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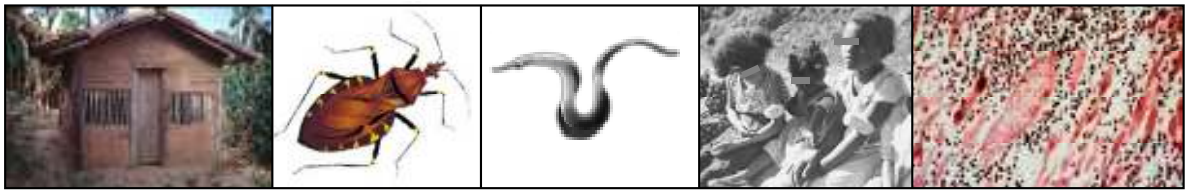

Universidade Federal de Minas Gerais

Belo Horizonte – MG

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Tese apresentada ao Programa de Pós-graduação em Patologia da Faculdade de Medicina da Universidade Federal de Minas Gerais/UFMG, como requisito parcial para obtenção do título de Doutor em Patologia.

Orientadora: Doutora Silvana Maria Elói Santos
Co-orientador: Doutor Olindo Assis Martins Filho

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Este trabalho foi desenvolvido nos laboratórios de Doença de Chagas do Centro de Pesquisa René Rachou-FIOCRUZ, sob orientação da doutora Silvana Maria Elói Santos* e do doutor Olindo Assis Martins Filho**, e contou com as colaborações de:

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“Não há limite para como os coisas podem ser complicadas, uma coisa sempre levando a outra”.

E. B. White

“Fomos uma correção do que ainda antes nós éramos, porque procuramos aperfeiçoar-nos. Somos um resumo do que fomos, porque nos simplificamos. E seremos um outro rascunho, porque a renovação se fará necessária”.

Wanducci Ilário da Costa

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SUMÁRIO

LISTA DE TABELAS

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

BCR – Receptor de células B

BFA – Brefeldina A

BSA – Albumina sérica bovina

Bz- benzonidazol

C3 – Componente da cascata de complemento

CD – "Cluster of differentiation"

CMF – Canal médio de fluorescência

CMSP – Células mononucleares do sangue periférico

E-IND – Crianças portadoras da forma crônica indeterminada recente da doença de Chagas

$E\text{-}IND_T$ – Crianças portadoras da forma crônica indeterminada recente da doença de Chagas submetidas ao tratamento com benzonidazol

EDTA – Etilenodiaminotetracético

EPI – Formas epimastigotas do *T. cruzi*

FITC – Isotiocianato de Fluoresceína

GIPLs – Glicoinositolfosfolipideos

GPI – Glicosilfosfatidilinositol

HAI – Hemaglutinação indireta

IFI – Imunofluorecência indireta

IMF – Intensidade Média de Fluorescência

LPS – Lipopolisacarídeo

LIT – Meio de cultura para as formas epimastigotas do *T. cruzi* (Liver Infusion Tryptose)

mAbs – Anticorpos monoclonais

MHC – Complexo principal de histocompatibilidade

NI – Grupo de indivíduos não infectado

NO – Óxido Nítrico

PBS – Tampão Fosfato Salínico

PBS-W – Tampão Fosfato Salínico com 0,5% de BSA

PBS-P – Tampão Fosfato Salínico com 0,5% de BSA e 0,5% de saponina

TCR – Receptor de células

TGF-β – Fator de Crescimento Tumoral beta

TLR – "Toll-like receptor"

TNF-α – Fator de Necrose Tumoral α

ABSTRACT

The immune 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 to determine the impact of Bz-treatment on major and minor peripheral blood leukocyte subpopulations in *T. cruzi*-infected children, characterizing the early stages of the indeterminate clinical form of Chagas' disease (E-IND). Firstly, we characterized the E-IND immune profile and compared with non infected children (NI). Our results indicated significant alterations in E-IND when compared with NI, including increased values of pre-NK cells, and higher values of pro-inflammatory monocytes. The higher values of activated B lymphocytes contrasted with impaired T cell activation and the cytokine secretion; a decreased frequency of regulatory cells was also observed. These findings reinforce the hypothesis that simultaneous activation of innate and adaptive immunity mechanisms in addition to modulation of T cell immune response occur during early events of Chagas' disease. Furthermore, we favour the hypothesis that T-cell-mediated immunity, during the early indeterminate clinical form, may represent a phenomenon restricted to the cardiac and lymph node compartments, not detectable in the peripheral blood. Besides establishing the immune profile for E-IND, we evaluated the impact of Bz-treatment on the circulating leukocytes during the early-indeterminate Chagas' disease. Our results demonstrated that Bz-treatment led to higher activation status of circulating monocytes but negatively correlated with the number of IL-12^{+}CD14^{+} cells. Moreover, Bz-treatment triggered high frequency of circulating pre-NK cells, besides outstanding activation status of CD16^{+} cells, correlated with a type-1 modulated cytokine pattern. Additionally, Bz-treatment induced substantial T and B-cell activation status associated with an overall IL-10 modulated type-1 cytokine profile. Taken together, these findings add new concepts in the context of immune activation status following the etiological treatment of Chagas disease suggesting that more than increased number of activated leukocytes in the peripheral blood, Bz-treatment may also involves a qualitative change in their functional capacity that guide their activation state toward a modulated cytokine profile that may count for the benefits of etiological treatment of Chagas disease.

