . Interestingly, recombinant ADI still stimulated the secretion of TNF- α by mast cells in a arginine-free medium, although in a lower extend that in the presence of arginine, indicating that either ADI itself can stimulate mast cells or through its metabolic product, citrulline.

Keywords *Giardia lamblia* · Mast cells · Activating molecules · Arginine deiminase

Introduction

Giardiasis is caused by the anaerobic protozoan *Giardia lamblia* (syn. *Giardia intestinalis*, *Giardia duodenalis*), and it is one of the major diarrhea agents in human and animals distributed worldwide (Ankarklev et al. 2015). It is estimated that approximately 200 million people

worldwide are infected with *G. lamblia* and in children under 5 years, giardiasis may produce severe acute diarrhea (Certad et al. 2017). Because of its epidemiological and public health implications, giardiasis was included in the WHO's Neglected Disease Initiative in 2004 (Savioli et al. 2006). In developing countries, the prevalence of giardiasis commonly ranges from 20 to 30% and from 2 to 3% in industrialized nations (Fletcher et al. 2012). Most *Giardia* infections in humans are asymptomatic, whereas others become acute or chronic disease associated with severe diarrhea, malabsorption, and weight loss. Multiple factors have been proposed to account for the disease variability, such as the host nutritional status, age, strain genotype, and the host immune system (Faubert 2000).

It has been demonstrated that both innate and adaptive immune responses play an important role during *Giardia* infection. Antimicrobial peptides, nitric oxide, mast cells, and dendritic cells are the first line of defense against *Giardia* (Saghaug et al. 2016). Moreover, T cells (CD4⁺) are essential for the early control of giardiasis in mice and long-term CD4⁺

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T cell memory response and IgA production, play a protective role in humans (Dann et al. 2015; Fink and Singer 2017).

The role for mast cells in controlling parasite infection has been demonstrated (Erlach et al. 1983; Hardin et al. 1997; Li et al. 2004). Mast cell-deficient mice infected either with *G. muris* or *G. lamblia* developed chronic giardiasis, whereas their wild-type littermates controlled *Giardia* infection rapidly (Erlach et al. 1983; Li et al. 2004). In jejunal tissue from Mongolian gerbils infected with *G. lamblia*, mast cell counts were correlated with increased epithelial permeability and macromolecular uptake, suggesting a link between development of these responses and mast cell recruitment (Hardin et al. 1997). Furthermore, smooth muscle contractions and changes in intestinal motility following *G. lamblia* infection are mediated through the release of mast cell granule contents. Indeed, mast cell responses coupled with nitric oxide-mediated muscle relaxation promote intestinal transit and parasite elimination in mice (Li et al. 2007). Consistent with these observations, inhibition of the mast cell products, histamine and serotonin, enhanced the intensity of *G. muris* infection in mice (Venkatesan et al. 1997). Recent data from a transcriptomic analysis of the host response to *Giardia* infection confirmed the importance of mast cell responses in parasite control. Outstandingly, mast cell genes were the most highly induced in the small intestine of *G. lamblia*-infected mice. Indeed, mast cell proteases 1 and 2, mast cell carboxypeptidase A3, mast cell chymase and the mast cell high affinity receptor, FcεRI, represented the transcripts with the largest induction (Tako et al. 2013).

In a previous study, we demonstrated (Munoz-Cruz et al. 2010) that mast cells were directly activated by both *G. lamblia* live trophozoites and total soluble extract (TSE) of trophozoites, inducing an increase of mast cell tryptase expression, release of histamine, and production of IL-6 and TNF-α, two key cytokines required for control of *G. lamblia* infection (Zhou et al. 2007; Zhou et al. 2003).

Since mast cells are crucial for controlling *G. lamblia* infections, we sought to identify the parasite proteins in TSE that may be involved in the direct activation of mast cells. For this, TSE was fractionated into three fractions (F1–F3) and tested for their ability to induce the release of TNF-α and IL-6. Furthermore, mounting evidence indicates that Toll-like receptors (TLRs) are implicated in the recognition of protozoan parasites (Ghosh and Stumhofer 2013); therefore, we investigated whether TLR2 and TLR4 could be involved in the release of IL-6 and TNF-α by mast cells stimulated either with *Giardia* TSE or fractions derived from it. In addition, to determine *Giardia*-specific proteins that participate in direct mast cell activation, we tested mast cell response to recombinant *Giardia* enolase (rGIENO) and arginine deiminase (rGIADI), two major immunodominant proteins secreted in vivo and in vitro during *G. lamblia* infection.

Materials and methods

Mast cells

The mast cell line HRMC (Hybrid rat mast cells), with a mucosal phenotype, was grown at 37 °C in a 5% CO₂ atmosphere, in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies). HRMC cell line was used for determination of tryptase exposure after activation. Rat peritoneal mast cells (PMCs), with a connective tissue phenotype, were used for histamine release assays because they have higher histamine content than HRMC cell line. PMC were obtained by lavage of the peritoneal cavity of noninfected male Sprague Dawley rats, as previously described (Munoz-Cruz et al. 2010). PMC purity was > 98% as determined by staining with toluidine blue, and cell viability was established as > 98% by trypan blue dye exclusion. Protocol for mast cell isolation from animals had approval of the Ethics Committee of the Mexican Institute of Social Security.

G. lamblia trophozoites culture

G. lamblia trophozoites (WB strain ATCC no. 30957) were cultured in TYI-S-33 medium supplemented with 10% calf serum and 10 µg/mL streptomycin, 10,000 UI/mL penicillin. Trophozoites in logarithmic growth phase were used for all the experiments.

Preparation of *G. lamblia* trophozoites total soluble extract

Parasite total soluble extract (TSE) was obtained as previously described (Chaudhuri et al. 1997), with slight modifications. *Giardia* trophozoites were harvested by chilling on ice for 30 min followed by centrifugation at 800g for 10 min. Pooled trophozoites were washed five times in sterile phosphate-buffered saline (PBS) pH 7.4, lysed by three freeze/thaw cycles and then sonicated three times with 30-s bursts. The suspension was centrifuged at 10,000g for 20 min at 4 °C. The clear supernatant containing the TSE was collected, and the protein concentration was determined by Bio-Rad DC protein assay (Bio-Rad Laboratories). The absence of endotoxin was tested using the Lit E-Toxate kit (Sigma Chemicals). The integrity and protein pattern of TSE (40 µg) was analyzed by 10% SDS-PAGE, gel dimensions 6.2 cm × 8 cm × 1.5 mm (height × length × thickness), run at a constant voltage (70 V) in cold room for 90 min 10%. Gel was Commassie blue stained.

