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Toxocara canis extract fractions promote mainly the production of Th1 and regulatory cytokines by human leukocytes *in vitro*

Dra Ana Lúcia Moreno Amor [a,*], Leonardo Nascimento Santos [b], Eduardo Santos Silva [b], Marina Borges Rabêlo de Santana [b], Emília Maria Medeiros de Andrade Belitardo [b], Flávia de Araújo Sena [b], Lain Pontes-de-Carvalho [c], Camila A. Figueiredo [b], Neuza Maria Alcântara-Neves [b]

[a] *Laboratório de Parasitologia, Centro de Ciências da Saúde, Universidade Federal do Recôncavo da Bahia, Avenida Carlos Amaral, 1015 - Cajueiro, Santo Antônio de Jesus, Bahia 44430-622, Brazil*
[b] *Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Bahia, Brazil*
[c] *Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, Brazil*

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ABSTRACT

Helminths possibly down-modulate immune responses to airborne allergens through the induction of a regulatory network. The identification of helminths bioactive molecules is highly desirable, given their immunomodulatory potential which could be used in immunotherapies for allergy and autoimmune diseases. To investigate the immunoregulatory potential of the adult *Toxocara canis* crude extract and ten protein fractions of its extract, human peripheral blood mononuclear cells (PBMC) from 10 allergic and 9 non-allergic individuals were cultivated, *in vitro,* in the presence or absence of these antigens, and their supernatants were evaluated for cytokine production (TGF-β, IL-10, IL-12, TNF-α, IL-6, IL-5, IL13, and IL-17). To determine the cell viability, the PBMC were cultivated for 24 h in the presence of the antigens and, following, they were subjected to a cytotoxicity assay. The viability of the PBMC was not affected by incubation with the *T. canis* antigens. As some fractions stimulated the production of immunoregulatory (TGF-β and/or IL-10), IL-12 and Th1 (TNF-α) cytokines, without stimulating Th2 cytokines (IL-5 and IL13) and IL-17, it was proposed that they would be potential candidates for further studies, especially involving the purification and characterization of specific proteins, which could be tested separately to evaluate their specific role as adjuvants in immunotherapy for inflammatory diseases.

1. Introduction

The prevalence of allergies and autoimmune diseases are increasing steadily in recent decades, and about 300 million people worldwide have asthma (Platts-Mills, 2015). On the other hand, it is estimated that 3 to 5% of the world population have autoimmune diseases (Quintero-Ronderos and Montoya-Ortiz, 2012). Researchers estimate that by 2050, two-thirds of the world population will live in cities and will have little contact with nature and biodiversity, and as a result, an increase in chronic diseases such as allergies and respiratory disorders is expected (Hanski et al., 2012). Up to now, there are no good curative or prophylactic drugs for these diseases and immunotherapy is an important alternative to the available therapies (Pfaar et al., 2018). It has as goals improving symptoms, increasing quality of life, favorably altering the course of disease. Moreover, it achieves these goals with a good benefit-to-risk ratio and is cost-effective (Nguyen and Casale, 2011).

Asthma and rhinitis (allergic atopic disorders) are caused by a deregulated immune response, involving T helper type 2 (Th2) cytokines, including interleukin (IL)-4, IL-5 and IL-13 (Cardoso et al., 2010). Th-17, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) have also been implicated in the pathogenesis of these diseases (Araújo et al., 2010). Some feasible strategies to down-modulate the allergic immune response are proposed by several studies, and are based on different mechanisms: (a) Th1 immune responses against viral and bacterial

* Corresponding author: Laboratório de Parasitologia. Centro de Ciências da Saúde, Universidade Federal do Recôncavo da Bahia, Avenida Carlos Amaral, 1015 - Cajueiro, 44430-622, Santo Antônio de Jesus, Bahia, Brazil
E-mail address: ana_amor@ufrb.edu.br (D.A.L.M. Amor).

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products (Th1 deviation), leading to the control of the allergic Th2 immune response (which may, however, increase the susceptibility to Th1-mediated diseases) (Hessle et al., 2005); and (b) induction of regulatory mechanisms ($CD4^{+}CD25^{+}$ regulatory T cells; expression of cytotoxic T-lymphocyte antigen 4 - CTLA-4; expression of IL-10 or of TGF-β) (Araújo et al., 2010).

Epidemiological studies have shown a lower prevalence of atopy (Alcântara-Neves et al., 2012; Rodrigues et al., 2008) and allergic diseases in countries that are endemic for helminthic infections (Cooper, 2009). These findings are explained by the down-modulated host protective immune responses caused by helminths, which have evolved mechanisms that modulate the immune system of their hosts by stimulating the production of IL-10 and TGF-β by regulatory T cells that actively suppress inflammatory effector cells (Logan et al., 2018; McSorley et al., 2013). These cytokines create a balance among different types of antibodies (Adjobimey and Hoerauf, 2010) i.e. an increase in the production of IgG, IgG4, IgA and a decrease in specific IgE levels (Robinson et al., 2004), that enable the helminths to survive in their hosts. This immune response has the effect of reducing the inflammatory damage of allergic and autoimmune origins (Maizels and Yazdanbakhsh, 2003). These parasites or their molecules have been studied and used in humans to treat chronic inflammatory diseases with varying results (Ryan et al., 2020; Santos et al., 2017). Advances in knowledge, in order to clarify these contradictions and identification of the molecules and extracts that can regulate specific pathways of the immune response *in vitro* are needed (Imai and Fujita, 2004). Some studies have shown that molecules of *Schistosoma mansoni* and filarial parasites are able to reduce atopy and allergy in experimental animal models, decreasing Th2 cytokine and increasing IL-10 production by human cells *in vitro* (Cardoso et al., 2010). Another relevant report is the therapeutic benefit of infection with the porcine whipworm *Trichuris suis*, for patients with inflammatory bowel diseases, reducing clinical manifestations of ulcerative colitis and Crohn's disease (Summers et al., 2005a, 2005b, Weinstock et al., 2002). Another report revealed that protein fractions of *Trichuris trichiura* (human whipworm) induced the production of IL-10 and decreased levels of Th2 cytokines in peripheral blood mononuclear cells from healthy donors (Santos et al., 2013).

Previous studies from this group have also shown that fungal extracts (candidine or trichophytin) elicit memory immune responses that can inhibit a Th2 response; stimulated the production of regulatory cytokines (TGF-β and / or IL-10), accompanied or not by stimulation of the production of cytokines associated with the Th1 response (TNF-α and IFN-γ) and IL-12 but without cytokine Th2 stimulation (IL-5 and IL-13) and IL-17, by peripheral blood mononuclear cells of most allergic and non-allergic individuals (Amor et al., 2014).

The human infection by the dog and cat roundworms *Toxocara canis* and *T. cati* is highly prevalent in low-income populations of developing countries. It is mostly asymptomatically or it causes the visceral larva migrans syndrome (Campos et al., 2003), in which the *Toxocara* spp larvae migrate to the lung causing asthma-like symptoms or to other organs and viscera causing polymorphic clinical manifestations including ocular symptoms (Pinelli and Aranzamendi, 2012). As we have found that this infection occurs in 46–65% of the low class population in Salvador, a large city in the Northeast of Brazil (Dattoli et al., 2011; Souza et al., 2011) and that the seropositivity for this infection was found associated to low skin prick test reactivity for aeroallergens (Mendonça et al., 2013), we decided to investigate whether human human peripheral blood mononuclear cells (PBMC) would respond to the stimulation with *T. canis* adult extract anion-exchange chromatographic fractions with the production of cytokines.

