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Brief Definitive Report

A proteomic approach to identify proteins from *Trichuris trichiura* extract with immunomodulatory effects

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SUMMARY

Infections with Trichuris trichiura *and other trichurid nematodes have been reported to display protective effects against atopy, allergic and autoimmune diseases. The aims of the present study were to investigate the immunomodulatory properties of* T. trichiura *adult worm extract (TtE) and its fractions (TtEFs) on the production of cytokines by peripheral blood mononuclear cells and to identify their proteinaceous components. Fourteen TtEFs were obtained by ion exchange chromatography and tested for effects on cytokine production by peripheral blood mononuclear cells. The molecular constituents of the six most active fractions were evaluated using nano-LC/mass spectrometry. The homology between* T. trichiura *and the related nematode* Trichinella spiralis *was used to identify 12 proteins in TtEFs. Among those identified, fructose biphosphate aldolase, a homologue of macrophage migration inhibitory factor and heat-shock protein 70 may contribute to the immunomodulatory effects of TtEFs. The identification of such proteins could lead to the development of novel drugs for the therapy of allergic and other inflammatory diseases.*

Keywords *cytokine, immunoregulation, peripheral blood mononuclear cells, proteomics,* Trichuris *spp*

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RESEARCH NOTE

There are compelling epidemiological data showing that the prevalence of allergic and autoimmune diseases have increased considerably over recent decades among children living in urban centres of low- and middle-income countries (1). The hygiene hypothesis provides an explanation for such temporal trends in the prevalence of inflammatory diseases (2, 3). Some intestinal helminth infections have been associated with protection against inflammatory diseases (3), among them, epidemiological studies and clinical trial using *Trichurid* infection have reported protection against atopy, asthma and autoimmune diseases (4–6). In addition, helminth extracts and their molecules regulating specific pathways of the immune response *in vitro* have been identified (7, 8).

In this study, the immunomodulatory effects of fractions from *T. trichiura* adult worms extract on cytokine responses by human peripheral blood monocytes (PBMCs) *in vitro* were investigated and mass spectrometry was used to identify the proteinaceous content within the active fractions that could be mediating these effects. To our knowledge, no similar research has been done using extracts isolated from this human trichuroid helminth.

T. trichiura adult worms were isolated from infected Ecuadorian children (9) treated with pyrantel pamoate and bisacodyl. Ethical approval for obtaining of stool sample from the children was provided by the Ethical Committee of the Universidad San Francisco de Quito, Ecuador. The written informed consent was provided by the parents or guardians. The worms (5 g – wet weight) were washed in 0.15 M phosphate-buffered saline pH 7.4 and their extract was prepared in a tissue grinder in the presence of zirconium/silica beads (BioSpec Products, Inc.,

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Bartlesville, OK, USA) and 20 mM TRIS-HCl pH 8, containing a mix of protease inhibitors (1 mM phenylmethanesulphonyl fluoride, 50 μM tosyl phenylalanyl chloromethyl ketone, 50 μM tosyllysinechloromethylketone and 2 mM ethylenediaminetetracetate; SIGMA-Aldrich, St. Louis, MO, USA), yielding 200 mg of protein. The extract was subjected to ion exchange chromatography using a salt gradient elution, on a Mono Q 5/50 column (GE Healthcare, São Paulo, SP, Brazil). The 14 collected fractions (TtEF-1 to TtEF-14) were dialysed against RPMI 1640.

PBMCs, obtained from eight 21- to 40-year-old healthy adults, were incubated in 96-well plates (2×10^5 cells/well) in a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (GIBCO, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (GIBCO), 1% glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 20 μg/mL polymyxin B (SIGMA-Aldrich, St. Louis, MO, USA). Subsequently, the cells were cultured in the presence and absence of 50 μg/mL of protein from each TtEF or TtE. This amount was the optimal concentration obtained in an assay using 12.5-100 μg/mL of these antigens to test IL-10 production by stimulated PBMCs (data not shown). Ethical approval for collecting blood from the donors was provided by the Ethical Committee of the Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Proposal 179/2008, Project no. 277. All subjects provided written informed consent. Lipopolysacharide (LPS) from *E. coli* (4 UE/mL; SIGMA-Aldrich) and phytohemagglutinin (PHA, 5 μg/mL; SIGMA-Aldrich) were used as positive controls. The inhibitory effects of TtEFs on cytokine production were assessed by coculturing the PBMCs with each TtEF or TtE in the presence of either LPS (4 UE/mL), for evaluating IL-10 and TNF-α; or LPS (4 UE/mL) plus IFN-γ (100 ng/mL; BD Bioscience, San Diego, CA, USA), for IL-12 p40; or PHA (5 μg/mL), for IL-5 and IL-13. PBMCs cultured with LPS or PHA in the absence of TtEF or TtE were used as controls.