Fractionation of *G. lamblia* total soluble extract and passive elution from polyacrylamide gels

To maintain the proteins secondary structure and their ability to activate mast cells, proteins contained in the TSE were separated by and recovered from native-PAGE according to the method previously described (Scheer and Ryan 2001), with slight modifications. Briefly, TSE proteins (500 µg) were separated by 10% native-PAGE, under nonreducing and nondenaturing conditions (without 2-mercaptoethanol and SDS) under the same conditions already described. A vertical slice of the gel was Coomassie blue stained to assess the presence of proteins. The unstained gel was sliced horizontally into three fractions (2 cm sections), named F1, F2, and F3. Each gel fraction was cut in small pieces using a new razor blade, placed into a 3.0-cm³ syringe and forced through the opening, without a needle, into a second syringe. PBS (1 mL) was added for five times to the gel material from each fraction and collected in a 1.5-mL tube; the mixture was vortexed for 30 s and incubated overnight at 4 °C. The gel material was centrifuged at 2000g for 5 min, and the supernatants containing the eluted proteins were collected, then concentrated with Amicon ultra-10 centrifugal filters (Millipore), aliquoted and stored at −70 °C and used to stimulate mast cells. To determine their protein patterns, F1–F3 were separated by 12% SDS-PAGE, under reducing conditions. Fixed gels were stained with Coomassie blue. Gel image was taken using the ChemiDoc™ XRS imaging system (Bio-Rad Laboratories), the molecular weight, number, and intensity of the bands detected in each protein fraction were determined using the 1-D analysis software Quantity One (BioRad Laboratories).

Detection of mast cell tryptase exposure by immunofluorescence

Tryptase exposure was determined by immunofluorescence as an indicator of mast cell activation. For this, HRMC (2.5×10^4 cells/200 µL) were stimulated with 100 ng/mL of each *G. lamblia* protein fractions (F1–F3) or parasite TSE for 30 min at 37 °C. Nonstimulated cells were included as control. After stimulation, cells were fixed in 4% paraformaldehyde, non-specific binding was blocked with 10% FBS plus 3% bovine serum albumin, and cells were stained with rabbit polyclonal antibody against mast cell tryptase (Santa Cruz Biotechnology) followed by FITC-conjugated goat anti-rabbit antibody (Invitrogen). Images were obtained using an Olympus BX52 Fluorescence microscope and the Image Pro Plus 6.2 software.

Mast cell histamine release

Histamine release was determined as a quantitative marker of mast cell activation. PMC (2.5×10^4 cells/200 µL) were

incubated with 100 ng/mL of each *G. lamblia* protein fractions (F1–F3) or the parasite TSE for 30 min at 37 °C. Nontreated PMC were included as control of basal release. The supernatants and cell pellets were separated by centrifugation at 3000g for 5 min at 4 °C. Histamine content was measured by a fluorometric method previously described (Bachelet et al. 2006), using a microplate fluorescent reader (Fluoroskan Ascent, Labsystems). In brief, histamine was detected in supernatant and cell samples by coupling to *ortho*-phthalaldehyde under alkaline conditions. The histamine-*ortho*-phthalaldehyde complex was stabilized and fluorescent intensity increased by HCl addition, to form a fluorescent product with an excitation of 340 nm and emission of 450 nm. Histamine release was calculated as percentage of the total cellular histamine content by the following formula:

$$\% \text{ Histamine release} = (\text{histamine in supernatant} / \text{histamine in supernatant and pellet}) \times 100.$$

Cytokine release

Since TNF-α and IL-6 have an important role in *Giardia* infection control and we have previously reported that *G. lamblia* TSE induced direct activation of mast cells with the release of these two proinflammatory cytokines, in the present study, it was our interest to determine the release of IL-6 and TNF-α by mast cells stimulated with F1–F3 and recombinant proteins GIENO and GIADI.

For this, HRMC (2.5×10^5 cells/200 µL) were incubated at 37 °C, with the different *G. lamblia* stimuli in serum-free medium and cell-free supernatants were collected after 6 and 24 h of exposure for the measurement of TNF-α and IL-6 release, respectively. Cytokine concentrations were determined using the rat TNF-α and IL-6 ELISA kits (Peprotech), according to supplier recommendations.

Flow cytometry analysis of mast cell surface TLR2 and TLR4 expression

Surface TLRs expression on unstimulated HRMC or treated with the different *Giardia* stimuli was analyzed by flow cytometry. For this, cells were stained with APC-conjugated antibody against TLR2 (e-bioscience) and FITC-conjugated antibody against TLR4 (Abcam), for 20 min at 4 °C. Appropriate isotype controls for each of the anti-TLRs were included. Cells were analyzed using a Becton Dickinson FACS canto II flow cytometer (BD Bioscience) and Infinicity I software.

Blocking experiment for TLR2 and TLR4

To search for the participation of TLR2 and TLR4 as receptors for *Giardia* TSE or F2, mast cells were preincubated for 60 min with 10 µg/mL anti-TLR2 or anti-TLR4 polyclonal

antibodies (Santa Cruz Biotechnology), for blocking assay. Cells were washed and incubated with 100 ng/mL TSE, 100 ng/mL F2 fraction, or medium alone (untreated), for 6 and 24 h to measure TNF- α and IL-6 release, respectively. Cytokine concentrations in supernatants were determined using the TNF- α and IL-6 ELISA kits (Peprotech), according to supplier recommendations. Peptidoglycan (PGN) from *Staphylococcus aureus* (Sigma-Aldrich) at 10 μ g/mL and lipopolysaccharide (LPS) from *E. coli* (Sigma-Aldrich) at 1 μ g/mL in the presence of 10% of FBS were included as control ligands of TLR2 and TLR4, respectively. IL-6 and TNF- α release by mast cells without TLR2 and TLR4 blocking was also determined.

Identification of proteins in F2 fraction by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

Since F2 showed mast cell stimulating activity greater than F1 and F3, unique and high-intensity protein bands from F2 were excised with a clean razor blade from a 12% SDS-PAGE stained with Coomassie blue. Proteins in the selected bands were identified by LC-MS/MS, at the Proteomic Mass Spectrometry Laboratory of the Institute of Biotechnology, National Autonomous University of Mexico. For this, cysteine residues in proteins were in-gel reduced with DTT and alkylated with iodoacetamide. The gel slices were dehydrated with acetonitrile, and in-gel digestion of proteins with trypsin was performed. Tryptic peptides extracted from gel slices were prepared for LC-MS analysis as previously described (Demesa Balderrama et al. 2011). The LC-MS system features an Accela Ultra High-Pressure Liquid Chromatography (Thermo Fisher Scientific) coupled with LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). The ions were generated by nano-electrospray ionization (nano-ESI) and were acquired in positive ion mode. Dissociation of all observed charge state ions was performed by collision-induced dissociation (CID) and high-energy collision dissociation (HCD) fragmentation methods. MS/MS data were searched against the NCBI nr database using the peptide mass fingerprinting analysis software Matrix science (Mascot Search result) and Protein Prospector (Batch-Tag Web).