2. Material and methods

2.1. Blood donors

All blood donors, aged between 21 and 40 years, signed an informed consent to participate in the research. They were classified into allergic (n = 10) and non-allergic (healthy) (n = 9) donors, based in the history of allergic symptoms, results of skin prick tests using extracts from six commonest regional allergens (*Blomia tropicalis, Dermatophagoides pteronyssinus, Blattella germanica and Periplaneta americana*, cat and dog epithelia; Alergolatina, Rio de Janeiro, Brazil), and measurement of specific IgE concentrations to these aeroallergens using ImmunoCAP assay (Phadia/Thermo Fisher Diagnostics AB, Uppsala, Sweden).

Although we are aware of the small sample size, we believe our preliminary results are relevant, precursors and a starting point in the identification of potential immunoregulatory proteins. In previous studies published by our group, we have also used similar sample size (Amor et al., 2014; Santos et al., 2013). One of such studies was continued by a transcriptomic analysis (Santos et al., 2016), which confirmed our preliminary identification using a smaller sample size for PBMC culture.

This research was approved by the Ethical Committee of the Maternidade Climério de Oliveira, Universidade Federal da Bahia, Proposal 195.933/2013 and by the Ethics Committee on Animal Use of the Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Bahia, Brazil (Proposal 014/2010).

2.2. Preparation of Toxocara canis antigens

T. canis adult worms were isolated from infected dogs treated with piperazine citrate (0,3 g/kg body weight). The worms were collected from feces with forceps up to 24 h after treatment and were washed in saline and cryopreserved at – 70 °C until use.

The parasite lysate and its chromatographic fractions were obtained according to Santos and collaborators [2013]. Briefly, the adult worms were lysed in a tissue grinder in the presence of zirconium/silica beads (BioSpec Products, Inc., Bartlesville, USA) and 20 mM TRIS - HCl pH 8.0, containing a mix of protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 50 mM tosyl phenylalanyl chloromethyl ketone, 50 mM tosyl lysine chloromethyl ketone, and 2 mM ethylenediaminetetraacetate) (Sigma-Aldrich, St. Louis, MO, USA). Then, the extract was centrifuged at 13,400 g for 20 min at 4°C and the supernatant sterilized by filtration and stored at -70°C. The extract was fractionated with a salt gradient elution by fast protein liquid chromatography using a Mono Q 5/50 column (GE Healthcare, São Paulo, SP, Brazil). The adult *Toxocara canis* crude extract (TcE) and ten protein fractions of its extract (TcEF01-TcEF10) were dialyzed against RPMI 1640 medium (Gibco, Grand Island, NY, USA) in order to use in cell cultures.

The protein concentration was measured using the Folin reagent by Lowry's method (Lowry et al., 1951), yielding the following protein concentrations: TcE (138 mg/mL), TcEF01 (33.5 mg/mL), TcEF02 (22.9 mg/mL), TcEF03 (31.9 mg/mL), TcEF04 (23.6 mg/mL), TcEF05 (24.1 mg/mL), TcEF06 (50.5 mg/mL), TcEF07 (34.7 mg/mL), TcEF08 (52.8 mg/mL), TcEF09 (40.3 mg/mL), and TcEF10 (24. 7 mg/mL). And the electrophoretic profile visualized in polyacrylamide gel (SDS-PAGE) by staining with Coomassie blue with the antigens of protein fractions, presenting molecular weight values ranging from 14 to 100 kDa determined by gel electrophoresis with predominance of bands between 30 and 100 kDa. 70 kDa in these products (Fig. 1A).

2.3. Choice of antigen and mytogen concentrations

To determine the optimal concentration of the crude extract of *T. canis* and ten of its protein fractions, PBMC, obtained from allergic and non-allergic volunteers, were isolated from venous blood by centrifugation on HISTOPAQUE 1077® solution (Sigma-Aldrich, St. Louis, MO, USA). The cells (10^6 / well), were cultivated in 96-well plates (200 μl/well) in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), at 37°C and 5% CO_2 and

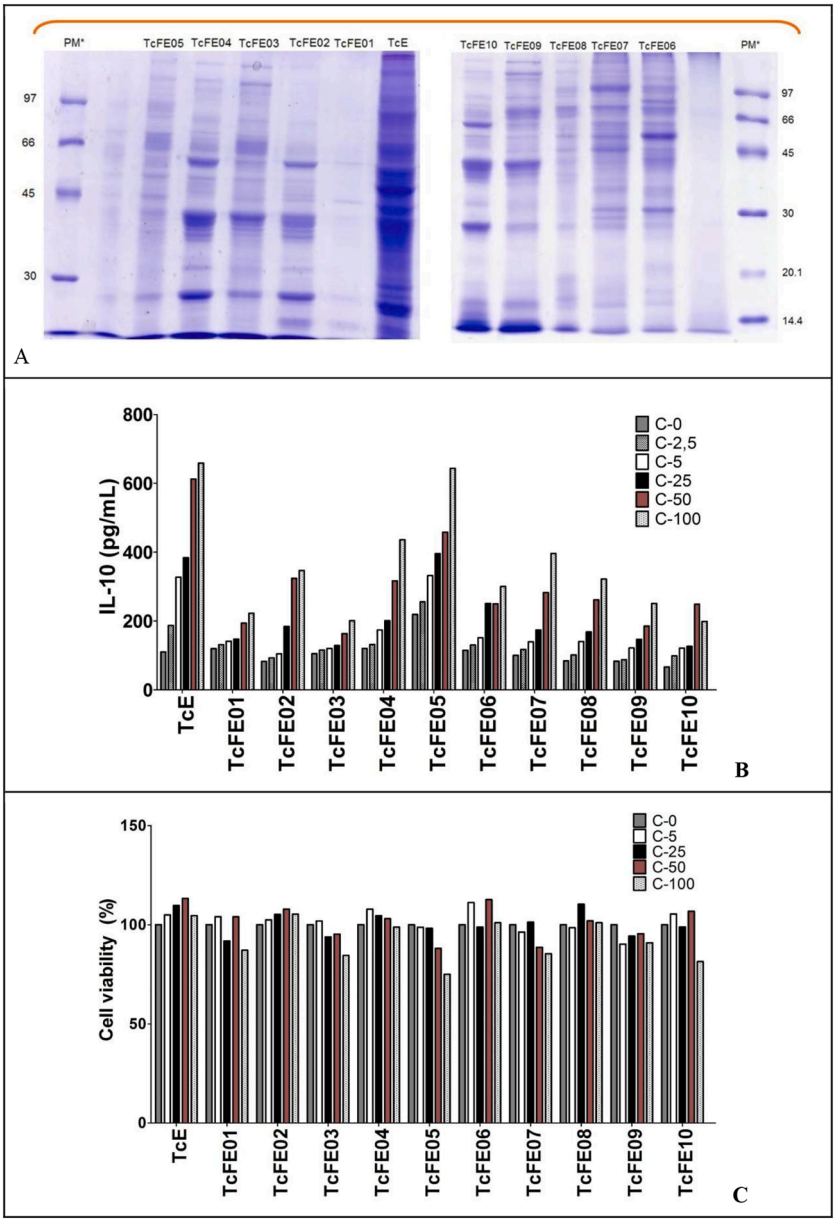


Fig. 1. *T. canis* antigens and ten of their protein fractions: (A) Characterization regarding the production and protein composition of the tested products arranged in gels (SDS-PAGE) stained with coomassie blue. (B) Determination of the optimal concentration after PBMC stimulation and subsequent measurement of IL-10 and (C) Cell viability via cytotoxicity assay using MTT.

