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Biologia Celular e Molecular

TESE DE DOUTORADO

ASPECTOS IMUNOLÓGICOS CELULARES E HUMORAIS NA FASE CRÔNICA DA DOENÇA DE CHAGAS

Danielle Marquete Vitelli Avelar

Orientador: Dr. Olindo Assis Martins Filho

Co-orientadora: Dra. Silvana Maria Elói Santos

Laboratório de Biomarcadores de Diagnóstico e Monitoração - IRR/FIOCRUZ

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"Todo o interesse na doença e na morte é apenas
uma outra forma de interesse pela vida"
Thomas Mann (1875-1955)

RESUMO

Diversos estudos que avaliam os mecanismos imunológicos associados à manifestação de diferentes formas clínicas da doença de Chagas têm sugerido eventos multifatoriais. Com esse estudo, nosso objetivo foi adicionar novos elementos à complexa rede imunológica que envolve a interação parasita-hospedeiro e as distintas manifestações clínicas da doença de Chagas crônica, indeterminada (IND), cardíaca (CARD) e digestiva (DIG). Para esse objetivo, realizamos uma análise detalhada de características fenotípicas da resposta imune com ênfase na análise imunofenotípica de células mononucleares do sangue periférico, no perfil de citocinas intracitoplasmáticas de leucócitos circulantes bem como na magnitude da resposta de IgG anti-Trypanosoma cruzi. Nossos principais achados demonstraram elevadas freqüências de células reguladoras $CD4^{+}CD25^{high}$ e NKT, associadas com níveis aumentados de células NK citotóxicas circulantes em IND, enquanto percentuais aumentados de linfócitos T $CD8^{+}HLA\text{-}DR^{+}$ foram observados em CARD e DIG. Adicionalmente, nossos dados demonstraram valores aumentados de células pré-NK, além de elevados valores de monócitos pró-inflamatórios e linfócitos B ativados, contrastando com a ativação diminuída de células T e diminuição da freqüência de células T reguladoras $CD4^{+}CD25^{high}$ marcos do estágio recente (E-IND) da doença de Chagas crônica. Análise comparativa transversal entre E-IND, IND e CARD ainda sugeriu que uma mudança em direção a altos valores de "macrófagos-like" e níveis basais de monócitos pró-inflamatórios além de elevados valores de células NK maduras, NKT e $CD4^{+}CD25^{high}$ podem resultar na forma clínica IND. Por outro lado, altos níveis de células $CD8^{+}HLA\text{-}DR^{+}$ paralelo a níveis basais de células NK maduras, NKT e $CD4^{+}CD25^{high}$ podem levar a doença cardíaca. Além disso, freqüência aumentada de linfócitos totais alto-produtores de IL-4, IL-10 e IL-13 e um perfil misto de citocinas derivadas de NK e neutrófilos foram marcos do grupo IND, enquanto freqüências aumentadas de monócitos alto-produtores de TNF-α e baixas freqüências de linfócitos T $CD8^{+}IL\text{-}10^{+}$ foram as principais características de CARD. A avaliação do perfil de citocinas de leucócitos circulantes aplicando a estratégia de visão panorâmica demonstrou que enquanto a maioria dos IND apresentam um perfil de citocinas reguladoras, a maioria dos CARD apresentam um micro-ambiente de citocinas inflamatórias. Apesar dos nossos achados corroborarem com relatos prévios de perfis distintos de anticorpos IgG anti-T. cruzi entre IND e CARD, eles indicaram que marcadores da resposta imune humoral são, sem dúvida, mais aplicáveis para diagnóstico sorológico e monitoração de critério de cura. Em suma, nossos dados demonstram que, mais que uma mudança em direção a um padrão polarizado, um fino balanço é relevante para direcionar os mecanismos imuno-mediados essenciais para a definição da doença de Chagas.

ABSTRACT

Studies on immunological mechanisms associated with different clinical forms of Chagas disease have suggested multifactorial events for the disease development. Herein, we intended to add new elements to the complex immunological network underlying the parasite-host relationship and the distinct clinical outcome of chronic Chagas disease, referred as indeterminate (IND), cardiac (CARD) and digestive (DIG) clinical forms. For this purpose, we have performed a detailed analysis of phenotypic features of the immune response emphasizing the ex vivo immunophenotype of peripheral blood mononuclear cells, the intracitoplasmatic cytokine profile of circulating leukocyte subsets as well as the magnitude of anti-Trypanosoma cruzi IgG immune response. Our major findings demonstrated that higher frequency of both $CD4^{+}CD25^{high}$ and NKT regulatory cells, associated with increased levels of circulating cytotoxic NK cells was observed in IND whereas increased percentage of $CD8^{+}$HLA-DR^{+} T-lymphocytes subset was exclusively associated with CARD and DIG. Additionally, our data demonstrated that increased values of pre-NK-cells besides higher values of proinflammatory monocytes and activated B lymphocytes contrasting with the impaired T- lymphocytes activation and decreased frequency of $CD4^{+}CD25^{high}$ regulatory T lymphocytes was the hallmark of early stage (E-IND) of chronic Chagas disease. Comparative cross-sectional analysis among E-IND, IND and CARD further suggested that a shift toward high values of macrophage-like cells and basal levels of proinflammatory monocytes besides high values of mature NK cells, NKT and regulatory T cells may account for IND clinical form outcome. On the other hand, the upsurge of high levels of $CD8^{+}$HLA-DR^{+} cells parallel with basal levels of mature NK cells, NKT and $CD4^{+}CD25^{high}$ cells might lead to cardiac disease. Moreover, enhanced frequency of total lymphocytes high producers of IL-4, IL-10 and IL-13 besides a mixed pattern of NK and neutrophil-derived cytokines was the hallmark of IND, whereas enhanced frequency of TNF-α producing monocytes and lower frequency of IL-10 producing $CD8^{+}$ T-cells was the major features of CARD. The assessment of the cytokine profile of circulating leukocytes applying the panoramic overview strategy demonstrated that while most IND presented a regulatory cytokine profile, the majority of CARD displayed a particular inflammatory cytokine milieu. Despite our findings corroborate previous reports of distinct profiles of anti-T. cruzi IgG between IND and CARD, they indicated that the humoral immune response biomarkers are indeed more applicable to the serological diagnosis and cure criteria monitoring. Altogether, our data demonstrated that more than shift toward a polarized pattern, a fine balance is relevant to drive the resultant immune-mediated mechanisms underlying the chronic Chagas disease outcome.

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ADCC – Citotoxicidade celular dependente de anticorpos
AL – Anticorpos líticos
APCs – Células apresentadoras de antígenos (antigen presenting cells)
ASC – Anticorpos da sorologia convencional
B1– Linfócitos B $CD5^+$
BSA – Albumina sérica bovina
CARD – Indivíduos portadores da forma clínica cardíaca
CD14 – Molécula expressa na superfície de monócitos
CD16 – Molécula expressa em células Natural Killer e monócitos
CD19 – Molécula expressa em linfócitos B
CD25 – Cadeia α do receptor para a citocina IL-2
CD3 – Molécula expressa em linfócitos T
CD4 – Molécula expressa em linfócitos T auxiliares
CD5 – Molécula expressa em linfócitos B1
CD8 – Molécula expressa em linfócitos T citotóxicos
CD56 – Molécula de adesão presente em células Natural Killer
DIG – Indivíduos portadores da forma clínica digestiva
ECG – Eletrocardiograma
EDTA – Etilenodiaminotetracético
EPI – Formas epimastigotas do Trypanosoma cruzi
FITC – Isotiocianato de fluoresceína
FL – Fluorescência
FSC – Tamanho (forward scatter)
HAI – Reação de hemaglutinação indireta
HLA – Antígeno Leucocitário Humano
Id – Anticorpo anti-idiotipo
IFI – Imunofluorescência indireta
IFN-γ – Interferon gama
Ig – Imunoglobulina

IgG – Imunoglobulina G

IL – Interleucina

IMF – Intensidade Média de Fluorescência

IND – Indivíduos portadores da forma clínica indeterminada

LIT – Meio de cultivo para formas epimastigotas do Trypanosoma cruzi (Liver Infusion Tryptose)

MFF – Solução Fixadora

MHC – Complexo principal de histocompatibilidade

NI – Grupo não infectado

NK – Células Natural Killer

NKT – Subpopulação de células T que expressa em sua superfície receptores de células NK

NO – Óxido Nítrico

PBS – Tampão Fosfato Salínico

PBS-P – PBS-W a 0,5% de saponina

PBS-W – PBS a 0,5% de albumina sérica bovina

PE – Ficoeritrina

PPFP – Percentual de Parasitos Fluorescentes Positivos

RPMI – Meio de cultura (Rosweel Park Memorial Institute)

SBF – Soro bovino fetal

SES – Secretaria de Estado de Saúde de Minas Gerais

SSC – Granulosidade (side scatter)

TCR – Receptor de célula T

TGF-β – Fator de Crescimento Transformante beta

TNF-α – Fator de Necrose Tumoral alfa

WHO – Organização Mundial da Saúde (World Health Organization)

1. INTRODUÇÃO

Carlos Chagas, médico brasileiro, descobriu a doença de Chagas a aproximadamente um século atrás e publicou sua primeira descrição em 1909. A descoberta dessa doença consistiu em uma das mais importantes conquistas médicas do Brasil. Carlos Chagas descreveu tanto aspectos da biologia do agente etiológico, o Trypanosoma cruzi, como da clínica, epidemiologia e patogenia da doença (CHAGAS, 1909). Entretanto, apesar do avanço nos estudos da doença de Chagas nas últimas décadas, questionamentos ainda permanecem relativos à interação parasito-hospedeiro, progressão clínica da infecção e mecanismos indutores de proteção e patologia.

Como parte das comemorações do centenário da descoberta da doença de Chagas, a FIOCRUZ criou o Programa Integrado de Doença de Chagas-PIDC que tem como pilares (1) estimular a cooperação intra-FIOCRUZ visando dar retorno à sociedade quanto aos desafios atuais da doença de Chagas; (2) assegurar contribuição efetiva da FIOCRUZ para o controle da doença de Chagas no século XXI, estabelecendo metas de pesquisa e desenvolvimento tecnológico para 2009; (3) possibilitar a retomada de liderança da FIOCRUZ no campo da pesquisa e intervenção em doença de Chagas no seu segundo centenário, fortalecendo as interações extramuros da FIOCRUZ; (4) articular projetos visando o estabelecimento de compromissos de geração de inovação na pesquisa em doença de Chagas e a captação de financiamentos. Esse estudo, além de outros desenvolvidos no Instituto René Rachou, vem contribuir com os ideais do PIDC, uma vez que investiga mecanismos imunológicos envolvidos no estabelecimento/manutenção de diferentes formas clínicas da doença de Chagas.

A doença de Chagas ocorre em áreas rurais e peri-urbanas do México, América Central e América do Sul. Dados da Organização Mundial da Saúde mostram a ocorrência de vinte e uma mil mortes e a incidência de duzentos mil novos casos ao ano na América Latina

(WORLD HEALTH ORGANIZATION, 2002), com 12 a 14 milhões de pessoas infectadas na América Latina (DIAS, 2007).

No Brasil, a doença de Chagas é a quarta causa de morte entre as doenças infecto-parasitárias, ocorrendo em uma área de três milhões de quilômetros quadrados. São cerca de 2.450 municípios, envolvendo uma população de mais de 28 milhões de pessoas expostas ao risco de contaminação e uma população de aproximadamente cinco milhões de indivíduos infectados (DIAS et al., 1997). Nos últimos 5 anos foram notificados mais de 470 casos de infecção aguda, quase 90% deles ocorreram na Amazônia Legal, sendo 75% no Pará (BRASIL, s.d.).

Em regiões onde os programas de controle do triatomíneo, inseto vetor da doença, são ineficazes, a infecção se dá principalmente pelo contato da pele ou mucosa do hospedeiro vertebrado com fezes e/ou urina do triatomíneo contaminado pelo protozoário T. cruzi. Os vetores se infectam quando formas tripomastigotas sangüíneas são ingeridas durante a sucção do sangue do hospedeiro vertebrado infectado. Uma vez no intestino posterior do inseto, as formas tripomastigotas se transformam em formas epimastigotas, que se multiplicam por divisão binária e, posteriormente, passam por alterações morfológicas e fisiológicas transformando-se em tripomastigotas metacíclicos no reto do inseto, estando, então aptas a penetrar em células do hospedeiro mamífero. Essas formas, após o repasto sangüíneo, são eliminadas juntamente com as fezes do vetor, penetrando na pele lesada pela picada ou em mucosas íntegras. Na região de penetração do parasito, ocorre a infecção de células principalmente do sistema fagocítico - mononuclear e/ou do próprio tegumento. Após ciclo focal de poucos dias, o parasito se difunde por via hematogênica e linfática, com multiplicação em vários órgãos e tecidos, ocorrendo como característica fundamental uma intensa parasitemia, detectável por exames parasitológicos diretos. No interior de células, as formas tripomastigotas se transformam em amastigotas, que conseguem escapar do vacúolo

parasitófago para o citoplasma, onde se inicia um processo de divisão binária. Após a multiplicação, as formas amastigotas transformam-se em tripomastigotas que rompem a célula e, uma vez na circulação sangüínea, podem infectar novas células ou serem ingeridas por outro inseto vetor, dando continuidade ao ciclo biológico do parasito (BRENER et al., 1973; GARCIA et al., 1991; DIAS, 2000).

Quando se estudam os mecanismos de transmissão, observa-se que, além da transmissão vetorial, casos de transmissão alternativos, como a via transfusional (YOUNG et al., 2007), congênita (DORN et al., 2007; GÜRTLER et al., 2003), por transplantes de órgãos (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2006) e acidentes laboratoriais podem ser identificados. A via transfusional é considerada a segunda via mais freqüente de infecção, tendo especial importância epidemiológica, uma vez que pode levar a doença para áreas sem transmissão natural (WENDEL, 1997; SCHOFIELD & DIAS 1999; VINHAES & DIAS, 2000). Além disso, em regiões como na Amazônia, existem mecanismos excepcionais de transmissão (vetorial domiciliar sem colonização, vetorial extradomiciliar) e a ocorrência de surtos episódicos de transmissão oral (BRASIL, 2005a, COURA, 2006). Recentemente, ocorreram surtos de doença de Chagas com forma aguda e morte por ingestão de formas tripomastigotas dissolvidas em bebidas, como suco de cana e açaí, em que os insetos vetores silvestres, provavelmente, foram triturados durante o preparo ou suas fezes contaminaram o alimento, conforme divulgado amplamente na mídia e no Guia de Vigilância Epidemiológica do Ministério da Saúde (BRASIL, 2005b).

A análise clínica e laboratorial de pacientes portadores da doença de Chagas permite classificar a infecção em duas fases: aguda e crônica. A fase aguda caracteriza-se por alterações teciduais degenerativas e inflamatórias focais, devido a uma intensa multiplicação local do parasito. Manifestações sistêmicas, como febre, mal estar, astenia, edema subcutâneo, linfadenomegalia, esplenomegalia e hepatomegalia, podem também ser observadas

(LARANJA, 1953; REZENDE & RASSI, 1994; BRASIL, 2004). Entretanto, em geral, esses sinais são atenuados e a fase inicial da doença passa desapercebida, confundindo-se com uma "gripe" ou "mal estar" passageiro.

A evolução da fase aguda para a fase crônica, que pode durar de poucas semanas a meses, é acompanhada pelo gradativo desaparecimento das manifestações clínicas, diminuição da parasitemia e elevação de anticorpos específicos da classe IgG. Os indivíduos portadores da infecção crônica podem ser classificados do ponto de vista clínico, como pertencentes às formas indeterminada (IND), cardíaca (CARD), digestiva (DIG) e cardiodigestiva. A imensa maioria dos pacientes evolui para uma forma crônica indeterminada, caracterizada pela sorologia positiva, ausência de sinais, sintomas da doença e eletrocardiograma-ECG e raio-X (coração, esôfago e cólon) normais. Os pacientes apresentam de forma geral, bom estado de saúde, desconhecendo, muitas vezes, a presença da infecção. Estima-se que cerca de 50-60% dos indivíduos infectados apresentam essa forma clínica. Embora reconhecendo que o prognóstico é favorável, sabe-se que, anualmente, cerca de 2 a 5% desses indivíduos evoluem para formas sintomáticas da doença. Após 20-30 anos de infecção, cerca de 20-30% dos indivíduos portadores da forma clínica IND desenvolvem a forma CARD, resultante de danos progressivos do miocárdio, secundários à miocardite fibrosante (DIAS, 1989; PRATA, 2001). A infecção chagásica pode levar ainda a dilatações do esôfago e cólon, principais manifestações da forma clínica DIG. Essa forma clínica ocorre em menos de 10% dos indivíduos portadores da doença, sendo resultado, principalmente, da formação de fibrose e da destruição dos neurônios do sistema nervoso autônomo do trato gastrointestinal (KOEBERLE & NADOR, 1955; DIAS, 1989; DA SILVEIRA et al., 2007a,b). Pessoas infectadas pelo T. cruzi com o sistema imune debilitado (AIDS, quimioterapia, terapias imunossupressoras) podem reativar a doença de Chagas com abundante parasitemia sanguínea e tecidual. Devido à longa duração da forma assintomática

da infecção, a doença de Chagas é considerada uma doença "matadora silenciosa" (MAGUIRE, 2006), esse constitui um dos vários motivos pelos quais a doença não atrai a atenção da mídia (TARLETON et al., 2007).

O benzonidazol é o composto disponível para o tratamento da doença de Chagas no Brasil. Esse composto apresenta sérios efeitos colaterais, requer administração por longos períodos de tempo sob supervisão médica e há grande variação na susceptibilidade de isolados do parasito à ação dessa droga, sendo recomendado o tratamento de pacientes agudos e crônicos recentes, nos quais se observam resultados positivos, principalmente em crianças menores de 15 anos (URBINA & DOCAMPO, 2003). Apesar da maioria dos estudos revelarem uma baixa eficiência desse fármaco durante a terapia de pacientes crônicos, avaliações recentes têm sugerido o tratamento de modo a retardar, ou mesmo evitar, a evolução da doença crônica (ANDRADE et al., 2004; DE CASTRO et al., 2006; VIOTTI et al., 2006).

Uma questão chave que envolve a doença de Chagas é o diagnóstico. Sem um diagnóstico correto, indivíduos infectados não podem ser identificados, conseqüentemente, tratados e a eficiência do tratamento não pode ser corretamente avaliada. O efeito de campanhas de controle não pode ser avaliado sem ferramentas de diagnóstico competentes. Na fase crônica, os níveis de parasitemia encontram-se baixos e assim o diagnóstico se baseia na detecção da resposta sorológica do hospedeiro. A maioria dos testes sorológicos disponíveis emprega preparações de antígeno bruto de formas epimastigotas do T. cruzi, única forma do ciclo de vida do parasito que não é encontrada no hospedeiro vertebrado, podendo ser responsável pelos resultados falso-positivos. A especificidade desses ensaios tem sido questionada devido à freqüência de infecções com outros tripanosomatídeos que circulam na mesma área geográfica do T. cruzi (Leishmania spp e Trypanosoma rangeli) e são responsáveis pela antigenicidade cruzada e resultados falso-positivos. A ausência de um teste

padrão-ouro torna difícil a avaliação da sensibilidade de testes sorológicos que podem ser úteis na avaliação rápida da eficácia do tratamento, do diagnóstico de infecção congênita ou para determinar o impacto de métodos de controle de transmissão, compondo base para o avanço de todos os campos de pesquisa na doença de Chagas (TARLETON et al., 2007).

Os mecanismos específicos envolvidos com o estabelecimento/manutenção das diferentes formas clínicas da doença de Chagas são complexos. Não se sabe explicar como alguns indivíduos infectados desenvolvem formas graves da doença e nem o fato das manifestações clínicas serem tão heterogêneas. Acredita-se que as manifestações patológicas tanto na fase aguda, quanto na fase crônica da doença de Chagas sejam conseqüência de mecanismos multifatoriais relacionados tanto ao parasito quanto ao hospedeiro vertebrado. Dentre os fatores relacionados ao parasito, análises em camundongos revelaram que a variabilidade das cepas, o tropismo, a antigenicidade e o tamanho do inóculo são aspectos relevantes (VAGO, 2000). Quanto ao hospedeiro, é importante ressaltar o estado nutricional, a faixa etária, o sexo e, especialmente, as características genéticas e imunológicas (DIAS, 2000; ARANTES et al.; 2007; CAMPBELL et al., 2004).

A resposta imune durante a infecção humana inicial pelo T. cruzi (aguda recente, aguda tardia e crônica recente) não é completamente entendida, apesar da sua função crucial no direcionamento das diferentes formas clínicas da infecção crônica. Estudos demonstram que o T. cruzi induz uma forte ativação do sistema imune durante a infecção aguda e que os diferentes mecanismos imunes induzidos durante estágios iniciais da fase crônica indeterminada da infecção representam componentes essenciais da atividade imune presente na fase crônica tardia (ANDRADE, 1991; BRENER & GAZZINELLI, 1997; MARINHO et al., 1999; SATHLER-AVELAR et al., 2003; GOLGHER & GAZZINELLI, 2004). Na busca de identificar diferenças na resposta imune relacionadas com o estabelecimento/manutenção das distintas formas clínicas da fase crônica tardia da doença de Chagas, é de suma

importância caracterizar subpopulações leucocitárias majoritárias e minoritárias do sangue periférico de indivíduos durante os eventos iniciais da infecção, assim como comparar com as alterações observadas em indivíduos na fase crônica tardia da doença. Estudo fenotípico realizado durante a infecção recente pelo T. cruzi, após classificação com base na sorologia, descreveu no grupo agudo recente uma supressão da atividade de linfócitos T, ativação de linfócitos B e o favorecimento da migração de monócitos para o foco inflamatório. Na fase aguda tardia, foi observada a ativação de células NK independente de células T e na fase crônica recente foi encontrado um aumento na população de linfócitos B ao lado de um aumento na população dos monócitos pró-inflamatórios sugerindo uma natureza independente de linfócitos T (SATHLER-AVELAR et al., 2003). Esses autores acreditam que a imunidade mediada por células T durante a forma clínica indeterminada recente da doença de Chagas pode representar um fenômeno restrito aos focos inflamatórios, considerando relatos prévios da presença de células T, principalmente linfócitos T $CD8^+$, no infiltrado inflamatório do tecido cardíaco de indivíduos infectados pelo T. cruzi (FUENMAYOUR et al., 2005). Estudo realizado em modelo experimental murino para a infecção pelo T. cruzi demonstrou que o aparecimento no coração de células T produtoras de citocinas está correlacionado com o aumento local da expressão de moléculas de adesão celular, o que seria consistente com a migração de linfócitos T para o foco inflamatório (FUENMAYOUR et al., 2005). Nesse estudo, os autores propõem que a inflamação crônica no coração chagásico é altamente ativa e está relacionada com um padrão imunológico estável que se estende desde a fase aguda recente até estágios tardios da fase crônica.

HIGUCHI et al. (1993a) utilizando técnicas de imunohistoquímica estabeleceram uma associação entre a presença de antígenos do T. cruzi e a formação de infiltrados inflamatórios no tecido cardíaco de pacientes com miocardite chagásica. Esse mesmo grupo avaliou o fenótipo das células do infiltrado inflamatório, encontrando um predomínio de células T $CD8^+$

(HIGUCHI et al., 1993a,b). Estudos desenvolvidos por REIS et al. (1993) mostram ainda que estas células $CD8^+$ expressavam granzima A, um marcador para linfócitos T citolíticos. Esses autores atribuem à célula T $CD8^+$ e a seus produtos, a fibrose e a citólise observadas no tecido cardíaco nos pacientes portadores de miocardite chagásica.

No contexto da forma clínica DIG, os estudos que avaliam os elementos envolvidos nos mecanismos imunopatológicos no tubo digestivo são ainda escassos. LEMOS et al., (1998), em estudo pioneiro, demonstraram um predomínio de linfócitos $CD4^+$ no infiltrado inflamatório do esôfago e do cólon de pacientes chagásicos portadores de mega decorrentes da forma DIG. DA SILVEIRA et al. (2007a,b) demonstram ainda que o desenvolvimento de megacólon após a infecção aguda pelo T. cruzi está associado com a manutenção da invasão de gânglios entéricos por linfócitos T citotóxicos e perda da inervação de músculos.

Devido a limitações éticas de se estudar o perfil fenotípico do infiltrado inflamatório e lesões teciduais decorrentes da infecção crônica da doença de Chagas, vários pesquisadores têm utilizado o sangue periférico como estratégia para avaliar a resposta imunológica desencadeada durante a fase crônica da doença. O estudo do sangue periférico de pacientes na fase crônica da doença de Chagas demonstra grande número de linfócitos T ativados e B circulantes, além de diminuição significativa no percentual de linfócitos T $CD3^+$. Linfócitos B $CD5^+$ estão presentes em alto percentual na circulação destes pacientes (DUTRA et al., 1994). Após estimulação in vitro por antígenos do T. cruzi ou por anticorpos humanos anti-epimastigotas, através de estimulação idiotípica, observa-se proliferação diferenciada das populações celulares. Dessa forma, a estimulação por antígenos de T. cruzi leva à expansão preferencial de linfócitos T ativados, enquanto a estimulação idiotípica estimula preferencialmente linfócitos B $CD5^+$ e linfócitos T $CD8^+$ (DUTRA et al., 2000). Na forma clínica DIG, inversão da razão CD4/CD8 foi descrita contrastando com resultados em CARD nos quais a razão é normal. Ainda em pacientes da forma DIG redução do percentual de

células $CD4^+CD28^+$ e um aumento na expressão de linfócitos T ativados ($HLA\text{-}DR^+$) foram observados (LEMOS et al., 1998).

Considerando a complexidade das interações imunológicas desencadeadas durante a infecção pelo T. cruzi, ainda são necessários esclarecimentos mais detalhados a respeito das populações e subpopulações celulares diretamente envolvidas nos mecanismos de resistência e morbidade da doença. A detecção de moléculas expressas na superfície de células, através da citometria de fluxo, empregando sistemas de análises mais elaborados e protocolos experimentais atuais, que permitem a análise simultânea de um maior número de moléculas por superfície celular, trouxe a possibilidade de identificar e/ou caracterizar novas sub-populações, trazendo assim, informações adicionais que podem enriquecer os conhecimentos acerca das diferentes formas clínicas da doença de Chagas.

Ao analisar macrófagos, seja em humanos seja em camundongos, observam-se duas populações fenotipicamente distintas: $CD14^{++}CD16^-$ e $CD14^+CD16^+$. Em indivíduos saudáveis, as células $CD14^+CD16^+$ correspondem aproximadamente a 10% da população de monócitos. Entretanto, em indivíduos com infecções graves, o número de monócitos $CD14^+CD16^+$ encontra-se consideravelmente aumentado (NOCKHER et al., 1998; SKRZECZYÑSKA et al., 2002). Tais monócitos expressam altos níveis da molécula HLA-DR em sua superfície, sintetizam elevados níveis de TNF-α e quantidades basais de citocinas reguladoras como IL-10, sendo denominados monócitos pró-inflamatórios (BELGE et al., 2002). Os altos níveis de TNF-α provavelmente decorrem da elevada expressão de receptores dirigidos a componentes estruturais dos agentes infecciosos, como exemplo temos o LPS atuando sobre os receptores "Toll-like 4" (TLR-4) em células dendríticas ativadas (THOMA-USZYNSKI et al., 2000). CAMPOS et al. (2001) demonstraram que glicoproteínas (GPIs) e glicolipídios (GIPLs) derivados de T. cruzi podem se ligar ao receptor "Toll-like 2" de monócitos e induzir a síntese de IL-12, TNF-α e óxido nítrico (NO). OLIVEIRA et al. (2004),

estudando a função de TLR-4 na doença de Chagas, constataram que camundongos deficientes para este receptor são altamente susceptíveis à infecção pelo T. cruzi, com elevada parasitemia e taxa de mortalidade. Isso se deve à falha na interação de GIPLs do parasita com os receptores TLR-4 presentes em monócitos, indicando sua importância na ativação da resposta imune protetora. Atualmente, atenção especial tem sido dada aos TLR devido ao seu papel na ativação de células NK durante infecções bacterianas e por protozoários. LAUZON et al. (2003) demonstraram que antígenos derivados de Leishmania sp. ao se ligarem aos receptores TLR-2 aumentam a produção das citocinas IFN-γ e TNF-α e a translocação nuclear de NF-κB pelas células NK. Esse mesmo grupo, recentemente, verificou que as células NK possuem diversos TLRs, que estão diretamente relacionados com o aumento da produção de citocinas e citotoxicidade celular (LAUZON et al., 2006). No contexto da doença de Chagas, pouco se sabe sobre a interação de antígenos do T. cruzi e os TLRs presentes em NK, mas é pertinente sugerir que a interação desses antígenos com esses receptores pode ser importante na ativação de células NK na fase aguda e crônica da doença de Chagas.

É bem aceito que a ausência de patologia em indivíduos infectados pelo T. cruzi está associada com a capacidade do indivíduo em regular a resposta anti-T. cruzi, responsável pelo controle da parasitemia persistente e do dano inflamatório tecidual, característicos da doença de Chagas (BRENER et al., 1997). Certamente, o dano tecidual frente à inflamação deve ser mais grave na ausência de mecanismos reguladores que envolvem tanto a resposta imune inata eficaz quanto a adaptativa. Estudos têm demonstrado que existem vários tipos de células com função reguladora, algumas induzidas em resposta a um estímulo infeccioso e outras consideradas reguladoras naturais (BLUESTONE & ABBAS, 2003; PICCIRILLO & SHEVACH, 2004; BELKAID & ROUSE, 2005). As células T reguladoras foram descritas como uma população única $CD4^+CD25^+$ que regulam respostas imunes inata e adaptativa e que possuem a capacidade de controlar respostas imunes efetoras excessivas ou mal

direcionadas, incluindo respostas contra patógenos ou antígenos próprios (MALOY & POWRIE, 2001; GAVIN et al., 2001; SHEVACH, 2002; TRZONKOWSKI et al., 2004). Vários mecanismos pelos quais as células T reguladoras realizam suas atividades moduladoras têm sido propostos, incluindo a inibição da atividade citotóxica pela regulação negativa de IL-12 e CD25 em linfócitos T $CD8^+$ ou através de contato direto com essas células efetoras (PICCIRILLO & SHEVAC, 2001; GROSSMAN et al., 2004; O´GARRA & VIEIRA, 2004; TRZONKOWSKI et al., 2004; CASSIS et al., 2005,). Embora em modelos experimentais murinos, as células T $CD4^+$ reguladoras sejam caracterizadas por apresentarem o fenótipo $CD4^+CD25^+$, sendo todas possuidoras de potencial papel regulador, caracterizado por uma diminuição de produção de citocinas e habilidade de inibir proliferação celular in vitro, em humanos, apenas as $CD4^+CD25^{HIGH}$ apresentam esta habilidade. No contexto da doença de Chagas, ARAÚJO e colabores (2007) demonstraram freqüência aumentada de células $CD4^+CD25^{HIGH}FOXP3^+$ em pacientes da forma IND quando comparados com pacientes portadores da forma clínica cardíaca, indicando que estas células teriam função importante na manutenção da forma clínica indeterminada da doença de Chagas.

Semelhantemente às células T reguladoras, as células NKT têm sido descritas como uma subpopulação relevante tanto na função inata (GODFREY et al., 2000; KRONENBERG & GAPIN, 2002), quanto na atividade anti-tumoral (GODFREY & KRONENBERG, 2004; VAN DER VLIET et al., 2004) e reguladora (HAMMOND & KRONENBERG, 2003; MATSUDA et al., 2003), apresentando atividades sob o controle de células acessórias e fatores solúveis do micro-ambiente local (LEITE-DE-MORAES et al., 1997; GODFREY & KRONENBERG, 2004). Estudos fenotípicos demonstraram que células NKT compreendem uma subpopulação de células T, distinta das células T convencionais, por expressarem receptores de células NK como o CD16, CD56 e/ou CD161, sendo o CD56 o melhor marcador para definir os subgrupos de células NKT (ORTALDO et al., 1991; GODFREY et

al., 2000). As funções reguladoras das células NKT previnem doenças auto-imunes e cooperam com respostas protetoras contra patógenos (GODFREY et al., 2000; GODFREY & KRONENBERG, 2004). Células NKT inibem várias doenças auto-imunes, incluindo diabetes tipo 1, lupus eritromatoso sistêmico e artrite reumatóide. A população de células NKT está diminuída nessas doenças, impedindo sua ação de prevenir respostas imunes autodestrutivas (KOJO et al., 2003; GODFREY & KRONENBERG, 2004; VAN DER VLIET et al., 2004). Durante infecções, células NKT secretam citocinas pró-inflamatórias que estimulam respostas inata e adaptativa que eliminam o patógeno (EMOTO et al., 2003). Por outro lado, células NKT secretam citocinas antiinflamatórias que limitam infecções induzidas por patógenos (TANIGUCHI et al., 2003; VAN KAER, 2004). Ainda é obscuro como as células NKT exacerbam respostas inflamatórias para controlar patógenos durante infecções, enquanto favorecem uma resposta antiinflamatória em outras infecções, prevenindo dano tecidual induzido pela infecção. Recentemente, foi demonstrado em modelo experimental murino de infecção pelo T. cruzi, que as células NKT apresentam função importante em ambas as respostas, pró-inflamatória e antiinflamatória (DUTHIE et al., 2005). Esses autores sugerem que células NKT invariantes limitam respostas inflamatórias, enquanto células NKT variantes aumentam respostas inflamatórias que contribuem para a morbidade e mortalidade. Assim, subpopulações de células com funções distintas podem preferencialmente direcionar eventos imunomoduladores, enquanto outras direcionam para respostas imunes inflamatórias. Essas observações sugerem que o sucesso no controle da infecção pelo T. cruzi e da doença de Chagas envolve o desenvolvimento de uma resposta imune modulada suficiente para controlar a infecção na ausência de extensa destruição tecidual.

Ainda no contexto da imunomodulação, a rede de citocinas é um importante circuito que determina a morbidade da doença de Chagas. Estudos demonstram uma correlação entre a secreção de IFN-γ e o desenvolvimento de doença cardíaca grave e o papel de IL-10

controlando a imunopatologia (BAHIA-OLIVEIRA et al., 2000; GOMES et al., 2003). Entretanto, o fato de uma grande proporção de indivíduos indeterminados também produzirem níveis elevados de IFN-γ, em adição a outras citocinas inflamatórias levantou a hipótese de que mais do que uma mudança para um perfil de citocinas polarizadas, um fino balanço de citocinas inflamatórias e reguladoras derivadas de diferentes fontes possa contribuir para os mecanismos imuno-mediados relacionados às diferentes formas clínicas da doença. De fato, tem sido demonstrado que enquanto monócitos de pacientes das formas clínicas IND e CARD são capazes de produzir IL-10, e alguns IND produzem altos níveis de IFN-γ por linfócitos T, monócitos de IND produzem níveis mais altos de IL-10, quando comparados com monócitos de pacientes da forma clínica cardíaca (CORRÊA-OLIVEIRA et al., 1999; GOMES et al., 2003; SOUZA et al., 2004). Esses achados enfatizam que o balanço de citocinas representa um elemento chave no estabelecimento/manutenção das diferentes formas clínicas. Assim, avaliar a produção de diferentes citocinas com características inflamatórias e reguladoras produzidas por subpopulações de leucócitos da imunidade inata e adaptativa representa estratégia adequada para caracterização do perfil de indivíduos portadores das diferentes formas clínicas da doença de Chagas.

A indução e/ou modulação da resposta do hospedeiro a antígenos derivados do parasito não deve ser avaliada de forma limitada, restrita a funções imunológicas celulares. Existem evidências do envolvimento da resposta humoral nos eventos que acompanham a evolução da doença de Chagas. Anticorpos anti-T. cruzi são amplamente detectados em portadores da doença de Chagas (BRENER, 1980). Os estudos da imunidade humoral foram revolucionados por KRETTLI & BRENER (1982), quando foi demonstrada a existência de duas categorias de anticorpos, funcionalmente distintos, no soro de hospedeiros infectados pelo T. cruzi, os quais foram denominados anticorpos da sorologia convencional (ASC) e anticorpos líticos (AL). Os ASC, também denominados não protetores, são encontrados no

soro de hospedeiros infectados pelo T. cruzi mesmo após quimioterapia eficaz. Esses anticorpos são identificados in vitro por técnicas sorológicas de rotina. Por outro lado, anticorpos da categoria AL, também conhecidos como anticorpos protetores, são encontrados apenas no soro de hospedeiros com infecção ativa, desaparecendo após a cura por intervenção terapêutica específica para a doença de Chagas. Esses anticorpos são específicos para epitopos presentes na superfície de formas tripomastigotas vivas do T. cruzi, sendo identificados in vitro através das técnicas de lise mediada pelo complemento (GALVÃO et al., 1993) e, mais recentemente, pela citometria de fluxo (MARTINS-FILHO et al., 1995). Por serem capazes de induzir a lise mediada pelo complemento de formas tripomastigotas sangüíneas, os anticorpos da categoria AL têm sido considerados importantes no controle da parasitemia na fase crônica da infecção pelo T. cruzi, e assim, a sua presença, indireta ou diretamente, poderia estar associada ao estabelecimento da forma clínica IND. Entretanto, esse mecanismo hipotético necessita ainda de investigações complementares. Considerando que os estudos sorológicos baseados na citometria de fluxo constituem um campo com grandes possibilidades de crescimento, devido ao aumento da sensibilidade de detecção, em relação a outros métodos (MARTINS-FILHO et al., 1995), a avaliação da resposta humoral por meio da reatividade de anticorpos que se ligam a formas epimastigotas e tripomastigotas do T. cruzi por citometria de fluxo (FC-AFEA e FC-ALTA) permitirá ampliar o conhecimento desses eventos imunológicos. É importante salientar que esse sistema é isento de variabilidades metodológicas inerentes ao analista e com sensibilidade e especificidade muito superiores aos diferentes protocolos de detecção e revelação convencionais, podendo assim trazer novas perspectivas para os estudos sorológicos aplicados ao diagnóstico, prognóstico, e critério de cura da doença de Chagas (MARTINS-FILHO et al., 2002).

Em suma, considerando o exposto, podemos observar que diversos mecanismos da resposta imune têm sido propostos e apresentados como elos no estabelecimento/manutenção

das diferentes formas clínicas na fase crônica da doença de Chagas. Entretanto, o grande avanço em técnicas imunológicas tem permitido a elaboração de perguntas ainda mais refinadas e o estabelecimento de novas hipóteses que podem estar associadas ao complexo conjunto de interações que envolvem a doença de Chagas humana. Nesse sentido, acreditamos que o estudo de características fenotípicas adicionais de populações e subpopulações leucocitárias do sangue periférico, através do uso de novas estratégias de análises, bem como a investigação do panorama de citocinas intracitoplasmáticas de leucócitos circulantes, em paralelo ao estudo da reatividade de anticorpos IgG anti-T. cruzi, apresenta-se como uma estratégia atual e relevante para subsidiar o entendimento do fenômeno da cronificação diferenciada na doença de Chagas.

2. OBJETIVOS

2.1. Objetivo Geral

Investigar aspectos imunológicos celulares e humorais na fase crônica da doença de Chagas com ênfase no perfil fenotípico e panorama de citocinas intracitoplasmáticas de leucócitos circulantes e na reatividade de anticorpos IgG anti-Trypanosoma cruzi.

2.2. Objetivos Específicos

1. Caracterizar aspectos fenotípicos de leucócitos circulantes a fim de estabelecer biomarcadores capazes de decodificar diferentes formas clínicas da infecção crônica pelo T. cruzi.

2. Instituir um novo modelo para a análise do perfil panorâmico de citocinas intracitoplasmáticas de leucócitos do sangue periférico em portadores das formas clínicas indeterminada e cardíaca.

3. Explorar as aplicabilidades de técnicas não convencionais para o estudo da reatividade sorológica anti-T. cruzi aplicada em estudos clínicos da doença de Chagas.

2.3. Tópicos de Investigação

- Estudar de forma descritivo-analítica o perfil fenotípico de leucócitos do sangue periférico de indivíduos portadores das formas clínicas indeterminada, cardíaca e digestiva, com ênfase na análise de células T $CD4^+$ e $CD8^+$ ativadas, subpopulações de células NK e NKT e na freqüência de células T reguladoras (Artigo 1);
- Caracterizar o perfil fenotípico de leucócitos do sangue periférico de indivíduos portadores da fase crônica recente, focalizando em subpopulações de células T e B, marcadores de ativação e de adesão celular, subpopulações de monócitos, células NK e NKT e na freqüência de células T reguladoras (Artigo 2);
- Correlacionar os principais achados imunofenotípicos de leucócitos do sangue periférico observados em portadores das formas clínicas indeterminada e cardíaca (Artigo 2);
- Estabelecer uma nova estratégia para análise panorâmica do perfil de citocinas intracitoplasmáticas de leucócitos circulantes associados à imunidade inata e adaptativa (Artigo 3).;
- Caracterizar o impacto da estimulação in vitro com antígenos de formas tripomastigotas do T. cruzi no panorama de citocinas intracitoplasmáticas de leucócitos circulantes associados à imunidade inata e adaptativa (Artigo 3).
- Avaliar a aplicabilidade da pesquisa de anticorpos IgG anti-formas epimastigotas fixadas do T. cruzi (FC-AFEA) no diagnóstico sorológico da Doença de Chagas (Artigo 4);
- Caracterizar o potencial da pesquisa de anticorpos IgG1 anti-formas tripomastigotas vivas do T. cruzi (FC-ALTA) e da FC-AFEA para fins prognósticos na monitoração clínica da infecção crônica pelo T. cruzi (Artigo 4);
- Avaliar o desempenho da FC-AFEA como técnica complementar na monitoração de cura pós-terapêutica etiológica da Doença de Chagas (Artigo 4).

3. MATERIAL E MÉTODOS

3.1. População de estudo

Os dados que compõem as publicações 1 "Chagasic patients with indeterminate clinical form of the disease have high frequencies of circulating $CD3^{+}CD16^{-}CD56^{+}$ natural killer T cells and $CD4^{+}CD25^{High}$ regulatory T lymphocytes" e 2 "Are increased frequency of macrophage-like and natural killer (NK) cells, together with high levels of NKT and $CD4^{+}CD25^{high}$ T cells balancing activated $CD8^{+}$ T cells, the key to control Chagas' disease morbidity?" correspondem ao objetivo específico 1.

Para cumprir o proposto no objetivo específico 1, foram selecionados 54 indivíduos, sendo 13 crianças e 41 adultos. Todos os participantes foram submetidos a testes de sorologia convencional específicos para o diagnóstico da doença de Chagas. Os indivíduos pertencentes ao grupo de pacientes chagásicos apresentaram resultados concomitantemente positivos nas reações de hemaglutinação indireta (HAI) e imunofluorescência indireta (IFI) para o T. cruzi. Considerando que esse estudo teve como objetivo realizar uma análise de diferentes parâmetros imunológicos associados à morbidade da infecção pelo T. cruzi, todos pacientes foram submetidos a exames clínicos, radiológicos e eletrocardiográficos que permitiram agrupá-los como pacientes portadores da forma clínica indeterminada recente ("Early INDeterminate chronic phase" – E-IND), indeterminada crônica (IND), cardíaca crônica (CARD) e digestiva crônica (DIG). Indivíduos dos grupos E-IND e IND foram selecionados a partir da comprovação da infecção chagásica na ausência de manifestações clínicas, eletrocardiográficas ou radiológicas de acometimento cardíaco ou digestivo. A segregação entre os indivíduos crônicos recentes (E-IND) e tardios (IND) foi feita de acordo com o Consenso Brasileiro em Doença de Chagas (2005) que considera como recente o período de cinco a doze anos após a infecção inicial. Os portadores da forma clínica CARD foram caracterizados pela presença de sintomas, sinais físicos e alterações em exames laboratoriais (eletrocardiograma /ECG e radiografia de tórax). O grupo DIG foi definido a partir do

diagnóstico radiológico de megaesôfago e megacólon, característica desta forma clínica, baseando-se essencialmente no exame clínico e radiológico - raio X de tórax em posições PA e PE, raio X contrastado de esôfago em OAD (oblíqua anterior direita), pela técnica de HADDAD E GODOY (1963) e enema opaco (REZENDE & MOREIRA, 2000).

Sete crianças da mesma área, assintomáticas e sem sinais de outra enfermidade, foram submetidas a testes sorológicos que confirmaram a ausência de infecção pelo T. cruzi, constituindo assim o grupo não infectado pelo T. cruzi (NI-1) usado como parâmetro de comparação para avaliação de alterações imunofenotípicas observadas no grupo E-IND. Já como parâmetro de comparação para as análises realizadas no grupo IND foram selecionados adultos saudáveis com resultados negativos para os testes de sorologia convencional supracitados, constituindo o grupo NI-2.

Todos os indivíduos foram voluntários, sendo provenientes das cidades Bambuí, Berilo e José Gonçalves de Minas do estado de Minas Gerais. O consentimento escrito foi obtido de todos os participantes. No caso das crianças, os pais ou responsáveis foram esclarecidos quanto ao estudo e, aqueles que concordaram em participar, assinaram o Termo de Consentimento Livre e Esclarecido para Pacientes Voluntários. A conduta clínico-laboratorial adotada nesse estudo foi previamente aprovada pelo Comitê de Ética do IRR/FIOCRUZ (Protocolo CEPSH/CPqRR #09/2003 e #11/2004). Todos os procedimentos, incluindo os estudos eletrocardiográficos, radiológicos e a assistência médico-laboratorial dada aos pacientes, bem como o acompanhamento ambulatorial e a coleta de sangue esteve sob a responsabilidade do Dr. João Carlos Pinto Dias, do Dr. João Soares Moreira Magalhães e da Dra. Marta de Lana. A Tabela 1 sumariza as informações referentes aos grupos de pacientes estudados.

Os dados que compõem a terceira publicação " New strategy to assess the overall cytokine profile of circulating leukocytes and decode distinct clinical status of human chagas disease" correspondem ao objetivo específico 2.

O grupo de pacientes portadores da doença Chagas crônica foi o mesmo empregado para o primeiro e segundo artigo excluindo-se o grupo de indivíduos com comprometimento digestivo. Para a análise pontual do efeito do tratamento foram selecionados 18 indivíduos, sendo 5 indivíduos do grupo IND selecionados a partir da comprovação da infecção chagásica na ausência de manifestações clínicas, eletrocardiográficas ou radiológicas de acometimento cardíaco ou digestivo e 13 indivíduos portadores da forma clínica CARD, caracterizados pela presença de sintomas, sinais físicos e alterações em exames laboratoriais (eletrocardiograma e radiografia de tórax). Os indivíduos foram voluntários provenientes do Ambulatório de Doença de Chagas do Hospital das Clínicas de UFMG. O consentimento escrito foi obtido de todos os participantes. A conduta clínico-laboratorial adotada nesse estudo foi previamente aprovada pelo Comitê de Ética do IRR/FIOCRUZ (IRR/FIOCRUZ-Minas protocolos #09/2003) e da Universidade Federal de Minas Gerais (UFMG-COEP protocolo #ETIC 070/00). Todos os procedimentos, incluindo os estudos eletrocardiográficos, radiológicos e a assistência médico-laboratorial dada aos pacientes, bem como o tratamento dos indivíduos infectados com benzonidazol (5mg/kg/peso por dia, durante 60 dias consecutivos) estiveram sob a responsabilidade da Dra. Silvana Maria Elói Santos e da Dra. Eliane Dias Gontijo. Para o estudo longitudinal, foram feitas duas coletas (antes e após um ano do término do tratamento) de amostras de sangue periférico coletadas em tubos Vacutainer®, contendo EDTA ou heparina sódica como anticoagulantes. A Tabela 1 sumariza as informações concernentes aos grupos de pacientes estudados.