Cloning and expression of *G. lamblia* enolase (GIENO) and arginine deiminase (GIADI)

Genomic DNA from *Giardia lamblia* strain WB-C6 was used for cloning enolase (ORF GL50803_11118) and ADI (ORF GL50803_112103) genes by PCR. Genes were amplified using the primers *Gleno* F (5'-GGCG G AAT TCA ATG GAG GCT CCG TCT ACG ATC AAG-3'), *Gladi* F (5'-AGA AGA GAA TTC ATG ACT GAC TTC TCC AAG GAT-3') including a *Eco*RI restriction site (underlined) and

Gleno R (5'-TCT TCT GC GGC CGC TCA CTT CCA GGC CTC GAA ACC ATA-3'), *Gladi* R (5'-TCT TCT GCG GCC GCT TCA CTT GAT ATC GAC GCA G-3') including a *Not*I restriction site (underlined). The amplified PCR products were cloned using Zero Blunt PCR Cloning kit vector (Invitrogen, Carlsbad, CA) and sequenced. Subsequently, DNA was subcloned into pET28a(+) vector (Novagen) and finally, the constructs pET28- *Gleno* or pET28- *Gladi* were introduced into *E. coli* BL21 (DE3) pLysS cells (Novagen, Madison, WI, USA) for gene overexpression. Cells were grown in LB containing 50 μ g/mL kanamycin at 37 °C until the absorbance at 600 nm reached 0.5. The overexpression was induced by the addition of isopropyl thio- β -D-galactoside (IPTG) at a final concentration of 1 mM for 3 h at 37 °C. Cells were harvested by centrifugation, and the pellet was washed twice with 100 mM Tris-HCl (pH 8.0). The cells were resuspended in lysis buffer (NaH₂PO₄ 50 mM, NaCl 300 mM, imidazole 10 mM, the protease inhibitor PMSF 200 μ M, pH 8.0) and lysed by sonication. After centrifugation, the supernatant was passed through Ni-NTA affinity column and the recombinant proteins (rGIENO and rGIADI) were purified with a gradient of 50–300 mM imidazole. Protein concentration was determined by the method of Bradford. Purified rGIENO and rGIADI were run in 12 and 10% SDS-PAGE, respectively, under the same conditions described for TSE electrophoretic analysis. The absence of endotoxin was tested using the Lit E-Toxate kit (Sigma Chemicals).

Release of TNF- α and IL-6 by mast cells stimulated with *G. lamblia* recombinant enolase (rGIENO) and ADI (rGIADI)

HRMC (2.5×10^5 /200 μ L) were incubated at 37 °C, with rGIENO or rGIADI at 50 ng/mL in serum-free culture medium and cell-free supernatants were collected after 6 and 24 h of exposure for the measurement of TNF- α and IL-6 release, respectively. Cytokine concentrations were determined using rat TNF- α and IL-6 ELISA kits (Peprotech), according to supplier recommendations.

Release of TNF- α by mast cells stimulated with *G. lamblia* recombinant ADI (rGIADI) and its metabolic products

We analyzed if rGIADI products, citrulline and ammonium, were responsible for mast cell activation. Experiments were performed in a final volume of 200 μ L; therefore, rGIADI was used at a final concentration of 10 and 20 ng corresponding to 50 and 100 ng/mL, citrulline at 1.5 and 3 μ M (citrulline concentrations produced by ADI at 10 and 20 ng, respectively, as determined by the Citrulline Assay Kit provided by Cell Biolabs, Inc.) and ammonium at 2 mM (NH₄⁺ levels produced

by 4 μg ADI). HRMC (2.5×10^5 cells) were stimulated with rGLADI and its products in RPMI-1640 medium. In addition, stimulation of mast cells by rGLADI in arginine-free HTB medium (Tyrode's buffer (130 mM NaCl, 10 mM HEPES (pH 7.4), 1 mM MgCl_2 , 5 mM KCl, 1.4 mM CaCl_2 , 5.6 mM glucose, and 1 mg/mL bovine serum albumin) was evaluated. Cells were incubated at 37 °C in serum-free culture medium, and cell-free supernatants were collected after 6 h of exposure for the measurement of TNF- α release. Cytokine concentration was determined using rat TNF- α ELISA kit (Peprotech), according to supplier recommendations.

Data analysis and statistics

In all experiments data represent means $\pm$ SE of at least three independent experiments. Histamine and cytokine release in treated cells in comparison to untreated cells, were analyzed using one-way ANOVA followed by Tukey's multiple comparison test (GraphPad Prism 6). Statistical significance between the amounts of cytokine secretion by mast cells stimulated in the presence or absence of arginine was analyzed by two-way ANOVA followed by Tukey's multiple comparison test (GraphPad Prism 6).

Results

G. lamblia-TSE fractionation

The integrity of *G. lamblia* TSE was observed by SDS-PAGE (Fig. 1a). The protein pattern of TSE includes several proteins with a broad molecular weight range. Then, TSE (500 μg) was separated under native conditions (nonreducing and nondenaturing conditions) to avoid toxic components such as SDS and 2-mercaptoethanol, and Coomassie blue stained. In Fig. 1b, protein enrichment is observed in the upper part of the gel. It is worth mentioning that in native PAGE, proteins maintain both their native conformation and charge, so proteins with the same charge but different molecular weight can be localized in the same region of the native gel. TSE fractions (F1–F3) were eluted from the native gel and concentrated with Amicon ultra-10 centrifugal filters and further analyzed by 12% SDS-PAGE (Fig. 2a). It can be observed that many protein bands were overlapped among fractions F1–F3. This is explained taken into account that each fraction was initially separated by charge (native conditions), and later on, they were SDS-PAGE electrophoretic separated by size then in F1–F3 can be presented proteins that have similar molecular weight. The presence/absence and intensity differences of protein bands present in the three fractions were analyzed using the 1-D analysis software Quantity One (Bio-Rad). Densitometric analysis revealed a different pattern of bands in the three fractions (Fig. 2b). F2 contained at least 20

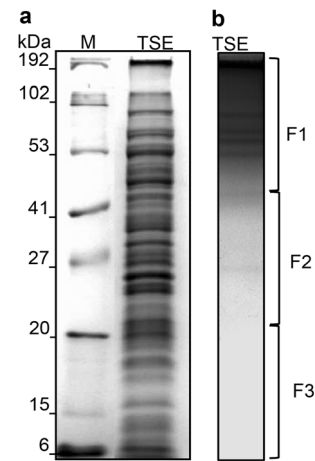


Fig. 1 Electrophoretic profile of *G. lamblia* total soluble extract (TSE). **a** SDS-PAGE profile of proteins from TSE (40 μg) under reducing condition. **b** Native-PAGE (nonreducing, nondenaturing conditions) of TSE (500 μg). All fixed gels were stained with Coomassie blue (Bio-Rad Laboratories). M molecular weight marker

Coomassie blue bands, with moderate to high intensity, in the range of 7 to 73 kDa. It is worth noticing that five unique protein bands (numbered 1, 4, 5, 7, and 12) were detected in F2, with approximate molecular masses of 73, 58, 44, 36, and 10 kDa. In addition, seven protein bands (numbered 2, 3, 6, 8, 9, 10, and 11), with approximate molecular masses of 65, 62, 42, 28, 25, 18, and 16 kDa were shared with F1 and F3, but present with higher intensity in F2 (Fig. 2a, b).

F2 fraction activates mast cell in a greater extent than F1 and F3 fractions

The ability of each fraction to induce mast cell degranulation and histamine release was analyzed. F2 fraction triggered mast cell degranulation resulting in a greater tryptase exposure than F1 and F3 fractions, and similar to that elicited by the *Giardia* TSE (Fig. 3a). In addition, F2 induced histamine release of 29%, which was significantly ($P < 0.01$) higher than histamine release (6.5%) from untreated mast cells, while fractions F1 and F3 produced a moderate histamine release of 18 and 10%, respectively, which was not significantly higher than basal histamine release in untreated mast cells (Fig. 3b). The histamine release induced by F2 was slightly lower than that induced by the *Giardia* TSE (36%), but higher than that induced by F1 and F3 (Fig. 3b). Altogether, these results suggest that some proteins contained in F2 fraction are the main responsible for the direct activation of mast cells.