Culture supernatants were collected after 24 h of culture for IL-10, IL-12 p40 and TNF-α production, and after 120 h for IL-5 and IL-13. Cytokines were measured using capture ELISA kit according to the manufacturers instructions (Pharmingen, BD Biosciences, San Diego, CA, USA). Production of cytokines by stimulated or inhibited by TtE or TtEFs PBMCs was compared with those control cultures using paired analyses, with Wilcoxon signed rank test. P values ≤ 0.05 were considered statistically significant.

For mass spectrometry analysis, aliquots of the six most active TtEFs corresponding to 150 μg of proteins each were dried and resuspended in 100 μL of NH_4HCO_3 (50 mM, pH 9.7) in duplicate, reduced by incubation with 5 μL of 200 mM dithiothreitol in 100 mM NH_4HCO_3 at 95°C for 5 min and alkylated with 4 μL of 500 mM iodoacetamide in 100 mM NH_4HCO_3 for 45 min at room temperature in the dark. The reaction was halted with 5 μL of 200 mM dithiothreitol in 100 mM NH_4HCO_3. Digestion was done using sequencing-grade trypsin (Promega Biotecnologia, São Paulo, SP, Brazil) at 1/50 (w/w) trypsin/protein at 37°C overnight. Tryptic peptides were desalted using Macro Trap™ cartridges following the manufacturers instructions (Michrom BioResources, Auburn, CA, USA).

Peptide separation was carried out by liquid chromatography (nanoACQUITY UPLC™, Waters Corporation, Milford, MA, USA) in duplicate. Peptides from the trypsin digest (3 μL) were loaded at a flow rate of 5 μL/min onto a Symmetry C_{18} trap column (Waters Corporation) for 2 min, using 0.1% formic acid (FA)/3% acetonitrile (ACN) in water as eluent. Peptides were eluted from the trap column with a step gradient using solvent B (0.1% FA in ACN) and separated on a BEH 130 C_{18} column (Waters Corporation) at a flow rate of 0.6 μL/min.

Mass detection of peptides was carried out on a hybrid quadrupole-time-of-flight mass spectrometer (Q-TOF *micro*, Micromass, Alliedscienpro, Quebec, Canada) equipped with a nanoelectrospray Z spray source. Data directed analyses acquired in survey mode were processed using MassLynx (v 4.1, Waters Corporation) and searched against SwissProt-UniProt database of the Trichocephalida order, downloaded in August 2011. Data searches used the Protein Lynx Global Server v 2.5 (PLGS). PLGS minimizes false positive results arising from the existence of homologue proteins in the database searched by combining several filtering and scoring factors. Search parameters were set for peptide tolerance of 100 ppm, fragment mass tolerance of 0.1 Da and estimated calibration error of 0.005 Da. The enzyme entry was set for trypsin and the maximum number of missed cleavages to 1. IAA alkylation of cysteine (carbamidomethyl C) and methionine oxidation were set as fixed and variable modifications respectively. Search results were automatically validated when at least three consecutive measured fragment ions of a peptide matched theoretical *b*- or *y*-fragment ions of a known protein sequence tag. The entire data set of identified proteins was further selected by considering only the proteins that replicated in both technical instrument duplicates with a likelihood of identification higher than 95% and PLGS score higher than 9 (a statistical measurement calculated by PLGS search engine using Monte Carlo algorithm that indicates the identification accuracy. It depends on several factors, and among them is the number of entries in the database, the peak area, the number of matched/unmatched peptides, fragmentation data, etc.). The highest PLGS score value obtained in this experiment was 9.696. Only the proteins

that had two or more identified peptides were considered to be present in the fraction.