Os dados que compõem a quarta publicação " Non-conventional flow cytometry approaches to detect anti-Trypanosoma cruzi immunoglobulin G in the clinical laboratory." correspondem ao objetivo específico 3.

Para esse estudo foram empregadas 4 coortes de amostras de soro: (1) de pacientes portadores da doença de Chagas crônica tardia, de leishmaniose visceral (VL), leishmaniose tegumentar (LCL), toxoplasmose (TX), malária (MA) e esquistossomose (SCH). As amostras de soros de pacientes portadores da doença de Chagas crônica tardia e os controles não infectados foram os mesmos dos objetivos específicos 1 e 2. Já as amostras de soros de indivíduos portadores de outras infecções foram obtidas a partir de sorotecas previamente montadas e consistiram de indivíduos adultos que por decisão do comitê de ética em pesquisas não foram identificados; (2) de pacientes portadores da doença de Chagas crônica tardia segregados nas diferentes formas clínicas (IND, CARD e DIG) e controles não infectados, sendo os mesmos empregados nos objetivos específicos 1 e 2; (3) de pacientes portadores da doença de Chagas crônica tardia segregados nas diferentes formas clínicas. A coleta desses soros foi realizada no Centro de treinamento e Referência de doenças Infecciosas e Parasitárias (CTRDIP) do Hospital das Clínicas da Faculdade de Medicina da Universidade Federal de Minas Gerais, sob a supervisão do Dr. Manoel Otávio da Costa Rocha. As amostras de soros dos pacientes DIG foram enviadas para Belo Horizonte pela Dr^{a}. Sheila Jorge Adad do Departamento de Patologia Cirúrgica da Faculdade de Medicina do Triângulo Mineiro (FMTM). A distribuição etária dessa coorte iniciou uma dispersão etária na faixa de 24-77 anos de idade; (4) de pacientes chagásicos não tratados (NT), tratados não curados (TNC) e tratados curados (TC). Essa coorte contém 60 indivíduos com idades de 6 meses a 68 anos, acompanhados pelo Dr. Anis Rassi da Faculdade de Medicina da Universidade Federal de Goiás. Estes pacientes tiveram exames sorológicos (IFA e HA) e parasitológico (hemocultura) avaliados em um estudo retrospectivo, que variou de três a 26

anos após tratamento etiológico, e foram classificados em três diferentes categorias como descrito por MARTINS-FILHO et al. (2002). O tratamento foi realizado durante a fase aguda, sub-aguda, ou crônica da infecção. O critério de cura utilizado para a classificação dos grupos baseou-se na negativação conjunta dos testes sorológicos convencionais IFI, HAI e ELISA e do xenodiagnóstico. Indivíduos NT e TNC persistiram com resultados positivos tanto nos testes parasitológicos como sorológicos. Pacientes foram considerados TC somente quando ambos exames foram consistentemente negativos pelo menos em 8 ensaios de coleta serial de amostras de sangue. Nesse estudo não foi dada ênfase aos diferentes esquemas de tratamento uma vez que não foi nosso objetivo. A Tabela 1 sumariza as informações concernentes aos grupos de pacientes estudados.

Tabela 1: População de estudo

Grupo		Nº de indivíduos	Idade	Sexo (M/F)
Artigo 1				
Não infectado*	NI	12	20-53	3/9
Indeterminado*	IND	08	44-67	3/5
Cardíaco*	CARD	13	50-70	5/8
Digestivo*	DIG	08	45-65	5/3
Artigo 2				
Crianças não infectadas	NI-1	07	9-14	6/1
Adultos não infectados*	NI-2	12	20-59	3/9
Indeterminado recente	E-IND	06	9-14	4/2
Indeterminado*	IND	08	44-67	3/5
Cardíaco*	CARD	13	50-70	5/8
Artigo 3				
Coorte 1				
Não infectado*	NI	11	20-53	3/8
Indeterminado*	IND	06	44-67	3/3
Cardíaco*	CARD	08	50-70	2/6
Coorte 2				
Indeterminado	IND	05	35-46	1/4
Cardíaco	CARD	13	29-58	7/6
Artigo 4				
Coorte 1				
Não infectado*	NI	12	20-53	3/8
Doença de Chagas*	CH	28	44-70	13/15
Leishmaniose visceral	VL	26	-	-
Leishmaniose tegumentar	LCL	20	-	-
Toxoplasmose	TX	20	-	-
Malária	MA	20	-	-
Esquistossomose	SCH	20	-	-
Coorte 2				
Não infectado*	NI	12	20-53	3/9
Indeterminado*	IND	06	44-67	3/3
Cardíaco*	CARD	13	50-70	5/8
Digestivo*	DIG	07	45-65	4/3
Coorte 3				
Indeterminado	IND	10	24-59	8/12
Cardíaco	CARD	10	26-71	15/12
Digestivo	DIG	10	30-77	19/10
Coorte 4				
Não tratados	NT	19	34-68	10/9
Tratados não curados	TNC	17	12-66	9/8
Tratados curados	TC	24	6 meses-54	6/18

M= masculino; F= feminino. * Representam a mesma população

3.2. Análise do fenótipo celular dos leucócitos do sangue periférico

3.2.1. Protocolo de imunofenotipagem celular por citometria de fluxo

Os ensaios de imunofenotipagem dos leucócitos do sangue periférico foram feitos segundo protocolo proposto pelo fabricante, modificado conforme descrito a seguir.

Em tubos de poliestireno 12x75mm, foram adicionados 5µL do anticorpo monoclonal específico para o marcador de superfície celular de interesse marcado com fluorocromo (Tabela 2). Combinações específicas de anticorpos monoclonais marcados com fluorocromos distintos foram utilizadas para a análise simultânea de marcadores de superfície celular necessários para a caracterização de subpopulações celulares de interesse. Para cada combinação de anticorpos monoclonais, foram adicionadas alíquotas de 100µL de sangue periférico total coletado em EDTA. Após homogeneização em vórtex, as preparações foram incubadas por 30 minutos, à temperatura ambiente e ao abrigo da luz. Após o período de incubação, as amostras foram submetidas à lise dos eritrócitos, utilizando 2ml de solução de lise comercial (FACS® Lysing Solution – Becton Dickinson) diluída 10 vezes em água destilada. Após nova homogeneização em vórtex, as preparações foram incubadas por 10 minutos a temperatura ambiente e então submetidas à centrifugação (400×g, 10 minutos a 18°C). O sobrenadante foi descartado e os leucócitos lavados com 2mL de PBS (0,015M pH 7,4), empregando-se as mesmas condições de centrifugação anteriormente citadas. Numa etapa final, os leucócitos foram fixados com 200µL de solução fixadora (10g/L de paraformaldeído, 1 % de cacodilato de sódio, 6,65g/L de cloreto de sódio, pH 7,2). Após um período de pelo menos 15 minutos a 4°C, os parâmetros fenotípicos e morfométricos das células presentes em cada tubo foram determinados no citômetro de fluxo (FACScalibur® – Becton Dickinson). O programa CELLQuest® foi utilizado para a aquisição de dados e para a análise dos resultados empregando diferentes estratégias.

Tabela 2: Anticorpos monoclonais marcados com fluorocromos utilizados para análise de populações, subpopulações celulares e moléculas de superfície.

Anticorpo	Fluorocromo	Clone	Fenótipo alvo no estudo
Anti-CD3	FITC, PE	UCHT1	Linfócitos T, NKT
Anti-CD4	FITC, PE	13B8.2 ou RPA-T4	Linfócitos T auxiliares
Anti-CD5	FITC	L17F12	Linfócitos B1
Anti-CD8	FITC, TC	B9.11 ou M-L233	Linfócitos T citotóxicos
Anti-CD14	TC	TüK4	Monócitos
Anti-CD16	FITC, TC	3G8	Células NK, NKT, "Macrófago-like"
Anti-CD18	FITC	YF118.3	Linfócito T ativado
Anti-CD19	FITC, PE, TC	HID19 ou 4G7,	Linfócitos B
Anti-CD23	PE	M-L233	Subpopulações de Linfócito B
Anti-CD25	PE	3G10	Célula T reguladora
Anti-CD28	PE	15E8	Linfócito T ativado
Anti-CD38	PE	AT13/5	Linfócito T ativado
Anti-CD54	FITC	15.2	Linfócito T ativado
Anti-CD56	PE	B159	Células NK, NKT
Anti-CD62L	FITC	DREG-56	Linfócito T ativado
Anti-HLA-DR	PE	Tü36	Linfócito T ativado, Monóc. pró-inflamatórios

FITC = isotiocianato de fluoresceína; PE = ficoeritrina; TC = tricolo

3.2.2. Estratégias para análises imunofenotípicas de leucócitos circulantes

Os dados referentes à imunofenotipagem dos leucócitos do sangue periférico foram analisados através de diversas estratégias, dependendo do fenótipo celular rastreado. Assim, empregando os recursos múltiplos do programa CELLQuest™, foram adotadas diferentes estratégias para análise fenotípica denominadas: análise convencional (SATHLER-AVELAR et al., 2003); análise de células T reguladoras (BAECHER-ALLAN et al., 2001); análise do marcador CD56 em subpopulações de células $CD3^{-}CD16^{+}$ (GADDY et al., 1997), análise de subpopulações de células $CD3^{-}CD56^{+}$ (COOPER et al., 2001); análise de células NKT (DOHERTY, et al., 1999); análise combinada "gated" (SATHLER-AVELAR et al., 2003) e análise de monócitos pró-inflamatórios (BELGE et al., 2002).

3.2.2.1. Análise convencional

A análise convencional foi realizada segundo proposto por SATHLER-AVELAR et al. (2003). A Figura 1 ilustra a seqüência de passos para a análise convencional. Esse tipo de análise consistiu na seleção da população celular de interesse baseada em aspectos morfométricos, realizada através de gráficos de distribuição puntual de tamanho (FSC) versus granulosidade (SSC) (Figura 1A). Após a seleção da região de interesse (R1), o percentual de subpopulações celulares fluorescentes, dentro da população selecionada, foi obtido em gráficos bidimensionais de distribuição puntual de fluorescência, incluindo as modalidades FL1 versus FL2, FL2 versus FL3 ou FL1 versus FL3 (Figura 1B).

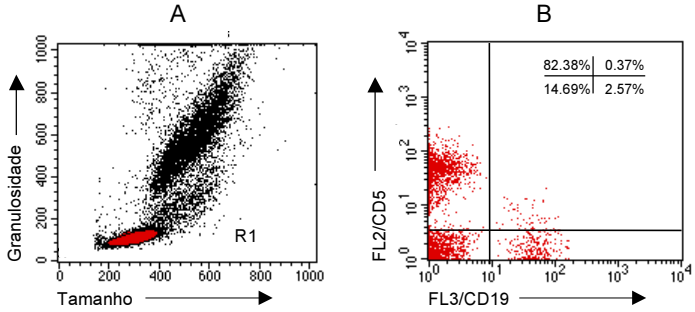


Figura 1: Seqüência de procedimentos utilizados para as análises dos percentuais de populações celulares por citometria de fluxo. (A) Gráfico de distribuição puntual FSC versus SSC utilizado para a seleção da população de interesse, nesse caso linfócitos pequenos – R1. (B) Gráfico de distribuição puntual FL1 versus FL2 utilizado para quantificar o percentual das populações ou subpopulações celulares específicas, confinadas em R1.

3.2.2.2. Análise de células T reguladoras

A análise de células T reguladoras foi realizada segundo proposto por BAECHER-ALLAN et al. (2001). A Figura 2 ilustra a seqüência de procedimentos para a análise de

células T reguladoras com fenótipo $CD4^{+}CD25^{HIGH}$. Após a seleção da região de interesse (R1), baseada em aspectos morfométricos, realizada através de gráficos de distribuição puntual de tamanho (FSC) versus granulosidade (SSC) (Figura 2A), gráficos de FL1/CD4 versus FL2/CD25 foram construídos, permitindo identificar a segregação da população $CD4^{+}$ em 3 subpopulações: $CD4^{+}CD25^{-}$ (R2), $CD4^{+}CD25^{LOW}$ (R3) e $CD4^{+}CD25^{HIGH}$ (R4). A fração celular confinada em R4, representa o valor percentual de células T reguladoras na população de linfócitos totais (Figura 2B).

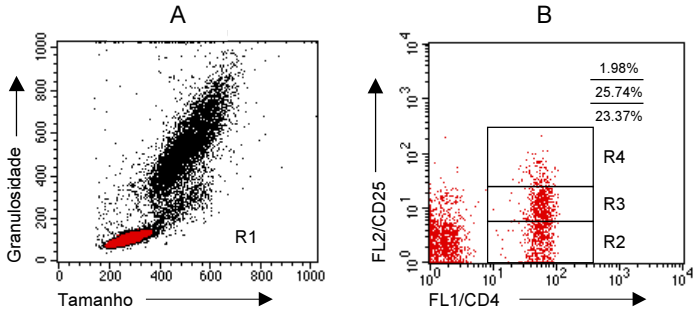


Figura 2: Seqüência de procedimentos utilizados para as análises dos percentuais de células T reguladoras ($CD4^{+}CD25^{HIGH}$) por citometria de fluxo. (A) Gráfico de distribuição puntual FSC versus SSC utilizado para a seleção da população de interesse, nesse caso linfócitos pequenos – R1. (B) Gráfico de distribuição puntual FL1/CD4 versus FL2/CD25 utilizado para quantificar o percentual de células T reguladoras ($CD4^{+}CD25^{HIGH}$), confinadas em R4.

3.2.2.3. Análise do marcador CD56 em subpopulações de células $CD3^{-}CD16^{+}$

A análise de subpopulações de células $CD3^{-}CD16^{+}$ foi realizada segundo proposto por GADDY et al. (1997). A Figura 3 ilustra a seqüência de procedimentos para a análise das subpopulações de células pré-NK ($CD3^{-}CD16^{+}CD56^{-}$) e células NK maduras ($CD3^{-}CD16^{+}CD56^{+}$). Após a seleção da região de interesse (R1), baseada em aspectos morfométricos, realizada através de gráficos de distribuição puntual de tamanho (FSC) versus granulosidade (SSC) (Figura 3A), foram construídos gráficos de FL1/CD3 versus FL3/CD16, onde uma nova região R2 foi construída, confinando a população $CD3^{-}CD16^{+}$ (Figura 3B).

Posteriormente, gráficos de FL1/CD3 versus FL2/CD56 foram empregados, onde uma nova região (R3) foi construída, confinando a população $CD3^{-}CD56^{+}$. Em seguida, após a combinação das regiões R1, R2 e R3 através das fórmulas "G2=R2+R3 e G3=R1 and G2" onde "+" representa o somatório de células confinadas nas regiões R1 e R2 e "and" designa a intercecção dos eventos presentes simultaneamente em G2 e R1. Em seguida gráficos de FL3/CD16 versus FL2/CD56, contendo as células confinadas em G3, foram utilizados para quantificar os percentuais das subpopulações $CD3^{-}CD16^{+}CD56^{-}$ e $CD3^{-}CD16^{+}CD56^{+}$.

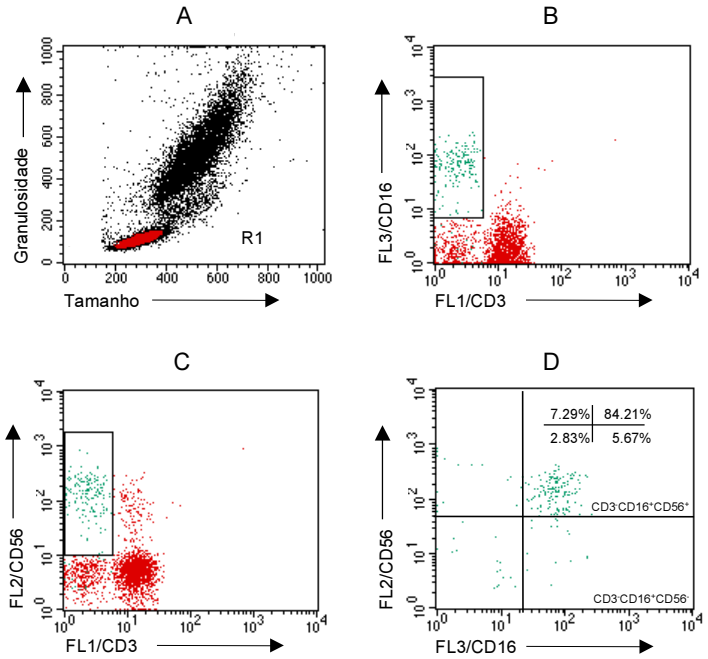
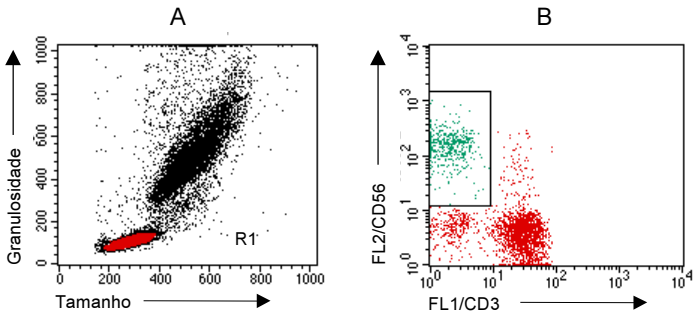


Figura 3: Seqüência de procedimentos utilizados para as análises dos percentuais das subpopulações de células NK: pré-NK ($CD3^{-}CD16^{+}CD56^{-}$) e NK madura ($CD3^{-}CD16^{+}CD56^{+}$) por citometria de fluxo. (A) Gráfico de distribuição puntual FSC versus SSC utilizado para a seleção da população de interesse, nesse caso linfócitos pequenos – R1. (B) Gráfico de distribuição puntual FL1/CD3 versus FL3/CD16 utilizado para selecionar a população celular $CD3^{-}CD16^{+}$ (R2). (C) Gráfico de distribuição puntual FL1/CD3 versus FL2/CD56 utilizado para selecionar a população celular $CD3^{-}CD56^{+}$ (R3). (D) Após a combinação das regiões R1, R2 e R3 através das fórmulas "G2=R2 + R3 e G3=R1 and G2" um gráfico de FL3/CD16 versus FL2/CD56 contendo as células confinadas em G3 foi utilizado para quantificar os percentuais das subpopulações de células NK.

3.2.2.4. Análise de subpopulações de células $CD3^{-}CD56^{+}$

A análise de células $CD3^{-}CD56^{+}$ foi realizada segundo proposto por COOPER et al. (2001). A Figura 4 ilustra a seqüência de procedimentos para a análise de células $CD3^{-}CD56^{DIM}CD16^{-/+}$ e $CD3^{-}CD56^{BRIGHT}CD16^{-/+}$. Após a seleção da região de interesse (R1), baseada em aspectos morfométricos, realizada através de gráficos de distribuição puntual de tamanho (FSC) versus granulosidade (SSC) (Figura 4A), foram construídos gráficos de FL1/CD3 versus FL2/CD56, onde uma região (R2) foi construída selecionando a população $CD3^{-}CD56^{+}$ (Figura 4B). O próximo passo consistiu na combinação das regiões R1 e R2 através da fórmula "G2=R1 and R2", onde "and" designa a interseção dos eventos presentes simultaneamente em R1 e R2. Em seguida, gráficos de FL3/CD16 versus FL2/CD56, contendo as células confinadas em G2, foram utilizados para quantificar os percentuais das subpopulações $CD3^{-}CD56^{DIM}CD16^{-/+}$ e $CD3^{-}CD56^{BRIGHT}CD16^{-/+}$ (Figura 4C).



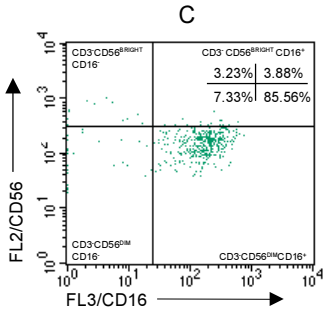


Figura 4: Seqüência de procedimentos utilizados para as análises dos percentuais das subpopulações $CD3^{-}CD56^{DIM}CD16^{-/+}$ e $CD3\text{-}CD56^{BRIGHT}CD16^{-/+}$ por citometria de fluxo. (A) Gráfico de distribuição puntual FSC versus SSC utilizado para a seleção da população de interesse, nesse caso linfócitos pequenos – R1. (B) Gráfico de distribuição puntual FL1/CD3 versus FL2/CD56 (R2) utilizado para selecionar a população celular $CD3^{-}CD56^{+}$ - R2. (C) Após a combinação das regiões R1 e R2, através da fórmula " G2= R1 and R2" , um gráfico de FL3/CD16 versus FL2/CD56, contendo as células confinadas em G2, foi empregado para quantificar o percentual das subpopulações $CD3^{-}CD56^{DIM}CD16^{-/+}$ e $CD3\text{-}CD56^{BRIGHT}CD16^{-/+}$.

3.2.2.5. Análise de células NKT

A análise de células NKT foi realizada segundo proposto por DOHERTY el al. (1999) e modificado por MARTINS-FILHO (2000). A Figura 5 ilustra a sequência de procedimentos para a análise de células NKT ($CD3^{+}CD16^{-/+}CD56^{-/+}$). Após a seleção da região de interesse (R1), baseada em aspectos morfométricos, realizada através de gráficos de distribuição puntual de tamanho (FSC) versus granulosidade (SSC) (Figura 5A), foram construídos gráficos de FL1/CD3 versus FL3/CD16, onde uma região (R2) foi construída selecionando a população $CD3^{+}CD16^{-/+}$ (Figura 5B). O próximo passo consistiu na combinação das regiões R1 e R2 através da fórmula "G2=R1andR2", onde "and" designa a interseção dos eventos presentes simultaneamente em R1 e R2. Em seguida, gráficos de FL3/CD16 versus FL2/CD56, contendo as células confinadas em G2, foram utilizados para quantificar os percentuais das seguintes subpopulações, por nós definidas: NKT1 ($CD3^{+}CD16^{+}CD56^{-}$), NKT2 ($CD3^{+}CD16^{-}CD56^{+}$) e NKT3 ($CD3^{+}CD16^{+}CD56^{+}$) (Figura 5C).

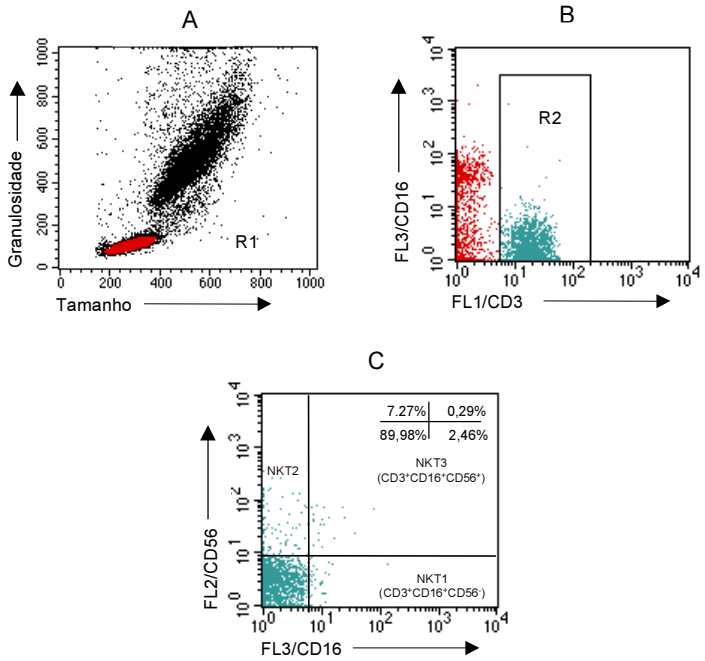


Figura 5: Seqüência de procedimentos utilizados para as análises dos percentuais das subpopulações NKT1 ($CD3^+CD16^+CD56^-$), NKT2 ($CD3^+CD16^-CD56^+$) e NKT3 ($CD3^+CD16^+CD56^+$) por citometria de fluxo. (A) Gráfico de distribuição puntual FSC versus SSC utilizado para a seleção da população de interesse, nesse caso linfócitos pequenos – R1. (B) Gráfico de distribuição puntual FL1/CD3 versus FL3/CD16 (R2) utilizado para selecionar a população celular $CD3^+$ - R2. (C) Após a combinação das regiões R1 e R2, através da fórmula "G2=R1andR2", um gráfico de FL3/CD16 versus FL2/CD56, contendo as células confinadas em G2, foi empregado para quantificar o percentual das subpopulações NKT1, NKT2 e NKT3.

3.2.2.6. Análise combinada "gated"

A análise de células $CD4^+$ e $CD8^+$ ativadas ($HLA\text{-}DR^+$) foi realizada segundo proposto por SATHLER-AVELAR (2003). A Figura 6 ilustra a seqüência de procedimentos para a análise das subpopulações de células T ativadas. Após a seleção da região de interesse, baseada em aspectos morfométricos, realizada através de gráficos de distribuição pontual de tamanho (FSC) versus granulosidade (SSC) (Figura 6A), foram construídos histogramas

individuais de FL1/CD4 ou FL1/CD8, onde uma nova região R2 foi construída, confinando a população $CD4^+$ ou $CD8^+$ (Fig.6B). O próximo passo consistiu na combinação das regiões R1 e R2 através da fórmula "G2=R1 and R2", onde "and" designa a interseção dos eventos presentes simultaneamente em R1 e R2. Em seguida, histogramas unidimensionais de FL2/HLA-DR, contendo as células presentes em G2, foram utilizados para quantificar os percentuais das subpopulações $CD4^+HLA\text{-}DR^+/CD4^+$ ou $CD8^+HLA\text{-}DR^+/CD8^+$ (Figura 6C).

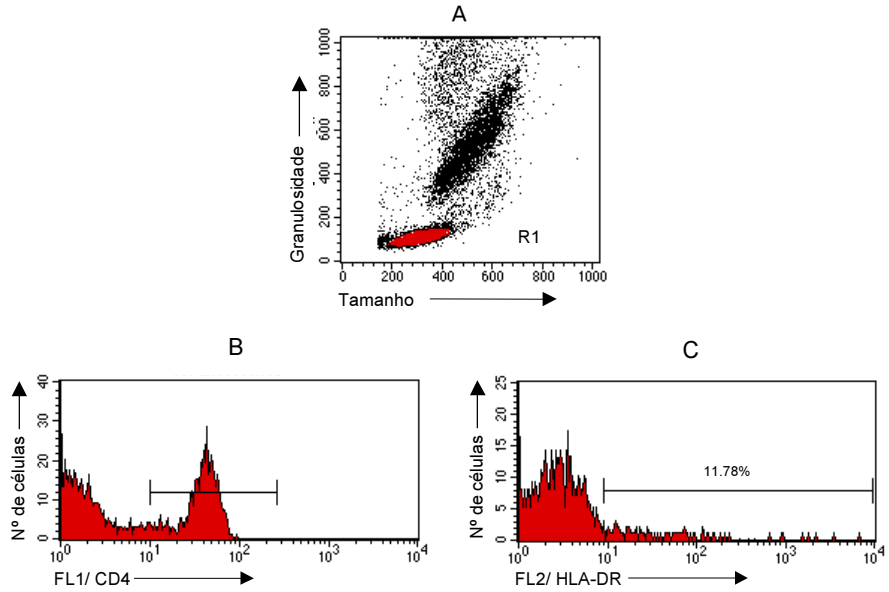


Figura 6: Seqüência de passos utilizados para as análises de subpopulações de células T $CD4^+$ ou $CD8^+$ ativadas ($HLA\text{-}DR^+$) por citometria de fluxo. (A) Gráfico de distribuição puntual FSC versus SSC utilizado para a seleção da população de interesse, nesse caso linfócitos pequenos – R1. (B) Histograma unidimensional de fluorescência FL1/CD4 utilizado para seleção da população de interesse – R2. (C) Após a combinação das duas regiões selecionadas (R1 "and" R2) garantindo a presença simultânea da população de interesse nas duas regiões selecionadas, o percentual da subpopulação celular foi identificado em histograma unidimensional de fluorescência – M1.

3.2.2.7. Análise de monócitos pró-inflamatórios

A análise de monócitos pró-inflamatórios ($CD14^{+}CD16^{+}HLA\text{-}DR^{++}$) foi realizada segundo proposto por BELGE et al. (2002). A Figura 7 ilustra a seqüência de procedimentos para a análise das subpopulações de monócitos pró-inflamatórios. Após a seleção da região de interesse (R1), baseada em aspectos morfométricos e imunofenotípicos, realizada através de gráficos de distribuição puntual de FL3/CD14 versus granulosidade (SSC) (Figura 7A), foram construídos gráficos de FL3/CD14 versus FL1/CD16, onde uma região (R2) foi construída selecionando a população $CD14^{+}CD16^{+}$ (Figura 7B). O próximo passo consistiu na combinação das regiões R1 e R2 através da fórmula "G2=R1andR2", onde "and" designa a interseção dos eventos presentes simultaneamente em R1 e R2. Em seguida, gráficos de FL3/CD14 versus FL2/HLA-DR, contendo as células confinadas em G2, foram utilizados para quantificar o percentual de células $CD14^{+}CD16^{+}HLA\text{-}DR^{++}$ (Figura 7C).

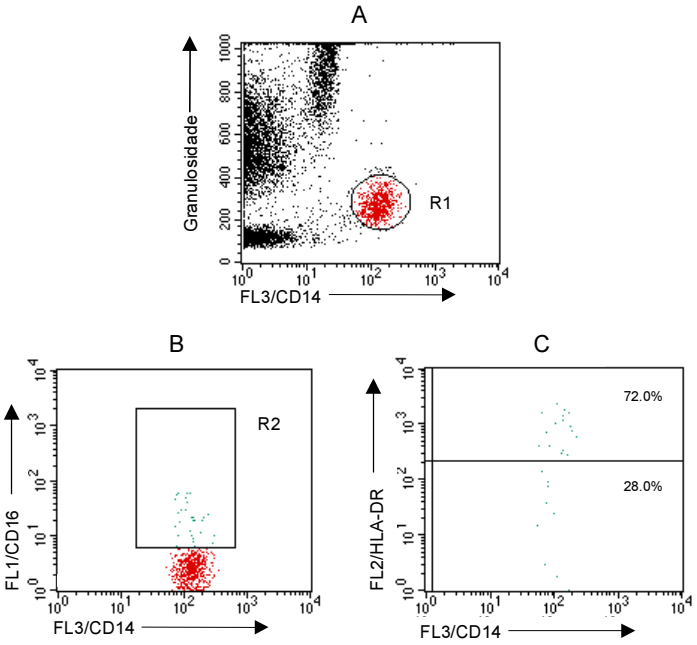


Figura 7: Seqüência de procedimentos utilizados para as análises dos percentuais de monócitos pró-inflamatórios ($CD14^{+}CD16^{+}HLA\text{-}DR^{++}$) por citometria de fluxo. (A) Gráfico

de distribuição puntual FL3/CD14 versus granulosidade (SSC) utilizado para a seleção da população de interesse, nesse caso células $CD14^+$ de granulosidade intermediária, correspondente aos monócitos – R1. (B) Gráfico de distribuição puntual FL3/CD14 versus FL1/CD16 (R2) utilizado para selecionar a população $CD14^+CD16^+$ – R2. (C) Após a combinação das regiões R1 e R2, através da fórmula " G2=R1 and R2", um gráfico de FL3/CD14 versus FL2/HLA-DR, contendo as células confinadas em G2, foi empregados para quantificar o percentual de monócitos pró-inflamatórios.

3.3. Avaliação do padrão de produção de citocinas citoplasmáticas em leucócitos circulantes

3.3.1. Obtenção do antígeno solúvel de formas tripomastigotas do T. cruzi

3.3.1.1. Cultivo de formas tripomastigotas do T. cruzi

Formas tripomastigotas de cultura de tecido foram obtidas a partir do sobrenadante de culturas de células LLCMK2 infectadas pelo T. cruzi. As células foram semeadas em frascos de cultura de tecido de $75cm^2$ (Falcon) com meio DMEM-5% SBF. Após a adesão e formação da monocamada, as células foram infectadas com tripomastigotas sanguíneos da cepa CL do T. cruzi na proporção de 10 parasitos/célula. As garrafas foram mantidas em estufa a 37°C, 5% de CO_2, 95% de umidade por 24h para favorecer a infecção. Após esse período, as monocamadas foram lavadas para retirar os parasitos remanescentes e mantidas com meio DMEM-5% SBF a 33°C, 5% de CO_2, 95% de umidade (BERTELLI et al. 1977). O meio foi trocado diariamente, e a partir do sexto dia de cultura formas tripomastigotas puderam ser coletadas no sobrenadante. Os parasitos foram separados dos restos celulares, por centrifugação diferencial. Inicialmente, a suspensão de parasitos foi centrifugada em tubos cônicos de 50ml à temperatura ambiente, 100g por 10 minutos. Posteriormente, os tubos foram mantidos a temperatura ambiente por 30 minutos para que as formas flageladas pudessem se deslocar do sedimento para o sobrenadante. O sobrenadante foi coletado e centrifugado a 4°C, 1.000g por 10 minutos para obtenção de formas tripomastigotas.

3.3.1.2. Preparo do antígeno solúvel de formas tripomastigotas do T. cruzi

As formas tripomastigotas obtidas de cultura de tecido foram lavadas 3 vezes em PBS-1X nas condições anteriores. Após a obtenção da massa de parasitos procedeu-se o preparo do antígeno solúvel. Essa foi transferida para o cone de vidro do aparelho Virtex e submetida a 10 ciclos de 30 rpm. Em seguida, o extrato foi transferido para um tubo Falcon de 50mL e levado ao nitrogênio líquido por 1 minuto e ao banho-maria a 37°C por 10 min. Este procedimento foi realizado 3 vezes. Uma alíquota do extrato antigênico foi observada em microscópio óptico, a fim de verificar o total rompimento dos parasitos e a formação de massa antigênica uniforme. Posteriormente, a massa antigênica foi centrifugada a 37.000g, 4°C por 70 minutos e o sobrenadante foi dialisado em PBS e filtrado em membrana 0,22µm. Uma alíquota foi empregada para a dosagem de proteínas pelo método de LOWRY et al., (1951). A preparação antigênica foi conservada a -70°C em alíquotas com concentração de 1mg/mL, para uso posterior nos ensaios de avaliação do perfil de produção de citocinas após estimulação curta antígeno-específica in vitro.

3.3.2. Protocolo de cultura de curta duração in vitro empregando amostras de sangue total

A metodologia proposta foi adaptada segundo protocolos originais descritos por PICKER et al. (1995), CARROCK-SEWELL et al. (1997) e SUNI et al. (1998).

Para a identificação das citocinas intracitoplasmáticas, alíquotas de 500µL de sangue periférico, coletado a vácuo em tubos de 10mL contendo heparina sódica, foram adicionadas a 2 tubos de polipropileno de 14mL (Falcon 2059), correspondentes às culturas controle e estimulada com antígeno solúvel das formas tripomastigotas do T. cruzi (TRIPO).

A cultura controle recebeu 500µL de meio de cultura RPMI 1640 (GIBICO – Grand Island, NY) e 10µL de Brefeldina A-BFA (Sigma), numa concentração de 10 µg/mL e foram incubadas por 4 horas em estufa a 37°C, 5% de CO_2. A BFA promove a retenção da citocina no complexo de Golgi, permitindo a sua detecção no interior dos leucócitos.

A cultura estimulada com antígeno solúvel foi previamente incubada, por 1 hora em estufa a 37°C, 5% de CO_2, na presença de 100µL do antígeno solúvel de formas tripomastigotas do T. cruzi , numa concentração final de 20µg/mL. Em seguida foram adicionados 10µL de BFA, numa concentração final de 10 µg/mL e a cultura re-incubada por 4 horas em estufa a 37°C, 5% de CO_2.

Culturas controle positivo foram realizadas com o objetivo de avaliar a viabilidade das amostras. Em tubos de polipropileno de 14mL (Falcon 2059) foram adicionados 500µL de sangue periférico, 500µL de RPMI 1640 mais Phorbol 12-Myristate 13-Acetate (Sigma, St Louis. MO, USA) na concentração de 25ng/ml, ionomicina (Sigma, St Louis. MO, USA) na concentração de1µg/mL e 10µL de Brefeldina A-BFA (Sigma) na concentração de 10 µg/mL. As culturas foram incubadas por 4 horas em estufa a 37°C, 5% de CO_2. Os padrões de citocinas observados nas culturas controle positivo confirmaram a viabilidade das amostras de sangue, como demonstrado pelos elevados níveis de células IFN-γ^+ e TNF-α^+ (dado não mostrado). Ao término da incubação, as culturas foram tratadas com 110µl EDTA (Sigma), numa concentração final de 2mM, e incubadas por 15 minutos à temperatura ambiente. Este procedimento bloqueia o processo de ativação posterior das células e garante a obtenção de resultados padronizados.

3.3.3. Protocolo de marcação de citocinas intracitoplasmáticas em leucócitos do sangue periférico após cultura de curta duração in vitro

Alíquotas de sangue total submetidas ao protocolo de cultura de curta duração in vitro foram lavadas duas vezes com 6mL de tampão de lavagem – PBS-W (0,015M de PBS 1X, 0,5% albumina sérica bovina – BSA e 0,1% de azida sódica), por centrifugação a 600×g durante 7 minutos a 18ºC. Após a última lavagem, a células foram ressuspendidas em 1,5mL de PBS-W. Após a centrifugação, o sedimento foi ressuspendido em 1,5mL de PBS-W e alíquotas de 400µL foram distribuídas em tubos de poliestireno de 5mL (Falcon- 2052) contendo (Tubo 1) 20µL de anticorpo monoclonal de camundongo anti-CD4-TC e anti-CD8-FITC, e alíquotas de 200µL em tubos contendo respectivamente, (Tubo 2) 15µL de anti-CD14-TC e (Tubo 3) 15µL de anti- CD5-FITC e 15µL de anti CD19-TC (Tabela 3). As amostras foram incubadas por 30 minutos à temperatura ambiente e ao abrigo da luz. Após a etapa da identificação das populações celulares, procedeu-se à lise dos eritrócitos e à fixação dos leucócitos pelo tratamento com 2mL de solução de lise (FACS^{TM} Lysing Solution – BD) por 10 minutos à temperatura ambiente e ao abrigo da luz. Após a fixação, a suspensão de leucócitos foi centrifugada a 600×g durante 7 minutos a 18ºC, o sobrenadante descartado e as células permeabilizadas com 2mL de solução permeabilizante – PBS-P (PBS-W e 0,5% de saponina – Sigma), por 10 minutos à temperatura ambiente e ao abrigo da luz. Após a permeabilização, as suspensões de leucócitos foram centrifugadas a 600×g durante 7 minutos, o sobrenadante descartado e as células lavadas com 3mL de PBS-W. As células foram ressuspendidas em dois volumes de PBS-W: 200µL, para os tubos contendo anti-CD4 TC e anti-CD8 TC e 100µL para os tubo contendo anti-CD14 TC e anti-CD19 TC. Após a ressuspensão das células, procedeu-se à marcação das citocinas intracitoplasmáticas em placas de 96 poços e fundo em "U" (Thomas 9383-A90). Para isso, alíquotas de 30µL das suspensões celulares, contendo aproximadamente 300.000 células, foram incubadas por 30 minutos à temperatura ambiente, ao abrigo da luz na presença de 20µL da suspensão de anticorpos anti-citocinas humanas, conjugados com o fluorocromo PE (anti-IFN-γ, anti-TNF-

α, anti-IL-4, anti-IL-10, anti-IL-12, anti-IL-13 – Tabela 4) previamente diluídos a 1:100 em PBS-P estéril. Após a incubação, as células foram lavadas com 150µl de PBS-P e, em seguida, com 200µl de PBS-W. As preparações celulares foram então fixadas em 200µl de solução fixadora - MFF (10g/L de paraformaldeído, 1% de cacodilato de sódio, 6,67g/L de cloreto de sódio, pH 7,2). As amostras contendo a suspensão celular a ser analisada foram transferidas para tubos de 500µL (Thomas laboratory Specialities) e estocadas a 4ºC ao abrigo da luz até a sua leitura no citômetro de fluxo dentro de 24 horas.

Tabela 3: Anticorpos monoclonais marcados com fluorocromos utilizados para análise de populações e subpopulações de leucócitos do sangue periférico.

Anticorpo	Fabricante	Clone	Concentração	População Alvo
Anti-CD4 TC	Caltag	S3.5	0,5µg/mL	Linfócito T auxiliar
Anti-CD8 FITC	BD	HIT8a	0,5µg/mL	Linfócito T citotóxico
Anti-CD14 TC	Caltag	Tük4	0,5µg/mL	Monócitos
Anti-CD5 FITC	BD	L17F12	0,5µg/mL	Linfócito B1
Anti-CD19 TC	Caltag	4G7/SJ25-C1	0,5µg/mL	Linfócitos B

Tabela 4: Anticorpos monoclonais utilizados para identificação de citocinas intracelulares em leucócitos do sangue periférico.

Anticorpo	Fabricante	Clone	Concentração	População Alvo
Anti-IFN-γ PE	BD	4S.B3	0,25µg/mL	Neutrófilos, Linfócitos
Anti-TNF-α PE	BD	Mab11	0,25µg/mL	Monócitos, Neutrófilos, Células NK, Linfócitos, Células B e B1
Anti-IL-4 PE	BD	8D4-8	0,25µg/mL	Neutrófilos, Células NK, Linfócitos
Anti-IL-10 PE	BD	JES3-19F1	0,25µg/mL	Monócitos, Neutrófilos, Linfócitos, Células B e B1
Anti-IL-12 PE	BD	C11.5	0,25µg/mL	Neutrófilos, Linfócitos

Anti-IL-13 PE	BD	JES10-5A2	0,25µg/mL	Neutrófilos, Linfócitos

3.3.4. Aquisição de dados para avaliação do perfil de expressão de citocinas intracelulares

Um total de 30.000 eventos/tubo foram obtidos usando o citômetro de fluxo FACScalibur® flow cytometer (Becton Dickinson, San Jose, CA, USA). Para a aquisição e análise dos dados utilizamos o software CellQuest software (Franklin Lakes, NJ, USA), como recomendado pelo fabricante.

3.3.5. Estratégias de análises de citocinas intracitoplasmáticas em subpopulações de leucócitos circulantes

Os dados referentes à pesquisa de citocinas intracitoplasmáticas em leucócitos do sangue periférico, foram analisados através de diversas estratégias, dependendo do fenótipo celular rastreado. Assim, empregando os recursos múltiplos do programa CELLQuest™, foram adotadas diferentes estratégias para análise fenotípica denominadas: análise de citocinas intracitoplasmáticas em monócitos (PERUHYPE-MAGALHÃES et al., 2006); análise de citocinas intracitoplasmáticas em neutrófilos (PERUHYPE-MAGALHÃES et al., 2006); análise de citocinas intracitoplasmáticas em células NK, análise de citocinas intracitoplasmáticas em linfócitos totais (SATHLER-AVELAR et al., 2003); análise de citocinas intracitoplasmáticas em subpopulações de linfócitos T e B (PERUHYPE-MAGALHÃES et al., 2006) e análise de citocinas intracitoplasmáticas em linfócitos B1.

3.3.5.1. Análise de citocinas intracitoplasmáticas em monócitos

A análise da produção de citocinas por monócitos foi feita a partir da construção de gráficos de fluorescência FL3/anti-CD14 TC versus granulosidade (SSC) e, os monócitos

discriminados como células $SSC^{low}CD14^{high}$ (Figura 8A). Já a análise da expressão de citocinas por estas células foi determinada em gráficos bidimensionais de distribuição pontual de fluorescência FL3/anti-CD14 TC versus FL2/anti-citocinas-PE (Figura 8B).

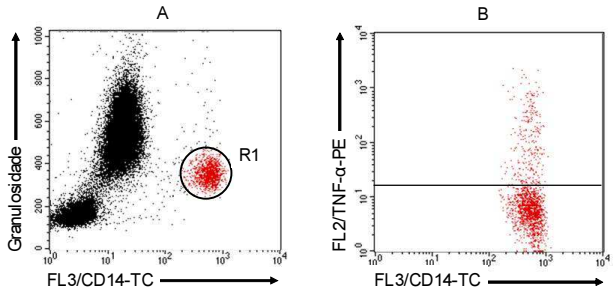


Figura 8: Análise da produção de citocinas citoplasmáticas por monócitos do sangue periférico por citometria de fluxo. (A) Gráfico de distribuição pontual FL3/anti-CD14 TC versus SSC utilizado para a seleção da população de monócitos – R1. (B) Gráfico de distribuição pontual FL3/anti-CD14-TC versus FL2/anti-TNF-α-PE utilizado para quantificar o percentual de células produtoras de citocinas em R1.

3.3.5.2. Análise de citocinas intracitoplasmáticas em neutrófilos

A análise da produção de citocinas por neutrófilos foi feita a partir da construção de gráficos de FL3/anti-CD14-TC versus SSC e, os neutrófilos discriminados como células $CD14^{low+}$ (R1). Já a análise da expressão de citocinas por estas células foi determinada em gráficos bidimensionais de distribuição pontual de SSC versus FL2/anti-citocinas-PE (Figura 9).

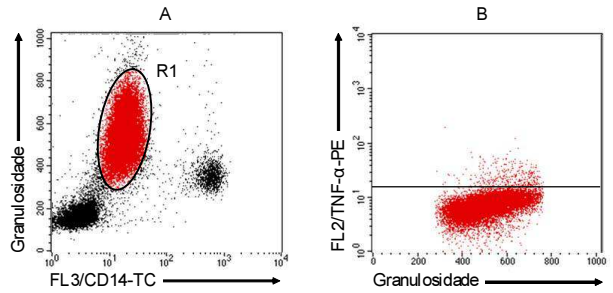


Figura 9: Análise da produção de citocinas citoplasmáticas por neutrófilos do sangue periférico por citometria de fluxo. (A) Gráfico de distribuição pontual FL3/anti-CD14 TC versus SSC utilizado para a seleção da população de neutrófilos – R1. (B) Gráfico de distribuição pontual SSC versus FL2/anti-TNF-α-PE utilizado para quantificar o percentual de células produtoras de citocinas em R1.

3.3.5.3. Análise de citocinas intracitoplasmáticas em células NK

A análise da produção de citocinas por células NK foi feita a partir da construção de gráficos de fluorescência tamanho (FSC) versus granulosidade (SSC) e, os linfócitos discriminados como células $FSC^{low}SSC^{low}$ (R1). Em seguida, foi realizada seleção da população $CD19^{-}CD5^{-}$ em gráficos de fluorescência FL3/anti-CD19-TC versus FL1/anti-CD5-FITC (R2). Essa estratégia foi definida uma vez que antígeno solúvel de formas tripomastigotas do T. cruzi leva à queda da expressão de CD16 na presença de soro autólogo, o que dificulta a identificação de células NK pela estratégia clássica. Além disso, essa estratégia garante a exclusão tanto de células B ($CD19^{+}$) e B1 quanto de células T e NKT ($CD5^{+}$). Uma vez definida as especificações do gate (G1) definido como "G1=R1 and R2", a freqüência de células NK produtoras de citocinas foi calculada dentro de G1 em gráficos de FSC versus FL2/anti-citocina-PE (Figura 10).

