UNIVERSIDADE FEDERAL DA BAHIA
INSTITUTO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA

LEONARDO NASCIMENTO SANTOS

EFEITOS MODULATÓRIOS DE FRAÇÕES DO EXTRATO SOMÁTICO DE *Trichuris trichiura* EM CÉLULAS MONONUCLEARES DO SANGUE PERIFÉRICO

Salvador
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Dissertação de mestrado apresentada ao programa de Pós-graduação em Imunologia do Instituto de Ciências da Saúde da Universidade Federal da Bahia, como requisito parcial para obtenção do título de Mestre em Imunologia.

Orientadora: Profª. Drª. Neuza Maria Alcântara Neves
Co-orientador: Prof. Dr. Lain Carlos Pontes de Carvalho

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UNIVERSIDADE FEDERAL DA BAHIA
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ATA DA SESSÃO PÚBLICA DO COLEGIADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA PARA JULGAMENTO DO TRABALHO DE DISSERTAÇÃO DO MESTRANDO **LEONARDO NASCIMENTO SANTOS**

Aos quatro dias do mês de fevereiro do ano de dois mil e onze, às oito horas, no auditório III, 2º andar no Instituto de Ciências da Saúde , a Banca Examinadora composta pelos Professores: **Dra. Neuza Maria Alcântara, Orientadora, Dra.Patrícia Sampaio Tavares Veras, Dr.Roberto José Meyer Nascimento,** se reúne com a finalidade de discutir, avaliar e julgar o trabalho de Dissertação intitulado: **"Efeitos Modulatórios de Frações do Extrato Somático de *Trichuris trichiura* em Células Mononucleares do Sangue Periférico"** do Mestrando **Leonardo Nascimento Santos.** Após a apresentação, foram feitos os comentários pelos examinadores. Havendo cumprido as exigências para Banca Examinadora conclui que o pós-graduando teve a sua defesa de Dissertação APROVADO , emitindo pareceres individuais que serão anexados à ata. Nada mais havendo a tratar, encerra-se a sessão, da qual é lavrada a presente ata que após lida e aprovada vai assinada pelas componentes da Banca examinadora, pela Mestrando e pela Coordenadora do Programa de Pós-Graduação. Salvador, quatro de fevereiro do ano de dois mil e onze.

Profa. Dra. Neuza Maria Alcântara Neves
Orientador

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"... the idea is to try to give **all of the information** to help others to judge the value of your contribution; not just the information that leads to judgment in one particular direction or another."

Richard Feynmann

RESUMO

Estudos epidemiológicos e experimentais indicam que helmintos regulam respostas imunes a aeroalérgenos através da estimulação de uma rede de regulatória. Tem sido relatado que infecção por *T. trichiura* e outros trichuroides levam à proteção contra atopia, doenças alergicas e auto-imunes. O objetivo deste estudo foi identificar frações do extrato somático de *T. trichiura* (TtEFs) com atividade imunomodulatória, avaliando seus efeitos sobre a produção de citocinas por células mononucleares do sangue periférico (CMSP) de doadores saudáveis. Quatorze TtEFs (TtEF1 a TtEF14) foram obtidos pelo fracionamento do extrato somático de *T. trichiura* por cromatografia líquida de troca iônica. Para avaliar o efeito estimulatório das TtEFs sobre a produção de citocinas (IL-10, IL-12 p40, TNF-α, IL-5 e IL-13) por CMSP, essas células foram obtidas de doadores saudáveis e cultivados na presença das TtEFs (50 ug de proteína/mL). Efeitos inibitórios foram avaliados por incubação das células com as TtEFs e: (a) uma concentração subótima de LPS (4 UE/mL), para IL-10 e TNF-α, (b) LPS e interferon (IFN) γ (100 ηg/mL), para IL-12 p40, e (c) fitohemaglutinina (5 μg/mL), para IL-5 e IL-13. As citocinas foram quantificados por ELISA de captura. Oito das frações (TtEF6, TtEF8 a TtEF14), bem como o extrato somático de *T. Trichiura*, induziram a produção de IL-10, TNF-α e, em menor quantidade, IL-12 p40. Três das frações (TtEF8, TtEF9 e TtEF10) não induziram significantemente a produção de IL-12 p40. Nenhuma das frações estimulou a produção de IL-13. Estas oito frações que estimularam a produção de IL-10 foram analisados por eletroforese em gel de poliacrilamida na presença de dodecilsulfato de sódio e para a atividade inibitória sobre a produção de citocinas por CMSP estimuladas. Todas as frações inibiram a produção de pelo menos uma citocina, mas nenhuma delas inibiu a produção de IL-10. Apenas uma das frações (TtEF9) inibiu significativamente a produção de todas as outras citocinas analisadas. TtEF10 inibiu a produção de TNF-α, IL-5 e IL-13; TtEF11 reduziu a produção de ambos TNF-α e IL-12 p40; três frações (TtEF6, TtEF12 e TtEF13) inibiram apenas a produção de TNF- α e duas frações (TtEF8 e TtEF14) inibiram significativamente apenas a produção de IL-5. Os resultados obtidos indicam que *T. trichiura* produz mais de uma molécula com atividade imunomodulatórias. TtEF9, que, além de estimular a produção de IL-10, sub-regula a produção de todas as outras citocinas estudadas, é o melhor candidato para conter uma molécula imunoregulatória que deve ser caracterizada e purificada à homogeneidade, a fim de investigar a sua utilização como um agente terapêutico para doenças imunomediadas.

Palavras-chave: Imunomodulação; *Trichuris trichiura* - Frações do extrato; CMSP; Citocinas.

ABSTRACT

Epidemiological and experimental studies indicate that helminthes downregulate immune responses to aeroallergen through the stimulation of an immunoregulatory network. It has been reported that *T. trichiura* and other trichuroid infections lead to protection against atopy, allergy and autoimmune diseases. The objective of this study was to identify *T. trichiura* extract fractions (TtEFs) with immunomodulatory activity by evaluating their effects on the production of cytokines by healthy donors' peripheral blood mononuclear cells (PBMC). Fourteen TtEFs (TtEF1 to TtEF14) were obtained by fractionation of the *T. trichiura* somatic extract by anion-exchange liquid chromatography. To disclose a stimulatory effect of the TtEFs on cytokine (IL-10, IL-12 p40, TNF-α, IL-5 and IL-13) production by PBMC, these cells were obtained from healthy donors and cultivated in the presence of the TtEFs (50 μg of protein/mL). Inhibitory effects were assessed by incubating the cells with the TtEFs and: (a) a suboptimal concentration of LPS (4 UE/mL), for IL-10 and TNF-α; (b) LPS and interferon (IFN-)γ (100 ηg/mL), for IL-12 p40; and (c) phytohemoagglutinin (5 μg/mL), for IL-5 and IL-13. The cytokines were quantified by capture ELISA. Eight of the fractions (TtEF6 and TtEF8 to TtEF14), as well as the *T. trichiura* somatic extract, induced the production of IL-10, TNF-α and, in lower amounts, IL-12 p40. Three of the fractions (TtEF8, TtEF9 and TtEF10) did not consistently induce the production of IL-12 p40, and, therefore, did not lead to statistically significant production of this cytokine chain. None of the fractions stimulated the IL-13 production. These eight fractions that stimulated IL-10 production were further analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and for inhibitory activity on the production of cytokines by stimulated PBMC. All fractions inhibited the production of at least one cytokine, but none of them inhibited the production of IL-10. Only one of the fractions (TtEF9) significantly inhibited the production of all other the assayed cytokines. TtEF10 inhibited the production of TNF-α, IL-5, and IL-13; TtEF11 reduced the production of both TNF-α and IL-12 p40; three fractions (TtEF8, TtEF12, and TtEF13) inhibited only the production of TNF-α; and two fractions (TtEF8 and TtEF14) significantly inhibited only the production of IL-5. The obtained results indicate that *T. trichiura* produces more than one molecule with immunomodulatory activities. TtEF9, which, in addition to stimulating the production of IL-10, down-regulated the production of all studied cytokines, is the best candidate to contain an immunoregulatory molecule that should be characterized and purified to homogeneity, in order to investigate its use as a therapeutic agent for immunomediated diseases.