Our research group has demonstrated previously that whole blood from children infected with *T. trichiura* produces IL-10 when stimulated with parasite antigen (10); that *T. trichiura* infections are inversely associated with skin prick test (SPT) reactivity to common allergens (4) and that infections in early life are associated with a reduced prevalence of SPT reactivity later in childhood (5). These observations are now extended in this study, in which is shown that some fractions of *T. trichiura* somatic extract (TtEF-6 and TtEF-8 through TtEF-14) are able to stimulate the production of IL-10 (Table 1; Figure S1) and induce the production of TNF-α and IL-12 p40 by PBMCs from naïve donors in stimulatory assays (Table 1), despite inhibiting them in inhibitory assay (Table 1; Figure S2 and Figure S3). These findings and the chromatographic profile of TtE (Fig. 1) indicate that the assessed fractions of the extract may contain distinct molecules capable of inducing dissimilar inflammatory effects, which is in agreement with the known inflammatory manifestations of helminth infection that are observed particularly during early infections (11–13). None of the fractions stimulated the production of the Th2 cytokines IL-5 and IL-13 (data not shown), but fractions TtEF-8 to TtEF-10 significantly inhibited the production of IL-5 by PBMCs stimulated with PHA (Table 1; Figure S4). Downregulation of IL-5 have been described as a feature of a modified Th2 response, which would lead to reduced eosinophil involvement (14). A modified Th2 response appears to be a mechanism to lessen potentially damaging Th2-associated inflammation (15) and would indirectly decrease an allergic response. TtEF-9 and TtEF-10 inhibited the PHA-induced IL-13 production (Table 1; Figure S5). This cytokine is involved in the induction of pulmonary inflammation and especially in mucus production by airway goblet cells (16, 17). TtEF-9 had the most potent regulatory properties among the tested fractions and was also associated with inhibition of LPS-induced TNF-α and IL-12 p40 (Table 1). This work shows the advantage of using fractions of TtE because this approach can separate parasite components with low or no immune regulatory activity, such as TtEF-13 that did not inhibit cytokine production, from components with intermediate effect and components with high immunoregulatory potential. In the whole extract, molecules with immune regulatory activity maybe masked by other inactive components.

Because there are very limited data available for the *T. trichiura* genome, the identification of most proteins in the active fractions of TtEF was accomplished by comparing homology with *Trichinella spirallis*, a nematode from the Trichocephalida order, that shows marked antigenic cross-reactivity with *T. trichiura* (18) and has also demonstrated regulatory activity against autoimmune diseases in experimental animal models (19, 20). Approximately 1300 proteins (probability > 95%) were identified from the six most active fractions (TtEF-6, TtEF-8 through TtEF-12), but only 13 proteins were doubly identified in replicate samples and their identification validated according to the search engine parameters specified in the methodology (Table S1). Some proteins were

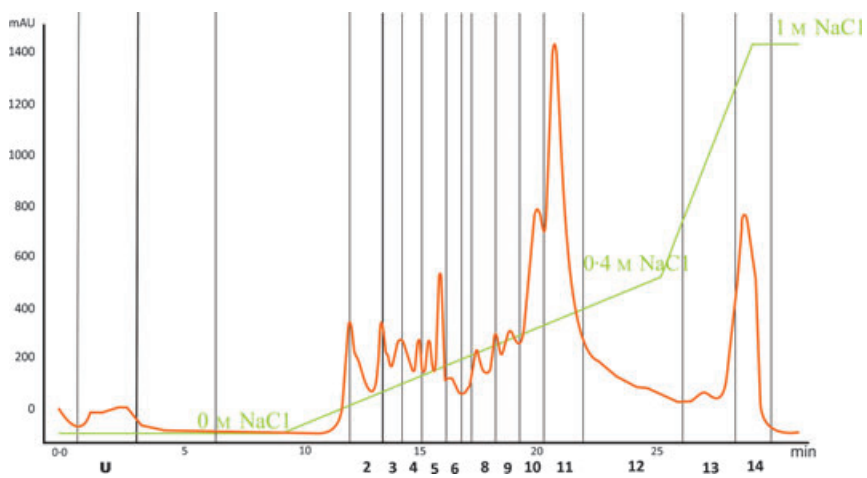

Figure 1 Chromatographic profile of *T. trichiura* somatic extract obtained by ion exchange fractionation. Green legend represents the salt gradient elution (0–1M NaCl). Absorbance was monitored at 280 ηm in milli-absorbance unit (mAU). U and respective numbers correspond to the collected fractions TtEf-2 to TtEF-14, which are delimited by vertical straight lines.