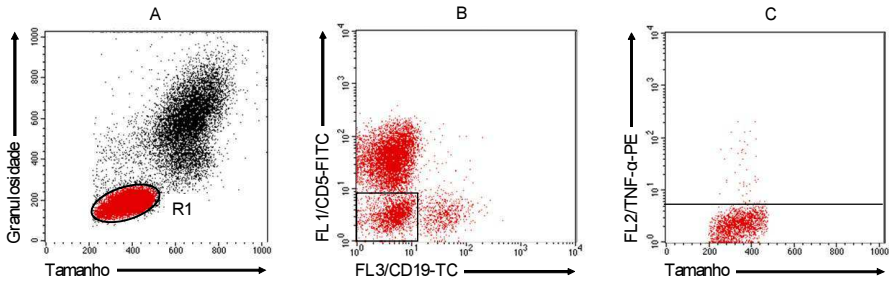


Figura 10: Análise da produção de citocinas citoplasmáticas por células NK do sangue periférico por citometria de fluxo. (A) Gráfico de distribuição pontual FSC versus SSC utilizado para a seleção da população de linfócitos – R1. (B) Gráfico de distribuição pontual FL1/anti-CD5-FITC versus FL3/anti-CD19-TC utilizado para a seleção da população de

células NK.- $CD19^{-}CD5^{-}$ (C) Gráfico de distribuição pontual FSC versus FL2/TNF-α-PE utilizado para quantificar o percentual de células produtoras de citocinas em "G1=R1 and R2".

3.3.5.4. Análise de citocinas intracitoplasmáticas em linfócitos totais

A análise da produção de citocinas por linfócitos totais foi feita a partir da seleção da população de interesse em gráficos de distribuição pontual de FSC versus SSC, sendo os linfócitos discriminados como células $FSC^{low}SSC^{low}$ (R1) Após a seleção correta da população de interesse, a freqüência de linfócitos totais produtores de citocinas foi quantificada em gráficos de FSC versus FL2/anti-citocinas-PE (Figura 11).

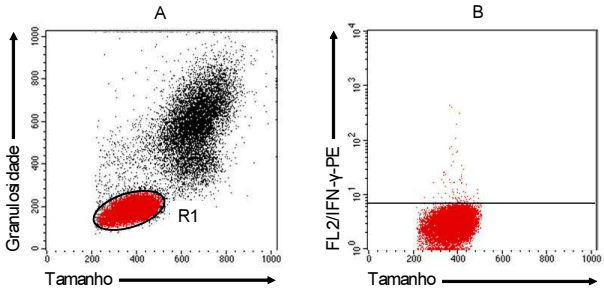


Figura 11: Análise da produção de citocinas citoplasmáticas por linfócitos totais do sangue periférico por citometria de fluxo. (A) Gráfico de distribuição pontual de FSC versus SSC utilizado para a seleção da população de linfócitos totais – R1. (B) Gráfico de distribuição pontual FSC versus FL2/anti-IFN-γ-PE utilizado para quantificar o percentual de células produtoras de citocinas em R1.

3.3.5.5. Análise de citocinas intracitoplasmáticas em subpopulações de linfócitos T e por linfócitos B

A análise da produção de citocinas por subpopulações de linfócitos T e B foi feita a partir da construção de uma região (R1) na população celular de interesse em gráficos de SSC versus FL1/anti-CD4-FITC, ou FL3/anti-CD8-TC ou FL3/anti-CD19-TC. Após a seleção correta da população de interesse, a freqüência de células B produtoras de citocinas foi quantificada em gráficos de FSC versus FL2/anti-citocinas-PE (Figura 12)

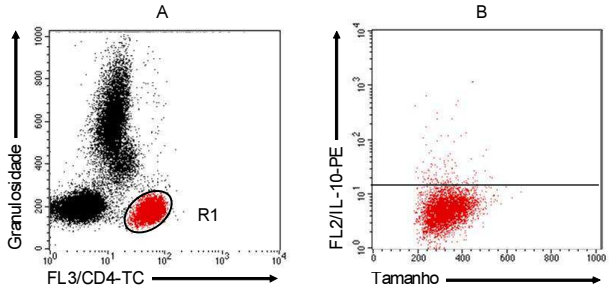


Figura 12: Análise da produção de citocinas citoplasmáticas por células T-CD4$^+$ do sangue periférico por citometria de fluxo. (A) Gráfico de distribuição pontual SSC versus FL3/CD4-TC utilizado para a seleção da população de linfócitos T CD4$^+$ – R1. (B) Gráfico de distribuição pontual FSC versus FL2/anti-IL-10-PE utilizado para quantificar o percentual de linfócitos T CD4$^+$ produtores de citocinas em "G1=R1".

3.3.5.6. Análise de citocinas intracitoplasmáticas em células B1

A análise da produção de citocinas por células B1 foi feita a partir da definição de uma região (R1) em gráficos de FSC versus SSC. Em seguida, células B1 (CD19$^+$CD5$^+$) foram selecionadas em uma segunda região (R2) em gráficos de FL3/anti-CD19 versus FL1/anti-CD5. Uma vez definidas as especificações do gate (G1) como "G1=R1 and R2", a freqüência de células B1 produtoras de citocinas foram quantificadas em gráficos de SSC versus FL2/anti-citocina-PE (Figura 13).

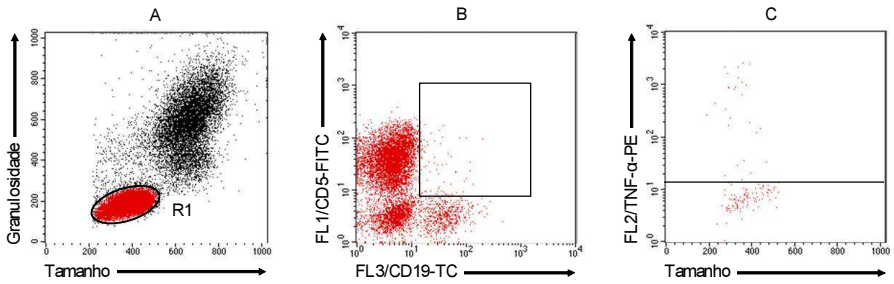


Figura 13: Análise da produção de citocinas citoplasmáticas por células B1 do sangue periférico por citometria de fluxo. (A) Gráfico de distribuição pontual FSC versus SSC utilizado para a seleção da população de linfócitos – R1. (B) Gráfico de distribuição pontual FL3/CD19-TC versus FL2/anti-CD5-FITC utilizado para a seleção da população de células B1 – $CD19^{+}CD5^{+}$. (C) Gráfico de distribuição pontual FSC versus FL2/anti-TNF-α-PE utilizado para quantificar o percentual de células produtoras de citocinas em "G1=R1 and R2".

3.3.6. Modelo panorâmico de análise imunofenotípica por Citometria de Fluxo

Inicialmente o percentual de células citocinas^{+} obtidos por citometria de fluxo para uma dada população celular foi empregado numa estratégia de análise que consistiu de 5 etapas. 1ª- Calcular a mediana global: para cada subpopulação de células citocinas^{+} foi considerado todo o conjunto de valores obtidos para os grupos clínicos inseridos no estudo para calcular a mediana global; 2ª- Categorizar baixo e alto produtores de citocinas: para cada subpopulação celular a mediana global de células citocinas^{+} foi utilizada como o ponto de corte para identificar indivíduos baixo (<mediana global) e alto (≥ mediana global) produtores de citocinas; 3ª- Compilar dados em diagramas multicor: os dados referentes as categorias supracitadas foram compilados em diagramas, empregando cores específicas para designar baixo produtores (□), alto produtores de citocinas inflamatórias (■) e alto produtores de citocinas reguladoras (▭) para cada subpopulação leucocitária avaliada. 4ª- Calcular o balanço de citocinas para cada subpopulação de leucócitos: os dados obtidos nos diagramas multicolor foram empregados para definir o balanço de citocinas para cada população celular, definindo quatro categorias de acordo com o predomínio de baixo produtores, alto produtores

de citocinas inflamatórias, alto produtores de citocinas reguladoras e perfil misto (▭) em situações de equivalência entre alto produtores de citocinas inflamatórias e reguladoras. 5ª- Estabelecer o panorama de citocinas dos leucócitos circulantes: foi calculado para a população total de leucócitos o perfil global de citocinas representando a proporção do balanço de citocinas predominantes compilando os perfis individuais inflamatórios, reguladores ou mistos de todas as subpopulações avaliadas.

3.4. Estudo da reatividade de IgG total anti-EPI (FC-AFEA) e IgG1 anti-TRIPO (FC-ALTA) por citometria de fluxo

3.4.1. Obtenção de preparações antigênicas do T. cruzi

3.4.1.2. Cultivo de formas epimastigotas do T. cruzi

Formas epimastigotas (EPI) foram obtidas a partir da semeadura de 1,0 x 10^7 tripomastigotas (cepa CL do T. cruzi) sanguíneos em um volume de 12ml de meio LIT (Liver Infusion Tryptose: 5,0g/l de infuso de fígado-Difco, 5,0g/l de triptose-Difco, 4,0g/l de NaCl-Synth, 0,4g/l de KCl-Synth, 8,0g/l de Na2HPO4-Synth, 2,0g/l de glicose-Sigma, 0,01g/l de hemina-Sigma, 100ml de SBF-Sigma, sendo o pH do meio ajustado para 7,2 com HCl-Merck) em erlenmeyer de 125ml e incubadas a 28°C (CAMARGO, 1964). Após 4 dias, a cultura com aproximadamente 8,0 x10^7 parasitos/ml foi repicada e mantida por passagens sucessivas em meio LIT. Para obtenção da massa de parasitos, a cultura foi transferida para um erlenmeyer de 250ml e aproximadamente 70ml de LIT foi adicionado. Os flagelados foram coletados na fase exponencial de crescimento, em tubos cônicos de 50ml, centrifugados (4°C, 1.000g por 10min), lavados 3 vezes com PBS e ressuspendidos em igual volume de PBS e solução fixadora para citometria. As formas EPI fixadas foram quantificadas em câmara de Neubauer e estocadas a 4°C por um período de até 30 dias. A concentração da suspensão de EPI foi

ajustada para 5,0 x 10^6/ml em PBS-3% SBF no momento da realização do ensaio de imunofluorescência.

3.4.1.3. Cultivo de formas tripomastigotas do T. cruzi

Formas tripomastigotas de cultura de tecido obtidas conforme descrito no item 3.3.1.1. Os parasitos foram lavados 3 vezes em PBS-10% SBF por centrifugação a 4°C, 1.000g por 10 minutos. Para os ensaios de imunofluorescência as formas tripomastigotas vivas foram quantificadas em câmara de Neubauer e a concentração da suspensão dos parasitos foi ajustada para 5,0 x 10^6/ml em PBS-10% SBF.

3.3.2. Protocolo de imunofluorescência indireta por citometria de fluxo

Os ensaios de citometria de fluxo para o estudo de anticorpos anti-T. cruzi presentes em plasmas de pacientes portadores da doença de Chagas foram realizados segundo protocolo descrito por MARTINS-FILHO et al. (1995), e adaptado para microplaca (CORDEIRO et al., 2001). Nas placas de 96 poços com fundo em "U" (Nunc, Denmark) foram adicionados 50µl do soro diluído e previamente aquecido a 56°C por 30 min em PBS-10% SBF e 50µl da suspensão de parasitos (5 x 10^6 parasitos/mL/poço). A mistura foi incubada a 37°C por 30 min, lavada 2 vezes com 150µl de PBS-10% SBF, centrifugada (4°C, 1.000g por 10min) e o sobrenadante desprezado.

Para a análise de IgG total, os parasitos foram reincubados (37°C por 30 min, ao abrigo da luz) na presença de 50µl da diluição de anticorpo anti-IgG humana marcado com isotiocianato de fluoresceína-FITC (Sigma). Os parasitos foram lavados 2 vezes com 150µl de PBS-10% SBF, cetrifugados (4°C, 1.000g, 10 min) e o sobrenadante desprezado.

Para análise de IgG1 os parasitos foram submetidos a 2 etapas de incubação. Na primeira etapa, 50µl de anticorpos anti-IgG1 biotinilados (Sigma) diluídos em PBS-10% SBF foram adicionados a cada poço da placa e a mistura incubada a 37°C por 30 min. Após a incubação, os parasitos foram lavados e centrifugados 2 vezes como descrito acima e o sobrenadante desprezado. Na etapa seguinte, os parasitos foram reincubados (37°C, 30 min, ao abrigo da luz) com 10µl de estreptavidina conjugada com ficoeritrina-SAPE (Gibco) diluída 1:400 em PBS-10% de SBF. Após a incubação os parasitos foram novamente lavados, centrifugados e o sobrenadante desprezado.

Após essas etapas os parasitos foram fixados com 200µl de solução fixadora para citometria (10,0g/l de paraformaldeído, 10,2g/l de cacodilato de sódio e 6,65g/l de cloreto de sódio, pH 7,2) e incubados por 30 min a 4°C. As amostras foram mantidas a 4°C, ao abrigo de luz, até o momento da leitura no citômetro de fluxo (FACScalibur - Becton Dickinson).

Para cada ensaio foi realizado um controle da reação, onde os parasitos foram incubados na ausência de soro humano, porém na presença do anticorpo secundário. Em todos os testes foram incluídas amostras controles de soros positivos e negativos para a doença de Chagas.

3.3.3. Estratégias de análises dos resultados

Os dados referentes à pesquisa da reatividade de IgG total anti-EPI (FC-AFEA) e IgG1 anti-TRIPO (FC-ALTA) por citometria de fluxo foram analisados para a determinação do percentual de parasitos fluorescentes positivos – PPFP (MARTINS-FILHO et al., 1995; CORDEIRO et al., 2001).

A Figura 14 ilustra a seqüência de procedimentos para a análise da reatividade de IgG anti-epimastigotas fixadas ou de IgG1 anti-tripomastigotas vivas de T. cruzi. Inicialmente, a

população de interesse – R1 foi selecionada, em gráficos de distribuição pontual de tamanho (FSC) versus granulosidade (SSC), com base em aspectos morfométricos dos parasitos (Figura 14A). Formas EPI e TRIPO do T. cruzi apresentam uma distribuição característica e homogênea em gráficos de FSC versus SSC, o que permite o posicionamento da região R1. Em seguida, histogramas individuais de intensidade de fluorescência em função do número de parasitos, foram utilizados para determinar o percentual de parasitos fluorescentes positivos (PPFP) para cada diluição das amostras utilizadas. Para tal, inicialmente foi determinado um limiar de ligação inespecífica do conjugado anti-IgG humano marcado com FITC, utilizando-se histogramas de fluorescência obtidos a partir do tubo controle do conjugado, realizado para cada bateria experimental (Figura 14B). O posicionamento do marcador do limiar seguiu sempre o critério de se obter no máximo 2% de PPFP para o controle interno da reação. Em seguida, empregando-se o mesmo marcador foram obtidos os valores de PPFP para cada diluição das amostras testadas (Figura 14C e Figura 14D). Para cada bateria experimental eram realizados ensaios empregando amostras controle positivo e controle negativo.

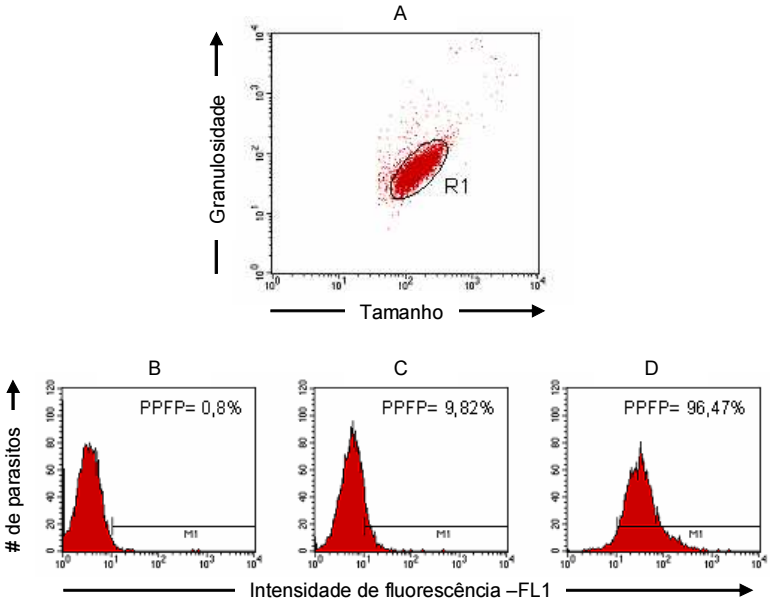


Figura 14: Seqüência de passos utilizados para as análises da reatividade de IgG anti-EPI e IgG1 anti-TRIPO do T. cruzi por citometria de fluxo. (A) Gráfico de distribuição puntual FSC versus SSC utilizado para seleção da população de interesse, nesse caso formas epimastigotas do T. cruzi – R1. (B) Histograma individual utilizado para posicionar o limiar de ligação inespecífica equivalente a um valor de PPFP≤2%, utilizando o controle interno da reação. (C) Histograma individual representativo do valor de PPFP obtido após a incubação com uma amostra controle negativo (D) Histograma individual representativo do valor de PPFP obtido após a incubação com uma amostra controle positivo.

3.5. Análise estatística

As análises estatísticas empregadas no estudo do perfil imunofenotípico de leucócitos circulantes foram feitas primeiramente pelo programa Minitab (versão 13.20) para testar as três hipóteses: independência, normalidade e variância dos grupos de dados. Os dados que apresentaram como verdadeiras as três hipóteses foram considerados paramétricos e avaliados por análise de variância (ANOVA) seguida de teste de Turkey usando o programa estatístico GraphPad Prism 5.0 (San Diego, CA, USA). Amostras consideradas não paramétricas foram

analisadas pelo teste de Kruskal-Wallis seguido de teste de Dunn´s. Análises de correlação foram feitas por testes de Pearson e Spearman, respectivamente. As diferenças foram consideradas significativas quando o $p<0,05$.

As medianas globais dos percentuais de células citocinas$^+$ foram calculadas pelo programa GraphPad Prism 5.0 (San Diego, CA, USA). Para comparações entre as freqüências de alto e baixo produtores de citocinas de NI, CARD e DIG foi realizado teste de χ^2. As diferenças foram consideradas significativas quando o $p<0,05$. As áreas dos polígonos obtidas nos gráficos de radar foram comparadas entre os grupos e entre as categorias de produtores de citocinas. Áreas duas vezes maiores ou menores foram consideradas significativamente diferentes. As diferenças entre os grupos foram indicadas pelas letras "a", 'b" e "c" em relação aos grupos NI, IND e CARD, respectivamente. Diferenças entre as categorias de produtores de citocinas foram indicadas pelo símbolo "*" quando havia diferença em relação às demais categorias e pelo símbolo "#" quando havia diferença em relação a alto produtores de citocinas com perfil misto.

A análise do desempenho da pesquisa de anticorpos anti-T. cruzi por citometria de fluxo (FC-AFEA-IgG) foi realizada empregando o programa estatístico MedCalc 2.3 (versão gratuita disponível na internet http://medcalc.be/download.php), sendo avaliados os seguintes índices estatísticos: sensibilidade = [verdadeiros positivos ÷ (verdadeiros positivos + falso negativos)] x 100; especificidade = [verdadeiros negativos ÷ (verdadeiros negativos + falso positivos)] x 100; valor preditivo positivo/VPP = [verdadeiros positivos ÷ total de positivos] x 100 e valor preditivo negativo/VPN = [verdadeiros negativos ÷ total de negativos] x 100 (YOUDEN, 1950). A curva ROC (receiver operating characteristic curve) foi construída a partir da representação gráfica da sensibilidade no eixo vertical, e do complemento da especificidade no eixo horizontal. A curva ROC foi usada para a definição de pontos de coorte que permitissem discriminar valores fracamente e altamente positivos de PPFP. A área

sob a curva ROC/ASC é o indicador de acurácia global do teste. Quanto maior a ASC, o que significa mais próximo do valor 1, melhor é o desempenho do método avaliado. Conforme sugere SWETS (1988), em função do valor da ASC podemos classificar o teste de diagnóstico como: sem valor (ASC=0,5), de baixa acurácia (0,5<ASC≤0,7), de moderada acurácia (0,7<ASC≤0,9), de elevada acurácia (0,9<ASC<0,1) e como teste perfeito (ASC=1). Outra forma de abordagem do desempenho de testes diagnósticos, particularmente daqueles cujos resultados são expressos em escala contínua, consiste na determinação das razões de verossimilhança (RVs) para diferentes resultados. As RVs para um resultado positivo ou negativo dos testes diagnósticos foram determinadas, respectivamente, pelas relações [verdadeiros positivos ÷ (verdadeiros positivos + falsos negativos)] ÷ [falsos positivos ÷ (falsos positivos + verdadeiros negativos)], e [falsos negativos ÷ (verdadeiros positivos + falsos negativos)] ÷ [verdadeiros negativos ÷ (falsos positivos + verdadeiros negativos)]. Como proposto por JAESCHKE et al. (1994), RVs superiores a 10 praticamente confirmam o diagnóstico da doença e RVs inferiores a 0,1 praticamente excluem o diagnóstico da doença.

4. ARTIGOS

4.1. ARTIGO 1 E 2:

Os artigos científicos apresentados a seguir correspondem ao objetivo específico 1.

Título: Chagasic patients with indeterminate clinical form of the disease have high frequencies of circulating $CD3^{+}CD16^{-}CD56^{+}$ natural killer T cells and $CD4^{+}CD25^{High}$ regulatory T lymphocytes.

Autores: Vitelli-Avelar DM, Sathler-Avelar R, Dias JC, Pascoal VP, Teixeira-Carvalho A, Lage PS, Elói-Santos SM, Corrêa-Oliveira R, Martins-Filho OA.

Revista: Scand J Immunol. 2005 Sep; 62(3):297-308.

DOI: 10.1111/j.1365-3083.2005.01668.x

Título: Are increased frequency of macrophage-like and natural killer (NK) cells, together with high levels of NKT and $CD4^{+}CD25^{high}$ T cells balancing activated $CD8^{+}$ T cells, the key to control Chagas' disease morbidity?

Autores: Vitelli-Avelar DM, Sathler-Avelar R, Massara RL, Borges JD, Lage PS, Lana M, Teixeira-Carvalho A, Dias JC, Elói-Santos SM, Martins-Filho OA.

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doi: 10.1111/j.1365-3083.2005.01668.x

Chagasic Patients with Indeterminate Clinical Form of the Disease have High Frequencies of Circulating $CD3^{+}CD16^{-}CD56^{+}$ Natural Killer T Cells and $CD4^{+}CD25^{High}$ Regulatory T Lymphocytes

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Abstract

Several studies have demonstrated that different clinical manifestations of human Chagas' disease are associated with distinct and complex host–parasite relationships directly involving the immune system. In this context, it has been proposed that tissue damage might be more severe in the absence of regulatory mechanisms that involve both innate and adaptive immune responses. Herein, we describe a descriptive phenotypic profile focusing on the frequency of major regulatory T cells [$CD4^{+}CD25^{high}$ and natural killer T (NKT) lymphocytes] in different clinical forms of Chagas' disease. Ex vivo immunophenotyping of whole blood demonstrated that the indeterminate clinical form displays a higher frequency of both $CD4^{+}CD25^{high}$ and NKT regulatory cells ($CD3^{+}CD16^{-}CD56^{+}$), associated with increased levels of circulating cytotoxic NK cells ($CD3^{-}CD16^{+}CD56^{+}$ and $CD3^{-}CD16^{+}CD56^{dim}$ NK cells). By contrast, the increased percentage of activated $CD8^{+}HLA\text{-}DR^{+}$ T-cell subset was exclusively associated with severe clinical forms of Chagas' disease. We hypothesize that regulatory T cells may be able to control the deleterious cytotoxic activity in the indeterminate clinical form by inhibiting the activation of $CD8^{+}HLA\text{-}DR^{+}$ T cells. The lack of regulated populations in cardiac and digestive clinical forms could account for impaired immune response that culminates in strong cytotoxic activity and tissue damage.

Introduction

Trypanosoma cruzi is an obligate intracellular parasite that causes American trypanosomiasis (Chagas' disease), a chronic and debilitating syndrome that affects approximately 18 million individuals in Latin America [1]. Most infected individuals survive the acute phase and then remain apparently asymptomatic for long periods of time or throughout their lifetimes. These patients are characterized by positive serological and parasitological tests for T. cruzi and are considered to present the indeterminate clinical form of disease. However, epidemiological studies in endemic areas show that 30–40% of infected individuals develop severe clinical forms of Chagas' disease, a chronic inflammatory illness that commonly results in cardiomyopathy (cardiac form) and/or gastrointestinal tract dysfunction (digestive form) [2]. In this context, the specific mechanisms associated with the establishment/maintenance of distinct clinical outcomes of Chagas' disease appear to be complex. Why some infected individuals develop severe Chagas' disease and others do not and why the clinical manifestations are highly heterogeneous are unclear. Several studies have demonstrated that different clinical manifestations are associated with distinct and complex host–parasite relationships directly involving the immune system. In fact, it is well accepted that the absence of chagasic pathology is associated with an individual's ability to regulate the anti-T. cruzi immune response that controls persistent parasitism, which can also contribute to

the inflammatory damage that causes Chagas' disease [3]. This inflammatory tissue damage might be more severe in the absence of regulatory mechanisms that involve both innate and adaptive effective responses. Indeed, it has been demonstrated that several types of regulatory cells exist, some of which are induced in response to infectious challenge and some of which are considered to be natural regulators [4–6]. Regulatory T cells have been recently described as a unique population of $CD4^{+}CD25^{+}$ T cells, a class of T cells that regulates innate and adaptive immune responses and has the capacity to control the excessive or misdirected effector immune response, including response to pathogens or self-antigens [7–10]. Several mechanisms in which regulatory T cells may perform their modulatory activity have been proposed, including inhibition of cytotoxic activity by downregulation of interleukin (IL)-12 and CD25 on $CD8^{+}$ T cells or through direct antigen-presenting cell-independent contact with these cells [10–14].

Similar to murine models, human natural killer T (NKT) cells have been described as another relevant subset of regulatory T lymphocytes, believed to play innate [15, 16], antitumor [17, 18] and regulatory roles [19–21], with multiple activities that are under the control of the accessory cells and soluble factors in local microenvironments [17, 22]. Phenotypic studies demonstrate that NKT cells are a subset of T lymphocytes, distinct from conventional T cells in that they express CD16, CD56 and/or CD161 NK receptors, with the expression of CD56 being a better marker to define the subsets of NKT cells than the expression of CD161 [15, 23]. The regulatory functions of NKT cells have been shown to prevent autoimmune disease and to contribute to protective responses against pathogens [15, 17]. NKT cells have been shown to suppress several human autoimmune diseases, including diabetes, systemic lupus erythematosus and rheumatoid arthritis. The NKT cell population appears to be diminished in these diseases and is unable to prevent self-damaging responses [17, 18, 24]. During infections, NKT cells have been shown to secrete proinflammatory cytokines that stimulate the innate and adaptive responses that eliminate pathogens [25]. In contrast, NKT cells have been shown to secrete anti-inflammatory cytokines that limit infection-induced pathology [26, 27]. It remains unclear how NKT cells during infections augment proinflammatory responses to control pathogens, whereas in other infections, they inhibit inflammatory responses to prevent infection-induced tissue damage. Recently, it has been proposed in an experimental murine model of T. cruzi infection that NKT cells play important roles in both proinflammatory and anti-inflammatory responses. These authors suggested that invariant NKT cells limit the inflammatory response and prevent tissue damage, whereas variant NKT cells increase the inflammatory response that contributes to morbidity and mortality [28]. Thus, a functionally distinct NKT cell subset may better trigger immunomodulatory events, while others are better at promoting immune response. These observations suggest that successful control of T. cruzi infection and Chagas' disease involves a balance between the generation of immune response sufficient to control the infection and the regulation of this response to prevent extensive destruction of host tissues.

We carried out a detailed, descriptive immunophenotyping study focusing on the frequency of these major regulatory T cells in different clinical forms of Chagas' disease. Ex vivo immunophenotyping of whole blood from chronic T. cruzi-infected patients demonstrated that the indeterminate clinical form displays a higher frequency of both $CD4^{+}CD25^{high}$ and NKT regulatory cells associated with increased levels of circulating NK cells ($CD3^{-}CD16^{+}CD56^{+}$ and $CD3^{-}CD16^{+}CD56^{dim}$ NK cells). By contrast, an increased percentage of activated $CD8^{+}$ HLA-DR^{+} T-cell subset was exclusively associated with severe clinical forms of Chagas' disease. Our findings support the hypothesis that the population of $CD4^{+}CD25^{high}$ regulatory T cells and NKT cells ($CD3^{+}CD56^{+}$) is able to control the deleterious cytotoxic activity in the indeterminate clinical form by inhibiting the activation of $CD8^{+}$ HLA-DR^{+} T cells. The lack of regulatory T-cell and NKT cell populations in cardiac and digestive patients could account for impaired immune response that culminates in strong cytotoxic activity and tissue damage. Taken together, these findings suggest that strong activation of $CD8^{+}$ HLA-DR^{+} T cells could result in tissue damage leading to the development of cardiomyopathy and/or digestive megas [29, 30]. By contrast, the ability to build up NK-mediated cell cytotoxicity modulated by $CD4^{+}CD25^{high}$ and NKT cells seems to play a pivotal role in the generation of effective, nondeleterious inflammatory mechanisms.

Further studies to functionally characterize these populations will be of great interest and may provide a better understanding of the immunological complexities that control the course of the indeterminate form of Chagas' disease. Moreover, an understanding of the immunological mechanisms involved in the control of parasite replication and development of chagasic cardiac/digestive disease may contribute new insights on how to prevent or treat the symptomatic forms of Chagas' disease.

Patients, materials and methods

Study population. The inclusion of all subjects in our investigation had the approval of the Ethics Committees of the FIOCRUZ. The patients included in this study ranged from 20 to 70 years of age. All infected individuals were from the state of Minas Gerais, Brazil, and had received a positive diagnosis for Chagas' disease. The diagnosis was based on standard serological tests, including indirect immunofluorescence assay and haemagglutination. In this study, we

used 29 samples from chagasic patients with chronic disease. According to their clinical records, the chagasic patients were divided into three different categories, namely indeterminate (IND), cardiac (CARD) and digestive (DIG). Patients presenting asymptomatic T. cruzi infection, classified as IND (n¼8), had no clinical manifestations of the disease other than their positive serology. Patients with cardiac dysfunction, CARD (n¼13), presented with dilated cardiomyopathy and were identified by a detailed clinical examination, including electrocardiography, 24-h Holter examination and chest X-ray. Chagasic patients with gastrointestinal disease, DIG (n¼8), presented with clinical radiological status of megacolon and/or megaesophagus. Twelve nonchagasic individuals, with negative results on serological tests for Chagas' disease, were included in this study as negative controls (NI). All were living in an endemic area for Chagas' disease (Table 1).

Flow cytometric analysis of peripheral blood. White blood cell phenotypes were analysed following an immunofluorescence procedure recommended by Becton Dickinson (Mountain View, CA, USA) and modified as follows: 100-ml samples of peripheral blood, which had been collected in Vacutainer tubes containing EDTA (Becton Dickinson), were mixed in 12 × 75 mm tubes with 5 ml of undiluted monoclonal antibodies specific for several cell-surface markers; the tubes were incubated in the dark for 30 min at room temperature. Following incubation, erythrocytes were lysed using 2 ml of fluorescence-activated cell sorter (FACS) Lysing Solution (Becton Dickinson Biosciences Pharmigen, San Diego, CA, USA). After incubation, the cells were washed twice with 2 ml of phosphate-buffered saline containing 0.01% sodium azide. Cell preparations were fixed in 200 ml of FACS fix solution (10 g/l paraformaldehyde, 1% sodium-cacodylate, 6.65 g/l sodium chloride, 0.01% sodium azide). Cytofluorimetric data acquisition was performed with a Becton Dickinson FACScalibur instrument. CELLQUEST™ software provided by the manufacturer was used for data acquisition and analysis.

Specific monoclonal antibodies used for immunophenotyping. Mouse anti-human monoclonal antibodies, conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and tri-colour (TC), specific for cell-surface markers were used simultaneously for two- or three-colour immunocytometric assays. In this study, we used anti-human FITC-conjugated monoclonal antibodies including anti-CD3 (UCHT1), anti-CD4 (13B8.2), anti-CD5 (L17F12), anti-CD8 (B9.11), anti-CD16 (3G8), anti-CD19 (HID19) and mouse IgG1 for isotypic control (679.1Mc7), all purchased from Becton Dickinson. As second colour reagents, we used anti-human PE-conjugated monoclonal antibodies anti-CD25 (3G10), anti-CD23 (M-L233), anti-CD56 (B159) anti-HLA-DR (TÜ36) and mouse IgG2a for the isotypic control (UCTH-1), all purchased from Becton Dickinson. The third colour parameter was evaluated using TC-conjugated monoclonal antibodies and included anti-CD14 (TÜK4), anti-CD16 (3G8) and anti-CD19 (4G7), all purchased from Caltag Laboratories (Burlingame, CA, USA).

Table 1 Patient characterization

Group	Number of individuals	Age range (years)	Male	Female
Noninfected (NI)	12	20–53	3	9
Indeterminate (IND)	8	44–67	3	5
Cardiac (CARD)	13	50–70	5	8
Digestive (DIG)	8	45–65	5	3

Statistical analysis. Differences between groups were first evaluated using the program MINITAB to test three hypotheses: independence, normality and variance. Samples that fit all the three hypotheses represent parametric data and were evaluated by ANOVA, followed by the Tukey test, using the PRISM 3.0 program. Samples that did not fit at least one of these three hypotheses represent nonparametric data and were evaluated by the Kruskal–Wallis test, followed by the Dunn's test. Significance was defined in both cases as $P < 0.05$.

Results

Phenotype analysis of T-cell and B-cell subsets

The analysis of T-cell and B-cell populations and their subsets is summarized in Table 2. The mean percentage of circulating T lymphocytes ($CD3^{+}$) was significantly lower in the IND and CARD groups than in the NI group ($P < 0.05$). No significant differences were found in the mean values of T-cell subsets, both $CD4^{+}$ and $CD8^{+}$, among the four groups. Analysis of B cells and their major subsets $CD19^{+}CD5^{-}$ (conventional B cells) and $CD19^{+}CD5^{+}$ (B1 lymphocytes), as well as their activation status based on CD23 expression, was carried out using two-colour flow cytometry immunostaining procedures. No significant differences were observed for the B-cell compartment among the four groups evaluated in this study.

Increased percentage of activated cytotoxic T-cell subset is associated with severe clinical forms of chronic Chagas' disease

Double-labelling studies using anti-CD4 FITC or anti-CD8 FITC and anti-HLA-DR PE were carried out to evaluate the percentages of activated circulating T-cell subsets (Fig. 1). An increased frequency of $CD4^{+}HLA\text{-}DR^{+}$ T cells (Fig. 1A) in all chagasic patients was seen in comparison with the NI group ($P < 0.05$). Despite the higher frequency of circulating $CD4^{+}$-activated T cells

Table 2 Phenotypic analyses of T- and B-lymphocyte subsets in chagasic patients and noninfected individuals

Cell phenotype	Groups* Noninfected [NI (n¼12)]	Indeterminate [IND (n¼8)]	Cardiac [CARD (n¼13)]	Digestive [DIG (n¼8)]
Total T lymphocytes ($CD3^+$)	71.17 ± 8.12	63.28 ± 5.55y	62.56 ± 12.19y	65.76 ± 5.25
$CD4^+$	42.01 ± 8.77	43.41 ± 5.13	42.79 ± 12.19	43.60 ± 6.93
$CD8^+$	24.22 ± 9.04	19.14 ± 4.30	21.70 ± 6.82	18.76 ± 6.11
Total B lymphocytes ($CD19^+$)	10.32 ± 5.87	8.98 ± 5.33	8.99 ± 4.91	13.07 ± 2.97
$CD19^+CD5^-$	8.32 ± 4.10	7.79 ± 4.72	7.61 ± 3.13	9.94 ± 1.76
$CD19^+CD5^+$	1.74 ± 1.99	1.38 ± 1.11	1.39 ± 1.34	3.63 ± 3.50
$CD19^+CD23^+$	50.01 ± 13.66	57.64 ± 14.44	50.79 ± 1.29	55.72 ± 16.05

*The results are expressed as mean percentage ± SD.
ySignificant differences at $P < 0.05$ in comparison with NI.

observed in all individuals chronically infected with T. cruzi, an increased percentage of activated cytotoxic T cells was exclusively associated with severe clinical forms of the disease. It was relevant that only CARD and DIG patients presented higher levels of activated $CD8^+$ T cells (Fig. 1B) compared with the NI group ($P < 0.05$). Figure 1(C) shows representative dot plots illustrating the differential T-cell activation status observed in the different clinical forms of chronic Chagas' disease.

Cytotoxic NK cell subsets are expanded in the indeterminate clinical form of Chagas' disease

In order to quantify the frequency of circulating NK cells and their subsets, including $CD3^-CD16^+CD56^-$ (precursor NK cell) and $CD3^-CD16^+CD56^+$ (mature cytotoxic NK cell), a three-colour flow cytometry analysis using anti-CD3 FITC, anti-CD56 PE and anti-CD16 TC was performed, as proposed by Gaddy et al. [31]. We observed a higher frequency of circulating NK cells in all groups of chagasic patients ($P < 0.05$). However, considering that $CD56^+$ NK cells are primarily cytotoxic [32], it was remarkable that the mean level of circulating $CD3^-CD16^+CD56^+$ cytotoxic NK cells was higher in IND patients than in the NI group (Fig. 2C). Consequently, a lower level of $CD3^-CD16^+CD56^-$ precursor NK cells was observed in the IND group than in the NI group (Fig. 2B). Figure 2(D) illustrates, in representative dot plots, the differential frequency of NK cells bearing the $CD3^-CD16^+CD56^+$ cytotoxic phenotype in chagasic patients.

The semiquantitative analysis of CD56 density on the cell surface of NK cells was further employed as an approach to enumerate the frequency of NK cells expressing lower levels of CD56, namely $CD56^{dim}$, which Cooper et al. characterizes as a more cytotoxic subset against NK-sensitive targets. We observed a higher frequency of $CD3^-CD56^{dim}CD16^+/CD3^-CD56^{dim}$ in the IND group than in the NI group (Fig. 3A); this additional phenotypic characteristic emphasizes the higher cytotoxic capacity of NK cells in IND patients. No significant difference was observed in $CD3^-CD56^{bright}CD16^+/CD3^-CD56^{bright}$ frequencies among the four groups evaluated (Fig. 3B).

Expansion of NKT cells is associated with the indeterminate clinical form of Chagas' disease

NKT cells are a subset of T cells distinct from conventional T cells and NK cells in that they express surface receptors of both cell types. They can provide protection against infections by rapidly producing cytokines, through their cytolytic activity or via stimulation of other cell populations [17]. In order to quantify the frequency of circulating NKT cells, we used the same three-colour flow cytometry platform previously described for enumeration of NK cell subpopulations. We observed a higher frequency of circulating NKT cells ($CD3^+CD16^{-/+}CD56^{-/+}$) in the IND group than in the NI group (Fig. 4A). Data analysis was further performed classifying NKT cells as NKT1 ($CD3^+CD16^+D56^-$), NKT2 ($CD3^+CD16^-CD56^+$) and NKT3 ($CD3^+CD16^+CD56^+$). On the basis of distinct phenotypic features of NKT cell subsets, our results revealed a significantly higher frequency of NKT2 ($CD3^+CD16^-CD56^+$) subset in IND patients than in NI patients (Fig. 4C). No other differences were observed for NKT1 and NKT3 subsets (Fig. 4B,D). The representative dot plots in Fig. 4(E) illustrate the higher frequency of NKT2 cells observed in IND patients.

Increment of circulating $CD4^+$ $CD25^{high}$ regulatory T cells is restricted to the indeterminate clinical form of Chagas' disease

It has been proposed that whereas the entire population of $CD54^+CD25^+$ T cells expressing both low and high CD25 levels exhibits regulatory function in mice, only the $CD4^+CD25^{high}$ population exhibits a similarly strong regulatory function in humans, comprising approximately 1–2% of circulating $CD4^+$ T cells [33]. Enumeration of $CD4^+CD25^{high}$ regulatory T cells was carried out by first

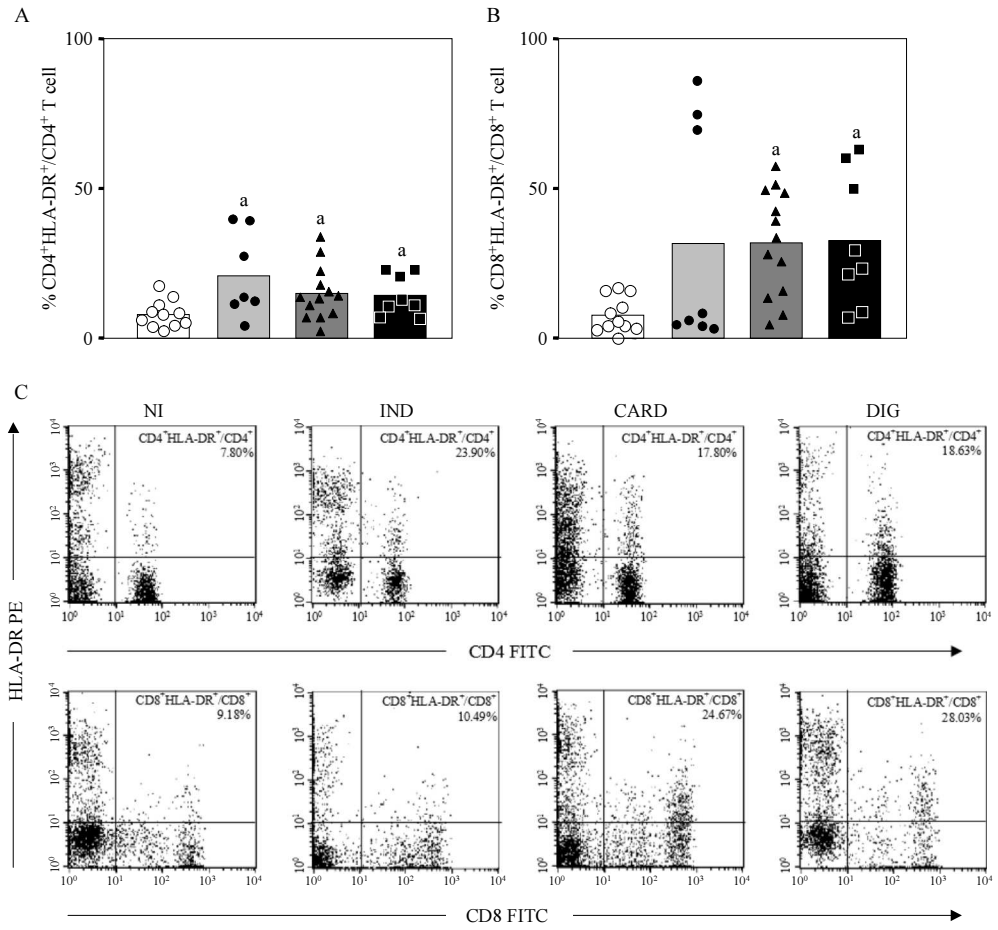

Figure 1 Analysis of activated $CD4^b$ and $CD8^b$ T lymphocytes in the peripheral blood of Trypanosoma cruzi-infected individuals including IND (.), CARD (~) and DIG (&) and noninfected individuals, NI (*). Phenotypic studies were carried out using a double-labelling protocol involving anti-CD4 or anti-CD8 fluorescein isothiocyanate (FITC) and anti-HLA-DR phycoerythrin (PE) to identify (A) $CD4^b$ HLA-DR^b/$CD4^b$ cells and (B) $CD8^b$ HLA-DR^b/$CD8^b$ cells. The results are expressed as scattering of individual values and mean percentage of activated cells within $CD4^b$ and $CD8^b$ lymphocytes. Significant differences at $P < 0.05$ are identified by the letter 'a' in comparison with the NI group. (C) Representative dot plots illustrating the higher frequency of $CD4^b$ HLA-DR^b/$CD4^b$ in all chagasic individuals in comparison with NI individuals, and a restricted higher frequency of $CD8^b$ HLA-DR^b/$CD8^b$ in CARD and DIG patients in comparison with the NI group. Quadrant statistics were used for data analysis, and the results are expressed as the percentage of positive cells within the selected gate.

gating on lymphocytes based on their morphometric features on forward scatter versus side scatter dot plots, followed by the selection of $CD4^b$ cells with high CD25 expression, as proposed by Baecher-Allan et al. [33]. Our results showed an increased frequency of $CD4^b$ $CD25^{high}$ regulatory T cells exclusively in the IND group in comparison with all other groups ($P < 0.05$; Fig. 5A). Figure 5(B) shows representative dot plots demonstrating these results.

Discussion

The chronic nature of Chagas' disease strongly suggests the development of effective immunological mechanisms that modulate influences contributing to the establishment/maintenance of a relatively balanced host–parasite relationship in asymptomatic carriers. The role of distinct lymphoid cell populations has been extensively explored in regard to cellular events related to induction and

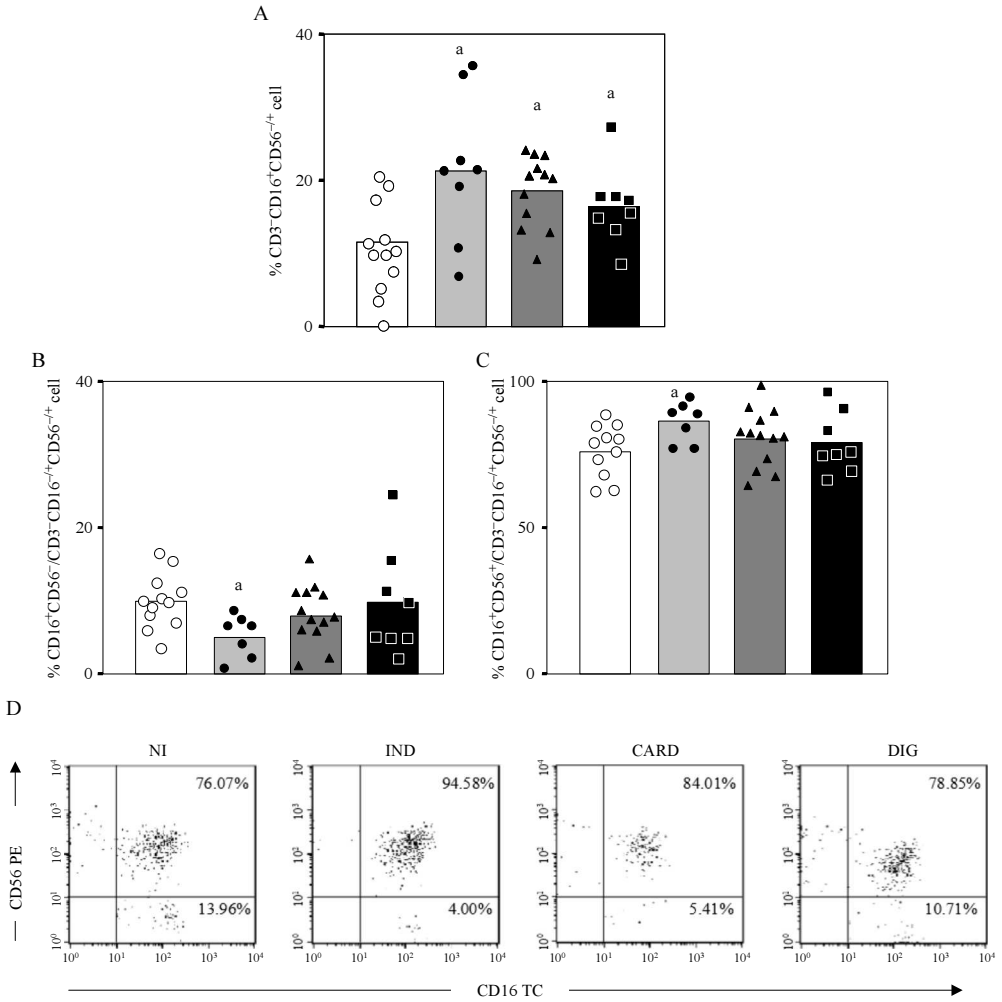

Figure 2 Analysis of total natural killer (NK) cell and subsets in the peripheral blood of Trypanosoma cruzi-infected individuals including IND (.), CARD (~) and DIG (&) and noninfected individuals, NI (*). Phenotypic studies were carried out using a triple-labelling protocol anti-CD3 fluorescein isothiocyanate (FITC), anti-CD56 phycoerythrin (PE) and anti-CD16 tri-colour (TC) to identify (A) $CD3^{-}CD16^{b}CD56^{-/b}$ (NK cells), (B) $CD3^{-}CD16^{b}CD56^{-}$ (precursor NK cells) and (C) $CD3^{-}CD16^{b}CD56^{b}$ (mature NK cells). The results are expressed as scattering of individual values and mean percentage of NK cells within total lymphocytes and its subsets within NK cells. Significant differences at $P < 0.05$ are identified by letter 'a' in comparison with NI. (D) Representative dot plots illustrating the higher frequency of mature NK cells in IND in comparison with NI individuals, and consequently a lower frequency of precursor NK cells in IND patients in comparison with NI group. Quadrant statistics were used for data analysis, and the results are expressed as the percentage of positive cells within the selected gate.

modulation of immunopathology in severe and indeterminate clinical forms, respectively [3].

