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Imunomodulação de células mononucleares do sangue periférico *in vitro* por produtos de patógenos (*Candida albicans, Trichophyton rubrum* e *Toxocara canis*)

Ana Lúcia Moreno Amor

SALVADOR – BAHIA

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Orientadora: Prof[a]. Dra. Neuza Maria Alcântara-Neves

Co-orientador: Prof. Dr. Lain Carlos Pontes-de-Carvalho

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ATA – DEFESA DE TESE

Ata de Defesa de Tese de Doutorado da aluna **Ana Lúcia Moreno Amor.** Aos vinte e cinco dias do mês de agosto do ano de dois mil e quatorze, às oito horas no Auditório III segundo andar no Instituto de Ciências da Saúde **(ICS-UFBA)** se reúne em sessão pública, a Banca Examinadora de Defesa de Tese composta pelos Professores e Doutores: **Neuza Maria Alcântara Neves** orientadora, **Valdirene Leão Carneiro, Fred da Silva Julião, Márcia Cristina Aquino Teixeira, Ricardo Wagner Dias Portela** perante o qual a Doutoranda **Ana Lúcia Moreno Amor,** aluno regularmente matriculado no Curso de Doutorado em Biotecnologia da Rede Nordeste de Biotecnologia – RENORBIO, Ponto Focal Bahia, defendeu, para preenchimento do requisito de Doutor, sua tese intitulada **"Imunomodulação de células mononucleares do sangue periférico *in vitro* por produtos de patógenos (*Candida albicans, Trichophyton rubrum* e *Toxocara canis*".** A defesa da referida tese ocorreu, das oito horas às 10:30, tendo a Doutoranda **Ana Lúcia Moreno Amor** sido submetido à sabatina, dispondo cada membro da Banca do tempo para tal. Finalmente, a Banca reuniu-se em separado e concluiu por considerar a Doutoranda ANA LÚCIA MORENO AMOR por sua tese e sua defesa terem, por unanimidade, recebido o conceito SATISFATÓRIO.

Eu, Neuza Maria Alcântara Neves, que presidi a Banca de Tese, assino a presente Ata, juntamente com os demais membros e dou fé. Em Salvador, vinte e cinco de agosto de 2014.

Profa. Dra. Neuza Maria Alcântara Neves – UFBA
(Orientadora)

Prof. Dr. Ricardo Wagner Dias Portela (UFBA)
(Examinador)

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(Examinador)

Profa Dra. Valdirene Leão Carneiro (UNEB)
(Examinadora)

Profa. Dra. Márcia Cristina Aquino Teixeira (UFBA)
(Examinadora)

Dedicatória

Aos meus pais, Geudete e Hildete, meus primeiros mestres e doutores da vida: afetos verdadeiros são reais.

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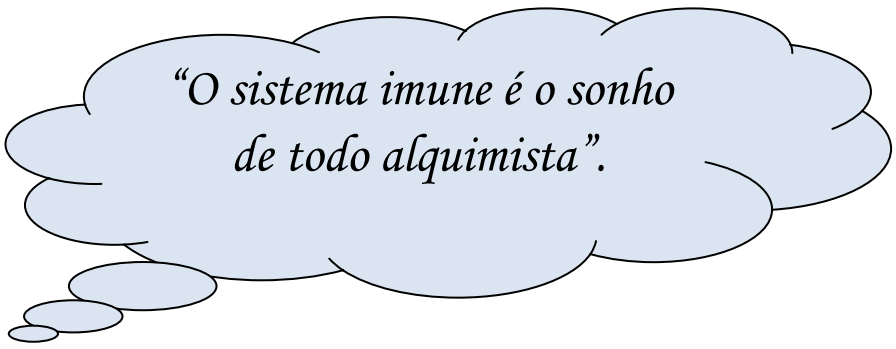
“O sistema imune é o sonho
de todo alquimista”.

RESUMO

A prevalência de asma alérgica e de outras alergias está aumentando mundialmente. Terapias farmacológicas eficazes e livres de efeitos colaterais indesejáveis ainda não estão disponíveis para estas doenças. O controle das respostas imunitárias na alergia ocorre principalmente por meio de diferentes subtipos de células reguladoras e supressoras. A exposição a patógenos e/ou a seus produtos, fungos e helmintos em particular, parecem proteger contra o desenvolvimento de doenças autoimunes e alérgicas. O objetivo deste estudo foi investigar se extratos de *Candida* spp (candidina), de *Trichophyton rubrum* (tricofitina), de *Toxocara canis* (TcE) e dez frações protéicas deste (TcEF01-TcEF10) ou se antígenos secretados-excretados de larvas de *T. canis* [(na forma nativa (TES) e recombinantes (rTES30E e rTES30P)], poderiam promover respostas imunes que inibissem a resposta imune Th2. As células mononucleares do sangue periférico (PBMC) de indivíduos alérgicos e não alérgicos foram cultivadas *in vitro* na presença ou ausência destes antígenos. Tricofitina ou candidina, ou ambos, TcE, TcEF01, TcEF02, TcEF06 e TcEF08-TcEF10 e recombinantes do TES, estimularam a produção de citocinas regulatórias (TGF-β e / ou IL-10), acompanhadas ou não pela estimulação da produção de citocinas associada com a resposta Th1 (TNF, IL-12 e IFN-γ), mas sem estimulação de citocinas do tipo Th2 (IL-5 e IL-13) e de IL-17, por PBMC de indivíduos não-alérgicos e, principalmente, de alérgicos. Estes resultados indicam que estes antígenos poderiam ser utilizados em terapia antialérgica. Além disso, eles justificam a realização de investigações que visam identificar as moléculas nestes produtos que possam induzir exclusivamente respostas imunes Treg e / ou Th1 passíveis de serem obtidas de forma recombinante e serem testadas em imunoterapia e imunoprofilaxia de doenças imunomediadas.

Palavras-chave: Tricofitina, Candidina, *Toxocara canis*, Imunomodulação, Alergia

ABSTRACT

The prevalence of asthma and other allergies is increasing worldwide. Effective pharmacological therapies and free from undesirable side effects are not yet available for these diseases. The control of immune responses in allergy occurs primarily through different subtypes of regulatory and suppressor cells. Exposure to pathogens and / or their products, in particular fungi and helminths, appear to protect against the development of autoimmune and allergic diseases. The objective of this study was to investigate whether candidin or trichophytin, or adult *Toxocara canis* crude extract (TcE) and ten protein fractions of its extract (TcEF01-TcEF10) and excreted-secreted *T. canis* larval antigens [(native (TES) and recombinant (rTES30E and rTES30P)], could promote immune responses that could potentially inhibit Th2 responses. Peripheral blood mononuclear cells from allergic and non-allergic individuals were cultivated *in vitro* in the presence or absence of these antigens. Trichophytin or candidin, or both, TcE, TcEF01, TcEF02, TcEF06 and TcEF08-TcEF10, TES-native and TES recombinant molecules stimulated the production of regulatory cytokines (TGF-β and/or IL-10), accompanied or not by stimulation of production of cytokines associated with the Th1 response (TNF, IL-12 and IFN-γ), but without stimulation of Th2 (IL-5 and IL-13) and IL-17 cytokines, by PBMC from non-allergic and, mainly from allergic individuals. These results indicate that these antigens could be used as adjuvants in anti-allergic therapy. In addition, they justify future investigations aiming at identifying molecules in these products that might exclusively induce Treg and/or Th1 immune responses and maybe obtain as recombinant to be used as tools for immunotherapy and immunoprofilaxy of immune-mediated diseases.

Key-words: Trichophytin, Candidin, *Toxocara canis,* Immunomodulation, Allergy

LISTA DE SIGLAS E ABREVIATURAS

AL e NAL – doadores alérgicos e não-alérgicos
APC – células apresentadoras de antígenos
C - candidina e T - tricofitina
C. albicans – *Candida albicans*
CD – cluster de diferenciação
CN e CP – controle negativo e controle positivo
ELISA – ensaio imunoenzimático
FPLC – cromatografia líquida rápida de proteína
IFN-γ - interferon gama
Ig – imunoglobulina
IL – interleucina
kDa – quilo dalton
LPS – lipopolissacarídeo
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBMC – células mononucleares do sangue periférico
PBS – tampão fosfato-salino
PHA - fitohemaglutinina
rTES30E – proteína recombinante do TES de 30 kDa, produzida em *Escherichia coli*
rTES30P – proteína recombinante do TES de 30 kDa, produzida em *Pichia pastoris*
SDS-PAGE – eletroforese em gel de poliacrilamida
T. canis – *Toxocara canis*
TcE – extrato somático de *Toxocara canis*
TcEF – fração do extrato somático de *Toxocara canis*
TES – antígeno excretório-secretório larval do *Toxocara canis*
TGF-β – fator de transformação do crescimento beta
Th – células T auxiliares e Treg – células T regulatórias
TNF - fator de necrose tumoral
T. rubrum – *Trichophyton rubrum*

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com antígenos do TES antigens (TES- native, rTES30E e rTES30P).

Figura 8. Razão de citocinas [(TGF-β, IL-10, TNF, IL-5, IL-13, IL-17 ou IL-6) / (IFN-γ)] em PBMC de doadores não-alérgicos (A, B, C) vs. alérgicos (D, E, F) estimuladas com antígenos do TES antigens (TES- native, rTES30E e rTES30P). **135**

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1. INTRODUÇÃO

As prevalências da asma alérgica, de outras alergias e de doenças autoimunes estão aumentando em todo o mundo (Woolcock e Peat, 1997; Ludvigsson, 2006; Czirják et al., 2007, Yazdanbakhsh et al., 2002). Estima-se que 300 milhões de pessoas tenham asma, com a predominância de crianças que vivem em países industrializados e, mais recentemente, nos países em desenvolvimento (Cooper e Rodrigues, 2009). Este aumento pode estar ligado a mudanças ambientais associados à urbanização e à aquisição de um estilo de vida "moderno" ou “ocidentalizado” (Pearce et al., 2007; Cooper e Rodrigues, 2009). A asma alérgica e outras alergias influenciam a qualidade de vida dos indivíduos (Matos e Machado, 2007), muitas vezes interferindo no trabalho ou na escola, levando-os à hospitalizações recorrentes, o que, consequentemente, leva a um alto custo, seja para o governo ou para o indivíduo.

Tratamentos disponíveis no momento para as doenças alérgicas (asma e rinite) são acompanhados de efeitos secundários indesejáveis (Araújo et al., 2010). O desenvolvimento de protocolos imunomoduladores com efeitos benéficos sobre alergias e doenças autoimunes é prioritário, e novas armas para o arsenal terapêutico contra estas enfermidades necessitam serem desenvolvidas.

Certamente, os fatores associados com o aumento destas doenças são diversos. Apesar de fatores genéticos serem importantes para explicar a variabilidade da prevalência de asma no mundo, a genética não explica o acentuado crescimento recente da prevalência de asma, rinite alérgica, eczema e reatividade ao teste alérgico cutâneo verificado em vários países. Entretanto, a hipótese da higiene constitui uma explicação razoável (Strachan, 1989). Esta hipótese tem o respaldo de publicações que demonstram associação inversa entre alergia e exposição a infecções virais, bacterianas e helmínticas (Araújo et al., 2000; Wills-Karp et al., 2001). A hipótese da higiene teoriza que diversos aspectos relacionados ao modo de vida moderno (exposição reduzida a patógenos no meio ambiente, uso difundido de antibióticos, menor número de familiares, menor duração da amamentação e imunizações por vacinas, entre

outros) contribuem para o aumento nos últimos anos da incidência das doenças alérgicas, pois levaria a ausência de infecções na infância resultando em alteração na imunorregulação.

O nosso grupo recentemente descreveu a primeira evidência em estudo de base populacional do potencial regulador da IL-10. Neste estudo verificamos que crianças que vivem em regiões pobres e com falta de saneamento básico (escassa coleta de lixo, falta de esgoto e falta de pavimentação das ruas) produzem menos citocinas Th1 e Th2 em cultura, sendo esta supressão de citocinas significantemente maior entre as crianças que produzem IL-10 quando comparadas aquelas que não produzem IL-10, indicando que a IL-10 pode modular esse efeito (Figueiredo et al., 2011). Acreditamos que o principal agente infeccioso que poderia estar envolvido neste processo de supressão de citocinas sejam os helmintos, por possuírem forte potencial imunomodulador (Araujo et al., 2004; Ponte et al., 2006). Esta intensa imunomodulação, induzida nos indivíduos infectados, pode explicar a proteção descrita na literatura contra o desenvolvimento de doenças imunomediadas. Fatos que corroboram esta hipótese são oriundos de estudos observacionais que associam um aumento da prevalência de doenças alérgicas em indivíduos infectados quando são tratados com drogas antiparasitárias (Lynch et al., 1993) e que os sintomas de asma são menos graves em indivíduos infectados (Medeiros et al., 2003).

Desta forma, objetivou-se, no presente estudo, investigar, se extratos de fungos (candidina ou tricofitina), produtos do helminto *Toxocara canis* [extrato do adulto (TcE) e dez frações protéicas deste obtidas por cromatografia de troca iônica (TcEF01-TcEF10) ou antígenos secretados-excretados de larvas de *T. canis* na forma nativa (TES) e recombinantes - rTES30E e rTES30P), provocam aumento de uma resposta imune regulatória e inibição de uma resposta Th2 alérgica.

2. REVISÃO DA LITERATURA

A patogênese das doenças alérgicas está relacionada ao envolvimento dos linfócitos T CD4 + (células T helper) os quais desempenham um papel central como reguladores e efetores da resposta imune (Liew, 2002). A ativação anormal de células Th1 é considerada evento crítico para a maioria das doenças autoimunes órgãos específicas; e a ativação de respostas aberrantes envolvendo citocinas tipo Th2, em resposta a antígenos inócuos, é responsável por efeitos deletérios em doenças inflamatórias alérgicas e asma (Brandtzaeg, 2010). As células Th2 estão envolvidas tanto na resposta IgE-específica quanto na resposta eosinofílica nestes processos patológicos (Fallon e Mangan, 2007). Dermatite atópica (ou eczema), rinite alérgica e asma alérgica são as principais manifestações clínicas da atopia, que é causada principalmente por uma inflamação dependente de IgE.

Anticorpos IgE, por outro lado, participam da imunidade contra infecções helmínticas, que são indutoras potentes destes anticorpos por meio da produção de citocinas Th2 (IL-4, IL-5 e IL-13). A IL-4 induz os linfócitos B a produzir anticorpo IgE e a IL-5 atrai e ativa os eosinófilos *in vivo* (Stone et al., 2010). A coordenação desses fatores solúveis e de células, especialmente orquestradas pelos linfócitos Th2, é que determina a patogenia das doenças alérgicas dentre elas, a asma. O controle destas respostas aberrantes pelo sistema imunológico se dá através de diferentes subtipos de células regulatórias e supressoras que desempenham importante papel na tolerância periférica (Mottet e Golshayan, 2007). As células T regulatórias (Tregs) CD4+ CD25+ FOXP3+ são as células de maior destaque e suprimem respostas imunológicas especialmente através de interações célula-célula e / ou a produção de Fator de transformação do crescimento beta- (TGF- β) e IL-10 (Figueiredo et al., 2011; Read, 2001).

Alterações nos níveis de citocinas reguladoras como a IL-10 e TGF-β, parecem possuir um importante papel na mediação de alergias (Yazdanbakhsh et al., 2002). A exposição a patógenos e seus produtos, helmintos em particular, parecem proteger contra o desenvolvimento de doenças autoimunes e

alérgicas em modelos experimentais (Burkhart et al., 1999; Nookala et al., 2004; Mingomataj et al., 2006; Ponte et al., 2006; Cooper, 2008; Cooper et al., 2009; Pacífico et al., 2009), e em humanos (Burkhart et al., 1999; Araujo et al., 2004; Mingomataj et al., 2006; Cooper et al., 2009). Esta proteção vem sendo explicada pela capacidade da IL-10, especialmente derivada de células T regulatórias, de inibir a hiper-estimulação da via Th2 promovendo um equilíbrio entre as respostas Th1 e Th2 (Robinson et al., 2004).

Em apoio à hipótese da higiene, pesquisadores têm observado que, crianças criadas em ambientes de fazendas e granjas estão menos sujeitas às doenças atópicas que as residentes nos centros urbanos. Nascida no ambiente rural, a criança teria mais contato com animais e maiores chances de exposição a endotoxinas (produtos bacterianos), potentes estimuladoras da resposta Th1, direcionando as respostas futuras do organismo para um padrão Th1 (Wills-Karp et al., 2001). Entretanto, segundo Horak et al (2002), crianças em idade escolar e atópicas que passam a viver numa fazenda, também passam a apresentar uma menor reação cutânea aos testes de hipersensibilidade, sugerindo que o organismo, a qualquer momento e não apenas no começo da infância, pode ser levado a uma resposta menos alérgica pela influência de fatores do ambiente rural, ou pela distância de fatores alergizantes do estilo de vida urbano.

Dados recentes obtidos pelo nosso grupo no Programa SCAALA (Social Change of Asthma and Allergy in South América), financiado pela Fundação Wellcome (Barreto et al., 2006) mostraram que crianças que tinham sido fortemente infectadas com *Trichuris trichiura* durante os três primeiros anos da infância têm um risco reduzido de desenvolvimento de atopia na infância tardia (Rodrigues et al., 2008), resultando na primeira evidência epidemiológica que infecções helmínticas intestinais na infância podem reduzir o risco de atopia posteriormente. Cooper et al (2009) relataram que crianças de mães infectadas com *T. trichiura* apresentam redução na prevalência de eczema. Além disso, pode explicar a associação inversa, já referida, entre infecções helmínticas intestinais e risco de atopia em crianças de idade escolar que vivem em áreas endêmicas para essas infecções, sendo apoiada por dados experimentais que

demonstram helmintos imunomodulando reações inflamatórias (Maizels et al., 2004).

Existem alguns indícios de que, em seres humanos, a infecção por ingestão de ovos de *Trichuris suis*, um helminto de suínos, pode reduzir manifestações clínicas de colite ulcerativa e doença de Crohn (Weinstock et al., 2002; Summers et al., 2005a; Summers et al., 2005b). Rodrigues et al (2008), revelaram uma associação inversa entre a infecção com o helminto *Trichuris trichiura* e doenças alérgicas, sendo tal inversão dependente da idade em que a infecção ocorreu e da carga parasitária. Outro estudo, de caráter experimental, revelou que frações proteicas de *Trichuris trichiura* induziram a produção de IL-10, bem como reduziram os níveis de citocinas do perfil Th2 em células mononucleares do sangue periférico (Santos et al., 2013).

Outros autores relataram que proteínas recombinantes de *Ascaris lumbricoides* (Tami et al., 2005) e de *Dirofilaria immitis*, um filarídeo de caninos (Imai e Fujita, 2004) inibiram respostas alérgicas e autoimunes, respectivamente, quando injetadas em camundongos. Trabalhos epidemiológicos, nesta área, encontraram altas concentrações de IL-10 em indivíduos infectados (Figueiredo et al., 2011); ou em animais experimentais injetados com produtos helmínticos (Cardoso et al., 2010), e estes resultados têm sido atribuído ao estímulo das células T regulatórias pelos helmintos (Pacífico et al., 2009). No entanto, Trujillo Vargas et al (2007), demonstraram que fatores excretados-secretados por *Nippostrongylus brasilienses* podem inibir reações alérgicas, inclusive as que regulam a produção de IgE, em mecanismo independente de IL-10. Eles também demonstraram que o fator ativo foi resistente à proteinase K, o que sugere que ele não seja de natureza protéica. Estes estudos apóiam a idéia da existência de diferentes mecanismos subjacentes à modulação do sistema imune por helmintos, ou seja, a imunomodulação pode ou não depender de IL-10.

Algumas espécies de helmintos apresentam importante papel na prevenção da patogênese da asma, como relatado acima, mas particularmente infecções por ancilostomídeos merecem a realização de maior investigação em decorrência de apresentar maior redução no risco de desenvolver asma nos indivíduos

parasitados (Dagoye et al., 2003). Infecção por ancilostomídeos foi associada com redução de asma em indivíduos atópicos na Etiópia (Dagoye et al., 2003), na inibição da reatividade ao teste alérgico cutâneo no Vietnã (Flohr et al., 2006) e na melhora de colite experimental em camundongos (Ruyssers et al., 2009). Estudos clínicos de infecções experimentais com ancilostomídeos em indivíduos com doenças atópicas estão em progresso (Falcone e Pritchard, 2005). Achados de infecção com ancilostomídeos (*Necator americanus*) indicam que eles produzem metaloprotease que digere eotaxina – importante para a migração eosinofílica (Culley et al., 2000), na resposta imunológica contra o parasito e que eles podem induzir apoptose em células T ativadas (Chow et al., 2000).

Patel et al (2007) demonstraram que camundongos BALB/c tratados com Na-ASP-2 (proteína secretada pela larva no estádio L3 de *Necator americanus*) ocorreu inibição do recrutamento celular (eosinófilos) quando comparados com camundongos controle tratados com PBS.

Outros parasitos que podem imunomodular populações de baixa renda dos países em desenvolvimento são *Toxocara canis* e *T. cati,* parasitos intestinais de canídeos e felinos, respectivamente (Campos Jr et al., 2003). A doença humana é causada por estágios larvais que migram para vísceras, pulmões, fígado, musculatura, cérebro e globo ocular causando a síndrome bem caracterizada “larva migrans visceral” (VLM) ou “larva migrans ocular” (OLM) (Schantz, 1989). Os mecanismos imunes parecem ser ineficazes na eliminação da infecção (Maizels, 2013). Estudos do nosso grupo mostraram que esta infecção ocorre em 46-65% da população de classe social baixa em Salvador, cidade do Nordeste do Brasil (Dattoli et al., 2011; Souza et al., 2011; Mendonça et al., 2013). No entanto, a infecção assintomática por este helminto foi encontrada associada à diminuição de reatividade cutânea a aeroalérgenos nas crianças da coorte do Programa “*Social Changes, Asthma and Allergy in Latin America*” (SCAALA) (Mendonça et al., 2012).

Antígenos excretados-secretados de larvas de *T. canis* (TES), obtidos a partir do cultivo de larvas do parasito são constituídos, na sua forma nativa, por múltiplos componentes de diferentes massas moleculares, destacando as

proteínas de massa molecular 26, 30, 45, 55, 70, 120 e 400 kDa (Maizels et al., 1984). São, pelo menos, 50 macromoléculas distintas, representadas, principalmente, por um conjunto relativamente simples de glicoproteínas, constituída por três famílias de genes que expressam três mucinas e duas lectinas do tipo C (Meghji e Maizels, 1986; Hewitson et al., 2009). Estas proteínas são glicosiladas e antigênicas (Meghji et al., 1986) e atuam na regulação imune (com potencial imunomodulador). Foi demonstrado que esses antígenos localizam-se na epicutícula das larvas e são receptores importantes para anticorpos (Lambertucci et al., 2001; Altcheh et al., 2003). Uma vez que as larvas trocam periodicamente a cutícula, liberando tais moléculas na circulação sanguínea, dificultam a ação dos anticorpos do hospedeiro, proporcionando ao parasito um ambiente bem sucedido para sua sobrevivência (Loukas et al., 2000).

Em um estudo experimental, a imunização prévia de camundongos com o TES antes da infecção experimental causou mobilização e recrutamento de células Foxp3+ para o fígado, e aumento da expressão de transcritos Foxp3 no baço. Estes resultados sugerem um papel importante de células reguladoras que expressam Foxp3 na evolução dos eventos imunopatológicos durante infecção por *T. canis* (Othman et al., 2011).

Antígenos de excreção-secreção de outro helminto intestinal, o *Heligmosomoides polygyrus,* induziram a produção de células T reg tanto *in vitro* em cultura de células quanto *in vivo* em um modelo de inflamação alérgica (Grainger et al., 2010).

Antígenos de fungos, como os extratos candidina (purificado da levedura *Candida albicans*) e tricofitina (filtrado bruto de *Trichophyton* spp) já foram injetados por via intradérmica em seres humanos ao longo de décadas para desencadear reações imunes Th1 cutâneas sem reações adversas, estes resultados indicam que esses extratos de fungos poderiam ser usados como adjuvantes em vacinas terapêuticas personalizadas em uma boa proporção de indivíduos e justificam a realização de investigações que identifiquem as moléculas nestes extratos que podem exclusivamente induzir Treg e / ou respostas imunes Th1 (Simark-Mattssonl et al., 1999; Costa et al., 2011). Por

exemplo, Cenci et al (1990) observaram que as células do baço de camundongos imunizados com candidina produziram elevados níveis de IFN-γ *in vitro* em resposta aos antígenos de *Candida* sp.