Key words: Immunomodulation; *Trichuris trichiura* - extract fractions; PBMC; Cytokines.

LISTA DE FIGURAS

LISTA DE SIGLAS E ABREVIATURAS

ES	Excretory/Secretory
FPLC	Fast Protein Liquid Chromatography
IFN-γ	Iterferon-gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG4	Immunoglobulin G4
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-10	Interleukin-10
IL-12 p40	Interleukin-12 (subunit 40)
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Salin
PHA	Phytohaemagglutinin
RPMI	Roswell Park Memorial Institute
SDS	Sodium Dodecyl Sulphate
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor-alpha
Tris-HCl	Tris-hidroxymetil amino metano - hydrochloride
TtEF	*Trichuris trichiura* – extract fractions

SUMÁRIO

DIFFERENT FRACTIONS OF *Trichuris trichiura* SOMATIC EXTRACT MODULATE CYTOKINE PRODUCTION BY HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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Author´s contribution

Conceived and designed the experiments: NMAN, LCPC,LNS. Performed the experiments: LNS, ESS. Analyzed the data: LNS. Contributed to development of the laboratory assays: MLB, PJC, CAVF; Wrote the paper: LNS. Revised the paper:LCPC, NMAN.

ABSTRACT

Epidemiological and experimental studies indicate that helminthes downregulate immune responses to aeroallergen through the stimulation of an immunoregulatory network. It has been reported that *T. trichiura* and other trichuroid infections lead to protection against atopy, allergy and autoimmune diseases. The objective of this study was to identify *T. trichiura* extract fractions (TtEFs) with immunomodulatory activity by evaluating their effects on the production of cytokines by healthy donors' peripheral blood mononuclear cells (PBMC). Fourteen TtEFs (TtEF1 to TtEF14) were obtained by fractionation of the *T. trichiura* somatic extract by anion-exchange liquid chromatography. To disclose a stimulatory effect of the TtEFs on cytokine (IL-10, IL-12 p40, TNF-α, IL-5 and IL-13) production by PBMC, these cells were obtained from healthy donors and cultivated in the presence of the TtEFs (50 μg of protein/mL). Inhibitory effects were assessed by incubating the cells with the TtEFs and: (a) a suboptimal concentration of LPS (4 UE/mL), for IL-10 and TNF-α; (b) LPS and interferon (IFN-)γ (100 ηg/mL), for IL-12 p40; and (c) phytohemoagglutinin (5 μg/mL), for IL-5 and IL-13. The cytokines were quantified by capture ELISA. Eight of the fractions (TtEF6 and TtEF8 to TtEF14), as well as the *T. trichiura* somatic extract, induced the production of IL-10, TNF-α and, in lower amounts, IL-12 p40. Three of the fractions (TtEF8, TtEF9 and TtEF10) did not consistently induce the production of IL-12 p40, and, therefore, did not lead to statistically significant production of this cytokine chain. None of the fractions stimulated the IL-13 production. These eight fractions that stimulated IL-10 production were further analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and for inhibitory activity on the production of cytokines by stimulated PBMC. All fractions inhibited the production of at least one cytokine, but none of them inhibited the production of IL-10. Only one of the fractions (TtEF9) significantly inhibited the production of all other the assayed cytokines. TtEF10 inhibited the production of TNF-α, IL-5, and IL-13; TtEF11 reduced the production of both TNF-α and IL-12 p40; three fractions (TtEF8, TtEF12, and TtEF13) inhibited only the production of TNF-α; and two fractions (TtEF8 and TtEF14) significantly inhibited only the production of IL-5. The obtained results indicate that *T. trichiura* produces more than one molecule with immunomodulatory activities. TtEF9, which, in addition to stimulating the production of IL-10, down-regulated the production of all studied cytokines, is the best candidate to contain an immunoregulatory molecule that should be characterized and purified to homogeneity, in order to investigate its use as a therapeutic agent for immunomediated diseases.

Key words: Immunomodulation; *Trichuris trichiura* - extract fractions*;* PBMC; Cytokines.

INTRODUCTION

There is compelling epidemiological data indicating that the prevalence of allergic diseases has increased considerably over recent decades, mainly among young people living in developed countries, but also in populations living in urban centres of developing countries, possibily due to the acquisition of a "Western" lifestyle [1; 2]. Similarly, there may also have been an increase in the prevalence of autoimmune diseases [3]. The hygiene hypothesis has explained these temporal trends in the prevalence of inflammatory diseases in terms of improved living conditions that have been associated with a reduction in infectious diseases of childhood and a consequent failure to develop adequate immune regulation leading to a failure to control inflammatory processes.

Intestinal helminth parasites are estimated to infect 2 billion humans worldwide and are considered to be important inducers of immune regulation in early childhood in areas where these parasites are endemic. Infections with intestinal helminths have been associated with protection against inflammatory diseases including allergic and autoimune diseases in experimental animal models and also in some epidemiological studies [4]. However, helminth infections such as *Ascaris lumbricoides* and *Toxocara sp.* have been associated also with an increased prevalence of asthma symptoms and bronchial hyperresponsiveness in some populations [5]. The differential effects of intestinal helminthes on host inflammation have been attributed to the age and intensity of infection and the parasite involved.

Acute helminth infection is characterized by a strong Th2 response, driven by IL-4, IL-5 and IL-13, which lead to eosinophilia, increased production of IgG4, IgA, IgE, mast cells, eosinophils and mucus secretion, besides physiological changes in the intestine, such as increased mucosal permeability and smooth muscle contractility [6; 7]. However, chronic infection has been associated with " a modified Th2 response" characterized by increased production of the immune regulatory cytokine IL-10 in the presence of IL-5 and increased parasite-specific IgG4 antibodies [8; 9; 10], Further, IL-10 and IgG4 antibodies have been associated with successful immunotherapy with aeroallergens and the production of IL-10 may be deficient in asthmatic subjects [10; 11; 12].

Helminth parasites have been used experimentally in humans to treat inflammatory diseases and with mixed results. Therapeutic benefit from infection with the pig trichurid parasite, *Trichuris suis*, has been reported in patients with inflammatory bowel disease [13; 14; 15]

but not allergic rhinitis [16]. No apparent clinical benefits for asthma [17] or allergic rhinitis[18] were reported after experimental infections of patients with hookworm in dose-finding randomized trials designed primarily to evaluate safety. *Trichinella spiralis*, another trichuroid helminth, has also shown to down-immunoregulate type 1 diabetes and ulcerative colitis in experimental models [19; 20].