It is important to reiterate that the current studies have been performed with human patients and that these groups, even though generally categorized by clinical presentation, are extremely heterogeneous. Therefore, initially we attempted to characterize basic phenotypic aspects of peripheral blood leucocytes as previously described for chagasic patients [34–36] in order to normalize our study population on well-established immunological features in spite of the intrinsic heterogeneous clinical status of unsynchronized chronically infected patients.

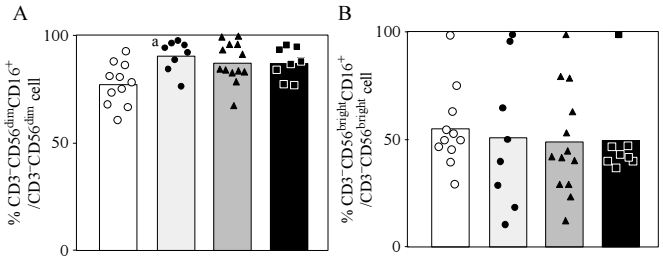

Figure 3 Analysis of natural killer (NK) cell subsets $CD3^{-}CD56^{dim}CD16^{b}$ and $CD3^{-}CD56^{bright}CD16^{b}$ within $CD3^{-}CD56^{dim}$ and $CD3^{-}CD56^{bright}$, respectively. Phenotypic studies were carried out using a triple-labelling protocol involving anti-CD3 fluorescein isothiocyanate (FITC), anti-CD56 phycoerythrin (PE) and anti-CD16 tri-colour (TC) to identify (A) $CD3^{-}CD56^{dim}CD16^{b}/CD3^{-}CD56^{dim}$ cells and (B) $CD3^{-}CD56^{bright}CD16^{b}/CD3^{-}CD56^{bright-}$ cells in the peripheral blood of Trypanosoma cruzi-infected individuals including IND (.), CARD (~) and DIG (&) and noninfected individuals, NI (*). The results are expressed as scattering of individual values and mean percentage of $CD3^{-}CD56^{dim}CD16^{b}/CD3^{-}CD56^{dim}$ and $CD3^{-}CD56^{bright}/CD3^{-}CD56^{bright}$ cells. A significant difference at $P < 0.05$ is identified by the letter 'a' in comparison with NI. Quadrant statistics were used for data analysis, and the results are expressed as the percentage of positive cells within the selected gate.

As previously described, we found that chagasic patients with IND and CARD clinical forms have lower percentages of $CD3^{b}$ T lymphocytes in their peripheral blood despite no significant differences on $CD4^{b}$ and $CD8^{b}$ T-cell subsets and normal levels of $CD19^{b}$ B cells [34]. Although no clinical classification according to severity of the digestive disease has been used in this study, most DIG patients presented with moderately increased levels of organ enlargement. Therefore, the normal frequency of circulating T cells ($CD3^{b}$, $CD4^{b}$ and $CD8^{b}$) and B cells ($CD19^{b}$) observed in our DIG group was as documented in previous reports because lower absolute counts of $CD3^{b}$ T cells and $CD4^{b}$ T cells have been observed only in patients exhibiting extreme stages of chagasic megaesophagus and/or megacolon [36].

Chagasic lesions are mainly concentrated in the cardiac tissue or digestive tract and clearly involve T-cell activation [29, 37, 38]. Despite earlier investigations focusing attention on the high activation status of peripheral blood T-cell subsets in IND and CARD, those studies did not indicate any significant differences between these two patient groups [34]. Recently, Lemos et al. [36] reported increased levels of circulating $CD4^{b}$ and $CD8^{b}$ T cells coexpressing the activation marker HLA-DR in DIG patients, regardless of the stage of the disease. We also observed an increased percentage of $CD4^{b}$ HLA-DR^{b} in all infected individuals; a higher frequency of activated $CD8^{b}$ T cells was restricted to CARD and DIG patients presenting severe clinical forms of Chagas' disease. The gating strategies applied to analyse the frequency of activated T-cell subsets may explain these observations. The previous studies usually expressed the frequency of activated T cells, taking into account the whole lymphocyte population, whereas more recent investigations have adopted the combined gating strategy to determine the ratio of T cells expressing a particular activation marker within a given cell subpopulation.

The elevated frequency of circulating $CD8^{b}$ HLA-DR^{b} T cells we observed in CARD patients agrees with data from Reis et al. [29], who found a high number of activated T cells, albeit primarily $CD8^{b}$ T cells, within cardiac lesions of chagasic patients. Interestingly, a small number of IND patients also presented with a high frequency of $CD8^{b}$ HLA-DR^{b} T cells. Because the current study has been carried out on patients living in endemic areas, the IND group, even though generally categorized by absence of clinical presentation, is extremely heterogeneous. The patients in this group are of mixed ages and certainly asynchronous in their infection stages. The 44–67-year age span of IND patients, which probably represents a span of 5–67 years of infection [39], offers manifold possibilities for a wide range of immunological statuses within a given group of patients. It is likely that IND patients presenting with a high frequency of activated $CD8^{b}$ T cells are prone to develop cardiomyopathy sooner than those showing low frequencies of circulating $CD8^{b}$ HLA-DR^{b} T cells.

It is well known that T. cruzi infection simultaneously triggers multiple elements of the innate and adaptive immune system, leading to a systemic synthesis of proinflamatory cytokines and activation of innate immune responses mediated by NK cells and macrophages in conjunction with activation of T cells during the acute phase of the disease [3, 40]. However, very little information is currently available regarding the involvement of innate immunity during chronic Chagas' disease. Here, in a pioneering study, we have demonstrated that an increased frequency of circulating NK cells ($CD3^{-}CD16^{b}CD56^{-/b}$) can be found in the peripheral blood of all patients chronically infected with T. cruzi. However, only IND patients showed a higher percentage of $CD3^{-}CD16^{b}CD56^{b}$ and $CD3^{-}CD16^{b}CD56^{dim}$ NK cells. Human NK cells are generally defined as being membrane $CD3^{-}CD16^{b}CD56^{-/b}$ lymphocytes [41]. In humans,

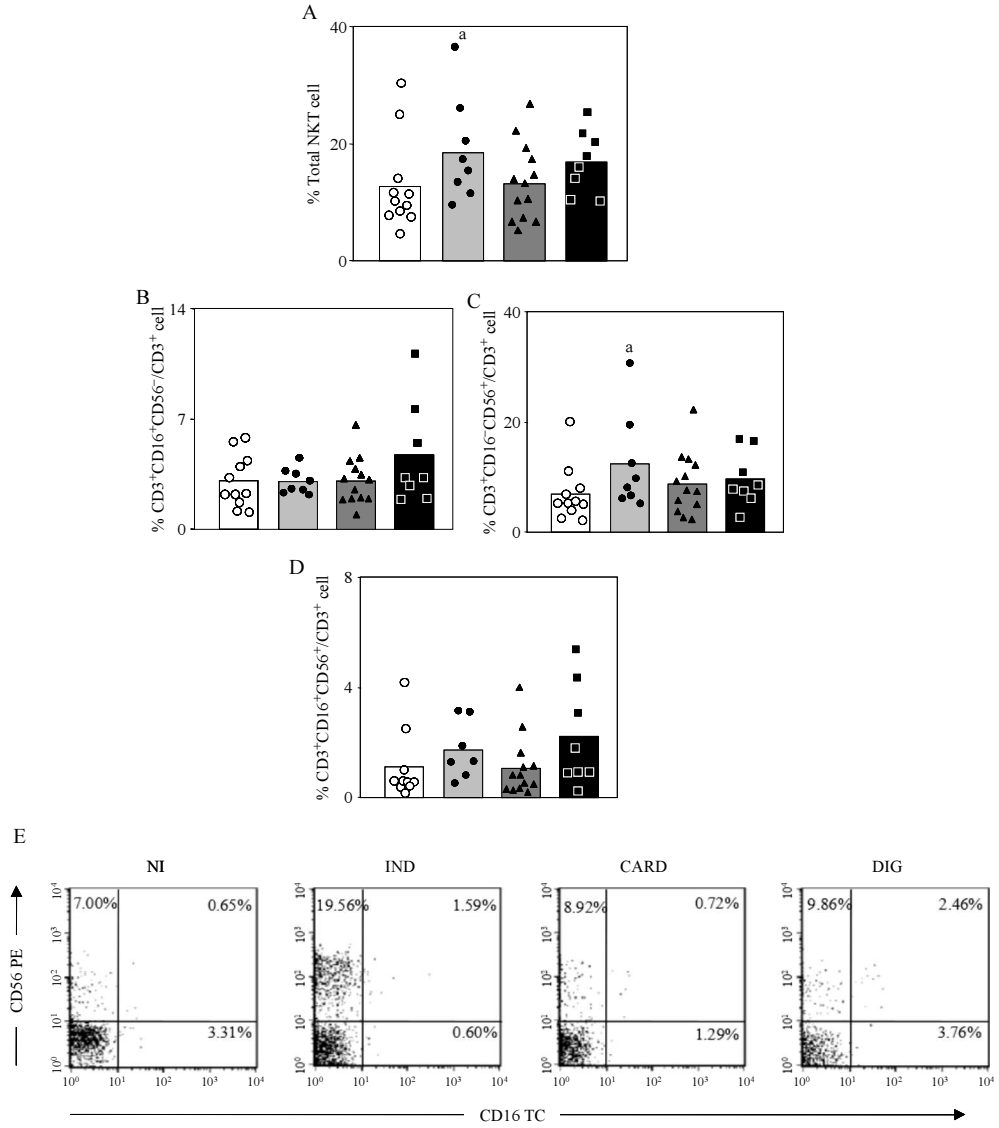

Figure 4 Analysis of total natural killer T (NKT) cells and subsets in the peripheral blood of Trypanosoma cruzi-infected individuals including IND (▪), CARD (▴) and DIG (▾) and noninfected individuals, NI (•). Phenotypic studies were carried out using a triple-labelling protocol involving anti-CD3 fluorescein isothiocyanate (FITC), anti-CD56 phycoerythrin (PE) and anti-CD16 tri-colour (TC) to identify (A) total NKT cells ($CD3^{b}CD16^{-/b}CD56^{-/b}$), (B) NKT1 cells ($CD3^{b}CD16^{b}CD56^{-}$), (C) NKT2 cells ($CD3^{b}CD16^{-}CD56^{b}$) and (D) NKT3 cells ($CD3^{b}CD16^{b}CD56^{b}$). The results are expressed as scattering of individual values and mean percentage of NKT cells within gated $CD3^{b}$ lymphocytes. A significant difference at $P < 0.05$ is identified by the letter 'a' in comparison with the NI group. (E) Representative dot plots illustrating the higher frequency of NKT2 cells in the IND group in comparison with the NI group. Quadrant statistics were used for data analysis, and the results are expressed as the percentage of positive cells within the selected gate.

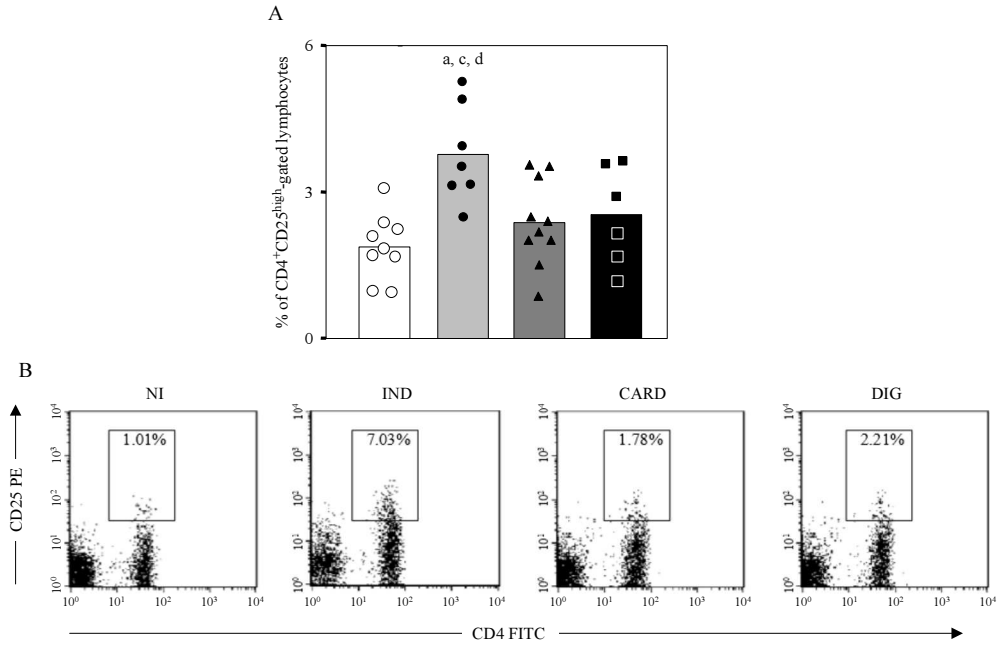

Figure 5 Analysis of $CD4^{þ}CD25^{high}$ regulatory cells in the peripheral blood of Trypanosoma cruzi-infected individuals including IND (.), CARD (~) and DIG (&) and noninfected individuals, NI (*). A double-labelling panel involving anti-CD4 fluorescein isothiocyanate (FITC) and anti-CD25 phycoerythrin (PE) was used to identify regulatory cells. The results are expressed as scattering of individual values and mean percentage of $CD4^{þ}CD25^{high}$ regulatory cells within gated lymphocyte. Significant differences at $P < 0.05$ are identified by letters 'a', 'c' and 'd' in comparison with NI, CARD and DIG groups, respectively. (B) Representative dot plots illustrating the higher frequency of $CD4^{þ}CD25^{high}$ regulatory cells in the IND group in comparison with the NI, CARD and DIG groups. Quadrant statistics were used for data analysis, and the results are expressed as the percentage of positive cells within the selected gate.

the majority in the peripheral blood is $CD16^{þ}CD56^{þ}$ [31] and little is known about the properties of $CD16^{þ}CD56^{-}$ NK cells. It has been suggested that those cells are functionally and phenotypically immature, capable of maturation and possible precursors to mature $CD16^{þ}CD56^{þ}$ NK cells [31]. Functional studies have demonstrated that $CD16^{þ}CD56^{-}$ cells showed higher proliferative capacity, whereas $CD16^{þ}CD56^{þ}$ were mainly cytotoxic [32]. Additionally, Cooper et al. [42] have proposed that human NK cells could be categorized into two distinct subsets that express different levels of surface CD56. Detailed phenotype functional studies have demonstrated that the $CD56^{dim}$ NK cells are more cytotoxic against NK-sensitive targets than $CD56^{bright}$ NK cells. We hypothesize that the higher cytotoxic activity of these NK cell subsets in IND patients could be important in helping to suppress parasitaemia to very low levels, resulting in avoidance of the development of a strong acquired immune response against parasite-specific antigens and the outcome of severe chagasic disease [43]. This parasite–host equilibrium allows prolonged host survival in the absence of symptoms or signs of disease, leading to a lifelong chronic phase in about 80% of the patients [44].

Taken together, these findings suggest that a strong activation of $CD8^{þ}$ HLA-$DR^{þ}$ T cells could result in tissue damage leading to the development of cardiomyopathy and/or megaesophagus or megacolon [29, 30]. By contrast, the ability to build up NK-mediated cell cytotoxicity seems to play a pivotal role in the generation of effective, nondeleterious inflammatory mechanisms. When parasitaemia and tissue parasitism are controlled, myocarditis, gastrointestinal damage and lymphocyte activation are attenuated during the chronic asymptomatic stage of infection. Considering the complexity of the human immune response, this insight suggests that other immunoregulatory mechanisms come into play to control the intense immune activity and are apparently necessary to prevent a deleterious effect of the excessive stimulation of the immune system. Therefore, the regulation of the cytotoxic effect of NK cells as well as the absence of activation of $CD8^{þ}$ T cells observed in IND patients would be a

consequence of additional regulatory mechanisms highlighted by current concepts of immunoregulation.

Recent reports suggest that the production of IFN-g and perforin as well as the NK and $CD8^{+}$ T-cell cytotoxicity are decreased by $CD4^{+}CD25^{+}$ regulatory T cells [10]. Whereas the entire population of $CD4^{+}CD25^{+}$ T cells expressing both low and high CD25 levels exhibits regulatory function in the mouse, only the $CD4^{+}CD25^{high}$ population, the natural regulatory T cells [11, 12], exhibits a similarly strong regulatory function in humans [11, 13]. These cells mediate their suppressive effects in vitro in a cell contact-dependent manner controlling disease processes, whereas adaptive regulatory T cells suppress immune responses by producing anti-inflammatory cytokines such as IL-10 and TGF-b [45]. We observed a higher frequency of $CD4^{+}CD25^{high}$ regulatory T cells exclusively in patients from the IND group. These findings clearly reinforce our hypothesis that additional immunoregulatory events may be important in driving NK cell activity and suppressing the activation of $CD8^{+}$ T cells in IND patients, thereby controlling the morbidity of Chagas' disease. The ability of $CD4^{+}CD25^{high}$ regulatory T cells to inhibit IFN-g synthesis confirms previous observations of low levels of IFN-g production by mononuclear cells from patients presenting with the asymptomatic chronic phase of Chagas' disease [46]. Further studies defining the role of regulatory cells during T. cruzi infection would contribute new insights into preventing or controlling the inflammatory process and the immunopathologies observed in chagasic patients.

The functions of human NKT cells are not totally understood. These cells are particularly abundant in the normal human liver, representing one-third of all hepatic $CD3^{+}$ cells but only approximately 2% of peripheral blood T cells [47]. Interestingly, it has been proposed that in humans these NKT cells may play an important role in eliminating autologous cells in the liver, an important site for the elimination of activated T cells via apoptosis. Huang et al. [48, 49] reported that following activation, circulating $CD8^{+}$ T cells are cleared from the lymph nodes and spleen and transported to the liver, where they undergo apoptosis. On the basis of these previous reports, we used a three-colour platform immunophenotyping technique in this study to identify NKT cells as $CD3^{+}CD16^{-/+}CD56^{-/+}$. In this context, we were able to perform the analysis of distinct NKT cell subsets, herein named as follows: NKT1 ($CD3^{+}CD16^{+}CD56^{-}$), NKT2 ($CD3^{+}CD16^{-}CD56^{+}$) and NKT3 ($CD3^{+}CD16^{+}CD56^{+}$). We observed a higher frequency of NKT2 cells ($CD3^{+}CD16^{-}CD56^{+}$) only in the IND group in comparison with noninfected individuals. These findings are consistent with the lower frequency of activated $CD8^{+}$ T cells in the peripheral blood of the IND group observed in our study [28]. In this context, we believe that in the indeterminate clinical form of Chagas' disease, NKT cells provide complementary functions in the liver, associated with the elimination of activated $CD8^{+}$ T cells from peripheral blood and thereby controlling the inflammatory process. However, recent studies using the experimental murine model of T. cruzi infection have proposed that NKT cells play important roles in both proinflammatory and anti-inflammatory responses. Those authors suggested that invariant NKT cells limit the inflammatory response and prevent tissue damage, whereas the variant NKT cells increase the inflammatory response that contributes to morbidity and mortality [28]. Thus, some functionally distinct NKT cell subsets may trigger immunomodulatory events, while others promote immune response. Our data did not, however, reveal any such phenotypic/functional differences in peripheral blood NKT cells among the clinical groups studied.

In conclusion, we demonstrated a new set of relevant phenotypic features, pointing out distinct peripheral blood cell subpopulations in patients with different clinical forms of Chagas' disease. These features are undoubtedly of major importance to understanding the cellular immune response triggered by human chronic T. cruzi infection. In this context, we have documented for the first time the presence of a higher frequency of $CD4^{+}CD25^{high}$ regulatory T cells in addition to higher levels of circulating NKT2 cells ($CD3^{+}CD56^{+}$) in the indeterminate clinical form than in the CARD and DIG forms. The elevated frequencies of these cell types together seem to play an important role in controlling $CD8^{+}$ T-cell-mediated cytotoxicity, regulating T-cell activation and the production of proinflamatory cytokines such as TNF-a and IFN-g as well as inducing the upregulation of anti-T. cruzi antibody response. The lack of regulated cell populations in CARD and DIG patients could account for the impaired immune response that culminates in strong cytotoxic activity and tissue damage. Further studies to functionally characterize these populations will be of great interest and may provide a better understanding of the immunological complexities that are involved in the IND form of Chagas' disease. Moreover, a better understanding of the immunological mechanisms involved in the control of parasite replication and development of chagasic cardiac/digestive disease may contribute new insights on how to prevent or treat the symptomatic forms of Chagas' disease.

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References

1 Moncayo A. Progress towards the elimination of transmission of Chagas disease in Latin America. World Health Stat Q 1997;50:195–8.

2 WHO Expert Committee. Control of Chagas disease. World Health Organ Tech Rep Ser 2002;905: i–vi, 1–109, back cover.

3 Brener Z, Gazzinelli RT. Immunological control of Trypanosoma cruzi infection and pathogenesis of Chagas' disease. Int Arch Allergy Immunol 1997;114:103–10.

4 Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. Nat Rev Immunol 2003;3:253–7.

5 Piccirillo CA, Shevach EM. Naturally-occurring CD4þ CD25þ immunoregulatory T cells: central players in the arena of peripheral tolerance. Semin Immunol 2004;16:81–8.

6 Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. Nat Immunol 2005;6:353–60.

7 Shevach EM. CD4þ CD25þ suppressor T cells: more questions than answers. Nat Rev Immunol 2002;2:389–400.

8 Gavin MA, Clarke SR, Negrou E, Gallegos A, Rudensky A. Homeostasis and anergy of CD4(þ)CD25(þ) suppressor T cells in vivo. Nat Immunol 2002;3:33–41.

9 Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. Nat Immunol 2001;2:816–22.

10 Trzonkowski P, Szmit E, Mysliwska J, Dobyszuk A, Mysliwski A. CD4þ CD25þ T regulatory cells inhibit cytotoxic activity of T CD8þ and NK lymphocytes in the direct cell-to-cell interaction. Clin Immunol 2004;112:258–67.

11 O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. Nat Med 2004;10:801–5.

12 Cassis L, Aiello S, Noris M. Natural versus adaptive regulatory T cells. Contrib Nephrol 2005;146:121–31.

13 Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target Cell death. Immunity 2004;21:589–601.

14 Piccirillo CA, Shevach EM. Control of CD8^{b} T cell activation by CD4þ CD25þ immunoregulatory cells. J Immunol 2001;167:1137–40.

15 Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. Immunol Today 2000;21:573–83.

16 Kronenberg M, Gapin L. The unconventional lifestyle of NKT cells. Nat Rev Immunol 2002;2:557–68.

17 Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. J Clin Invest 2004;114:1379–88.

18 Van der Vliet HJ, Molling JW, von Blomberg BM et al. The immunoregulatory role of CD1d-restricted natural killer T cells in disease. Clin Immunol 2004;112:8–23.

19 Matsuda JL, Gapin L, Baron JL et al. Mouse Va14i natural killer T cells are resistant to cytokine polarization in vivo. Proc Natl Acad Sci USA 2003;100:8395–400.

20 Vincent MS, Gumperz JE, Brenner MB. Understanding the function of CD1-restricted T cells. Nat Immunol 2003;4:517–23.

21 Hammond KJL, Kronenberg M. Natural killer T cells: natural or unnatural regulators of autoimmunity? Curr Opin Immunol 2003;15:683–9.

22 Leite-de-Moraes MC, Herbelin A, Gombert JM, Vicari A, Papiernik M, Dy M. Requirement of IL-7 for IL-4-producing potential of MHC class I-selected CD4^{-}CD8^{-}TCRab^{b} thymocytes. Int Immunol 1997;9:73–9.

23 Ortaldo JR, Winkler-Pickett RT, Yagita H, Young HA. Comparative studies of CD3^{-} and CD3^{b}CD56^{b} cells: examination of morphology, functions, T cell receptor rearrangement, and pore-forming protein expression. Cell Immunol 1991;136:486–95.

24 Kojo S, Tsutsumi A, Goto D, Sumida T. Low expression levels of soluble CD1d gene in patients with rheumatoid arthritis. J Rheumatol 2003;30:2524–8.

25 Emoto M, Miyamoto M, Emoto Y et al. Highly biased type 1 immune responses in mice deficient in LFA-1 in Listeria monocytogenes infection are caused by elevated IL-12 production by granulocytes. J Immunol 2003;171:3970–6.

26 Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao HR. The regulatory role of Va14 NKT cells in innate and acquired immune response. Annu Rev Immunol 2003;21:483–513.

27 Van Kaer L. Regulation of immune responses by CD1d-restricted natural killer T cells. Immunol Res 2004;30:139–53.

28 Duthie MS, Kahn M, White M, Kapur RP, Kahn SJ. Critical proinflammatory and anti-inflammatory functions of different subsets of CD1d-restricted natural killer T cells during Trypanosoma cruzi infection. Infect Immun 2005;73:181–92.

29 Reis DD, Jones EM, Tostes S Jr. et al. Characterization of inflammatory infiltrates in chronic chagasic myocardial lesions: presence of tumor necrosis factor-aþ cells and dominance of granzyme Aþ, CD8þ lymphocytes. Am J Trop Med Hyg 1993;48:637–44.

30 Reis DD, Lemos ML, Silva GC et al. Phenotypic characterization of the inflammatory cells in chagasic mega-oesophagus. Trans R Soc Trop Med Hyg 2001;95:177–8.

31 Gaddy J, Broxmeyer HE. Cord blood CD16þ 56– cells with low lytic activity are possible precursors of mature natural killer cells. Cell Immunol 1997;180:132–42.

32 Sondergaard SR, Ullum H, Pedersen BK. Proliferative and cytotoxic capabilities of CD16þ CD56– and CD16þ /– CD56þ natural killer cells. APMIS 2000;108:831–7.

33 Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4þ CD25 high regulatory cells in human peripheral blood. J Immunol 2001;167:1245–53.

34 Dutra WO, Martins-Filho OA, Cancado JR et al. Activated T and B lymphocytes in peripheral blood of patients with Chagas' disease. Int Immunol 1994;6:499–506.

35 Dutra WO, Martins-Filho OA, Cancado JR et al. Chagasic patients lack CD28 expression on many of their circulating T lymphocytes. Scand J Immunol 1996;43:88–93.

36 Lemos EM, Reis D, Adad SJ, Silva GC, Crema E, Correa-Oliveira R. Decreased CD4(þ) circulating T lymphocytes in patients with gastrointestinal Chagas disease. Clin Immunol Immunopathol 1998;88:150–5.

37 Higuchi Mde L, Gutierrez PS, Aiello VD et al. Immunohistochemical characterization of infiltrating cells in human chronic chagasic myocarditis: comparison with myocardial rejection process. Virchows Arch A Pathol Anat Histopathol 1993;423:157–60.

38 Corrêa-Oliveira R, Gomes J, Lemos EM et al. The role of the immune response on the development of severe clinical forms of human Chagas disease. Mem Inst Oswaldo Cruz 1999;94 (Suppl. 1):253–5.

39 Brener Z. Recent developments in the field of Chagas' disease. Bull World Health Organ 1982;60:463–73.

40 Golgher D, Gazzinelli RT. Innate and acquired immunity in the pathogenesis of Chagas disease. Autoimmunity 2004;37:399–409.

41 Lanier LL, Phillips JHJ, Tutt M, Kumar V. Natural killer cells: definition of a cell type rather than a function. J Immunol 1986;137:2745–9.

42 Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol 2001;22:633–40.

43 Lieke T, Graefe SE, Klauenberg U, Fleischer B, Jacobs T. NK-cells contribute to the control of Trypanosoma cruzi infection by killing free parasites by perforin-independent mechanisms. Infect Immun 2004;72:6817–25.

44 Andrade ZA. Immunopathology of Chagas' disease. Mem Inst Oswaldo Cruz 1999;94:71–80.

45 Dittmer U, He H, Messer RJ et al. Functional impairment of CD8(þ) T cells by regulatory T cells during persistent retroviral infection. Immunity 2004;20:293–303. Erratum in: Immunity 2004: May; 20: 653.
46 Gomes JA, Bahia-Oliveira LM, Rocha MO, Martins-Filho OA, Gazzinelli G, Correa-Oliveira R. Evidence that development of severe cardiomyopathy in human Chagas' disease is due to a Th1-specific immune response. Infect Immun 2003;71:1185–93.
47 Norris S, Doherty DG, Collins C et al. Natural T cells in the human liver, cytotoxic lymphocytes with dual T cell and natural killer cell phenotype and function are phenotypically heterogeneous and include Va24-JaQ and gd T receptor bearing cells. Hum Immunol 1999;60:20–31.
48 Huang L, Soldevila G, Leeker M, Flavell R, Crispe IN. The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. Immunity 1994;1:741–9.
49 Huang L, Sye K, Crispe IN. Proliferation and apoptosis of $B220^{p}CD4^{-}CD8^{-}TCRa/b^{int}$ T cells in the liver of normal mice: implications for Ipr pathogenesis. Int Immunol 1994;6:533–40.

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Are increased frequency of macrophage-like and natural killer (NK) cells, together with high levels of NKT and $CD4^+CD25^{high}$ T cells balancing activated $CD8^+$ T cells, the key to control Chagas' disease morbidity?

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Summary

The immunological response during early human Trypanosoma cruzi infection is not completely understood, despite its role in driving the development of distinct clinical manifestations of chronic infection. Herein we report the results of a descriptive flow cytometric immunophenotyping investigation of major and minor peripheral blood leucocyte subpopulations in T. cruzi-infected children, characterizing the early stages of the indeterminate clinical form of Chagas' disease. Our results indicated significant alterations by comparison with uninfected children, including increased values of pre-natural killer (NK)-cells ($CD3^-$ $CD16^+$ $CD56^-$), and higher values of proinflammatory monocytes ($CD14^+$ $CD16^+$ HLA-DR^{++}). The higher values of activated B lymphocytes ($CD19^+$ $CD23^+$) contrasted with impaired T cell activation, indicated by lower values of $CD4^+$ $CD38^+$ and $CD4^+$ HLA-DR^+ lymphocytes, a lower frequency of $CD8^+$ $CD38^+$ and $CD8^+$ HLA-DR^+ cells; a decreased frequency of $CD4^+$ $CD25^{HIGH}$ regulatory T cells was also observed. These findings reinforce the hypothesis that simultaneous activation of innate and adaptive immunity mechanisms in addition to suppression of adaptive cellular immune response occur during early events of Chagas' disease. Comparative cross-sectional analysis of these immunophenotypes with those exhibited by patients with late chronic indeterminate and cardiac forms of disease suggested that a shift toward high values of macrophage-like cells extended to basal levels of proinflammatory monocytes as well as high values of mature NK cells, NKT and regulatory T cells, may account for limited tissue damage during chronic infection favouring the establishment/maintenance of a lifelong indeterminate clinical form of the disease. On the other hand, development of an adaptive cell-mediated inflammatory immunoprofile characterized by high levels of activated $CD8^+$ cells and basal levels of mature NK cells, NKT and $CD4^+$ $CD25^{HIGH}$ cells might lead to late chronic pathologies associated with chagasic heart disease.

Keywords: Chagas' disease, flow cytometry, peripheral blood, recent infection.

Introduction

Chagas' disease or American trypanosomiasis is a protozoan infection caused by the haemoflagellate protozoan Trypanosoma cruzi. It is one of the most important public health problems in Latin America, affecting 16–18 million people in South and Central America [1].

Human T. cruzi infection evolves from a usually oligosymptomatic acute phase to a chronic disease, where patients can be grouped into distinct categories based on clinical status. The great majority of the patients that progress to the chronic phase remain clinically asymptomatic for many years; this condition characterizes the indeterminate (IND) clinical form of the disease. About 30–40% of patients progress to cardiac (CARD) or digestive symptomatic disease. It is estimated that 30% of all infected individuals will eventually develop heart disease [2].

The factors that underlie and determine the distinct clinical outcomes, mild or severe disease, are not completely understood. However, there is a general consensus

that the host immune response plays a pivotal role associated with the pathogenesis as well as the protective events that control chagasic tissue damage [3]. It is also well accepted that T. cruzi induces a strong activation of the immune system during acute infection and that the different immunological mechanisms triggered during the early indeterminate (E-IND) stages of T. cruzi infection may represent an essential component of the immune activity observed during ongoing, clinically distinct chronic infection [4].

In the search to identify differences in the immunological response related to the development/maintenance of distinct chronic disease, we have focused on major and minor peripheral blood leucocyte subsets during E-IND and late chronic IND and CARD Chagas' disease [5,6]. We have reported previously that an expansion of natural killer (NK) cells before the development of T cell-mediated immunity, in addition to enhancement of circulating activated B cells, are the hallmarks of human immune response during early T. cruzi infection. Moreover, we have reported that an increase of pre-NK cells ($CD16^+$ $CD56^-$), as well as a persistent expansion of activated B cells and down-regulation of CD54 on T cells, are also observed during initial stages of chronic T. cruzi infection [5]. Here, we discuss the hypothesis that T cell-mediated immunity during the early stages of T. cruzi infection may represent a phenomenon restricted to the cardiac and lymph node compartment, and may not be detectable in the peripheral blood.

Despite the T cell-independent nature of the immune response triggered in early Chagas' disease, we have demonstrated that T cells play an important role in the dynamics of chronic Chagas' disease [6–8]. Previous reports from our group showed that despite their clinical status, chronic chagasic patients display a high frequency of peripheral blood activated T cells ($HLA\text{-}DR^+$) as well as lack of CD28 expression on many of their circulating T lymphocytes [7,8]. More recently, ex vivo immunophenotyping demonstrated that IND patients display a higher frequency of both $CD4^+$ $CD25^{HIGH}$ and NKT ($CD3^+$ $CD16^-$ $CD56^+$) regulatory cells associated with increased levels of circulating 'cytotoxic' NK cells ($CD3^-$ $CD16^+$ $CD56^+$ and $CD3^-$ $CD16^+$ $CD56^{DIM}$ NK cells) [6]. On the other hand, an increased percentage of activated $CD8^+$ $HLA\text{-}DR^+$ T cell subset was associated exclusively with severe clinical forms of Chagas' disease [6]. We hypothesize that regulatory T cells control the deleterious cytotoxic activity in the indeterminate clinical form, inhibiting the activation of $CD8^+$ $HLA\text{-}DR^+$ T cells. The lack of regulated populations in CARD disease patients could account for exacerbated immune response that culminates in strong cytotoxic activity and tissue damage.

Relevant findings regarding histopathological alterations in biopsies from chagasic patients showed that tissue $CD4^+$ and $CD8^+$ T cells increase simultaneously during early infection but not in the chronic phase, supporting the hypothesis of compartmentalized T cell-mediated immune response during early disease and suggesting an immunological imbalance of T cell profile in late chronic Chagas' disease. In the chronic phase, patients with heart failure present with higher levels of $CD8^+$ T cells than $CD4^+$ T cells, leading to a lower tissue $CD4^+/CD8^+$ T cell ratio [9,10].

More recently, data have been reported suggesting that monocytes from IND patients display modulatory characteristics related to low HLA-DR and high IL-10 expression, whereas monocytes from CARD patients may be committed to induction of inflammatory responses related to high tumour necrosis factor (TNF)-α expression [11–13].

Increasing numbers of novel cellular parameters and surface markers have been examined as conventional flow cytometry-based investigations, i.e. 'look and conclude' analyses, have adopted new gating strategies to analyse immunophenotypes at the single-cell level in a semiquantitative manner. Indeed, flow cytometry has emerged as the methodology of choice for enumerating and characterizing of novel leucocyte subsets using three- and four-colour platform technology. With this technology, several novel phenotypic features of leucocyte subsets are characterized routinely in parallel by their in vitro and in vivo functional properties, such as NKT cells ($CD3^+$ $CD56^+$) [14], functionally distinct NK subsets ($CD3^-CD16^{-/+}$ $CD56^{-/+}$) [15,16], regulatory T cells ($CD4^+$ $CD25^{HIGH}$) [17], macrophage-like monocytes ($CD14^+$ $CD16^+$) [18] and proinflammatory monocytes ($CD14^+$ $CD16^+$ $HLA\text{-}DR^{++}$) [19].

We have performed a descriptive flow cytometric immunophenotyping investigation based on these new gating strategies to enumerate major and minor leucocyte subpopulations in the peripheral blood of T. cruzi-infected children, characterizing the E-IND stages of Chagas' disease. Comparative cross-sectional analyses of the predominant immunophenotypes were also performed in those patients exhibiting late chronic IND or CARD disease. Our results suggest that a shift toward high levels of macrophage-like cells ($CD14^+$ $CD16^+$) and NK cells, besides high frequency of regulatory lymphocytes (NKT and $CD4^+$ $CD25^{HIGH}$ cells), may favour the establishment/maintenance of the lifelong indeterminate clinical form of the disease. On the other hand, maintenance of major cell phenotypic features observed during early infection as well as the development of an adaptive cell-mediated inflammatory immunoprofile characterized by high levels of activated $CD8^+$ cells and basal frequency of mature NK cells, NKT and $CD4^+$ $CD25^{HIGH}$, might lead to a late chronic disease associated with cardiac pathological events.

Patients, materials and methods

Study area

Berilo and José Gonçalves de Minas are located in Jequitinhonha Valley in the north-east of Minas Gerais State, Brazil.

Jequitinhonha Valley comprises 970 km^2. Chagas' disease was formerly endemic in the area. Together, these two municipalities have 17 632 inhabitants, with 78·43% of these individuals living in rural areas with an economy based on agriculture and cattle ranching [IBGE.Cidades@http://URL:http://www.ibge.gov.br/cidadesat/default.php (search for Berilo and José Gonçalves de Minas); accessed 4 January 2006].

Bambui is located in the south-west of Minas Gerais State, Brazil. It comprises 1455 km^2 and is another area in which Chagas' disease was formerly endemic. It has 22 274 inhabitants, 80% of them in the urban area of the municipality [IBGE.Cidades@http://www.ibge.gov.br/cidadesat/default.php (search for Bambuí); accessed 4 January 2006].

Study population

School children enrolled in a cross-sectional study performed at 37 communities from Berilo and José Gonçalves de Minas, including 39 school units, participated in a serological screening trial to detect anti-T. cruzi antibodies by enzyme-linked immunosorbent assay (ELISA) using blood eluate from filter paper. The screening immunoassay identified 2·69% of school children with positive results for anti-T. cruzi IgG. Confirmatory immunodiagnosis for Chagas' disease was performed by ELISA, EIE-Rec-ELISA (Biomanguinhos/FIOCRUZ), indirect immunofluorescence assay (IFA) and haemaglutination (HA) tests. Considering the World Health Organization and Brazilian Health Ministry criteria that recommend the use of at least two serological tests, with distinct principles, to confirm the diagnosis of Chagas' disease, we confirmed six of the 38 cases first identified with positive results by the screening ELISA, leading to a total prevalence of 0·42%. The seropositive cases included four males and two females, with ages ranging from 9 to 14 years. The clinical and physical examination revealed that all children were asymptomatic, showing normal conventional electrocardiograms (except no. 701, who showed enlargement of Pri−0·22) and unaltered thoracic X-ray (RX) (Table 1). The haemoculture was positive in all children examined (six of six), generally within the first month of blood cultivation in liver infusion tryptose (LIT) media supplemented with 10% fetal calf serum. All seropositive children were treated with benznidazol (Rochagan®, Roche) and are currently under evaluation following the protocol recommended by the Brazilian Health Ministry [20]. Seven non-infected (NI) schoolchildren were included as a control group. The NI-1 group consisted of age-matched schoolchildren with negative serology for anti-T. cruzi IgG immunodiagnosis (ELISA and IFA). The NI-1 group included one male and six females, with ages ranging from 9 to 14 years (mean = 12·4).

Table 1. Patient characteristics.

Group	No. of individuals	Age range (years)	Sex (male/female)
Non-infected children (NI-1)	7	9–14	6/1
Non-infected adults (NI-2)	12	20–59	3/9
Early indeterminate (E-IND)	6	9–14	4/2
Chronic indeterminate (IND)	8	44–67	3/5
Chronic cardiac (CARD)	13	50–70	5/8

Cross-sectional analyses of late chronic chagasic patients compared with uninfected adult controls were carried out to evaluate major immunophenotypic features. All late chronic infected individuals as well as the uninfected adult controls were from Bambuí, Minas Gerais State, Brazil, and participated in serological examination to confirm the positive or negative diagnosis for T. cruzi infection, respectively. The diagnosis was based on standard serological tests, including IFA and HA tests. In this study, we used 21 samples from chagasic patients with late chronic disease. According to their clinical records, the late chronic chagasic patients were divided into two categories, namely IND and CARD clinical forms. Patients presenting asymptomatic T. cruzi infection, classified as indeterminate (n = 8), had no clinical manifestations of the disease other than their positive serology. Patients with cardiac dysfunction, CARD (n = 13), presented dilated cardiomyopathy and were diagnosed by a detailed clinical examination, including eletrocardiography (ECG), 24-h Holter examination and chest X-ray. Twelve seronegative adults were included in this study as negative controls 2 (NI-2 = 12). All were living in an area endemic for Chagas' disease (Table 1).

Informed written consent was obtained from all participants or through their parents or legal guardians in the case of the school children. This work complied with resolution number 196/1996 from the National Health Council for research involving humans and was approved by the Ethical Committee at Centro de Pesquisas René Rachou (CPqRR/FIOCRUZ protocol 11/2004), Belo Horizonte, Minas Gerais, Brazil.

Blood samples

A 5-ml sample of peripheral blood was collected from each subject using ethylenediamine tetraacetic acid (EDTA) as the anticoagulant. The samples were collected by trained professionals in an ambulatory hospital. After the collection, the whole peripheral blood was analysed by flow cytometry.

Specific monoclonal antibodies used for immunophenotyping

Mouse anti-human monoclonal antibodies (mAbs), conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or tri-colour (TC) and specific for cell-surface markers

were used simultaneously for two- or three-colour flow cytometric assay. In this study, we used anti-human FITC-conjugated mAbs including anti-CD3 (UCHT1), anti-CD4 (RPA-T4), anti-CD5 (L17F12), anti-CD8 (B9·11), anti-CD16 (3G8), anti-CD18 (YF118·3), anti-CD54 (15·2), anti-CD62L (DREG-56) and mouse IgG1 as the isotypic control (679·1Mc7). The following second-colour reagents were used: anti-human PE-conjugated mAbs anti-CD3 (UCHT1), anti-CD4 (RPA-T4), anti-CD19 (4G7), anti-CD23 (M-L233), anti-CD25 (3G10), anti-CD28 (15E8), anti-CD38 (AT13/5), anti-CD56 (B159), anti-HLA-DR (TÜ36) and mouse IgG2a as the isotypic control (UCTH-1). All antibodies were purchased from Becton-Dickinson (Mountain View, CA, USA). The third colour parameter was evaluated using TC-conjugated mAbs and included anti-CD8 (M-L233), anti-CD14 (TüK4), anti-CD16 (3G8) and anti-CD19 (4G7), all purchased from Caltag Laboratories (Burlingame, CA, USA).

Flow cytometric analysis of peripheral blood

White blood cell phenotypes were analysed following an immunofluorescence procedure recommended by Becton-Dickinson, modified as follows: 100 µl peripheral blood which had been collected in Vacutainer tubes containing EDTA (Becton Dickinson) was mixed in 12 × 75 mm tubes with 5 µl undiluted mAbs specific for several cell surface markers; the tubes were incubated in the dark for 30 min at room temperature. Following the incubation, erythrocytes were lysed with 2 ml FACS lysing solution (Becton Dickinson Biosciences Pharmigen, San Diego, CA, USA). The remaining cells were then washed twice with 2 ml phosphate-buffered saline containing 0·01% sodium azide. Cell preparations were fixed in 200 µl FACS Fix solution (10 g/l paraformaldehyde, 1% sodium-cacodylate, 6·65 g/l sodium-chloride, 0·01% sodium azide). Cytofluorimetric data acquisition was performed with a Becton-Dickinson FACScalibur instrument. cellquest™ software provided by the manufacturer was used for data acquisition and analysis.

Statistical analysis

Differences between groups were first evaluated by minitab software (release 13·20) to evaluate the independence, normality and variance of data sets. Those data sets meeting the three criteria were considered parametric and were compared further by analysis of variance (anova) followed by the Tukey test, using the prism 3·0 program. Non-parametric data were analysed by the Kruskal–Wallis test followed by Dunn's test. Correlation analysis was performed by Pearson's and Spearman's tests, respectively. Significance was defined in both cases at $P < 0·05$.

Results

Low values of $CD3^+$ T lymphocytes, mainly $CD8^+$ T lymphocytes and impaired T cell activation, are the hallmark of the early indeterminate clinical form of Chagas' disease

The percentage of T cell populations and the major subsets $CD4^+$ and $CD8^+$ are shown in Fig. 1. Statistical analysis demonstrated a lower percentage of circulating T lymphocytes ($CD3^+$) in children with E-IND Chagas' disease in comparison to non-infected children (NI-1) (Fig. 1a). Further analysis revealed that the decrease in $CD3^+$ T cells was correlated with a significant decrease in the $CD8^+$ T lymphocyte subset ($r = 1$, $P = 0·0167$) (Fig. 1c). No significant differences were found in the mean values of the circulating $CD4^+$ T cell subset (Fig. 1b).