Desde a sua introdução no tratamento da rinite alérgica sazonal há quase um século, na Inglaterra (Noon, 1911; Freeman, 1911), a imunoterapia alérgeno-específica tem sido usada por muitos anos em casos de doenças alérgicas mediadas por IgE, incluindo alergias respiratórias (asma e rinossinusite) (Nelson, 2007). A sua eficácia foi estabelecida, por exemplo, em ensaios de rinossinusite alérgica a pólens e fungos (Horst et al., 1990). Tradicionalmente, extratos alergênicos obtidos de fontes naturais têm sido utilizados para diagnóstico e tratamento. Os problemas associados ao uso de extratos naturais incluem dificuldades com a avaliação da potência e inconsistências inerentes à produção de extratos com conteúdo equivalente de alérgeno. Apesar dos avanços nas técnicas de purificação e padronização de alérgenos, estes extratos permanecem produtos heterogêneos, misturas de diferentes proteínas, com contaminantes não alergênicos (ou de outras fontes), muitas vezes, quantidades indefinidas e até mesmo ausentes dos alérgenos relevantes, e podem conter enzimas proteolíticas com capacidade de reduzir a concentração de alérgeno no extrato (Chapman et al., 2000; Geraldini et al., 2008). Através da tecnologia recombinante, proteínas de extratos alergênicos estão sendo identificadas e modificadas para reduzir a alergenicidade, sem reduzir a sua imunogenicidade, mostrando menos efeitos colaterais (Nelson, 2007).

Ou seja, alérgenos recombinantes têm sido produzidos com alto grau de pureza, em grandes quantidades, com capacidade similar de ligação à IgE quando comparados aos seus homólogos naturais, ou com modificações de modo a reduzir a reatividade à IgE (hipoalérgenos) (Linhart e Valenta, 2012).

Mais recentemente, o uso de alérgenos recombinantes de pólen de bétula (rBet v 1) e de gramas (coquetel de 5 alérgenos) em imunoterapia foi relatado como seguro e eficaz, com resultados comparáveis aos obtidos usando extratos naturais, em pacientes com rinoconjuntivite alérgica a polens (Arruda et al., 2013). A melhora clínica foi acompanhada por um aumento de 4.000 vezes nas

concentrações de IgG4 e pela ausência de novas sensibiliações ao final do tratamento (Jutel et al., 2005).

Outra questão a ser considerada, é que, por exemplo, a presença de adjuvantes em vacinas imunoterapêuticas aumenta as respostas imunes a alérgenos (Mothes et al., 2003; Francis e Durham, 2004), na terapia antialérgica.

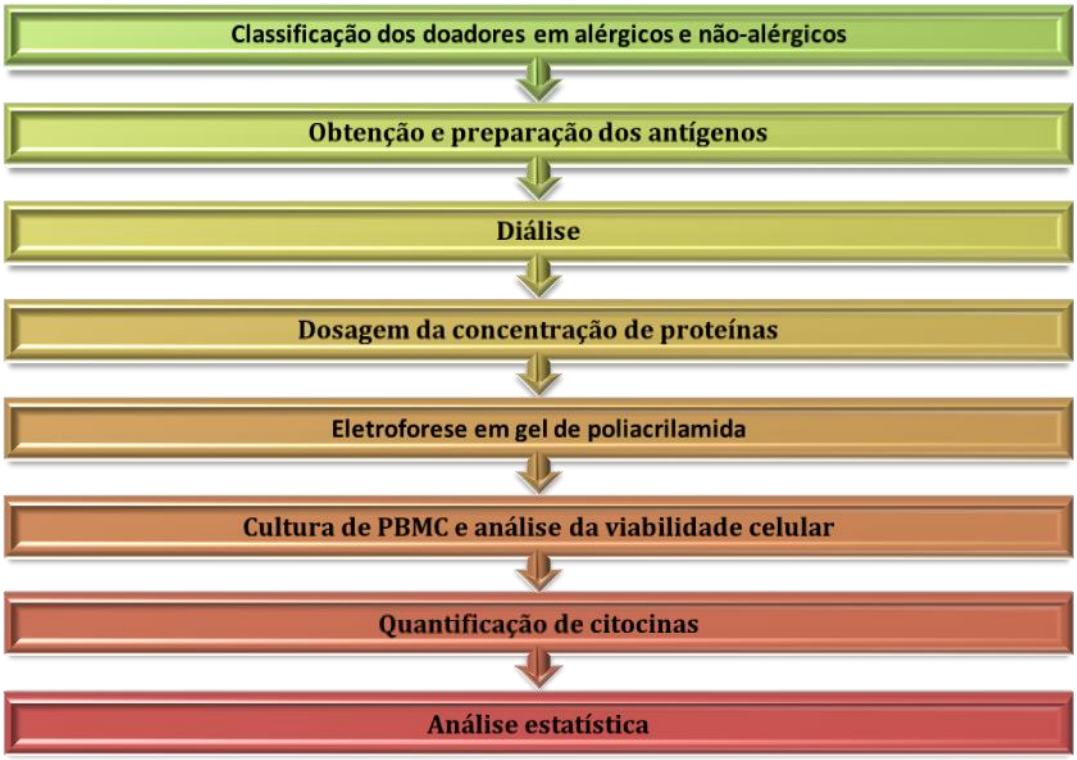
Estudos pré-clínicos com adjuvantes que estimulam uma resposta imune Th1, como oligodesoxinucleotideos CpG, têm produzido resultados promissores (Xu et al., 2008).

Provavelmente, o conhecimento das propriedades intrínsecas dos alérgenos e dos mecanismos patológicos permitirá o desenvolvimento de novas abordagens terapêuticas. É possível antecipar um uso cada vez maior dos alérgenos recombinantes em estudos clínicos. Essa nova situação exigirá a produção de alérgenos em condições de boas práticas de fabricação, e a aprovação dos órgãos reguladores, permitindo o uso de alérgenos recombinantes tanto para aplicações diagnósticas como terapêuticas. Já foram publicados estudos clínicos de vacinas de peptídeos derivados de alérgenos, conjugados de alérgeno-oligonucleotídeo, moléculas recombinantes hipoalergênicas, alérgenos recombinantes modificados para administração intralinfática, etc., demonstrando eficácia clínica e bom perfil de segurança. Portanto, é possível prever a ocorrência de uma contínua incorporação de alérgenos recombinantes aos estudos de imunoterapia, que deverão resultar em vacinas mais eficazes para doenças alérgicas estabelecidas e, em longo prazo, com a perspectiva da imunização profilática (Arruda et al., 2013).

Desta forma, justifica-se a investigação proposta neste trabalho de pesquisa, visando fornecer subsídios para o uso de candidina e / ou tricofitina como adjuvantes em preparações imunoterápicas antialérgicas ou de possibilitar pesquisas em produtos (proteínas) de *T. canis* com potencial imunomodulador. No presente trabalho, investigou-se a natureza das citocinas que foram produzidas por doadores alérgicos e não-alérgicos (saudáveis), nas células mononucleares do sangue periférico humano (PBMC), quando estas foram

incubadas *in vitro* com os produtos dos fungos e do helminto, pesquisando se eles iriam provocar respostas imunes que possibilitasse a inibição da reação de hipersensibilidade tipo I, sem obter uma resposta imune potencialmente patogênica Th17 ou Th2.

3. DESENHO EXPERIMENTAL



4. **RESULTADOS**

Os dados gerados neste estudo estão apresentados na forma de artigos científicos. Esta forma de apresentação visa propiciar uma divulgação objetiva e rápida dos resultados obtidos.

O Artigo 1 ("*Candidin and trichophytin stimulate the production of Th1 and regulatory cytokines by peripheral blood mononuclear cells. Implication for their use as adjuvants in immunotherapy*") investiga se extratos de fungos (candidina ou tricofitina) podem promover uma resposta imune que potencialize inibir uma resposta Th2. Este trabalho está formatado segundo as normas do periódico *Journal of Immunotherapy* (fator de impacto = 2.393), ao qual foi submetido, aprovado e está na fase de ajustes junto ao editor para publicação em outubro de 2014.

O Artigo 2 ("*Fractions from the extract of* ***Toxocara canis*** *promote mainly the production of Th1 and regulatory cytokines by human leukocytes in vitro*") descreve os efeitos do extrato do adulto de *Toxocara canis* (TcE) e dez frações protéicas deste (TcEF01 a TcEF10) obtidas por meio de cromatografia de troca iônica, em células mononucleares do sangue periférico de humanos. Este trabalho foi submetido ao *BioMed Research International* (fator de impacto = 2.880).

O Artigo 3 ("*Immune response of human peripheral blood mononuclear cells to excreted-secreted Toxocara canis larval antígenos (TES) native and recombinants*") pesquisa se antígenos secretados-excretados de larvas de *Toxocara canis* (na forma nativa e recombinantes – rTES30E e rTES30P) produzem resposta imune que potencialize inibição de uma resposta do perfil Th2, em células mononucleares do sangue periférico de indivíduos alérgicos e não-alérgicos. Este trabalho está formatado segundo as normas do periódico *Journal of Immunology Research* (fator de impacto = 3.064), ao qual será submetido.

No Anexo 1, encontram-se os dados quanto à caracterização do indivíduos em alérgicos e não-alérgicos, onde todos os indivíduos positivos a aeroláergenos pelo teste cutâneo, apresentaram também soropositividade para IgE específica para aeroalérgenos.

No Anexo 2, há a representação gráfica dos dados gerados nos ensaios de viabilidade celular (citotoxicidade) dos produtos testados). Verifica-se, no geral, que os produtos apresentaram baixa citotoxicidade, isto é, a adição dos produtos dos patógenos pesquisados, em concentrações variadas, nas culturas de células mononucleares do sangue periférico, não afetou significativamente a viabilidade celular.

No Anexo 3, é mostrada a caracterização da produção e composição proteica dos produtos testados (extrato bruto, frações proteicas e TES) disposta em géis (SDS-PAGE), corada com azul de comassie. Após avaliação da composição proteica, percebe-se, nos SDS-PAGE, predominância das bandas entre 30 a 70 kDa nestes produtos. Evidenciando a possibilidade de moléculas comuns entre as fases de larva à adulto.

4.1 ARTIGO 1

TÍTULO:

Candidin and trichophytin stimulate the production of Th1 and regulatory cytokines by peripheral blood mononuclear cells. Implication for their use as adjuvants in immunotherapy

Submetido e aprovado para publicação no *Journal of Immunotherapy* – Fator de impacto = 2,393. No **Anexo 4** encontra-se cópia de e-mail informando sobre o aceite do trabalho.

Este **Artigo** objetivou investigar se extratos de fungos (candidina ou tricofitina) poderiam provocar resposta imune regulatória que poderia inibir uma resposta Th2 alérgica, a partir da estimulação de células mononucleares do sangue periférico de nove indivíduos alérgicos e de sete não-alérgicos na presença ou na ausência destes extratos de fungos.

Dados obtidos mostraram que tricofitina ou candidina, ou ambos, estimularam a produção de citocinas regulatórias (TGF-β e / ou IL-10), acompanhada ou não pela estimulação da produção de citocinas associadas com a resposta do tipo Th1 (TNF, IL-12 e IFN-γ), mas sem estimulação de citocinas Th2 (IL-5 e IL-13) e IL-17, mais nas PBMC de indivíduos alérgicos do que em não-alérgicos.

Os resultados indicaram que estes extratos de fungos poderiam ser utilizados como adjuvantes em vacinas terapêuticas personalizadas em uma boa proporção de indivíduos. Além disso, eles justificam a realização de investigações que visam identificar as moléculas nestes extratos que podem exclusivamente induzir respostas imunes Treg e / ou Th1.

Preliminary communication

Candidin and trichophytin stimulate the production of Th1 and regulatory cytokines by peripheral blood mononuclear cells. Implication for their use as adjuvants in immunotherapy

Short title: Response of human leukocytes to fungal antigen stimulation.

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Abstract

Aim. This study objective was to investigate whether candidin or trichophytin could elicit recall immune responses that could potentially inhibit a Th2 response.

Materials and Methods. Peripheral blood mononuclear cells from nine allergic and seven non-allergic individuals were cultivated in vitro in the presence or absence of these fungal extracts.

Results. Trichophytin or candidin, or both, stimulated the production of regulatory cytokines (TGF-β and/or IL-10), accompanied or not by stimulation of production of cytokines associated with the Th1 response (TNF, IL-12 and IFN-γ), but without stimulation of Th2 cytokines (IL-5 and IL-13) and IL-17, by PBMC of most allergic and non-allergic individuals.

Conclusions. These results indicate that these fungal extracts could be used as adjuvants in personalized therapeutic vaccines in a fair proportion of individuals. In addition, they justify the carrying out of investigations aimed at identifying molecules in these extracts that might exclusively induce Treg and/or Th1 immune responses.

Keywords: Cytokine - Candidin - Trichophytin - Immune response – Immunotherapy

Introduction

The prevalence of allergic asthma and other allergies is increasing worldwide [1, 2].

Immunotherapy is an important alternative to the available therapies [3, 4], as pharmacological therapies that are both effective and free from undesirable side effects are still unavailable for these diseases.

Since its introduction for treating seasonal allergic rhinitis almost a century ago in England [5, 6], allergen-specific immunotherapy has been used for many years in cases of IgE-mediated allergic diseases, including respiratory allergies (rhinosinusitis and asthma) [7, 8].

The most common type of allergic diseases in humans are associated with the Gell and Coombs' type I hypersensitivity reaction, with an IgE-dependent activation of mast cells and basophils, predominance of Th2 cells and tissue eosinophilia [9].

Among the cytokines produced by Th2 cells during the allergic reactions, interleukin-4 (IL-4) and IL-13 stimulate IgE synthesis [10, 11], prolong the survival of mast cells in tissues [12] and determine the selective recruitment of eosinophils by increasing vascular cellular adhesion molecule-1 expression in the vascular endothelium. IL-5 and IL-33 are the primary determinants of differentiation, recruitment, activation, and survival of eosinophils [10, 13]. IL-13 plays an important role in mucus secretion and in the hyperresponsiveness of airway smooth muscle [10, 14].

There seems to be two distinct and perhaps sequential immunologic responses

to immunotherapy, namely immune deviation from Th2 to Th1 responses and generation of regulatory T (Treg) cells. Inhibition of the Th2 immune response accompanied by an augmentation of a Th1 cellular immune response with IFN-γ production has been reported in allergic patients following immunotherapy [15-18].

On the other hand, other authors have reported that anti-allergy immunotherapy leads to activation of Treg, with production of the regulatory cytokines IL-10 and/or transforming growth factor (TGF)-β [18-22].

The presence of adjuvants in immunotherapeutic vaccines enhances the immune responses to allergens [23, 24]. Alum is the traditional adjuvant in "depo" extracts widely used in Europe [25]. However, similarly to what happens with preparations without adjuvants, alum containing vaccines require several years of repeated applications, a fact that reduces patients' compliance [26, 27]. Preclinical studies with adjuvants that stimulate Th1 immune responses, such as CpG oligodesoxynucleotides, have produced promising results [28]. However, a better understanding of the molecular and cellular mechanisms that lead to immune deviation and/or to the differentiation and activation of Treg cells following an effective anti-allergy immunotherapy, in addition to further clinical trials utilizing new adjuvants, is still required.

Candidin is a purified extract of the yeast *Candida albicans* cultivated in synthetic culture media [29], whereas trichophytin is a crude filtrate of *Trichophyton* spp [30].

There are many pieces of direct and indirect evidence in the literature indicating that these preparations may be used as adjuvants in anti-allergy

immunotherapy. For instance, the intradermal injections of candidin and trichophytin elicit recall Th1 cell-mediated immune reactions, and, in fact, these antigens have been widely used to induce cellular immune responses for the assessment of immune competence [31, 32].

Contrary to these data, other published results support the use of candidin as an adjuvant that promotes the production of regulatory cytokines. For example, fungal polysaccharides are known to promote chronic mucocutaneous candidiasis through the production of IL-10 [33].

A major caveat, however, may hinder the use of crude fungal extracts in anti-allergy immunotherapy. The exposure to *C. albicans* polarized the T-cell immune response in an arthritis mouse model towards a Th17 response, resulting in more destructive arthritis [34]. A major concern in using fungus antigens as adjuvants is, therefore, the possibility of inducing pathogenic Th17 immune responses. Th17 immune responses have indeed been associated with respiratory allergy [35, 36].

As candidin and trichophytin have been injected in human beings for decades without undesirable consequences, they very likely could be used as adjuvants associated with allergens without raising untoward reactions.

The facts described above justify the carrying out of research aimed at providing support for the use of candidin and/or trichophytin as adjuvants in immunotherapeutic anti-allergy preparations.

In the present work, the nature of the cytokines that were produced by allergic patients' and healthy individuals' peripheral blood mononuclear cells (PBMC), when they were incubated in vitro with candidin or trichophytin, was

investigated. This was carried out to find out whether these fungal extracts would elicit recall immune responses that could potentially inhibit a type I hypersensitivity reaction without eliciting a potentially pathogenic Th17 immune response. This was indeed observed with the PBMC from most donors.

Materials & Methods

Blood donors

All blood donors, aged between 21 and 40 years, signed an informed consent to participate in the present research. They were classified into allergic (n = 9) and non-allergic (healthy individuals) (n = 7) donors based on the results of skin prick tests using extracts from six common allergens (Alergolatina Produtos Alergênicos Ltda., Rio de Janeiro, Brazil), on the history of allergic symptoms and according to measurement of the levels of specific IgE to ***Blomia tropicalis***, ***Dermatophagoides pteronyssinus***, ***Periplaneta americana***, ***Blattella germanica*** and ***Ascaris lumbricoides*** using the ImmunoCAP assay (Phadia Diagnostics AB, Uppsala, Sweden).

The research was approved by the institutional Ethics Committee for Research in Human Subjects.

Preparation of candidin and trichophytin

Trichophyton rubrum strains 1 and 2 and *C. albicans* strains A, B and CT were from the fungi collection of the National Institute of Quality Control in Health,

Oswaldo Cruz Foundation, Ministry of Health, Rio de Janeiro, Brazil. *C. albicans* was cultivated in Sabouroud medium in a shaker at 25^{o} C for 48 hours (strains A and B) and 72 hours (strain CT). The yeast was collected and washed by centrifugation in endotoxin-free 0.15 M phosphate-buffered saline, pH 7.2 (PBS), and stored at -70^{o} C until use. *T. rubrum* was cultivated in the same conditions for 14 days, collected from the medium and washed with PBS. The fungi were lysed using an electrical triturator in the presence of silica beads (BioSpec Products, Inc, Bartlesville, OK, USA) and of PBS. Following centrifugation at 10,000 g for 20 minutes at 4° C, the supernatants of the *C. albicans* lysate (candidin) and of the *T. rubrum* lysate (trichophytin) were collected, filtered through a polystirene filter with 40 μm-diameter pores, aliquoted and stored at -70^{o} C until used. Protein concentrations were measured using the Folin reagent (Bio-Rad Laboratories, Richmond, CA, USA).

Stimulation of PBMC by fungal extracts

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by centrifugation on HISTOPAQUE 1077® solution (Sigma-Aldrich, St. Louis, MO, USA). PBMC (2 x 10^{6} cells/well) were stimulated by fungal extracts containing 5 or 50 μg of protein per mL 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, 100 μg/mL streptomycin and 20 μg/mL polymyxin B (Sigma-Aldrich, St. Louis, MO, USA), for 24 hours [for quantification of IL-10, tumor necrosis factor (TNF), IL-6, IL-12, and transforming growth factor (TGF-)β] or for 120

hours (for quantification of IL-5, IL-17, and IL-13), at 37° C and 5% CO_2. Polymixyn B was not used in wells stimulated by LPS. In negative control cultures, the cells were incubated with supplemented medium alone.

Cell viability assay

After cell culture for 24 hours and collection of the supernatant, nine randomly chosen PBMC were subjected to a cytotoxicity assay utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA). Cell viability values were expressed as the absorbance percentage in relation to those of negative control (not containing fungal extract) cultures.

Cytokine measurements

The concentrations of cytokines were assessed using commercially available enzyme-linked immunosorbent assay (ELISA) duo-sets (Pharmingen, BD Biosciences, San Diego, CA, USA), according to manufacturer's instructions, and a 3,3, 5,5-tetramethylbenzidinedihydrochloride substrate system (Amresco, LLC, Ohio, USA). The reactions were stopped by the addition of 50 μL of 2 N sulfuric acid (Sigma-Aldrich, St. Louis, MO, USA) and the absorbances of 450 nm wavelength light were measured. The increases in cytokine concentrations in a stimulated PBMC culture were calculated by subtracting the cytokine levels in the non-stimulated culture from the levels in the stimulated culture. For most cytokines, the levels in unstimulated cultures were relatively low: a 34 to 179 pg range was observed for IL-10; a 19 to 382 pg range for TNF, with a 663 pg

outsider; a 46 to 243 pg range for IL-5; a 25 to 83 pg range for IL-13; a 9 to 185 pg range for IL-17; and a 20 to 251 pg range for IL-12. The concentrations of TGF-β were much higher than those of these other cytokines in all cultures, ranging from 2,146 to 4,236 pg, with an outsider of 8,290 pg. As for IFN-γ, there was a great variation in the spontaneous production of the cytokine, whose concentrations in cultures supernatants ranged from 8 to 927 pg.

Statistical analysis

The statistical significance of differences in cytokine concentrations between stimulated and non-stimulated cultures were determined by Friedman's test, followed by Dunn's post-test. Differences of $p \leq 0.05$ were considered significant.

Fisher's exact probability test was used to assess the statistical significance of differences in the proportion that fungal antigen-stimulated and unstimulated PBMC presented a given pattern of produced cytokines and in the frequencies of production of IL-17 in candidin- and trichophytin-stimulated cultures.

Results

Cell viability in fungal extract-stimulated peripheral blood mononuclear cell (PBMC) cultures.

The mean percentages of viable cells in PBMC cultures to which 50 μg/mL of candidin and trichophytin extracts were added, in relation to untreated cultures,

were 98.2 and 99.0, respectively.

Cytokine production by fungal extract-stimulated PBMC

The fungal extract concentrations (5 and 50 μg/mL) that were utilized in the present work did not differ in terms of the patterns of cytokine level increases that they induced in the different PBMC cultures, although the best concentration was not always the same for the different PBMC (data not shown). The results shown in figures 1 and 2 were those obtained with the best concentration (either 5 and 50 μg/mL) for each PBMC.

The addition of candidin significantly increased the production of IL-10, TNF, and IL-12 by healthy individuals' PBMC, and of IL-10, TGF-β, IL-17, IFN-γ and IL-12 by allergic patients' PBMC (Table 1), whereas trichophytin significantly increased the production of IL-12 by healthy individuals' PBMC, and of TGF-β by allergic patients' PBMC (Table 1). Although statistically significant increases could be observed for some cytokines in cultures of allergic donors' PBMC and not in cultures of non-allergic donors' PBMC, or vice versa (Table 1), there were no statistically significant differences between the results obtained in cultures of allergic and non-allergic donors' PBMC.

The addition of trichophytin increased regulatory cytokine (TGF-β and/or IL-10) levels in 6 out of 7 cultures of healthy individuals' PBMC and in 8 out of 9 cultures of allergic patients PBMC. This also happened with the addition of candidin in all 16 individuals (seven healthy and nine allergic)'s PBMC (Fig. 1). In most cultures, however, these increases in regulatory cytokines were accompanied by increases in proinflammatory cytokines (Fig. 1 and 2).

Trichophytin increased regulatory cytokines levels exclusively, without augmenting any of the investigated proinflammatory cytokines, in 2 out of 7 non-allergic individuals' PBMC (non-allergic donors 2 and 4; Figs. 1 and 2) and in 3 out of 9 allergic patients' PBMC (allergic donors 2, 3, and 4; Figs. 1 and 2). An exclusive increase in regulatory cytokine levels were not seen in any culture of candidin-stimulated PBMC (Figs. 1 and 2).

Stimulation of the production of cytokines associated with the Th1 immune response (TNF, IL-12 and IFN-γ) by either trichophytin or candidin or by both was seen in 7 out of 8 healthy individuals' PBMC and in all nine allergic patients' PBMC (Figs. 1 and 2).

To be a feasible candidate for immunological adjuvant in anti-allergy immunotherapy, a substance should stimulate the production of Treg cytokines, Th1 cytokines or both, without stimulating the production of Th2 and Th17 cytokines. Stimulation of Treg cytokine (TGF-β and/or IL-10) production accompanied or not by stimulation of the production of cytokines associated with the Th1 immune response, and without stimulation of Th2 cytokines (IL-5 and IL-13) and IL-17, by either trichophytin or candidin or by both of them, was seen with 4 out of 7 healthy individuals' PBMC (non-allergic donors 1, 2, 4 and 6; Figs 1 and 2) and with 9 out of 9 allergic patients' PBMC (Fig. 1 and 2). This differed significantly from what was seen in unstimulated cultures ($p = 0.035$ and $p = 0.0002$, respectively; Fisher's exact probability test). Although a higher proportion of PBMC with these patterns of cytokine production was seen in the allergic patients' group than in the non-allergic group, this difference was not statistically significant ($p = 0.26$, two-tailed Fisher's exact probability test).

The levels of the allergy-associated Th2 cytokine IL-5 were increased in PBMC cultures from 3 out of 7 healthy individuals' (non-allergic donors 3, 5, and 7, Fig. 2) and in none of the alergic patients' PBMCs that were stimulated by candidin. Trichophytin, on the other hand, stimulated the production of IL-5 by just one of the non-allergic donors PBMC (non-allergic donor 7) and by none of the allergic donors PBMC.

The levels of IL-13, another allergy-associated cytokine, were not increased when candidin was added to cultures of non-allergic donors' PBMC (Fig. 2). Only a small increase was seen in one of the allergic patients' PBMC cultures due to the addition of that fungal extract (allergic donor 9; Fig. 2). As for trichophytin-stimulated PBMC, the level of IL-13 was increased in only one of the cultures of non-allergic individuals' PBMC (non-allergic donor 5; Fig. 2) and in none of the 9 allergic patients' PBMC cultures (Fig. 2).