G. lamblia F2 induces TNF- α and IL-6 release by mast cells

Since F2 fraction induced the release of histamine in a similar extend than TSE, it was of interest to investigate if F2 also induced the release of TNF- α and IL-6 by mast cells, as it had

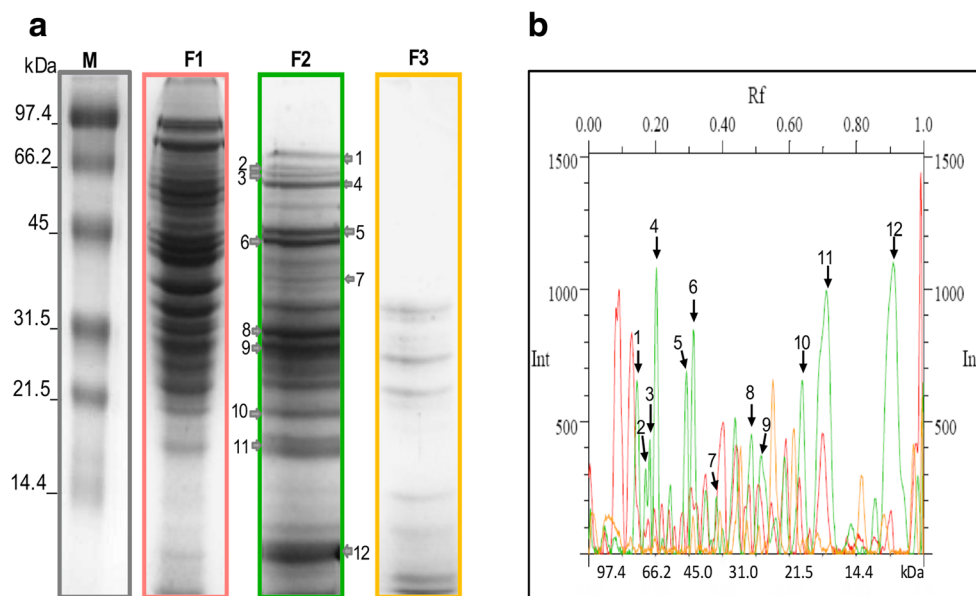


Fig. 2 Analysis of *G. lamblia* F1, F2, and F3 fractions. **a** Electrophoretic pattern of proteins from F1-F3, separated by 12% SDS-PAGE and Coomassie blue stained. Each fraction is represented by colored rectangles (F1 red, F2 green, and F3 yellow). Arrows in F2 lane indicate the 12 bands that were selected for LC-MS/MS analysis. **b** Densitometric analysis comparing proteins from F1 (red lane), F2

(green lane), and F3 (yellow lane) fractions. Arrows indicate proteins corresponding to selected bands of the F2 lane in **a**. The X-axis of the graph shows the molecular weight in kDa, and the Y-axis is the pixel intensity value at each point along the lane. Analysis was performed using Quantity One V4.62 software from Bio-Rad, Laboratories. These results are representative of three independent experiments

previously demonstrated for TSE of *G. lamblia* trophozoites (Munoz-Cruz et al. 2010). As shown in Fig. 4, significant ($P < 0.05$) release of IL-6 and TNF- α (73.5 and 129.3 pg/mL, respectively) was observed in mast cells treated with *G. lamblia* F2, in comparison to both cytokine release in untreated mast cells (27.6 pg/mL of IL-6 and 58.6 pg/mL of TNF- α); however, F2-treated mast cells released lower amount of IL-6 and TNF- α than TSE-treated mast cells (100 and 194.6 pg/mL for IL-6 and TNF- α , respectively), suggesting that some molecules present in the TSE but absent in F2 also contribute to the production of these proinflammatory cytokines.

Surface TLR2 and TLR4 expression on mast cells are not affected by *G. lamblia* TSE or F2

Since mast cells recognize pathogens by a variety of receptors, including TLRs (Sandig and Bulfone-Paus 2012), and previous studies have reported that surface TLRs expression on mast cells is up regulated after PAMP-TLR interaction (Konopka et al. 2010; Pietrzak et al. 2011), we aimed to elucidate whether TSE or F2 can affect mast cell surface TLR2 and TLR4 expression levels. To test this, HRMC were treated with *Giardia* TSE or F2 for 6 and 24 h and TLR2 and TLR4 surface expression was analyzed by flow cytometry. As shown in Fig. 5, *Giardia* TSE or F2 did not affect TLRs baseline surface expression on mast cells. Although F2 induced an average increase on TLR2 expression on mast cells after 24 h of treatment, it was not significantly greater than TLR2 expression on untreated mast cells (Fig. 5a, c).

Similarly, both F2 and TSE induced a moderate increase on surface mast cells TLR4 expression after 24 h of treatment (Fig. 5b, d), but again, it was not significantly greater than basal expression, indicating that *Giardia* TSE or F2 stimuli do not affect TLR2 or TLR4 expression.

TLR2 and TLR4 blockade does not affect TNF- α and IL-6 production by mast cells stimulated by *G. lamblia* TSE and F2

Participations of TLR2 and TLR4 in mast cell activation induced by TSE and F2 were evaluated. Blockade of TLR2 and TLR4 did not inhibit IL-6 or TNF- α secretion induced by *Giardia* TSE or F2 in mast cells (Fig. 6a, b). These results confirm that release of IL-6 and TNF- α induced in mast cells by *Giardia* TSE or F2 does not occur via TLR2 or TLR4.

G. lamblia immunodominant proteins as well as metabolic enzymes were identified among the major proteins present in the F2 fraction

To identify the proteins that may be responsible for mast cell activation, unique and high-intensity protein bands from F2 were excised from a SDS-PAGE gel (Fig. 2a) and identified by LC-MS/MS. No proteins were identified in the 9-kDa band, with any of the two search engines used, Mascot or Prospector. The 19 identified proteins are described in Table 1.