Analysis of activated T cells revealed a lower ratio of $CD4^+$ $HLA\text{-}DR^+$ and $CD4^+$ $CD38^+$ T cells with no changes in $CD8^+$ $HLA\text{-}DR^+$ and $CD8^+$ $CD38^+$ T cells, parallel to an unaltered profile of CD28 expression within $CD4^+$ and $CD8^+$ T cells (Table 2).

Higher values of activated B lymphocytes is observed of early indeterminate Chagas' disease

Phenotypic analysis at the single-cell level was used to analyse the frequency of B cell subsets (conventional B lymphocytes/$CD19^+$ $CD5^-$ and B1/$CD19^+$ $CD5^+$) as well as their activation status, using anti-CD23 PE and anti-CD19 FITC in a dual-platform to identify activated B cells ($CD19^+$ $CD23^+$) (Fig. 1). Our findings showed no significant differences in the mean percentage of B cells and their major subsets between E-IND and NI-1 children (Fig. 1d,e,f). Interestingly, analysis of $CD19^+$ B cells co-expressing the CD23 cell-surface activation marker showed an increased mean ratio of double-positive B lymphocytes within $CD19^+$ cells in E-IND in comparison to NI-1 children (Fig. 1g).

Despite the lack of activation phenotypes among circulating $CD8^+$ T cells, early indeterminate chagasic children displayed a high migratory potential of cytotoxic T cells

In order to quantify the frequency of circulating T cell subsets co-expressing surface selectin (CD62L) and integrins (CD18 and CD54), a three-colour flow cytometry analysis was carried out using a cocktail of monoclonal antibodies, including anti-CD62L, anti-CD18 or anti-CD54 FITC, plus anti-CD4 PE and anti-CD8 TC. Our results indicated $CD4^+$ T cell activation, demonstrated by the lower ratio of circulating $CD62L^+$ cells, despite unaltered levels of $CD18^+$ and $CD54^+$ cells, among $CD4^+$ T lymphocytes (Table 2).

On the other hand, regardless of no phenotypic changes signalling the activation of $CD8^+$ T cells (i.e. expression of HLA-DR, CD28, CD38, CD62L and CD18), our data demonstrated

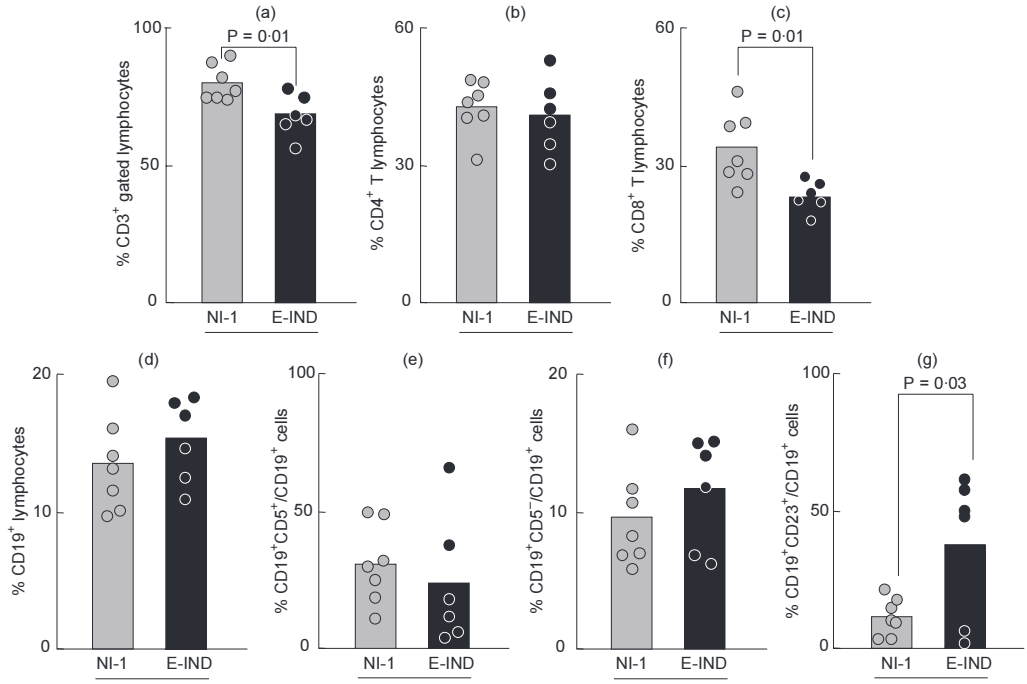

Fig. 1. Analysis of T and B cell subsets in the peripheral blood of early indeterminate Trypanosoma cruzi infected children (E-IND, ▨) and non-infected children (NI-1, ▩). Phenotypic studies were performed by a double-labelling protocol using anti-CD3 fluorescein isothiocyanate (FITC) and anti-CD4 phycoerythrin (PE) or anti-CD8-PE for T cell analysis. Anti-CD19 FITC and anti-CD5-PE or anti-CD23-PE were used to identify B cell subsets. The results are expressed as scattering of individual values and mean percentage of total T cells (a), T cell subsets (b, c), and total B cells (d) within gated lymphocytes. B lymphocyte subsets, including B-1 cells (e), conventional B cells (f) and activated B lymphocytes (g) were analysed within gated $CD19^+$ lymphocytes. Significant differences (connecting lines) and P-values are shown on figure.

that increased levels of circulating $CD8^+$ $CD54^+$ T cells would be an immunological event that suggest the increased migratory potential of this cytotoxic population, which is important in controlling tissue parasitism (Table 2).

Low levels of macrophage-like ($CD14^+$ $CD16^+$) cells and expansion of $CD14^+$ $CD16^+$ HLA-DR^{++} proinflammatory monocytes were observed in the early chronic T. cruzi-infected children

Ziegler-Heitbrock [18] suggested that, in humans, the expression of CD14 and CD16 by monocytes can be used to define at least two subsets of monocytes with distinct functional properties. In this context, $CD14^+$ $CD16^-$ cells are considered to be classical monocytes whereas $CD14^+$ $CD16^+$ cells are typically macrophage-like cells. Herein we have focused our analysis on major and minor circulating monocyte subpopulations. Our data demonstrated that E-IND

Table 2. Frequency of activation marker and adhesion molecule expression by peripheral blood $CD4^+$ and $CD8^+$ T cell subsets from early indeterminate Trypanosoma cruzi-infected children early indeterminate (E-IND) and non-infected children (NI-1).

	T cell subsets			
	$CD4^+$		$CD8^+$	
Phenotype[a]	NI-1	E-IND	NI-1	E-IND
HLA-DR^+	3·4 ± 0·9	1·3 ± 1·8*	5·3 ± 2·6	4·0 ± 2·8
$CD28^+$	94·2 ± 5·1	97·6 ± 0·8	54·8 ± 14·7	66·1 ± 11·3
$CD38^+$	61·3 ± 17·4	45·2 ± 10·2*	54·7 ± 8·2	56·2 ± 9·9
$CD62L^+$	78·8 ± 10·3	68·1 ± 7·4*	52·4 ± 13·0	50·3 ± 12·0
$CD18^+$	20·8 ± 9·7	27·8 ± 12·7	63·4 ± 14·5	55·2 ± 13·1
$CD54^+$	7·8 ± 10·0	2·9 ± 4·9	27·4 ± 15·2	70·6 ± 15·0*

[a]The results are expressed as proportion within a given T cell subset, e.g. ratio of $CD4^+$ HLA-DR^+ within the $CD4^+$ population, allowing the normalization of data when percentage of a given subset may differ. *Statistically significant differences ($P < 0·05$) in comparison to NI-1.

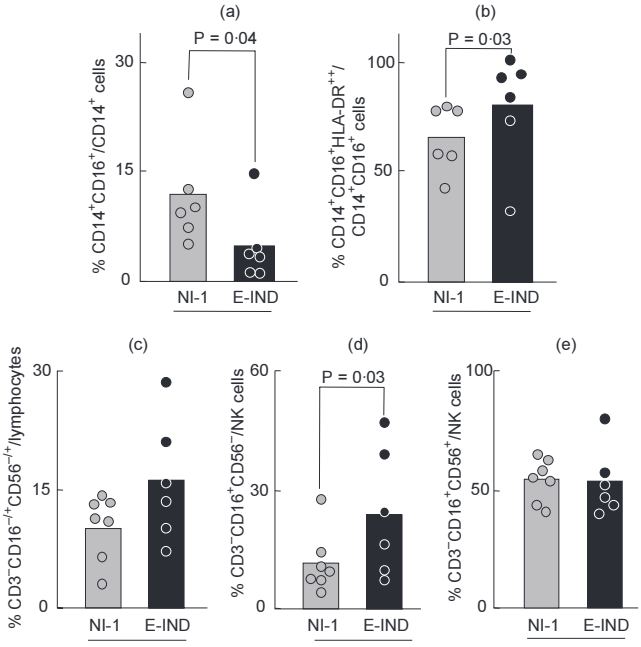

Fig. 2. Analysis of monocyte and natural killer (NK) cell subsets in the peripheral blood of early indeterminate Trypanosoma cruzi-infected early indeterminate (E-IND, ▨) children and non-infected children (NI-1, ▩). Monocyte subpopulation analysis was performed by a triple-labelling platform using anti-CD14 TC, anti-CD16 fluorescein isothiocyanate (FITC) and anti-HLA-DR phycoerythrin (PE) to identify macrophage-like cells ($CD14^+ CD16^+$) (a), proinflammatory monocytes ($CD14^+ CD16^+ HLA\text{-}DR^{++}$) (b). Natural killer (NK) phenotypic studies were performed by a triple-labelling protocol using anti-CD3 FITC, anti-CD56 PE and anti-CD16 tri-colour (TC) to identify total NK cells $CD3^- CD16^{-/+} CD56^{-/+}$ (c), pre-NK cells $CD3^- CD16^+ CD56^-$/$CD3^- CD16^{-/+} CD56^{-/+}$ (b), mature NK cells $CD3^- CD16^+ CD56^+$/$CD3^- CD16^{-/+} CD56^{-/+}$ (e). Data of monocyte subsets are expressed as scattering of individual values and mean percentage of cells within gated monocytes. The results of total NK cells were calculated within gated lymphocytes, whereas the frequency of NK cell subsets were reported within gated NK cells. Significant differences (connecting lines) and P-values are shown on figure.

samples displayed low levels of macrophage-like cells compared to NI-1 samples (Fig. 2a).

Within the $CD14^+ CD16^+$ macrophage-like cells, two monocyte populations can be distinguished: classical HLA-DR^+ monocytes and proinflammatory HLA-DR^{++} monocytes [19]. Quantification of the $CD14^+ CD16^+$ HLA-DR^{++} proinflammatory monocytes was carried out by first gating on the monocyte population identified on dot-plots based on their morphometric and immunophenotypic features, such as $SSC^{intermediate} CD14^+$, followed by the selection of $CD14^+ CD16^+$ cells (macrophage-like cells) and further enumeration of those $CD14^+ CD16^+$ cells with high expression of HLA-DR, as proposed by Belge et al. [19]. Our results showed a higher value of $CD14^+ CD16^+$ HLA-DR^{++} proinflammatory monocytes within $CD14^+ CD16^+$ monocytes in E-IND samples compared with NI-1 samples (Fig. 2b).

Pre-NK cells ($CD3^- CD16^+ CD56^-$) are expanded in peripheral blood in early indeterminate Chagas' disease

As proposed by Gaddy and Broxmeyer [21], distinct NK cell subsets can be identified based on the differential expression of two major NK cell markers: CD16 and CD56. In order to quantify the frequency of major NK cell subsets, pre-NK cells ($CD3^- CD16^+ CD56^-$) and mature NK cells ($CD3^- CD16^+ CD56^+$) were quantified on a three-colour platform using anti-CD16 TC, anti-CD56 PE and CD3 FITC to exclude NKT cells. Our data showed that despite absence of statistically significant differences in the percentage of total NK cells ($CD3^- CD16^{-/+} CD56^{-/+}$) (Fig. 2c) or of mature NK cells ($CD3^- CD16^+ CD56^+$) (Fig. 2e), E-IND children showed a higher percentage of pre-NK cells ($CD3^- CD16^+ CD56^-$) by comparison with NI-1 children (Fig. 2d).

Decreased percentages of NKT cells ($CD3^+ CD16^- CD56^+$) are associated with the early indeterminate clinical form of Chagas' disease

NKT cells are a unique T lymphocyte subpopulation, distinct from conventional T cells, because they express surface markers of both T cell and NK cell subsets. They can provide protection against infectious diseases by rapidly producing cytokines, through their cytolytic activity or via stimulation of other cell populations [22]. In order to quantify the frequency of circulating NKT cells, we used the same three-colour flow cytometry platform described for enumeration of NK cell subpopulations. Data analysis was performed by classifying NKT cells as NKT1 ($CD3^+ CD16^+ CD56^-$), NKT2 ($CD3^+ CD16^- CD56^+$) or NKT3 ($CD3^+ CD16^+ CD56^+$), as proposed by Vitelli-Avelar et al. [6]. Our results revealed a significantly lower frequency of the NKT2 ($CD3^+ CD16^- CD56^+$) subset in

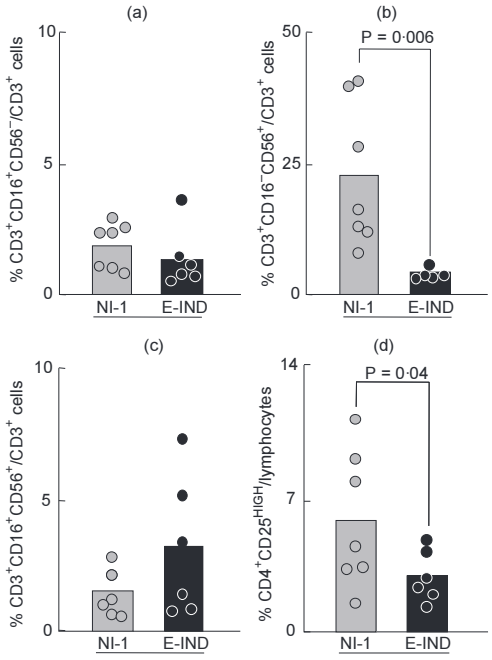

Fig. 3. Analysis of regulatory T cells [natural killer (NK) T and $CD4^+CD25^{HIGH}$] in the peripheral blood of early indeterminate Trypanosoma cruzi-infected children (E-IND, ▩) and non-infected children (NI-1, ▨). NKT phenotypic studies were performed by a triple-labelling protocol using anti-CD3 fluorescein isothiocyanate (FITC), anti-CD56 phycoerythrin (PE) and anti-CD16 TC to identify NKT subsets including NKT1 cells $CD3^+$ $CD16^+$ $CD56^-/CD3^+$ (a), NKT2 cells $CD3^+$ $CD16^-$ $CD56^+/CD3^+$ (b) and NKT3 cells $CD3^+$ $CD16^+$ $CD56^+/CD3^+$ (c) analysed within gated $CD3^+$ lymphocytes. Regulatory T cells were identified through a double staining procedure with anti-CD4 FITC and anti-CD25 PE monoclonal antibodies to identify regulatory $CD4^+$ $CD25^{HIGH}$ T cells (d) within gated lymphocytes. Data are expressed as scattering of individual values and mean percentage of cells. Significant differences (connecting lines) and P-values are shown on the figure.

E-IND samples by comparison with NI-1 samples (Fig. 3b). No differences were observed when the values of NKT1 and NKT3 subsets were evaluated (Fig. 3a,c).

Decrease of circulating $CD4^+$ $CD25^{HIGH}$ T cells highlights impaired immunoregulation in T. cruzi-infected children

In humans, it has been proposed that only the $CD4^+$ $CD25^{HIGH}$ population, comprising ~1–2% of circulating $CD4^+$ T cells, exhibits regulatory functions [17]. Enumeration of $CD4^+$ $CD25^{HIGH}$ regulatory T cells was carried out by first gating on lymphocytes based on their morphometric features on forward- versus side-scatter dot plots, followed by the selection of $CD4^+$ cells presenting high expression of CD25 [17]. Our results demonstrated that lower values of $CD4^+$ $CD25^{HIGH}$ regulatory T cells are observed in E-IND samples than in NI-1 samples (Fig. 3d).

Using the same gating strategy described previously to evaluate E-IND and NI-1 samples, we performed a parallel investigation of major peripheral blood leucocyte phenotypes of IND, CARD and NI-2 subjects, including: $CD14^+$ $CD16^+$ (macrophage-like), $CD14^+$ $CD16^+$ $HLA\text{-}DR^{++}$ (proinflammatory monocytes), $CD4^+$ $CD25^{HIGH}$ (regulatory T cells), $CD8^+$ $HLA\text{-}DR^+$ (activated $CD8^+$ T cells), $CD3^-$ $CD16^+$ $CD56^-$ (pre-NK cells), $CD3^-$ $CD16^+$ $CD56^+$ (mature NK cells) and $CD3^+$ $CD16^-$ $CD56^+$ (NKT2 cells) (Fig. 4). Our data demonstrated a higher value of circulating macrophage-like, regulatory T cells, mature NK cells and NKT cells in IND than in NI-2 samples (Fig. 4a,c,f,g, respectively). Interestingly, the value of regulatory T cells observed in IND samples was also significantly higher than that observed in samples from CARD patients (Fig. 4c). Basal values (reference average of cells observed in healthy individuals) of proinflammatory monocytes and low levels of pre-NK cells were also observed in IND samples compared to NI-2 samples (Fig. 4b,e). It was remarkable to note that the IND group presented divergent data regarding the value of activated $CD8^+$ T cells, with some individuals presenting low basal levels of activated $CD8^+$ T cells (median value = 3·9%) and others displaying extremely high levels of $CD8^+$ $HLA\text{-}DR^+$ cells/$CD8^+$ T cells (median value = 74·5%), suggesting the existence of distinct subgroups of individuals (Fig. 4d, dotted rectangles). Which immunological feature could compensate the high levels of cellular immune response in these individuals in order to maintain the asymptomatic disease? To answer this question, we assessed these phenotypic features at an individual level, which pointed out that all IND patients who presented with a high value of $CD8^+$ $HLA\text{-}DR^+$ cells also had low values of regulatory T cells (Fig. 5, left panel, top graph). Moreover, these individuals also presented immunophenotypes that suggest a more active role of innate cellular response, because they also displayed higher values of proinflammatory monocytes and mature NK cells (Fig. 5, left panel, top graph). Confirmatory analysis was carried out by correlation studies that further validated these findings, showing a negative correlation between $CD8^+$ $HLA\text{-}DR^+$ and $CD4^+$ $CD25^{HIGH}$ cells and a positive association between $CD8^+$ $HLA\text{-}DR^+$ and $CD14^+$ $CD16^+$ $HLA\text{-}DR^{++}$ cells, with the latter also correlated with the frequency of $CD3^-$ $CD16^+$ $CD56^+$ cells (Fig. 5, left panels, bottom graphs).

Increased percentage of activated $CD8^+$ T cells and basal values of NK, NKT and regulatory T cells are major phenotypes related to late cardiac Chagas' disease

Analysis of major cardiac Chagas' disease discriminatory immunophenotypic features revealed that high levels of

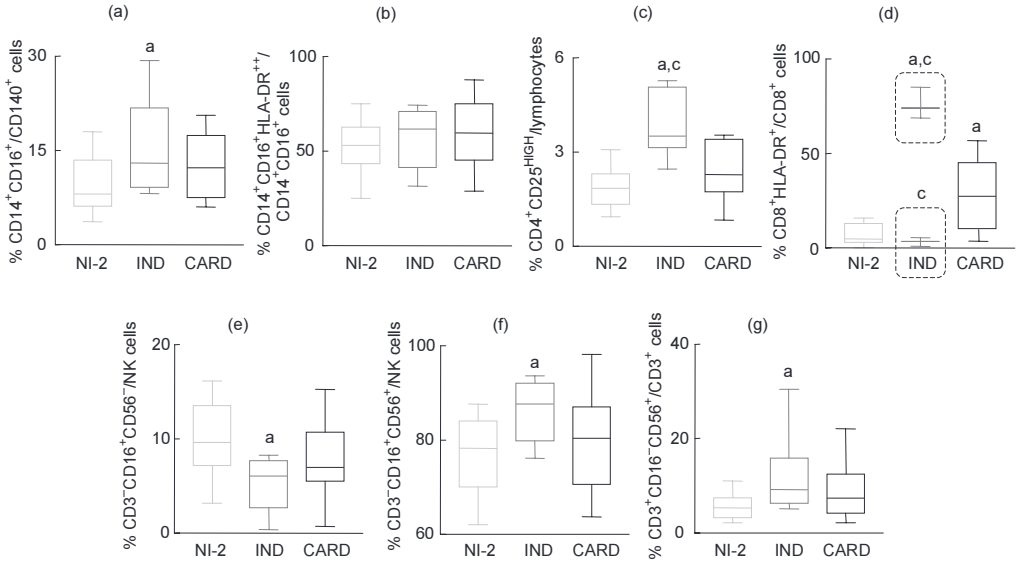

Fig. 4. Analysis of major discriminatory immunophenotypes among individuals with indeterminate disease (IND, ▭) or cardiac disease (CARD, ▭) and uninfected adults (NI-2, ▭). Phenotypic studies were performed using a double or triple-labelling protocol to identify macrophage-like $CD14^+$ $CD16^+$ cells (a), proinflammatory monocytes ($CD14^+$ $CD16^+$ $HLA\text{-}DR^{++}$, b), regulatory T cells ($CD4^+$ $CD25^{HIGH}$, c), activated $CD8^+$ $HLA\text{-}DR^+$ T cells (d), pre-natural killer (NK) cells ($CD3^-$ $CD16^+$ $CD56^{-/+}$/$CD3^-$ $CD16^{-/+}$ $CD56^{-/+}$, e), mature NK cells ($CD3^-$ $CD16^+$ $CD56^+$/$CD3^-$ $CD16^{-/+}$ $CD56^{-/+}$, f) and NKT2 cells ($CD3^+$ $CD16^-$ $CD56^+$, g). The results are expressed in box-plot format. The box stretches from the lower hinge (defined as the 25th percentile) to the upper hinge (the 75th percentile) and therefore contains the middle half of the scores in the distribution. The median is shown as a line across the box. Therefore 1/4 of the distribution is between this line and the top of the box and 1/4 of the distribution is between this line and the bottom of the box. Significant differences compared with NI-2 and CARD are indicated by letters a and c, respectively, at $P < 0{\cdot}05$.

circulating $CD8^+$ $HLA\text{-}DR^+$ cells is the hallmark of the CARD group by comparison with the NI-2 group (Fig. 4d). Additional analysis at an individual level further demonstrated that in the CARD group, individuals displaying higher levels of $CD8^+$ $HLA\text{-}DR^+$ cells (higher than median value = 27·5%) also presented lower levels of mature NK cells, and were confined within a subgroup showing a low value of NKT cells (Fig. 5, right panel, top graph). Confirmatory analysis by correlation studies validated these findings, showing a negative correlation between $CD8^+$ $HLA\text{-}DR^+$ cells and both mature NK and NKT cell subpopulations (Fig. 5, right panel, bottom graphs).

Discussion

Understanding the role of immune responses to T. cruzi and the mechanisms of injury in Chagas' disease has been a major challenge. T. cruzi infection simultaneously triggers multiple compartments of the innate and adaptive immune system. It is possible that the strong immune stimulation and the intense inflammatory process elicited during early infection by T. cruzi [3] could be not only a major determinant of the immunopathology of the late disease, but could also be a crucial factor in confining the aetiological agent to an intracellular site, controlling the consequences of life-long infection and preventing tissue damage [4,23–25]. However, the early stage of T. cruzi infection has been studied mainly in experimental mouse models, and the precise mechanism underlying the immunological events in humans is poorly understood [5,24].

The present studies involved a cross-sectional investigation of major and minor changes in peripheral blood leucocyte subpopulations during early and late phases of Chagas' disease. The subjects included T. cruzi-infected children in the early stages of the IND clinical form of disease as well as chagasic adults typifying late chronic Chagas' disease.

Our findings showed a lower value of T cells, due mainly to a drop in the value of $CD8^+$ T cells, in addition to an increase in activated B cells and impaired T cell activation are hallmarks of early indeterminate Chagas' disease (Fig. 1 and Table 2). The data presented here are consistent with our previous results from phenotypic characterization of peripheral blood leucocytes from early T. cruzi infection in Bolivian children [5]. This mixed activated/modulated immunological status can be explained partially by the action of distinct T. cruzi surface molecules that induce activation of B cells

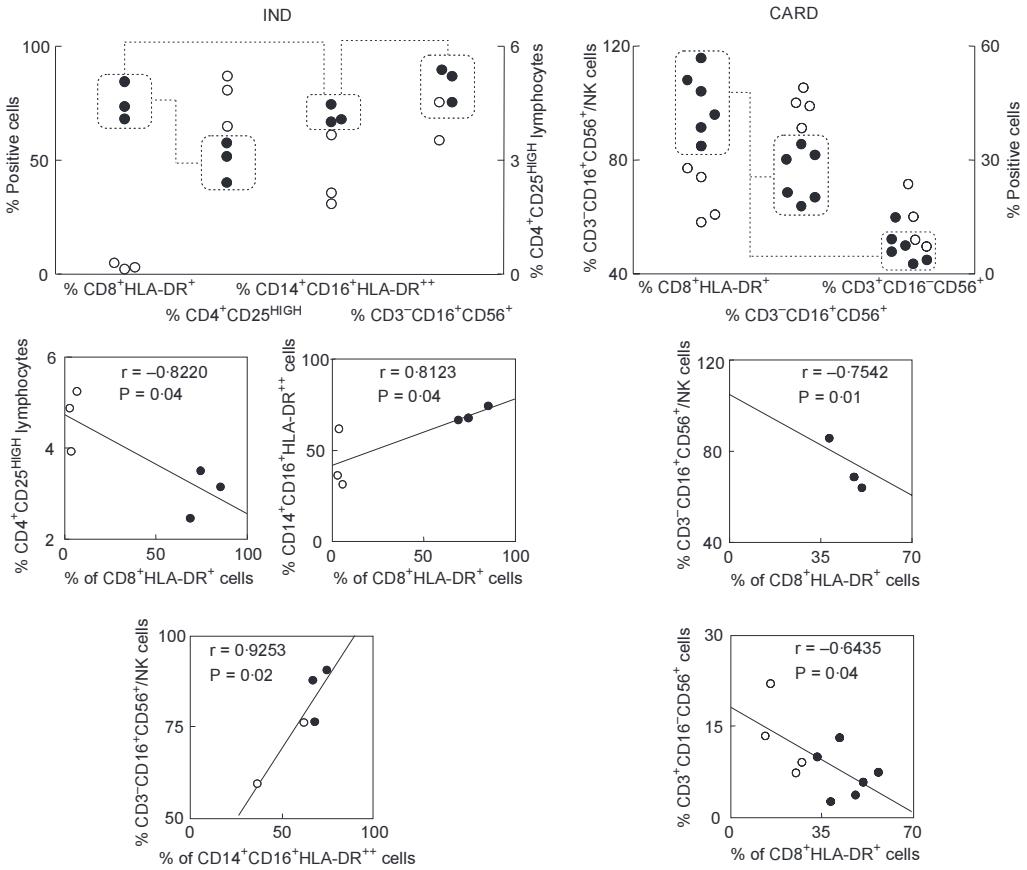

Fig. 5. Analysis of major discriminatory immunophenotypes of indeterminate (IND, left panels) and cardiac patients (CARD, right panels). Analysis of individual data from IND demonstrates an association (dotted rectangles and lines) between high frequency of $CD8^+$ HLA-DR^+ cells in IND (■) with low frequency of regulatory T cells $CD4^+$ $CD25^{HIGH}$, high frequency of proinflammatory monocytes ($CD14^+$ $CD16^+$ HLA-DR^{++}) and high levels of mature natural killer (NK) cells ($CD3^-$ $CD16^+$ $CD56^+$). Analysis of individual data from CARD demonstrates an association (dotted rectangles and lines) between the high frequency of $CD8^+$ HLA-DR^+ cells (■) with low levels of mature NK cells ($CD3^-$ $CD16^+$ $CD56^+$) and NKT cells ($CD3^+$ $CD16^-$ $CD56^+$). Confirmatory correlation analysis validates the negative association between $CD8^+$ HLA-DR^+ and $CD4^+$ $CD25^{HIGH}$ cells and a positive association between $CD8^+$ HLA-DR^+ and $CD14^+$ $CD16^+$ HLA-DR^{++} cells as well as $CD14^+$ $CD16^+$ HLA-DR^{++} cells and mature NK cells ($CD3^-$ $CD16^+$ $CD56^+$) in IND. Analysis performed with data obtained from CARD validates the negative association between $CD8^+$ HLA-DR^+ with mature NK cells ($CD3^-$ $CD16^+$ $CD56^+$) as well as NKT2 cells ($CD3^+$ $CD16^-$ $CD56^+$). Correlation analysis (r and P-values) are shown in the figure.

with non-specific Ig secretion [3,26,27] as well as suppression of T cell activation [28–30]. Consistent with this hypothesis, several studies have hypothesized that T. cruzi-derived glyco-inositol-phospholipids (GIPLs) and B cell activation could play a role in the conspicuous Ig production observed during infection, whereas membrane glycosyl-phosphatidyl-inositol (GPI)-anchored molecules are able to trigger suppression of human T cell response [29,30]. It has been suggested that the polyclonal activation of B cells and the T cell anergy may represent the mechanism of parasite evasion, i.e. misleading the immunological system and preventing the development of a strong adaptive immune response, thereby favouring disease onset and immunopathology [31].

The importance of NK cells in resistance to acute Chagas' disease is illustrated by studies showing that neutralization of endogenous interleukin (IL)-12 or interferon (IFN)-γ as well as depletion of NK cells renders animals more susceptible to infection with T. cruzi [32,33]. Thus, NK cells are an important source of IFN-γ, before development of T cell-mediated

immunity. Besides cytotoxic activity and cytokine secretion, NK cells can control B cell Ig secretion independent of T cell induction [27,34]. The higher frequency of pre-NK cells (Fig. 2b) reported here might be related to the early activation of B cells (Fig. 1g), contrasting with cell phenotypes pointing to impaired T cell activation (Table 2).

$CD16^+ CD56^-$ pre-NK cells have been considered to be precursors of functional and phenotypically distinct mature $CD16^+ CD56^+$ NK cells [21,35]. Pre-NK cells have higher proliferative capacity and are better sources of cytokines, whereas mature NK cells display mainly cytotoxic activities [35]. Our data suggest that the expansion of pre-NK cells might be related to important mechanisms of macrophage activation during early indeterminate Chagas' disease. Macrophages are efficiently activated by NK derived IFN-γ, which invokes nitric oxide production and controls parasite replication during the early stages of T. cruzi infection [36–39]. Despite the low levels of circulating macrophage-like cells, our results demonstrated an increased frequency of $CD14^+ CD16^+$ HLA-DR^{++} proinflammatory monocytes [19] among circulating $CD14^+ CD16^+$ cells in infected children (Fig. 2a,b).

It is important to point out that strong, uncontrolled activation of NK cells as well as proinflammatory monocytes may also lead to tissue damage leading to the development of cardiomyopathy and/or digestive megas [40,41]. Thus, the establishment of immunoregulatory mechanisms seems to be an important key to controlling immune activity and preventing deleterious effects of excessive stimulation of the immune system that may lead to fatality. Current and previous reports have suggested that, in human liver, NKT cells may play an important role in eliminating autologous cytotoxic T cells via apoptosis of activated $CD8^+$ T cells [42,43]. Moreover, it has been also proposed that IFN-γ and perforin production as well as NK and $CD8^+$ T cell cytotoxicity are efficiently regulated by $CD4^+ CD25^+$ regulatory T cells [44]. We have described here a lower value of NK T cells, as well as $CD4^+ CD25^{HIGH}$ regulatory T cells, in infected children (Fig. 3b,d), consistent with a higher levels of NK cells and proinflammatory monocytes (Fig. 2d,b). These findings suggest that the inability to shift the immune response toward higher levels of $CD4^+ CD25^{HIGH}$ may contribute to the development of cardiac tissue damage.

The low values of NKT and regulatory $CD4^+ CD25^{HIGH}$ cells during early Chagas' disease raise the question of why no phenotypic features related to T cell activation can be observed in the peripheral blood of E-IND patients. We believe that T cell-mediated immunity during the early indeterminate clinical form of Chagas' disease may represent a phenomenon restricted to the inflammatory sites, not detectable in the peripheral blood, considering previous reports describing the presence of these cells in the cardiac inflammatory infiltrate during early human Chagas' disease [10]. This hypothesis is supported by our findings of a higher percentage of $CD8^+$ T lymphocytes carried by CD54, an important adhesion molecule involved in migration pathways from the bloodstream to tissue inflammatory sites. We hypothesize here that the increased levels of circulating $CD8^+ CD54^+$ T cells reflect incipient immunological events during early chronic Chagas' disease, suggesting the enhanced migratory potential of this cytotoxic population to control tissue parasitism.

Once we determined the immunophenotypic profile of circulating leucocytes during early Chagas' disease, we then investigated the major discriminatory phenotypes during late chronic indeterminate and cardiac Chagas' disease. Comparative cross-sectional analysis of major immunophenotypes exhibited by late chronic chagasic patients with those exhibited by patients bearing early indeterminate disease suggested that a shift towards high values of macrophage-like cells, together with basal values of proinflammatory monocytes, regulatory $CD4^+ CD25^{HIGH}$ T cells and high levels of mature NK cells and NKT cells, would be responsible for development of late chronic asymptomatic disease (Fig. 4). On the other hand, the development of a cell-mediated inflammatory immunoprofile characterized by high levels of activated $CD8^+$ HLA-DR^+ T cells in the presence of basal levels of mature NK cells, NKT cells and regulatory $CD4^+ CD25^{HIGH}$ cells would account for the development of late chronic cardiac disease (Fig. 4).

It is important to observe that unlike the CARD patients (Fig. 5, right panel, top graph), the IND patients that present high levels of activated $CD8^+$ HLA-DR^+ T cells (Fig. 5, left panel, top graph) also count with high levels of mature NK cells ($CD3^- CD16^+ CD56^+$ cells) that may contribute to the establishment/maintenance of their asymptomatic clinical status. We have reported previously that blood samples from patients with the late indeterminate clinical form of Chagas' disease display a higher value of $CD4^+ CD25^{HIGH}$ and NKT ($CD3^+ CD16^- CD56^+$) regulatory cells, as well as increased levels of circulating NK cells. In the present study, we have also shown a correlation between the high levels of $CD4^+ CD25^{HIGH}$ T cells and the low frequency of activated $CD8^+$ T cells (Fig. 5, left panel, bottom graphs). We have also documented previously the existence of an increased frequency of activated $CD8^+$ HLA-DR^+ T cells and low levels of $CD4^+ CD25^{HIGH}$ in patients with severe clinical forms of Chagas' disease [6]. In the present study, we have addressed this issue further, demonstrating that patients bearing cardiac Chagas' disease display, in addition to the high levels of activated $CD8^+$ T cells, an opposite immunological profile of low values of NK and NKT cells (Fig. 5, right panel, top graph).

Taken together, our findings suggest that the expansion of proinflammatory monocytes $CD14^+ CD16^+$ HLA-DR^{++} as well as high values of pre-NK cells, in a microenviroment deficient in NKT cell and $CD4^+ CD25^{HIGH}$ cell populations, represent an important immunological profile that controls parasite load in E-IND Chagas' disease. However, the persistence of this immunophenotypic pattern parallels the establishment of a strong adaptive $CD8^+$ T cell activation that

could lead to late chronic disease associated with cardiac damage. On the other hand, the shift of this immunological pattern towards high values of macrophage-like cells, together with enhanced frequency of mature NK cells, NKT cells and regulatory $CD4^+$ $CD225^{HIGH}$ T cells, could be beneficial, limiting tissue damage and leading to lifelong persistence of the indeterminate form of Chagas' disease.

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References

1 WHO Expert Committee. Control of Chagas' disease. World Health Organ Tech Rep 2002; 905:1–109.
2 Umezawa ES, Stolf AM, Corbett CE, Shikanai-Yasuda MA. Chagas' disease. Lancet 2000; 357:797–9.
3 Brener Z, Gazzinelli RT. Immunological control of Trypanosoma cruzi infection and pathogenesis of Chagas' disease. Int Arch Allergy Immunol 1997; 114:103–10.
4 Marinho CR, D'Imperio Lima MR, Grisotto MG, Alvarez JM. Influence of acute-phase parasite load on pathology, parasitism, and activation of the immune system at the late chronic phase of Chagas' disease. Infect Immun 1999; 67:308–18.
5 Sathler-Avelar R, Lemos EM, Reis DD et al. Phenotypic features of peripheral blood leucocytes during early stages of human infection with Trypanosoma cruzi. Scand J Immunol 2003; 58:655–63.
6 Vitelli-Avelar DM, Sathler-Avelar R, Dias JCP et al. Chagasic patients with indeterminate clinical form of the disease have high frequencies of circulating $CD3^+CD16^-CD56^+$ natural killer T cells and $CD4^+CD25^{High}$ regulatory T lymphocytes. Scand J Immunol 2005; 62:297–308.
7 Dutra WO, Martins-Filho OA, Cancado JR et al. Activated T and B lymphocytes in peripheral blood of patients with Chagas' disease. Int Immunol 1994; 6:499–506.
8 Dutra WO, da Luz ZM, Cancado JR et al. Influence of parasite presence on the immunologic profile of peripheral blood mononuclear cells from chagasic patients after specific drug therapy. Parasite Immunol 1996; 18:579–85.
9 Higuchi MD, Ries MM, Aiello VD et al. Association of an increase in $CD8^+$ T cells with the presence of Trypanosoma cruzi antigens in chronic, human, chagasic myocarditis. Am J Trop Med Hyg 1997; 56:485–9.
10 Fuenmayor C, Higuchi ML, Carrasco H et al. Acute Chagas' disease: immunohistochemical characteristics of T cell infiltrate and its relationship with T. cruzi parasitic antigens. Acta Cardiol 2005; 60:33–7.
11 Gomes JA, Bahia-Oliveira LM, Rocha MO, Martins-Filho OA, Gazzinelli G, Correa-Oliveira R. Evidence that development of severe cardiomyopathy in human Chagas' disease is due to a Th1-specific immune response. Infect Immun 2003; 71:1185–93.
12 Souza PE, Rocha MO, Rocha-Vieira E et al. Monocytes from patients with indeterminate and cardiac forms of Chagas' disease display distinct phenotypic and functional characteristics associated with morbidity. Infect Immun 2004; 72:5283–91.
13 Vitelli DM. Study of immune response of patients carrying different clinical manifestations of Chagas' disease: focus in cellular and molecular aspects of peripheral blood [Thesis]. Belo Horizonte: Federal University of Minas Gerais, 2004.
14 Doherty DG, Norris S, Madrigal-Estebas L et al. The human liver contains multiple populations of NK cells, T cells, and $CD3^+CD56^+$ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. J Immunol 1999; 163:2314–21.
15 Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol 2001; 22:633–40.
16 Cooper MA, Fehniger TA, Turner SC et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56 (bright) subset. Blood 2001; 97:3146–51.
17 Baecher-Allan C, Brown JA, Freeman GJ. Hafler DA. $CD4^+CD25^{high}$ regulatory cells in human peripheral blood. J Immunol 2001; 167:1245–53.
18 Ziegler-Heitbrock HW. Heterogeneity of human blood monocytes: the $CD14^+$ $CD16^+$ subpopulation. Immunol Today 1996; 17:424–8.
19 Belge KU, Dayyani F, Horelt A et al. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. J Immunol 2002; 168:3536–42.
20 Fragata Filho AA, Luquetti AO, Prata A et al. Etiological treatment of Chagas' disease (The National Health Foundation of Brazil). Parasitol Today 1997; 13:127–8.
21 Gaddy J, Broxmeyer HE. Cord blood $CD16^+56^-$ cells with low lytic activity are possible precursors of mature natural killer cells. Cell Immunol 1997; 180:132–42.
22 Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. J Clin Invest 2004; 114:1379–88.
23 Hontebeyrie-Joskowicz M, Minoprio P. Chagas' disease: Trypanosoma cruzi vs. the host immune system. Res Immunol 1991; 142:125–6.
24 Samudio M, Montenegro-James S, Cabral M, Martinez J. Rojas de Arias A, James MA. Cytokine responses in Trypanosoma cruzi-infected children in Paraguay. Am J Trop Med Hyg 1998; 58:119–21.
25 Teixeira MM, Gazzinelli RT, Silva JS et al. Chemokines, inflammation and Trypanosoma cruzi infection. Trends Parasitol 2002; 18:262–5.
26 Bento CA, Melo MB, Previato JO, Mendonca-Previato L, Pecanha LM. Glycoinositolphospholipids purified from Trypanosoma cruzi stimulate Ig production in vitro. J Immunol 1996; 157:4996–5001.
27 Gao W, Wortis HH, Pereira MA. The Trypanosoma cruzi transsialidase is a T cell-independent B cell mitogen and an inducer of non-specific Ig secretion. Int Immunol 2002; 14:299–308.
28 Acosta-Serrano A, Almeida IC, Freitas-Junior LH, Yoshida N, Schenkman S. The mucin-like glycoprotein super-family of Trypanosoma cruzi: structure and biological roles. Mol Biochem Parasitol 2001; 114:143–50.
29 Argibay PF, Di Noia JM, Hidalgo A et al. Trypanosoma cruzi surface

mucin TcMuc-e2 expressed on higher eukaryotic cells induces human T cell anergy, which is reversible. Glycobiology 2002; 12:25–32.
30 Dos Reis GA, Freire-de-Lima CG, Nunes MP, Lopes MF. The importance of aberrant T cell responses in Chagas' disease. Trends Parasitol 2005; 21:237–43.
31 Golgher D, Gazzinelli RT. Innate and acquired immunity in the pathogenesis of Chagas' disease. Autoimmunity 2004; 37:399–409.
32 Aliberti JC, Cardoso MA, Martins GA, Gazzinelli RT, Vieira LQ, Silva JS. Interleukin-12 mediates resistance to Trypanosoma cruzi in mice and is produced by murine macrophages in response to live trypomastigotes. Infect Immun 1996; 64:1961–7.
33 Cardillo F, Voltarelli JC, Reed SG, Silva JS. Regulation of Trypanosoma cruzi infection in mice by gamma interferon and interleukin 10: role of NK cells. Infect Immun 1996; 64:128–34.
34 Blanca IR, Bere EW, Young HA, Ortaldo JR. Human B cell activation by autologous NK cells is regulated by CD40–CD40 ligand interaction. role of memory B cells and CD5+ B cells. J Immunol 2001; 167:6132–9.
35 Sondergaard SR, Ullum H, Pedersen BK. Proliferative and cytotoxic capabilities of $CD16^{+}CD56^{-}$ and $CD16^{+/-}$ CD56+ natural killer cells. APMIS 2000; 108:831–7.
36 Silva JS, Vespa GN, Cardoso MA, Aliberti JC, Cunha FQ. Tumor necrosis factor alpha mediates resistance to Trypanosoma cruzi infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages. Infect Immun 1995; 63:4862–7.
37 Camargo MM, Andrade AC, Almeida IC, Travassos LR, Gazzinelli RT. Glycoconjugates isolated from Trypanosoma cruzi but not from Leishmania species membranes trigger nitric oxide synthesis as well as microbicidal activity in IFN-gamma-primed macrophages. J Immunol 1997; 159:6131–9.
38 Campos MA, Almeida IC, Takeuchi O et al. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol 2001; 167:416–23.
39 Michailowsky V, Silva NM, Rocha CD, Vieira LQ, Lannes-Vieira J, Gazzinelli RT. Pivotal role of interleukin-12 and interferon-gamma axis in controlling tissue parasitism and inflammation in the heart and central nervous system during Trypanosoma cruzi infection. Am J Pathol 2001; 159:1723–33.
40 d'Avila Reis D, Lemos EM, Silva GC et al. Phenotypic characterization of the inflammatory cells in chagasic mega-oesophagus. Trans R Soc Trop Med Hyg 2001; 95:177–8.
41 Reis DD, Jones EM, Tostes S Jr et al. Characterization of inflammatory infiltrates in chronic chagasic myocardial lesions: presence of tumor necrosis factor-alpha+ cells and dominance of granzyme A^{+}, $CD8^{+}$ lymphocytes. Am J Trop Med Hyg 1993; 48:637–44.
42 Wack A, Corbella P, Harker N, Crispe IN, Kioussis D. Multiple sites of post-activation $CD8^{+}$ T cell disposal. Eur J Immunol 1997; 27:577–83.
43 Murray DA, Crispe IN. TNF-alpha controls intrahepatic T cell apoptosis and peripheral T cell numbers. J Immunol 2004; 173:2402–9.
44 Trzonkowski P, Szmit E, Mysliwska J, Dobyszuk A, Mysliwski A. $CD4^{+}CD25^{+}$ T regulatory cells inhibit cytotoxic activity of T $CD8^{+}$ and NK lymphocytes in the direct cell-to-cell interaction. Clin Immunol 2004; 112:258–67.

4.2. ARTIGO 3:

O artigo científico apresentado nesse tópico corresponde ao objetivo específico 2, e foi submetido à revista Journal of Immunological Methods em março desse ano.

Título: Stratagem to assess and correlate the overall cytokine profile of circulating leukocytes with distinct clinical status of human Chagas disease.

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STRATAGEM TO ASSESS AND CORRELATE THE OVERALL CYTOKINE PROFILE OF CIRCULATING LEUKOCYTES WITH DISTINCT CLINICAL STATUS OF HUMAN CHAGAS DISEASE

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Short Title:
Panoramic cytokine profile in human Chagas disease

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Abstract

It has been hypothesized that the deregulation of the immune system and indeed the resultant of a disturbed multifactorial immunological mechanisms are more likely to be involved in the pathogenesis of Chagas disease and therefore in the establishment of distinct clinical status of chronic Trypanosoma cruzi. In this context, impaired cytokine network has been pointed out as an important circuit determining disease morbidity. Herein we have described a novel stratagem to assess the overall cytokine profile of circulating leukocytes and correlate the dominant cytokine profile with the indeterminate-IND and cardiac-CARD clinical forms of Chagas disease. Taking the whole study population, we have first calculated the global median percentages of cytokine$^+$ leukocytes to establish, for each cytokine$^+$ cell population, the cutoff edge used to segregate "low" and "high" cytokine-producers as the start point to build color-diagrams and determine the panoramic cytokine overview. Using this approach our findings demonstrated that while most IND presented regulatory dominant cytokine profile, CARD displayed a prominent inflammatory panoramic cytokine overview. In addition, radar chart analysis confirmed the dichotomic cytokine balance between IND and CARD and further allowed the identification of the real contribution of each cell population for the overall cytokine pattern observed. Data analysis demonstrated that while CD4$^+$ T-cells were the major cell population accountable to define the regulatory profile in IND, monocytes and CD4$^+$ T-cells were determinants for the panoramic inflammatory cytokine pattern in CARD. Interestingly, in vitro booster with trypomastigote T. cruzi antigen was able to overturn the overall cytokine balance of IND and CARD. In fact, upon antigenic stimulation, disturbed IL-10 circuit of CD4$^+$ T-cells and monocytes seems to be the relevant key to drive IND toward an inflammatory pattern and CARD forward a regulatory cytokine profile. Preliminary data supported that this phenomenon may also occur in vivo after Chagas disease etiological treatment. Altogether, these findings added new insights to the complex cytokine

network underline the immunopathogenesis of Chagas disease re-enforcing the role of putative immunological biomarkers of disease severity and therapeutic response.