IL-17 levels were increased in cultures of trichophytin-stimulated PBMC from 1 out of 7 healthy individuals (donor 5, Fig. 2), and from none of the nine alergic patients (Fig. 2), whereas it was increased in cultures from candidin-stimulated PBMC from 4 out of 7 healthy individuals (non-allergic donors 1, 3, 5, and 7, Fig. 2), and from 5 out of 9 allergic patients (allergic donors 2-5 and 9; Fig. 2). Combining the data from allergic and non-allergic individuals for trichophytin (1 out of 16 individuals with PBMC producing IL-17) and for candidin (9 out of 16 individuals with PBMC producing IL-17), the difference observed between the frequencies of stimulation of IL-17 production by the two extracts was highly significant ($p = 0.0059$, two-tailed Fisher's exact probability test).

Discussion

The viability of the PBMC was not affected by incubation with up to 50 μg/mL of candidin or trichophytin for 24 hours, as shown by the absence of additional MTT reduction. Cell death, therefore, did not affect the variation in cytokine production caused by the stimulation of the cells by the fungal extracts.

The present work, although studying a relatively small sample of individuals, clearly show that trichophytin and/or candidin elicited the production of regulatory and/or Th1-associated cytokines without stimulating the production of the respiratory allergy-associated Th2 cytokines and IL-17 in a proportion of healthy (4 out of 7 in the present study) and allergic (9 out of 9 in the present study) individuals' PBMC. These allergic patients, therefore, are candidates for receiving anti-allergy immunotherapeutic preparations containing the allergen(s) and, as adjuvant, only candidin (allergic donor 8; Figs. 1 and 2); only trichophytin (allergic donors 2, 3, 4, 5, and 9; Fig. 2), or both (allergic donors 1, 6, and 7; Fig. 2). These results open, therefore, the possibility of using customized immunotherapeutic preparations that would contain only the adjuvant(s) that did not induce Th2 cytokine or IL-17 production in that particular patient. For obvious reasons, adjuvants that are candidates for inclusion in anti-allergy therapeutic vaccines should not induce the production of these cytokines, as they are directly associated with the pathogenesis of respiratory allergy [37]. A customized immunotherapy that would involve the previous realization of a cell culture procedure and the assessment of cytokine production would be relatively expensive. Its price, however, should be weighed

against that of carrying out a less effective immunotherapeutic procedure for years.

Candidin significantly stimulated the production of three cytokines by healthy individuals' PBMC (IL-10, TNF and IL-12), and of five cytokines by allergic patients' PBMC (IL-10, TGF-β, IL-17, IFN-γ and IL-12), whereas trichophytin significantly increased the production of only IL-12 by healthy individuals' PBMC, and of only TGF-β by allergic patients' PBMC (Table 1). Candidin, therefore, seems to be more stimulatory than trichophytin for human PBMC. In fact, candidin stimulated the production of IL-17 by the PBMC from a larger number of donors (9 out 16, Fig. 2) than trichophytin (1 out of 16; $p = 0.0059$, two-tailed Fischer's exact probability test). However, PBMC of a larger number of individuals, and in different geographic areas, should be tested in order to determine which of the two preparations, trichophytin or candidin, would be the best anti-allergy adjuvant candidate.

Although current knowledge highlights the role of T regulatory cell-mediated immune regulation, defined mechanisms that lead to successful clinical outcomes of allergen-specific immunotherapy still remains an open area of research [38].

An interesting finding, which may be relevant for the possible use of trichophytin and candidin as adjuvants in anti-allergy immunotherapy, is that these fungal extracts did not stimulate the production of Th-2 cytokines by allergic donor' PBMC more intensely than by healthy donor' PBMC. Allergic patients, therefore, do not seem to have an immune system biased towards the production of Th2 cytokines when stimulated by these fungal antigens.

As trichophytin and candidin have been injected for decades in human beings for the assessment of the cellular immune response [32], a candidin- and/or trichophytin-containing immunotherapeutic preparation would be more easily approved for clinical use.

Conclusions

Trichophytin and candidin stimulated the production of regulatory cytokines on the PBMC from nearly all donors (15 out of 16 and 16 out of 16, respectively). These finding justify the realization of studies aimed at investigating whether there are proteins in these fungus extracts that preferentially induce the production of regulatory cytokines. These proteins would be ideal Treg-inducing adjuvant candidates for inclusion in immunotherapeutic vaccines for inflammatory diseases, such as allergy and autoimmune diseases. An interesting possibility, therefore, would be the purification of molecules from candidin or trichophytin that would specifically stimulate an immune regulatory response that, in its turn, would control the allergic immune response, or alternatively, identify those molecules and have they produced by the recombinant DNA technology.

Future perspectives

New tolerogenic anti-allergy immunotherapies should be available in the next five to ten years. These immunotherapies will probably utilize preparations

containing Treg-inducing adjuvants. It is possible that molecules obtained from candidin or trichophytin might be used as tolerogenic adjuvants.

Summary points

- The identification of molecules that could be used as adjuvants in anti-allergy immunotherapeutic preparations is highly desirable.
- Anti-allergy immunotherapy has been associated with the induction of regulatory T cells and/or T helper 1 cells that could theoretically inhibit the development of allergy-inducing T helper 2 immune responses.
- In this study, it was investigated whether candidin or trichophytin could elicit recall immune responses that could potentially inhibit a Th2 response, in nine allergic and seven non-allergic individuals' peripheral blood mononuclear cells (PBMC).
- PBMC were cultivated in vitro in the presence or absence of these extracts at 37° C and 5% CO2 during 24 and 120 hours.
- In this way it was sought to obtain indirect evidence that they could be used as adjuvants aimed at inhibiting Th2 immune responses.
- The two extracts induced cytokine production in all PBMC preparations. Stimulation of the production of Treg cytokines (TGF-β and/or IL-10), accompanied or not by stimulation of production of cytokines associated with the Th1 response (TNF, IL-12 and IFN-γ), but without stimulation of Th2 cytokines (IL-5 and IL-13) and IL-17, by either trichophytin or candidin or by

both of them, was seen with 4 out of 7 healthy individuals' PBMC and with 9 out of 9 allergic patients' PBMC.

- As candidin and trichophytin have been injected intradermally in human beings for decades to trigger cutaneous Th1 immune reactions without adverse reactions, these results indicate that these fungal extracts could be used as adjuvants in personalized therapeutic vaccines in a fair proportion of individuals and justify the carrying out of investigations aimed at identifying molecules in these extracts that might exclusively induce Treg and/or Th1 immune responses.

Competing interests

The authors have no financial conflicts of interest.

Authors' contributions

A.L.M. Amor and L.N. Santos were involved in recruiting study patients, performing the experiments, acquisition, statistical analysis and interpretation of the data, and preparing a first draft of the manuscript. A.A. Galvão, E.M.M.A. Belitardo and E.S. Silva performed part of the experiments. N.M. Alcântara-Neves helped in devising the study, and designed and supervised the experiments. L.C. Pontes-de-Carvalho devised the study, designed and performed statistical analysis, interpreted the data and wrote the final version of the manuscript.

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FIGURE LEGENDS

Figure 1 - TGF-β, IL-10, TNF and IL-12 concentrations in cultures of healthy and allergic individuals' peripheral blood mononuclear cells (PBMC) stimulated with fungal extracts. A, PBMC from non-allergic individuals' donors. B, PBMC from allergic individuals' donors. Candidin (C) or trichophytin (T) were added to a final concentration of 5 or 50 μg/mL to the cells, which were incubated as described in the Material and Methods section. The amounts of cytokines detected in non-stimulated cultures were subtracted from the amounts detected in the stimulated cultures. Each symbol shows the results obtained with the peripheral blood mononuclear cells from an individual donor. The legends above the graphs identify the individual PBMC donors (NAl = non-allergic donor; Al = allergic donor). The results obtained from the same donors' PBMC are represented by identical symbols in Figures 1 and 2.

Figure 2 - IL-5, IL-13, IL-17, and interferon gamma (IFN-γ) concentrations in cultures of peripheral blood mononuclear cells (PBMC) stimulated with fungal extracts. A, PBMC from non-allergic individuals' donors. B, PBMC from allergic individuals' donors. Candidin (C) or trichophytin (T) were added to a final concentration of 5 or 50 μg/mL to the cells, which were incubated as described in the Material and Methods section. The amounts of cytokines detected in

non-stimulated cultures were subtracted from the amounts detected in the stimulated cultures. Each symbol shows the results obtained with the peripheral blood mononuclear cells from an individual donor. The legends above the graphs identify the individual PBMC donors (NAl = non-allergic donor; Al = allergic donor). The results obtained from the same donors' PBMC are represented by identical symbols in Figures 1 and 2.

Table 1. Statistical significance* of variations in cytokine production by fungal extract-stimulated peripheral blood mononuclear cells.

Produced cytokine	Peripheral blood mononuclear cells from	Comparison	
		Candidin-stimulated versus non-stimulated cultures	Trichophytin-stimulated versus non-stimulated cultures
IL-10	Allergic patients	$p < 0.0100$	NS
	Healthy individuals	$p < 0.0001$	NS
TNF	Allergic patients	NS	NS
	Healthy individuals	$p < 0.0500$	NS
IL-5	Allergic patients	NS	NS
	Healthy individuals	NS	NS
IL-13	Allergic patients	NS	NS
	Healthy individuals	NS	NS
IL-17	Allergic patients	$p < 0.0500$	NS
	Healthy individuals	NS	NS
TGF-β	Allergic patients	$p < 0.0001$	$p < 0.0500$
	Healthy individuals	NS	NS
IL-12	Allergic patients	$p < 0.0100$	NS
	Healthy individuals	$p < 0.0100$	$p < 0.0500$
IFN-γ	Allergic patients	$p < 0.0500$	NS
	Healthy individuals	NS	NS

*As assessed by Friedman's non-parametric test followed by Dunn's post-test.
NS = no statistical significance.

Figure 1

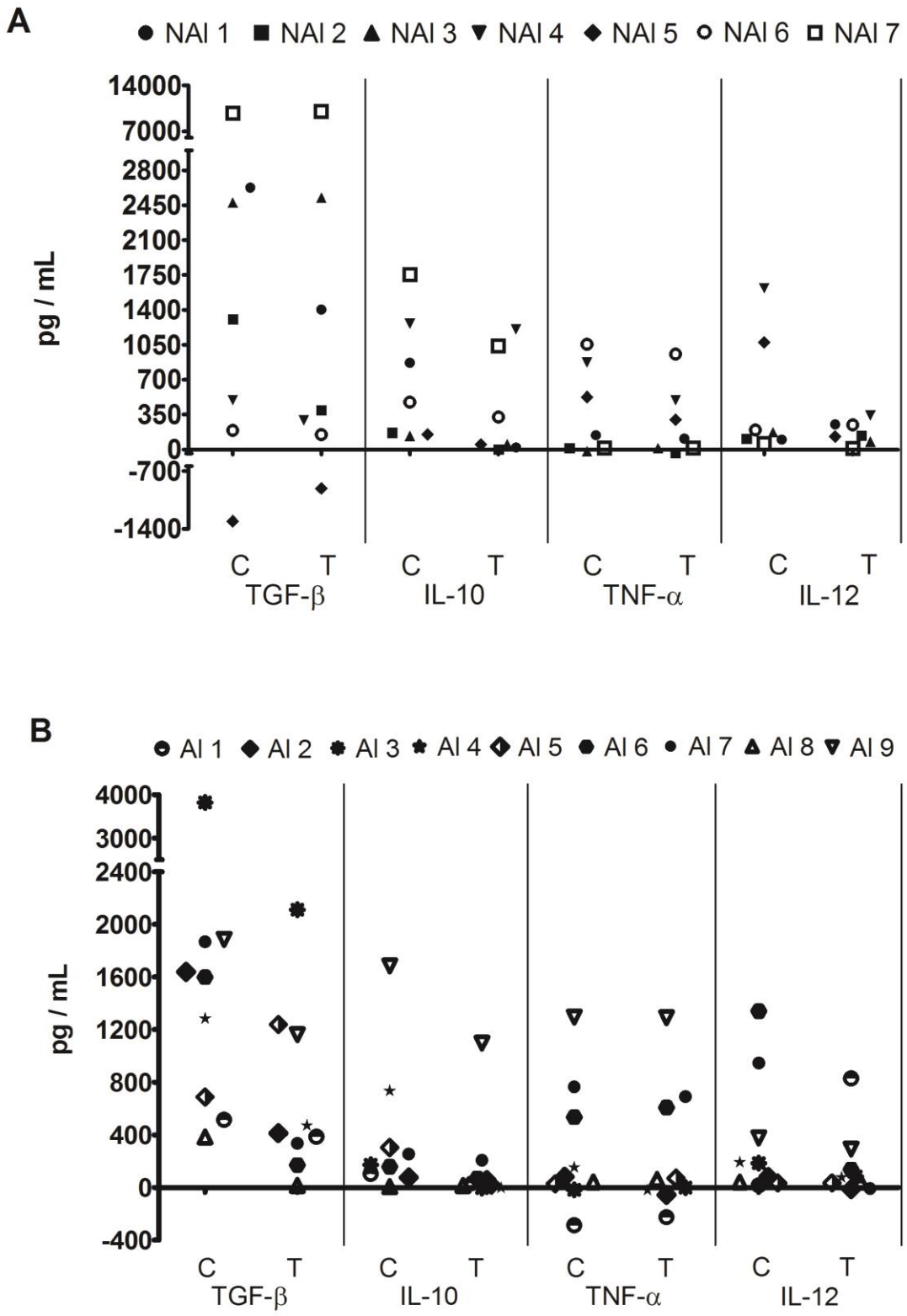
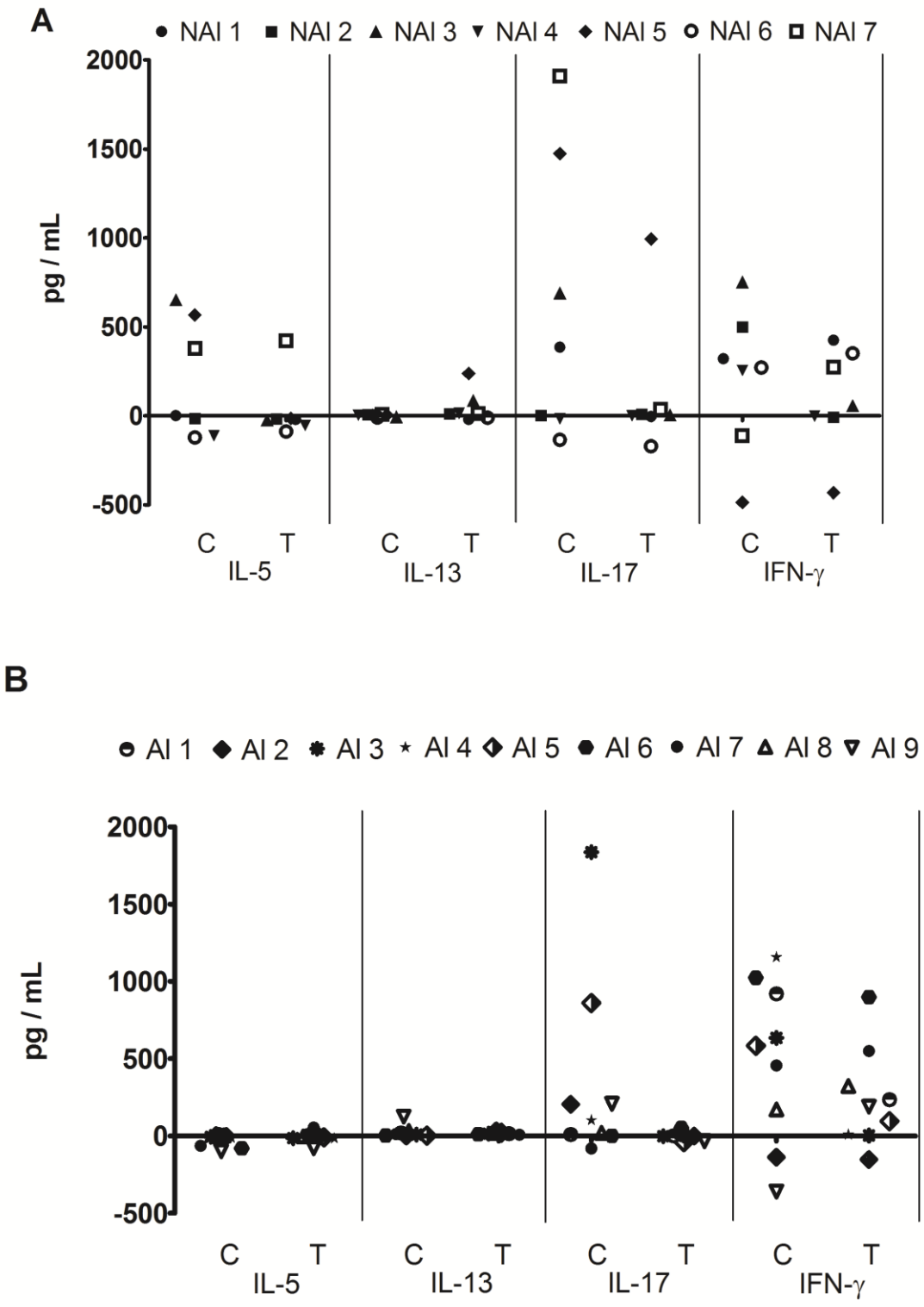

A
NAI 1
NAI 2
NAI 3
NAI 4
NAI 5
NAI 6
NAI 7
pg / mL
14000
7000
2800
2450
2100
1750
1400
1050
700
350
0
-700
-1400
C T C T C T C T
TGF-β IL-10 TNF-α IL-12
B
AI 1
AI 2
AI 3
AI 4
AI 5
AI 6
AI 7
AI 8
AI 9
pg / mL
4000
3000
2400
2000
1600
1200
800
400
0
-400
C T C T C T C T
TGF-β IL-10 TNF-α IL-12

Figure 2


A
NAI 1
NAI 2
NAI 3
NAI 4
NAI 5
NAI 6
NAI 7
2000
1500
1000
500
0
-500
pg / mL
C
T
IL-5
IL-13
IL-17
IFN-γ
B
AI 1
AI 2
AI 3
AI 4
AI 5
AI 6
AI 7
AI 8
AI 9
2000
1500
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500
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C
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IL-5
IL-13
IL-17
IFN-γ

4.2 ARTIGO 2

TÍTULO:

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

Manuscrito submetido para publicação no *BioMed Research International* – Fator de impacto = 2.880. No **Anexo 5** encontra-se cópia do envio *on line* do trabalho ao periódico.

Este **Manuscrito** investigou o potencial imunomodulatório do extrato bruto do adulto de *Toxocara canis* (TcE) e de dez frações proteicas deste extrato (TcEF01, TcEF02, TcEF03, TcEF04, TcEF05, TcEF06, TcEF07, TcEF08, TcEF09 e TcEF10) em células mononucleares do sangue periférico humano (PBMC) de 10 indivíduos alérgicos e de 09 indivíduos não alérgicos, cultivadas *in vitro*, na presença ou ausência destes antígenos, e os sobrenadantes foram avaliados para a produção de citocinas (TGF-β, IL-10, IL-12, TNF, IL-6, IL-5, IL-13 e IL-17).

Algumas frações estimularam a produção de citocinas regulatórias (TGF-β e / ou IL-10) e do tipo Th1 (IL-12, TNF), sem estimularem citocinas Th2 (IL-5 e IL13) e IL-17.

Foi proposto que o estudos com estas frações seriam adequados após purificação de suas moléculas, testando-as para avaliar o possível papel para imunoprofilaxia antialérgica.

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

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Key words: Bioactive molecules, *Toxocara canis*, Immunomodulation, Allergy.

Abstract

Helminths possibly downmodulate immune responses to airborne allergens through a regulatory network induction. The identification of bioactive molecules with immunomodulatory potential which could be used in immunotherapy of allergy and autoimmune diseases is highly desirable. To investigate the immunoregulatory potential of the adult *Toxocara canis* crude extract (TcE) and ten protein fractions of its extract (TcEF01, TcEF02, TcEF03, TcEF04, TcEF05, TcEF06, TcEF07, TcEF08, TcEF09 and TcEF10), 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 they supernatants were evaluated for cytokines production (TGF-β, IL-10, IL-12, TNF, IL-6, IL-5, IL13 and IL-17). To determinate the cell viability the PBMC were cultivated for 24 hours, in the presence of the antigens, and following, they were submitted to the 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) and Th1 (IL-12) cytokines without stimulating Th2 cytokines (IL-5 and IL13) and IL-17 it was proposed that these fractions would be more suitable for further studies aiming to purify their molecules and testing them separately to evaluate their possible role as adjuvant in immunotherapy and vaccines for inflammatory diseases.

Introduction

The prevalence of allergies and autoimmune diseases are increasing steadily in recent decades, where about 300 million people worldwide have asthma [1] and is estimated that 50 million Americans have autoimmune diseases [2]. Researchers estimate that by 2050 year, two-thirds of the world population will live in cities and will have little closeness to nature and biodiversity, and as a result, the increasing in chronic diseases such as allergies and respiratory disorders are expected [3]. Up to now there are no good curative or prophylactic drugs for these diseases and immunotherapy is an important alternative to the available therapies [4] and it has as goals improving symptoms, increasing quality of life, favorably altering the course of disease, and achieving these goals with a good benefit-to-risk ratio and yet being cost-effective [5].

Asthma and rhinitis (allergic atopic disorders) are caused by a deregulated immune response, involving the T helper type 2 (Th2) cytokines, including interleukin (IL)-4, IL-5 and IL-13 [6,]. More recently, Th-17 and IL-33 have been also implicated in the pathogenesis of these diseases [7]. Some feasible strategies to down-modulate the allergic immune response are proposed by several studies: (a) immune response toward a Th1 deviation from viral and bacterial products or exogenous IFN-γ or IL-12 production, probably leads to control the allergic Th2-response, however may increase susceptibility to Th1-mediated diseases [8-10]; and (b) induction of regulatory mechanisms (CD4+CD25+ T regulatory cells; expression of

cytotoxic T-lymphocyte antigen 4 - CTLA-4; IL-10; TGF-β) [7; 11-12] by helminth antigens associated or not with specific immunotherapy, which would culminate with the control of the exacerbated inflammatory allergic response.

Epidemiological studies have shown a lower prevalence of atopy [13-15] and allergic diseases in countries that are endemic for helminthic infections [16, 17]. 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 actions [18-21]. These cytokines create a balance among different types of antibodies [22] i.e. an increase in the production of IgG, IgG4, IgA and a decrease in specific IgE levels [23, 24], that enable them to survive in their hosts. This immune response has the effect of reducing the inflammatory damage of allergic and autoimmune origins [25]. These parasites or their molecules have been studied and used in humans to treat chronic inflammatory diseases with different results [26-27]. Advances, in order to clarify these contradictions and identification of these molecules and extracts that can regulate specific pathways of the immune response *in vitro* are needed [28]. Some studies have shown that molecules of *Schistosoma mansoni* and filarial parasites were able to reduce atopy and allergy in experimental animal models, decreasing Th2 cytokine and increasing IL-10 production by human cells *in vitro* [6, 29] Another relevant report is the therapeutic benefit of infection with the

porcine whipworm *Trichuris suis*, to patients with inflammatory bowel diseases, reducing clinical manifestations of ulcerative colitis and Crohn's disease [30-32]. 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 [33].

The human infection by the dogs and cats roundworms *Toxocara canis* and *T. cati* is highly prevalent in low-income populations of developing countries, mostly asymptomatically or causing the visceral larva migrans syndrome [34] where 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 [35; 36]. As we have found that this infection occurs in 46-65 % of the low class level population in Salvador, a large city of Northeast Brazil [37, 38] and that the seropositivity for this infection was found associated to low skin prick test reactivity for aeroallergens [39], we decided to investigated the role played *T. canis* adult extract ion-exchange chromatographic fractions upon human PMBC cells.

Material e methods

Blood donors

All blood donors, aged between 21 and 40 years, signed an informed consent to participate in the present 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 *Blomia tropicalis, Dermatophagoides pteronyssinus, Blattella germanica and Periplaneta americana* (using ImmunoCAP assay (Phadia/Thermo Fischer Diagnostics AB, Uppsala, Sweden). This research was approved by the Ethical Committee of the Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Proposal 179/2008, Project no. 277.

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 hours after treatment. After that, the parasites were washed in saline and cryopreserved at – 70^{o}C until use. To obtain these parasites, the dog owners were consulted and the project was approved by the Ethics Committee on Animal Use of the Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz - Bahia – Brazil (Proposal 014/2010).

The parasite lysate and its chromatographic fractions were obtained according to Santos et al [33]. Shortingly, the adult worms were lysated 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 tosyllysinechloromethylketone, and 2 mM ethylenediaminetetracetate) (Sigma- Aldrich, St. Louis, MO, USA). Then, the extract was centrifuged at 13,400 g for 20 minutes at 4 °C and the supernatant sterilized by filtration and stored at -70 °C. The extract was fractionated by ion exchange chromatography with a salt gradient elution, by fast protein liquid chromatography using a Mono Q 5/50 column (GE Healthcare, São Paulo, SP, Brazil). Ten fractions collected (TcEF01 to TCEF10) and the crude extract (TCE) 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 [40], yielding the following protein concentrations: TcE (138 mg), TcEF01 (33,5 mg), TcEF02 (22,9 mg), TcEF03 (31.9 mg), TcEF04 (23,6 mg), TcEF05 (24,1 mg), TcEF06 (50,5 mg), TcEF07 (34,7), TcEF08 (52,8 mg), TcEF09 (40,3 mg), and TcEF10 (24,7 mg).