RESUMO

A resposta imunológica durante a infecção recente pelo *Trypanosoma cruzi* não esta completamente entendida, apesar do seu papel no direcionamento do desenvolvimento das manifestações clínicas da infecção crônica e do sucesso terapêutico. Nesse trabalho reportamos os resultados de uma investigação imunofenotípica por citometria de fluxo para determinar o impacto do tratamento com benzonidazol nas subpopulações leucocitárias de crianças infectadas pelo *T. cruzi*, caracterizando os estágios iniciais da forma clínica indeterminada da doença de Chagas (E-IND). Primeiramente, caracterizamos o perfil imune do grupo E-IND e comparamos com o grupo das crianças não infectadas (NI). Nossos resultados indicaram alterações significativas em E-IND quando comparado com NI, incluindo valores aumentados de células pré-NK e monócitos pró-inflamatórios. Os valores elevados de células B ativadas contrastaram com a deficiência na ativação de células T e na secreção de citocinas; uma queda na frequência de células reguladoras também foi observada. Esses achados reforçam a hipótese que a ativação simultânea dos mecanismos da imunidade inata e adaptativa associados com uma modulação na resposta imune de células T ocorrem durante os eventos iniciais da doença de Chagas. Além disso, nossos dados contribuem para a hipótese de que a imunidade mediada por células T, durante a forma clinica indeterminada recente, possa representar um fenômeno restrito ao compartimento cardíaco e linfonodos, não detectados no sangue periférico. Após o estabelecimetno do perfil imune do E-IND, avaliamos o impacto do tratamento com benzonidazol nos leucócitos circulantes durante a doença de chagas indeterminada recente. Nossos resultados demonstraram que o tratamento com benzonidazol levou a um padrão ativado de monócitos circulantes, mas correlacionado negativamente como o número de células $CD14^{+}IL\text{-}12^{+}$. O tratamento com benzonidazol também induziu aumento na frequência de células pré-NK circulantes, assim como uma grande ativação das células $CD16^{+}$, correlacionada com um padrão de citocinas do tipo 1 modulado. Adicionalmente, o tratamento com benzonidazol induziu um estatus de ativação substancial para as células T e B associado com um perfil do tipo 1 global, modulado por IL-10. Nossos dados em conjunto adicionam nova concepção no contexto da ativação imune após o tratamento etiológico da doença de Chagas, sugerindo que mais do que um aumento no número de leucócitos ativados no sangue periférico, o tratamento com benzonidazol pode também levar a mudanças qualitativas na capacidade funcional que guia o seu estatus ativado para um perfil de citocinas modulado que pode contribuir com benefícios para o tratamento etiológico da doença de Chagas.

1. INTRODUÇÃO

1.1. Epidemiologia

A Tripanossomíase Americana ou doença de Chagas, descoberta em 1909 por Ribeiro Justiniano das Chagas (Chagas, 1909), é uma zoonose causada pelo hemoflagelado *Trypanosoma cruzi*, pertencente à ordem Kinetoplastida e à família *Tripanosomatidae*. Segundo a Organização Mundial de Saúde, a doença de Chagas representa um importante problema de saúde pública, sendo o quarto maior de impacto social entre todas as doenças infecciosas e parasitárias prevalentes na América Latina, estando distribuída desde o sul dos Estados Unidos até o sul da Argentina, afetando cerca de 13 milhões de indivíduos e com uma incidência de 200 mil novos casos por ano (WHO, 2003).

No Brasil, a prevalência da doença de Chagas humana abrange uma área de três milhões de quilômetros quadrados, desde o Maranhão até o Rio Grande do Sul. 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).