Keywords: Human Chagas disease; Trypanosoma cruzi; cytokines; radar graph.

1. Introduction

Chagas disease is a long-lasting infection, caused by the protozoa parasite Trypanosoma cruzi, endemic throughout the Latin America where an estimated 8 million people remain infected (New global effort to eliminate Chagas disease, 2007; Dias, 2006; Pérez-Gutiérrez et al., 2006). The pathogenesis of chronic Chagas disease is still partly understood although there is a general consensus that the host immune response plays pivotal role underling the distinct clinical aspects of the disease. There are several immunological events that seem to influence the outcome of chronic Chagas disease including the cytotoxic mechanisms, mediated by $CD8^+$ T-cells on the development of cardiac disease, the importance of regulatory cells such as $CD4^+CD25^{High}$ Treg and NKT-cells, as well as the protective role of cytotoxic NK-cells (Reis et al., 1993; Dutra et al., 2005; Vitelli-Avelar et al., 2005; Vitelli-Avelar et al., 2006; Korbel et al., 2004; Papazahariadou et al., 2007).

A direct correlation between the secretion of IFN-γ and the development of the severe cardiac Chagas disease besides the role of IL-10 controlling immunopathology have been already reported (Gomes et al., 2003; de Barros-Mazon et al., 2004). However, the fact that a large proportion of IND patients also produce high levels of IFN-γ, in addition to other inflammatory cytokines raises the hypothesis that more than shift toward a polarized cytokine pattern, the fine balance of inflammatory and regulatory cytokines derived from distinct cell sources may account for the resultant immune-mediated mechanisms that drive the disease outcome (Gomes et al., 2003; Bahia-Oliveira et al., 1998; Corrêa-Oliveira et al., 1999). In fact, it has been demonstrated that whereas monocytes from both IND and CARD are able to produce IL-10 and some IND patients can produce higher levels of IFN-γ, monocytes from IND produce higher levels of IL-10 than do monocytes from CARD (Gomes et al., 2003; Dutra et al., 1997; Souza et al., 2004). These findings emphasize that the cytokines balance represents a key element in the establishment of distinct clinical forms of chronic Chagas

disease.

Attempting to further contribute to this filed of investigation, herein we have described a novel strategy to characterize the cytokine overall profile from a range of innate and adaptive immunity cells and decode the distinct clinical status of human Chagas disease (Bahia-Oliveira et al., 1998). The panoramic cytokine overall profile of peripheral blood leukocyte subsets offered additional evidences concerning the immunological events relevant for clinical studies of Chagas disease. This approach would be of great interest and may provide a better understanding of the immunological complexities that control the outcome of chronic Chagas' disease and a useful tool to predict the immunological response following therapeutic intervention.

2. Materials and methods

2.1. Study population

This study enrolled 43 individuals grouped into two cohorts as following described:

2.1.1. Cohort 1

Consisted of chagasic patients classified as indeterminate-IND (03 males and 03 females, 43-67 years old with no clinical manifestations besides the positive serology) and cardic-CARD (02 males and 06 females, 50-70 years old presenting dilated cardiomyopathy besides the positive serology) as well as 11 non-infected individuals-NI (03 males and 08 females, 20-55 years old), from Bambuí, Minas Gerais, Brazil. Chagas disease diagnosis was based on standard serological tests to detect anti-T. cruzi IgG, including indirect immunfluoirescence assay and haemoaglutionation test.

2.1.2. Cohort 2

Consisted of 18 chagasic patients classified as IND (01 males and 04 females, 35-46 years old) and CARD (07 males and 06 females, 29-58 years old) analyzed prior and one-year after etiological treatment with benznidazole (Rochagan®; Roche, SP, Brazil).

This work was approved by the Ethical Committee at IRR/FIOCRUZ-Minas (11/2004) and UFMG-COEP (ETIC 070/00).

2.2. Short-term whole blood in vitro culture

Peripheral blood samples from 25 individuals were collected into Vacutainer tubes containing sodium heparin (BD Pharmingen, San Diego, CA, USA). For each collected sample, short-term in vitro cultures of whole blood samples were performed in two distinct platforms referred as "control" and "antigen-stimulated" cultures. Control cultures were performed in 14ml polypropylene tubes (Falcon®, BD Pharmingen, San Diego, CA, USA), using 500µl aliquots of heparinized whole blood samples, incubated in the presence of 500µl of RPMI-1640 (GIBCO, Grand Island, NY, USA) plus Brefeldin A (BFA) (Sigma, St Louis, MO, USA) at a final concentration of 10µg/ml for 4h at 37°C in a 5% CO_2 humidified incubator. This condition represented the "ex vivo" cytokine profile, particularly in the absence of exogenous stimuli which may reflect the dynamic events taking place in blood leukocyte subsets in vivo.

Additionally, the antigen-stimulated cultures were performed in 14ml polypropylene tubes (Falcon®, BD Pharmingen, San Jose, CA, USA) by previous incubation of 500µl aliquots of heparinized whole blood samples in the presence of 400µl of RPMI-1640 (GIBCO, Grand Island, NY, USA) plus 100µl of TRYPO soluble antigen prepared as described by Gomes et al. [10] at a final concentration of 20µg/ml, for 1 h at 37°C in a 5% CO_2 humidified incubator. Following antigen priming, cells were incubated in the presence of BFA at 10µg/ml for an additional period of 4h at 37°C in a 5% CO_2 humidified incubator.

Positive control cultures were also performed in order to evaluate the sample viability. For this purpose, 500µl of aliquots of whole blood samples were incubated in the presence of 500µl of RPMI-1640 plus Phorbol 12-Myristate 13-Acetate (Sigma, St Louis, MO, USA) at 25ng/ml, ionomycin (Sigma, St Louis, MO, USA) at 1µg/ml and BFA at 10µg/ml final concentration. Whole blood cultures were incubated for 4h at 37°C in a 5% CO_2 humidified incubator. Cytokine patterns observed in the positive control cultures, named "PMA-stimulated cultures" confirmed the cell viability of all blood samples, as demonstrated by high levels of IFN-γ^+ and TNF-α^+ cells (data not shown).

2.3. Immunostaining for cell subsets and intracellular cytokines analysis by flow cytometry

Following the short term stimulation in vitro, the cultures, previously treated with 2mM EDTA (Sigma, St Louis, MO, USA), were washed once with FACS buffer prepared as PBS 0.5% of bovine serum albumin and 0.1% sodium azide (Sigma, St Louis, MO, USA). After resuspension in 1ml of FACS buffer, 400µl aliquots of cell suspension from each whole blood culture were immunostained with FITC (BD Pharmingen, San Diego, CA, USA) or TC (Caltag, Burlingame, CA, USA).labeled mAbs anti-CD3, CD4, CD5, CD8, CD14, CD16 or CD19 in the dark for 30 minutes at room temperature. After lysing/fixation procedure, membrane-stained leukocytes were permeabilized with FACS perm-buffer, (FACS buffer 0.5% of saponin) and incubated, for 30 min at room temperature, in the dark in the presence of 20µl of PE-labelled anti-cytokine mAbs (IFN-γ, TNF-α, IL-12, IL-10, IL-4 and IL-13). After intracytoplasmatic cytokine staining, the cells were washed and then fixed in FACS FIX Solution and stored at 4°C prior flow cytometry acquisition and analysis. Distinct gating strategies were used to assess the percentage of cytokine$^+$ cell subsets as previous described by Peruhype-Magalhães et al. [16].

2.4. Assessment of overall cytokine profiling of circulating leukocytes

The percentage of cytokine$^+$ cells within a given leukocyte population obtained by flow cytomety quadrant statistics were further transformed using a five step platform that consisted of: 1st – to calculate the global median for each cytokine$^+$ cell subset considering the whole range of values obtained for NI, IND and CARD; 2nd – to establish for each cell subset the concept of "low" and "high" cytokine-producers using the global median percentage of cytokine$^+$ cells as the cutoff edge to segregate the individuals into two categories named as "low" and "high" cytokine-producers; 3rd – to create a color diagram representing low cytokine-producers (▭ for all cytokines), inflammatory high cytokine-producers (▬ for IFN-γ, TNF-α and IL-12) and regulatory high cytokine-producers (▬ for IL-10, IL-4 and IL-13) within each leukocyte subset; 4th – to calculate for each leukocyte subset the cytokine balance defined into four categories as the predominant proportion of low cytokine-producers, inflammatory, regulatory or mixed (▬) cytokine-producers and 5th – to determine for the whole leukocyte population the overall cytokine profile as the proportion of inflammatory, regulatory or mixed cytokines taking together all cell subsets evaluated.

2.5. Statistical analysis

χ^2 test was used for comparisons of low and high cytokine-producers frequencies among NI, IND and CARD and significance considered at $p<0.05$. Comparison of radar graphs axes and polygon areas were considered significant for ratio twice lower of higher in magnitude. Data analysis for the results presented in the radar chart format was performed by comparing the central polygon areas among cytokine-producers categories intra and inter clinical groups. Significant differences were considered for ratio indicating axes and polygon areas twice lower of higher in size. Significant differences among clinical groups are highlighted by letters "a", "b" and "c" as compared to NI, IND and CARD, respectively.

Significant differences among cytokine-producers categories within a given clinical groups are highlighted by * as compared to all other categories or # as compared to high mixed cytokine-producers.

3. Results

3.1. Establishing the global median percentages of cytokine$^+$ cells as the cut-off edge to define "low" and "high" cytokine-producers

It has been already reported that a detailed analysis of individual level of IFN-γ secretion allow the segregation of chagasic patients into low and high producers able to distinguish IND and CARD, with the latter group presenting enhanced frequency of high IFN-γ producers (Bahia-Oliveira et al., 1998). Herein, we intended to apply this concept to investigate a broader range of cytokines and assess the overall ex vivo cytokine profile of circulating leukocytes and decode the distinct clinical status of human Chagas disease. For this purpose, the global median for each cytokine$^+$ cell subset was calculated, taking the whole range of values obtained for NI, IND and CARD as illustrated for TNF-α$^+$ monocytes and IL-10$^+$ CD4$^+$ T-cells (Fig. 1). The global median percentage of each cytokine$^+$ cells population was used as the cut-off edge to segregate the individuals into two categories named "low" and "high" cytokine-producers. The frequencies of high cytokine-producers in the innate and adaptive compartments are presented in Table 1 and 2, respectively.

Analysis of cytokine$^+$ neutrophils demonstrated that all IND patients fell into a region of high IL-12$^+$ and IL-13$^+$-producers (Table 1). On the other hand, the analysis of the adaptive immunity compartment demonstrated that none IND individuals displayed values of circulating IL-12$^+$ within the total lymphocyte population above the cutoff edge, significantly lower than NI and CARD (Table 2).

On the other hand, our data demonstrated that most of CARD displayed values of

circulating TNF-α^+ monocytes above the cutoff edge, significantly higher than NI (Table 1). Interestingly, analysis of total lymphocytes demonstrated that none CARD individuals fell into a region of high IL-10^+-producers, unlike NI and IND confined above the global cut-off edge. Additional analysis revealed that a small proportion of CARD individuals displayed values of circulating IL-10^+ $CD8^+$ T-cells above the cutoff edge, significantly higher than NI (Table 2).

3.2. Distinct overall cytokine profile is a genuine approach to decode the distinct clinical status of human Chagas disease

To further characterize the cytokine profile of NI, IND and CARD we have created color diagrams to represent for each subject the three classes of cytokine-producers named as low cytokine-producers (▭ for all cytokines), high inflammatory cytokine-producers (▬ for IFN-γ, TNF-α and IL-12) and high regulatory cytokine-producers (▭ for IL-10, IL-4 and IL-13) within each leukocyte subsets (Fig. 2A). Data demonstrated a predominance of low cytokine-producers within neutrophils from CARD as compared to IND (Fig. 2A). Additionally, color diagrams reinforce that CARD presented a predominance of TNF-α high cytokine-producers within monocytes as compared to NI (Fig. 2A). Furthermore, decreased frequency of low cytokine-producers within NK-cells was observed in IND as compared to NI (Fig. 2A). No significant differences were observed among the three classes of cytokine-producers within T-cells subsets as well as B-cell compartment (Fig. 2A).

Additional analysis was carried out to characterize the cytokine balance defined as the predominant proportion of low cytokine-producers or high inflammatory, regulatory or mixed (▭) cytokine-producers of each leukocyte subset taking together the color diagram summary for each cell population (Fig. 2B).

As an example, we can consider the cytokine profile of IND 1. The analysis of B-cell cytokine profile revealed a predominant low cytokine-producer pattern, yielding a general low cytokine-producer balance. On the other hand, taking into account the cytokine profile of monocytes, a predominant high inflammatory greater than regulatory cytokine-producer pattern yielded a general high inflammatory cytokine-producer balance. Still, the analysis of NK-cells displayed a predominance of high regulatory cytokine-producer pattern, yielding a general high regulatory cytokine balance. In addition, considering the neutrophils cytokine profile, an equivalent high inflammatory and regulatory cytokine-producer pattern was observed, producing a resultant high mixed cytokine-producer balance.

The data obtained as the cytokine balance for each cell population was further compiled to determine the overall cytokine profile of peripheral blood leukocytes for NI, IND and CARD (Fig. 2C). The analysis of the overall cytokine profile suggests a predominance of mixed cytokine pattern in NI and IND. On the other hand, a predominance of inflammatory cytokine pattern in CARD was observed (Fig. 2C). Interestingly, the analysis of those individuals with dominant immune response toward inflammatory or regulatory profile demonstrated that while a equivalence between inflammatory/regulatory cytokine profile was the hallmark of NI, 75% of IND displayed a prominent regulatory cytokine profile whereas 80% of CARD showed predominance of an inflammatory cytokine pattern (Fig. 2C).

3.3. Radar graph further identified that distinct leukocyte subsets contributed to the overall cytokine profile balance of IND and CARD

Aiming to further characterize the high inflammatory and regulatory cytokine balance observed in IND and CARD, respectively, radar charts were employed to identify the contribution of distinct leukocyte subset for the overall cytokine balance. The radar graph is a method of graphically displaying a composite measure that has a number of axes emanating

from a central point. Each axis displays the proportion of each cytokine balance category within one cell population and the values on each of the axes may be joined to form a central polygon. The area within the polygon represents the performance of the cytokine balance on each category with increasing or decreasing areas reflecting better or poorer contribution of each cytokine balance category within IND or CARD.

Data analysis re-emphasized that IND presented a predominant area within the polygon regarding the regulatory cytokine balance whereas the analysis of the radar charts from CARD demonstrated a higher contribution of inflammatory cytokine-producers surrounding central polygon (Fig. 3).

It was interesting to notice that the higher central polygon area, illustrating the regulatory cytokine balance in IND, was particularly determined by $CD4^+$ T-cells axis (Fig. 3). On the other hand the higher central polygon area illustrating the inflammatory cytokine balance in CARD was particularly determined by monocytes as well as $CD4^+$ T-cells (Fig. 3).

3.4. Trypomastigote T. cruzi antigen booster in vitro overturn the cytokine balance of peripheral blood leukocytes driving IND toward an inflammatory pattern and CARD forward a regulatory profile

Once established the overall cytokine profile for NI, IND and CARD, we have moved up to another relevant question whether the in vitro booster with trypomastigote T. cruzi soluble antigens would alter the cytokine balance of the major circulating leukocyte subsets. To answer to this issue we have carried out an analysis of intracellular cytokine balance of circulating leucocytes following short-term whole blood in vitro culture in the presence of tripomastigote T. cruzi soluble antigen and calculate the impact of antigen stimulation as the ratio between antigen-stimulated/control cultures.

The analysis of the in vitro T. cruzi antigenic booster on the cytokine balance of whole leukocyte population is presented in radar graphs format in Fig. 4. Our findings demonstrated that the trypomastigotes T. cruzi antigen booster in vitro overturn the cytokine balance of peripheral blood leukocytes, enhancing the inflammatory cytokine balance in IND and driving CARD toward a predominant frequency of regulatory cytokine balance (Fig. 4).

In fact, upon in vitro T. cruzi antigen stimulation, the cytokine balance of IND revealed an increased inflammatory cytokine balance as compared to the ex vivo cytokine balance presented in Fig. 3. Indeed, this change was mainly due to the enhancement of regulatory cytokines derived from monocytes and $CD4^+$ T-cells (Fig. 4). The major impact observed in the regulatory cytokine balance in IND, mainly affected the $CD4^+$ T-cells axis despite the enlargement of the neutrophils and $CD8^+$ T-cells axes (Fig. 4) as compared to the ex vivo analysis (Fig. 3).

On the other hand, as compared to the cytokine balance displayed in Fig. 3, the lower central polygon area illustrating the inflammatory cytokine balance in CARD was particularly due to the cutback of monocytes and $CD4^+$ T-cells axes. Interestingly, despite the lower polygon area, the axis representing the $CD8^+$ T-cells still characterize a prominent source of inflammatory cytokines in CARD. On the other hand, the higher central polygon area illustrating the regulatory cytokine balance of CARD was mainly owed to enlarged neutrophils, monocytes and $CD4^+$ T-cells (Fig. 4).

3.5. The in vivo IL-10 circuit of monocytes and $CD4^+$ T-cells observed in IND and CARD after etiological treatment reproduce the overturning in the cytokine balance observed upon T. cruzi antigen booster in vitro

The impact of T. cruzi antigenic booster in vitro in the cytokine balance of circulating leukocyte subsets highlighted that the regulatory $CD4^+$ T-cells from IND and regulatory

monocytes from CARD represented the major overturned target. Aiming to substantiate whether this phenomena may also occur in vivo following the massive T. cruzi antigen release that occur during Chagas disease etiological treatment, herein we have performed a focal analysis of IL-10 circuit in $CD4^+$ T-cells in IND and the monocytes in CARD, before and after benznidazole-treatment. Our data pointed out that, likely the T. cruzi antigen stimulation in vitro, the etiological treatment led to remarkable in vivo changes in the regulatory cytokine pattern of monocytes and $CD4^+$ T-cells. Indeed, after etiological treatment, decreased levels of $IL\text{-}10^+$ $CD4^+$ T-cells can be observed in IND whereas enhanced frequency of $IL\text{-}10^+$ monocytes is observed in CARD, as compared to cytokine pattern observed before treatment (Fig. 5).

4. Discussion

It has been hypothesized that a deregulation of the immune system is more likely to be involved in the pathogenesis of Chagas disease and that the chronic clinical forms are indeed resultant of a disturbed multifactorial immunological mechanisms, where impaired cytokine network has been pointed out as an important circuit determining disease morbidity (Gomes et al., 2003; Bahia-Oliveira et al., 1998; Corrêa-Oliveira et al., 1999). As the conventional strategies may not capture the global cytokine imprint, and does not reflect the panoramic cytokine profile of the wide range of circulating leukocyte subsets, herein we described a novel strategy to assess the panoramic cytokine overview of circulating leukocytes and describe the dominant cytokine profile associated with indeterminate and cardiac clinical forms of Chagas disease.

Our results highlighted the mixed nature of the cytokine response of uninfected subjects which is agreement with previous reports (Peruhype-Magalhães et al., 2006). Moreover, our finding were capable of define a predominance of regulatory cytokine pattern

in IND contrasting with the dominant inflammatory profile of CARD (Fig. 3). The data presented as overall dominant cytokine profile of circulating leukocytes from chagasic patients are also in agreement with those previously reported for isolated peripheral blood mononuclear cells (Corrêa-Oliveira et al., 1999; Dutra et al. 2005; Golgher & Gazzinelli, 2004). The major goal of the present investigation was the opportunity to analyze the dominant cytokine pattern of circulating leukocytes jointly with complementary data set displayed in radar graphs that allowed to built a feasible hierarchical cytokine network and better understand the complex cytokine circuit of a range of peripheral blood cell subsets from innate and adaptive immune response.

Using the radar charts it was easy to notify not only the magnitude of each cytokine category, illustrated by the area of the central polygon but also to identify the major cell sources that contribute for the overall cytokine balance. It is important to emphasize that the area of the central polygon was as important as its overall shape since down or up-regulation in certain cytokine cell sources could be clearly identified. Using this novel strategy of analysis we have identified that the predominance of regulatory cytokine pattern in IND (Fig. 3) is mostly determined by up-regulation of modulatory cytokines derived from $CD4^+$ T-cells (Fig. 3). Furthermore, the lower frequency of inflammatory cytokines in IND (Fig. 3) was mainly due to a reduced frequency of inflammatory $CD4^+$ T-cells (Fig. 3). The lower regulatory cytokine-producers in CARD were particularly determined by the shift of monocytes towards an inflammatory phenotype (Fig. 3) and in lesser extension by the mixed cytokine profile of neutrophils, NK and B1-cells (Table 1).

Our findings demonstrated that peripheral blood leukocytes from patients with different clinical forms of Chagas disease display distinct cytokine profiles, with the most relevant findings of CARD individuals been the particular enhanced frequency of TNF-α producing monocytes and lower frequency of IL-10 producing $CD8^+$ T-cells. In fact,

previously studies have already reported that monocytes from cardiac-disease patients are able to produce higher levels of TNF-α, consistent with the establishment of strong cytotoxic $CD8^+$ T-cell response that trigger exacerbated inflammation and tissue damage (Reis et al., 1993; Souza et al., 2004). On the other hand, in IND individuals the enhanced frequency of total lymphocytes high producers of IL-4, IL-10 and IL-13 and the mixed pattern of neutrophil and NK-derived cytokines represented the major features of the panoramic cytokine overview. It has been demonstrated that cytokines produced in type-II responses such as IL-4 and IL-13 can indeed activate macrophages and antagonize the classic activation pathway that in addition with IL-10 stimulates phenotypes that some authors have proposed to define alternative activation of macrophages (Mantovani et al., 2004; Gordon, 2003). This activation pathway characterized by low production of pro-inflammatory cytokines may represent a relevant immunoregulatory mechanism that take place in IND patients (Noel et al., 2004). It is known that endogenous IL-4 has an important role in avoiding excessive inflammatory process and pathology elicited during chronic infection with T. cruzi (Soares et al., 2001). Previous reports have demonstrated that IL-13 is able to inhibit the production of inflammatory cytokines by monocytes (McKenzie et al., 1993). In fact, Dutra et al. (1997) have already proposed that circulating immune cells from chagasic patients, despite their clinical status, displayed increased levels of mRNA specific for IL-13 as compared to non-infected individuals. It is possible that the lack of differences between IND and CARD observed by these authors was mainly due to the fact that they have focused on the cytokine analysis on PBMCs and herein we have focused on a broader range of peripheral blood leukocytes subsets. The neutrophils are probably the most underappreciated immune cell among peripheral blood leukocytes and many neutrophil functions remain to be unrevealed (Chakravarti et al., 2007). Our data have pointed out that neutrophils are indeed the major supplier of IL-13, herein described for the first time as a relevant source of IL-13 exclusively

in IND group (Table 1). Thus, this cytokine may be involved in the control of the inflammatory responses and may be involved in the alternative macrophages activation pathway in IND. In fact, the ability of neutrophils to modify the monocyte/dendritic cells function, by inhibiting the cytokine release including TNF-α and impact on down-stream immunity such as T-cell proliferation has been recently reported (Eken et al., 2008).

Our data showed that the neutrophils from IND were also a relevant source of IL-12 (Table 1) displaying an overall mixed cytokine balance. The plasticity of neutrophils that explains its capacity to transdiferenciate depending on the local requirements of the immune response has been already reported (Chakravarti et al., 2007). It is possible that the enhanced frequency of high IL-12 producers within IND neutrophils may be a relevant loop to trigger the activation of cytotoxic NK-cells. Indeed, we have previously reported a predominance of $CD16^{+}CD56^{dim}$ NK-cells in IND which has been considered to display higher cytotoxic activity (Cooper et al., 2001).

In the adaptive compartment, it was interesting to notice that whereas the circulating lymphocytes from most IND patients were classified as high IL-10 producers, no lymphocytes from CARD individuals fell into a region of high IL-10 producer (Table 2). It is plausible to speculate that these high IL-10 producing lymphocytes from IND may represent Treg cells. In fact, we have previously demonstrated that IND display enhanced levels of $CD4^{+}CD25^{HIGH}$ lymphocytes (Vitelli-Avelar et al., 2006). It has been established that Treg cells can mediate suppressive effect in vitro in a cell contact-dependent manner controlling disease process, whereas adaptive regulatory T-cells suppress immune response by producing regulatory cytokines such as IL-10 (Dittmer et al., 2004). Functional studies with Treg enriched leukocyte populations are currently under evaluation in our laboratory.

One fascinating finding of the present investigation that trypomastigotes T. cruzi antigen booster in vitro was able to overturn the cytokine balance of peripheral blood

leukocytes driving impaired frequency of inflammatory cytokine in CARD forwards a prominent regulatory profile. On the other hand, the major impact observed in IND demonstrated an enhancement in the frequency of inflammatory with a clear down regulation of regulatory cytokine-producers (Fig. 4). We have previously proposed that the cytokine profile of peripheral blood leukocytes from untreated chagasic patients, artificially reproduced in vitro upon T. cruzi antigen stimulation, may reflect the cytokine synthesis in vivo triggered by the etiological treatment (Sathler-Avelar et al., 2008). Herein, preliminary data demonstrated that the etiological treatment led to decreased levels of IL-10^{+} $CD4^{+}$ in IND group, whereas increased frequency of IL-10^{+} monocytes could be observed in CARD, which corroborate our hypothesis (Fig. 5).

Taken together, the data presented here re-emphasize that a fine balance between inflammatory- and regulatory-cytokines represents a key element in the establishment of distinct clinical forms of chronic Chagas disease. Thus, the cytokine network especially the balance between regulatory and inflammatory cytokines appears to play a critical role in controlling T. cruzi infection and the fate of different clinical forms as previous suggested (Crema et al., 2006). It is clear that this novel strategy to analyze immunological data clarified important aspects of the immunopathology of Chagas disease and, possibly, revealed alternative immunological marker applicable in clinical follow-up studies of disease severity as well as therapeutic response.

Acknowledgements

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References

Bahia-Oliveira LM, Gomes JA, Rocha MO et al. IFN-gamma in human Chagas' disease: protection or pathology? Braz J Med Biol Res.1998; 31: 127-31.

Chakravarti A, Allaeys I, Poubelle PE. Neutrophil and immunity: is it innate or acquired? Med Sci. 2007; 23: 862-7.

Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001; 22: 633-40.

Correa-Oliveira R, Gomes J, Lemos EL, et al. The role of the immune response on the development of severe clinical forms of human Chagas disease. Mem Inst Oswaldo Cruz. 1999; 94: 253-5.

Crema E, Monteiro IO, Gomes MG, Silva AA, Rodrigues Júnior V. Evaluation of cytokines (MIG, IFN-gamma, TNF-alpha, IL-4, IL-5, and IL-10) during the different evolutive phases of chagasic esophagopathy. Clin Immunol. 2006; 119: 213-8.

de Barros-Mazon S, Guariento ME, da Silva CA, Coffman RL, Abrahamsohn IA. Differential regulation of lymphoproliferative responses to Trypanosoma cruzi antigen in patients with the cardiac or indeterminate form of Chagas disease. Clin Immunol. 2004; 111: 137-45.

Dias JC. Chagas disease: successes and challenges. Cad Saude Publica 2006; 22: 2020-1.

Dittmer U, He H, Messer RJ, Functional impairment of CD8(+) T cells by regulatory T cells during persistent retroviral infection. Immunity. 2004; 20: 293-303.

Dutra WO, Gollob KJ, Pinto-Dias JC, et al. Cytokine mRNA profile of peripheral blood mononuclear cells isolated from individuals with Trypanosoma cruzi chronic infection. Scand J Immunol. 1997; 45: 74-80.

Dutra WO, Rocha MO, Teixeira MM. The clinical immunology of human Chagas disease.

Trends Parasitol. 2005; 21: 581-7.

Eken C, Gasser O, Zenhaeusern G, Oehri I, Hess C, Schifferli JA. Polymorphonuclear neutrophil-derived ectosomes interfere with the maturation of monocyte-derived dendritic cells. J Immunol. 2008; 180: 817-24.

Golgher D, Gazzinelli RT. Innate and acquired immunity in the pathogenesis of Chagas disease. Autoimmunity 2004; 37: 399-409.

Gomes JA, Bahia-Oliveira LM, Rocha MO, Martins-Filho OA, Gazzinelli G, Correa-Oliveira R. Evidence that development of severe cardiomyopathy in human Chagas' disease is due to a Th1-specific immune response. Infect Immun. 2003; 71: 1185-93.

Gordon S. Alternative activation of macrophages. Nat Rev Immunol. 2003; 3: 23-35.

Korbel DS, Finney OC, Riley EM. Natural killer cells and innate immunity to protozoan pathogens. Int J Parasitol. 2004; 34: 1517-28.

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 2004; 25: 677-86.

McKenzie AN, Culpepper JA, de Waal Malefyt R, et al. Menon. Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. Proc Natl Acad Sci U S A. 1993; 90: 3735-9.

New global effort to eliminate Chagas disease. Weekly Epidemiological Records 2007; 82: 259-60.

Noel W, Raes G, Hassanzadeh Ghass G, De Baetselier P, Beschin A. Alternatively activated macrophages during parasite infections. Trends Parasitol. 2004; 20: 126–33.

Papazahariadou M, Athanasiadis GI, Papadopoulos E et al. Involvement of NK cells against

tumors and parasites. Int J Biol Markers. 2007; 22: 144-53.

Pérez-Gutiérrez E, Agrelo RS, Figueroa R. Technical recommendation on Chagas' disease epidemiology and prevention, focusing its transmission as a disease transmitted by food. Rev Soc Bras Med Trop. 2006; 39: 512-4.

Peruhype-Magalhães V, Martins-Filho OA, Prata A, et al. Mixed inflammatory/regulatory cytokine profile marked by simultaneous raise of interferon-gamma and interleukin-10 and low frequency of tumour necrosis factor-alpha(+) monocytes are hallmarks of active human visceral Leishmaniasis due to Leishmania chagasi infection. Clin Exp Immunol. 2006; 146: 124-32.

Reis DD, Jones EM, Tostes Jr S et al. Characterization of inflammatory infiltrates in chronic chagasic myocardial lesions: presence of tumor necrosis factor alpha^{+} cells and dominance of granzyme A^{+}, CD8^{+} lymphocytes. Am J Trop Med Hyg. 1993; 48: 637-44.

Sathler-Avelar R, Vitelli-Avelar DM, Massara RL. Etiological treatment during early chronic indeterminate Chagas disease incites an activated status on innate and adaptive immunity associated with a type 1-modulated cytokine pattern. Microbes Infect. 2008;10:103-13.

Soares MB, Silva-Mota KN, Lima RS, Bellintani MC, Pontes-de-Carvalho L, Ribeiro-dos-Santos R. Modulation of chagasic cardiomyopathy by interleukin-4: dissociation between inflammation and tissue parasitism. Am J Pathol. 2001; 159: 703-9.

Souza PE, Rocha MO, Rocha-Vieira E, et al. Monocytes from patients with indeterminate and cardiac forms of Chagas' disease display distinct phenotypic and functional characteristics associated with morbidity Infect Immun. 2004; 72: 5283-91.

Vitelli-Avelar DM, Sathler-Avelar R, Dias JC et al. Chagasic patients with indeterminate clinical form of the disease have high frequencies of circulating CD3^{+}CD16^{-}CD56^{+} natural killer T cells and CD4^{+}CD25^{High} regulatory T lymphocytes. Scand J Immunol.

2005; 62: 297-308.

Vitelli-Avelar DM, Sathler-Avelar R, Massara RL et al.. Are increased frequency of macrophage-like and natural killer (NK) cells, together with high levels of NKT and CD4+CD25high T cells balancing activated CD8+ T cells, the key to control Chagas' disease morbidity? Clin Exp Immunol. 2006; 145: 81-92.

FIGURE LEGENDS

Fig. 1. Representative scatter graphs of TNF-α^+ monocytes and IL-10^+ $CD4^+$ T-cells employed to establish the concept of low cytokine-producers (○) and high inflammatory (●) or regulatory (◍) cytokine-producers. Low cytokine-producers were defined for values of $cytokine^+$ cells lower than the global median whereas high cytokine-producers were defined for values of $cytokine^+$ cells higher or equal the global median cut-off.

Fig. 2. (A) Color diagrams representing low (▭), high inflammatory (▬) and high regulatory (▭) cytokine-producers for chagasic patients (IND and CARD) and non-infected individuals (NI). (B) Color diagrams representing the cytokine balance of low, high inflammatory, regulatory or mixed (▬) cytokine-producers for a range of cell subset from innate and adaptive immune response. (C) Overall cytokine profile representing the frequency of dominant inflammatory or regulatory cytokine profile within NI, IND and CARD.

Fig. 3. Radar graph representing the prevalence of ex vivo inflammatory (▬) or regulatory (▭) cytokine balance in a range of cell subsets of innate and adaptive immunity from IND and CARD chagasic patients. Each axis displays the proportion of each cytokine balance category within a given leukocyte subset. The values of each axis can be joined to form the central polygon area which represents the general inflammatory or regulatory cytokine balance. Increasing or decreasing central polygon areas reflect higher or lower contribution of inflammatory or regulatory cytokine balance in IND and CARD. The analysis of the radar chart axes highlights the contribution of distinct leukocyte subset for the overall cytokine balance.

Fig.4. Radar graph representing the impact of in vitro T. cruzi antigen stimulation calculated as the ratio between antigen-stimulated/control cultures in the prevalence of inflammatory (▬) or regulatory (▭) cytokine balance in a range of cell subsets of innate and adaptive immunity from IND and CARD chagasic patients. Each axis displays the proportion of each cytokine balance category within a given leukocyte subset. The analysis of the radar chart axes highlights the contribution of distinct leukocyte subset for the overall cytokine balance. The trypomastigotes T. cruzi antigen booster in vitro overturn the cytokine balance of peripheral blood leukocytes from IND and CARD as compared with the ex vivo cytokine balance.

Fig.5. Impact of etiological treatment on the ex vivo IL-10 circuit of $CD4^+$ T-cells from IND (▭) and monocytes from CARD (▬) individuals. The results are expressed as mean percentage of $cytokine^+$ cells ± standard error for IND and CARD groups before and after etiological treatment. Statistical significant differences at $p< 0.05$ are highlighted by * as compared to the data obtained before the etiological treatment.

Table 1. Frequency of cytokine high-producers subjects based on the global median cytokine cut-off detected in innate immunity cells*

Cytokine$^+$ Cell		Global Median Cut-off	% of High Cytokine-producers		
			NI	IND	CARD
Monocytes	TNF-α^+	20.1 (2.3-60.7)	27.3	50.0	87.5[a]
	IL-10$^+$	3.4 (0.9-8.2)	63.6	50.0	37.5
Neutrophils	IFN-γ^+	0.7 (0.2-1.5)	63.6	33.3	50.0
	TNF-α^+	0.8 (0.3-1.8)	45.5	66.7	50.0
	IL-12$^+$	0.8 (0.3-1.8)	36.4	100.0[a,c]	25.0
	IL-10$^+$	1.1 (0.6-2.1)	54.5	50.0	50.0
	IL-4$^+$	1.2 (0.2-3.5)	54.5	66.7	37.5
	IL-13$^+$	0.5 (0.1-1.3)	45.5	100.0[a]	50.0
NK Cells	TNF-α^+	7.6 (3.4-52.6)	36.4	83.3	50.0
	IL-4$^+$	22.5 (8.3-57.2)	27.3	83.3	62.5

* Data are expressed as percentage of individuals displaying percentage of cytokine$^+$ cells higher or equal the global median cut-off calculated for each cell population within the innate immunity cells. Statistical significance at $p<0.05$ (Chi-square) are represented by letters "*a*" and "c" for comparisons with NI, IND and CARD, respectively.

Table 2. Frequency of cytokine high-producers subjects based on the global median cytokine cut-off detected in adaptive immunity cells*

Cytokine$^+$ Cell			Global Median Cut-off	% of High Cytokine-producers		
				NI	IND	CARD
	Total Lymphocytes	IFN-γ^+	1.2 (0.4-2.4)	63.6	33.3	50.0
		TNF-α^+	1,0 (0.3-2.8)	63.6	50.0	37.5
		IL-12$^+$	1.2 (0.3-6.1)	72.7	0.0[a,c]	62.5
		IL-10$^+$	1.7 (0.2-12.7)	72.7	83.3	0.0[a,b]
		IL-4$^+$	1.7 (0.4-4.4)	63.6	66.7	25.0
		IL-13$^+$	0.8 (0.2-2.5)	63.6	66.7	25.0
Lymphocyte Subsets	CD4$^+$ T-cells	IFN-γ^+	1.1 (0.3-2.3)	63.6	50.0	37.5
		TNF-α^+	0.8 (0.1-4.3)	72.7	16.7	50.0
		IL-12$^+$	1.2 (0.3-9.8)	45.5	50.0	62.5
		IL-10$^+$	2.7 (0.5-10.6)	45.5	66.7	50.0
		IL-4$^+$	1.6 (0.3-6.7)	45.5	83.3	37.5
		IL-13$^+$	0.8 (0.2-5.0)	63.6	50.0	37.5
	CD8$^+$ T-cells	IFN-γ^+	1.1 (0.1-5.6)	63.6	33.3	50.0
		TNF-α^+	1.3 (0.2-6.0)	63.6	66.7	25.0
		IL-12$^+$	1.4 (0.3-8.2)	63.6	50.0	50.0
		IL-10$^+$	3.1 (0.4-12.1)	81.8	66.7	12.5[a]
		IL-4$^+$	2.2 (0.2-6.3)	54.5	33.3	62.5
		IL-13$^+$	1.6 (0.3-6.5)	63.6	50.0	37.5
	B-cells	TNF-α^+	1.9 (0.3-9.2)	45.5	66.7	37.5
		IL-10$^+$	7.5(3.1-54.0)	63.6	33.3	50.0
	B1-cells	TNF-α^+	19.2 (1.96-100.0)	54.5	33.3	50.0
		IL-10$^+$	41.2 (4.1-100.0)	54.5	50.0	50.0

* Data are expressed as percentage of individuals displaying percentage of cytokine$^+$ cells higher or equal the global median cut-off calculated for each cell population within the adaptive immunity cells. Statistical significance at $p<0.05$ (Chi-square) are represented by letters "a", "b" and "c" for comparisons with NI, IND and CARD, respectively.

Figure 1.

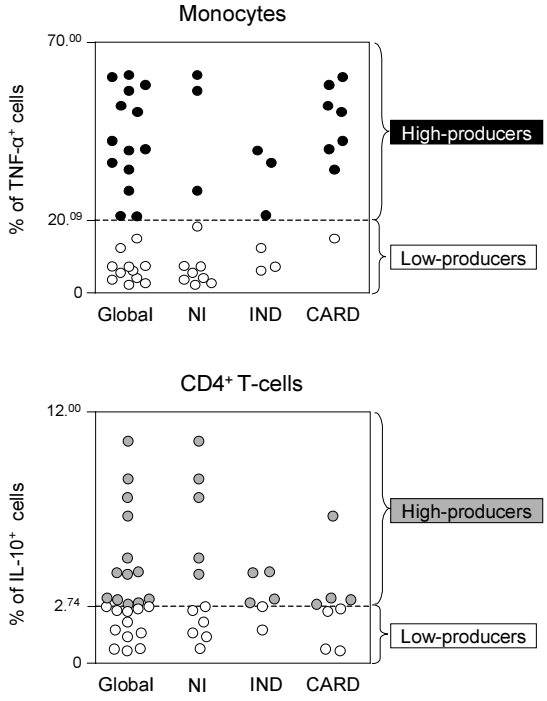

Monocytes
70.00
20.09
0
% of TNF-α+ cells
High-producers
Low-producers
Global
NI
IND
CARD
CD4+ T-cells
12.00
2.74
0
% of IL-10+ cells
High-producers
Low-producers
Global
NI
IND
CARD

Figure 2.

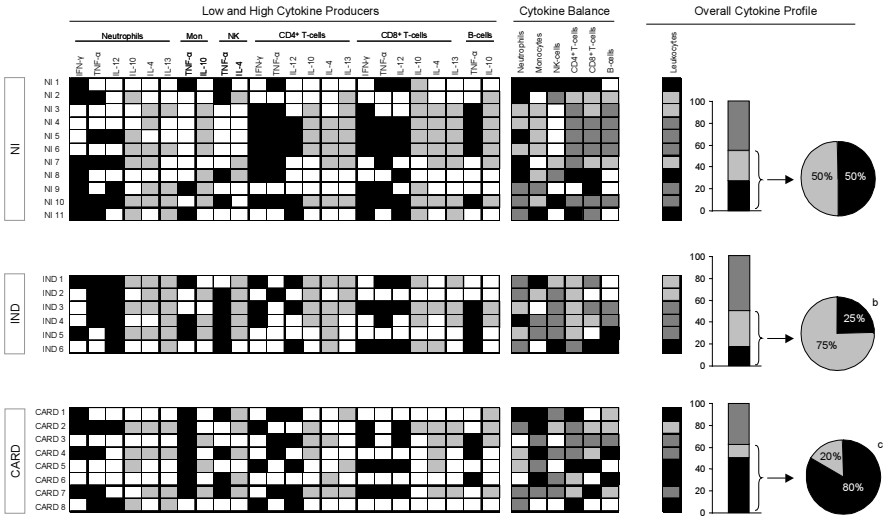

Low and High Cytokine Producers
Cytokine Balance
Overall Cytokine Profile
Neutrophils
Mon
NK
CD4+ T-cells
CD8+ T-cells
B-cells
IFN-γ
TNF-α
IL-12
IL-10
IL-4
IL-13
TNF-α
IL-10
TNF-α
IL-4
Neutrophils
Monocytes
NK-cells
CD4+ T-cells
CD8+ T-cells
B-cells
Leukocytes
NI
NI 1
NI 2
NI 3
NI 4
NI 5
NI 6
NI 7
NI 8
NI 9
NI 10
NI 11
IND
IND 1
IND 2
IND 3
IND 4
IND 5
IND 6
CARD
CARD 1
CARD 2
CARD 3
CARD 4
CARD 5
CARD 6
CARD 7
CARD 8
100
80
60
40
20
0
50%
50%
25%
75%
b
20%
80%
c

Figure 3.

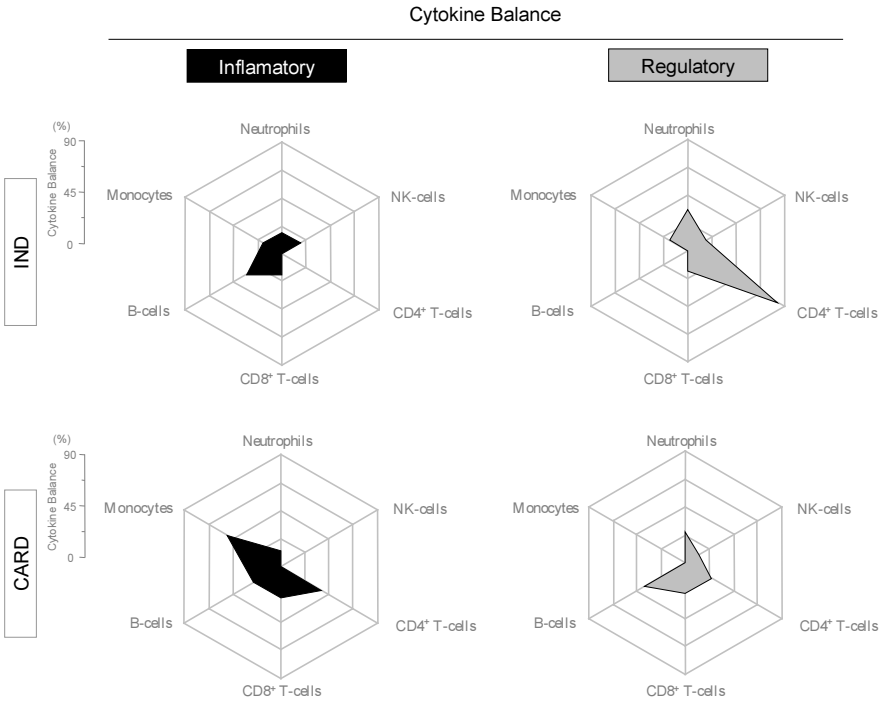

Cytokine Balance
Inflamatory
Regulatory
IND
CARD
(%)
90
45
0
Cytokine Balance
Neutrophils
NK-cells
CD4+ T-cells
CD8+ T-cells
B-cells
Monocytes

Figure 4.

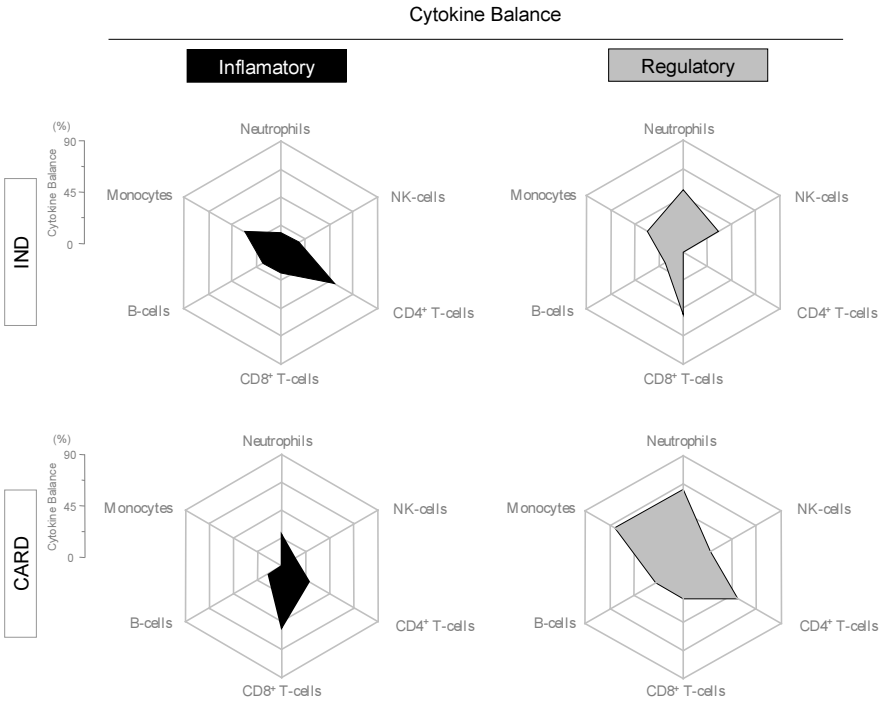
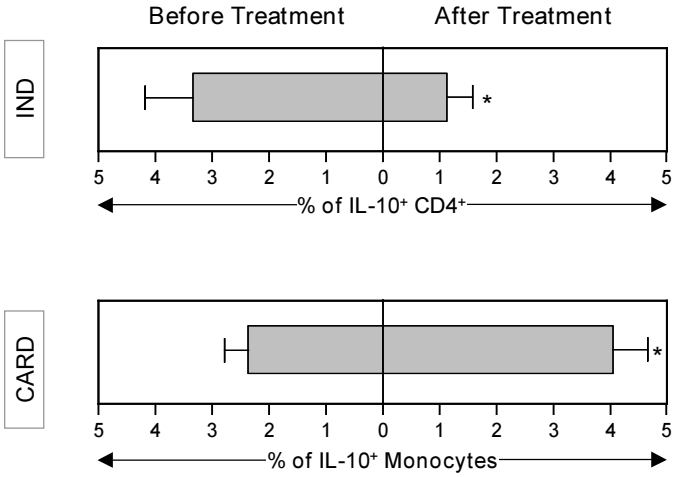

Cytokine Balance
Inflamatory
Regulatory
IND
CARD
(%)
90
45
0
Cytokine Balance
Neutrophils
NK-cells
CD4+ T-cells
CD8+ T-cells
B-cells
Monocytes

Figure 5.