Chosen of antigen and mytogen concentrations

To determinate the optimal concentration of the crude extract of *T. canis* and ten of its protein fractions, human peripheral blood mononuclear cells (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 stimulated or not with lipopolysacharide (LPS from *E. coli)* (8 UE/mL; Sigma-Aldrich, St. Louis, MO, USA) 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 hours. Following, the IL-10 production was measured in the cell supernatants. After collection of the supernatant, PBMC were submitted 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 et al [41]. Fifty µg/mL was proved to be the optimal concentration to stimulate the PBMC (Data not shown). The viability of the PBMC was not affected by incubation for 24 hours with 50 µg/mL of the crude extract of *T. canis* and by ten of its protein fractions (Data not shown).

Stimulatory and inhibitory assays

PBMC were stimulated by *T. canis* antigens containing 50 µg/mL of each 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) in the cell supernatant or 10 µg/mL of PHA for measuring IL-5, IL-13 and IL-17. Cell were cultivated as described above, for 48 hours (for quantification of IL-10, TNF, IL-12 and TGF-β or for 120 hours (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 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 mytogens (and IFN-γ) alone. Unstimulated PBMC were used as negative control in both assays.

Cytokines measurements

The cytokines concentrations (TGF-β, IL-10, IL-12, IL-6, TNF, IL-5, IL13 and IL-17) in PMBC 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). And the percentage (%) of inhibition of citokyne 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 showed in the Figures.

Statistical analysis

Data normality was assessed by the Kolmogorov-Smirnov test. Kruskal-Wallis analysis was performed to compare the cytokines production by PBMC of the allergic and non-allergic volunteer groups. The differences in

cytokines production into groups were determined using Friedman's test, followed by Wilcoxon signed rank test (paired by individuals) to evaluate differences between the stimulation (or inhibition) by each antigen and non-stimulated (non-inhibited) cultures. Data are presented as means ± SEM; $p \leq 0.05$ was considered to be significant. All statistical analysis was conducted using the Graphpad Prism 6 software.

Results

Cytokines production by PBMC stimulated with *T. canis* antigens

Figures 1 and 2 show the stimulatory assays. For regulatory cytokines, it was observed in PBMC from allergic donors, that *T. canis* extract and six of its protein fractions (TcEF01, TcEF09, TcEF04-07) induced in a statistically significant way TGF-β production (Figure 1A). IL-10 was induced on PBMC of allergic donors by TcE and 8 of its fractions (TcEF02-06 and TcEF08-10) while in non-allergics PBMC, this citokyne was induced by four fractions (TcEF06-TcEF09) (Figure 1C e 1D, respectively).

In respect to Th1 cytokines, the production of IL-12 increased significantly in PBMC cultures of allergic donors stimulated by TcE and four of its fractions (TcEF03-06; Figure 1E) while in non-allergics, it increased in TcE and three fractions stimulated cultures (TcEF06, TcEF08 and TcEF-10; Figure 1F). TNF increased significantly in the cultures of the non-allergic donors (Figure 1H) by TcE and four of its fractions (TcEF01, TcEF05-07).

Interestingly, for Th2 cytokines and Th17, the allergic donors PBMC did not increased production of IL-5, IL-13 and IL-17 except for the increase of IL-17 in cultures stimulated with TcEF05 (Figures 2A, 2C and 2E) and for non-allergic donors only IL-13 was more produced in cultures stimulated with TcEF03, TcEF04 and TcEF07 (Figures 2B, 2E and 2F).

Cytokines production by PBMC inhibited by *T. canis* antigens

The *T. canis* antigens did not inhibited the production of any regulatory or Th1 cytokines in both allergic and non-allergic donors PBMC in a statistically significant way, except for TcEF08 and TcEF09 which inhibited the allergic donors cells to produce IL-12 (Figure 3E). While for Th2 cytokines, TcE inhibited IL-5 in both allergic and non-allergic PBMC donors (4A and 4B); TcEF03 inhibited IL-13 in allergic and TcEF01-03 inhibited statistically IL-13 in non-allergic PBMC donors (Figures 4C and 4D). Meanwhile, IL-17 was inhibited by TcEF01-04 in allergic donnor and by TcE, TcEF01-02 of non-allergic PBMC donors (Figures 4E and 4F).

The summary seen in Table 1 show a panoramic picture of the total stimulation or inhibition of TcE and its protein fraction upon PBMC production of all cytokines by allergic and non-allergic donors based only on data with statistical significance. They show that, in most cultures, there were increased production of regulatory cytokines (TGF-β and / or IL-10) accompanied by an increase in pro-inflammatory Th1 cytokines (IL-12 and / or TNF), and no stimulation of Th2 (IL-5 and / or IL-13) cytokines. IL-17 was

observed only in stimulated PBMC with TcE, TcEF01, TcEF02, TcEF06, TcEF08, TcEF09 and TcEF10 antigens.

Discussion

The consensus for immunotherapy to treat allergy, consider two separate and sequential immune responses: an immune deviation from Th2 to type Th1 responses and the generation of regulatory T cells (Treg) [42]. Therefore, 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 [23; 43-46].

On the other hand, other authors reported that the anti-allergy immunotherapy leads to the activation of Treg cells, with production of regulatory cytokines [IL-10 and / TGF-β [23; 43-45]. 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 [7]. Indeed, it has been demonstrated that the success of allergen-specific immunotherapy is associated with the induction of IL-10 [47, 48].

In respect to the use of *T. canis* crude extract and / or its fractions in immunotherapy of allergic diseases and / or autoimmune diseases it shall be observed, the profile of each tested product with a differential response in the production of cytokines surveyed in stimulatory and inhibitory assays.

In this study it was found that the peripheral blood mononuclear cells from different donors did not respond with the same intensity for the same fractions, which may reflect genetic differences or differences in antigenic stimulation history of each 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 [37, 39].

This work, although has studied a relatively small sample of individuals, clearly shows the production of regulatory and / or Th1 cytokines, without stimulating the production of Th2 and IL-17 cytokines which are associated with respiratory allergy by PBMC of allergic individuals stimulated with *T. canis* antigens (TcE extract and the fractions TcEF01, TcEF02, TcEF06, TcEF08, TcEF09 and TcEF10). Meanwhile, the TcEF07 fraction stimulated regulatory cytokines and IL-13, without inhibiting Th2 cytokine, and TcEF09 fraction that stimulated only regulatory cytokines) might be used in experimental models for autoimmune diseases (colitis, and autoimmune encephalomyelitis experimental). Up based only on data with statistical significance.

Our data showed that the *T. canis* antigens were mostly good stimulators of IL-12 in allergic donors. For operational reasons, it was not possible to quantitate IFN-γ in cell cultures performed, since the time of collection of the supernatant 48 hours appears to have been insufficient for the dosage of this cytokine when compared to the production of IL-12 in PBMC of subjects in the study.

The research for drugs that stimulate the production of cytokine IL-12 and

IFN-γ including natural stimuli (extracts of helminths and vegetables, for example) is interesting for studies on allergic immunotherapy. Studies in peripheral blood mononuclear cells have suggested that a defect in the secretion of IFN-γ is the primary component of the atopic state [49]. Studies show a significant reduction of IFN-γ by mononuclear cells in patients with severe asthma [50] and other allergic diseases, such as allergy to cow's milk [51] and atopic dermatitis [52]. The antigen-presenting cells, presumably through the stimulation of pattern recognition receptors [53], can produce IL-12 which stimulates the differentiation of naive T lymphocytes to the Th1 rather than Th2 cells [42]. A major function of the cytokine is IL-12 induction of IFN-γ, since most of its pro-inflammatory effects is IFN-γ-dependent. In this respect the IL-12 produced by APC acts on T and NK cells, inducing the production of IFN-γ and this, in turn, acts on the APC activating them. 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 cellular immune response [54]. Indeed, inhibition of the Th2 cells is accompanied by an augmentation of a Th1 cellular immune response with IFN-γ production has been reported in allergic patients following immunotherapy [55-57]. IFN-γ is a cytokine that acts on CD4 T cells promoting the differentiation of Th1 and inhibiting the differentiation of Th2 lymphocytes [58].

Our results show that *T. canis* antigens activated the cytokine production of innate immunity, such as TNF. Proteins with regulatory functions but also inducing IL-12 and TNF transiently, without inhibition of IL-10 and

subsequent increase in IL-10 and TNF inhibition were also seen in other studies [59, 60].

The Th2 cells have essential role in the immunopathogenesis of allergic diseases such as asthma [61]. These cells secrete IL-4 and IL-13, which induce and maintain the production of IgE by B cells, and IL-5, which induces the recruitment, differentiation and infiltration of eosinophils in the bronchial mucosa [62]. Therefore, all caution should of taking in the screening of helminth products for allergy immunotherapy, since they are prominent Th2 stimulators. It also should be considered products that do not stimulate IL-17 cytokine because Th17 immune responses have been associated with respiratory allergy [7, 63-65].

There was no correlation of the data of IL-6 and TGF-β, with increased IL-17. Studies show that, in particular, IL-6 controls the balance between regulatory T cells (the production of TGF-β) and Th17 cells [66-67]. Suggest that blockade of IL-6 signaling is effective in treating experimental models of autoimmune and chronic inflammatory diseases such as inflammatory bowel diseases, diabetes, multiple sclerosis, asthma and rheumatoid arthritis as well as models of inflammation-associated cancer [68].

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

It was around the 1990s that researchers seriously considered the possibility of using helminth parasites in human therapeutic administration,

intentionally, in an attempt to treat autoimmunity or allergy [64]. Safety tests on humans was performed with the porcine whipworm, *Trichuris suis*, in the treatment of inflammatory bowel disease [30] and the parasite *Necator americanus* (hookworm human agent) for the treatment of asthma [65].

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* molecules 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 [33]. Since it is not feasible obtaining worms from infected dogs to purifiy 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 *Toxocara canis* [69, 70], for example, will favour this task.

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.

As some fractions stimulated the production of immunoregulatory cytokines (TGF-β and/or IL-10) and Th1 cytokines (IL-12 and/or TNF) without stimulating Th2 (IL -5 and IL-13) and IL-17 cytokines it is proposed to

continue this study with further analysis to obtaining candidate molecules for immunotherapeutic vaccines in inflammatory and autoimmune diseases. TcE, TcEF01, TcEF02, TcEF06, TcEF08, TcEF09 and TcEF10 were the most suitable products for studies on allergy and TcEF07 and TcEF09 on autoimmune diseases. Up based only on data with statistical significance.

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Disclosures

The authors have no financial conflicts of interest.

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Table 1. Summary of results of the citokynes production by allergic and non-allergic donor leukocytes stimulated or inhibited by TcE and TF01-10 (based only on data with statistical significance).

Assay	ALLERGIC DONORS ($p \leq 0.05$* and $p \leq 0.01$**)	ASSAY	NON-ALLERGIC DONNORS ($p \leq 0.05$* and $p \leq 0.01$**)
TcE			
Stimulatory	↑TGF-β*; ↑IL-10**; ↑IL-12**	**Stimulatory**	↑IL-12*; ↑TNF**
Inhibitory	↓IL-5*	**Inhibitory**	↓IL-5**; ↓IL-17**
TcEF01			
Stimulatory	↑TGF-β*	**Stimulatory**	↑TNF*
Inhibitory	↓IL-17**	**Inhibitory**	↓IL-13**; ↓IL-17*
TcEF02			
Stimulatory	↑IL-10**	**Stimulatory**	--
Inhibitory	↓IL-17**	**Inhibitory**	↓IL-13*
TcEF03			
Stimulatory	↑IL-10**; ↑IL-12**	**Stimulatory**	↑IL-13*
Inhibitory	↓IL-13;* ↓IL-17**	**Inhibitory**	↓IL-13*
TcEF04			
Stimulatory	↑TGF-β**; ↑IL-10**; ↑IL-12**	**Stimulatory**	↑IL-13*
Inhibitory	↓IL-17**	**Inhibitory**	--
TcEF05			
Stimulatory	↑TGF-β**; ↑IL-10*; ↑IL-12**; ↑IL-17*	**Stimulatory**	-
Inhibitory	--	**Inhibitory**	↑TNF*
TcEF06			
Stimulatory	↑TGF-β**; ↑IL-10**; ↑IL-12*	**Stimulatory**	↑IL-10**; ↑IL-12**; ↑TNF**
Inhibitory	--	**Inhibitory**	--
TcEF07			
Stimulatory	↑TGF-β*	**Stimulatory**	↑IL-10**; ↑TNF**; ↑IL-13**
Inhibitory	--	**Inhibitory**	--
TcEF08			
Stimulatory	↑IL-10*	**Stimulatory**	↑IL-10*; ↑IL-12**
Inhibitory	↓IL-12**	**Inhibitory**	--
TcEF09			
Stimulatory	↑TGF-β**; ↑IL-10*	**Stimulatory**	↑IL-10**
Inhibitory	↓IL-12*	**Inhibitory**	--
TcEF10			
Stimulatory	↑IL-10**	**Stimulatory**	↑IL-12*
Inhibitory	--	**Inhibitory**	--

*$p \leq 0.05$ or **$p \leq 0.01$ or ***$p \leq 0.001$ - Wilcoxon signed rank test.

LEGEND TO FIGURES

Figure 1 – Stimulatory effects on cytokine production (TGF-β, IL-10, IL-12 and TNF) in cultures of allergic (A, C, E and G) and non-allergic individuals' (B, D, F and H) peripheral blood mononuclear cell stimulated with either TcE or TcEF. Fifty μg/mL of the *T. canis* antigens were added to the cells, which were incubated as described in the Material and Methods section. PBMCs were cultured for 48 hours either in the absence (negative control - NC) or in the presence of the total extract or fraction (TcE or TcEF) indicated at the X axis. Columns and vertical bars represent the mean values and SEM of 19 different PBMC cultures of allergic and non-allergic donors. The differences in cytokines production into groups were determined using Friedman's test, followed by Wilcoxon signed rank test (paired by individuals) to evaluate differences between the stimulation by each antigen and non-stimulated cultures - *$p \leq 0.05$ or **$p \leq 0.01$ or ***$p \leq 0.001$.

Figure 2 – Stimulatory effects on cytokine production (IL-5, IL-13, IL-17 and IL-6) in cultures of allergic (A, C, E and G) and non-allergic (B, D, F and H) individuals' peripheral blood mononuclear cell stimulated with either TcE or TcEF. Fifty μg/mL of the *T. canis* antigens were added to the cells, which were incubated as described in the Material and Methods section. PBMCs were cultured for 120 hours either in the absence (negative control - NC) or in the presence of the total extract or fraction (TcE or TcEF) indicated at the X axis. Columns and vertical bars represent the mean values and SEM of 19 different PBMC cultures of allergic and non-allergic donors. The differences

in cytokines production into groups were determined using Friedman's test, followed by Wilcoxon signed rank test (paired by individuals) to evaluate differences between the stimulation by each antigen and non-stimulated cultures - *$p \leq 0.05$ or **$p \leq 0.01$ or ***$p \leq 0.001$.

Figure 3 – Inhibitory effects on cytokine production (TGF-β, IL-10, IL-12 and TNF) in cultures of allergic (A, C, E and G) and non-allergic (B, D, F and H) individuals peripheral blood mononuclear cell stimulated with either TcE or TcEF. PBMCs were cultured for 48 hours either in the presence of fifty μg/mL of the *T. canis* extract or fraction (TcE or TcEF) as indicated at the X axis in cultures with LPS and IFN-γ. As positive controls the mitogens and IFN-γ were added alone as described in the Material and Methods section. Columns and vertical bars represent the % of inhibition values of cytokines production by PBMC cultures from allergic and non-allergic donors, which showed any inhibition of the investigated cytokine production. The differences in cytokines production into groups were determined using Friedman's test, followed by Wilcoxon signed rank test (paired by individuals) to evaluate differences between the stimulation by each antigen and non-stimulated cultures - *$p \leq 0.05$ or **$p \leq 0.01$ or ***$p \leq 0.001$. The % of inhibition of citokyne production in the inhibitory assay was calculated as: [1 - (OD mean of well incubated with antigens and mitogen/OD mean of wells incubated with mitogen) x 100]; where OD = optical density.

Figure 4 – Inhibitory effects on cytokine production (IL-5, IL-13, IL-17 and

IL-6) in cultures of allergic (A, C, E and G) and non-allergic (B, D, F and H) individuals peripheral blood mononuclear cell stimulated with either TcE or TcEF.

PBMCs were cultured for 120 hours either in the presence of fifty μg/mL of the T. canis extract or fraction (TcE or TcEF) as indicated at the X axis in cultures with PHA. As positive controls the mitogens and IFN-γ were added alone as described in the Material and Methods section. Columns and vertical bars represent the % of inhibition values of cytokines production by PBMC cultures from allergic and non-allergic donors, which showed any inhibition of the investigated cytokine production. The differences in cytokines production into groups were determined using Friedman's test, followed by Wilcoxon signed rank test (paired by individuals) to evaluate differences between the stimulation by each antigen and non-stimulated cultures - *$p \leq 0.05$ or **$p \leq 0.01$ or ***$p \leq 0.001$. The % of inhibition of citokyne production in the inhibitory assay was calculated as: [1 - (OD mean of well incubated with antigens and mitogen/OD mean of wells incubated with mitogen) x 100]; where OD = optical density.

FIGURE 1

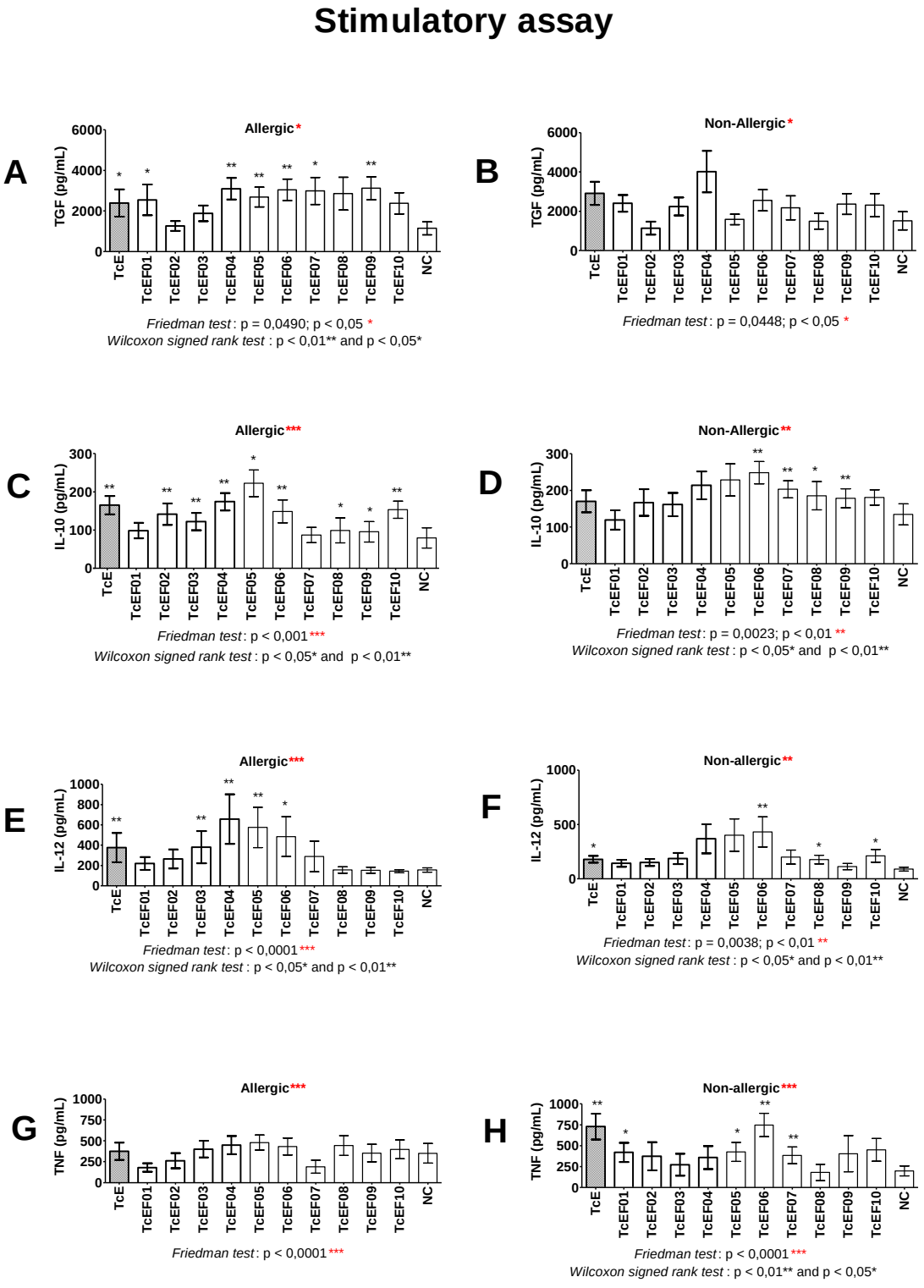

Stimulatory assay
A
Allergic*
TGF (pg/mL)
6000
4000
2000
0
TcE
TcEF01
TcEF02
TcEF03
TcEF04
TcEF05
TcEF06
TcEF07
TcEF08
TcEF09
TcEF10
NC
Friedman test: p = 0,0490; p < 0,05 *
Wilcoxon signed rank test : p < 0,01** and p < 0,05*
B
Non-Allergic*
TGF (pg/mL)
Friedman test: p = 0,0448; p < 0,05 *
C
Allergic***
IL-10 (pg/mL)
300
200
100
0
Friedman test: p < 0,001***
Wilcoxon signed rank test : p < 0,05* and p < 0,01**
D
Non-Allergic**
IL-10 (pg/mL)
Friedman test: p = 0,0023; p < 0,01 **
Wilcoxon signed rank test : p < 0,05* and p < 0,01**
E
Allergic***
IL-12 (pg/mL)
1000
800
600
400
200
0
Friedman test: p < 0,0001***
Wilcoxon signed rank test : p < 0,05* and p < 0,01**
F
Non-allergic**
IL-12 (pg/mL)
1000
500
0
Friedman test: p = 0,0038; p < 0,01 **
Wilcoxon signed rank test : p < 0,05* and p < 0,01**
G
Allergic***
TNF (pg/mL)
1000
750
500
250
0
Friedman test: p < 0,0001***
H
Non-allergic***
TNF (pg/mL)
Friedman test: p < 0,0001***
Wilcoxon signed rank test : p < 0,01** and p < 0,05*