In the case of the human trichurid worm, *T. trichiura*, epidemiological studies have clearly demonstrated inverse associations between infection and atopy [21; 22] and atopic asthma [22], with some evidence of greater protection being associated with higher parasite burdens [21; 22]. Several helminth molecules have already been isolated, identified and functionally analyzed, showing significant regulation of certain pathways of the immune response [23; 24]. Given that intestinal helminths may have both beneificial and harmful effects on the human host, the strategy to isolate the parasite-derived products that are responsible for their beneficial effects is likely to be optimal thus avoiding potentially harmful pro-inflammatory effects of live infection.

The objective of the present study was to identify fractions of somatic extract from *T. trichiura* adult worms with immunomodulatory properties as measuerd by their ability to modulate cytokine production by PBMCs from from healthy donors.

MATERIALS AND METHODS

Worms and *T. trichiura* somatic extract (TtE)

Adult *T. trichiura* worms were obtained by treatment of infected children, from Ecuador, with pyrantel pamoate, and collection of worms from stool samples collected 1-2 days post-treatment. The adult worms were washed carefully in 0.15 M phosphate-buffered saline, pH 7.4 (PBS) and cryopreserved until use. The parasite extract was prepared by crushing the frozen worms in a tissue grinder with the aid of zirconium/silica beads (BioSpec Products, Inc, Bartlesville, USA) using as diluent 20 mM Tris-hidroxymetil amino metano (TRIS-HCl), pH 8, containing the following protease inhibitors: 1 mM phenylmethanesulfonyl fluoride, 50 μM tosyl phenylalanyl chloromethyl ketone, 50 μM tosyl lysine chloromethyl ketone, and 2mM ethylenediaminetetraacetate (SIGMA- Aldrich, St Louis, MO, USA). The ground extract was centrifuged at 13,400 g for 20 minutes at 4° C, and

the supernatant was collected and stored at -70°C. Protein concentration was measured using the Lowry's method.

Obtaining of *T. trichiura* extract fractions (TtEFs)

The extract was purified by anion-exchange liquid chromatography, with mobile phases constituted by buffer A (20 mM TRIS-HCl, pH 8) and/or buffer B (20 mM TRIS-HCL, 1 M NaCl, pH 8). The extract was centrifuged at 22,500g, for 10 minutes at 4° C and the supernatant filtered with a 0.22 μm-diameter pore syringe filter. The filtered extract was chromatographed in a Mono Q 5/50 column using a Fast Protein Liquid Chromatography system (FPLC, AKTA Purifier, G&E, Pittsburgh, USA). The column was equilibrated in buffer A and the fractions were eluted by running two consecutive linear gradients: 0 to 40% of buffer B in buffer A, in 16 minutes, followed by 60 to 100% of buffer B in buffer A, in 4 minutes, at a flow rate of 1 mL/minute. The initially obtained 1-mL-volume FPLC fractions were pooled according to 280 nm-absorbance chromatographic peaks, as detailed in Fig. 1, producing 13 new fractions (a fraction that did not bind to the column, TtEF2 to TtEF6 and TtEF8 to TtEF14). These pooled fractions were concentrated, dialyzed against RPMI 1640 in dialysis tubes with membranes excluding > 12 kDa molecules (SIGMA-Aldrich, St Louis, MO, USA), aliquoted and stored frozen at -70° C till use. The protein concentration of fractions were determined using the Lowry method. Practically all detectable proteins, that produced 280-nm absorbance peaks, were contained in TtEF2 to TtEF6, TtEF8 to TtEF14, and in the fraction that did not bind to the column. These 13 fractions, therefore, were chosen to be evaluated in terms of stimulatory or inhibitory activities on cytokine production by leukocytes and of protein composition by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis (PAGE)

PAGE in the presence of 12% sodium-dodecyl-sulphate (Invitrogen, Auckland, NZ) (SDS PAGE) was carried as described by Laemmli [25]. Fractions were boiled at 100° C for 5 minutes in sample buffer (15% glycerol, 3% SDS, 0.125 M TRIS, pH 6.8) with 50mM 2-mercaptoethanol, and loaded at 3-10 pg of protein/lane. The bands were stained with Coomassie blue.

Effects of fractions on cytokine production by PBMCs from healthy donors

Peripheral blood mononuclear cells from peripheral blood of healthy adults aged between 21 and 40 were obtained by gradient centrifugation using Histopaque 1077® (SIGMA-Aldrich, St Louis, CA, USA). All volunteers were non-atopic as determined by allergen skin prick test reactivity to a panel of relevant aeroallergens and none had allergic symptoms. . PBMC cultures (2 x 105 cells/well) were incubated in 96-well plates with RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (GIBCO, Grand Island, NY), 1% glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 20 μg/ml polymyxin B (SIGMA- Aldrich, St Louis, CA, USA), the latter used as a depleting agent for endotoxin.

To evaluate the effect of protein fractions of *T. trichiura* (TtFr) on cytokine production (IL-10, IL-12 p40, TNF-α, IL-5 and IL-13), PBMCs were stimulated with 50μg/ml of protein fractions or with LPS from E. coli (4EU/ml; SIGMA-Aldrich, St. Louis, CA, USA) or with 5μg/ml of phytohemagglutinin (PHA, SIGMA-Aldrich, St. Louis, CA, USA). Inhibitory effects of TtFr were assessed by co-culture of PBMCs with a suboptimal dose of LPS (4UE/ml) for IL-10 and TNF-α, LPS (4UE/ml) plus IFN- γ (100ηg/ml) for IL-12 p40 or PHA (5μg/ml) for IL-5 and IL-13 measurements.

PBMCs were cultured in a humidified atmosphere of 5% CO2 (5%) at 37°C. Culture supernatants were harvested at 24h for IL-10, IL-12 p40 and TNF-α and for 120 h for IL-5 and IL-13. Supernatants were stored at -70ºC until analysis for cytokines using capture ELISA and following the manufacturers' instructions (Pharmingen, BD Biosciences, San Diego, CA, USA).