stimulated or not with lipopolysaccharide (LPS from *Escherichia coli)* (8 UE/mL; Sigma-Aldrich, St. Louis, MO, USA) and in the absence and presence of 2,5 to 100 μg/mL of protein from each antigen at 37°C and 5% CO2 during 24 h. Following, the IL-10 production was measured in the cell supernatants. After collection of the supernatant, PBMC were subjected to the cytotoxicity assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA), according to Mosmann and collaborators (Mossman, 1983). Fifty μg/mL was proved to be the optimal concentration to stimulate the PBMC and the viability of the PBMC was not affected by incubation for 24 h with 50 μg/mL of the crude extract of *T. canis* and by its protein fractions (Fig. 1B and C).

To determine the optimal concentration of the crude *T. canis* extract and ten of its protein fractions for PBMC stimulation, IL-10 production was measured, as well as cell viability after incubation with different concentrations of antigens. Cells were cultured in the absence and presence of 2.5 to 100 μg/ml protein of each antigen and induced the production of IL-10 cytokines in all PBMC preparations. The concentration of 50 μg/ml proved to be ideal for testing the production of IL-10 by stimulated PBMC (Fig. 1B). Similar to previous studies of our group (Santos et al., 2013), we determine the immunomodulatory effect of *T. trichiura* adult worm extract (TtE) and its fractions (TtEFs) on cytokine production by PBMC cultured in the presence of 50 μg/mL of TtE or TtEFs. PBMC viability was not affected by incubation with up to 50 μg/ml of the crude extract of *T. canis* and ten of its protein fractions for 24 h, demonstrated by the absence of further reduction in MTT (Fig. 1C).

2.4. Stimulatory and inhibitory assays

PBMC were stimulated by *T. canis* antigens containing 50 μg/mL of protein (TcE or TcEFs). As positive control it was used 8 UE/mL of LPS from *E. coli* for measuring IL-10, transforming growth factor (TGF-)β, IL-6, IL-12 and tumor necrosis factor (TNF-)α in the cell supernatant or 10 μg/mL of PHA for measuring IL-5, IL-13 and IL-17. Cells were cultivated

as described above, for 48 h (for quantification of IL-10, TNF-α, IL-12 and TGF-β) or for 120 h (for quantification of IL-5, IL-17, and IL-13), at 37°C and 5% CO_2.

The cells were incubated with 20 μg/mL polymyxin B (Sigma-Aldrich, St. Louis, MO, USA) as an endotoxin neutralizing agent, except for the wells incubated with LPS.

Regarding the inhibition assay, the PBMC were incubated with TcE and its fractions and suboptimal concentrations of LPS (4 UE/mL) to measure IL-10, TGF-β, and TNF-α; or with LPS 4 UE/mL plus IFN-γ 100 ng/mL to measure IL-12 and with PHA 5 μg/mL, to quantify IL-5, IL-13 and IL-17. As positive control the PBMC were incubated with the mitogens (and IFN-γ) alone. Unstimulated PBMC were used as negative controls in both assays.

2.5. Cytokines measurements

The cytokines (TGF-β, IL-10, IL-12, IL-6, TNF-α, IL-5, IL13 and IL-17) concentrations in PBMC culture supernatants were assessed using commercially available enzyme-linked immunosorbent assay (ELISA) duo-sets, according to manufacturer's instructions (Pharmingen, BD Biosciences, San Diego, CA, USA). The percentage of inhibition of cytokine production was calculated as: 1- (OD mean of wells incubated with antigens and mitogen/OD mean of wells incubated with mitogen) x 100; where OD = optical density. Only the cultures where inhibition occurred were shown in the Figures.

2.6. Statistical analysis

Data normality was assessed by the Pearson's and D'Agostino's test. Data normality was assessed by the Pearson's and D'Agostino's test. The statistical significance of differences in cytokine concentrations between stimulated and nonstimulated cultures were determined by: (i) Friedman's test with post test of Dunn's Multiple Comparison test, when data was non-parametric; (ii) ANOVA, followed by Bonferroni's Multiple Comparison test, when data was parametric. Differences of $p \leq 0.05$ were considered significant.

3. Results

3.1. Production of cytokines by PBMCs stimulated with T. canis antigens

Figs. 2 and 3 show the stimulatory assays. Regarding the differences between the antigens of each group, the following cytokines were produced by PBMC from allergic donors in a statistically significant way: TGF-β, IL-5, IFN and IL-17 ($p \leq 0.05$), IL-6 ($p \leq 0.01$), IL-10, IL-12 and TNF ($p \leq 0.001$). The color gradient shows a greater number of cultures stimulated by regulatory cytokines, IL-12 and the Th1 profile for allergic and non-allergic donors (Frame 1A and B).

All *T. canis* products stimulated the production of TGF-β and IL-10 in PBMC from allergic donors and IL-12 in non-allergic donors, however, in a statistically significant way, the following results are highlighted. The production of TGF-β was verified by stimulation with TcEF04 in non-allergic patients (Fig. 2B). It is noteworthy that this stimulation induced the production of this cytokine in all cultures of allergic donors (Frame 1A).

The adult *T. canis* crude extract (TcE) and two of its protein fractions (TcEF04 and TcEF05) induced IL-10 production by PBMCs from allergic donors (Fig. 2C), while in non-allergic PBMCs, the production of this cytokine was induced by TcEF06 (Fig. 2D). The TcE, TcEF02, TcEF03 and TcEF10 products induced the production of this cytokine in all ten cultures from the allergic donors (Frame 1A).

IL-12 production was induced in PBMC by two types of fractions per donor, TcEF04 and TcEF05 for allergic (Fig. 2E) and TcEF05 and TcEF06 for non-allergic (Fig. 2F). The products TcE and TcEF06 and TcEF04 and TcEF05 induced the production of this cytokine in all cultures from non-allergic and allergic donors, respectively (Frame 1A and B).

TNF production significantly increased in PBMC cultures of non-allergics donnor stimulated by TcE and one of its fractions (TcEF06) (Fig. 2H). The TcEF07 product induced the production of this cytokine in all nine cultures from non-allergic donors (Frame 1B).

Some fractions stimulate (Fig. 2I and J) and inhibit the production of IFN-γ, but without statistical significance.