FIGURE 2

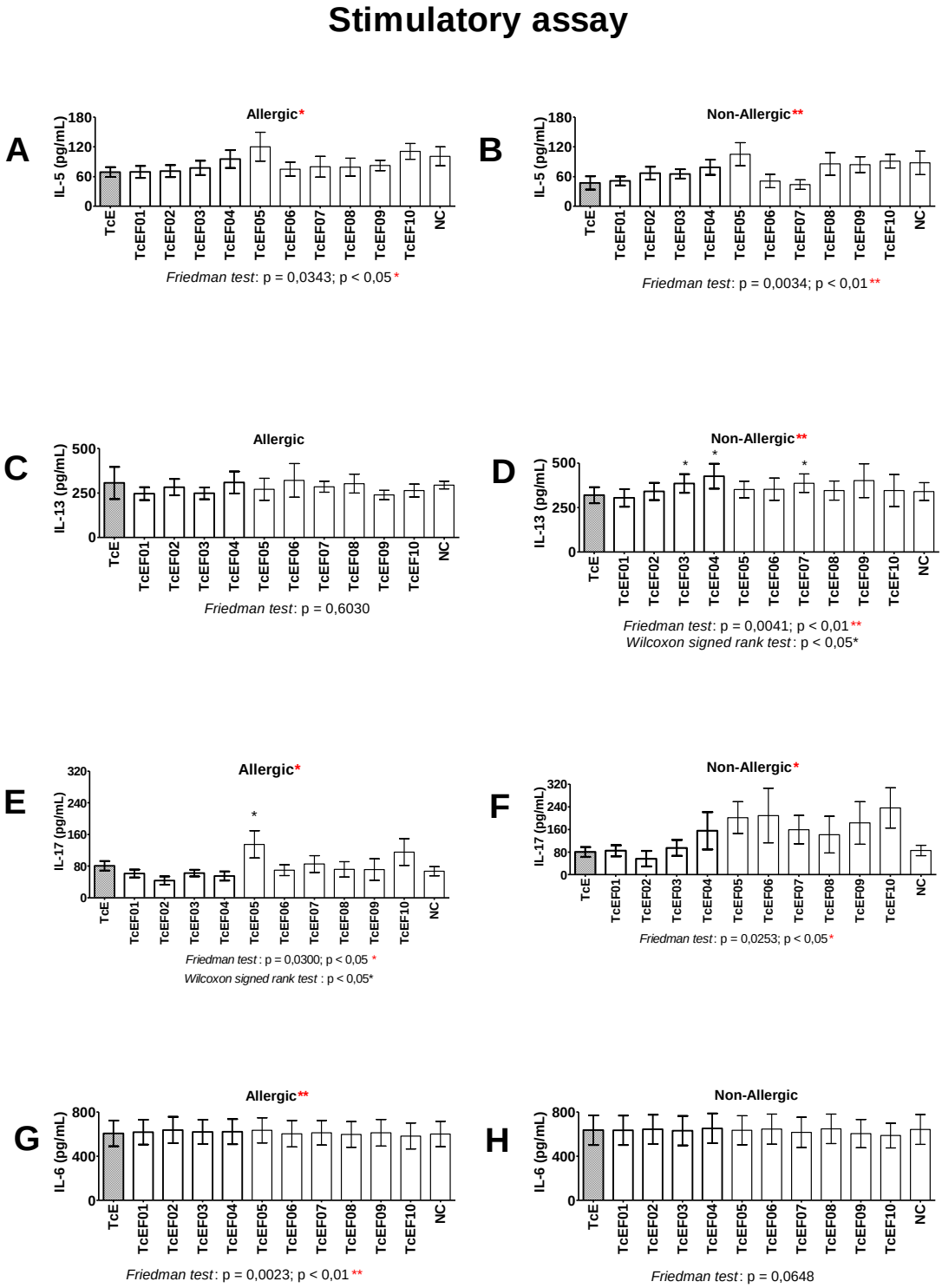

Stimulatory assay
A
Allergic*
IL-5 (pg/mL)
180
120
60
0
TcE
TcEF01
TcEF02
TcEF03
TcEF04
TcEF05
TcEF06
TcEF07
TcEF08
TcEF09
TcEF10
NC
Friedman test: p = 0,0343; p < 0,05*
B
Non-Allergic**
IL-5 (pg/mL)
Friedman test: p = 0,0034; p < 0,01**
C
Allergic
IL-13 (pg/mL)
500
250
0
Friedman test: p = 0,6030
D
Non-Allergic**
IL-13 (pg/mL)
Friedman test: p = 0,0041; p < 0,01**
Wilcoxon signed rank test: p < 0,05*
E
Allergic*
IL-17 (pg/mL)
320
240
160
80
0
Friedman test: p = 0,0300; p < 0,05 *
Wilcoxon signed rank test : p < 0,05*
F
Non-Allergic*
IL-17 (pg/mL)
Friedman test: p = 0,0253; p < 0,05*
G
Allergic**
IL-6 (pg/mL)
800
400
0
Friedman test: p = 0,0023; p < 0,01 **
H
Non-Allergic
IL-6 (pg/mL)
Friedman test: p = 0,0648

FIGURE 3

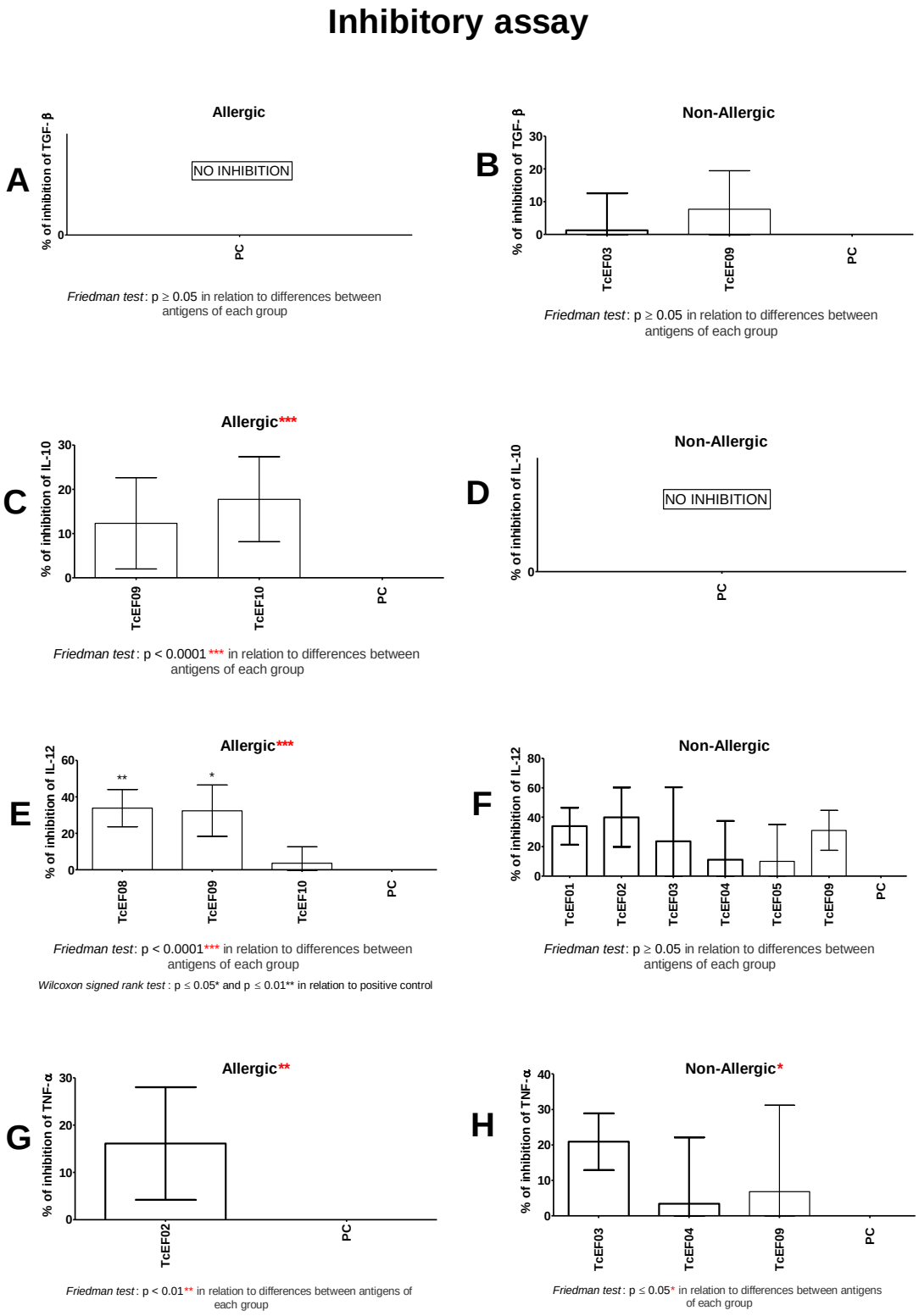
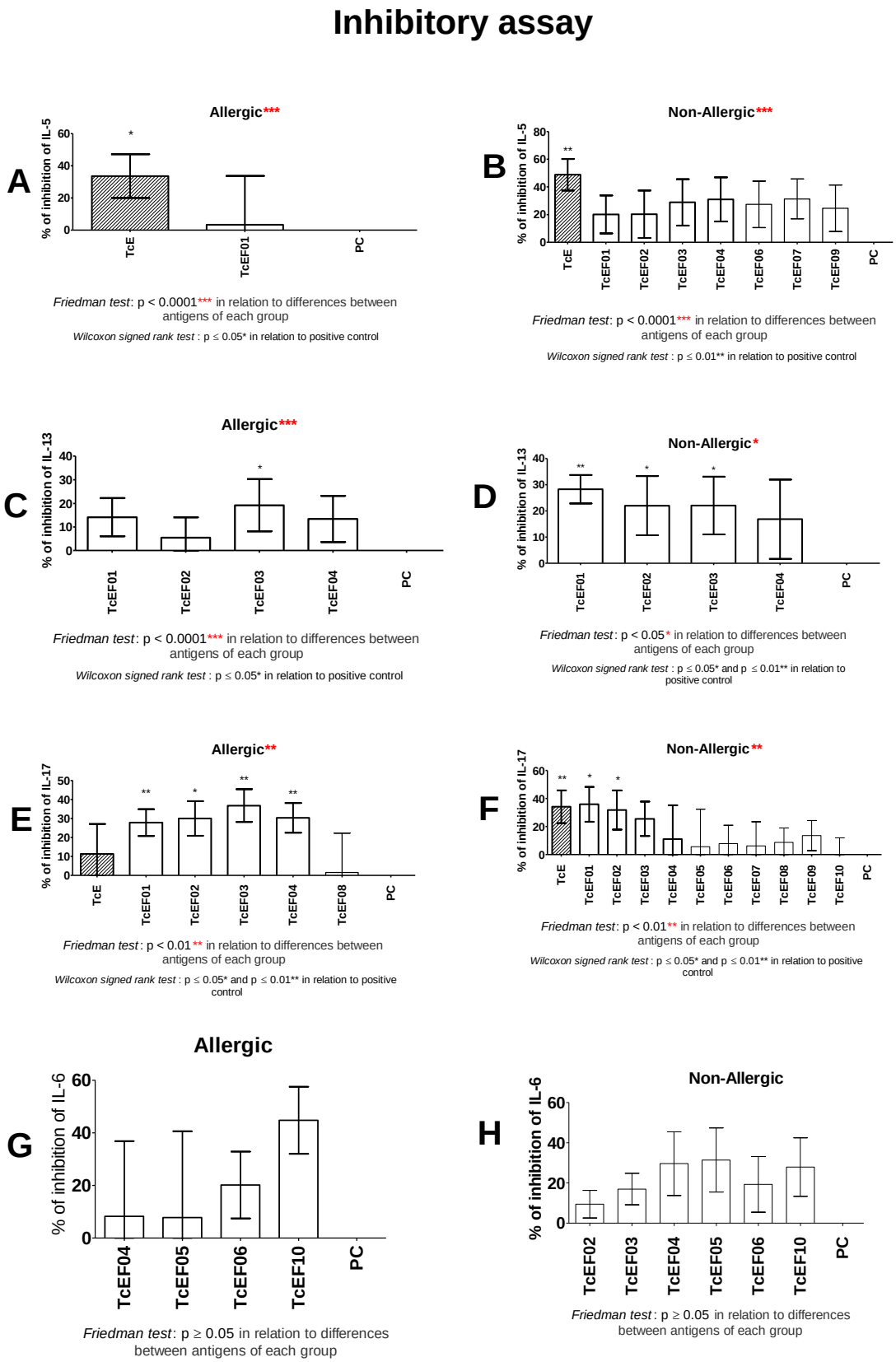

Inhibitory assay
A
Allergic
% of inhibition of TGF- β
NO INHIBITION
0
PC
Friedman test: p ≥ 0.05 in relation to differences between antigens of each group
B
Non-Allergic
% of inhibition of TGF- β
30
20
10
0
TcEF03
TcEF09
PC
Friedman test: p ≥ 0.05 in relation to differences between antigens of each group
C
Allergic***
% of inhibition of IL-10
30
20
10
0
TcEF09
TcEF10
PC
Friedman test: p < 0.0001*** in relation to differences between antigens of each group
D
Non-Allergic
% of inhibition of IL-10
NO INHIBITION
0
PC
E
Allergic***
% of inhibition of IL-12
60
40
20
0
**
*
TcEF08
TcEF09
TcEF10
PC
Friedman test: p < 0.0001*** in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05* and p ≤ 0.01** in relation to positive control
F
Non-Allergic
% of inhibition of IL-12
80
60
40
20
0
TcEF01
TcEF02
TcEF03
TcEF04
TcEF05
TcEF09
PC
Friedman test: p ≥ 0.05 in relation to differences between antigens of each group
G
Allergic**
% of inhibition of TNF- α
30
20
10
0
TcEF02
PC
Friedman test: p < 0.01** in relation to differences between antigens of each group
H
Non-Allergic*
% of inhibition of TNF- α
40
30
20
10
0
TcEF03
TcEF04
TcEF09
PC
Friedman test: p ≤ 0.05* in relation to differences between antigens of each group

FIGURE 4


Inhibitory assay
A
Allergic***
% of inhibition of IL-5
TcE
TcEF01
PC
Friedman test: p < 0.0001*** in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05* in relation to positive control
B
Non-Allergic***
% of inhibition of IL-5
TcE
TcEF01
TcEF02
TcEF03
TcEF04
TcEF06
TcEF07
TcEF09
PC
Friedman test: p < 0.0001*** in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.01** in relation to positive control
C
Allergic***
% of inhibition of IL-13
TcEF01
TcEF02
TcEF03
TcEF04
PC
Friedman test: p < 0.0001*** in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05* in relation to positive control
D
Non-Allergic*
% of inhibition of IL-13
TcEF01
TcEF02
TcEF03
TcEF04
PC
Friedman test: p < 0.05* in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05* and p ≤ 0.01** in relation to positive control
E
Allergic**
% of inhibition of IL-17
TcE
TcEF01
TcEF02
TcEF03
TcEF04
TcEF08
PC
Friedman test: p < 0.01** in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05* and p ≤ 0.01** in relation to positive control
F
Non-Allergic**
% of inhibition of IL-17
TcE
TcEF01
TcEF02
TcEF03
TcEF04
TcEF05
TcEF06
TcEF07
TcEF08
TcEF09
TcEF10
PC
Friedman test: p < 0.01** in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05* and p ≤ 0.01** in relation to positive control
G
Allergic
% of inhibition of IL-6
TcEF04
TcEF05
TcEF06
TcEF10
PC
Friedman test: p ≥ 0.05 in relation to differences between antigens of each group
H
Non-Allergic
% of inhibition of IL-6
TcEF02
TcEF03
TcEF04
TcEF05
TcEF06
TcEF10
PC
Friedman test: p ≥ 0.05 in relation to differences between antigens of each group

4.3 ARTIGO 3

TÍTULO:

Immune response of human peripheral blood mononuclear cells to excreted-secreted ***Toxocara canis*** *larval antígenos TES-native and recombinants*

Este **Manuscrito** será submetido ao Journal of Immunology Research – Fator de impacto = 3.064.

Neste estudo, foi investigado se antígenos excretados-secretados de larvas de *Toxocara canis* (TES) (nativo e proteínas recombinantes – rTES30E e rTES30P) poderiam provocar respostas imunes que poderiam inibir uma resposta Th2, em células mononucleares do sangue periférico (PBMC) de nove indivíduos alérgicos e de dez indivíduos não alérgicos. As PBMC foram cultivadas *in vitro* na presença ou ausência destes antígenos, e os sobrenadantes foram avaliados para a produção de citocinas (TGF-β, IL-10, IL-12, IFN-γ, IL-6, TNF, IL-5, IL13 e IL-17).

Os antígenos induziram a produção de citocinas regulatórias (TGF-β e / ou IL-10), acompanhada ou não pela estimulação da produção de citocinas associadas com a resposta do tipo Th1 (IL-12 e IFN-γ), mas sem estimulação de citocinas do tipo Th2 (IL -5 e a IL-13) e IL-17.

Immune response of human peripheral blood mononuclear cells to excreted-secreted *Toxocara canis* larval antígenos (TES) native and recombinants

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Abstract

In this study, it was investigated whether excreted-secreted *T. canis* larval antigens (TES) (native and recombinants – rTES30E and rTES30P) would elicit recall immune responses that could potentially inhibit a Th2 response, in nine allergic and ten non-allergic individuals' peripheral blood mononuclear cells (PBMC). PBMC were cultivated *in vitro* in the presence or absence of TES (natural and recombinants) at 37 °C and 5% CO2 during 48 and 120 hours and culture supernatants were evaluated for cytokine production (TGF-β, IL-10, IL-12, IFN-γ, TNF, IL-6, IL-5, IL-13 and IL-17). The TES induced Treg cytokines (TGF-β and/or IL-10) up-regulation from both allergic and non-allergic PBMC donors. We observed an *in vitro* down-modulation of Th2 cytokine by TES antigens (native and recombinants) and also Th17 (IL-17), in the inhibitory assays. The results indicate that TES could be used as adjuvants in personalized therapeutic vaccines in some individuals and also could justify carrying out investigations aiming at identifying others molecules in these products which might exclusively induce Treg and/or Th1 immune responses.

1. Introduction

The prevalence of allergic asthma and autoimmune diseases are increasing worldwide [1,2]. It is estimated that 300 million people worldwide have asthma, mainly children from industrialized countries and more recently in developing countries [3] and is estimated that 50 million Americans are affected by autoimmune disease [4]. This number can be linked to environmental changes associated with urbanization and the acquisition of a "modern" life style [3]. The hygiene hypothesis [5] links such environmental changes to an inverse association between allergy and exposure to viral, bacterial and helminth infections [6, 7]. The currently available treatments for allergic diseases are accompanied by undesirable side effects such as when using corticosteroids [8]. The development of immune modulatory protocols with beneficial effects on allergy and autoimmune diseases is therefore a priority.

The pathogenesis of allergic diseases is related to the involvement of CD4 + T cells (T helper) lymphocytes which play a central role as regulators and effectors of the immune response [9]. Abnormal activation of Th1 cells is considered critical event for most organ specific autoimmune diseases; and activation of aberrant Th2 responses involving cytokines in response to innocuous antigens, is responsible for deleterious effects in allergic inflammatory diseases such as asthma [10, 11]. Th2 cells are involved in both IgE-specific and eosinophilic responses in these pathological processes [12]. IgE antibodies and eosinophils, on the other hand, also participate in immunity against helminth infections, which are potent inducers of these antibodies and

cells, by inducing Th2 cytokines production (IL-4, IL-5 and IL-13) [12]. The control of any immune responses occurs mainly via different subtypes of regulatory and suppressor cells which play an important role in peripheral tolerance [13]. Regulatory T cells (Treg) are able to suppress immune responses mainly through cell-cell interactions and / or production of IL-10 and transforming factor beta (TGF- β) [14, 15].

The exposure to pathogens and their products, in particular helminths, appear to protect against the development of autoimmune and allergic diseases in experimental models [16, 17, 18, 19, 20, 21]. This protection has been explained by the ability of Treg, inhibiting hyper-stimulation of the Th2 response promoting a balance between Th1 and Th2 [22].

In this context, *Toxocara canis*, intestinal parasite of dogs [23] and *T. cati*, intestinal parasite of felines, [24] constitute important modulators of immune response in low-income populations in developing countries. Human disease is caused by larval stages invading musculature, brain and eyes, causing the well-characterized syndrome of visceral larva migrans [25], where the immune mechanisms appear to be ineffective at eliminating the infection [26]. The prevalence of such infection is high worldwide [27]. We have found that this infection occurs in 46-65 % of a low-income population in Salvador, a large city of Northeast Brazil [28, 29, 30] and also is negatively associated with skin reactivity to aeroallergens in the same population of children between 4 and 11 years old [31].

The excretory / secretory antigens of *T. canis* larvae (TES), obtained from the culture of larval *T. canis*, consists of five major molecules [32], all of which are

heavily glycosylated (i.e. are glycoproteins) and having molecular weights of 32, 55, 70, 120 and 400 kDa as determined by gel electrophoresis. There are, at least, 50 different macromolecules, mainly represented by a relatively simple set of glycoproteins, comprising three families of genes which express mucins and two C-type lectins [33, 34]. These proteins are glycosylated and highly immunogenic [33].

Unbalanced cytokines responses (i.e. Th2 vs. Th1 or pro-inflammatory vs. anti-inflammatory) are considered markers of various disease states such as asthma and atopy [35, 36] and immunotherapy is able to modify the natural course of the disease by altering the Th2/Th1 balance or stimulating regulatory T cells.

Although TES native are widely used in immune therapy, recombinant allergens have been produced with high purity in large quantities with similar IgE-binding capacity compared to their natural counterparts, or with modifications to reduce the reactivity to IgE (hypoallergens) [37]. In this way, the aim of this study was to evaluate the *in vitro* immune modulatory potential of the excreted-secreted *Toxocara canis* larval antigens (native TES and recombinants - rTES30E produced in *Escherichia coli* and rTES30P produced in *Pichia pastoris*) on human peripheral blood monocytes cultures (PBMCs) from allergic and non-allergic donors.

2. Material and Methods

2.1 - Blood donors

Blood donors, aged between 21 and 40 years, signed an informed consent to

participate in the present research. They were classified into allergic (n = 09) and non-allergic (healthy) (n = 10) donors, based in the history of allergic symptoms, the reactivity of skin prick tests using extracts from six common regional allergens (*Blomia tropicalis, Dermatophagoides pteronyssinus, Blattella germanica and Periplaneta americana*, cat and dog epithelia; Alergolatina, Rio de Janeiro, Brazil) and the presence of specific IgE to *Blomia tropicalis, Dermatophagoides pteronyssinus, Blattella germanica and Periplaneta americana* (using ImmunoCAP assay (Phadia/Thermo Diagnostics AB, Uppsala, Sweden) in serum. The research was approved by the Ethical Committee of the Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Proposal 179/2008, Project no. 277.

2.2 - Preparation of TES – native and TES-recombinant antigens

The parasites were obtained by collaboration with Dr. Philip J Cooper of the *Fundacion Ecuatoriana para Investigacion en Salud* (Ecuador).

The *T. canis* larvae antigen (TES) were obtained according to De Savigny [38], modified by Alcântara-Neves et al [39]. Briefly, female *T. canis* were subjected to removal the uterus, eggs were incubated in 2% formalin solution to become fertilized (approximately 28 days). The membranes were disrupted by incubation with 5% sodium hypochlorite and the released larvae, in conjunction with vigorous agitation with glass beads. The larvae were cultured in RPMI 1640 medium supplemented with penicillin (1.000 U/ml), streptomycin (1mg/ml), amphotericin (2.5 µg/mL) and gentamicin (0,2 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) and kept in an incubator with an atmosphere containing 5% CO2 and

temperature at 37 °C for obtaining TES for six months, with medium changes every 120 hours. Weekly, the supernatants were collected, added protease inhibitor fenilmetilsulfonamida fluoride (PMSF, Sigma, St. Louis, MO, USA) and stored at -70ºC until further use. By the time of the experiments, the protein content was concentrated by ultrafiltration using an Amicon filter (Millipore Corporate, MA, USA) at 4 °C. Afterwards, the material was dialyzed against phosphate buffered saline (PBS), pH 7.4.

Recombinant proteins rTES30E and rTES30P were obtained with Dr. Fabricio Rochedo Conceição and Dr. Paula de Lima Telmo of the Universidade Federal de Pelotas (Brazil). The *T. canis* TES30 antigen was produced in two systems of heterologous protein expression, a prokaryote, using *Escherichia coli* (rTES30E) and an eukaryote, using the methylotrophic yeast *Pichia pastoris* (rTES30P).

Briefly, the synthetic gene TES30 designed from the sequence of GenBank AB009305 was synthesized by Epoch Biolabs Company, Inc. (USA) containing *P. pastoris* preferred codons (codon usage), and restriction enzyme sites for cloning into the expression vector in *E. coli* pAE or expression vector in *P. pastoris* pPICZαB (Invitrogen) [40].

The protein concentration was measured using the Folin reagent (Bio-Rad Laboratories, Richmond, CA, USA) by the method of Lowry et al [41] and stored at -70 °C. Yielding 620 μg / mL for TES-native; 233 μg / mL for rTES30E and 355 μg / mL for rTES30P.

PBMC stimulations were performed using 50 μg/mL of all three TES antigen, from the use of a curve presenting optimal concentration to stimulate PBMC

using these antigens (Data not shown).

2.3 - Stimulatory and inhibitory assays

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by centrifugation on HISTOPAQUE 1077® solution (Sigma-Aldrich, St. Louis, MO, USA). The cells (1 x10^6/mL) were cultivated in 96-well plates (200 µl/well) and stimulated with 50 µg/mL of TES 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)

For stimulatory assays, as positive control, PBMC were stimulated with 8 UE/mL of lipopolysacharide (LPS from *Escherichia coli*) and we measured IL-10, TGF-β, IL-12, IL-6, TNF and IFN-γ production. Also, we stimulated PBMC with 10 µg/ml of phytohaemagglutinin (PHA) when IL-5, IL-13 and IL-17 were evaluated.

Both whole extract and recombinant TES were co-cultured with 20 µg/mL polymyxin B (Sigma-Aldrich, St. Louis, MO, USA) as an endotoxin neutralizing agent, as previously described [42].

In order to assess the inhibitory effect of TES in PBMC, both TES were incubated with suboptimal concentrations of LPS (4 UE/mL) and IL-10, TGF-β, TNF and IFN-γ were measured; to assess IL-12 production, in addition to the LPS (4 UE/mL), 100 ng/mL of IFN-γ was added to the culture; PHA at 5 µg/mL was used and IL-5, IL-13 and IL-17 were evaluated.

Unstimulated PBMC were used as negative control in both assays.

In stimulatory and inhibitory assays, cells were incubated at 37 °C, 5% CO2 during 48 hours [for quantification of IL-10, tumor necrosis factor (TNF), IL-12, IFN-γ and transforming growth factor (TGF-)β or for 120 hours [for quantification of IL-4, IL-5, IL-17 and IL-13]. After the incubation period, the supernatants were collected and stored at -70 °C for later quantification of cytokines.

We evaluated the cytotoxic effect of the concentrations used in both stimulatory and inhibitory assays using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA), according to Mosmann et al [43]. No significant differences were detected in cell viability of PBMC at the concentrations used (data not shown).

2.4 - Cytokines measurements

The cytokines concentrations (TGF-β, IL-10, IL-12, IFN-γ, TNF, IL-5 IL13, IL-17 and IL-6) in PMBC 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). And the percentage (%) of inhibition of cytokines 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.