Em outros países da América do Sul, como a Bolívia, observam-se índices mais elevados da doença. Segundo Valencia *et al.*, até 1990, 1,1 a 1,8 milhões de pessoas residentes na Bolívia poderiam estar infectadas com o *T. cruzi*, o que corresponderia a 15% da população total do país, com o surgimento anual de mais de 85 mil novos casos (VALENCIA *et al*., 1990).

A infecção chagásica é classicamente considerada como sendo de área rural. Entretanto, o panorama atual mostra um aumento dos casos de infecção chagásica também nos grandes centros urbanos, podendo estar relacionado a dois principais fatores: (1) a migração de pessoas contaminadas com o *T. cruzi* das áreas rurais para as cidades, com o conseqüente aumento do percentual de portadores da doença em áreas não endêmicas; (2) o

transporte do vetor junto aos pertences pessoais, durante o êxodo, favorecendo a formação de um ciclo urbano, uma vez que o vetor é capaz de se adaptar a novos ambientes (CARRASCO *et al*., 1990, DIAS 2007).

A principal forma de controle da doença faz-se através de ações de combate químico sistemático aos insetos vetores e ou melhorias habitacionais, complementadas por rigorosa seleção de doadores de sangue. No final do último século, ficou comprovado que medidas sistemáticas de controle e vigilância epidemiológica, em áreas endêmicas, podem levar à eliminação da maioria das populações de vetores domésticos, contribuindo para a interrupção da transmissão da doença. O impacto social do controle da doença pode agora ser demonstrado pela redução ou até mesmo o desaparecimento de casos agudos e de novas infecções em indivíduos mais jovens (DIAS *et al*., 2002). Apesar do progresso das últimas décadas, em algumas áreas endêmicas, a transmissão vetorial da doença de Chagas ainda permanece, principalmente onde a vigilância epidemiológica e o controle da doença são incipientes. O estado de Minas Gerais, que desde a descoberta da doença, tornou-se importante centro de investigações sobre a doença de Chagas, teve como cenário o desaparecimento do vetor *Triatoma infestans* e a não documentação de indivíduos menores de dez anos infectados pelo *T. cruzi*. Entretanto, com a descontinuidade dos programas de vigilância epidemiológica, estudos atuais vêm constatando o ressurgimento de novos casos de infecção pelo *T. cruzi* em crianças com idade inferior a 10 anos. Estes dados chamam a atenção para a importância de se adotar medidas de vigilância e controle vetorial sistêmicas. Além disso, é importante salientar que não basta somente o controle do surgimento de novos casos da doença. No Brasil, são cerca de 5 milhões de indivíduos infectados que correspondem a um sobrecarga para os órgãos de saúde pública e de previdência social, uma vez que a enfermidade ameaça e acomete basicamente as regiões pobres do país, priorizando populações de baixa expressão política, socialmente excluídas, de origem rural e pouco

letradas (DIAS, 2007). Por tudo isso, a atenção aos indivíduos infectados pelo *T. cruzi* geralmente pressupõem uma ação de Estado, o que gera clara dependência de políticas públicas conseqüentes e continuadas, a fim de minimizar os efeitos deletérios da doença

1.2. Ciclo Evolutivo

O ciclo evolutivo do *T. cruzi* inicia-se no hospedeiro invertebrado quando a forma tripomastigota sanguínea é ingerida pelo triatomíneo no momento do seu repasto sangüíneo. No trato digestório, as formas tripomastigotas transformam-se em epimastigotas que se multiplicam por fissão binária e se diferenciam em formas tripomastigotas metacíclicas, na porção posterior do tubo digestivo. Estas formas, quando eliminadas junto às fezes e urina, infectam o hospedeiro vertebrado através de mucosas íntegras ou lesões de continuidade na pele. Subseqüentemente, os tripomastigotas penetram por endocitose tanto nas células fagocitárias quanto nas não fagocitárias e completam o seu ciclo biológico. Em seguida, modificam-se em amastigotas, que conseguem escapar do vacúolo parasitófago, para o citoplasma das células hospedeiras. Após cerca de 35 horas, inicia-se um processo de divisão binária que pode durar vários dias, dependendo da característica da cepa de *T. cruzi* e da célula parasitada. Em torno de cinco dias, inicia-se um processo no qual as formas amastigotas se transformam em tripomastigotas e rompem a célula hospedeira, podendo infectar novas células ou permanecer na corrente sangüínea, e iniciam novo ciclo biológico, quando ingeridas pelo vetor (Figura 1) (BRENER *et al*., 1973; GARCIA *et al*., 1991).