Interestingly, for Th2 and Th17 cytokines, in PBMC cultures from donors allergic to IL-5 production, IL-13 and IL-17 did not present themselves to most T. canis stimuli (Fig. 3A, C and E). In relation to PBMC, IL-13 presented a higher number of induction in cultures from non-allergic donors and IL-17 in cultures from allergic donors (Frame 1A and B).

3.2. Cytokine production by PBMCs inhibited by T. canis antigens

Figs. 4 and 5 show the inhibitory assays. The color gradient shows a greater number of cultures inhibiting the production of Th2 and IL-17 cytokines (Frame 1), for allergic and non-allergic donors.

T. canis antigens did not statistically significantly inhibit the production of any regulatory cytokine or Th1 or IL-12 in PBMC from allergic and non-allergic donors (Fig. 4). When the inhibition profile is observed, it can be seen that, despite some cultures inhibiting the production of some cytokines (Table 1), the means of the antigen groups did not reflect this inhibition (Figs. 4 and 5).

As for Th2 cytokines, TcE inhibited IL-5 production in all PBMC cultures from non-allergic donors (Fig. 5B; Frame 1B).

On the other hand, IL-17 production by PBMC from allergic donors was inhibited by TcEF01-04 (Fig. 5E). The production of this cytokine by PBMCs from non-allergic donors was inhibited with all stimuli (Fig. 5F).

Table 1 shows an overview of stimulation or inhibition with statistically significant data and general data of these actions caused by TcE and their respective protein fractions in the production of all cytokines by PBMC from allergic and non-allergic donors.

It shows that, in most of the trials with statistical significance, there was an increase in the production of regulatory cytokines (TGF-β and/or IL-10) followed by an increase in pro-inflammatory Th1 cytokines (TNF-α) and IL-12, without stimulation of Th2 profile cytokines (IL-5 and/or IL-13 and/or IL-6) and IL-17. The actions of TcE, TcEF04, TcEF05 and TcEF06 products in allergic and/or non-allergic donors stand out for these actions.

4. Discussion

To be considered a promising candidate to function as primary or as adjuvant tool for immunotherapy of allergy, the product must stimulate the production of Treg cytokines, Th1 cytokines or both, without stimulating Th2 and Th17 cytokines production (Bohle et al., 2007; Jutel et al., 2003; Robinson et al., 2004).

On the other hand, other authors report that allergen-specific immunotherapy should lead to the activation exclusively of Treg cells, with production of regulatory cytokines [IL-10 and / TGF-β] (Bohle et al., 2007; Jutel et al., 2003; Robinson et al., 2004).

IL-10, a cytokine produced by many cell types, promotes a decrease in IgE production, and inhibits the release of histamine and other inflammatory mediators by mast cells, inhibiting allergic inflammatory responses (Araújo et al., 2010). Indeed, it has been demonstrated that the success of allergen-specific immunotherapy is associated with the induction of IL-10 (Burastero et al., 2008).

In respect to the use of *T. canis* crude extract and / or its fractions in immunotherapy of allergic diseases and / or autoimmune diseases, it should be taken into account the pattern of cytokine production by leukocytes stimulated with each tested product, ideally in stimulatory and inhibitory assays.

In this study it was found that the PBMC different donors did not respond with the same intensity to the same fractions, which may reflect genetic differences or differences in antigenic stimulation history of each

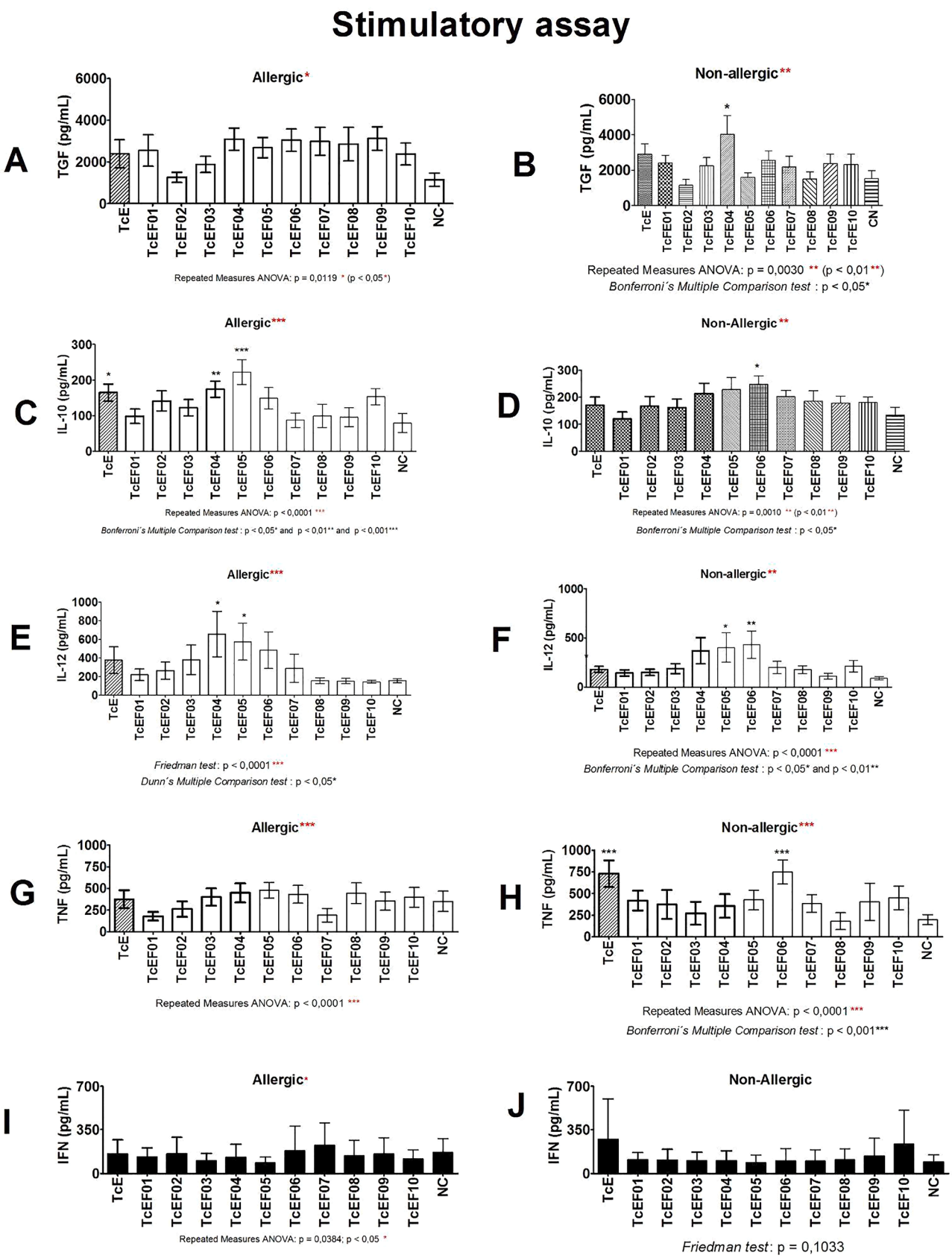


Fig. 2. Stimulatory effects on cytokine production (TGF-β, IL-10, IL-12. TNF-α and IFN) in cultures of PBMC of the allergic (A, C, E and G) and non-allergic individuals' (B, D, F and H) stimulated in the absence (negative control - NC) or in the presence of the TcE or TcEF.