Statistical analysis

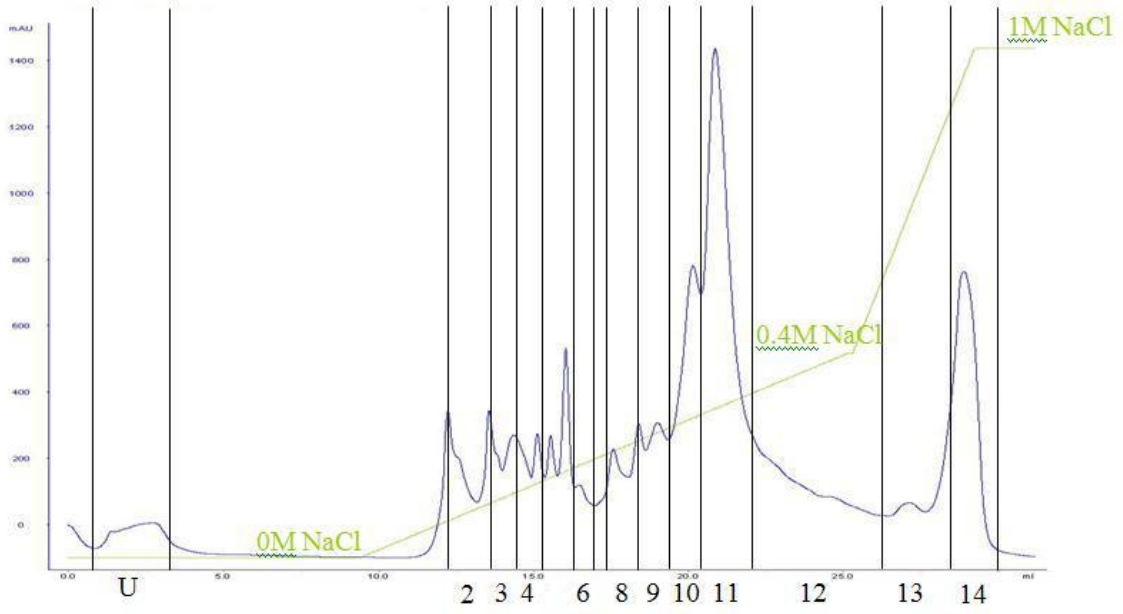
The Friedman' test was used to evaluate the difference of all studied fractions on the production of individual cytokines. When this test show difference, the statistical significance of the differences in cytokine concentrations in cultures of PBMC stimulated with somatic extract of *T. trichiura* or its fractions, in relation to control cultures (of non-stimulated PBMC, or of LPS- or PHA-stimulated PBMC), paired by PBMC donor, was assessed by the Wilcoxon's signed rank test. Statistical significance was inferred by at a level of $p < 0.05$.

RESULTS

TtEFs

When the TtE was subjected to anion-exchange liquid chromatography, using a G&E Mono Q 5/50 column, most proteins, as judged by peaks of 280 nm-absorbance, were eluted with NaCl concentrations ranging from 0 to 1 M (Fig. 1A). The predominant product of somatic extract was represented by a peak that was eluted with 0.28 M NaCl (Fig. 1A) and was represented by a major electrophoretic band - this band of apparent molecular weight of 50.5 KDa appeared in fractions TtFr8, TtFr9, TtFr10, TtFr11 and TtFr12 (Fig. 1B), indicating that this group of proteins with similar molecular weight were only partially separated by their electrical charges.

A



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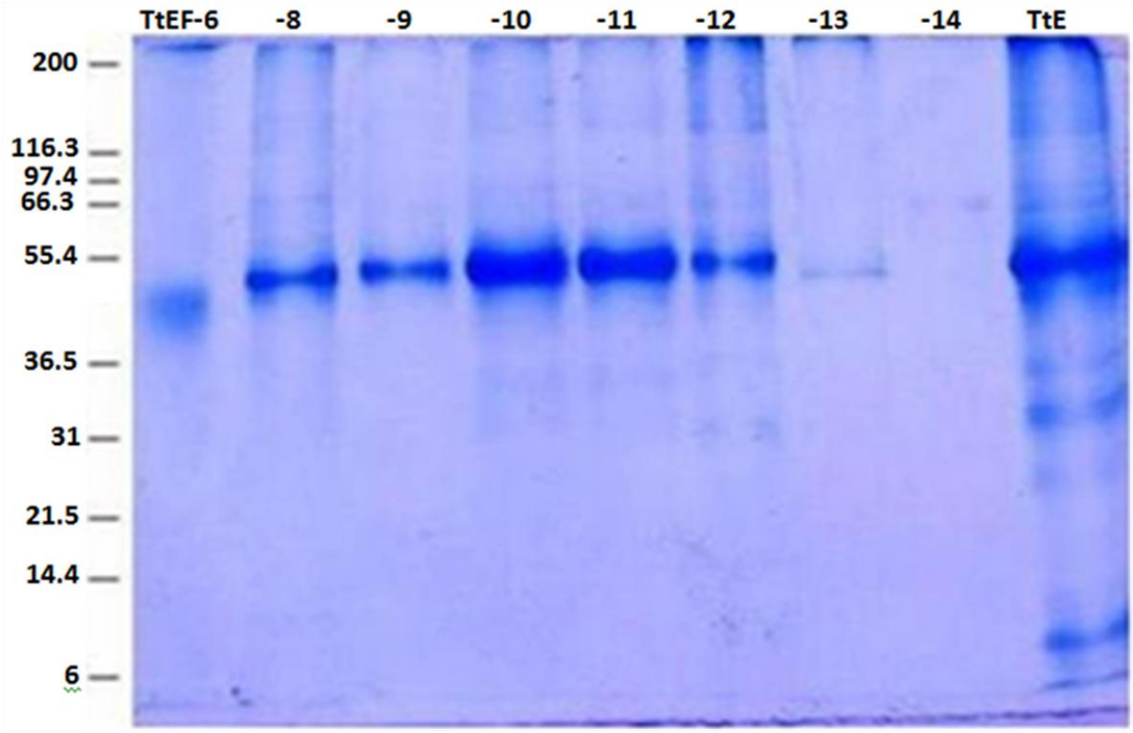

Figure 1. Chromatogram of the fractionation of a somatic extract of *T. trichiura* (TtE) by ion-exchange chromatography (A) and apparent molecular weights of the protein components of the TtE and its fractions (B).

Induction by TtEFs of cytokine production

Of 13 tested fractions, eight (TtEF6, TtEF8, TtEF9, TtEF10, TtEF11, TtEF12, TtEF13 and TtEF14) stimulated the production of IL-10 in a dose-dependent manner (data not shown and Fig. 2). These fractions also induced the production of TNF-α (Fig. 3) and IL-12 p40 chain (Fig. 4), although the stimulation of the latter by TtEF8, TtEF9, and TtEF10 was not statistically significant (Fig. 4). None of the fractions stimulated the production of the Th2 cytokines IL-5 and IL-13 by PBMC (not shown).

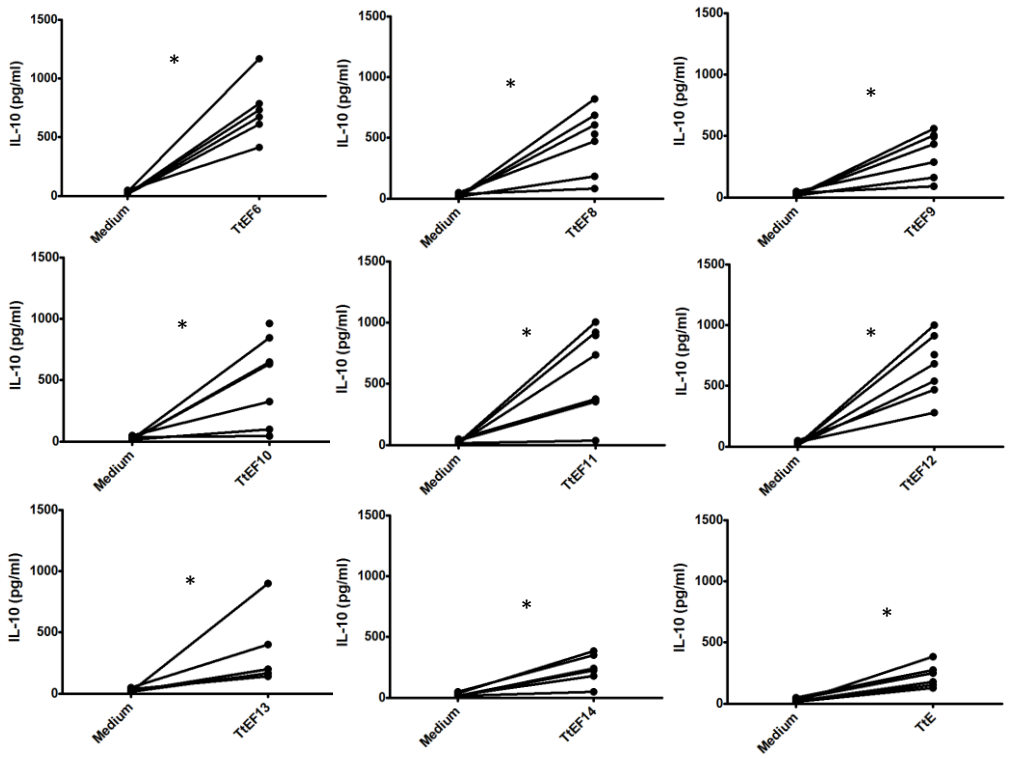

Figure 2: Induction of IL-10 production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMC) from healthy donors. *: $P < 0.05$ (in relation to the differences between the two groups of PBMC).