The cytokines ratios were calculated to examine the Th1 vs. Th2 balance and to examine the generation of a regulatory profile, using the following:

(a) [(TGF-β, IL-10, IL-12, TNF, IL-5, IL-13, IL-17 or IL-6) / IFN-γ];

(b) [(TGF-β, IL-12, IFN-γ, TNF, IL-5, IL-13, IL-17 or IL-6) / IL-10];

(c) [(IL-10; IL-12, IFN-γ, TNF, IL-5, IL-13, IL-17 or IL-6) / TGF-β].

2.5 - Statistical analysis

Statistical analysis was conducted in Graphpad Prism 6 software. Data normality was assessed by the Kolmogorov-Smirnov test. Kruskal-Wallis analysis was performed to compare the cytokines production by PBMC from allergic and non-allergic volunteers. Differences in cytokines production between groups of antigens were determined using Friedman's test, followed by Wilcoxon signed rank test (paired by individuals) to evaluate differences between the stimulation (or inhibition) by TES and non-stimulated (non-inhibited) cultures; $p \leq 0.05$ was considered statistically significant.

3. Results

3.1 Immune modulatory profile of TES-native in PBMC

The immune modulatory effect of excretory / secretory larvae antigen of *T. canis* (TES) was evaluated using three different human culture systems: a) peripheral blood mononuclear cells (PBMC); b) whole blood cultures and; c) monocytes-macrophages cultures.

We found in cultures stimulated with the TES, had a significant increase of IL-10 production ($p \leq 0.05$) on PBMC, and no stimulation of IL-4 production on whole blood cultures and PBMC cultures. In addition, there was a significant production of IFN-γ ($p \leq 0.05$) on whole blood and PBMC stimulated by TES **(Figure 1).**

Whereas cultures of peripheral blood mononuclear cells reflect a larger extent

as the dosage of cytokine in their supernatants, this culture was chosen for further experiments in the stimulatory and inhibitory over the implementation of cultures of whole blood and macrophages. IL-4 is a cytokine labile and usually with low-dose *in vitro* assays, we chose to analyze IL-5 and IL-13 as Th2 cytokine profile.

3.2 Cytokine production by TES (native and recombinants) in stimulated PBMC

Both TES (native and recombinants) tested induced the production of regulatory cytokines, TGF-β and IL-10 in PBMC from allergic and non-allergic donors, in the stimulatory assays being higher in allergic donors in relation to non-allergic ones ($p \leq 0.01$). On the other hand, inhibitory assays led to a down-modulation of IL-10 production in both allergic and non-allergic donors (except for cells stimulated with TES native in non-allergic individuals) and also there was impairment in the production of TGF-β in both donors (**Figs 2A-B and 4A-B; Table 1**).

Although the IL-13 cytokine is up-regulated in PBMC from both donors (allergic and non-allergic), TES stimulation induce inhibition of IL-5 production in the inhibitory assays (**Figs. 3D-E and 5D-E; Table 1**).

Comparing the data obtained with IL-17 between allergic and non-allergenic donors, it is interesting that the production of this cytokine was statistically significantly only for the non-allergic ($p \leq 0.05$ and $p \leq 0.01$) (**Figure 2C**), whereas in the inhibitory assays in both groups, this production was significantly inhibited (**Figure 4C; Table 1**).

On stimulatory assays, it was observed an increased significant production of IL-12, IFN-γ and TNF, with all TES antigens ($p \leq 0.05$ and $p \leq 0.01$), in PBMC from both donors, except to IFN-γ, in PBMC of non-allergic donors stimulated with rTES30P antigen (**Figure 3A-C**). There was in this inhibition of production of these cytokines in the inhibitory assays (except for IL-12 and TNF in PBMC from non-allergic donors and stimulated with rTES30E with rTES30P) (**Figure 5A-C; Table 1**).

Synthesizing, on stimulatory assay, the addition of TES antigens (native and recombinants), increased the production of TGF-β, IL-10, IL-6, IL-12, IFN-γ, TNF and IL-17 by healthy individuals' PBMC ($p \leq 0.05$) (except IL-10 and IFN-γ for rTES30P). The production of TGF-β, IL-10, IL-6, IL-12, IFN-γ and TNF was also up-regulated on PBMC from allergic donors ($p \leq 0.05$) (**Figures 2A-D and 3A-E**). The addition of TES antigens in the inhibitory assay, significantly inhibited the production of IL-13 and IL-17 by healthy individuals' PBMC ($p \leq 0.05$) (**Figures 5D-E; Table 1**). The addition of TES native in allergic donors PBMC significantly inhibited the production of IL-5, IL-13 and IL-17; rTES30E significantly inhibited the production of IL-5, IL-10, TNF and IL-17; whereas rTES30P significantly inhibited the production of IL-5, IL-10, IL-13 and IL-17 ($p \leq 0.05$) (**Figs.3B-C and 5C-E; Table 1**).

The production of IL-6 was not inhibited from stimulation of PBMC with the three antigens (**Figure 4D**), and proved their production with statistical significance in PBMC from both donors stimulated with TES-native (**Figure 2D; Table 1**).

Between allergic and non-allergic, on the stimulatory tests, TGF-β, IL-10, IL-12, IFN-γ and TNF were up-regulated in allergic group. Highlighting the increased

production of IFN-g in PBMC stimulated with recombinant antigens allergic compared to non-allergic (**Figure 3B; Table 1**).

3.3 Cytokines ratios profile induced by TES antigens-stimulated PBMC

Induction of Th1 and/or Treg cells by TES stimulation would indicate that such molecules might be effective on allergy immunotherapy. After stimulation of PBMC from allergic donors with TES antigens, it was observed an up-regulation of TGF-β / IFN-γ ratio without increase (IL-5, IL-13 and IL-17) / (IFN or IL-10) ratios for TES antigens suggesting a shift of Th2 cells to Th1 cells (**Figures 6-8**).

The production of TGF-β was superior to all other cytokines studied in PBMC from both donors, but mainly on allergic donor cells stimulated with recombinant antigens of TES (rTES30E and rTES30P) (**Figure 6**).

Comparing the cytokine ratios in the PBMC from allergic and non-allergic donors stimulated with TES-antigen, we observed a best down-modulation of Th2 cells from allergic donors (**Figures 6-8**). Regarding IL-10, a higher production was found in this respect to the Th2 cytokine and IL-17 (**Figure 7**).

Data concerning the Th1 can be seen the results obtained from the (Th2 and IL-17 cytokines) / (IFN-γ) ratio, where this profile was the best seen in PBMCs from allergic donors stimulated mainly by the recombinant rTES30P (**Figure 8**).

Greater production of IL-6 on ratio with IFN-γ for PBMC stimulated with the three antigens, was not accompanied by increased production of IL-17 in individuals analyzed in both groups (allergic and non-allergic) (**Figure 8**). The (IL-6) / (TGF-β) ratio visualized higher production of regulatory cytokine (Figure

6). PBMC stimulated with antigen rTES30E and TES-native produced more IL-6 in the ratio with IL-10 than seen in cells stimulated with rTES30P but were not accompanied with significant increase of IL-17 by these donors (**Figure 7**).

4. Discussion

TES antigen is obtained from the culture of *T. canis* larvae [39] and the 30 kDa protein fraction, is one of the most abundant larval surface antigen [44, 45]. TES-30 is a recombinant protein from *T. canis* second-stage larvae, corresponding to the 30 kDa antigen from TES and, with TES-32 [C-type lectins 1 – (CTL-1)] is one of the most prominent larval proteins, present not only as a major TES constituent [46], but also in the cuticular matrix of the larva [44]. The 219-amino acid sequence shows a carbohydrate-recognition domain with similarity to mammalian CTLs, and recombinant TES-32 has been shown to bind to monosaccharides (mannose or galactose) in a calcium-dependent manner [47]. Sequence analysis of cDNA revealed an open reading frame encoding a ~24 kDa protein with similarity to regions corresponding to epidermal growth factor-like and lectin-like domains of the core proteins of vertebrate chondroitin sulfate proteoglycans [45].

C-type lectins (CTLs) are known to be central players in pathogen recognition and cellular activation by the mammalian innate immune system [48]. The preponderance of active lectins, and the likelihood of additional lectins being present in the ‘secretome’ of *T. canis*, highlights the critical role of glyco-recognition in the immune response to this parasite [49].

Our initial experiments showed that the native TES increased the production of IL-10 in PBMC, whole blood and human macrophages culture *in vitro* without increasing IL-4 in PBMC culture and whole blood, indicating the potential value of this antigen as an immunomodulator of allergy and/or autoimmune diseases. Some researchers indicated that infection with *Toxocara canis* in dogs and rats increased the production of IL-10 by PBMCs *in vitro* [50, 51].

Increased IL-10 appears to be an important strategy for the treatment immune-mediated diseases such as allergies. It is a potent inhibitor of monocyte/macrophage function and also suppressing the production of many pro-inflammatory cytokines [52]. Importantly, successful allergen-specific immunotherapy in humans- which leads to a reduction in allergic symptoms—is associated with the emergence of IL-10–producing Treg cells [53].

We observed that both antigens used are able to significantly induce the production of regulatory cytokines (IL-10 and TGF-β) and Th1 cytokines (IL-12 and IFN-γ), both in PBMC from allergic donors as well as in PBMC from non-allergic donors, on stimulatory tests.

The production of Th1 cytokines, such as IFN-γ by PBMCs from atopic asthmatic patients can translate a possible mechanism of regulating allergic disease (decreasing production of IgE, eosinophils and Th2 cytokines) [54]. IL-12 is produced by B cells, macrophages and dendritic cells, and primarily regulates Th1 cell differentiation, while suppressing the expansion of Th2 cell clones.

In our study, low production and significant inhibition of the production of Th2 (IL-5 and IL-13) and IL-17 cytokines in allergic donors occurred, mainly for

allergic donor PBMC stimulated by recombinants antigens (rTES30P and/or rTES30E).

IL-10 suppresses the activity and function of eosinophils, as well as the production of IL-5 by Th2 lymphocytes [55]. Several studies have demonstrated the essential role of IL-5 and IL-13 in the development of airway hyperresponsiveness (AHR) and airway eosinophilia [56, 57]. In contrast, Th1 cytokines such as IL-12 and IFN-γ have been shown to down-regulate allergic responses *in vivo*, are potent negative regulators of Th2 responses [58, 59].

It was found differences on IL-13 and IL-5 production between the groups being lower in PBMC from allergic donors stimulated by TES antigens. On the inhibitory test, in both groups we observed a down-regulation of both cytokines. Modulation of both cytokines IL-13 and IL-5 is an important strategy for allergy immunotherapy.

No significant production of IL-17 was observed in our study on PBMC in allergic patients; highlighting the fact that in the inhibitory assays, inhibition of this production have shown statistical significance after stimulation of PBMC with TES antigen and recombinants.

In this study, cytokines ratio was calculated to examine the Th1 vs. Th2 balance of cytokines and the generation of Treg profile; no increase of Th2 cytokines in PBMC stimulated with TES antigens *in vitro* was observed. The rTES30P and rTES30E recombinants appear to induce a good immune response with production of Treg and / or Th1 response, indicating that these proteins may be used in anti-allergy therapies. Furthermore, by separately analyzing the individuals TES-native seems to stimulate further production of Th2 and IL-17 in

some than in others donors studied in relation to PBMC stimulated with recombinants. This may be due to the fact that TS-native compound is a wide range of molecular weight and the recombinant antigenic be a single molecule with a mass of 30 kDa. The recombinant antigens (unlike native TES antigen) are single or homogenous molecules [60].

Another issue may be related to the fact that, in contrast to the TES-native, highly glycosylated, recombinant produced in bacteria (rTES30E) is not glycosylated. The absence of glycosylation avoids, or at least decreases, the cross-reactivity with antibodies that recognize the sugar moieties of the TES antigens produced by *Toxocara canis* larvae [61].

An IL5 / IFN-γ increased ratio is a feature of allergic diseases. It was observed a reduction on (IL5, IL-13, IL-17) / IFN-γ ratio indicating a decrease of allergy-inducing Th2 immune responses, suggesting an immune deviation from Th2 to Th1 response. One of the primary mechanisms for the success of immunotherapy is the alteration of T cell function with reduced production of Th2 and / or cytokines for inducing "immune deviation" a pattern of Th2 cytokine for Th1 [62].

Induction of immune tolerance in peripheral T cells is another key step for immunotherapy. This tolerance is characterized mainly by the production of allergen-specific regulatory T lymphocytes (Treg) and the suppression of the Th2 response induced by allergen. The balance between Th2 and Treg may lead to the development of allergy or not [63]. Increased production of IL-10 and TGF-β was observed in our study.

There was no increased production of IL-6 to the detriment of decreased production of TGF-β and / or IL-17. Although the reason with IFN-γ, IL-6 have been shown in higher production in most subjects analyzed. Their study is made interesting by virtue of the provisions in the literature. The increased production of IL-6 and decreased production of IFN-γ of cord blood mononuclear cells appear to be the hallmark of newborns from the highrisk population of allergy [64]. In particular, IL-6 controls the balance between regulatory T cells and Th17 cells. Importantly, recent findings suggest that blockade of IL-6 signaling is effective in treating experimental models of autoimmune and chronic inflammatory diseases such as inflammatory bowel diseases, diabetes, multiple sclerosis, asthma and rheumatoid arthritis as well as models of inflammation-associated cancer [65].

Mice deficient in IL-17 are resistant to collagen-induced arthritis [66]. The development of the IL-17-producing T cells, Th17, was initially shown to depend on the presence, during antigen stimulation, of IL-23 produced by the antigen-presenting cells [67 – 70]. Although IL-23 has a key role in Th17-mediated inflammation *in vivo* [71], recent studies have demonstrated that the in vitro polarization of naive CD4+ T cells toward the Th17 lineage requires a combination of T cell antigen receptor stimulation and the cytokines transforming growth factor β (TGF-β) and IL-6, but that it is independent of IL-23 [72, 73]. Additionally, it was previously reported that in vivo polarization of naive T cells toward the Th17 lineage requires IL-6 and TGF-β [72, 73]. Importantly, it is reported that IL-6 signaling suppressed in vitro regulatory T cell (Treg) development, suggesting reciprocal development pathways for the generation

of Th17 and Tregs [72]. However, it has not been shown whether these reciprocal development pathways also exist in in vivo situation [74].

For the use of these antigens in antiallergic therapy, it is suggested previously, a greater number of PBMC from individuals and from different geographical areas are tested to determine which one (s) products can be used as candidates for this purpose. In this work, although using a relatively small sample of individuals, clearly shows the production of regulatory cytokines and / or Th1 (IL-12 and/or IFN-γ) associated, without stimulating the production of Th2 cytokines (IL-5 e IL-13) typical immune response on allergy and of IL-17 in PBMC of some individuals allergic stimulated with TES-native and / or TES-recombinant (antigens of *T. canis*).

More recently, the use of recombinant allergens from birch pollen (rBet v 1) and from grass pollen (a cocktail of five allergens) in immunotherapy has been reported to be safe and effective, with results comparable to those obtained with natural extracts in patients with rhino-conjunctivitis due to pollen allergy [75, 76], showed strong modulation of the responses of T and B lymphocytes to specific allergens [77, 78].

The ability in producing essentially unlimited quantities of recombinant allergens, the possibility of using higher doses of these hypoallergens and immune tolerance induction by T-cells without the risk of anaphylaxis and other adverse reactions, also allows us to consider the investigation of the use of prophylactic vaccination on disease allergic [79, 80]. Therefore, it is possible to predict the occurrence of a continuous incorporation of recombinant allergens for immunotherapy studies, which should lead to more effective vaccines for

allergic diseases and established in the long term, with the prospect of prophylactic immunization [81, 82].

5. Conclusions

The identification of molecules from helminths induces immunosuppression and the elucidation of the mechanisms involved is essential for the development of alternative strategies for the prevention and / or treatment of inflammatory diseases. The manipulation of inducible regulatory pathway can be therapeutically effective and beneficial for the treatment of allergic airway disease.

Attention can be given to anti-allergic immunotherapy using recombinant antigens, which may therefore be manipulated producing hybrid molecules with anti-allergic action or adjuvant action.

Finally, we focus on the benefit of these new perspectives for the future development of innovative therapies.

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Table 1 - Statistical significance (* or **) of variations in cytokine production by TES stimulated PBMC.

Produced cytokine	Peripheral blood mononuclear cells	Comparison					
		rTES30E-stimulated versus non-stimulated cultures		rTES30P-stimulated versus non-stimulated cultures		TES-native-stimulated versus non-stimulated cultures	
		Induction	Inhibition	Induction	Inhibition	Induction	Inhibition
TGF-β	Allergic patients	$p \leq 0.0100$	NS	$p \leq 0.0100$	NS	$p \leq 0.0100$	$p \leq 0.0500$
	Healthy individuals	NS	NS	NS	NS	NS	NS
IL-10	Allergic patients	$p \leq 0.0100$	$p \leq 0.0500$	$p \leq 0.0100$	$p \leq 0.0500$	$p \leq 0.0100$	NS
	Healthy individuals	$p \leq 0.0100$	NS	$p \leq 0.0100$	NS	$p \leq 0.0100$	NS
IL-12	Allergic patients	$p \leq 0.0100$	NS	$p \leq 0.0500$	NS	$p \leq 0.0500$	NS
	Healthy individuals	$p \leq 0.0100$	NS	$p \leq 0.0500$	NS	$p \leq 0.0100$	NS
IFN-γ	Allergic patients	$p \leq 0.0100$	NS	$p \leq 0.0100$	NS	$p \leq 0.0100$	NS
	Healthy individuals	$p \leq 0.0100$	NS	NS	NS	$p \leq 0.0100$	NS
IL-6	Allergic patients	NS	NS	NS	NS	$p \leq 0.0100$	NS
	Healthy individuals	NS	NS	NS	NS	$p \leq 0.0100$	NS
TNF	Allergic patients	$p \leq 0.0001$	$p \leq 0.0500$	$p \leq 0.0001$	NS	$p \leq 0.0001$	$p \leq 0.0500$
	Healthy individuals	$p \leq 0.0500$	$p \leq 0.0500$	$p \leq 0.0500$	NS	$p \leq 0.0100$	NS
IL-5	Allergic patients	NS	$p \leq 0.0100$	NS	$p \leq 0.0500$	NS	$p \leq 0.0100$
	Healthy individuals	NS	NS	NS	NS	NS	NS-
IL-13	Allergic patients	NS	NS	NS	$p \leq 0.0100$	NS	$p \leq 0.0500$
	Healthy individuals	NS	$p \leq 0.0100$	NS	$p \leq 0.0100$	NS	$p \leq 0.0100$
IL-17	Allergic patients	NS	$p \leq 0.0100$	NS	$p \leq 0.0100$	NS	$p \leq 0.0500$
	Healthy individuals	$p \leq 0.0100$	$p \leq 0.0100$	$p \leq 0.0100$	NS	$p \leq 0.0500$	$p \leq 0.0500$

*$p \leq 0{,}05$ and **$p \leq 0{,}01$ = statistical significance of cytokines production by non-allergic (healthy) or allergic donor, assessed by Friedman's non-parametric test followed by Wilcoxon signed rank test: rTES30E and/or rTES30P and/or TES-native stimulated versus non-stimulated with LPS (induction) or with phytohemoagglutinin (inhibition).
NS = no statistical significance.

LEGEND TO FIGURES

Figure 1 - Stimulatory effects on cytokine production. Peripheral blood mononuclear cells (PBMC), whole blood culture (WBC) and macrophage culture (MC), were cultured in the presence of 1000 µg/m of TES native; the results were expressed as the increase in cytokine concentrations in pg/mL plus the standard error of the mean, comparing antigen stimulated and non-stimulated cells. *p ≤ 0·05 in relation to negative control (non-stimulated cells) of each culture type using Wilcoxon signed rank test). Closed symbols, TES antigens-stimulated cultures; open symbols, non-stimulated cultures

Figure 2 - Stimulatory effects on cytokine production [TGF-β, IL-10 (B), IL-17 (C) and IL-6 (D)] in cultures of allergic and non-allergic individual's peripheral blood mononuclear cell stimulated with either TES antigens. The TES antigens were added to a final concentration of 50 µg/mL to the cells, which were incubated as described in the Material and Methods. PBMCs were cultured for 48 hours or 120 hours either in the absence (CN) or in the presence of the antigen indicated at the X axis. Columns represent the median with interquartile range values of 19 different PBMC cultures (allergic and non-allergic, respectively). The differences in cytokines production into groups were determined using Friedman's test, followed by Wilcoxon signed rank test (paired by individuals) to evaluate differences between the stimulation by each antigen and non-stimulated cultures - *p ≤ 0.05 or **p ≤ 0.01 or ***p ≤ 0.001.

The results obtained from the same donors' PBMC are represented by identical symbols in figures 2-5.

Figure 3 - Stimulatory effects on cytokine production [IL-12 (A), IFN-γ (B), TNF (C), IL-5 (D) and IL-13 (E)] in cultures of allergic and non-allergic individual's peripheral blood mononuclear cell stimulated with either TES antigens. The TES antigens were added to a final concentration of 50 μg/mL to the cells, which were incubated as described in the Material and Methods. PBMCs were cultured for 48 hours or 120 hours either in the absence (CN) or in the presence of the antigen indicated at the X axis. Columns represent the median with interquartile range values of 19 different PBMC cultures (allergic and non-allergic, respectively). The differences in cytokines production into groups were determined using Friedman's test, followed by Wilcoxon signed rank test (paired by individuals) to evaluate differences between the stimulation by each antigen and non-stimulated cultures - *$p \leq 0.05$ or **$p \leq 0.01$ or ***$p \leq 0.001$. The results obtained from the same donors' PBMC are represented by identical symbols in figures 2-5.

Figure 4 - Inhibitory effects on cytokine production [TGF-β, IL-10 (B), IL-17 (C) and IL-6 (D)] in cultures of allergic and non-allergic individuals peripheral blood mononuclear cell stimulated with either TES antigens. The TES antigens were added to a final concentration of 50 μg/mL to the cells, which were incubated as described in the Material and Methods. PBMCs were cultured for 48 or 120 hours either in the presence of fifty μg/mL of the antigens

as indicated at the X axis in cultures with PHA and IFN-γ. As positive controls the mitogens and IFN-γ were added alone as described in the Material and Methods section. Columns represent the median with interquartile range values of ten and nine different PBMC cultures (allergic and non-allergic, respectively), and vertical bars represent the standard deviations of the means. The differences in cytokines production into groups were determined using Friedman's test, followed by Wilcoxon signed rank test (paired by individuals) to evaluate differences between the stimulation by each antigen and non-stimulated cultures - *$p \leq 0.05$ or **$p \leq 0.01$ or ***$p \leq 0.001$. 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. The results obtained from the same donors' PBMC are represented by identical symbols in figures 2-5.

Figure 5 - Inhibitory effects on cytokine production [IL-12 (A), IFN-γ (B), TNF (C), IL-5 (D) and IL-13 (E)] in cultures of allergic and non-allergic individuals peripheral blood mononuclear cell stimulated with either TES antigens. The TES antigens were added to a final concentration of 50 μg/mL to the cells, which were incubated as described in the Material and Methods. PBMCs were cultured for 48 or 120 hours either in the presence of fifty μg/mL of the antigens as indicated at the X axis in cultures with PHA and IFN-γ. As positive controls the mitogens and IFN-γ were added alone as described in the Material and Methods section. Columns represent the median with interquartile range values of ten and nine different PBMC cultures (allergic and non-allergic,

respectively), and vertical bars represent the standard deviations of the means. The differences in cytokines production into groups were determined using Friedman's test, followed by Wilcoxon signed rank test (paired by individuals) to evaluate differences between the stimulation by each antigen and non-stimulated cultures - *p ≤ 0.05 or **p ≤ 0.01 or ***p ≤ 0.001. 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. The results obtained from the same donors' PBMC are represented by identical symbols in figures 2-5.