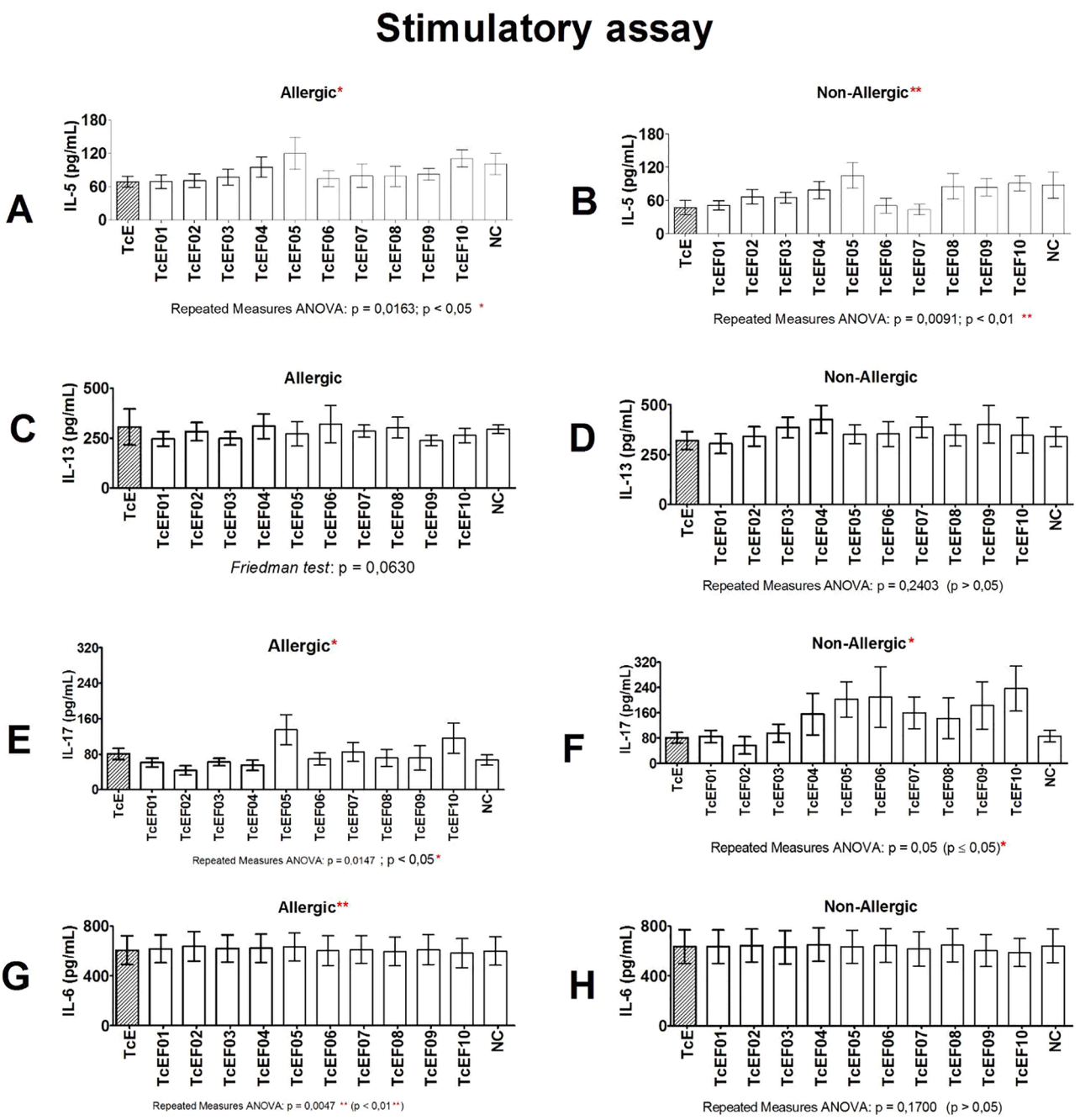


Fig. 3. Stimulatory effects on cytokine production (IL-5, IL-13, IL-17 and IL-6) in cultures of PBMC of the allergic (A, C, E and G) and non-allergic individuals' (B, D, F and H) stimulated in the absence (negative control - NC) or in the presence of the TcE or TcEF.

donor immune systems, including infection by *Toxocara* spp, especially in Salvador, Bahia, Brazil where this work took place and seroprevalence of *Toxocara* spp infection is high (Dattoli et al., 2011; Mendonça et al., 2013).

This work, although has studied a relatively small sample of individuals, clearly shows the production of regulatory and / or Th1 cytokines by PBMC of allergic individuals stimulated with *T. canis* antigens (TcE extract and the fractions TcEF01-10) without statistically significantly stimulating the production of Th2 and IL-17 cytokines, which are associated with respiratory allergy. Such findings are interesting and agree with previous studies with other helminth species. While some proteins of these nematodes have been identified and acted as allergens leading to Th2 responses (Acevedo et al., 2013; Ahumada et al., 2020; De las Vecillas et al., 2020), others displayed a regulatory role by inducing a shift in inflammatory and allergenic responses (Coronado et al., 2017; Coronado et al., 2019; Hartmann and Lucius, 2003). With statistically significant data in the assays, in addition to TcE, there are responses for a regulatory cytokine profile, Th1 or IL-12 for the stimulatory assays with TcEF04, TcEF05 and TcEF06. Therefore, it can be inferred that some fractions induced stronger response towards a specific immune profile because specific proteins were present in higher amounts in the final stimulatory concentration, whereas in TcE those proteins were strongly diluted and, in turn, would not be able to produce the same stimulatory behavior.

Our data showed that *T. canis* antigens were good stimulators of IL-12 in allergic donors, but with non-statistically significant production of IFN-γ. For operational reasons, IFN-γ was quantified in cell cultures with the supernatant collection time (48 h). Perhaps if we increased the