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Table 1 Summary of the production of cytokines by PBMCs stimulated with *Trichuris trichiura* somatic extract (TtE) and its fractions (TtEFs), and respective proteins identified by mass spectrometry comparing by homology with other parasites of the Trichocephalida order

Tested samples	[a]Stimulatory effects on cytokine productions			[b]Inhibitory effects on cytokine productions					[c]Protein content with potential immunoregulatory effect
	IL-10	TNF-α	IL-12 p40	IL-10	TNF-α	IL-12 p40	IL-5	IL-13	
TtE	**203.1 (37.8)**	**333 (110.2)**	**99.06 (39.5)**	62.9 (80.4)	**149.9 (59.5)**	**227.2 (68.7)**	85.5 (45.1)	96.93 (251.4)	–
TtEF-6	**704.7 (102.9)**	**404.4 (119.9)**	**122.1 (36)**	89.08 (63.1)	**450.2 (111.6)**	243 (122.4)	54.77 (48.3)	332.1 (297.9)	FBPA; MIFH; PEPCK
TtEF-8	**461.3 (102.5)**	**290.6 (94.5)**	94.77 (49.8)	101.4 (65.2)	543.5 (204.8)	317.9 (151.5)	**125.9 (63.8)**	360.9 (202.3)	FBPA; MIFH; HSP70; PEPCK
TtEF-9	**340.2 (73)**	**310.9 (132.7)**	78.13 (35.5)	78.31 (89.3)	**590 (149.5)**	**462.6 (183.2)**	**140.3 (59.6)**	**711 (253.3)**	FBPA; MIFH
TtEF-10	**486.7 (139.3)**	**402.2 (167.7)**	84.66 (49.3)	72.73 (48)	**803 (149.3)**	369.2 (160.9)	**77.68 (25)**	**485.2 (194.6)**	HSP70; PEPCK
TtEF-11	**595.2 (140.9)**	**448.8 (159.2)**	**53.87 (16.4)**	36.47 (60.3)	**643.8 (119.3)**	**374.1 (130.9)**	112 (59.1)	18.56 (174)	FBPA
TtEF-12	**641.5 (99.8)**	**618.2 (157.5)**	**178.6 (79.9)**	102.9 (89.2)	**490.1 (145.8)**	245.4 (112.9)	122.3 (65.4)	44.36 (242.9)	FBPA
TtEF-13	**309.1 (120.2)**	**258.6 (81.5)**	**143.9 (79.1)**	164.4 (90.7)	108.3 (45.7)	33.09 (37)	60.9 (73)	329 (297.1)	–
TtEF-14	**213 (45.4)**	**244.9 (82.1)**	**70.13 (25.4)**	91.71 (34.5)	**262.5 (38.2)**	142.4 (61.5)	**57.97 (20.5)**	323.4 (220.6)	–

[a]Stimulatory effects on cytokine production. Peripheral blood mononuclear cells (PBMCs) were cultured in the presence of 50 μg/mL of either TtE or TtEFs; the results were expressed as the increase in cytokine concentrations in pg/mL plus the standard error of the mean, comparing antigen-stimulated and nonstimulated cells; [b]Inhibitory effects on cytokine production. PBMCs were stimulated with either a suboptimal dose of LPS (4 UE/mL), for evaluating inhibition of IL-10 and TNF-α; or LPS (4 UE/mL) plus IFN-γ (100 ηg/mL), for IL-12 p40; or PHA (5 μg/mL), for IL-5 and IL-13, and cocultured with TtE or TtEFs. The results were expressed as the decrease in cytokine concentrations in pg/mL plus the standard error of the mean, comparing LPS and IFN-γ stimulated cells in the absence or presence of TtE and TtEFs. Bold numbers are those statistically significant at $P < 0.05$ (for Wilcoxon signed rank test); [c]FBPA, fructose biphosphate aldolase; MIFH, macrophage migration inhibitory factor homologue; HSP 70, heat-shock protein 70; PEPCK, phosphoenolpyruvate carboxykinase.