Outros mecanismos de transmissão, alternativos ao vetorial, têm sido descritos e incluem a transfusão sanguínea, a transmissão congênita, transplante de órgãos e acidentes laboratoriais (DIAS *et al*., 2000), com grande importância epidemiológica a transmissão através da transfusão sanguínea, pois possibilita a disseminação da doença nos centros urbanos (WENDEL, 1997). Hoje, no Brasil, estima-se que cerca de 70% dos indivíduos portadores da doença de Chagas vivam no espaço urbano, proporção esta que é menor para

outros países como Bolívia e Paraguai. Em áreas de grande endemicidade, como a Bolívia, a transmissão congênita atinge importância na saúde pública, pois os índices de contaminação chegam a 80% em áreas rurais. Estudos sobre a prevalência da infecção chagásica em gestantes e a incidência da transmissão nas Américas do Sul e Central, demonstram haver grandes variações regionais. No Brasil a transmissão congênita atinge 1,6% dos casos de crianças infectadas na cidade de Salvador-BA (AZOGUE *et al*., 1985; BITTENCOURT *et al.,* 1985).

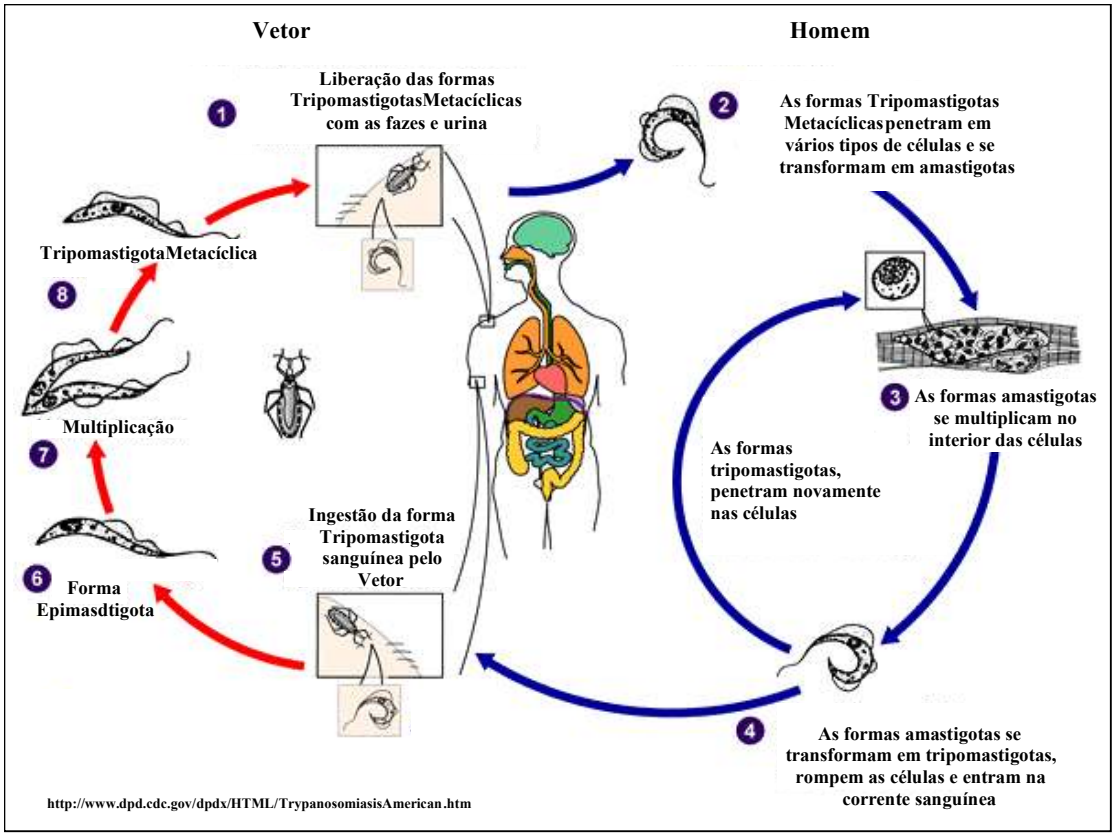

Figura 1: Ciclo Biológico do *Trypanosoma cruzi*

1.3. Patologia da infecção chagásica

Logo após a infecção, surge a fase aguda, de curta duração, seguida pela fase crônica da doença, que permanece por toda a vida do indivíduo. O *T. cruzi* pode indicar seu ponto de entrada no corpo humano através do surgimento de lesão inflamatória, o chagoma de inoculação – formação cutânea ligeiramente saliente, arredondada, eritematosa, de consistência firme, com alguns centímetros de diâmetro e geralmente indolor. Quando a porta de entrada se dá na região ocular, forma-se o sinal de Romaña – edema bipalpebral unilateral e indolor considerado, por muito tempo, como característico da doença de Chagas. A fase aguda é marcada por alterações teciduais degenerativas e inflamatórias focais, devidas a intensa multiplicação local do parasita (LARANJA, 1953). Manifestações sistêmicas, como febre, mal estar, astenia, edema subcutâneo, linfadenomegalia, esplenomegalia e hepatomegalia, são também observadas. Em cerca de 90% dos casos, as manifestações clínicas duram algumas semanas ou meses, regredindo espontaneamente. Nos demais pacientes, alterações cardíacas ou esofagianas podem permanecer além deste prazo e levar ao óbito. Em alguns casos, a fase aguda pode ainda apresentar-se assintomática (DUTRA *et al*., 2005).

A evolução da fase aguda para a fase crônica é acompanhada pelo gradativo desaparecimento das manifestações clínicas e diminuição da parasitemia. Acredita-se que alterações na fase crônica sejam conseqüências de mecanismos multifatoriais relacionados tanto ao parasita quanto ao hospedeiro vertebrado, assim como a fatores sócio-econômicos. Dentre os fatores relacionados ao parasita, a variabilidade das cepas, o tropismo, a antigenicidade e o tamanho do inóculo são aspectos relevantes (FRANCO *et al*., 2003; VAGO *et al*., 2000). Quanto ao hospedeiro, é importante ressaltar a faixa etária, o sexo e especialmente as características imunológicas e genéticas. Em relação aos fatores sócio-econômicos, destacam-se os aspectos nutricionais, uma vez que alguns micronutrientes