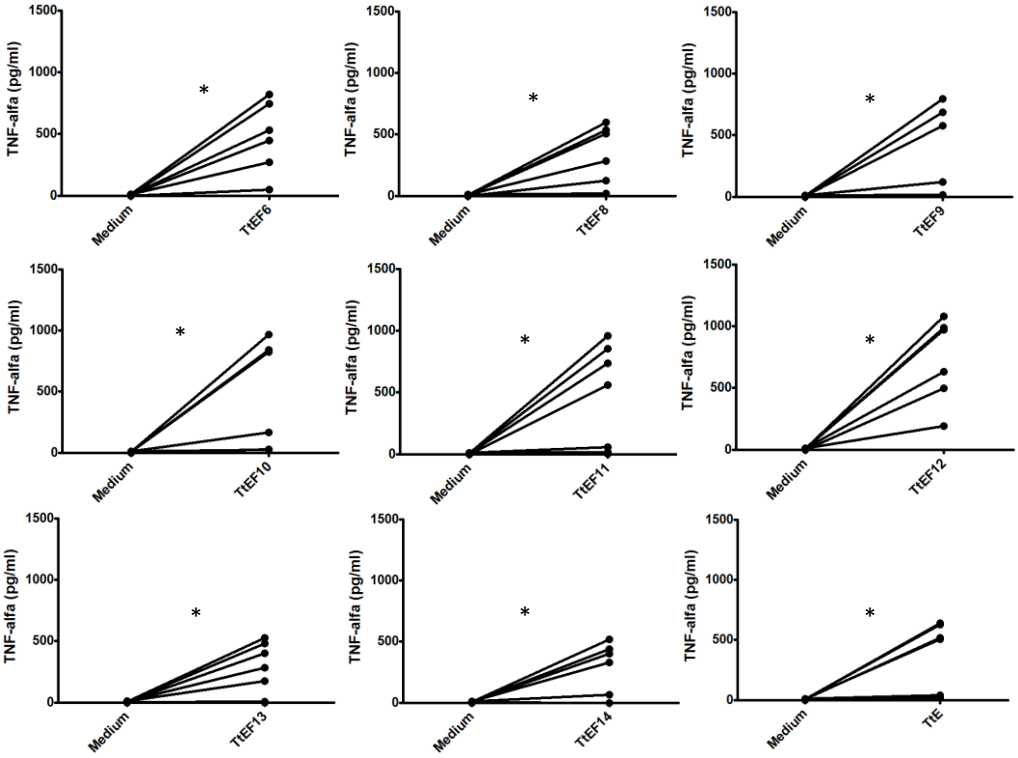

Figure 3: Induction of TNF-α production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMC) from healthy donors. *: $P < 0.05$ (in relation to the differences between the two groups of PBMC).

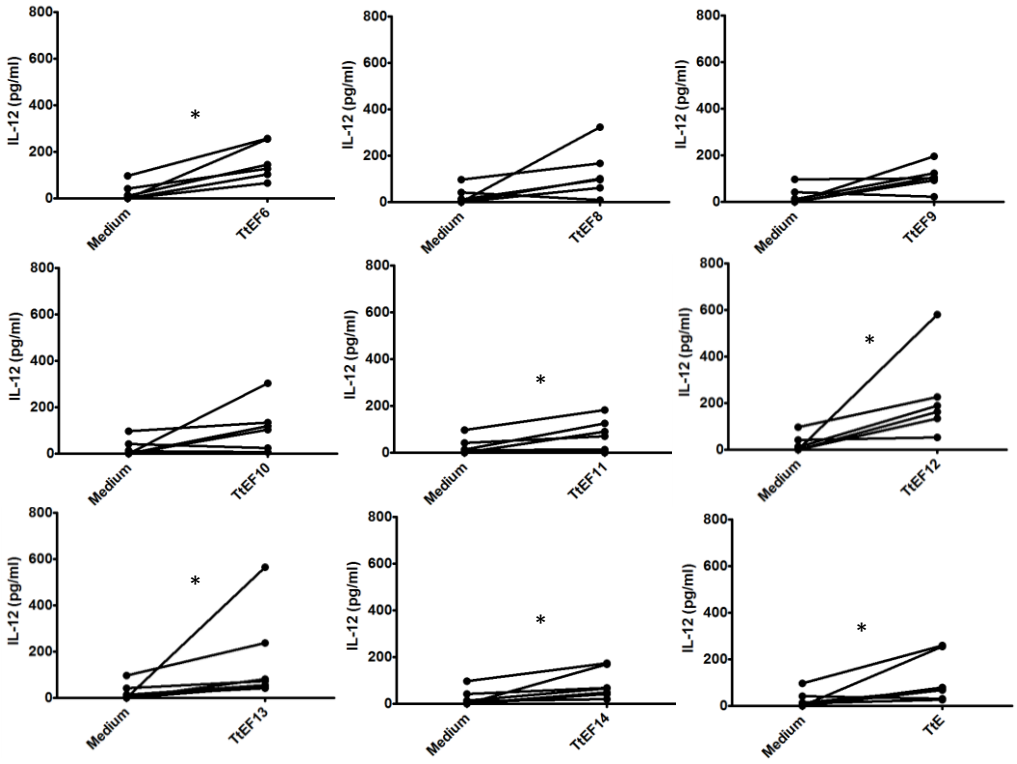

Figure 4: Induction of IL-12 p40 production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMC) from healthy donors. *: $P < 0.05$ (in relation to the differences between the two groups of PBMC).

Inhibition by TtEFs of cytokine production

None of the tested fractions significantly inhibited the production of IL-10 in cultures of PHA- or LPS-stimulated PBMC (Fig. 5). When the PBMC were stimulated by LPS, TtEF6, TtEF9, TtEF10, TtEF11, TtEF12, TtEF14, and TtE reduced the production of TNF-α (Fig. 6), and only TtEF9, TtEF11 and TtE inhibited the IL-12 p40 production in a statistically significant manner (Fig. 7). TtEF8, TtEF9, TtEF10 and TtEF14 inhibited the PHA-induced production of IL-5 (Fig. 8), and TtEF9 and TtEF10 inhibited the IL-13 production (Fig. 9).