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found in more than one of the active fractions. Macrophage migration inhibitory factor homologue (MIFH) was the only protein directly identified from *T. trichiura* and was present in TtEF-6, TtEF-8 and TtEF-9 (Table S1). MIFH has been shown to be produced by several parasites and is thought to have an important role in the evasion mechanism within the host, mimicking the action of the human MIF (15). MIFH from intestinal parasites has several recognized immune modulatory effects on intestinal and allergic airways inflammation (21, 22) and acts through the recruitment of monocytes and regulatory T cells (23, 24), decreasing Th2 cytokine production by PBMCs from atopic asthma patients and increasing IL-10 and TGF-β production (22, 25). Fructose biphosphate aldolase (FBPA), which was present in all fractions except TtEF-10, and phosphoenolpyruvate carboxykinase, present in TtEF-6, TtEF-8 and TtEF-10 (Table S1), are essential proteins involved in the parasite life cycle as well in the establishment of infection, and also may play a modulatory role in the immune response (26, 27). Likewise, the heat-shock protein 70 (HSP 70), present in TtEF-8 and TtEF-10, may have immune regulatory properties similar to those described for *Schistosoma japonicum* (28, 29).

The data shown in this work present evidence that protein fractions from *T. trichiura* have immune modulatory effects on PBMCs from naïve individuals and provide some important clues as to the proteins that may be relevant in mediating these effects. These proteins could be considered for further evaluation as novel therapeutic tools for the treatment or prevention of allergic and autoimmune diseases. In addition, they support the immune regulatory effects that have been associated with *T. trichiura* infection.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Induction of IL-10 production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMCs) from healthy donors. The IL-10 level in culture supernatants was assayed by ELISA. PBMCs were cultured for 24 h either in the absence (Medium) or in the presence of the fraction (TtEF) indicated at the X axis. The lines represent each individual. $*P < 0.05$ (in relation to the differences between the two groups of PBMC).

Figure S2. Inhibition of TNF-α production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMC) from healthy donors. All PBMC were stimulated with bacterial lipopolysaccharide. The TNF-α levels in culture supernatants were assayed by ELISA. The PBMC were cultured for 24 h either in the absence (Medium) or in the presence of the fraction (TtEF) indicated at the X axis. The lines represent each individual. $*P < 0{\cdot}05$ (in relation to the differences between the two groups of PBMC).

Figure S3. Inhibition of IL-12 p40 production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMCs) from healthy donors. All PBMC were stimulated with bacterial lipopolysaccharide. The IL-12 p40 level in culture supernatants were assayed by ELISA. PBMCs were cultured for 24 h either in the absence (Medium) or in the presence of the fraction (TtEF) indicated at the X axis. The lines represent each individual. $*P < 0{\cdot}05$ (in relation to the differences between the two groups of PBMC).

Figure S4. Inhibition of IL-5 production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMCs) from healthy donors. All PBMC were stimulated with phytohemoagglutinin. The IL-5 level in culture supernatants was assayed by ELISA. PBMCs were cultured for 5 days either in the absence (Medium) or in the presence of the fraction (TtEF) indicated at the X axis. The lines represent each individual. $*P < 0{\cdot}05$; $**P < 0.01$ (in relation to the differences between the two groups of PBMC).

Figure S5. Inhibition of IL-13 production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMCs) from healthy donors. All PBMC were stimulated with phytohemoagglutinin. The IL-5 level in culture supernatants were assayed by ELISA. PBMCs were cultured for 5 days either in the absence (Medium) or in the presence of the fraction (TtEF) indicated at the X axis. The lines represent each individual. $*P < 0{\cdot}05$ (in relation to the differences between the two groups of PBMC).

Table S1. Mass-spectrometry identified and validated proteins in the most active *Trichuris trichiura* somatic extract fractions (TtEF-6 and TtEF-8 to TtEF-12) obtained by ion-exchange chromatography.