Before Treatment
After Treatment
IND
*
5 4 3 2 1 0 1 2 3 4 5
% of IL-10+ CD4+
CARD
*
5 4 3 2 1 0 1 2 3 4 5
% of IL-10+ Monocytes

4.3. ARTIGO 4:

O artigo científico apresentado nesse tópico corresponde ao objetivo específico 3.

Título: Non-conventional flow cytometry approaches to detect anti-Trypanosoma cruzi immunoglobulin G in the clinical laboratory.

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Research paper

Non-conventional flow cytometry approaches to detect anti-Trypanosoma cruzi immunoglobulin G in the clinical laboratory

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Abstract

We have recently developed a flow cytometric approach to detect anti-live trypomastigote and anti-fixed epimastigote IgG antibodies (FC-ALTA and FC-AFEA) in sera from individuals infected by Trypanosoma cruzi. Here, we present the first evaluation of the applicability of FC-AFEA-IgG as a diagnostic tool for Chagas disease. Performance analysis demonstrated that FC-AFEA-IgG has a sensitivity of 82% and a specificity of 100%. The assessment for prognosis performed by FC-ALTA-IgG1 and FC-AFEA-IgG, after classification of chagasic patients as belonging to indeterminate (IND), cardiac (CARD) or digestive (DIG) clinical forms, showed that most of IND have higher amounts of IgG than individuals' carrying CARD or DIG Chagas disease. FC-AFEA-IgG was also evaluated as a method to monitor chemotherapy efficacy in individuals classified into three distinct categories: not treated (NT), treated but not cured (TNC), and treated and cured (TC). Performance analysis demonstrated that FC-AFEA-IgG has an extraordinary capacity as a serological criterion to assess cure after therapeutic intervention in Chagas disease. These results represent a great advance in the application of serological techniques for clinical investigations on Chagas disease, and they clearly define new directions and perspectives. We intend to continue this field research focusing our attention on the influence of the degree of clinical damage on the FC-ALTA-IgG1 and FC-AFEA-IgG reactivity.

Keywords: Chagas disease; Trypanosoma cruzi; Flow cytometry; Serology; FC-ALTA; FC-AFEA

1. Introduction

Chagas disease represents the third largest tropical disease burden after malaria and schistosomiasis. This is a chronic and ultimately fatal disease caused by protozoan Trypanosoma cruzi and constitutes a major

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public health problem in Latin America (Moncayo, 2003). Several countries in this area have well-established vector-control programs. However, contact with contaminated feces or urine from infected triatomine insects is still the main cause of infection. Moreover, endemic areas where parasite transmission is not yet controlled present a constant threat to neighbouring regions meaning that 120 million people are currently at risk of infection. The World Health Organization estimates that approximately 300,000 new cases of Chagas disease occur every year (World Health Organization, 2002).

In the acute phase of Chagas disease, when parasitemia is high, usually diagnosis can be easily made using conventional parasitological methods (xenodiagnosis and hemoculture). Nevertheless, detection of the acute phase in endemic areas is not always easy because most individuals are oligosymptomatic or asymptomatic. The great majority of the patients are identified during long lasting chronic disease. In this phase, serologic tests are used to detect antibody against T. cruzi and not the presence of the parasite itself. These methods can provide false results because of the lack of specificity or sensitivity. Consequently, the Pan American Health Organization suggests the use of at least two methods for the diagnosis of the disease. Conflicting results with conventional serology are frequent (Camargo et al., 1986). Furthermore, the antigens commonly used in serodiagnosis of Chagas disease are fixed epimastigote forms or complex mixtures of proteins and glycoconjugates extracted from whole parasites which lead to false positive results due to cross-reactions with other parasites, such as leishmaniasis (Carvalho et al., 1993; Umezawa et al., 2003; Amato Neto et al., 2005).

Although conventional serology remains the most widely used method for Chagas disease diagnosis, flow cytometry is becoming a reliable and powerful serological method for clinical investigations on Chagas disease. Therefore, in the area of disease prognosis and management/treatment, non-parasitological markers can be implemented as predictors of morbidity and treatment efficacy, and the risk-benefit of chemotherapy can be focused on different phases of Chagas disease. Furthermore, the proposed flow cytometry methodology is highly sensitive and can also be applied as a non-conventional serological diagnosis technique (Martins-Filho et al., 1995, 2002; Cordeiro et al., 2001).

The results of this double blind investigation re-emphasize the applicability of flow cytometry to detect anti-T. cruzi antibodies in human serum for diagnosis as well as for prognosis and assessment of cure.

2. Materials and methods

2.1. Study population

The inclusion of all subjects in our investigation had the approval of the Ethics Committees of the FIOCRUZ. To evaluate the efficacy of our test in prognosis, diagnosis, and assessment of cure we studied different cohorts.

2.1.1. Cohort 1

To evaluate our test in regard to diagnosis, 148 samples were selected comprising a total of 28 chagasic patients (CH), 26 with classic Kalazar–American Visceral Leishmanisasis (VL), 20 with American Localized Cutaneous Leishmaniasis (LCL), 20 toxoplasmosis patients (TX), 20 malarial patients (MA), 21 Schistosoma mansoni infected individuals (SCH) and 12 non-infected controls (NI). Chagasic patients were collected in Bambui, Minas Gerais/Brazil, an endemic area for Chagas disease and had received a positive diagnosis for Chagas disease (as determined by indirect immunofluorescence assay-IFA and hemagglutination-HA).

2.1.2. Cohort 2

The serum samples were the same as those used in cohort 1 from Bambuí, Minas Gerais/Brazil. According to their clinical records, the patients were divided into three different categories, namely indeterminate (IND), cardiac (CARD) and digestive (DIG). Patients presenting asymptomatic T. cruzi infection, classified as IND (n=6), had no clinical manifestations of the disease other than their positive serology. Patients with cardiac dysfunction, CARD (n=13), presented with dilated cardiomyopathy and were identified by a detailed clinical examination, including electrocardiography (ECG), 24-h Holter examination and chest X-ray. Chagasic patients with gastrointestinal disease, DIG (n=7), presented with clinical radiological status of megacolon and/or megaesophagus. Twelve non-chagasic individuals, with negative results on serological tests for Chagas disease, were included in this study as negative controls (NI). All were living in an endemic area for Chagas disease.

2.1.3. Cohort 3

Serum samples were collected from 30 chagasic seropositive patients (as determined by IFA and HA). As for cohort 2, we classified the individuals according to their clinical records, as IND (n=10), CARD (n=10) or DIG (n=10). The individuals were from Minas Gerais/Brazil and ranged from 24 to 77 years of age. Twenty

non-chagasic individuals, with negative results on serological tests for Chagas disease, were included in this study as negative controls (NI).

2.1.4. Cohort 4

This sub-cohort was composed of 60 individuals, ranging in age from 6 months to 68 years. These patients have been evaluated parasitologically (hemoculture) and serologically (IFA and HA) in a follow-up study from 3 up to 26 years. In this study we did not focus on the efficacy of the different therapeutic schemes that were used since that was not the major goal of our present investigation. After clinical, parasitological, and serological follow-up studies patients were classified into three different categories followed the description of Martins-Filho et al. (2002): not treated (NT, n=19); treated but not cured (TNC, n=17); treated and cured (TC, n=24). The treatments were carried out during acute, sub-acute or chronic phases. NT and TNC patients persisted with positive results on both serological and parasitological tests. Patients were considered TC only when both conventional serological and parasitological tests were consistently negative in at least eight assessments of serially collected blood samples.

2.2. Parasite preparations

2.2.1. Epimastigotes

Epimastigotes forms from the CL strain were obtained by inoculation of 1.0×10^7 bloodstream trypomastigotes from experimentally infected mice, in liver infusion tryptose medium-LIT (Camargo, 1964) with 10% of heat-inactivated fetal bovine serum (FBS, GIBCO, Grand Island, New York, USA) at 28 °C. After serial passages in vitro, the parasites were harvested during the log phase of growth. The organisms were washed three times with PBS supplemented with 3% FBS (1000 ×g for 15 min at 4 °C), resuspended immediately in equal volume of PBS and FACS fix solution [10.0 g/l of paraformaldehyde, 1% sodium cacodylate, 6.63 g/l of sodium chloride and 0.01% of sodium azide (Sigma Chemical), pH 7.2], and stored at 4 °C until use. The suspension of parasites was adjusted to 10×10^6/ml before used in the flow cytometric method to detect anti-fixed epimastigote antibodies (FC-AFEA).

2.2.2. Trypomastigotes

LLC-MK_2 cells were maintained in our laboratory by serial passages and kept frozen in liquid nitrogen. For the assays, 5×10^5 LLC-MK_2 cells were seeded in tissue culture flasks (Falcon 25 cm^2 or 75 cm^2) with 10 ml of DMEM medium (GIBCO, Grand Island, New York, USA) containing 10% FBS, and incubated at 37 °C in a humidified air containing 5% CO_2. After 2 or 3 days, the monolayer was infected with 5×10^6 trypomastigotes of T. cruzi-CL strain obtained from experimentally infected mice (Brener and Chiari, 1963). The cultures were maintained in DMEM, 10% of FBS at 33 °C in 5% of CO_2 at 95% humidity (Bertelli et al., 1977). After 5–6 days the trypomastigotes were harvested from the supernatant. Cell debris and amastigotes were removed by differential centrifugation at 100 ×g for 10 min at room temperature. The supernatant containing most of the parasites was centrifuged at 1000 ×g for 15 min at 4 °C. The pellet was washed three times in 0.15 M phosphate buffered saline, pH 7.2 (PBS) supplemented with 10% FBS. The suspension of parasites was adjusted to 10×10^6/ml before use in the flow cytometric method to detect anti-live trypomastigote antibodies (FC-ALTA) assays.

2.3. Immunofluorescence by flow cytometry

The serum samples were inactivated by heating for 30 min at 56 °C and kept at −20 °C until use. The inactivated sera were diluted in PBS containing 10% FBS and used to evaluate the presence of anti-T. cruzi antibodies by flow cytometry.

The parasite immunofluorescence staining was carried out as described by Martins-Filho et al. (1995), modified for U bottom 96 well plate (LINBRO, ICN Biomedicals, Inc. Aurora, Ohio) by Cordeiro et al. (2001). Briefly, 500,000 parasites/well were incubated at 37 °C for 30 min in the presence of different dilutions (1/128 to 1/16,384) of individual serum from all patients and controls selected for this study. After incubation with sera, the parasites were washed twice with 150 μl of PBS-10% FBS (1000 ×g for 10 min at 4 °C) and reincubated at 37 °C for 30 min in the dark, in the presence of fluorescein isothiocyanate (FITC) conjugated anti-human IgG antibody or with biotin-conjugated anti-human IgG1 subclass antibody (Sigma Chemical Corp., St. Louis, MO). The FITC-labeled parasites were fixed for 30 min with a FACS fix solution before analysis in the cytometer. The biotin-labeled parasites were incubated with 10 μl of streptavidin-phycoerythrin-SAPE (GIBCO, Grand Island, New York, USA) at 37 °C for 30 min in the dark. After being stained, the PE-labeled parasites were washed twice with PBS-10% FBS and fixed on ice for 30 min with FACS fix solution. Stained parasites were stored at 4 °C up to 24 h before cytofluorometric analysis.

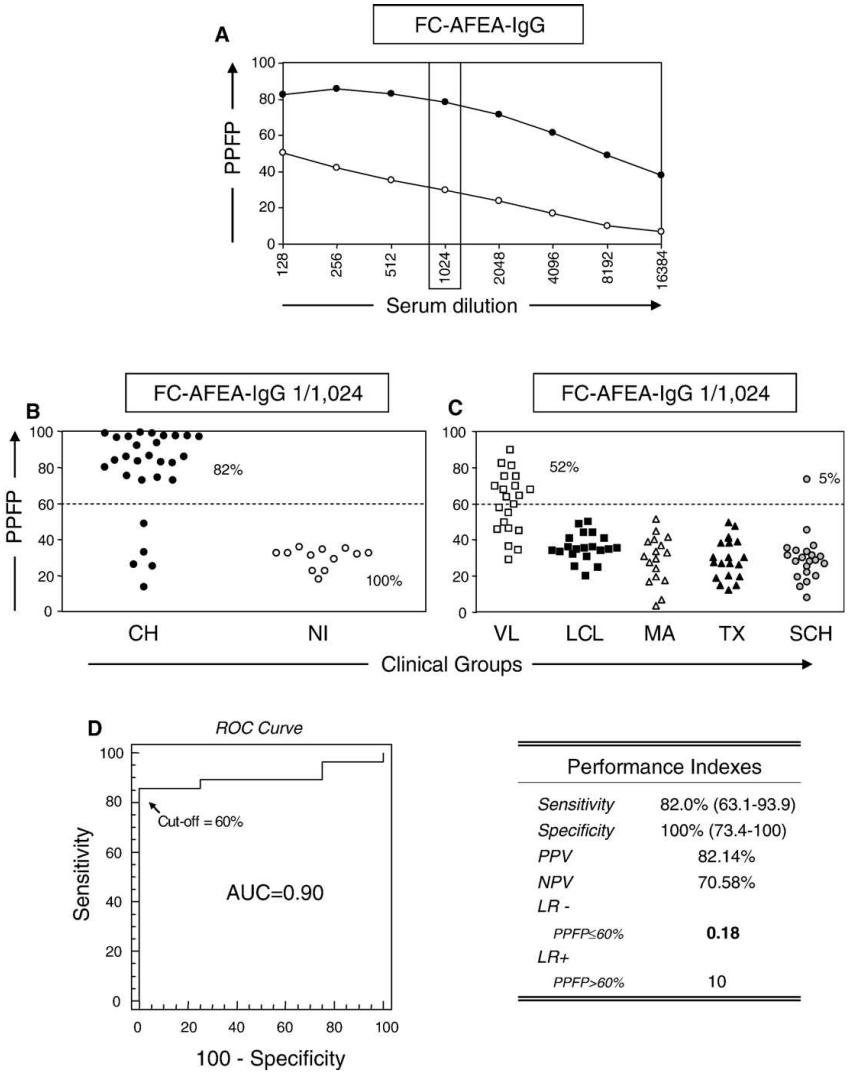

Fig. 1. Titration curve average of anti-fixed epimastigotes IgG reactivity (FC-AFEA-IgG) of serum samples from chagasic patients (CH ●, n=28) and non-infected individuals (NI ○, n=12). The continuous box marks the most promising serum dilution to discriminate CH from NI (A). FC-AFEA-IgG of individual serum samples from chagasic (CH) and non-infected (NI) individuals from cohort 1 at 1/1024 dilution (B). FC-AFEA-IgG of individual serum samples from classic Kalazar (VL), American Cutaneous Leishmaniasis (LCL), malarial (MA), toxoplasmosis (TX), Schistosoma mansoni (SCH) infected individuals from cohort 1 at 1/1024 dilution (C). The results are expressed as PPFP. The dotted line represents the cut-off between negative or low positive (PPFP≤ 60%) and high positive (PPFP>60%) PPFP values. ROC curve analysis was applied to establish the better cut-off edge to discriminate PPFP values from CH and NI (D). Additional performance indexes are also provided (inserted table).

2.4. FACScan data storage and analysis

Flow-cytometric measurements were performed on a Becton Dickinson FACScan interfaced to an Apple Quadra FACStation. The Cell-Quest software package was used in both data storage and analysis. Stained parasites were run in the cytometer, and 5000 events per sample were acquired. The parasites were identified on the basis of their specific forward (FCS) and side (SSC) light-scattering properties. Parasites were selected by gating on the FCS×SSC dot plot distribution. Parasites have a characteristic homogeneous distribution that allows selective analysis by creating a specific window over the parasite region. This profile was obtained by adjusting size and granularity gains, on a log scale, with values of 10 and 300, respectively. The relative FITC or PE fluorescence intensity for each parasite preparation was analyzed using a single histogram representation. A marker was set on the internal control for nonspecific binding of FITC or PE-conjugated antibody and used to determine for each serum sample the percentage of positive fluorescent parasites (PPFP). Data analysis was initially performed by establishing 20% and 50% of PPFP as the cut-off between negative (PPFP≤ 20%), low positive (20%bPPFP≤ 50%) and high positive (PPFPN50%) results as described by Martins-Filho et al. (1995) and Cordeiro et al. (2001). Additional cut-off edges were further identified by Receiver Operating Curve (ROC-curve), and PPFP=60% was used for most data analysis presented on this investigation.

2.5. Statistical analysis

Each test's performance was assessed by the following statistical indexes: sensitivity=[true positives÷(true positives+false negatives)]×100; specificity=[true negatives÷(true negatives+false positives)]×100; positive predictive value — PPV=[true positives÷total positives]×100 and negative predictive value — NPV=[true negatives÷total negatives]×100 (Youden, 1950). The receiver operating characteristic curve (ROC curve) was built applying the sensitivity values in the ordinate, and the complement of specificity in the abscissa. The curve was used to select the cut-off value to discriminate negative from low positive and high positive PPFP results. The tests' global accuracy was also evaluated, taking the area under the ROC curve (AUC) according to Swets (1988). Each test's performance was also evaluated by Likelihood Ratios (LR), taking LR for the positive result=[true positives÷(true positives+false negatives)]÷[false positives÷(falses positive+true negatives)], and LR for the negative result=[false negatives÷(true positives+false negatives)]÷[true negatives÷(false positives+true negatives)]. As proposed by Jaeschke et al. (1994), LRN10 practically confirms disease diagnosis and LRb0.1 practically excludes disease diagnosis.

3. Results

3.1. Establishment of the FC-AFEA-IgG as a non-conventional assay for the diagnosis of Chagas disease with minimal cross-reactivity with major tropical endemic diseases in Brazil

During the chronic phase of Chagas disease, due to the low parasitemia, diagnosis is usually performed by immunological methods in conjunction with clinical epidemiological evidence. In recent years, our groups have worked toward developing new diagnostic tests employing flow cytometry in serological diagnosis of Chagas disease. Here, we report the evaluation of anti-fixed epimastigotes IgG (FC-AFEA-IgG) for the diagnosis of T. cruzi infection (Cohort 1).

FC-AFEA-IgG results were evaluated using serial dilution of all sera samples, starting at 1/128 up to 1/16,384. The titration curve of the CH and NI averages allowed the identification of serum dilution 1/1024 as a promising experimental condition to better discriminate the CH from NI based in the superior segregation (Fig. 1A). Using the PPFP=60% as the cut-off edge, elected by the ROC curve analysis for serum dilution 1/1024 (Fig. 1D), we observed that 82% of CH showed high positive PPFP values and all NI presented low positive PPFP values (Fig. 1B). Performance analysis of FC-AFEA-IgG for diagnosis was also performed by MedCalc software package 7.3 further confirmed these finding demonstrating the outstanding performance indexes of FC-AFEA-IgG, including a sensitivity of 82.0% (63.1–93.9) and a specificity of 100% (73.4–100) with positive and negative predictive values of 82.14% and 70.58%, respectively (Table inserted in Fig. 1). The analysis of the area under ROC curve also showed a good performance of the test (AUC=0.90). Likelihood ratio (LR) confirmed that FC-AFEA-IgG has a good performance for Chagas disease diagnosis. Our data demonstrated that results of PPFP N60% is more than 10 times more likely to come from a CH patient than NI individual (Table inseted in Fig. 1). On the other hand a low PPFP result is unlikely to belong to a CH individual, strongly suggesting a negative diagnosis for Chagas disease.

Further studies searching for cross-reactivity were performed using serum samples from individuals with other infectious diseases (Fig. 1C). The analysis demonstrated

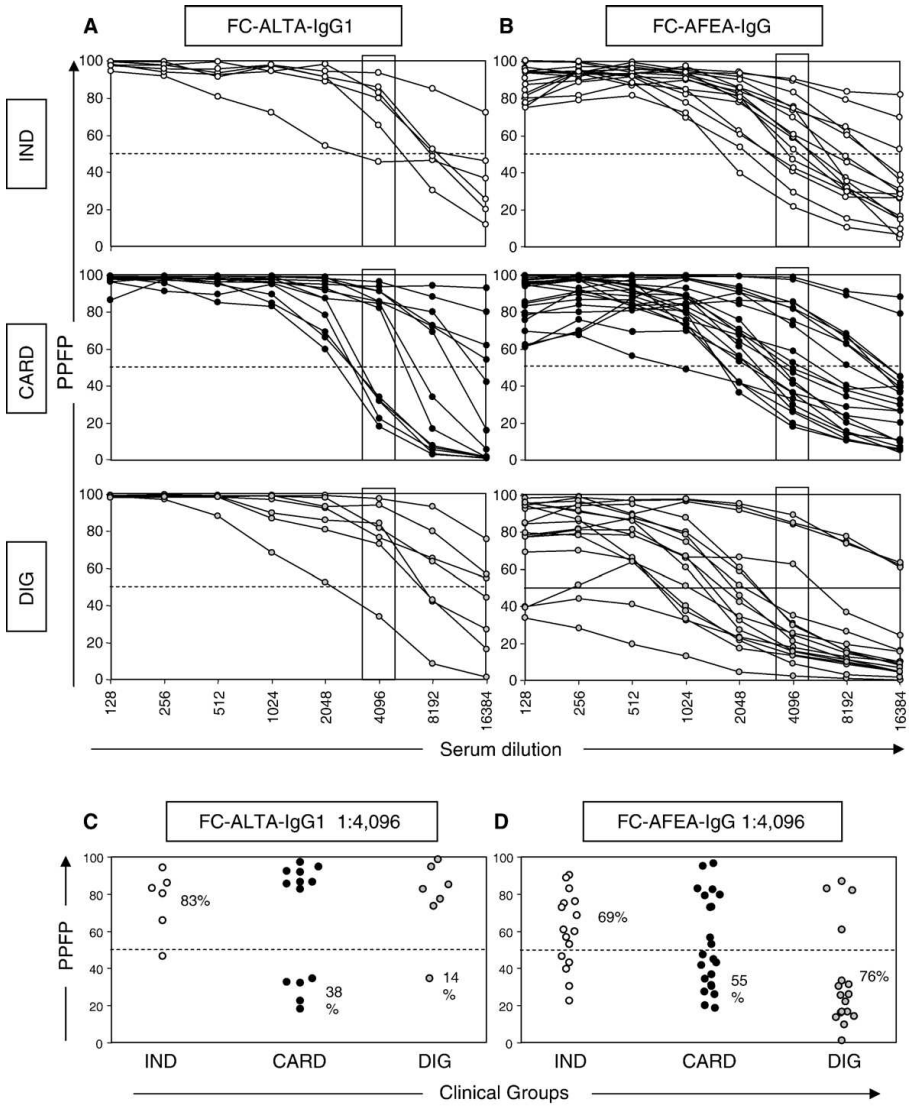

Fig. 2. Titration curve of FC-ALTA-IgG1 (A) and FC-AFEA-IgG (B) of individual serum samples from indeterminate (IND ○), cardiac (CARD ●) and digestive (DIG ◍) chagasic patients (subcohort 2 and subcohort 2+3, respectively). The results are expressed as PPFP. The dotted line represents the cut-off proposed by Cordeiro et al. (2001) to classify PPFP values as low positive (PPFP≤ 50%) and high positive results (PPFP>50%). The continuous rectangle highlights the most promising serum dilution 1/4096 selected to further identify the differential FC-ALAT-IgG1 and FC-AFEA-IgG reactivity between chronic chagasic patients (C and D). Using the proposed reactivity cut-off of 50%, the FC-ALTA-IgG1 showed high positive PPFP values for 83% of IND, whereas 38% of CARD and 14% of DIG had low positive PPFP values (C). FC-AFEA-IgG additionally showed that 69% of IND presented high positive PPFP values, whereas 55% of CARD and 76% of DIG had low PPFP values (D).

that in the conditions previously described only VL (52%) and SCH (5%) provided false-positive results on FC-AFEA-IgG. Despite the high frequency of cross-reactivity of samples from VL patients observed by FC-AFEA-IgG, it is important to mention that this situation could be resolved by the clinical status of VL patients, classically identified as an acute febrile hepatosplenic condition, not compatible with any clinical manifestation of chronic Chagas disease.

In order to further evaluate the performance of FC-AFEA-IgG applicability in Chagas disease diagnosis, focusing attention on improvement of sensitivity and specificity, we have suggested the possibility of using FC-AFEA-IgG subclasses as a useful tool to eliminate cross-reactivity in serological diagnosis of Chagas disease, mainly due to the high recognition of fixed T. cruzi epimastigotes by VL samples. For this purpose, we designed an experiment to investigate the potential of FC-AFEA-IgG1 to contribute on the discrimination of CH and VL samples. Our data demonstrate that, despite a similar PPFP profile observed for CH along the titration curve, as compared to IgG reactivity, no real gain on the discrimination of CH and VL was observed with only 3 samples from the VL group with negative results at a serum dilution of 1/1024 (data not shown).

3.2. Use of anti-live trypomastigote antibodies (FC-ALTA) by flow cytometry as a reliable method for serological diagnosis of distinct clinical manifestations of chronic Chagas disease

Cordeiro et al. (2001) suggested the potential use of anti-live tripomastigotes IgG1 subclasses (FC-ALTA-IgG1) by flow cytometry for prognostic purposes, for monitoring the progression of chronic Chagas disease and for predicting the risk of cardiac damage.

Aiming to confirm this report of a differential FC-ALTA-IgG1 reactivity between chagasic patients bearing distinct clinical manifestations of chronic disease, we have performed a blind FC-ALTA-IgG1 study on a sub-cohort of chagasic patients from Bambui, Minas Gerais/Brazil, an endemic area for Chagas disease (cohort 2). FC-ALTA-IgG1 reactivity was performed using serial dilutions from 1/128 to 1/16,384, and data were expressed as PPFP (Fig. 2A). We selected the serum dilution 1/4096 and PPFP=50% as the cut-off to discriminate IgG1 reactivity by FC-ALTA as proposed by Cordeiro et al. (2001). Our data demonstrated that 83% of IND presented high positive PPFP values, confirming our previous report of higher IgG1 reactivity on asymptomatic patients. The analysis of FC-ALTA-IgG1 data from CARD and DIG demonstrated that only 38% and 14% of them presented low positive PPFP values, respectively (Fig. 2C).

3.3. Development and evaluation of the performance of FC-AFEA-IgG by flow cytometry to detect differences between chagasic patients presenting different clinical forms of chronic Chagas disease

Continuing our efforts to detect differences between chagasic patients presenting different clinical forms of chronic disease, we searched for a differential profile of reactivity using FC-AFEA-IgG (Cohorts 2 and 3). We performed a FC-AFEA-IgG titration curve, from serum dilution 1/128 to 1/16,384, in order to identify the best sera dilution candidate for differential IgG reactivity between the three groups of patients evaluated. Our results confirmed that serum dilution 1/4096 and PPFP=50% as the cut-off were the most promising for detecting differential FC-AFEA-IgG reactivity (Fig. 2B). Our findings demonstrated that 69% of IND showed high positive PPFP values, whereas 55% and 76% of CARD and DIG presented low positive PPFP values, respectively (Fig. 2D).

3.4. Investigation of FC-AFEA-IgG as a non-conventional serological alternative to monitor cure after etiological treatment of chronic chagasic patients

One of the greatest concerns in Chagas disease is the absence of reliable methods for the evaluation of chemotherapy efficacy in treated patients. In this context, the identification of anti-T. cruzi antibodies by flow cytometry may be a promising tool for cure criterion. FC-AFEA-IgG reactivity was performed using serial dilution starting from 1/128 up to 1/16,384 and data expressed as PPFP. Initially, the cut-off selected to discriminate IgG reactivity by FC-AFEA were PPFP=20% and PPFP=50%. However, further investigations to justify the proposed cut-off was determined using the ROC-curve that had established that the cut-off of 20% and 60% of PPFP were the better choices to classify the samples as negative (PPFP≤ 20%), low positive (20%bPPFP≤ 60%) and high positive (PPFPN60%). Considering these established PPFP limits, we first determined the differential IgG reactivity on the sera samples included in this investigation, using the sera dilution 1/256 for FC-ALTA, as previously proposed by Martins-Filho et al. (1995, 2002). At PPFP=20% and PPFP=60% as cut-offs, all NT and TNC patients presented high positive IgG reactivity consistent with their clinical status. However, the results for TC patients were not consistent with their clinical status; 92% of

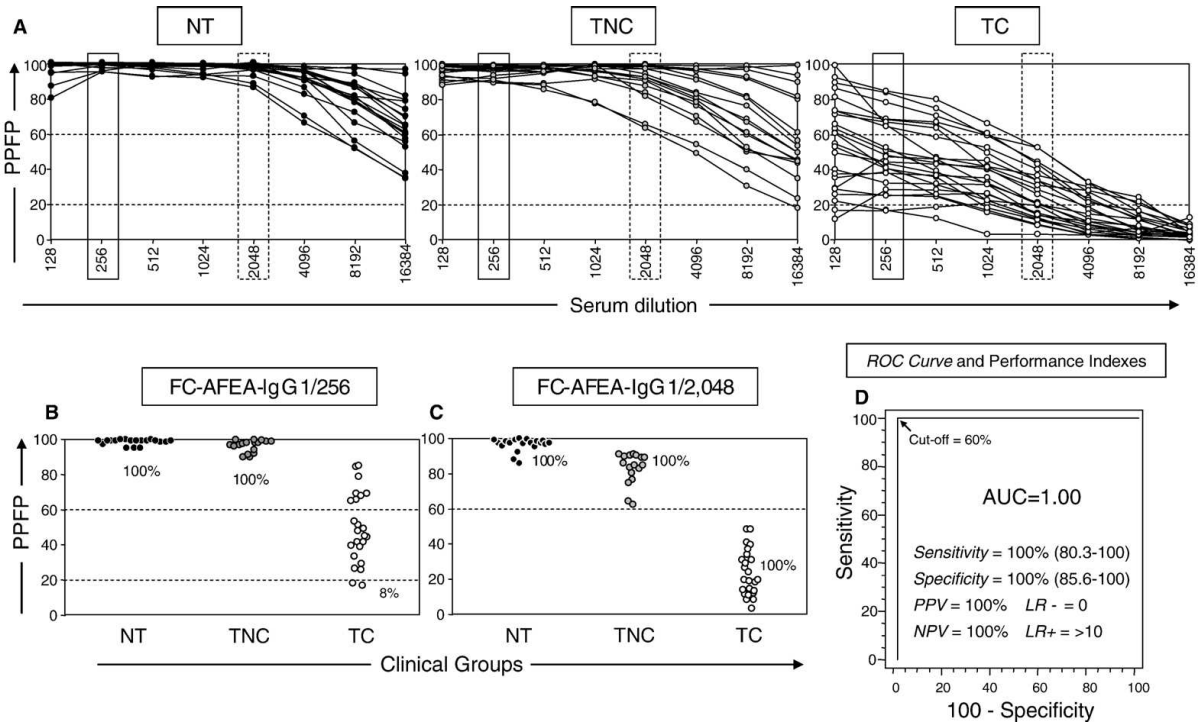

Fig. 3. Titration curve of anti-fixed epimastigote IgG reactivity (FC-AFEA-IgG) of individual serum samples from not-treated (NT ●, n=19), treated not-cured (TNC ◎, n=17) and treated cured (TC ○, n=24) individuals (A). Continuous and dotted rectangles represented the two promising serum dilutions: 1/2048 and 1/16,384, which were further evaluated (B and C). The results are expressed as PPFP. The dotted lines represent the cut-off between negative (PPFP≤ 20%), low positive (20%ÞPPFP≤ 60%) and high positive (PPFPN60%) PPFP values. Anti-fixed-epimastigote IgG reactivity (FC-AFEA-IgG) of individual serum samples from NT, TNC and TC individuals at 1/256 (B) and 1/2048 dilutions (C) demonstrated outstanding performance of FC-AFEA-IgG applied at serum dilution 1/2048, able to precisely discriminate the clinical status of the chagasic patients after etiological treatment. Additional ROC curve data and performance indexes are also provided (D) supporting the use of this tool to monitor cure after etiological treatment.

them had positive PPFP values (PPFP N 20%), and 33% of them had high positive IgG reactivity (PPFP N 60%) (Fig. 3A and B). Therefore, we investigated the FC-AFEA-IgG performance at higher serum dilutions. The titration curve allowed the identification of the 1/2048 as another promising serum dilution. Our data demonstrated that using the cut-off of PPFP=60% at serum dilution 1/2048, all NT and TNC samples showed high positive PPFP values, and all TC samples showed negative or low positive PPFP values (PPFP≤ 60%) (Fig. 3A and C).

Together, our data demonstrated that FC-AFEA-IgG performed at serum dilution 1/2048 was able to precisely discriminate the clinical status of the chagasic patients after etiological treatment and suggested the use of this tool to monitor cure after etiological treatment. If 1/2048 is used as the reference dilution, a high positive PPFP result observed after etiological treatment gives a precise conclusion of therapeutic failure. On the other hand, PPFP result ≤ 60% strongly suggests successful treatment efficacy after etiological therapeutics (Fig. 3C).

Performance analysis of FC-AFEA-IgG for cure criterion purposes was performed by MedCalc software package 7.3 and demonstrated that FC-AFEA-IgG has 100% of sensitivity (80.3–100) and specificity (85.6–100) with positive and negative predictive values of 100% (Fig. 3D). Likelihood ratio (LR) confirmed that FC-AFEA-IgG has an outstanding performance as a cure criterion after therapeutic intervention in Chagas disease (Fig. 3 D). As proposed by Jaeschke et al. (1994), LR+ N 10 practically confirms disease diagnosis and LR− b 0.1 practically excludes disease diagnosis. Based on these propositions, our data demonstrated that a low positive PPFP result is not likely to come from a TNC patient, confirming therapeutical effectiveness. On the other hand a high positive PPFP result is not likely to come from a TC, confirming therapeutic failure. We consider that despite the good performance of FC-AFEA-IgG to identify post-therapeutical cure at sera dilution 1/16,384, the lower specificity to identify therapeutic failure should rule out this serum dilution for clinical purposes (Fig. 3A right and middle panels, respectively).

4. Discussion

Although there has been an improvement in the diagnosis of chronic Chagas disease, the low sensitivity by conventional serology is a drawback to its application in diagnosis and post-therapeutic control (Portela-Lindoso and Shikanai-Yasuda, 2003). Here we report the establishment of the FC-AFEA-IgG as non-conventional assays for diagnosis, as well as, prognosis and cure criteria.

For the purpose of diagnosis, the titration curve of FC-AFEA-IgG allowed the identification of serum dilution of 1/1024 and the PPFP=60% as the cut-off to discriminate low positive and high positive PPFP values, considering NI individuals in comparison to CH, we found that 82% of chagasic patients and 100% of NI individuals presented high and low or negative values of PPFP, respectively. The good performance of FC-AFEA-IgG is supported by results obtained with the statistical program MedCalc software package 7.3.

Including serum samples from individuals carrying other parasitic diseases our data still suggested that the flow cytometry based methodology has good potential for the diagnosis of Chagas disease, with less cross-reactivity than conventional tests with major endemic tropical diseases in Brazil. The good performance of the test also confers it an advantage for screening purposes in epidemiological studies. However, the limited availability of the technique and its high cost limits its use in epidemiological surveys. Andrade et al. (1992) using conventional serology tests showed that comparison of results provided by blood banks with the reference laboratory's results indicated a relative sensitivity of 77%, which ranged from 50% to 100% depending on the blood bank studied. This data, re-emphasize the limited applicability of this conventional serology for Chagas disease diagnosis purposes. The sensitivity of the diagnostic method depends on multifactorial aspects such as type, source, and purity of the antigen employed as well as the detection system intrinsic to given technology. In this context, our data support the hypothesis that working selectively with membrane antigens associated with the high performance of the flow cytometric fluorescence due to photomultiplier detectors, was probably the major feature responsible for the higher sensitivity of FC-AFEA-IgG. Besides its sensitivity, the capacity of flow cytometry to count 10,000 parasites per assay and the possibility to use intact parasites as source of antigen improved the confidence of this methodology over other techniques.

In order to further evaluate the performance of FC-AFEA-IgG applicable on Chagas disease diagnosis, focusing attention on sensitivity and specificity improvement, we have suggested the possibility of using of FC-AFEA-IgG subclasses as a useful tool to eliminate cross-reactivity in serological diagnosis of Chagas disease, mainly due to the high recognition of fixed T. cruzi epimastigotes by VL samples. Nevertheless, our data demonstrate that, despite a similar PPFP profile

observed for CH along the titration curve, as compared to IgG reactivity, no real gain on the discrimination of CH and VL could be observed. Actually, the use of a more sensitive development system for IgG1 reactivity, based on the use of biotin/PE-labeled-streptavidin, has been shown to increase the cross-reactivity previously observed through IgG along the titration curve. Therefore, no promising dilution could be identified for the use of IgG1 anti-fixed epimastigotes for the purpose of clarifying the cross-reactivity between VL samples when using FC-AFEA. Considering recent findings from our laboratory in regard to Leishmaniasis IgG subclass reactivity (Rocha et al., 2006), the IgG2 subclass has been shown to have higher power to discriminate CH and VL cross reactivity when LCL is the major diagnostic tool. These findings will be further evaluated by our group regarding its applicability for Chagas disease diagnosis. Besides, most of the false-positive cases, correspond to VL individuals could be solved by clinical analysis, since the acute febrile hepatosplenic clinical condition is not compatible with any clinical manifestation of chronic Chagas disease, which would rule out any indication to perform FC-AFEA-IgG for those VL patients.

Analyses of the reactivity of IgG subclasses in human T. cruzi infection remain scarce. As Chagas disease manifests itself in a wide diversity of clinical forms, there is even less information relevant to the possibility that differences in antibody subclass secreted during the course of infection may influence the pathological manifestation. In this context, Cordeiro et al. (2001) showed differential FC-ALTA-IgG1 reactivity between chagasic patients bearing distinct clinical manifestations of chronic Chagas disease. In order to validate the findings of Cordeiro et al. (2001), we performed a blind FC-ALTA-IgG1 study on a new cohort of chagasic patients. Selecting the serum dilution 1/4096 and PPFP = 50% as the cut-off to discriminate IgG1 reactivity by FC-ALTA-IgG1, our data demonstrated that 83% of IND presented high positive PPFP values, confirming the previous report of higher IgG1 reactivity on asymptomatic patients. The analysis of FC-ALTA-IgG1 data from CARD demonstrated that only 38% of them presented low positive PPFP values. Although, these data appear to be inconsistent with those of Cordeiro et al. (2001) CARD patient present different degrees of heart damage, so it is possible that the high PPFP values came from CARD patients with minor electrocardiographic alterations. Another hypothesis is that differential IgG1 reactivity could be a consequence of host-strain-specific features as previously reported for anti-live trypomastigote antibody reactivity by the complement-mediated lysis approach (Zulantay et al., 1998). Those authors demonstrated that the antibody-dependent complement-mediated lysis test has strain dependence on the detection of lytic antibodies in chronic chagasic sera. Western blot analysis confirmed that chagasic samples recognize different antigens depending on the strain used.

As a subsequent aim we attempted to search for a similar profile of reactivity using FC-AFEA-IgG. Our findings demonstrated that 69% of IND showed high positive PPFP values, whereas 55% and 76% of CARD and DIG presented low positive PPFP values, respectively. The FC-AFEA-IgG reactivity profile was similar in the group IND when compared with FC-ALTA-IgG1. Furthermore, low reactivity was observed for CARD and DIG samples, strongly suggesting its applicability for prognosis. We believe that IND individuals with low values of IgG are probably in clinical evolution and, as mentioned above, the higher levels of IgG presented by some CARD and DIG individuals could be relationed with moderate degree of cardiac and digestive damage. The development of longitudinal FC-AFEA-IgG as well as FC-ALTA-IgG1 would further support our hypothesis. Moreover, we are currently focussing the levels of FcγRI, FcγRII and FcγRIII expression by peripheral blood leukocytes from chagasic patients bearing distinct clinical forms to further address the dynamic on immunological interaction at the interface between the cellular and humoral immune response in Chagas diasease.

The treatment of Chagas disease in both acute and recent chronic infections may prevent pathologic effects in the later stages of disease (Ferreira, 1990). The indication of such treatment in the chronic phase is still controversial because most treated patients continue to have positive conventional serology, even though their hemocultures become less frequently positive than those of the untreated, chronically infected patients (Galvão et al., 1993).

Performance analysis demonstrated that FC-AFEA-IgG for cure criterion has a sensitivity (80.3–100) and specificity (85.6–100) of 100% with positive and negative predictive values of 100%. Likelihood ratio (LR) confirmed that FC-AFEA-IgG has an extraordinary performance as a cure criterion after therapeutic intervention in Chagas disease. As proposed by Jaeschke et al. (1994), LR > 10 practically confirms diseases diagnosis and LR < 0.1 practically excludes disease diagnosis. Based on these propositions, our data demonstrated that a low positive PPFP result has little chance of coming from a TNC patient confirming therapeutical effectiveness. On the other hand a high

positive PPFP result has little chance of coming from a TC, confirming therapeutic failure. We considered that despite the good performance of FC-AFEA-IgG at serum dilution 1/16,384, the lower specificity to identify therapeutic failure should rule out this serum dilution for clinical purposes.

We conclude that this flow cytometric approach is a major advance in serological assessments for clinical investigations on Chagas disease. We intend to continue this field of research by focusing our attention on the influence of the degree of heart and mega damage on FC-ALTA-IgG1 and FC-AFEA-IgG reactivity, with appropriate caution regarding the choice of anti-human IgG antibodies.

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References

Amato Neto, V., De Marchi, C.R., Ferreira, C.S., Ferreira, A.W., 2005. Observations on the use of TESA blot for the serological diagnosis of Chagas' disease. Rev. Soc. Bras. Med. Trop. 38, 534.

Andrade, A.L., Martelli, C.M., Luquetti, A.O., de Oliveira, O.S., Almeida e Silva, S., Zicker, F., 1992. Serologic screening for Trypanosoma cruzi among blood donors in central Brazil. Bull. Pan Am. Health Organ. 26, 157.

Bertelli, M.S., Golgher, R.R., Brener, Z., 1977. Intraspecific variation in Trypanosoma cruzi: effect of temperature on the intracellular differentiation in tissue culture. J. Parasitol. 63, 434.

Brener, Z., Chiari, E., 1963. Variações morfológicas observadas em diferentes amostras de Trypanosoma cruzi. Rev. Inst. Med. Trop. 5, 220.

Camargo, E.P., 1964. Growth and differentiation in Trypanosoma cruzi. I. Origin of metacyclic trypanosomes in liquid media. Rev. Inst. Med. Trop. 6, 93.

Camargo, M.E., Segura, E.L., Kagan, I.G., Souza, J.M., Carvalheiro, J.R., Yanovsky, J.F., Guimarães, M.C., 1986. Three years of collaboration on the standardization of Chagas' disease in the Americas: an appraisal. PAHO Bull. 20, 233.

Carvalho, M.R., Krieger, M.A., Almeida, E., Oeleman, W., et al., 1993. Chagas' disease diagnosis: evaluation of several tests in blood bank screening. Transfusion 33, 830.

Cordeiro, F.D., Martins-Filho, O.A., Da Costa Rocha, M.O., Adad, S.J., Correa-Oliveira, R., Romanha, A.J., 2001. Anti-Trypanosoma cruzi immunoglobulin G1 can be a useful tool for diagnosis and prognosis of human Chagas' disease. Clin. Diagn. Lab. Immunol. 8, 112.

Ferreira, H.O., 1990. Treatment of the undetermined form of Chagas disease with nifortimox and benzonidazole. Rev. Soc. Bras. Med. Trop. 23, 209.

Galvão, L.M., Nunes, R.M., Cancado, J.R., Brener, Z., Krettli, A.U., 1993. Lytic antibody titre as a means of assessing cure after treatment of Chagas disease: a 10 years follow-up study. Trans. R. Soc. Trop. Med. Hyg. 87, 220.

Jaeschke, R., Guyatt, G.H., Sackett, D.L., 1994. Users' guides to the medical literature. III. How to use an article about a diagnostic test. B. What are the results and will they help me in caring for my patients? The Evidence-Based Medicine Working Group. JAMA 271, 703.

Martins-Filho, O.A., Pereira, M.E.S., Carvalho, J.F., Cançado, J.R., Brener, Z., 1995. Flow cytometry, a new approach to detect anti-live trypomastigote antibodies and monitor the efficacy of specific treatment in human Chagas' disease. Clin. Diagn. Lab. Immunol. 2, 569.

Martins-Filho, O.A., Eloi-Santos, S.M., Carvalho, A.T., Oliveira, R.C., Rassi, A., Luquetti, A.O., Rassi, G.G., Brener, Z., 2002. Double-blind study to evaluate flow cytometry analysis of anti-live trypomastigote antibodies for monitoring treatment efficacy in cases of human Chagas' disease. Clin. Diagn. Lab. Immunol. 9, 1107.

Moncayo, A., 2003. Chagas disease: current epidemiological trends after the interruption of vectorial and transfusional transmission in the Southern Cone countries. Mem. Inst. Oswaldo Cruz 98, 577.

Portela-Lindoso, A.A., Shikanai-Yasuda, M.A., 2003. Chronic Chagas' disease: from xenodiagnosis and hemoculture to polymerase chain reaction. Rev. Saude Publica 37, 107.

Rocha, R.D., Gontijo, C.M., Eloi-Santos, S.M., Teixeira-Carvalho, A., Correa-Oliveira, R., Ferrari, T.C.A., Marques, M.J., Mayrink, W., Martins-Filho, O.A., 2006. Clinical value of anti-live Leishmania (Viannia) braziliensis immunoglobulin G subclasses, detected by flow cytometry, for diagnosing active localized cutaneous leishmaniasis. Trop. Med. Int. Health 11, 156.

Swets, J.A., 1988. Measuring the accuracy of diagnostic systems. Science 240, 1285.

Umezawa, E.S., Bastos, S.F., Coura, J.R., Levin, M.J., Gonzalez, A., Rangel-Aldao, R., Zingales, B., Luquetti, A.O., da Silveira, J.F., 2003. An improved serodiagnostic test for Chagas' disease employing a mixture of Trypanosoma cruzi recombinant antigens. Transfusion 43, 91.

World Health Organization, 2002. Control of Chagas disease. WHO Technical Report Series, vol. 905, p. 109.

Youden, W.J., 1950. Index for rating diagnostic tests. Cancer 3, 32.

Zulantay, I., Venegas, J., Apt, W., Solari, A., Sanchez, G., 1998. Lytic antibodies in Trypanosoma cruzi-infected persons with low parasitemia. Am. J. Trop. Med. Hyg. 58, 775.