Eight out of 19 proteins identified in F2 were uncharacterized proteins of unknown function, such as hypothetical

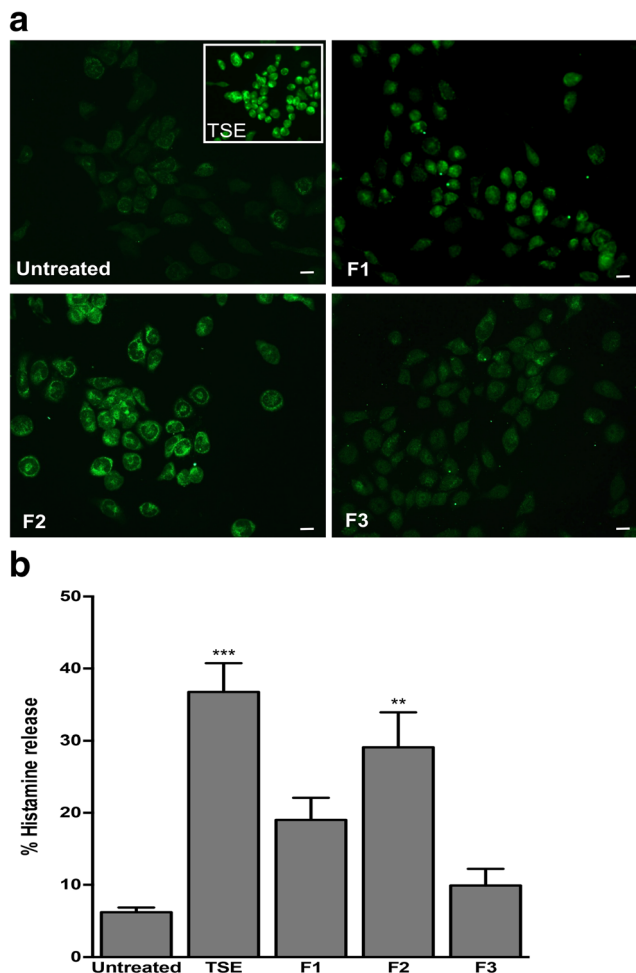


Fig. 3 Activation of mast cells induced by *Giardia* total soluble extract (TSE) and fractions F1–F3. **a** Immunofluorescence detection of mast cell tryptase in HRMC incubated with 100 ng/mL of each *G. lamblia* protein fractions (F1–F3) or TSE (insert), for 30 min at 37 °C. The exposure of tryptase, assessed by the increase of immunoreactivity (green), is an indicator of mast cell activation. Images were obtained at $\times 10$ magnification using an Olympus BX52 fluorescence microscope with image analysis software Image Pro 6.2. (bars = 10 μ m). **b** Histamine release by rat peritoneal mast cells (PMC) incubated with 100 ng/mL of each fraction (F1–F3) or TSE, for 30 min at 37 °C. Basal histamine release was determined in untreated PMC. The percentage of histamine released was determined in each case. Values are means $\pm$ SEM of three independent experiments in triplicate. ** $P < 0.01$; *** $P < 0.001$ compared to untreated cells

proteins (GL50803_10708 and GL50803_5810), three protein members of the poorly described Protein 21.1 family; Hybrid cluster protein lateral transfer candidate, FixW, and the immunodominant protein GTA-1.

Additionally, enzymes such as protein disulfide isomerase 5 (PDI5) and peroxiredoxin were identified. They are involved in protein transport and oxidative stress resistance, respectively (McArthur et al. 2001; Vicente et al. 2009). Two enzymes of unknown function were also identified, NEK-unclassified protein kinase (GL50803_15409), and Xaa-Pro dipeptidase. Particularly, Nek-unclassified protein

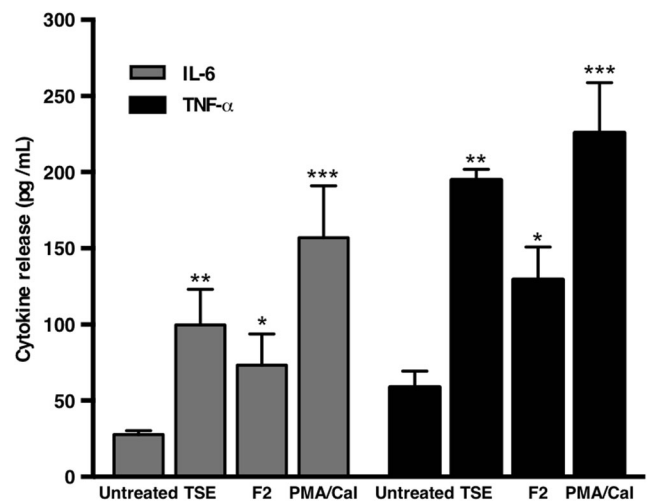


Fig. 4 Mast cell IL-6 and TNF- α production induced by *Giardia* total soluble extract (TSE) and Fraction 2 (F2). HRMC were stimulated with TSE or F2 at 100 ng/mL. Cell-free supernatants from each condition were harvested, after 6 and 24 h, respectively, to measure IL-6 and TNF- α by ELISA. Untreated cells incubated in medium alone were analyzed for constitutive cytokine production. Cells treated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) plus 5 mM Calcium Ionophore A23187 (Cal), were used as positive control. Results are presented as the mean values ($\pm$ SE) of three independent experiments performed in duplicate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, with respect to untreated cells

kinase, is catalytically inactive, localized diffusely throughout the cytoplasm and to an anterior region that is plasma membrane associated; however, its function remains unclear (Manning et al. 2011).

Three proteins with an important role during different cellular processes, including cell cycle and encystment were also identified: 14-3-3 protein, dipeptidyl-aminopeptidase IV (DPP) and the binding immunoglobulin protein (BiP) (Lalle et al. 2010; Lujan and Touz 2003; Touz et al. 2002).

In addition, alpha-1 and alpha-2 giardins were also detected in F2. These *Giardia*-specific proteins have been reported as common immunoreactive proteins of about 30 kDa in several studies (Faubert 2000; Palm et al. 2003; Weiland et al. 2003). These two molecules localize at the plasma membrane of *Giardia* trophozoites. In particular, alpha-1 giardin localizes at the surface of excyzoites playing a role in the early host–parasite interactions by ensuring attachment of the excyzoite to host epithelium while the ventral adhesive disk is being assembled (Palm et al. 2003; Weiland et al. 2003). In relation to alpha-2 giardin, it has been identified as an assemblage A-specific protein of human infective *G. lamblia*; however, its exact function is unknown (Steuart et al. 2008).

It is worth noticing that the metabolic enzymes, enolase and ADI, were also identified in F2 fraction. Enolase and ADI are recognized by sera from humans naturally infected with *G. lamblia* and from experimental mouse infections (Palm et al. 2003; Ringqvist et al. 2008). Interestingly, both enzymes are released when *G. lamblia* trophozoites interact with epithelial cells (Ringqvist et al. 2008), a feature that