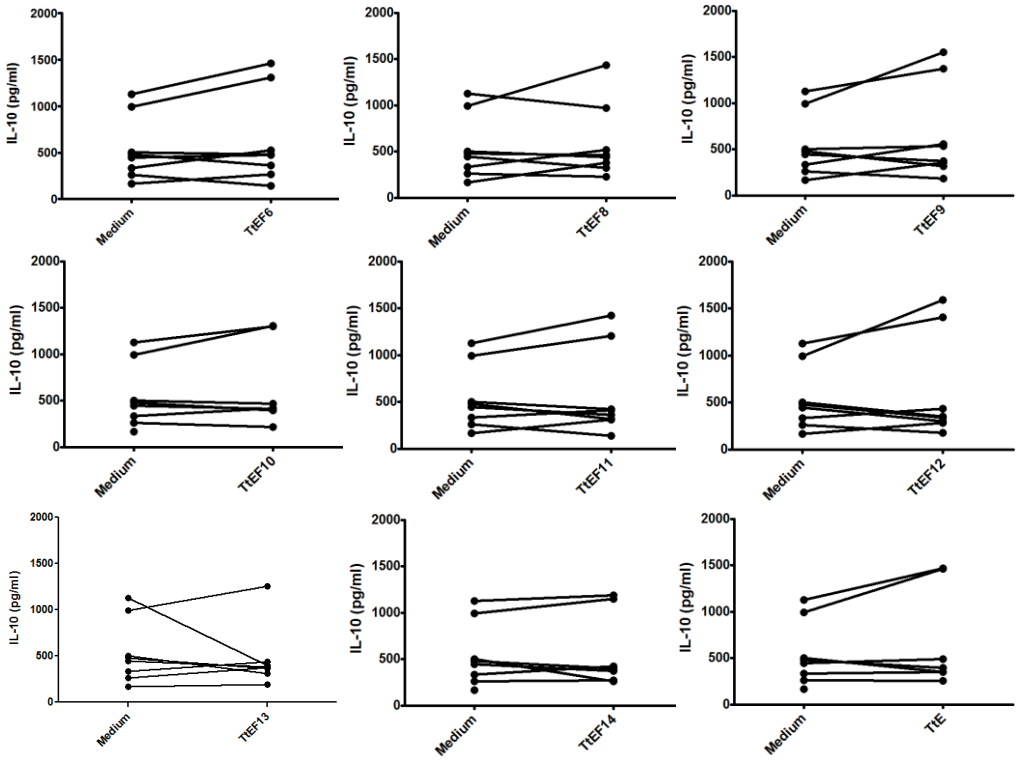

Figure 5. Inhibition of IL-10 production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMC) from healthy donors. *: P < 0·05 (in relation to the differences between the two groups of PBMC).

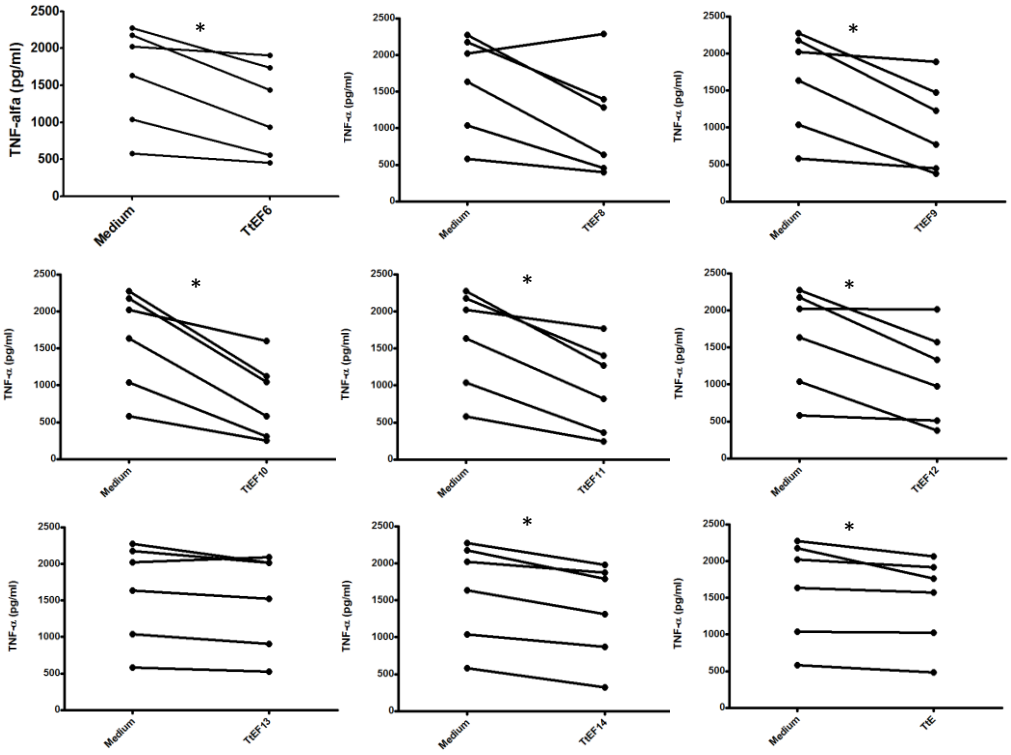

Figure 6. Inhibition of TNF-α production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMC) from healthy donors. *: P < 0·05 (in relation to the differences between the two groups of PBMC).

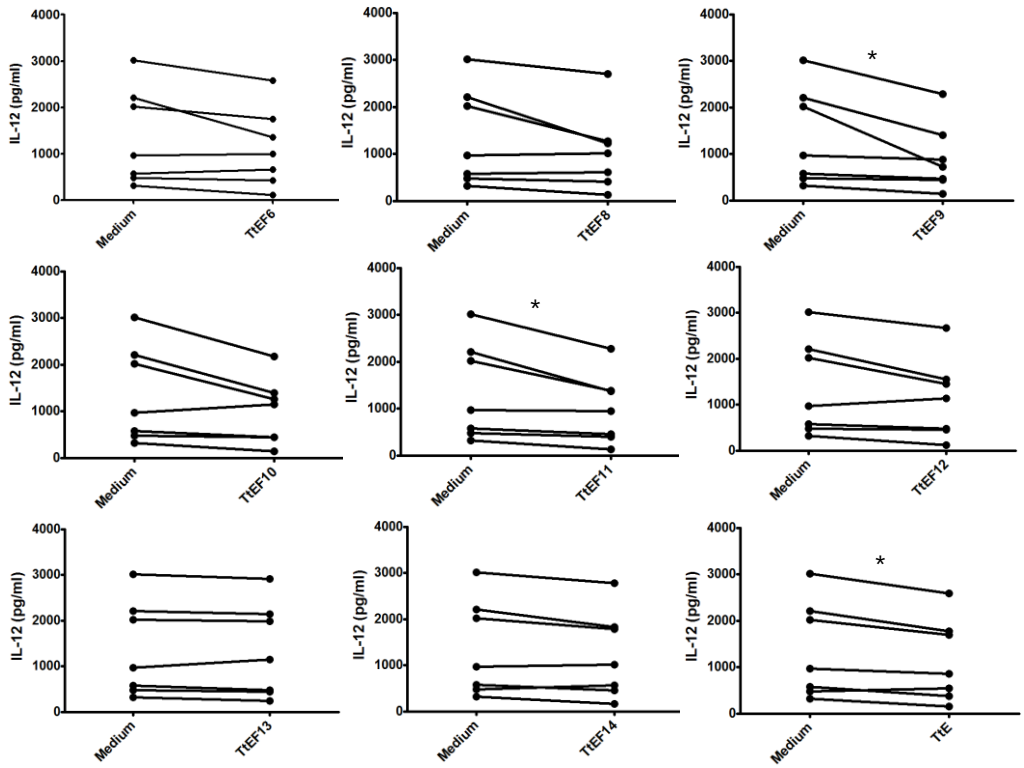

Figure 7. Inhibition of IL-12 p40 production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMC) from healthy donors. *P: < 0·05 (in relation to the differences between the two groups of PBMC).