ingeridos parecem estar relacionados a quadros clínicos específicos (DE SOUZA *et al*., 2003; RIVERA *et al*., 2002; ARANTES *et al*., 2007).

A maioria dos que entram na fase crônica permanecem assintomáticos, e a doença é detectada somente por exames laboratorias. Esses pacientes são classificados como indeterminados, a presença da doença e confirmada através da positividade de pelo menos dois testes sorológicos específicos, porque os pacientes não apresentam sintoma ou sinal específico da doença de Chagas. Segundo critério adotado pela organização mundial de saúde, os pacientes portadores da forma crônica indeterminada também apresentam exames de eletrocardiograma, raio X de tórax, esôfago e colon normais (WHO, 2002). Um percentual significativo do indivíduos infectados desenvolverão sintomas clínicos ou apresentar alterações em exames laboratorias (por exemplo, aumento nos níveis de hormônio natriurético) e ou de imagens (mudanças no ecocardiograma) avaliados. Alguns pacientes chagásicos podem apresentar-se com probroblemas digestivos, relacionados com a destruição da rede neuronal mioentérica, mais frequente no esôfago e no intestino grosso (MENEGHELLI, 2004). Estes problemas frequentemente relacionados com a motilidade e disfunção digestiva e, em casos extremos, pode levar à morte. Entre 20% e 30% dos pacientes cronicamente infectados desenvolve a forma clínica cardíaca da doença de Chagas, que pode variar de grau moderado de alterações da função do coração (detectado somente através do uso de métodos laboratorias sensíveis) à grave da doença. Os casos mais graves com alterção na condutividade de impulsos nervosos, os quais podem estar associados com falha na contatibilidade do musculo estriado cardíaco, levam à cardiomiopatia chagásica crônica e uma alta taxa de mortalidade (ROCHA *et al*., 2003). A classificação clínical publicada recentemente (ROCHA *et al*., 2003) está baseada na avaliação das funções do coração utilizando técnicas bem padronizadas (ecocardiograma e radiografia) e mais sensíveis (“Doppler” eletrocardiografia, Holter 24-horas e testes ergométricos). Outra classificação

frequentemente empregada é aquela sugerida pela Organização Mundial de Saúde que é baseada na presença e gravidade de sintomas, e não pelo emprego de metodologias refinadas. Apesar de sua grande aplicabilidade na classificação para estudos epidemiológicos, muitos pacientes que apresentam exames normais monstram alteração cardíacas por métodos mais sensíveis (PEREZ *et al* 2003).