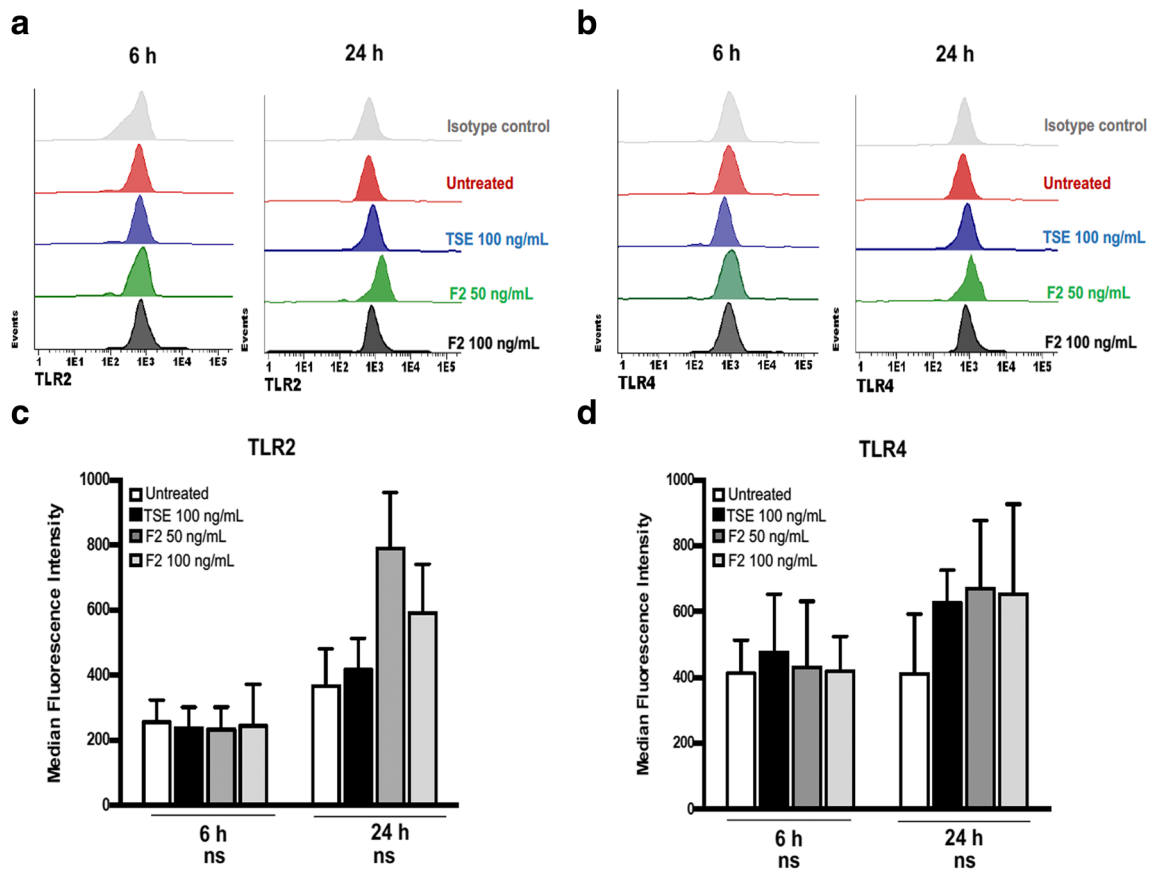


Fig. 5 TLR2 and TLR4 expression on mast cells treated with *Giardia* total soluble extract (TSE) and Fraction 2 (F2). HRMC were treated with *Giardia* TSE or F2 at the indicated concentrations for 6 and 24 h. After treatment, HRMC were stained with anti-TLR2 (a, c) and anti-TLR4 (b, d) monoclonal antibodies and analyzed with a FACSscan flow cytometer.

These results are representative of three independent experiments. Bars represent mean $\pm$ SEM of three independent experiments performed in duplicate. ns no significance relative to untreated cells

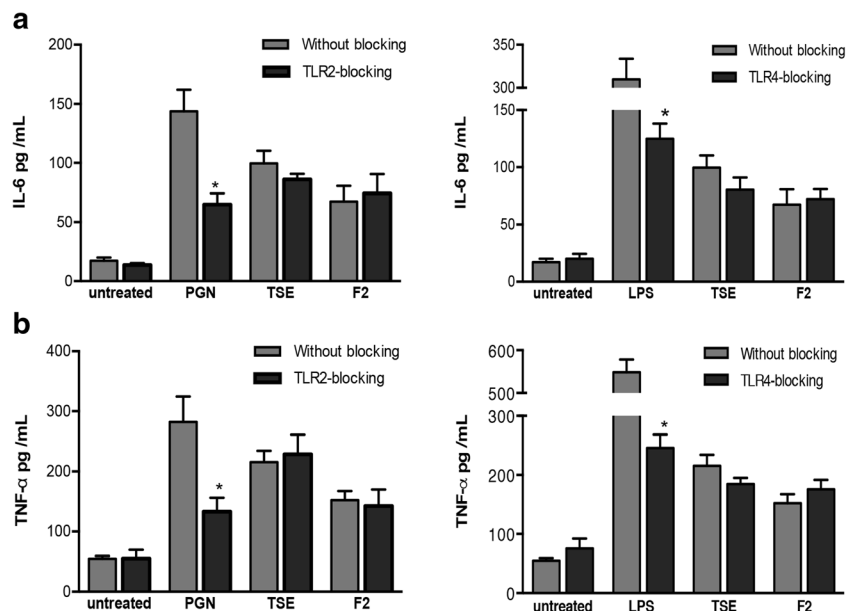


Fig. 6 IL-6 and TNF- α release by TSE and F2 treated mast cells preincubated with anti-TLR2 or anti-TLR4 blocking antibodies. HRMC were preincubated with anti-TLR2 or anti-TLR4 blocking antibodies or medium alone (without TLR blocking) for 60 min, washed and incubated with *Giardia* TSE, F2, or medium alone (untreated). **a** IL-6 and **b** TNF- α were measured by ELISA, after 6 and 24 h, respectively, in cell-free

supernatants. TLR2 and TLR4 agonists, peptidoglycan from *Staphylococcus aureus* (PGN) and lipopolysaccharide from *E. coli* (LPS), respectively, were used as controls. Values are means $\pm$ SEM of three independent experiments in duplicate. * $P < 0.05$ significant inhibition

Table 1 *G. lamblia* proteins identified in selected bands of F2 fraction

Band number	Experimental MW (kDa)	Proteins identified by LC-MS/MS	Theoretical MW (kDa)	Gene ID ^a
1 ^b	73	Dipeptidyl-aminopeptidase IV	87.6	GL50803_6148
		Hypothetical protein	75	GL50803_10708
		BIP	74.3	GL50803_17121
2 ^c	65	Protein 21.1	71.12	GL50803_16220
		Protein 21.1	66.23	GL50803_17060
3 ^c	62	Arginine deiminase	64.13	GL50803_112103
		Hybrid cluster protein lateral transfer candidate	62.54	GL50803_3042
4 ^b	58	NEK kinase	57.01	GL50803_15409
		Hybrid-cluster protein lateral transfer candidate	62.54	GL50803_3042
5 ^b	44	Xaa-Pro dipeptidase	49.67	GL50803_17327
		Enolase	48.22	GL50803_11118
6 ^c	42	Protein 21.1	36.63	GL50803_9030
7 ^b	36	Alpha-1 giardin	33.89	GL50803_11654
		Alpha-2 giardin	33.93	GL50803_7796
8 ^c	28	NEK kinase	57.01	GL50803_15409
		14–3–3 protein	28.57	GL50803_6430
9 ^c	25	Protein 21.1	36.63	GL50803_9030
		Peroxiredoxin	22.54	GL50803_16076
		Giardia trophozoite antigen GTA-1	20.73	GL50803_17090
		Giardia trophozoite antigen GTA-1	20.73	GL50803_17090
10 ^c	18	Giardia trophozoite antigen GTA-1	20.73	GL50803_17090
11 ^c	16	Hypothetical protein	14.5	GL50803_5810
		FixW protein, putative	14.95	GL50803_6289
		Protein disulfide isomerase 5	14.59	GL50803_8064
12 ^b	10	Not identified		

MW molecular weight

^a GiardiaDB Giardia Genomics Resource

^b Bands present only in F2

^c High-intensity bands

makes enolase and ADI potential mast cell activating molecules. In fact, ADI and enolase have been suggested as potential virulence and pathogenicity factors of *G. lamblia* (Ringqvist et al. 2011; Ringqvist et al. 2008).