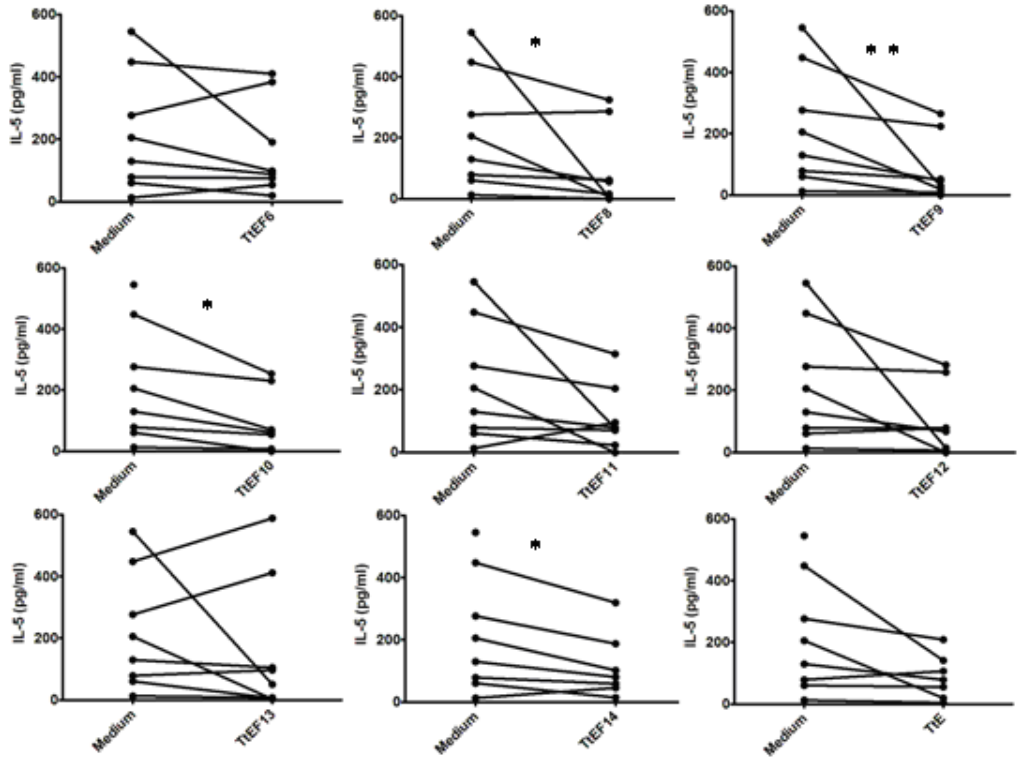

Figure 8. Inhibition of IL-5 production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMC) from healthy donors. *: P < 0·05; **: P<0.01 (in relation to the differences between the two groups of PBMC).

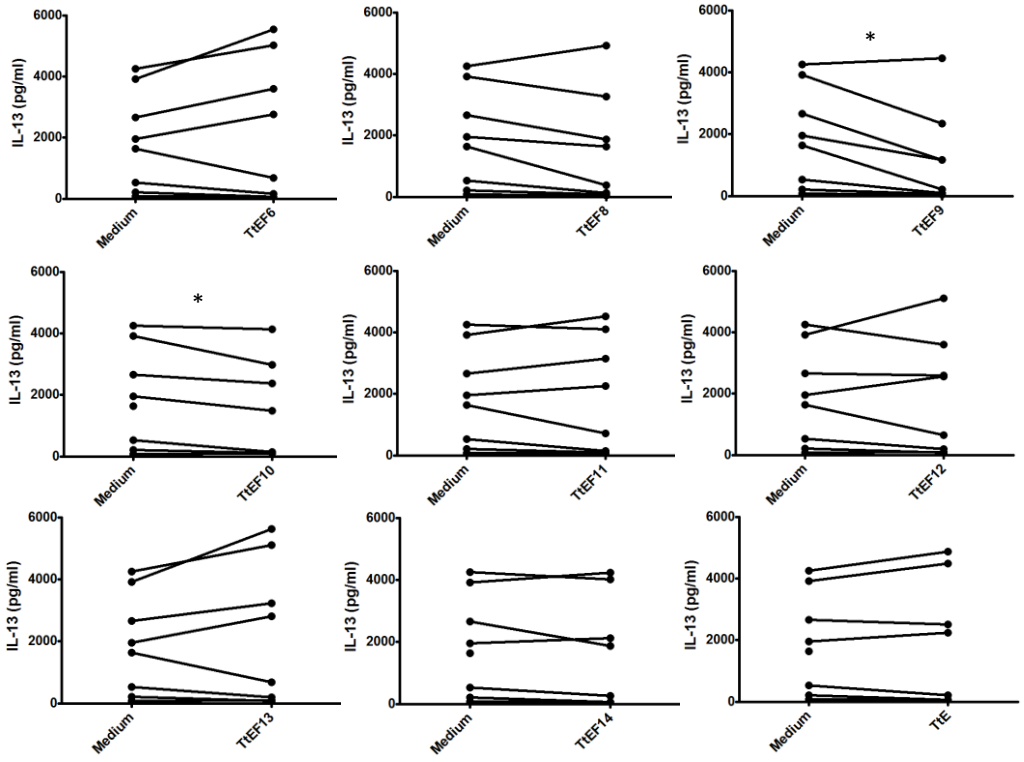

Figure 9. Inhibition of IL-13 production by somatic extract of T. *trichiura* (TtE) or its fractions in peripheral blood mononuclear cells (PBMC) from healthy donors. *: $P < 0{\cdot}05$ (in relation to the differences between the two groups of PBMC).

DISCUSSION

Helminth parasites including intetsinal helminths have been shown to have potent effects in the modulation of inflammation. The immune modulatory effects of extracts from helminth parasites are of great potential interest because such molecules may have considerable potential for the development of novel treatments for inflammatory diseases. Previous studies have provided evidence that trichurid worms may be protective against inflammatory diseases in experimental animal models and also in humans [14; 20; 21]. To our knowledge, there are no previous published studies investigating the immunoregulatory properties of partially purified extracts or molecules isolated from T. trichiura.

The major component of T. trichiura somatic extract was a 50,5 kDa band, which when separated by ion exchange chromatography appeared in fractions TtFr8 to TtFr12. Previously

a major band from T. trichiura adult worm extract and excretory/secretory (ES) products has been reported to be 49-51 kDa by SDS-PAGE [26], and likely represents the major band reported in the present study. The 49-51 kDa band was considered to be the same as a 47kDa antigen identified from metabolically-labelled T. trichiura somatic extract. The latter was poorly represented in metabolically-labelled ES products suggesting that it is either synthesized at a slow rate or is stored in parasite [26]. The major component of somatic extract of T. muris (43kDa) appeared to be structurally and functionally related to a 47 kDa band of T. trichiura [26; 27; 28]. The 43kDa band was more abundant in ES products of adult than L3 from T. muris and seemed to be absent in earlier stages; adult and the late larval stages are thought to be immunomodulatory, but the early larval stages are not [29], suggesting that some component of this band may be responsible for immunomodulation.