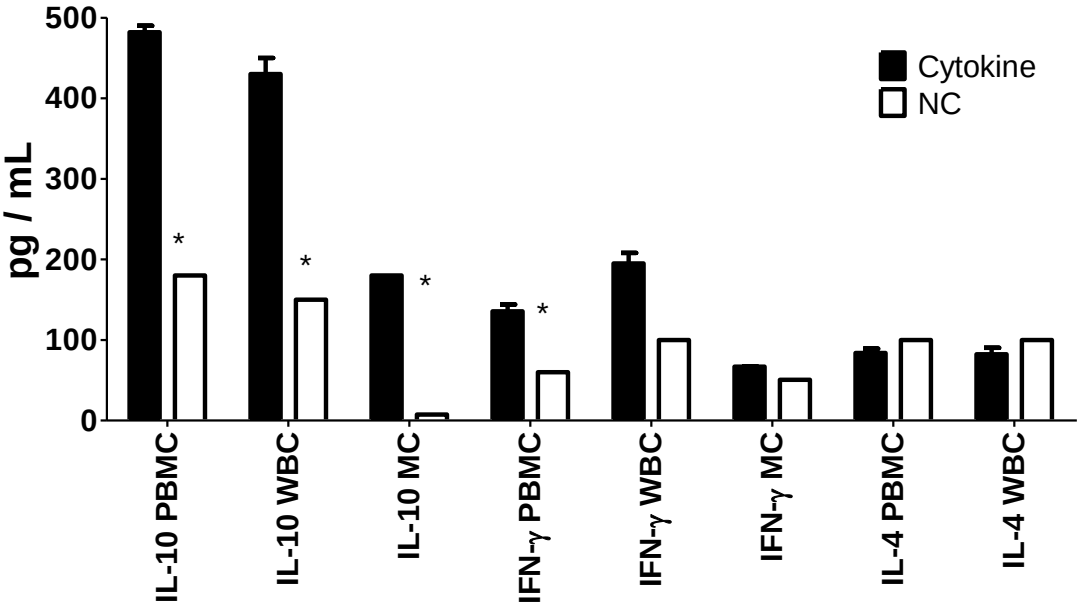
Figure 6 - Ratios of cytokine [(IL-10, IL-12, IFN-γ, TNF, IL-5, IL-13, IL-17 or IL-6) / (TGF-β] concentration in non-allergic (A, B, C) vs. allergic donors (D, E, F) PBMC stimulated with TES antigens (TES- native, rTES30E and rTES30P). The cytokine measurements were as described in the Material and Methods. The amounts of cytokines detected in blank of cultures were subtracted from the amounts detected in the stimulated cultures. Each symbol shows the results obtained with the peripheral blood mononuclear cells from an individual donor. The legends above the graphs identify the individual PBMC donors (NAl = non-allergic donor; Al = allergic donor). The results obtained from the same donors' PBMC are represented by identical symbols in figures 6-8.

Figure 7 - Ratios of cytokine [(TGF-β, IL-12, IFN-γ, TNF, IL-5, IL-13, IL-17 or IL-6) / (IL-10)] concentration in non-allergic (A, B, C) vs. allergic donors (D, E, F) PBMC stimulated with TES antigens (TES- native, rTES30E and

rTES30P). The cytokine measurements were as described in the Material and Methods. The amounts of cytokines detected in blank of cultures were subtracted from the amounts detected in the stimulated cultures. Each symbol shows the results obtained with the peripheral blood mononuclear cells from an individual donor. The legends above the graphs identify the individual PBMC donors (NAl = non-allergic donor; Al = allergic donor). The results obtained from the same donors' PBMC are represented by identical symbols in figures 6-8.

Figure 8 – Ratios of cytokine [(TGF-β, IL-10, TNF, IL-5, IL-13, IL-17 or IL-6) / (IFN-γ)] concentration in non-allergic (A, B, C) vs. allergic donors (D, E, F) PBMC stimulated with TES antigens (TES- native, rTES30E and rTES30P). The cytokine measurements were as described in the Material and Methods. The amounts of cytokines detected in blank of cultures were subtracted from the amounts detected in the stimulated cultures. Each symbol shows the results obtained with the peripheral blood mononuclear cells from an individual donor. The legends above the graphs identify the individual PBMC donors (NAl = non-allergic donor; Al = allergic donor). The results obtained from the same donors' PBMC are represented by identical symbols in figures 6-8.

Figure 1



Wilcoxon signed rank test: $p < 0.05$* in relation to negative control (NC)

Figure 2

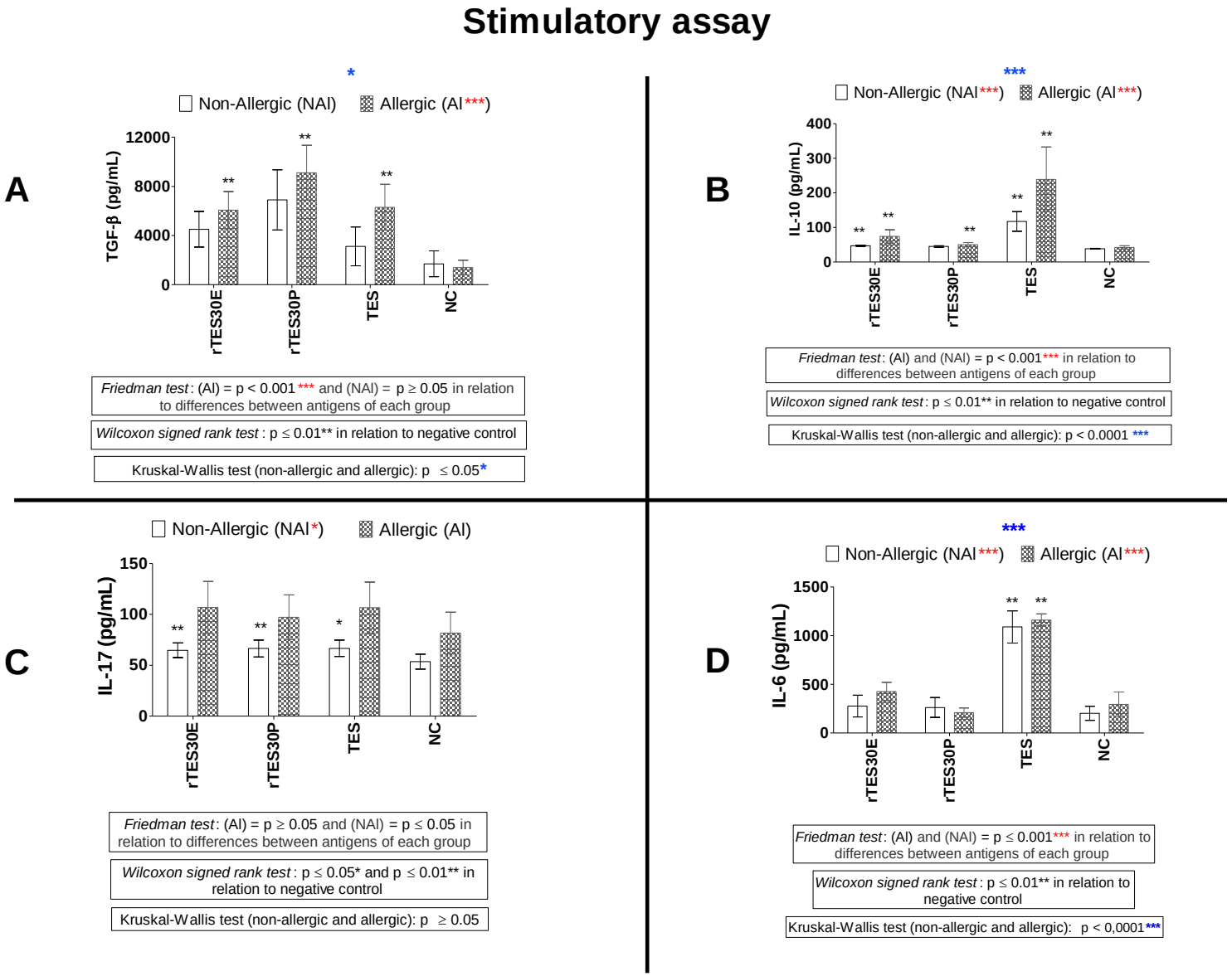

Stimulatory assay
A
*
Non-Allergic (NAI)
Allergic (AI***)
TGF-β (pg/mL)
12000
8000
4000
0
rTES30E
rTES30P
TES
NC
Friedman test: (AI) = p < 0.001*** and (NAI) = p ≥ 0.05 in relation to differences between antigens of each group
Wilcoxon signed rank test: p ≤ 0.01** in relation to negative control
Kruskal-Wallis test (non-allergic and allergic): p ≤ 0.05*
B
***
Non-Allergic (NAI***)
Allergic (AI***)
IL-10 (pg/mL)
400
300
200
100
0
Friedman test: (AI) and (NAI) = p < 0.001*** in relation to differences between antigens of each group
Wilcoxon signed rank test: p ≤ 0.01** in relation to negative control
Kruskal-Wallis test (non-allergic and allergic): p < 0.0001 ***
C
Non-Allergic (NAI*)
Allergic (AI)
IL-17 (pg/mL)
150
100
50
0
Friedman test: (AI) = p ≥ 0.05 and (NAI) = p ≤ 0.05 in relation to differences between antigens of each group
Wilcoxon signed rank test: p ≤ 0.05* and p ≤ 0.01** in relation to negative control
Kruskal-Wallis test (non-allergic and allergic): p ≥ 0.05
D
***
Non-Allergic (NAI***)
Allergic (AI***)
IL-6 (pg/mL)
1500
1000
500
0
Friedman test: (AI) and (NAI) = p ≤ 0.001*** in relation to differences between antigens of each group
Wilcoxon signed rank test: p ≤ 0.01** in relation to negative control
Kruskal-Wallis test (non-allergic and allergic): p < 0,0001***

Figure 3

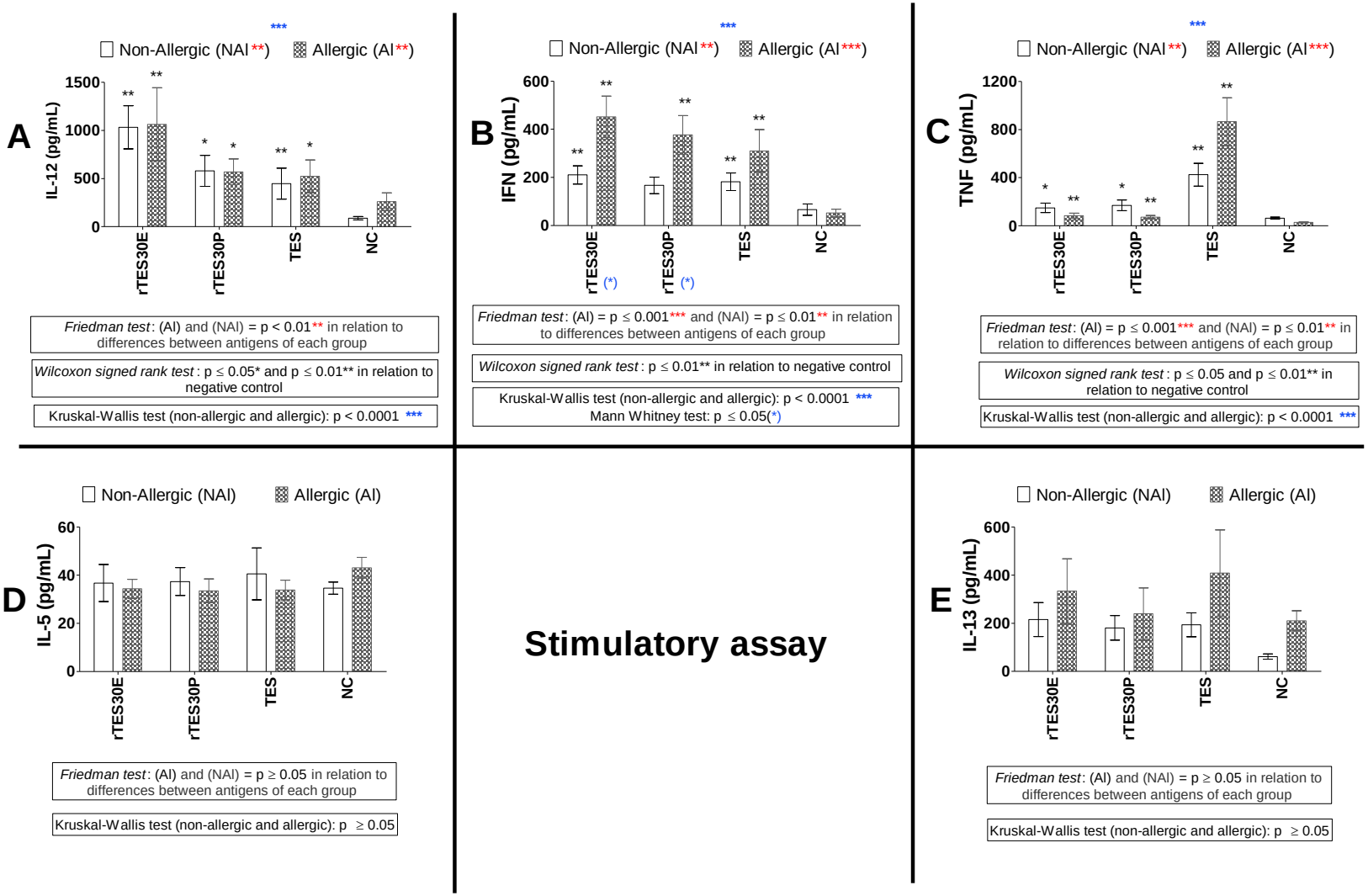

A
***
Non-Allergic (NAI **)
Allergic (AI **)
IL-12 (pg/mL)
1500
1000
500
0
rTES30E
rTES30P
TES
NC
Friedman test: (AI) and (NAI) = p < 0.01** in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05* and p ≤ 0.01** in relation to negative control
Kruskal-Wallis test (non-allergic and allergic): p < 0.0001 ***
B
***
Non-Allergic (NAI **)
Allergic (AI ***)
IFN (pg/mL)
600
400
200
0
(*)
(*)
Friedman test: (AI) = p ≤ 0.001*** and (NAI) = p ≤ 0.01** in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.01** in relation to negative control
Kruskal-Wallis test (non-allergic and allergic): p < 0.0001 ***
Mann Whitney test: p ≤ 0.05(*)
C
***
Non-Allergic (NAI **)
Allergic (AI ***)
TNF (pg/mL)
1200
800
400
0
Friedman test: (AI) = p ≤ 0.001*** and (NAI) = p ≤ 0.01** in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05 and p ≤ 0.01** in relation to negative control
Kruskal-Wallis test (non-allergic and allergic): p < 0.0001 ***
D
Non-Allergic (NAI)
Allergic (AI)
IL-5 (pg/mL)
60
40
20
0
Friedman test: (AI) and (NAI) = p ≥ 0.05 in relation to differences between antigens of each group
Kruskal-Wallis test (non-allergic and allergic): p ≥ 0.05
Stimulatory assay
E
Non-Allergic (NAI)
Allergic (AI)
IL-13 (pg/mL)
600
400
200
0
Friedman test: (AI) and (NAI) = p ≥ 0.05 in relation to differences between antigens of each group
Kruskal-Wallis test (non-allergic and allergic): p ≥ 0.05

Figure 4

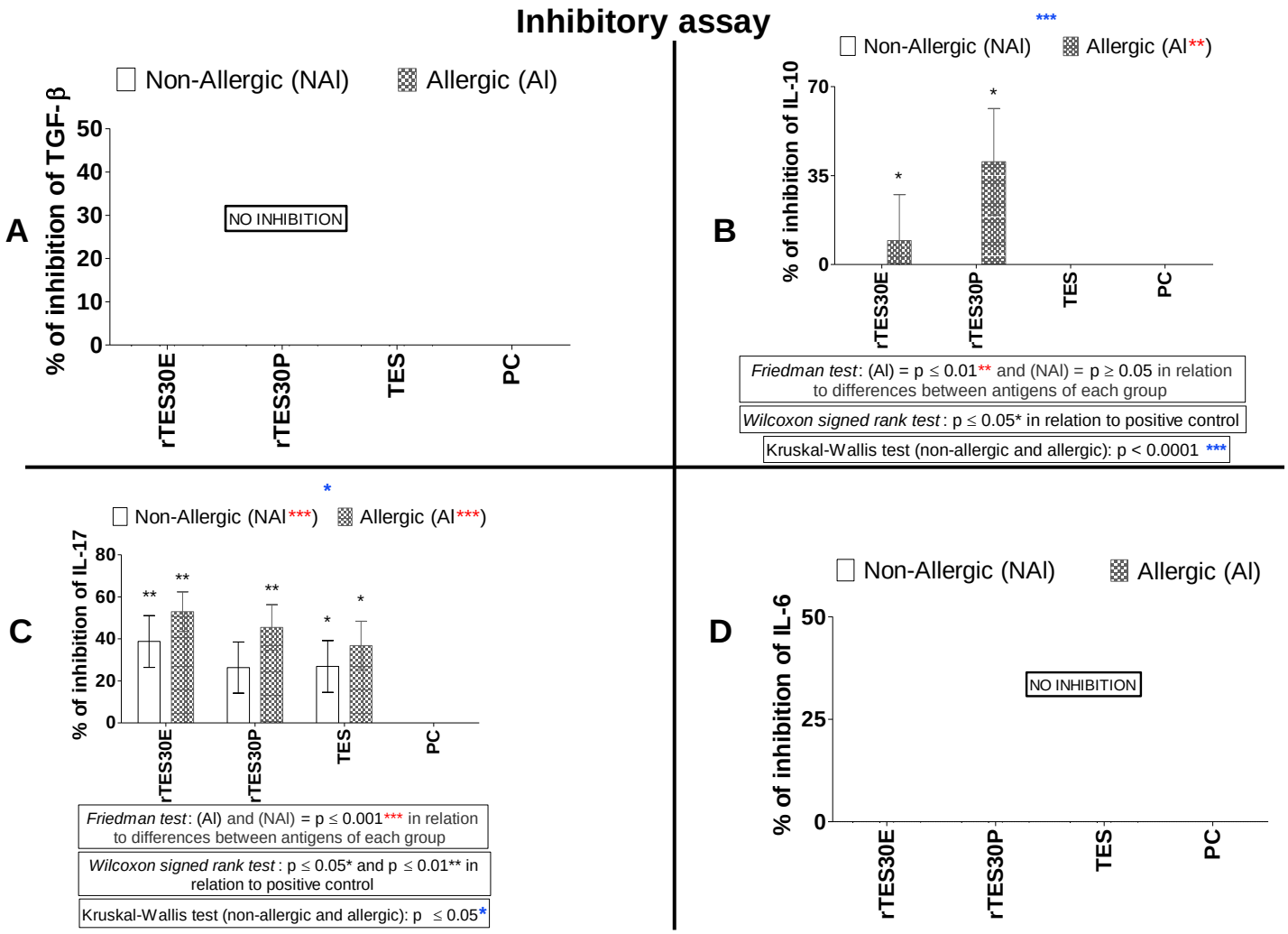

Inhibitory assay
A
Non-Allergic (NAI)
Allergic (AI)
% of inhibition of TGF-β
50
40
30
20
10
0
NO INHIBITION
rTES30E
rTES30P
TES
PC
B
***
Non-Allergic (NAI)
Allergic (AI**)
% of inhibition of IL-10
70
35
0
*
*
rTES30E
rTES30P
TES
PC
Friedman test: (AI) = p ≤ 0.01** and (NAI) = p ≥ 0.05 in relation to differences between antigens of each group
Wilcoxon signed rank test: p ≤ 0.05* in relation to positive control
Kruskal-Wallis test (non-allergic and allergic): p < 0.0001 ***
C
*
Non-Allergic (NAI***)
Allergic (AI***)
% of inhibition of IL-17
80
60
40
20
0
**
**
**
*
*
rTES30E
rTES30P
TES
PC
Friedman test: (AI) and (NAI) = p ≤ 0.001*** in relation to differences between antigens of each group
Wilcoxon signed rank test: p ≤ 0.05* and p ≤ 0.01** in relation to positive control
Kruskal-Wallis test (non-allergic and allergic): p ≤ 0.05*
D
Non-Allergic (NAI)
Allergic (AI)
% of inhibition of IL-6
50
25
0
NO INHIBITION
rTES30E
rTES30P
TES
PC

Figure 5

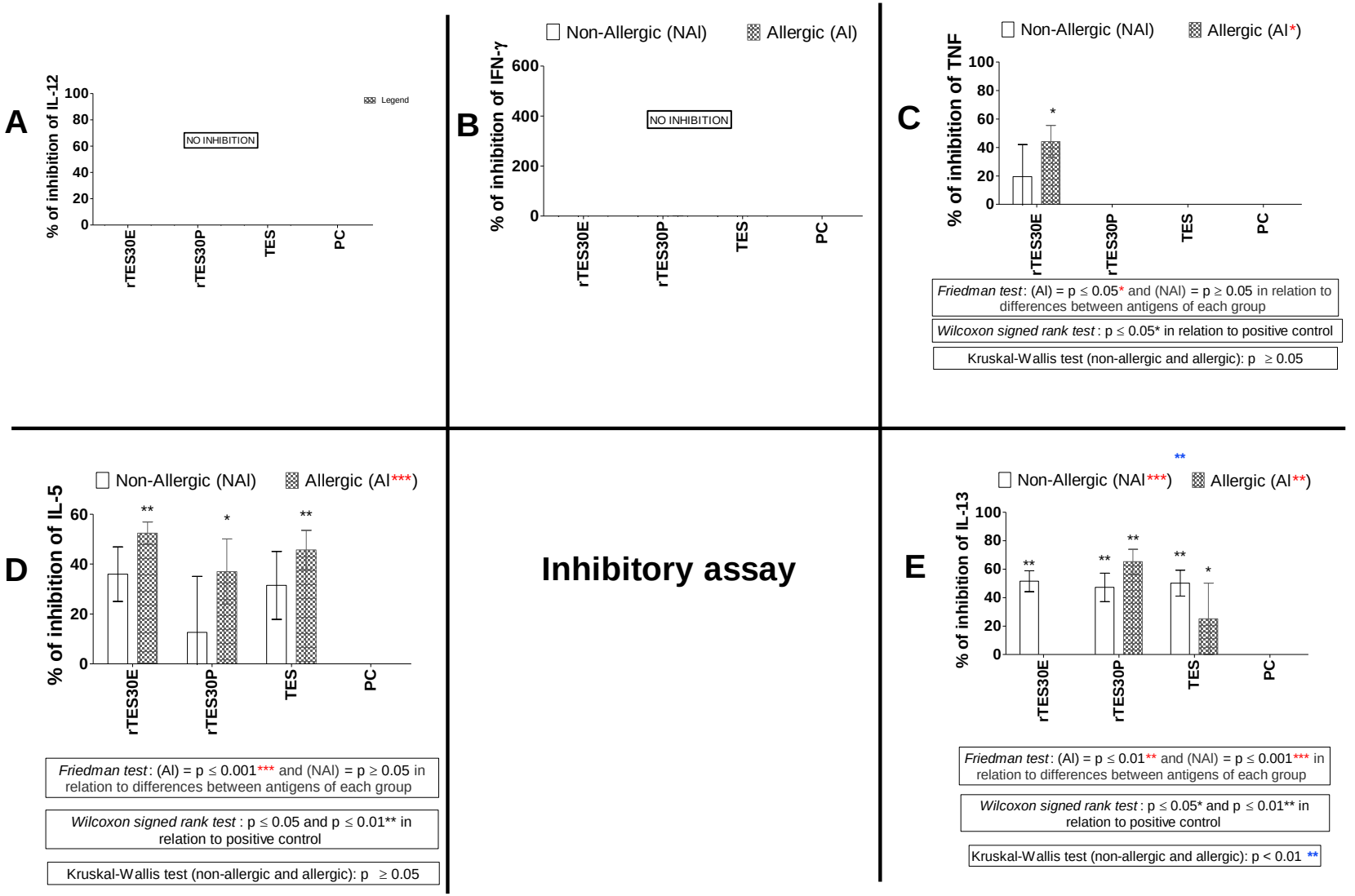

A
% of inhibition of IL-12
Legend
NO INHIBITION
rTES30E
rTES30P
TES
PC
B
Non-Allergic (NAI)
Allergic (AI)
% of inhibition of IFN-γ
NO INHIBITION
C
Non-Allergic (NAI)
Allergic (AI*)
% of inhibition of TNF
Friedman test: (AI) = p ≤ 0.05* and (NAI) = p ≥ 0.05 in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05* in relation to positive control
Kruskal-Wallis test (non-allergic and allergic): p ≥ 0.05
D
Non-Allergic (NAI)
Allergic (AI***)
% of inhibition of IL-5
Friedman test: (AI) = p ≤ 0.001*** and (NAI) = p ≥ 0.05 in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05 and p ≤ 0.01** in relation to positive control
Kruskal-Wallis test (non-allergic and allergic): p ≥ 0.05
Inhibitory assay
E
Non-Allergic (NAI***)
Allergic (AI**)
% of inhibition of IL-13
Friedman test: (AI) = p ≤ 0.01** and (NAI) = p ≤ 0.001*** in relation to differences between antigens of each group
Wilcoxon signed rank test : p ≤ 0.05* and p ≤ 0.01** in relation to positive control
Kruskal-Wallis test (non-allergic and allergic): p < 0.01 **