5. CONSIDERAÇÕES FINAIS

Diversos estudos que avaliam os mecanismos imunológicos associados à manifestação de diferentes formas clínicas têm sugerido eventos multifatorais e a combinação de parâmetros imunológicos diferentes, dando suporte à hipótese de que mecanismos complexos estão envolvidos na indução e/ou regulação da patogenia da doença de Chagas. Embora alguns dos elementos envolvidos no fenômeno de cronificação diferenciada na doença de Chagas já tenham sido descritos, estudos adicionais fazem-se ainda necessários para a compreensão da dinâmica desse processo. O estudo da participação de diferentes populações celulares nos eventos protetores e/ou patológicos da doença chamam a atenção para o envolvimento de ambos compartimentos da resposta imune, o celular e o humoral. Nesse contexto, a detecção de moléculas expressas na superfície de células, bem como de imunoglobulinas ligantes de alvos antigênicos, via reação de imunofluorescência indireta, através da citometria de fluxo, têm permitindo um grande avanço na pesquisa científica aplicada no estudo de doenças infectoparasitárias humanas. Empregando sistemas de análises mais elaborados e protocolos experimentais mais atuais, torna-se possível ainda a análise simultânea de um maior número de moléculas por superfície celular, trazendo a possibilidade de identificação e/ou caracterização de novas sub-populações, oferecendo assim, informações adicionais que podem enriquecer os conhecimentos acerca das diferentes formas clínicas da doença de Chagas.

O estudo de forma descritivo-analítica do perfil imuno fenotípico de leucócitos circulantes indivíduos portadores das formas clínicas indeterminada, cardíaca e digestiva, exposto no ARTIGO 1, documentou pela primeira vez que indivíduos portadores da forma clínica indeterminada apresentam freqüências aumentadas de células T reguladoras $CD4^{+}CD25^{high}$ e NKT associadas com níveis aumentados de células NK circulantes ($CD3^{-}CD16^{+}CD56^{+}$ e $CD3^{-}CD16^{+}CD56^{dim}$). Por outro lado, foi demonstrado um percentual aumentado de células $CD8^{+}HLA\text{-}DR^{+}$ exclusivamente relacionado com as formas graves da

doença de Chagas. Nossos achados dão suporte à hipótese de que populações de células reguladoras são capazes de controlar a atividade citotóxica deletéria na forma clínica indeterminada pela inibição da ativação de células T-$CD8^+HLA\text{-}DR^+$. Já a ausência de células T reguladoras em pacientes CARD e DIG pode contribuir para uma resposta imune deletéria que culmina em forte atividade citotóxica e dano tecidual. Em conjunto nossos dados corroboram com a hipótese de que uma forte ativação de células $CD8^+HLA\text{-}DR^+$, na ausência de mecanismos imunomoduladores, pode resultar em dano tecidual levando ao desenvolvimento de cardiomiopatia e/ou megas do cólon e esôfago. Contrariamente, a habilidade de montar uma resposta citotóxica mediada por células NK, modulada por células T reguladoras, parece apresentar papel fundamental na montagem de resposta inflamatória efetiva e não deletéria.

Considerando o ARTIGO 2, que abordou o estudo descritivo do perfil imuno fenotípico de leucócitos de crianças portadoras da forma clínica indeterminada recente, os resultados indicaram alterações significativas, incluindo valores aumentados de células pré-NK e monócitos pró-inflamatórios ($CD14^+CD16^+HLA\text{-}DR^{++}$) quando comparados com crianças não infectadas. Valores aumentados de linfócitos B ativados ($CD19^+CD23^+$) contrastaram com a deficiência de células T ativadas, indicada pelos reduzidos valores de $CD4^+CD38^+$, $CD4^+HLA\text{-}DR^+$, $CD8^+CD38^+$, $CD8^+HLA\text{-}DR^+$ e células T reguladoras $CD4^+CD25^{high}$. Análise comparativa transversal entre E-IND, IND e CARD ainda sugeriu que mudança em direção a altos valores de "macrófagos-like" e níveis basais de monócitos pró-inflamatórios além de elevados valores de células NK maduras, NKT e $CD4^+CD25^{high}$ podem resultar na forma clínica IND. Por outro lado, altos níveis de células $CD8^+HLA\text{-}DR^+$ paralelo a freqüência basal de células NK maduras, NKT e $CD4^+CD25^{high}$ parece estar relacionado ao desenvolvimento de uma fase crônica tardia associada com eventos patológicos no coração.

No ARTIGO 3 descrevemos uma nova estratégia para obtenção do balanço de citocinas produzidas por subpopulações de leucócitos da imunidade inata e adaptativa do sangue periférico, partindo do conceito previamente descrito de indivíduos baixo e alto produtores de citocinas (BAHIA-OLIVEIRA et al., 2000), para caracterizar o perfil de citocinas global. A realização de tal trabalho justifica-se uma vez que uma desregulação do sistema imune está envolvida na patogênese da doença de Chagas e as diferentes formas clínicas são resultantes de distúrbios múltiplos de mecanismos imunes como demonstrado anteriormente pelo estudo do perfil imuno fenotípico. Acreditamos que estratégias convencionais de análise da produção de citocinas podem não alcançar a representação global, podendo não refletir o perfil panorâmico de citocinas. A ação das citocinas é pleiotrópica e redundante, sendo importante analisar de forma cuidadosa as mudanças no micro ambiente de citocinas na tentativa de obter o perfil sistêmico. A quantificação de diferentes citocinas com efeitos reguladores ou inflamatórios produzidos por leucócitos, pode gerar uma melhor caracterização do ambiente sistêmico de indivíduos portadores das diferentes formas clínicas da doença de Chagas. Nossos resultados demonstraram um predomínio de um padrão de citocinas reguladoras em IND contrário ao perfil predominante de citocinas inflamatórias em CARD. Mas um dos pontos mais relevantes dessa investigação foi a oportunidade de analisar o padrão de citocinas dominantes em gráficos de radar, uma vez que esse gráfico permite a construção de uma rede organizada de citocinas e o melhor entendimento do circuito complexo de citocinas produzidas por uma variedade de células da resposta imune. Usando esse gráfico foi simples detectar não apenas a magnitude de cada categoria de citocinas – ilustrada pela área central do polígono, obtida com a conexão dos pontos de cada eixo, que representam as diferentes populações celulares avaliadas – mas também identificar as principais fontes que contribuíram para o balanço global de citocinas. Usando essa estratégia observamos que o predomínio de um padrão de citocinas reguladoras em IND era devido

principalmente aos linfócitos totais e T-CD4$^+$, as principais fontes de citocinas reguladoras. Além disso, uma mudança de monócitos e linfócitos totais em direção a produção de citocinas inflamatórias foi a principal causa do reduzido predomínio do padrão de citocinas reguladoras em CARD.

Nossos resultados demonstraram que leucócitos do sangue periférico de pacientes portadores de diferentes formas clínicas apresentam diferentes perfis de citocinas, sendo os achados mais relevantes em CARD o aumento da freqüência de monócitos produtores de TNF-α^+ e a menor freqüência de TCD8$^+$IL-10$^+$. Por outro lado, no grupo IND foram observadas elevadas freqüências de linfócitos totais IL-4$^+$, IL-10$^+$ e IL-13$^+$, além de um perfil misto de citocinas reguladoras e inflamatórias em neutrófilos e células NK.

Uma vez definido o panorama global do perfil de citocinas para NI, IND e CARD, uma relevante questão foi formulada: "O antígeno solúvel de formas tripomastigotas do T. cruzi possui impacto diferencial no perfil panorâmico de citocinas em indivíduos portadores das formas clínicas IND e CARD?". É importante chamar a atenção que estudos anteriores do perfil de citocinas produzidas por indivíduos portadores da doença de Chagas foram realizados in vitro frente à estimulação com antígenos de formas epimastigotas do T. cruzi. Portanto, a condição experimental escolhida para essa investigação poderia dar informações relevantes em relação a resposta imune gerada frente a maciça liberação de antígenos que ocorre in vivo em conseqüência da liberação de antígenos de ninhos de parasitas ou a descarga substancial de antígenos derivados do parasito que ocorre frente ao tratamento etiológico. Um achado fascinante foi que o antígeno de T. cruzi in vitro foi capaz de estimular relevante produção de citocinas, levando a uma menor freqüência de indivíduos baixo produtores de citocinas em todas as formas clínicas avaliadas. Além disso, a estimulação maciça com antígenos solúveis do T. cruzi foi capaz de transformar o balanço de citocinas mudando a direção de um perfil inflamatório prejudicial observado em CARD para um perfil de citocinas

predominantemente regulador. Ainda, dados preliminares demonstraram que o tratamento etiológico leva à diminuição da freqüência de IL-$10^{+}CD4^{+}$ em IND, enquanto freqüência aumentada de IL-$10^{+}CD14^{+}$ pode ser observado em CARD, corroborando com a hipótese de que o perfil de citocinas produzidas por leucócitos de indivíduos chagásicos não tratados sob a estimulação antigênica especifica, possa reproduzir a síntese de citocinas direcionadas in vivo frente o tratamento etiológico.

O panorama do perfil global de citocinas de leucócitos do sangue periférico ofereceu evidências adicionais relacionadas a eventos relevantes para estudos da doença de Chagas. Essa estratégia forneceu um melhor entendimento das complexidades que controlam o curso da doença de Chagas crônica e uma ferramenta útil para predizer as respostas imunes que surgem após intervenção terapêutica.

Além de questões no campo do estabelecimento/manutenção das diferentes formas clínicas da doença de Chagas investigadas por nosso grupo a partir de análise imuno fenotípicas e do padrão de produção de citocinas intracitoplasmáticas, com destaque para o perfil panorâmico de citocinas, existem ainda questões relacionadas ao diagnóstico, prognóstico e critério de cura pós-terapêutica na doença de Chagas que merecem destaque nos estudos da imunidade humoral, o que nos conduziu a investigar no ARTIGO 4 aspectos inerentes ao perfil de imunoglobulinas anti-T. cruzi e sua aplicabilidade em estudos clínicos da doença de Chagas. Embora os métodos convencionais sejam os mais amplamente usados na rotina laboratorial, metodologias alternativas apresentam-se como importantes ferramentas que visam preencher lacunas importantes não contempladas pelas técnicas convencionais. A sorologia por citometria de fluxo têm se tornado um método seguro e poderoso para emprego em investigações clínicas. Portanto, na área de prognóstico e avaliação do tratamento, marcadores não parasitológicos podem ser empregados como preditores de morbidade e da eficácia do tratamento e do risco-benefício da quimioterapia podendo ser avaliado nas

diferentes fases da doença de Chagas. Além disso, essa metodologia é altamente sensível e pode ser aplicada como uma metodologia de sorologia não convencional para diagnóstico. Nossos resultados obtidos a partir de estudo duplo-cego re-enfatizam a aplicabilidade da citometria de fluxo para detectar anticorpos anti-T. cruzi no soro de humanos para diagnóstico, bem como, prognóstico e critério de cura.

A detecção de anticorpos anti-formas tripomastigotas vivas do T. cruzi por citometria de fluxo tem demonstrando potencial como critério de cura (MARTINS-FILHO et al., 1995; 2002) e para fins prognósticos (CORDEIRO et al., 2001). Entretanto, devido às limitações de se trabalhar com a forma tripomastigota viva do T. cruzi, forma infectante e de cultivo que requer profissionais esclarecidos em cultivo de células de tecido, surgiu o interesse de avaliar o perfil de reatividade de soros de indivíduos portadores da doença de Chagas tratados ou não anti-formas epimastigotas fixadas do T. cruzi, técnica denominada FC-AFEA–"Flow Cytometry – Anti-Fixed Epimastigotes IgG Antibodies". As vantagens de se trabalhar com a forma epimastigota do T. cruzi são a facilidade do cultivo que é acelular, o fato dessa forma não ser infectante e, além disso, trabalhar com a forma epimastigota fixada permite o preparo de um volume grande de parasitos que podem ser empregados em uma grande amostragem de soros.

Para fins diagnósticos, observamos que a FC-AFEA apresenta bom potencial, com menos reatividade cruzada que os testes convencionais quando empregadas amostras das principais doenças endêmicas do Brasil: leishmanioses, malária, toxoplasmose e esquistossomose. Acreditamos que a maior sensibilidade do teste está relacionada a detecção seletiva de antígenos de superfície, associada com a alta performance da citometria de fluxo.

CORDEIRO e colaboradores (2001) demonstraram reatividade diferencial de anticorpos anti-formas tripomastigotas vivas do T. cruzi em indivíduos portadores das diferentes formas clínicas. Realizamos esse ensaio em uma nova sub-coorte com o intuito de

validar esse achado e testar a aplicabilidade da FC-AFEA. A maioria dos indivíduos IND apresentou uma elevada reatividade de anticorpos (83%) anti-formas tripomastigotas vivas. Esperávamos detectar baixos títulos de anticorpos no grupo CARD, mas apenas 38% dos indivíduos CARD ficaram em uma região de baixa reatividade, o que nos fez acreditar que a maioria do grupo CARD apresentava a forma mais branda da doença cardíaca. Já a análise por FC-AFEA demonstrou que 69% dos IND apresentaram alta reatividade enquanto 55% dos CARD apresentaram baixa reatividade. Esses resultados sugerem a aplicabilidade da FC-AFEA como exame adicional para fins prognósticos. Finalmente, a análise da performance da FC-AFEA para fins de critério de cura mostrou valores de sensibilidade e especificidade de 100%.

Apesar dos nossos achados corroborarem com relatos prévios de perfis distintos de anticorpos IgG anti-T. cruzi entre IND e CARD, eles indicaram que marcadores da resposta imune humoral são sem dúvida mais aplicáveis para diagnóstico sorológico e monitoração de critério de cura. Concluímos então que a citometria de fluxo trouxe avanços na sorologia para investigações clínicas e nosso grupo pretende dar continuidade a esse campo de investigação com foco na definição de graus diferentes de comprometimento cardíaco e digestivo, assim como na escolha de anticorpos anti-IgG. Além disso, nossa idéia é associar a análise da reatividade de IgG pela FC-AFEA, à técnica FC-AFPA (Flow Cytometry – Anti-Fixed Promastigote IgG Antibodies), a qual contribuiria para a exclusão de indivíduos portadores de leishmaniose tegumentar e visceral, tendo uma importante aplicabilidade em hemocentros como uma técnica confirmatória para o esclarecimento das situações diagnósticas não-negativas (inconclusivas) e indeterminadas (zona cinza) para doença de Chagas, que constituem uma realidade dispendiosa. Por empregar três preparações antigênicas diferentes (T. cruzi, Leishmania amazonensis e Leishmania chagasi) essa metodologia será denominada “triplex”.

Em suma, nossos dados demonstram que mais que uma mudança em direção a um padrão polarizado, um fino balanço é relevante para direcionar os mecanismos imuno-mediados essenciais para a definição da doença de Chagas. Com base nos trabalhos gerados, podemos constatar a complexidade dos fatores envolvidos na evolução da doença de Chagas. Embora muitos conhecimentos tenham sido adquiridos em relação à resposta imune de pacientes chagásicos, importantes aspectos ainda não foram esclarecidos. Estudos adicionais para caracterizar funcionalmente as populações celulares que demonstraram papel crucial no estabelecimento/manutenção das diferentes formas clínicas da doença de Chagas são de grande interesse do nosso grupo. Esses estudos poderão fornecer um melhor entendimento das complexidades imunológicas que controlam a gravidade da doença em indivíduos indeterminados. Além disso, o entendimento dos mecanismos envolvidos no controle da replicação do parasito e no desenvolvimento da doença cardíaca e digestiva poderá contribuir para a área de tratamento e prevenção das formas sintomáticas da doença de Chagas. Acreditamos que o aprofundamento do estudo da resposta imune nas diferentes formas clínicas da doença de Chagas é importante e poderá direcionar a busca de novas terapias preventivas e curativas, beneficiando a população infectada ou em risco de infecção. Entretanto, é possível que mecanismos imunológicos envolvidos na interação conjunta de células do sistema imune, além da susceptibilidade genética diferencial do hospedeiro, gerem uma patologia altamente complexa, impondo dificuldades para o desenvolvimento de vacinas e imunoterapias eficientes. O desenvolvimento de estratégias terapêuticas visando a regulação da funcionalidade celular e da modulação de componentes inflamatórios, associado às drogas anti-parasitárias, seriam alvos importantes no tratamento da doença de Chagas e na busca do delicado balanço entre proteção versus doença.

6. REFERÊNCIAS BIBLIOGRÁFICAS

ANDRADE AL, MARTELLI CM, OLIVEIRA RM, SILVA SA, AIRES AI, SOUSSUMI LM, COVAS DT, SILVA LS, ANDRADE JG, TRAVASSOS LR, ALMEIDA IC. Short report: Benznidazole efficacy among Trypanosoma cruzi-infected adolescents after a six-year follow-up. Am J Trop Med Hyg 71: 594-597, 2004.

ANDRADE ZA. Pathogenesis of Chagas' disease. Res Immunol. Feb;142(2):126-9, 1991. Review.

ARANTES JM, PEDROSA ML, MARTINS HR, VELOSO VM, DE LANA M, BAHIA MT, TAFURI WL, CARNEIRO CM. Trypanosoma cruzi: treatment with the iron chelator desferrioxamine reduces parasitemia and mortality in experimentally infected mice. Exp Parasitol. Sep;117(1):43-50. Epub 2007 Mar 24, 2007.

ARAUJO FF, GOMES JA, ROCHA MO, WILLIAMS-BLANGERO S, PINHEIRO VM, MORATO MJ, CORREA-OLIVEIRA R. Potential role of CD4+CD25HIGH regulatory T cells in morbidity in Chagas disease. Front Biosci. May 1;12:2797-806, 2007.

BAECHER-ALLAN C., BROWN J.A., FREEMAN G.J., HAFLER D.A. $CD4^{+}CD25^{high}$ regulatory cells in human peripheral blood. Immunol. Aug 1;167(3):1245-53, 2001.

BAHIA-OLIVEIRA LM, GOMES JA, CANÇADO JR, FERRARI TC, LEMOS EM, LUZ ZM, MOREIRA MC, GAZZINELLI G, CORREA-OLIVEIRA R. Immunological and clinical evaluation of chagasic patients subjected to chemotherapy during the acute phase of Trypanosoma cruzi infection 14–30 years ago. J. Infect. Dis. 182, 634–638, 2000.

BELGE KU, DAYYANI F, HORELT A, SIEDLAR M, FRANKENBERGER M, FRANKENBERGER B, ESPEVIK T, ZIEGLER-HEITBROCK L. The proinflammatory $CD14^{+}CD16^{+}DR^{++}$ monocytes are a major source of TNF. J Immunol. 168(7):3536-42, 2002.

BELKAID Y, ROUSE BT. Natural regulatory T cells in infectious disease. Nat Immunol. Apr;6(4):353-60, 2005.

BERTELLI MS, GOLGHER RR, BRENER Z. Intraspecific variation in Trypanosoma cruzi: effect of temperature on the intracellular differentiation in tissue culture. J Parasitol. Jun;63(3):434-7, 1977.

BLUESTONE JA, ABBAS AK. Natural versus adaptive regulatory T cells. Nat Rev Immunol. Mar;3(3):253-7, 2003.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. A a Z: Glossário de Doenças. Doença de Chagas. Informações Técnicas. Disponível em Portal da Saúde: < http://portal.saude.gov.br/portal/saude/visualizar_texto.cfm?idtxt=27876 >. Acesso em: 26 fev. 2008.

BRASIL. Ministério da Saúde. Secretaria Nacional de Vigilância em Saúde. Sistema de Notificação de Agravos de Notificação (SINAN) (2004) Doença de Chagas Aguda: Manual prático de subsídio à notificação obrigatória no SINAN. Brasília: MS/SVS. 20 p. Disponível em: <http://bvsms.saude.gov.br/bvs/publicacoes/manual_chagas.pdf> Acesso em: 26 fev. 2008.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde (2005a) Consenso Brasileiro em Doença de Chagas. Revista da Sociedade Brasileira de Medicina Tropical. 38(Suplemento III): 7-29.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde (2005b) Guia de Vigilância Epidemiológica. 6 ed. Brasília: Ministério da Saúde. 816 p. (Série A. Normas e Manuais Técnicos).

BRENER Z. Biology of Trypanosoma cruzi. Annu Rev Microbiol. 27:347-82, 1973.

BRENER Z. Immunity to Trypanosoma cruzi.Adv Parasitol. 18:247-92, 1980.

BRENER Z, GAZZINELLI R.T. Immunological control of Trypanosoma cruzi infection and pathogenesis of Chagas'disease. Int Arch Allergy Immunol. 114(2):103-10, 1997.

CAMARGO EP. Growth and differentiation in Trypanosoma cruzi. I. Origin of metacyclic trypanosomes in liquid media. Rev Inst Med Trop Sao Paulo. May-Jun;12:93-100, 1964.

CAMPBELL DA, WESTENBERGER SJ, STURM NR. The determinants of Chagas disease: connecting parasite and host genetics. Curr Mol Med. Sep;4(6):549-62, 2004.

CAMPOS MA, ALMEIDA IC, TAKEUCHI O, AKIRA S, VALENTE EP, PROCOPIO DO, TRAVASSOS LR, SMITH JA, GOLENBOCK DT, GAZZINELLI RT. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol. 167(1):416-23, 2001.

CARROCK SEWELL, WA, NORTH, ME; WEBSTER, AD; FARRANT, J. Determination of intracellular cytokines by flow cytometric following whole-blood culture. J Immunol Methods. 206-67, 1997.

CASSIS L, AIELLO S, NORIS M. Natural versus adaptive regulatory T cells. Contrib Nephrol.;146:121-31, 2005. Review.

CENTERS FOR DISEASE CONTROL AND PREVENTION (2006) Chagas disease after organ transplantation - Los Angeles, California, 2006. MMWR Morb Mortal Wkly Rep 55: 798-800. Disponível em: <http://www.cdc.gov/MMWR/preview/mmwrhtml/mm5529a3.htm>. Acesso em: 26 fev. 2008.

CHAGAS C. Nova espécie mórbida do homem produzida por um Trypanosoma (Trypanosoma cruzi): Nota prévia. Brasil Médico. 23: 161, 1909.

CONSENSO BRASILEIRO EM DOENÇA DE CHAGAS. Secretaria de Vigilância em Saúde do Ministério da Saúde. Revista da Sociedade Brasileira de Medicina Tropical. Vol. 38: Suplemento III, 2005.

COOPER MA, FEHNIGER TA, TURNER SC, CHEN KS, GHAHERI BA, GHAYUR T, CARSON WE, CALIGIURI MA. Human natural killer cells: a unique innate immunoregulatory role for the CD56 (bright) subset. Blood. 97(10):3146-51, 2001.

CORDEIRO FD, MARTINS-FILHO OA, DA COSTA ROCHA MO, ADAD SJ, CORRÊA-OLIVEIRA R, ROMANHA AJ. Anti-Trypanosoma cruzi IgG1 can be a useful tool for diagnosis and prognosis of human Chagas disease. Clin. Diag. Lab.Immunol. 8(1):112-8, 2001.

CORRÊA-OLIVEIRA R, GOMES J, LEMOS EM, CARDOSO GM, REIS DD, ADAD S, CREMA E, MARTINS-FILHO OA, COSTA MO, GAZZINELLI G, BAHIA-OLIVEIRA LM. The role of the immune response on the development of severe clinical forms of human Chagas disease. Mem Inst Oswaldo Cruz. 94 Suppl 1:253-5, 1999.

COURA JR. Transmissão da infecção chagásica por via oral na história natural da doença de Chagas. Rev Soc Bras Med Trop 39 (Supl. IV): 113-117, 2006.

DA SILVEIRA AB, ADAD SJ, CORREA-OLIVEIRA R, FURNESS JB, D'AVILA REIS D. Morphometric study of eosinophils, mast cells, macrophages and fibrosis in the colon of chronic chagasic patients with and without megacolon. Parasitology. 2007 Jun;134(Pt 6):789-96. Epub Feb 9, 2007.

DA SILVEIRA AB, LEMOS EM, ADAD SJ, CORREA-OLIVEIRA R, FURNESS JB, D'AVILA REIS D. Megacolon in Chagas disease: a study of inflammatory cells, enteric nerves, and glial cells. Hum Pathol. Aug;38(8):1256-64. Epub 2007 May 8, 2007.

DE CASTRO AM, LUQUETTI AO, RASSI A, CHIARI E, GALVAO LM. Detection of parasitemia profiles by blood culture after treatment of chronic Trypanosoma cruzi infection. Parasitol. Res 99: 379-383, 2006.

DIAS J.C. The indeterminate form of human chronic Chagas disease. A cinical epidemiological review. Ver. Soc. Bras. Med. Trop. 22: 147-156, 1989.

DIAS J.C.P. Epidemiologia. In: Brener Z, Andrade ZA, Barral-Neto M. Trypanosoma cruzi e doença de Chagas. Rio de Janeiro: Guanabara Koogan. P.48-74, 2000.

DIAS J.C.P. Globalização, iniquidade e doença de Chagas. Caderno de Saúde Pública, Rio de Janeiro, 23 Sup 1:S13-S22, 2007.

DOHERTY DG, NORRIS S, MADRIGAL-ESTEBAS L, MCENTEE G, TRAYNOR O, HEGARTY JE, O'FARRELLY C. The human liver contains multiple populations of NK

cells, T cells, and $CD3^+CD56^+$ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. J Immunol. Aug 15;163(4):2314-21, 1999.

DUTHIE MS, KAHN M, WHITE M, KAPUR RP, KAHN SJ. Critical proinflammatory and antiinflammatory functions of different subsets of CD1d-restricted natural killer T cells during Trypanosoma cruzi infection. Infect Immun. Jan;73(1):181-92, 2005.

DUTRA W D, MARTINS-FILHO OA, CANCADO JR, PINTO-DIAS JC, BRENER Z, FREEMAN JUNIOR GL, COLLEY DG, GAZZINELLI G, PARRA JC. Activated T and B lymphocytes in peripheral blood of patients with Chagas' disease. Int Immunol. 6(4):499-506, 1994.

DUTRA WO, COLLEY DG, PINTO-DIAS JC, GAZZINELLI G, BRENER Z, PEREIRA ME, COFFMAN RL, CORREA-OLIVEIRA R, CARVALHO-PARRA JF. Self and nonself stimulatory molecules induce preferential expansion of CD5+ B cells or activated T cells of chagasic patients, respectively. Scand J Immunol. Jan;51(1):91-7, 2000.

EMOTO M, MIYAMOTO M, EMOTO Y, YOSHIZAWA I, BRINKMANN V, VAN ROOIJEN N, KAUFMANN SH. Highly biased type 1 immune responses in mice deficient in LFA-1 in Listeria monocytogenes infection are caused by elevated IL-12 production by granulocytes. J Immunol. Oct 15;171(8):3970-6, 2003.

FUENMAYOR C, HIGUCHI ML, CARRASCO H, PARADA H, GUTIERREZ P, AIELLO V, PALOMINO S. Acute Chagas' disease: immunohistochemical characteristics of T cell infiltrate and its relationship with T. cruzi parasitic antigens. Acta Cardiol., 60:33–7, 2005.

GADDY J, BROXMEYER HE. Cord blood $CD16^+56^-$ cells with low lytic activity are possible precursors of mature natural killer cells. Cell Immunol. 180:132-142, 1997.

GALVAO LM, NUNES RM, CANCADO JR, BRENER Z, KRETTLI AU. Lytic antibody titre as a means of assessing cure after treatment of Chagas disease: a 10 years follow-up study. Trans R Soc Trop Med Hyg. 87(2):220-3, 1993.

GARCIA E.S., AZAMBUJA P. Development and interactions of Trypanosoma cruzi within the insect vector. Parasitol Today. 7: 240-244, 1991.

GAVIN MA, CLARKE SR, NEGROU E, GALLEGOS A, RUDENSKY A. Homeostasis and anergy of $CD4(^+)CD25(^+)$ suppressor T cells in vivo. Nat Immunol. 2002 Jan;3(1):33-41. Epub Dec 10, 2001.

GODFREY DI, HAMMOND KJ, POULTON LD, SMYTH MJ, BAXTER AG. NKT cells: facts, functions and fallacies. Immunol Today. Nov;21(11):573-83, 2000. Review.

GODFREY DI, KRONENBERG M. Going both ways: immune regulation via CD1d-dependent NKT cells. J Clin Invest. Nov;114(10):1379-88, 2004. Review.

GOLGHER D, GAZZINELLI RT. Innate and acquired immunity in the pathogenesis of Chagas disease. Autoimmunity. Aug;37(5):399-409, 2004.

GOMES JA, BAHIA-OLIVEIRA LM, ROCHA MO, MARTINS-FILHO OA, GAZZINELLI G, CORREA-OLIVEIRA R. Evidence that development of severe cardiomyopathy in human Chagas' disease is due to a Th1-specific immune response. Infect Immun. 71(3):1185-93, 2003.

GROSSMAN WJ, VERBSKY JW, BARCHET W, COLONNA M, ATKINSON JP, LEY TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity. Oct;21(4):589-601, 2004.

GUIA DE VIGILÂNCIA EPIDEMIOLÓGICA / Ministério da Saúde, Secretaria de Vigilância em Saúde – 6 ed. – Brasília: Ministério da Saúde, 2005. 816 p – (Série A. Normas e Manuais Técnicos).

GÜRTLER RE, SEGURA EL, COHEN JE. Congenital transmission of Trypanosoma cruzi infection in Argentina. Emerg Infect Dis 9: 29-32, 2003.

HADDAD,N. & GODOY, R. A. Valor da medida do tempo e trânsito esofágico como meio de diagnóstico precoce da disperistalse do esôfago em indivíuos chagásicos. Ver. Goiana Med. 9: 45-60, 1963.

HAMMOND KJ, KRONENBERG M. Natural killer T cells: natural or unnatural regulators of autoimmunity? Curr Opin Immunol. Dec;15(6):683-9, 2003. Review.

HIGUCHI ML, GUTIERREZ PS, AIELLO VD, PALOMINO S, BOCCHI E, KALIL J, BELLOTTI G, PILEGGI F. Immunohistochemical characterization of infiltrating cells in human chronic chagasic myocarditis: comparison with myocardial rejection process. Virchows Arch A Pathol Anat Histopathol. 423(3):157-60, 1993a.

HIGUCHI ML, BRITO T, REIS MM, BARBOSA A, BELLOTTI G, PEREIRA-BARRETO, PILEGGI F. Correlation between Trypanosoma cruzi parasitism and mtocardial inflammatory infiltrate in human chronic chagasic myocarditis: Light microscopy and immunohistochemical findings Cardiovasc Pathol. 2:101-106, 1993b.

JAESCHKE R, GUYATT GH, SACKETT DL. Users' guides to the medical literature. III. How to use an article about a diagnostic test. B. What are the results and will they help me in caring for my patients? The Evidence-Based Medicine Working Group. JAMA. Mar 2;271(9):703-7, 1994.

KOEBERLE F. & NADOR E. Etiologia e patogenia do megaesôfago no Brasil. Rev. Paulista Med. 47: 643-661, 1955.

KOJO S, TSUTSUMI A, GOTO D, SUMIDA T. Low expression levels of soluble CD1d gene in patients with rheumatoid arthritis. J Rheumatol. Dec;30(12):2524-8, 2003.

KRETTLI AU, BRENER Z. Resistance against Trypanosoma cruzi associated to anti-living trypomastigote antibodies. J Immunol. May;128(5):2009-12, 1982.

KRONENBERG M, GAPIN L. The unconventional lifestyle of NKT cells. Nat Rev Immunol. Aug;2(8):557-68, 2002. Review.

LARANJA F.S. Aspectos clínicos da moléstia de Chagas. Rev. Bras. Med. 10: 482, 1953.

LAUZON NM, MIAN F, MACKENZIE R, ASHKAR AA. The direct effects of Toll-like receptor ligands on human NK cell cytokine production and cytotoxicity. Cell Immunol. 241:102-12, 2006

LEITE-DE-MORAES MC, HERBELIN A, GOMBERT JM, VICARI A, PAPIERNIK M, DY M. Requirement of IL-7 for IL-4-producing potential of MHC class I-selected CD4-CD8-TCR alpha beta+ thymocytes. Int Immunol. Jan;9(1):73-9, 1997.

LEMOS EM, REIS D, ADAD SJ, SILVA GC, CREMA E, CORREA-OLIVEIRA R. Decreased CD4(+) circulating T lymphocytes in patients with gastrointestinal chagas disease. Clin Immunol Immunopathol. Aug;88(2):150-5, 1998.

LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. Nov;193(1):265-75, 1951.

MAGUIRE JH. Chagas' disease—Can we stop the deaths? N Engl J Med 355: 760-761, 2006.

MALOY KJ, POWRIE F. Regulatory T cells in the control of immune pathology. Nat Immunol. Sep;2(9):816-22, 2001.

MARINHO CR, D'IMPÉRIO LIMA MR, GRISOTTO MG, ALVAREZ JM. Influence of acute-phase parasite load on pathology, parasitism, and activation of the immune system at the late chronic phase of Chagas' disease. Infect Immun. Jan;67(1):308-18, 1999.

MARTINS-FILHO OA, PEREIRA ME, CARVALHO JF, CANÇADO JR, BRENER Z. Flow cytometry, a new approach to detect anti-live trypomastigote antibodies and monitor the efficacy of specific treatment in human Chagas' disease. Clin. Diagn. Lab. Immunol. 2: 569-573, 1995.

MARTINS-FILHO OA Course on flow cytometry. XXV Meeting of the Brazilian Society of Immunology. Florianópolis, SC, Brazil. August 11th-12th, 2000.

MARTINS-FILHO OA, ELOI-SANTOS SM, TEIXEIRA CARVALHO A, OLIVEIRA RC, RASSI A, LUQUETTI AO, RASSI GG, BRENER Z. Double-blind study to evaluate flow

cytometry analysis of anti-live trypomastigote antibodies for monitoring treatment efficacy in cases of human Chagas' disease. Clin Diagn Lab Immunol. 9(5):1107-13, 2002.

MATSUDA JL, GAPIN L, BARON JL, SIDOBRE S, STETSON DB, MOHRS M, LOCKSLEY RM, KRONENBERG M. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. Proc Natl Acad Sci U S A. Jul 8;100(14):8395-400, 2003.

NOCKHER WA, SCHERBERICH JE. Expanded CD14+ CD16+ monocyte subpopulation in patients with acute and chronic infections undergoing hemodialysis. Infect Immun. 66(6):2782-90, 1998.

O'GARRA A, VIEIRA P. Regulatory T cells and mechanisms of immune system control. Nat Med. Aug;10(8):801-5, 2004. Review.

OLIVEIRA AC, PEIXOTO JR, DE ARRUDA LB, CAMPOS MA, GAZZINELLI RT, GOLENBOCK DT, AKIRA S, PREVIATO JO, MENDONÇA-PREVIATO L, NOBREGA A, BELLIO M. Expression of functional TLR4 confers proinflammatory responsiveness to Trypanosoma cruzi glycoinositolphospholipids and higher resistance to 1: J Immunol. 1;173(9):5688-96, 2004.

ORTALDO JR, WINKLER-PICKETT RT, YAGITA H, YOUNG HA. Comparative studies of $CD3^-$ and $CD3^+$ $CD56^+$ cells: examination of morphology, functions, T cell receptor rearrangement, and pore-forming protein expression. Cell Immunol. Sep;136(2):486-95, 1991.

PERUHYPE-MAGALHÃES V, MARTINS-FILHO OA, PRATA A, SILVA LDE A, RABELLO A, TEIXEIRA-CARVALHO A, FIGUEIREDO RM, GUIMARÃES-CARVALHO SF, FERRARI TC, VAN WEYENBERGH J, CORREA-OLIVEIRA R. Mixed inflammatory/regulatory cytokine profile marked by simultaneous raise of interferon-gamma and interleukin-10 and low frequency of tumour necrosis factor-alpha($^+$) monocytes are hallmarks of active human visceral Leishmaniasis due to Leishmania chagasi infection. Clin Exp Immunol. Oct;146(1):124-32, 2006.

PICCIRILLO CA, SHEVACH EM. Cutting edge: control of $CD8^+$ T cell activation by $CD4^+CD25^+$ immunoregulatory cells. J Immunol. Aug 1;167(3):1137-40, 2001.

PICCIRILLO CA, SHEVACH EM. Naturally-occurring $CD4^+CD25^+$ immunoregulatory T cells: central players in the arena of peripheral tolerance. Semin Immunol. Apr;16(2):81-8, 2004.

PICKER LJ, SINGH MK, ZDRAVESKI Z, TREER JR, WALDROP SL, BERGSTRESSER PR, MAINO VC. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. Blood. Aug 15;86(4):1408-19, 1995.

PRATA A. Clinical and epidemiological aspects of Chagas disease. Lancet Infect Dis 1: 92-100; 2001.

REIS DD, JONES EM, TOSTES S JR, LOPES ER, GAZZINELLI G, COLLEY DG, MCCURLEY TL. Characterization of inflammatory infiltrates in chronic chagasic myocardial lesions: presence of tumor necrosis factor-alpha$^+$ cells and dominance of granzyme A$^+$, CD8$^+$ lymphocytes. Am J Trop Med Hyg. 48(5):637-44, 1993.

REZENDE J M & MOREIRA H. Forma Digestiva da Doença de Chagas. In: BRENER Z, ANDRADE ZA & ABARRAL-NETTO M. (eds). Trypanosoma cruzi e Doença de Chagas. Rio de Janeiro: Guanabara Koogan, 297-343, 2000.

REZENDE J.M. & RASSI A. Doença de Chagas. In: Castro LP, Cunha AS, Rezende JM. Protozooses Humanas. São Paulo: Fundo Editorial Byk. P: 38-77, 1994.

SATHLER-AVELAR R, LEMOS EM, REIS DD, MEDRANO-MERCADO N, ARAUJO-JORGE TC, ANTAS PR, CORREA-OLIVEIRA R, TEIXEIRA-CARVALHO A, ELOI-SANTOS SM, FAVATO D, MARTINS-FILHO OA. Phenotypic Features of Peripheral Blood Leucocytes During Early Stages of Human Infection with Trypanosoma cruzi. Scand J Immunol. Dec;58(6):655-663, 2003.

SCHOFIELD C.J. & DIAS J.C. The Southern Cone Initiative against Chagas disease. Adv Parasitol;42:1-27, 1999.

SHEVACH EM. CD4$^+$ CD25$^+$ suppressor T cells: more questions than answers. Nat Rev Immunol. Jun;2(6):389-400, 2002.

SKRZECZYNSKA J, KOBYLARZ K, HARTWICH Z, ZEMBALA M, PRYJMA J. CD14$^+$CD16$^+$ monocytes in the course of sepsis in neonates and small children: monitoring and functional studies. Scand J Immunol. 55(6):629-38, 2002.

SOUZA PE, ROCHA MO, ROCHA-VIEIRA E, MENEZES CA, CHAVES AC, GOLLOB KJ, DUTRA WO. Monocytes from patients with indeterminate and cardiac forms of Chagas' disease display distinct phenotypic and functional characteristics associated with morbidity. Infect Immun. 2004 Sep;72(9):5283-91.

SUNI MA, PICKER LJ, MAINO VC. Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. J. Immunol. Methods. Mar 1;212(1):89-98, 1998.

SWETS JA. Measuring the accuracy of diagnostic systems. Science. Jun 3;240(4857):1285-93, 1988. Review.

TANIGUCHI M, HARADA M, KOJO S, NAKAYAMA T, WAKAO H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. Annu Rev Immunol.;21:483-513. Epub 2001 Dec 19, 2003. Review.

TARLETON RL, REITHINGER R, URBINA JA, KITRON U, GÜRTLER RE. The challenges of Chagas Disease- grim outlook or glimmer of hope. PLoS Med. Dec;4(12):e332, 2007.

THOMA-USZYNSKI S, KIERTSCHER SM, OCHOA MT, BOUIS DA, NORGARD MV, MIYAKE K, GODOWSKI PJ, ROTH MD, MODLIN RL. Activation of toll-like receptor 2 on human dendritic cells triggers induction of IL-12, but not IL-10. J Immunol. 165(7):3804-10, 2000.

TRZONKOWSKI P, SZMIT E, MYŚLIWSKA J, DOBYSZUK A, MYŚLIWSKI A. $CD4^{+}CD25^{+}$ T regulatory cells inhibit cytotoxic activity of T $CD8^{+}$ and NK lymphocytes in the direct cell-to-cell interaction. Clin Immunol. Sep;112(3):258-67, 2004.

URBINA JA, DOCAMPO R. Specific chemotherapy of Chagas disease: Controversies and advances. Trends Parasitol 19: 495-501, 2003.

VAGO AR, ANDRADE LO, LEITE AA, D'AVILA REIS D, MACEDO AM, ADAD SJ, TOSTES S JR, MOREIRA MC, FILHO GB, PENA SD. Genetic characterization of Trypanosoma cruzi directly from tissues of patients with chronic Chagas disease: differential distribution of genetic types into diverse organs. Am J Pathol. May;156(5):1805-9, 2000.

VAN DER VLIET HJ, MOLLING JW, VON BLOMBERG BM, NISHI N, KÖLGEN W, VAN DEN EERTWEGH AJ, PINEDO HM, GIACCONE G, SCHEPER RJ. The immunoregulatory role of CD1d-restricted natural killer T cells in disease. Clin Immunol. Jul;112(1):8-23, 2004. Review.

VAN KAER L. Regulation of immune responses by CD1d-restricted natural killer T cells. Immunol Res.;30(2):139-53, 2004.

VINHAES M.C. & DIAS J.C. Chagas disease in Brazil. Cad Saude Publica;16 Suppl 2:7-12, 2000.

VIOTTI R, VIGLIANO C, LOCOCO B, BERTOCCHI G, PETTI M, ALVAREZ MG, POSTAN M, ARMENTI A.. Longterm cardiac outcomes of treating chronic Chagas disease with benznidazole versus no treatment: A nonrandomized trial. Ann Intern Med 144: 724-734, 2006.

WENDEL S. Doença de Chagas transfusional. In: Dias JCP, Coura JR (Ed). Clinica e terapêutica da doença de Chagas. Uma abordagem prática para o clínico geral. Rio de Janeiro: FIOCRUZ Editora, 411-428, 1997.

WORLD HEALTH ORGANIZATION (2002) Control of Chagas Disease: Second report of the WHO Expert Committee. Geneva: WHO. 120 p. (WHO Technical Report Series, 905). ISBN: 92 4 120905 4. ISSN: 0512-3054. Disponível em: <http://whqlibdoc. who.int/trs/WHO_TRS_905.pdf >. Acesso em: 26 fev. 2008.

YOUDEN WJ. Index for rating diagnostic tests. Cancer. 1950 Jan;3(1):32-5.

YOUNG C, LOSIKOFF P, CHAWLA A, GLASSER L, FORMAN E. Transfusion acquired Trypanosoma cruzi infection. Transfusion 47: 540-547, 2007.

7. ANEXO

Durante o período da Iniciação Científica (2000-2002)

Primeiro lugar na IX Reunião Anual de Iniciação Científica do Centro de Pesquisas René Rachou/FIOCRUZ de 14-16 de maio de 2001 na apresentação do trabalho intitulado "Análise da reatividade de IgG anti-formas epimastigotas fixadas do Trypanosoma cruzi em indivíduos portadores de diferentes doenças parasitárias."

Durante o período do Mestrado (2002-2004)

Publicação do artigo "Estabelecimento de novas metodologias para o estudo sorológico, por citometria de fluxo, na fase crônica da doença de Chagas: ênfase em anticorpos da sorologia convencional e da categoria de anticorpos líticos, visando o controle de cura pós-terapêutica" na Revista da Sociedade Brasileira de Medicina Tropical, v. 35, n. Suplemento, p. 162-165, 2002.

Durante o período do Doutorado (2004-2008)

Doutorado Sandwich na Southwest Foundation for Biomedical Research-Texas/USA, sob a orientação do Dr. John L. VandeBerg, com o intuito de validar alterações imunológicas identificadas em humanos para modelo experimental de infecção em primatas não-humanos visando estudos complementares (CAPES – Programa de Doutorado com Estágio no Exterior/PDEE). Período: 19 de março de 2006 a 20 de março de 2007.

Publicação como primeira autora dos artigos:

"Chagasic Patients with Indeterminate Clinical Form of the Disease have High Frequencies of Circulating $CD3^{+}CD16^{-}CD56^{+}$ Natural Killer T Cells and

$CD4^+CD25^{High}$ Regulatory T Lymphocytes" na revista Scandinavian Journal of Immunology 62, 297–308, 2005.

"Are increased frequency of macrophage-like and natural killer (NK) cells, together with high levels of NKT and $CD4^+CD25^{high}$ T cells balancing activated $CD8^+$ T cells, the key to control Chagas' disease morbidity?" na revista Clinical and Experimental Immunology 145(1):81-92, 2006.

"Non-conventional flow cytometry approaches to detect anti-Trypanosoma cruzi immunoglobulin G in the clinical laboratory" na revista Journal of Immunological Methods 10;318(1-2):102-12, 2007.

Publicação como co-autora dos artigos:

"Benznidazole treatment during early-indeterminate Chagas' disease shifted the cytokine expression by innate and adaptive immunity cells toward a type 1-modulated immune profile" na revista Scandinavian Journal of Immunology 64(5):554-63, 2006.

"Impact of dual infections on chemotherapeutic efficacy in BALB/c mice infected with major genotypes of Trypanosoma cruzi" na revista Antimicrobial Agents Chemotherapy 51(9):3282-9, 2007.

"Etiological treatment during early chronic indeterminate Chagas disease incites an activated status on innate and adaptive immunity associated with a type 1-modulated cytokine pattern", Microb Infect (2007), doi:10.1016/j.micinf.2007.10.009

"Persistence of positive tissue-PCR in benznidazole-treated mice with negative blood parasitological and serological tests in dual-infections with Trypanosoma cruzi stocks from different genotypes", Journal of Antimicrobial Chemotherapy.

Elaboração e submissão como primeira autora do artigo:

"Strategy to assess and correlate the cytokine profile of circulating leukocytes and clinical status of human Chagas disease" a revista Journal of Immunological Methods em março de 2008.

Elaboração e submissão de capítulo de livro como co-autora:

"Innate immune response in human trypanosomatidae infections" a editora Nova Science em 24 de janeiro de 2008.

Primeiro lugar no XXI Prêmio Jovem Cientista-2005 tema "Sangue Fluido da Vida", na categoria estudante de ensino médio, como orientadora da estudante Natália Évelin Martins no trabalho intitulado: "Análise da reatividade de IgG anti-epimastigotas fixadas de Trypanosoma cruzi em soros de pacientes portadores de diferentes doenças parasitárias".

Segundo lugar nas apresentações orais de projetos na XIII Jornada de Iniciação Científica e II Jornada do Programa de Vocação Científica do Centro de Pesquisas René Rachou FIOCRUZ, realizada de 23-25 de maio de 2005, como orientadora da estudante Natália Évelin Martins no projeto "Uso da citometria de fluxo na análise de subclasses de IgG anti-formas epimastigotas do Trypanosma cruzi aplicada ao diagnóstico da doença de Chagas".

Orientação da monografia da estudante Paula Souza Lage, intitulada "Impacto da estimulação antígeno-específica na expressão de receptores Fcγ em leucócitos do sangue periférico de pacientes portadores de diferentes formas clínicas da doença de Chagas", aprovada em 21 de junho de 2005 no Instituto de Ciências Biológicas e da Saúde, da Pontifícia Universidade Católica de Minas Gerais.

Participação da banca de monografia do estudante Rodrigo Lima Massara, intitulada "Estudo do perfil da resposta imune em crianças portadoras de infecção recente pelo Trypanosoma cruzi" em 28 de junho de 2005, no Instituto de Ciências Biológicas da Pontifícia Universidade Católica de Minas Gerais, campus Betim.