Interestingly, the fractions able to induce high IL-10 production shared the 49-52 kDa band, except TtFr6, suggesting that those fractions contained the component or components responsible for immunoregulatory activity of this worm. Particular importance was given to IL-10, because it is considered to be the most important cytokine involved in the regulatory network. It is produced by several cells types and has a diversity of functions relating to immunoregulation such as: the induction of T cell hyporresponsiviness with consequent inhibition of the production of Th1, Th2 and Th17 cytokines; inhibition of chemokine production; suppression of costimulatory molecules and MHC class II expression; and preferential production of IgG4 over IgE [30].

Although IL-10 has been shown to significantly suppress IL-12 p40 and LPS-induced production of TNF-α and IL-1β by peripheral blood mononuclear cells [31; 32; 33], some of the Tt fractions in our study induced also TNF-α and IL-12 p40 production which may be induced by initial exposure of macrophages to these fractions before the later induction of inhibitory effects, as has been observed previously [34; 35; 36] or because the fractions contain distinct molecules with pro-inflammatory and regulatory functions. Fractions TtFr8, TtFr9 and TtFr10 did not induced IL-12 p40 that is produced by macrophages and dendritic cells. Stimulation of TLR-2, TLR-4 and TLR-9 receptors on macrophages stimuli can induce the production of both IL-12p40 and TNF-α [37]. The linkage of innate to adaptive immunity through monocyte/macrophages is considered to be an important step in the modulation of immunity: T. trichiura could modulate immune responses through effects on TLR signalling pathways [34].

None of the fractions inhibited LPS-induced IL-10, but all inhibited LPS-induced TNF-α, and fractions TtFr9 and TtFr11 also inhibited LPS-induced IL-12. Similar inhibitory effects

on cytokine production have been described for specific molecules including glatiramer acetate (an immunomodulating polypeptide approved for the treatment of multiple sclerosis) on microglial cells [38], ES-62 (a secreted glycoprotein of the rodent filarial nematode Acanthocheilonemaviteae) on macrophages and dendritic cells [36] and Onchocystatin (a secreted cysteine protease inhibitor of Onchocerca volvulus) on PBMCs [39]. Consistent with our own observations for some *T. trichiura* fractions, onchocystatin has been shown to induce an initial increase of TNF-α at 6 h in PBMCs followed by a significant increase of IL-10 at 24 h and 48 h.

Th2 cytokines are involved in the pathogenesis of allergic diseases such as asthma [40; 41]. Our data shows that fractions TtFr8, TtFr9 and TtFr10 decreased PHA-induced IL-5, a cytokine that is responsible for eosinophil activation and which contributes to tissue injury caused by eosinophils [42]. Decreased levels of IL-5 has been described as a feature of the modified Th2 response, a tolerized Th2 response that may protect against allergic inflammation [10]. Inhibition of IL-5 could be mediated by elevated IL-10 [43], but because not all fractions that induced IL-10 inhibited IL-5, this effect may also be mediated through an alternate pathway such as through inhibition the NF-κB activation or inactivation of Rho-kinase, a putative inhibitor of IL-5 and IL-13 in bronchoalveolar lavage fluid from ovalbumin-challenged mice [44; 45]. TtFr9 and TtFr10 also inhibited PHA-induced IL-13 [46] that is involved in the induction of pulmonary inflammation, emphysema and asthma, and which increases mucus production by airways globet cells [41; 47]. TtFr9 was the fraction with the greatest downregulatory properties, since it induced IL-10 production, inhibited LPS-induced TNF-α and IL-12 p40, inhibited PHA-induced IL-5 and IL-13, without inducing IL-12 production.

CONCLUSION

Different fractions of the T. trichiura somatic antigen, obtained by anion-exchange chromatography, induced the production of IL-10 by otherwise unstimulated PBMC, and inhibited the production by stimulated PBMC of cytokines from the Th1- and Th2-dependent immune responses, indicating that the parasite produces more than one different molecule with immunomodulatory activity. One of the fractions, which eluted with 0.28 M NaCl from a Mono Q column, had a more marked down-regulatory activity than the other fractions and

should be the focus of further studies. These studies could lead to the characterization and further purification at molecular level of one or more than one bioactive molecules, with the potential of being used as tools for the development of protocols for the immunotherapy or immunoprophylaxis of allergic and autoimmune diseases.

ACKNOWLEDGEMENTS

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ANEXO A

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisas Gonçalo Moniz

Comitê de Ética em Pesquisas
Pesquisa Ética
CEP FIOCRUZ-BA

Comitê de Ética em Pesquisa – CPqGM/FIOCRUZ

PARECER Nº 179/2008

Protocolo: 277

Projeto de Pesquisa: **"Investigação sobre prováveis efeitos imunomedulatórios de produtos de *Trichuris trichuira* e *Toxocara canis* em populações celulares do sistema imune *in vitro* e em modelos experimentais de alergia respiratória e doença auto-imune".**

Pesquisador Responsável: **Dr. Lain Carlos Pontes de Carvalho**

Instituição ou Departamento: **LPBI/CPqGM-FIOCRUZ**

Considerações:

Após análise ética do projeto e realização dos esclarecimentos solicitados ao responsável, o CEP considera que o projeto atende aos princípios éticos de autonomia, beneficência, não maleficência, equidade e justiça.

Diante do exposto, o Comitê de Ética em Pesquisas do Centro de Pesquisas Gonçalo Moniz da Fundação Oswaldo Cruz (CEP-CPqGM/FIOCRUZ), conforme atribuições conferidas pela CONEP/CNS/MS (Carta Doc.32-04/97), com base na Resolução 196/96 e suas complementares, julga **aprovado** o projeto supracitado.

O CEP/CPqGM-FIOCRUZ especifica, abaixo, o período de vigência, bem como, determina as datas para o envio dos relatórios semestral e final, referentes ao desenvolvimento do protocolo de pesquisa aprovado.

Vigência: **18/12/2008 a 18/06/2011.**

Envio dos Relatórios Semestrais em: **18/06/2009 – 18/12/2009 – 18/06/2010.**

Relatório Final: **18/07/2011.**

*The present study, entitled **"Investigation of modulatory effects of purified fractions from Trichuris sp. Lysates and from Toxocara canis excretory-secretory molecules on immune cell populations in vitro and on experimental models of respiratory allergy and autoimmunity" (protocol number 277)** has been approved by the* Comitê de Ética em Pesquisa do Centro de Pesquisa Gonçalo Moniz - FIOCRUZ *(**IORG**0002090 / **IRB**000026120) in November 27th 2008 meeting. The protocol and procedures presented in the project are in accordance with the ethical standards of the responsible committee on human subject (institutional) and with the Helsinki Declaration of 1975, as revised in 2000. In the present version, this project is licensed and valid until June 18th 2011.*

Salvador, 18 de dezembro de 2008.

Drª Maria Fernanda Rios Grassi
Coordenadora do Comitê de Ética em Pesquisa
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