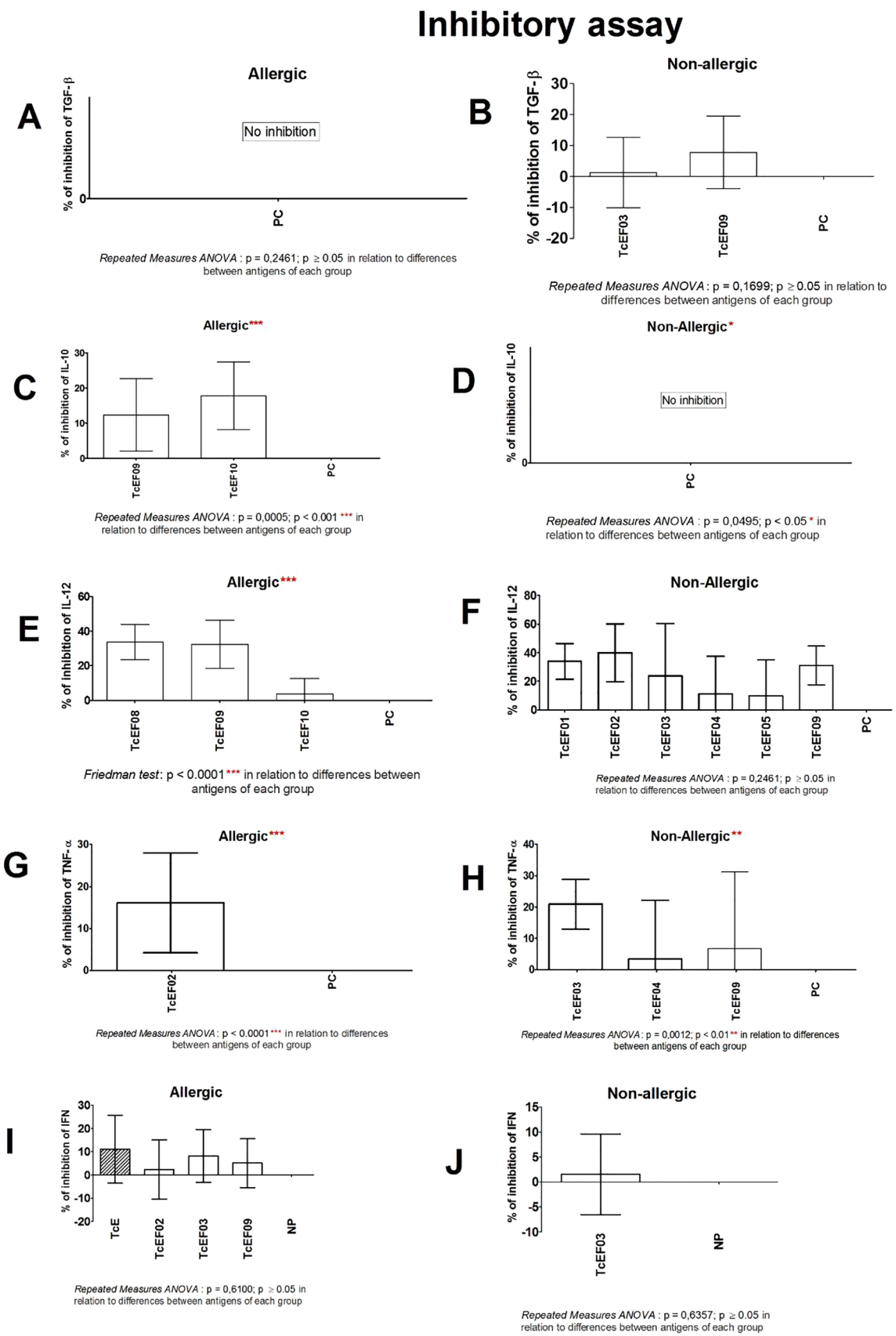


Fig. 4. Inhibitory effects on cytokine production (TGF-β, IL-10, IL-12, TNF-α and IFN) in cultures of PBMC of the allergic (A, C, E and G) and non-allergic individuals' (B, D, F and H) stimulated in the presence of the TcE or TcEF.

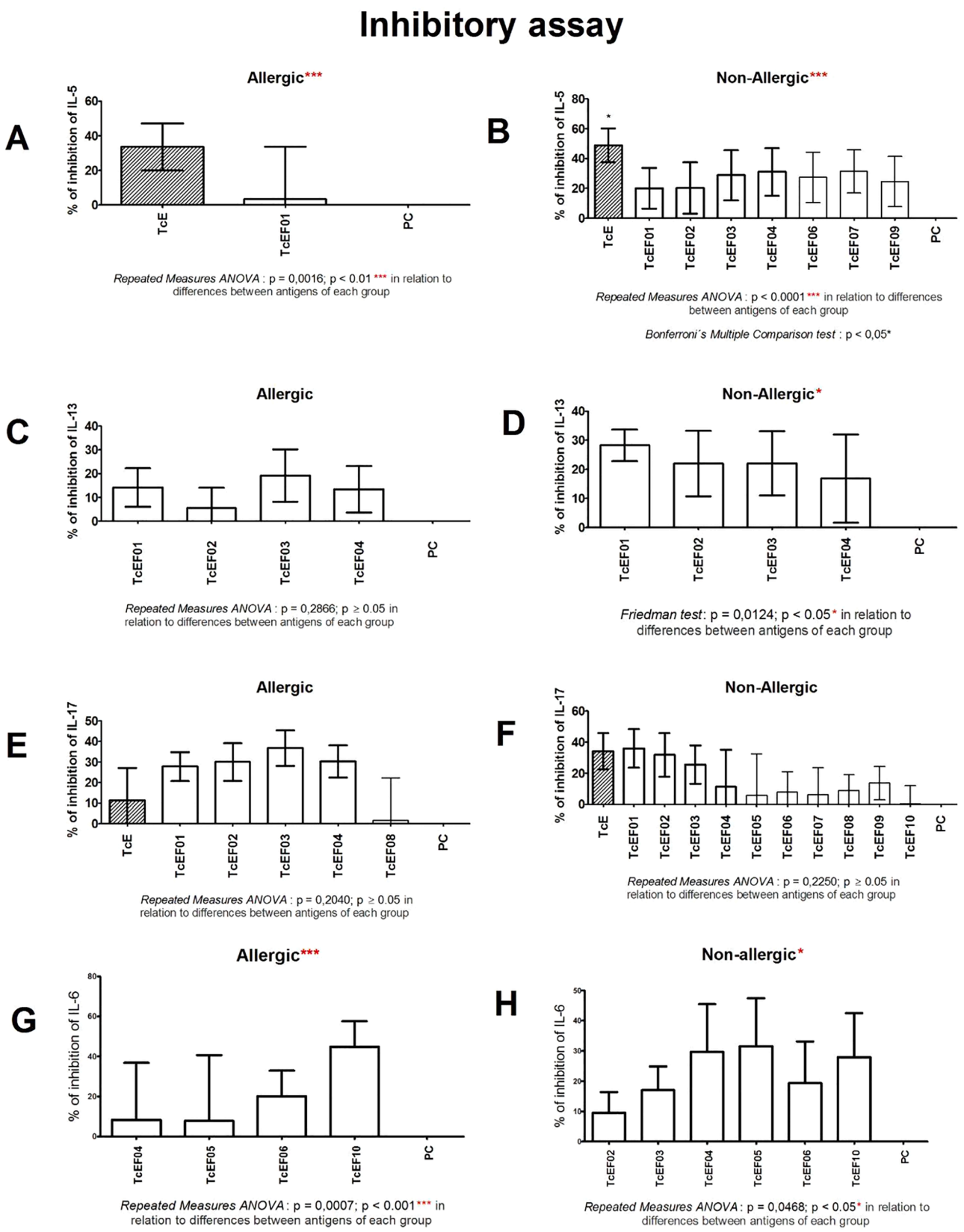
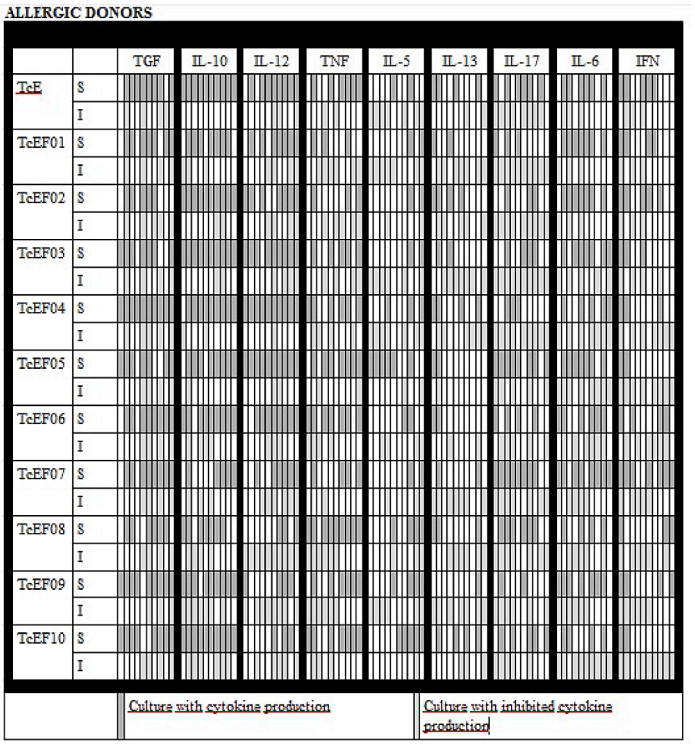
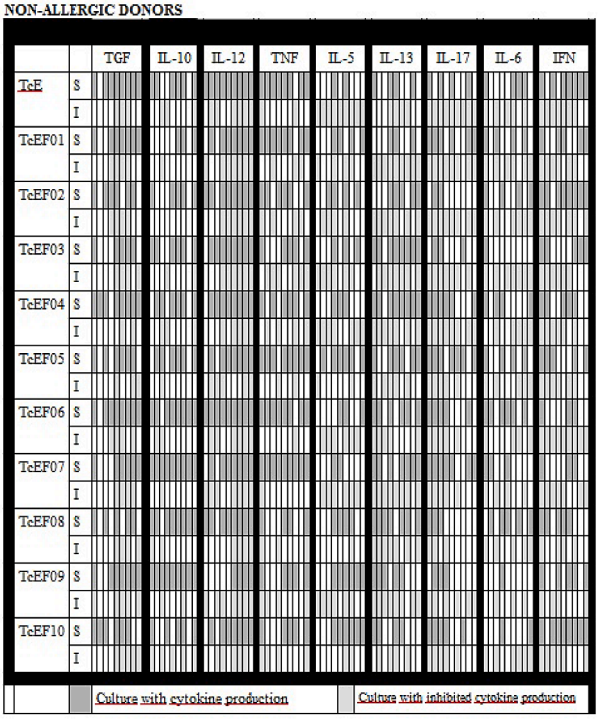