Recombinant *Giardia* ADI induced mast cell production of TNF- α and IL-6

Previous studies have demonstrated that ADI and enolase play a crucial role during *Giardia* infection, and since both enzymes were identified in F2, those are two of the most promising proteins involved in mast cell activation. Purified rGIADI and rGIENO (Fig. 7a) were used to stimulate HRMC and production of TNF- α and IL-6 was analyzed. For this, mast cells were stimulated with 50 ng/mL of each recombinant protein, for 6 and 24 h to measure TNF- α and IL-6, respectively. Although the endotoxin levels of recombinant proteins were low (≤ 1 EU/mL), mast cells were stimulated in serum-free medium to avoid mast cell endotoxin

responsiveness through the presence of soluble CD14 provided in vitro by serum (Brenner et al. 2014). As shown in Fig. 7b, incubation of HRMC with rGIADI resulted in the secretion of 58 pg/mL IL-6 and 245 pg/mL TNF- α that was significantly different ($P < 0.01$ and $P < 0.001$, respectively) from release in untreated mast cells. In contrast, rGIENO induced the production of low amounts of IL-6 (15 pg/mL) and TNF- α (32 pg/mL), which were not significantly different from untreated cells. These results highlight that ADI is one of the active components in F2 that specifically induced the secretion of IL-6 and TNF- α by mast cells.

Recombinant GIADI and citrulline induced TNF- α production by mast cells

Activation of mast cells by rGIADI and/or its metabolic products was evaluated. For this, HRMC production of TNF- α induced by rGIADI, citrulline, and ammonium was analyzed in RPMI-1640 medium. Mast cell activation with rGIADI was

also performed in HTB medium (arginine free). As shown in Fig. 8, TNF- α secretion by HRMC stimulated with rGIADI at 50 and 100 ng/mL in RPMI-1640 medium resulted in 245 and 528 pg/mL, respectively, that was significantly different ($P < 0.01$ and $P < 0.001$, respectively) from release in untreated mast cells. For citrulline at 1.5 μ M, mast cells secrete 81 pg/mL, which was not significantly different from untreated cells. However, citrulline at 3 μ M induced TNF- α levels of 185 pg/mL, significantly different with respect to untreated mast cells ($P < 0.01$). On the other hand, 2 mM ammonium stimulated HRMC to release 55 pg/mL of TNF- α ; however, it was not significantly different with respect to control cells. Decreased TNF- α secretion by HRMC stimulated with rGIADI was observed when arginine HTB medium was used. In this case, TNF- α levels of 199 and 339 pg/mL were determined at 50 and 100 ng/mL, respectively, significantly different ($P < 0.01$ and $P < 0.001$, respectively) with respect to control untreated mast cells. Then, rGIADI induced TNF- α secretion by HRMC in either the presence or absence of arginine.

Discussion

The importance of mast cell in the control of *Giardia* infections has been clearly elucidated (Erlach et al. 1983; Hardin et al. 1997; Li et al. 2007; Li et al. 2004; Venkatesan et al. 1997); however, the understanding of how *Giardia* activates mast cell response is incomplete. Particularly concerning to how mast cell recognizes the parasite and the identification of the parasite molecules involved in the activation of mast cell.

In the present study, we aimed to identify some of the *G. lamblia* molecules that activate mast cells and the mast cell receptors that could be involved in this activation. We identified a fraction from trophozoite-derived TSE, named here F2, with a high ability to activate mast cells in vitro. *G. lamblia* F2 induced tryptase exposure and a significant release of histamine, IL-6 and TNF- α by mast cells. IL-6 and TNF- α have a very important role in the early control of giardiasis as has been previously demonstrated in *Giardia* infection using mouse models (Bienz et al. 2003; Zhou et al. 2007; Zhou et al. 2003). Also, in human studies, elevated levels of IL-6 and TNF- α have been found in serum of patients with giardiasis compared to that in healthy individuals (Matowicka-Karna et al. 2009). This is consistent with elevated TNF- α levels in sera of *Giardia* infected children (Bayraktar et al. 2005). Interestingly, a recent study demonstrated that peripheral blood mononuclear cells, from *Giardia* infected individuals, stimulated in vitro with *Giardia* soluble proteins, showed higher levels of CD4⁺ effector memory T cells co-producing TNF- α and IL-17A (Saghaug et al. 2015). Relevantly, IL-17A has a significant role in generating a protective immune response in *Giardia*-infected animals (Dann et al. 2015; Dreesen et al. 2014; Grit et al. 2014) and its production in response to *Giardia* infection is dependent of IL-6

production (Dann et al. 2015). Therefore, increased production of IL-6 by mast cells after *Giardia* stimulation could be important to promote a protective Th17 response.

TLRs are important for recognizing a variety of pathogens, including protozoan parasites. TLR2 and TLR4 have been reported to recognize PAMPs from *Leishmania major*, *Trypanosoma cruzi*, and *Plasmodium falciparum* (Ghosh and Stumhofer 2013). Other TLRs also recognize protozoan parasites, such as TLR11, which recognizes *Toxoplasma gondii*, and TLR9, which recognizes genomic DNA from *T. brucei* and *T. cruzi* (Gazzinelli and Denkers 2006). Herein, it was found that TLR2 and TLR4 were not involved in *Giardia* F2 or TSE-induced proinflammatory cytokine production by mast cells. So far, only one *G. lamblia* molecule, the binding immunoglobulin protein (BiP), has been reported to induce proinflammatory cytokine production in dendritic cells via TLR4/Myd88-dependent pathway through activation of NF- κ B (Lee et al. 2014). In this study, high levels of *Giardia* BiP (ranging from 0.1 to 10 μ g/mL) were employed to activate TNF- α and IL-6 production through TLR4. It is worth noting that BiP protein was identified as a molecule present in F2 and further studies will be carried out to analyze its possible role in the activation of mast cell.

Enolase and ADI were identified in F2; both molecules are secreted during the interaction of parasite with the epithelial intestinal cells and are recognized by patients' serum (Palm et al. 2003) (Ringqvist et al. 2008). In addition, enolase is a glycolytic enzyme and it is also localized on the surface of trophozoites and cysts (Castillo-Romero et al. 2012). Regarding ADI, it participates in the hydrolysis of L-arginine into citrulline and ammonium, as part of the arginine dehydrolase pathway, an important metabolic route that produces ATP in this amitochondrial organism. By depleting arginine, ADI interferes with host nitric oxide production, reduces the proliferation of intestinal epithelial cells, and decreases the secretion of IL-10 and IL-12p40 by dendritic cells (Stadelmann et al. 2013; Banik et al. 2013). ADI also citrullinates the arginine of variant-specific proteins, affecting their recognition by the host immune response, and regulates the expression of specific encystation genes (Touz et al. 2008). Enolase and ADI are considered as parasite virulence factors.