Figure 6

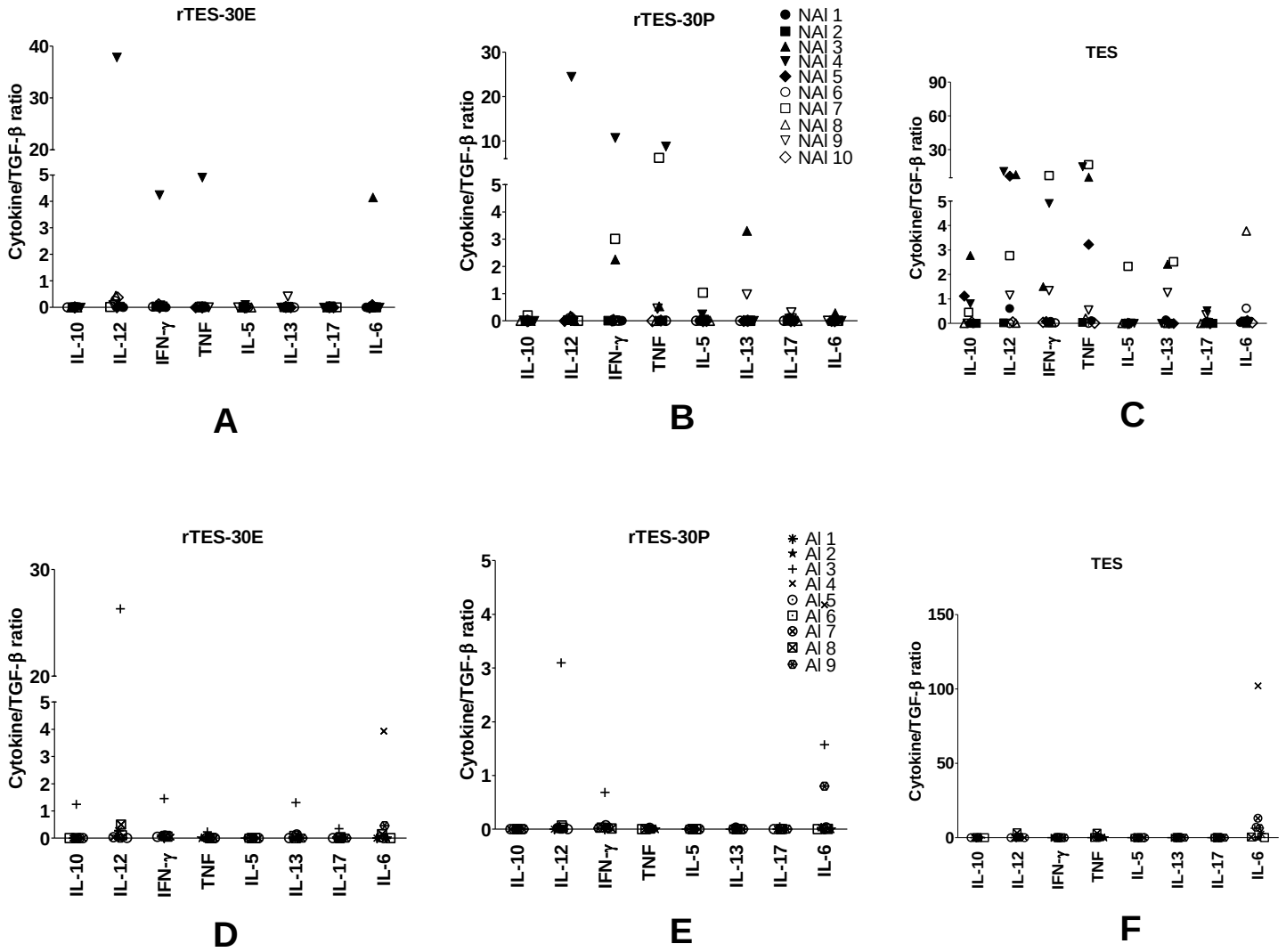

rTES-30E
Cytokine/TGF-β ratio
IL-10
IL-12
IFN-γ
TNF
IL-5
IL-13
IL-17
IL-6
A
rTES-30P
NAI 1
NAI 2
NAI 3
NAI 4
NAI 5
NAI 6
NAI 7
NAI 8
NAI 9
NAI 10
B
TES
C
AI 1
AI 2
AI 3
AI 4
AI 5
AI 6
AI 7
AI 8
AI 9
D
E
F

Figure 7

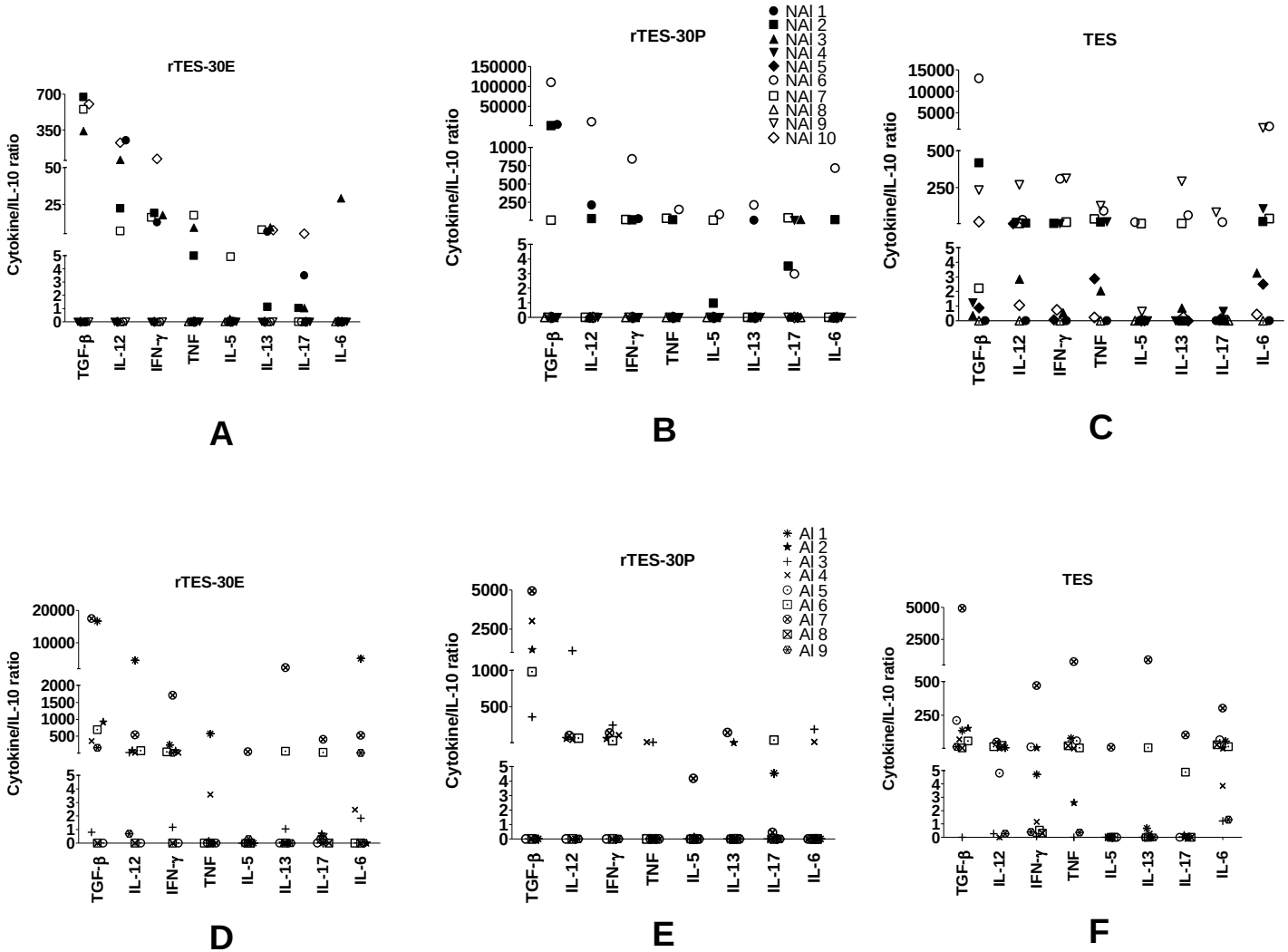
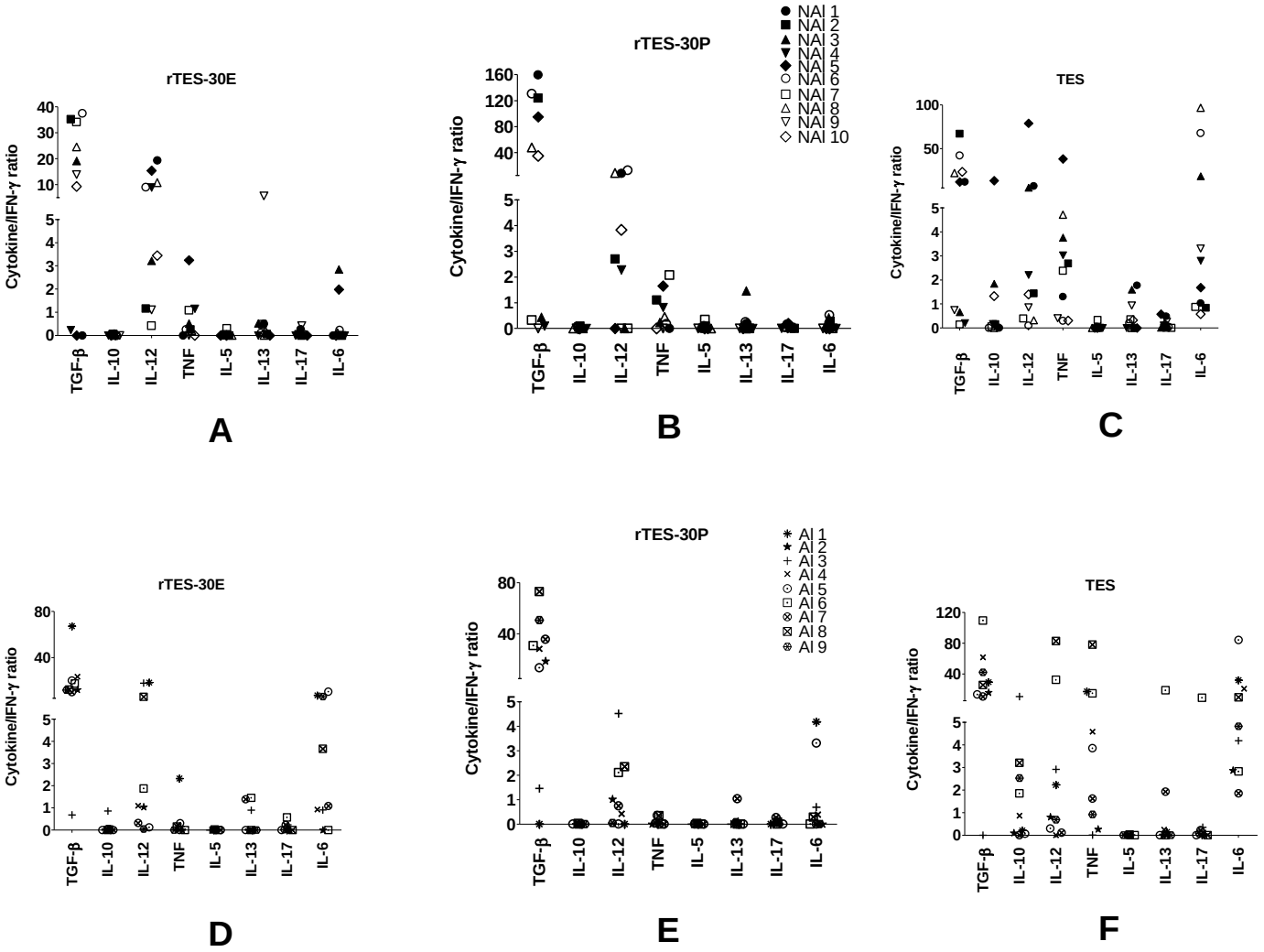

rTES-30E
Cytokine/IL-10 ratio
700
350
50
25
5
4
3
2
1
0
TGF-β
IL-12
IFN-γ
TNF
IL-5
IL-13
IL-17
IL-6
A
rTES-30P
NAI 1
NAI 2
NAI 3
NAI 4
NAI 5
NAI 6
NAI 7
NAI 8
NAI 9
NAI 10
150000
100000
50000
1000
750
500
250
B
TES
15000
10000
5000
500
250
C
rTES-30E
20000
10000
2000
1500
1000
500
D
rTES-30P
AI 1
AI 2
AI 3
AI 4
AI 5
AI 6
AI 7
AI 8
AI 9
5000
2500
1000
500
E
TES
F

Figure 8


rTES-30E
Cytokine/IFN-γ ratio
TGF-β
IL-10
IL-12
TNF
IL-5
IL-13
IL-17
IL-6
A
rTES-30P
NAI 1
NAI 2
NAI 3
NAI 4
NAI 5
NAI 6
NAI 7
NAI 8
NAI 9
NAI 10
B
TES
C
AI 1
AI 2
AI 3
AI 4
AI 5
AI 6
AI 7
AI 8
AI 9
D
E
F

5 SUMÁRIO DOS ACHADOS E CONCLUSÕES

Os produtos testados (de helmintos e fungos), em geral, estimularam a produção de citocinas reguladoras nas células dos doadores, em especial nos alérgicos.

Candidina e tricofitina estimularam a produção de citocinas Th1 e regulatórias por células mononucleares do sangue periférico.

Os resultados indicam que os extratos de fungos poderiam ser utilizados como adjuvantes em vacinas terapêuticas personalizadas.

Quanto ao extrato do adulto de *Toxocara canis* e suas frações TcE, TcEF01, TcEF02, TcEF06, TcEF08, TcEF09 e TcEF10 foram os produtos mais adequados para estudos sobre alergia e TcEF07 e TcEF09 para doenças autoimunes.

Os dados justificam a realização de estudos para identificação de moléculas presentes nos produtos testados que possam exclusivamente induzir respostas imunes Treg e/ou Th1 para terapia antialérgica ou a indução de respostas imunes Treg e/ou Th2 para terapia autoimune.

Os recombinantes rTES30P e rTES30E parecem induzir uma boa resposta imune, com a produção de Treg e / ou a resposta do tipo Th1, o que indica que estas proteínas podem ser usadas em terapia antialérgica.

Para a utilização dos antígenos recombinantes testados (rTES30E ou rTES30P) em terapia antialérgica, um maior número de PBMC de indivíduos e de diferentes áreas geográficas deverão ser testados para esta finalidade.

6 PERSPECTIVAS FUTURAS E CONSIDERAÇÕES FINAIS

Uma vez que não é viável a obtenção contínua de extratos de fungos, antígenos larvais e extrato de helmintos para purificar moléculas naturais presentes nestes, a forma mais prática seria a escolha da proteína a partir destes produtos com capacidade imunomoduladora, utilizando ferramentas da biologia molecular.

A manipulação de uma via reguladora (com produção de citocinas regulatórias) pode ser terapeuticamente eficaz e benéfica para o tratamento de doença alérgica das vias respiratórias.

Nesta perspectiva, nosso grupo pretende caracterizar bioquimicamente os produtos utilizados neste trabalho através de espectrometria de massas e com o uso de ferramentas de biotecnologia obter moléculas recombinantes que possuam mais especificidade das ações mostradas neste estudo do que os produtos que as continham. Além disso, estes antígenos são passiveis de se obter em média ou larga escala.

Estas moléculas serão testadas *in vitro* em PBMC e em modelo de alergia a ácaros de poeira assim como em linhagens celulares de diabetes e de colite ulcerativa assim como em modelos experimentais destas doenças.

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8. ANEXOS

ANEXO 1 - Caracterização dos doadores pesquisados

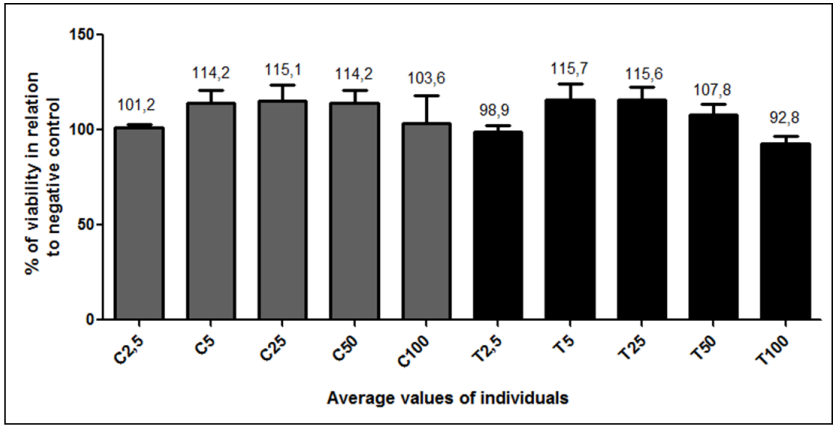
Quadro 1 – Caracterização dos 68 doadores pesquisados em alérgicos e não-alérgicos baseada na análise do teste cutâneo e na medição das concentrações de IgE específicos para aeroalérgenos – 2013.

SEXO	PHADIATOP e IMUNOCAP*		Teste cutâneo**	
	≥ 0,70 KU	< 0,70 KU	(+)	(-)
FEMININO	19	21	19	21
MASCULINO	18	10	18	10
TOTAL	37	31	37	31

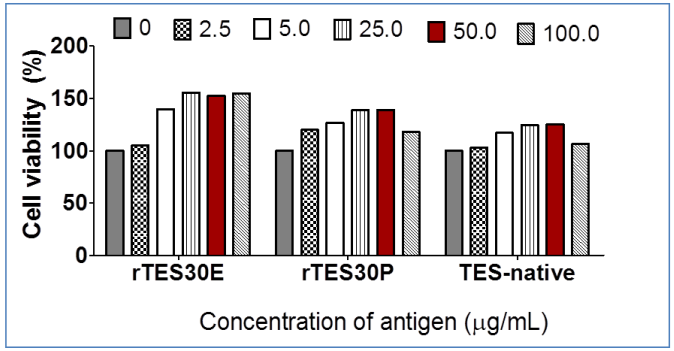
*Medição, no soro, das concentrações de IgE específicas para aeroalérgenos (*Blomia tropicalis, Dermatophagoides pteronyssinus, Blattella germanica e Periplaneta americana*): concentração maior ou igual a 0,70 KU, o indivíduo é considerado alérgico.
**Testes cutâneos utilizando extratos dos seis mais comuns alérgenos regionais (*Blomia tropicalis, Dermatophagoides pteronyssinus, Blattella germanica e Periplaneta americana*, pelo de gato e de cão): (+) = positivo; (-) = negativo.

ANEXO 2 - Pesquisa da viabilidade celular aos estímulos com antígenos

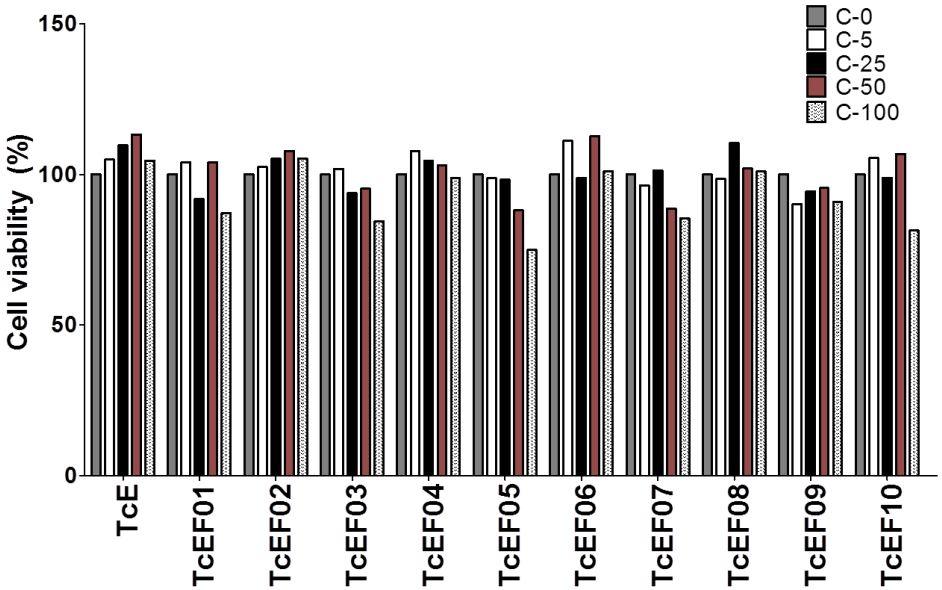
(A) FUNGOS



(B) TES

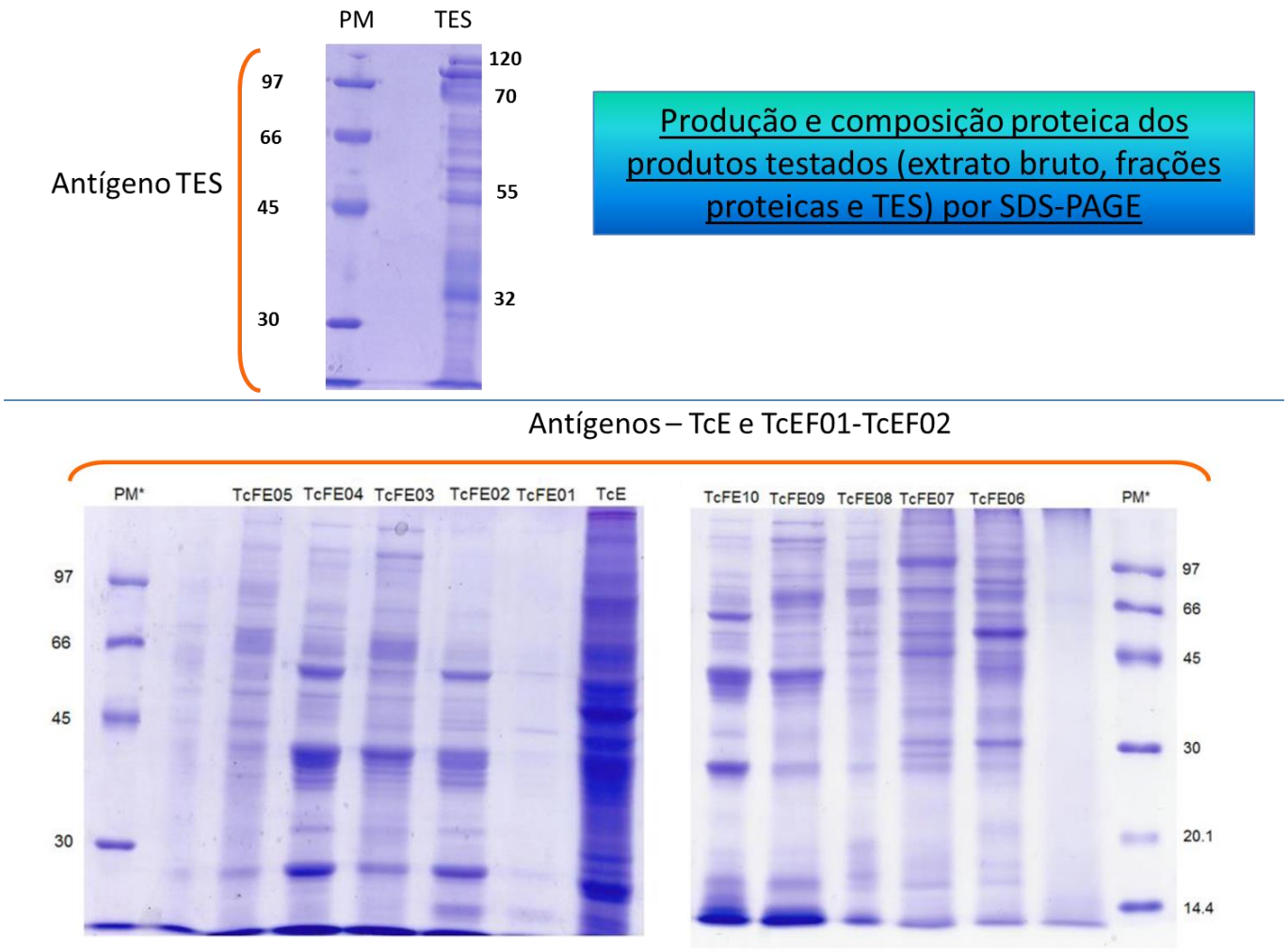


(C) Extrato bruto e frações do *Toxocara canis*



Anexo 2 - Viabilidade celular (citotoxicidade dos produtos testados): (A) extratos de fungos (C = candidina; T = tricofitina); (B) antígenos de TES (native e recombinantes) e (C) extrato bruto do *Toxocara canis* (TcE) e suas frações (TcEF01-TcEF10).

ANEXO 3



Anexo 3 - Caracterização quanto à produção e composição proteica dos produtos testados (extrato bruto, frações proteicas e TES) dispostos em géis (SDS-PAGE) corados com azul de comassie.

ANEXO 4 - Carta de aceite do Artigo 1.

------- Original message -------
From: Jonathan Wilkinson
Date:11/08/2014 12:21 (GMT-03:00)
To: Lain Carvalho
Subject: RE: Resubmission of manuscript by Amor et al.

Dear Lain,

I hope this email finds you well. Thanks again for sending me your revised manuscript. I have now been through it and can see that you have thoroughly addressed the reviewer comments. Therefore, I am happy to accept your manuscript for publication in *Immunotherapy*. Your article is provisionally scheduled for the October issue and a member of our production team will be in contact with you in due course regarding galley proofs. However, if you have any questions in the meantime, please don't hesitate to contact me.

We appreciate your contribution and look forward to seeing the final article.

Best wishes,

Jonathan

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ANEXO 5 - Comprovante de submissão do Artigo 2.

Hindawi Publishing Corporation

582381.v1 (Research Article)

Title	Fractions from the extract of Toxocara canis promote mainly the production of Th1 and regulatory cytokines by human leukocytes in vitro
Journal	BioMed Research International
Subject Area	Allergy
Issue	Regular
Additional Files	Cover Letter
Manuscript Number	582381 (Research Article)
Submitted On	2014-08-18
Author(s)	ANA AMOR, Leonardo Nascimento Santos, Eduardo Santos Silva, Marina Borges Rabêlo de Santana, Emilia Maria Medeiros de Andrade Belitardo, Flávia de Araújo Sena, Lain Pontes-de-Carvalho, **Neuza Maria Alcantara-Neves Alcantara-Neves**
Editor	
Status	Under Review
Supplementary Materials	View Supplementary Materials