Os danos do tecido cardíaco foram investigados por muitos anos sem sucesso, uma vez que o reduzido número de parasitas nas fibras cardíacas não poderia ser responsável pelo surgimento dos focos inflamatórios (GOGHER & GAZZINELLI, 2004). Essas observações levaram à proposição de fenômenos de auto-agressão com destruição de células normais, possivelmente por uma reação cruzada entre antígenos do *T. cruzi* e as fibras cardíacas. Esta hipótese foi reforçada pela presença de anticorpos capazes de interagir com endocárdio, vasos e interstício e também pela presença de linfócitos incrustados nas células do músculo cardíaco, produzindo lise celular nos indivíduos com cardiomiopatia chagásica (REIS *et al*., 1993). Com os avanços nas técnicas de biologia molecular, tornou-se possível a identificação de fragmentos de DNA de *T. cruzi* em tecido cardíaco dos cardiopatas chagásicos crônicos (JONES *et al*., 1993; HIGUCHI, 1995), demonstrando assim uma correlação positiva entre o número de parasitos e a severidade da doença.

Grande parte das manifestações clínicas no hospedeiro deve-se à resposta imune dirigida contra o parasita. No homem, bem como em modelos experimentais, a infecção pelo *T. cruzi* mobiliza diferentes compartimentos do sistema imune, levando ao aparecimento de respostas humorais e celulares específicas contra o parasita (BRENER & GAZZINELLI, 1997). A estimulação do sistema imune é crucial na redução da carga parasitária, mas, por outro lado, pode contribuir para o agravamento dos sintomas clínicos. Entender as diferenças da resposta imune envolvida na lesão tecidual é o atual desafio no estudo sobre a imunologia na doença de Chagas.

1.4. Aspectos imunológicos na doença de Chagas

Vários estudos destacam forte ativação do sistema imune inato durante a fase aguda, como sendo a primeira linha de defesa contra a invasão do microorganismo (FEARON *et al.,* 1996; GAZZINELLI *et al.,* 1998; JANEWAY *et al.,* 2001). Nesse período, observa-se ativação inespecífica, bem como de macrófagos (ORTIZ *et al.,* 1976) e células natural killer-NK (HATCHER *et al.* 1981), acompanhadas por uma resposta policlonal de linfócitos B e T. O *T. cruzi*, assim como outros microorganismos patogênicos, é capaz de induzir prontamente a ativação de proteínas e enzimas efetoras da cascata de complemento que destroem formas epimastigotas não infectantes. Porém, formas tripomastigotas infectantes são resistentes à ação lítica da cascata de complemento (NOGUEIRA *et al*., 1975). Esta resistência parece estar relacionada à expressão de proteínas semelhantes ao DAF humano (decay-acelerating factor) na superfície da forma tripomastigota. Tal proteína, denominada T-DAF, atua desestabilizando a C3 convertase depositada na membrana do parasita, impedindo a lise (RIMOLDI *et al*., 1988; TAMBOURGI *et al*., 1993).

A infecção pelo *T. cruzi* também induz considerável aumento da secreção de proteínas hepáticas de fase aguda, como a proteína C reativa. Esta, em conjunto com outras proteínas séricas, se liga a compostos antigênicos do *T. cruzi* que são eficientemente endocitadas por macrófagos, uma vez que estes expressam, em suas superfícies, receptores para tais proteínas (ISAAC *et al*., 1990). Contudo, até o momento, os dados fornecidos não esclarecem se a ligação dessas proteínas a compostos antigênicos propicia a eliminação do parasita ou se relaciona ao mecanismo de invasão e replicação dentro de macrófagos (LUZ *et al*., 1995; MORROT *et al*., 1997).

Os macrófagos têm papel essencial no controle da infecção. Secretam uma variedade de mediadores químicos e citocinas que regulam diferentes compartimentos do sistema imunológico. Eles capturam, processam e apresentam antígenos para as células T e são uma

fonte de moléculas co-estimulatórias para a ativação destas células. Uma vez estimulados por antígenos derivados de formas tripomastigotas e amastigotas do *T. cruzi*, podem atuar como células efetoras para uma atividade citotóxica, no caso parasiticida, com produção de metabólitos reativos de nitrogênio e oxigênio e citocinas pró-inflamatórias como IL-1β, IL-6, IL-12 e TNF-α (CAMARGO *et al.,* 1997; CHANDRASEKAR *et al.,* 1998). Entretanto, sabe-se que alguns glico-inositol-fosfolipídios (GIPLs), extraídos da membrana celular de formas epimastigotas e tripomastigotas metacíclicas do *T. cruzi*, exercem função supressiva na ativação de macrófagos e células dendríticas, inibindo a secreção de TNF-α e IL-12 (SILVA *et al.,* 1998; VAN OVERTVELT *et al.,* 1999; ZAMBRANO-VILLA *et al.,* 2002). GIPLs também induzem diminuição da expressão de moléculas co-estimulatórias na superfície das células apresentadoras de antígenos (BRODSKYN *et al.,* 2002) que, associadas às citocinas pró-inflamatórias, são essenciais para a ativação dos sistemas imunes inato e adaptativo com conseqüente eliminação do parasita. Desta forma, tais atividades supressivas e imuno-regulatória dos GIPLs são potencialmente importantes para a evasão do sistema imune e o estabelecimento da infecção (BRODSKYN *et al.,* 2002; ZAMBRANO-VILLA *et al.,* 2002).