Fig. 5. Inhibitory effects on cytokine production (IL-5, IL-13, IL-17 and IL-6) in cultures of PBMC of the allergic (A, C, E and G) and non-allergic individuals' (B, D, F and H) stimulated in the presence of the TcE or TcEF.

A



B



Frame 1. Profile of cytokine production per individual (S = stimulatory assay; I = inhibitory assay): A. Cultures from allergic donors; B. Cultures from non-allergic donors.

Table 1
Summary of results of the cytokines production by allergic and non-allergic donor leukocytes stimulated (S) or inhibited (I) by TcE and TcEF01-10.

Product	Assay	ALLERGIC DONORS	Assay	NON-ALLERGIC DONORS
TcE	**S**	↑IL-10*; ↑TGF-β; ↑IL-12; ↑TNF-α; ↑IL-17	**S**	↑TNF-α*** ↑TGF-β; ↑IL-10; ↑IL-12; ↑IFN
	I	↓IL-5; ↓IL-17; ↓IFN	**I**	↓IL-5*; ↓IL-17
TcEF01	**S**	↑TGF-β; ↑IL-10; ↑IL-12; ↑IL-6	**S**	↑TGF-β; ↑IL-12; ↑IFN; ↑TNF-α
	I	↓IL-5; ↓IL-13; ↓IL-17	**I**	↓IL-12; ↓IL-5; ↓IL-13; ↓IL-17
TcEF02	**S**	↑TGF-β; ↑IL-10; ↑IL-12; ↑IL-6	**S**	↑IL-10; ↑IL-12; ↑IFN; ↑TNF-α; ↑IL-13
	I	↓IFN; ↓TNF-α; ↓IL-13; ↓IL-17	**I**	↓IL-12; ↓IL-5; ↓IL-13; ↓IL-17; ↓IL-6
TcEF03	**S**	↑TGF-β; ↑IL-10; ↑IL-12; ↑IL-6; ↑TNF-α	**S**	↑TGF-β; ↑IL-10; ↑IL-12; ↑IFN; ↑TNF-α; ↑IL-13
	I	↓IFN; ↓IL-13; ↓IL-17	**I**	↓TGF-β; ↓IL-12; ↓TNF-α; ↓IFN; ↓IL-5; ↓IL-13; ↓IL-17; ↓IL-6
TcEF04	**S**	↑IL-10**; ↑IL-12* ↑TGF-β; ↑TNF-α	S	↑TGF-β*; ↑IL-10; ↑IL-12; ↑IFN; ↑TNF-α; ↑IL-13; ↑IL-17
	I	↓IL-13; ↓IL-17; ↓IL-6	**I**	↓IL-12; ↓TNF-α; ↓IL-5; ↓IL-13; ↓IL-17; ↓IL-6
TcEF05	**S**	↑IL-10***; ↑TGF-β; ↑IL-12; ↑IL-6; ↑TNF-α; ↑IL-5; ↑IL-17	**S**	↑IL-12*; ↑TGF-β; ↑IL-10; ↑TNF-α; ↑IL-5; ↑IL-13; ↑IL-17
	I	↓IL-6	**I**	↓IL-12; ↓IL-17; ↓IL-6; ↓IFN
TcEF06	**S**	↑TGF-β; ↑IL-10; ↑IL-12; ↑IFN; ↑IL-6; ↑TNF-α	**S**	↑IL-10*; ↑IL-12**; ↑TNF-α*** ↑TGF-β; ↑IFN; ↑IL-6; ↑IL-13; ↑IL-17
	I	↓IL-6	**I**	↓IL-5; ↓IL-17; ↓IL-6
TcEF07	**S**	↑TGF-β; ↑IL-12; ↑IFN; ↑IL-17	**S**	↑TGF-β; ↑IL-10; ↑IL-12; ↑IFN; ↑TNF-α; ↑IL-13; ↑IL-17
	I	–	**I**	↓IL-5; ↓IL-17
TcEF08	**S**	↑TGF-β; IL-10; ↑TNF-α; ↑IL-13	**S**	↑IL-10; ↑IL-12; ↑IFN; ↑IL-6; ↑IL-13
	I	↓IL-10; ↓IL-12; ↓IL-17	**I**	↓IL-17
TcEF09	**S**	↑TGF-β; ↑IL-10; ↑IL-6; ↑TNF-α	**S**	↑TGF-β; ↑IL-10; ↑IL-12; ↑IFN; ↑IL-13
	I	↓IL-12; ↓IFN	**I**	↓TGF-β; ↓IL-12; ↓TNF-α; ↓IL-5; ↓IL-17
TcEF10	**S**	↑TGF-β; IL-10; ↑TNF-α; ↑IL-5; ↑IL-17	**S**	↑TGF-β; IL-10; ↑IL-12; ↑IFN; ↑TNF-α; ↑IL-5; ↑IL-17
	I	↓IL-10; ↓IL-12; ↓IL-6	**I**	↓IL-17; ↓IL-6

*p≤0.05 or **p≤0.01 or ***p≤0.001 - Bonferroni's Multiple Comparison Test or Dunn's Multiple Comparison Test.

incubation time to 72h it could be enough for this more robust detection in PBMC cultures, but during the period of the experiments, due to laboratory logistics, it was not possible to perform such an analysis for this period of time.