In *Giardia* infection, the intestinal barrier function is affected, implicating disruption of tight junctional proteins that are caspase-3-dependent. Parasite cysteine proteases are also involved in the proteolytic disruption of epithelial villin, an important constituent of brush border microvilli (Buret et al. 2015). Therefore, parasite secreted enolase and ADI could reach intestinal mast cells and induce their activation. Herein, it was demonstrated that only rGIADI induced a significant release of IL-6 and TNF- α by mast cells. Then, it was of interest to assess if GIADI itself activates mast cell. Our data showed that rGIADI induced the TNF- α secretion by HRMC in either the presence or absence of arginine; however,

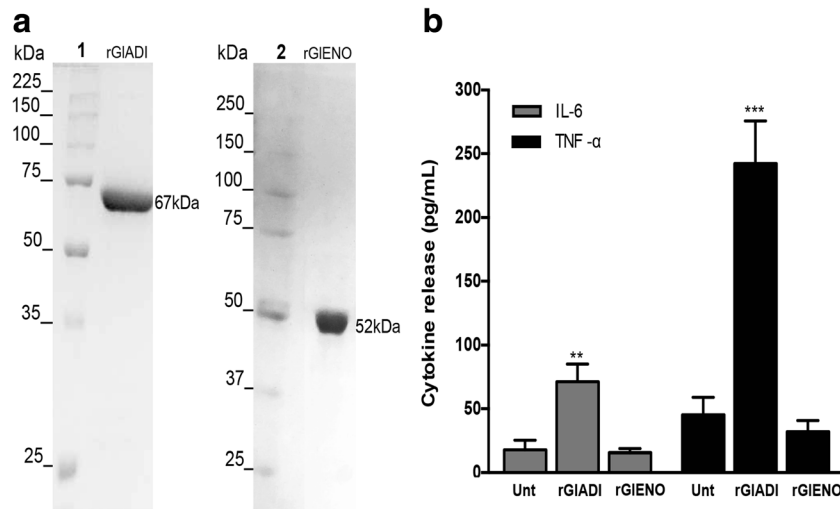


Fig. 7 IL-6 and TNF- α production by mast cells stimulated with *G. lamblia* recombinant arginine deiminase (rGIADI) and enolase (rGIENO). **a** Visualization of purified rGIADI and rGIENO, on 12 and 10% SDS-PAGE, respectively, using Coomassie blue staining. Molecular weight estimation was made by comparison using the broad range protein standards, V8491 from Promega (lane 1) and Precision Plus™ from BioRad (lane 2). **b** Cytokine release by HRMC stimulated with 50 ng/

mL of each recombinant protein in serum free medium. Cell-free supernatants from each condition were harvested and analyzed for IL-6 and TNF- α by ELISA. Untreated cells (Unt) incubated in medium alone were analyzed for basal cytokine production. Results are presented as the mean values ($\pm$ SE) and are representative of three independent experiments done in duplicate. ** P < 0.01; *** P < 0.001 compared to untreated cells

TNF- α levels decreased in an arginine-free medium, suggesting that GIADI metabolic products participate in the induction

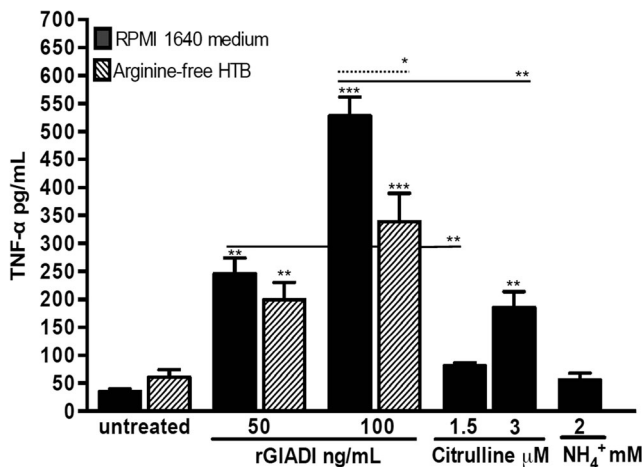


Fig. 8 TNF- α production by mast cells stimulated with *G. lamblia* recombinant arginine deiminase (rGIADI), citrulline, and ammonium. HRMC were stimulated for 6 h with the different stimuli at the indicated concentrations in either RPMI-1640 medium or arginine-free HTB medium. Cell-free supernatants from each condition were harvested and analyzed for TNF- α by ELISA. Untreated cells incubated in medium alone were analyzed for basal cytokine production. Results are presented as the mean values ($\pm$ SE) and are representative of three independent experiments done in duplicate. TNF- α release by mast cells stimulated with the different stimuli was statistically compared to that secreted by untreated cells in the corresponding medium. In addition, the statistical significance between TNF- α secreted by cells stimulated with rGIADI and citrulline at the different concentrations is indicated by a solid line. Dotted lines correspond to significance between TNF- α secreted by cells stimulated with rGIADI in RPMI medium vs arginine-free medium. * P < 0.05; ** P < 0.01; *** P < 0.001

of this cytokine. In fact, TNF- α release was achieved when mast cells were stimulated with citrulline, but ammonium did not affect the cytokine secretion. Then, rGIADI itself and citrulline are able to induce the secretion of TNF- α by mast cells. ADI has been also shown to increase the TNF- α secretion by LPS-activated human monocyte-derived dendritic cells; however, high levels of TNF- α were maintained in the absence of ADI and arginine, indicating that cytokine secretion was mainly due to arginine depletion (Banik et al. 2013). In contrast, our data demonstrated that untreated mast cells maintained in arginine-free medium do not increase secretion of TNF- α , but it increases in the presence of rGIADI, supporting the direct role of ADI in mast cell activation.

It is important to mention that ADI is one of the active components in *Giardia* F2, but there are other potential molecules such as plasma membrane proteins that could be natural mast cells activators. In this regard, alpha-1 giardin, another protein present in F2, related to annexins, displays a Ca(2+)-dependent binding to glycosaminoglycans (GAGs) present in intestinal and mast cells of human small intestine (Weiland et al. 2003). Then, alpha-1 giardin could trigger mast cell activation through the binding to GAG as has been observed with other parasites, such as *T. cruzi* that possesses a cell-surface protein that binds to specific GAG molecules on macrophages and triggers cell activation (Weeratunga et al. 2012). A future step is to define the participation of alpha-1 giardin in mast cell activation and production of the key cytokines IL-6 and TNF- α .

In addition, it was recently demonstrated that recognition of *G. lamblia* by the manose-binding lectin (MBL) resulted in

activation of the lectin pathway of complement leading to mast cell recruitment and parasite control, indicating that complement is a key factor in the recognition of *Giardia* and control of the infection (Li et al. 2016). However, it remains to determine the specific role of lectin pathway and the pattern recognition molecules in *Giardia* that participates in the activation of mast cell.

In summary, in the present study, the direct activation of mast cells by *Giardia* TSE, F2 fraction, and rGLADI was demonstrated, with the subsequent release of IL-6 and TNF- α . It was also shown that the production of TNF- α by mast cells was elicited by ADI itself and its metabolic product, citrulline. The specific mechanism for the activation of mast cells by these parasite molecules has yet to be elucidated. The activation of mast cell by other molecules identified in F2 such as BiP protein and alpha-1 giardin, either alone or together with ADI needs to be analyzed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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