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 anti-inflamatórias 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 2" (TLR-2) em células dendríticas ativadas (YOSHIMURA *et al.,* 1999; THOMA-USZYNSKI *et al.,* 2000; BELGE *et al.,* 2002; OUAISSI *et al.,* 2002). 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.

As células NK possuem um papel indispensável na resposta imune anti-*T. cruzi* através da produção de IFN-γ mesmo na ausência de células T. São as primeiras células a produzirem esta citocina antes do desenvolvimento da resposta imune mediada por células T, favorecendo precocemente uma resposta do tipo 1, essencial para o controle do parasita durante os estágios iniciais da infecção chagásica (SILVA *et al.,* 1992; TORRICO *et al.,* 1991). O mecanismo clássico proposto para a ativação de células NK, durante a fase aguda,

sugere que as formas tripomastigotas e amastigotas do *T. cruzi* induzem a síntese de IL-12 por macrófagos que, em associação com TNF-α e IL-1β, promovem a produção de IFN-γ por aquelas células (ALIBERTI *et al.,* 1996; BRENER *et al.,* 1997; GAZZINELLI *et al.* 1997). Além disso, estudos *in vitro* e *in vivo* mostram que macrófagos ativados por citocinas liberadas por células com o fenótipo do tipo 1 iniciam o controle da infecção (CAMARGO *et al.,* 1997; De DIEGO *et al*., 1997; MICHAILOWSKY *et al.,* 2001). Tem sido relatado que IFN-γ e TNF-α potencializam sinergisticamente a atividade dos macrófagos para eliminar o *T. cruzi* através da síntese de NO, que restringe eficientemente a replicação do parasita (DENICOLA *et al.,* 1993; PETRAY *et al.,* 1994; VESPA *et al.,* 1994; CHANDRASEKAR *et al.,* 2000). Silva *et al.,* (1992) e Torrico *et al.,* (1991) utilizando, respectivamente, anticorpos anti-IFN-γ e camundongos deficientes para produção de IFN-γ, observaram maior susceptibilidade à infecção, demonstrando que esta citocina é imprescindível nos estágios iniciais da resposta imune. Outra citocina associada ao controle da infecção pelo *T. cruzi* é a IL-18 que, assim como a IL-12, induz síntese de IFN-γ, proliferação de células do tipo 1 e aumenta atividade das células NK (HYODO *et al.,* 1999). Esse aumento na atividade das células NK, independente de IL-2, é de extrema importância, já que camundongos infectados com *T. cruzi* apresentam uma redução na síntese desta citocina e na expressão de seu receptor, podendo ser então a IL-18 uma possível via de eliminação do parasita (ROTTENBERG *et al.,* 1989; NABORS *et al.,* 1991). Sabe-se que a IL-18 não ativa diretamente linfócitos T, por não possuírem receptores expressos constitutivamente em suas superfícies para esta citocina, o que demonstra a sua acuidade na ativação do sistema imune inato. Antúnez *et al.* (2001) demonstraram uma ação conjunta de IL-18 com IL-12 no controle da replicação do parasita em camundongos infectados pelo *T. cruzi*. Aqui a resposta imune do tipo 1 pode induzir reação inflamatória intensa, sendo necessária a presença de citocinas do moduladoras, como

IL-10, para contrabalançar a atividade da resposta inflamatória, reduzindo danos teciduais induzidos pelo processo inflamatório frente à infecção pelo *T. cruzi*.