The search for drugs that stimulate the production of the cytokines IL-12 and IFN-γ (including extracts of helminths and vegetables, for example) is interesting for studies on allergen-specific immunotherapy. A major function of the cytokine IL-12 is the induction of IFN-γ, since most of its pro-inflammatory effects are IFN-γ-dependent (Fahey et al., 2007). In this respect the IL-12 produced by APC acts on T and NK cells, inducing the production of IFN-γ that in turn, acts activating APC. Thus, the interaction between IL-12 and IFN-γ leads to a strong positive feedback mechanism, which is important for inflammation control mediated by the Th2 cellular immune response (Frucht et al., 2001). Indeed, inhibition of the Th2 cells accompanied by an augmentation of a Th1 cellular immune response with IFN-γ production has been reported in allergic patients following immunotherapy (Arkwright and David, 2001).

Our results show that *T. canis* antigens activated the cytokine production of innate immunity, such as TNF-α. Proteins with regulatory functions but also inducing IL-12 and TNF-α transiently, without inhibition of IL-10 were also seen in other studies (Goodridge et al., 2005).

Some studies have shown that IL-6 controls the balance between regulatory T cells (the production of TGF-β) and Th17 cells (Bettelli et al., 2006; Veldhoen et al., 2006) and they suggest that blockade of IL-6 signaling is effective in treating experimental models of chronic inflammatory diseases such as colitis, diabetes, meningoencephalitis, rheumatoid arthritis, and asthma (Neurath and Finotto, 2011). Although not statistically significant for our trials, our study recorded the percentage of IL-6 inhibition when stimulated by some T. canis products in allergic and/or non-allergic donors (TcEF02-06 and TcEF10).

Previous study also showed that T cells from healthy humans produce significant amounts of IL-4 and IL-5, but no IL-2 or IFN-γ after stimulation with products secreted / secreted by *T. canis* larvae (TES) (Del Prete et al., 1991). On the other hand, Inuo et al. (1995) reported that human PBMC proliferate in response to adult *Toxocara* worm antigen (TcAg) and that their differentiation into Th2 cells is not obvious, as IL-2 and IFN-γ mRNA expression was observed along with the expression of IL-4 and IL-5 mRNA. Thus, more efforts should be made to reveal the mechanisms of interaction of parasitic products of *Toxocara* with human immune cells, given the diversity of molecules present in these, in order to ensure a better understanding of the complex infection process during human toxocariasis (Długosz et al., 2019).

These results, therefore, allow for the possibility of exploring further these fractions to obtain molecules for use as primary tools or as adjuvant for designing immunotherapeutic preparations that do not induce Th2 and IL-17 cytokines.

Safety tests in humans have already been performed with the porcine whipworm, *Trichuris suis*, in the treatment of inflammatory bowel disease (Weinstock et al., 2002) and the parasite *Necator americanus* (hookworm human agent) for the treatment of asthma (Mortimer et al., 2006).

Although a handful of individual proteins with immunoregulatory properties have been identified from excretory/secretory products (ESPs), helminth-secreted proteomes still present a relatively untapped pharmacopeia (Ryan et al., 2020) and much less is known about parasitic helminth metabolomes. The parasitic helminth metabolomes have begun to reveal small-molecule metabolites in parasitic helminth somatic extracts and ESPs (Becker et al., 2017; Kokova and Mayboroda, 2019).

Molecular biology tools have contributed to the identification of helminth proteins responsible for down-modulating their host's immune system. The strategies for their identification and produc-tion are diverse, from random discovery to the active and rationalsearch for immunoregulatory molecules, together with the devel-opment of suitable expression systems, mostly by different strains of *E. coli* (Santos et al., 2017).

More recent work by our group identified in silico two recombinant proteins as promising vaccine candidates for canine toxocariasis, with results in reducing larval loads in mice challenged with *T. canis* by 54.3% and 53.9% compared to unimmunized controls (Salazar Garcés et al., 2020).

Also in this study, proteomic analysis of protein antigens derived from immunomodulatory products secreted / secreted by *T. canis* larvae (TES) were also candidates for vaccines against this infection (Salazar Garcés et al., 2020).

In the present study, the immunomodulatory effects of fractions of the extract of *T. canis* adults worms on the responses of cytokines by human peripheral blood monocytes (PBMC) were investigated *in vitro* and it showed interesting results, which may be relevant to the possible use of *Toxocara* products such as TcE and/or TcEFs as adjuvants in anti-allergy immunotherapy. An interesting possibility would therefore be to use mass spectrometry to identify the protein content within the active fractions that could mediate these effects (Santos et al., 2013). Since it is

not feasible obtaining worms from infected dogs to purify natural molecules constantly, the most factible way would be to select protein from these fractions with immunomodulatory capacity and cloning their genes. The complete mitochondrial genomes of the *T. canis* (Maizels et al., 2000; Ming-Wei et al., 2008), for example, may favor this task.

5. General considerations

The products of the parasite *T. canis* (TcE and TcEFs) may contain anti-inflammatory molecules that suppress the activation of a harmful immune response that leads to inflammatory diseases.

The fractions that stimulated the production of immunoregulatory cytokines (TGF-β and/or IL-10), IL-12 and Th1 cytokines (TNF-α) without stimulating Th2 (IL -5 and IL-13) and IL-17 cytokines should be further studied to obtaining candidate molecules for immunotherapeutic vaccines in inflammatory and autoimmune diseases. The actions of TcE, TcEF04, TcEF05 and TcEF06 products in allergic and/or non-allergic donors stand out for these actions.

As for one of the limitations of this study, we inform that the number of samples did not allow obtaining purer fractions.

Since it is not feasible to continuously obtain antigens via helminth extract to purify natural molecules present in them, the most practical way would be to choose the protein from these products with immunomodulatory capacity, and subsequently produce them in the recombinant form by heterologous expression.

In this perspective, our group intends to biochemically characterize the products used in this work through mass spectrometry, as we have been previously successful and with the use of molecular biology obtain recombinant molecules that will be tested *in vitro* and in vivo by PBMC culture and in mice models of allergy and autoimmune diseases, respectively. With that, we can finally settle which *T. canis* proteins can be used as adjuvants or therapeutic molecules for immunotherapy of inflammatory illnesses.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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