Como mencionado anteriormente, as células NK são indispensáveis no controle da infecção pelo *T. cruzi*, por serem fonte de IFN–γ. No entanto, suas atividades não se restringem à produção de citocinas. Elas também apresentam atividade citotóxica que pode variar conforme a subpopulação (SONDERGAARD *et al*., 2000). Atualmente, são descritos dois subtipos de células NK humanas, identificadas pela intensidade da expressão da molécula CD56. Aproximadamente 90% das células NK apresentam baixa expressão de CD56 ($CD56^{dim}$), expressam em sua superfície altos níveis de CD16 e possuem alta capacidade citotóxica, enquanto cerca de 10% das células NK apresentam alta expressão de CD56 ($CD56^{bright}$), baixo ou nenhum nível de CD16, com reduzida atividade citotóxica, mas com acentuada atividade proliferativa (SONDERGAARD *et al.,* 2000; COOPER *et al.,* 2001). Entretanto, Gaddy *et al.* (1997), ao estudarem a ontogenia das células NK utilizando sangue de cordão umbilical, demonstraram a existência de uma nova subpopulação de células NK ($CD16^{+}CD56^{-}$) com baixa atividade lítica. Esta célula é considerada como célula pré-NK, possível precursora de células NK maduras, uma vez que quando cultivada na presença da IL-2, modifica-se para um fenótipo $CD16^{+}CD56^{+}$ e na presença da IL-12 transforma-se em $CD16^{+}CD56^{+}$ e ou $CD16^{-}CD56^{+}$.

Além de sua importância na produção de citocinas e na atividade citotóxica, estudos demonstram a interação entre células NK e linfócitos B com conseqüente ativação policlonal e aumento dos níveis de imunoglobulinas IgM e IgG2a em camundongos infectados pelo *T. cruzi* (DE ARRUDA HINDIS *et al.,* 2001). Esses autores observaram que a ativação das células B não ocorre quando estas são cultivada na ausência das células NK, indicando que a sua ativação é provavelmente dependente do contato com células NK. Para tanto, moléculas co-estimulatórias atuam na interação célula-célula e na ativação de suas várias vias efetoras.

A molécula CD28 desempenha uma importante função na indução de atividade citotóxica e secreção de citocinas pelas células NK, quando em contato com CD80 ou CD86, seus ligantes em outras células (MARTIN-FONTECHA *et al.,* 1999; HUNTER *et al.,* 1997). Porém, de acordo com De Arruda Hindis *et al.*(2001), a ativação de linfócitos B pelas células NK é independente do contato CD28-CD86. Já Blanca *et al.* (2001) constataram que a ativação policlonal de células B depende da interação entre CD40, presente na superfície de células NK, e o seu ligante CD40L na superfície de linfócitos B.

A infecção pelo *T. cruzi* também mobiliza outros elementos celulares envolvidos na resposta inata do hospedeiro. Algumas subpopulações linfocitárias são estimuladas no modelo murino de infecção, como por exemplo as células NKT (DUTHIE *et al*., 2002). Essas células, apesar de rearranjarem somaticamente os genes de seus receptores antigênicos (TCR), apresentam um repertório de especificidade restrito. Os determinantes antigênicos reconhecidos são, em geral, estruturas comuns a diferentes microorganismos ou ainda determinantes do próprio hospedeiro, cuja expressão ou reconhecimento são induzidos pelo processo infeccioso. Dessa forma, essas células se situam a meio caminho entre a resposta inata e adquirida.

As células NKT são uma subpopulação de linfócitos T distintos dos linfócitos T convencionais e das células NK, expressando receptores de ambos os tipos celulares, como o receptor de células T (TCR) e o NK1. 1 de células NK. Diferente das células T convencionais, as células NKT são estimuladas por glicolipídios apresentados via CD1, uma molécula com função semelhante ao MHC de classe I (GODFREY *et al*., 2000). Desta forma, promovem a proteção contra agentes infecciosos através da rápida produção de IFN-γ e IL-4, da atividade citotóxica e da ativação de células NK (EBERL *et al*., 2000). Estas células, além de atuarem contra agentes infecciosos, podem estar relacionadas a mecanismos protetores contra respostas autoimunes (HONG *et al*., 1999; GODFREY *et al*., 2000). Duthie *et al*. (2002), ao

estudarem seu papel em camundongos infectados com a cepa CL do *T. cruzi*, constataram que durante a fase aguda, células NKT atuam no controle da parasitemia e na fase crônica, são responsáveis pelo aumento da secreção de anticorpos específicos. Assim, as células NKT podem, possivelmente, atuar no controle da progressão clínica da doença de Chagas.

Uma vez que, os estágios iniciais da doença de Chagas humana são, em sua maioria, assintomáticos, os estudos referentes às alterações imunológicas nestas fases são escassos (SAMUDIO *et al*.1998). Estudos recentes realizados em nosso laboratório, com o objetivo de identificar alterações fenotípicas em leucócitos do sangue periférico durante os estágios iniciais da infecção pelo *T. cruzi* em crianças, demonstraram um microambiente imune independente de células T (SATHLER-AVELAR *et al*. 2003), compatível com aqueles previamente descritos em modelos experimentais (BRENER *et al*., 1997). De fato, estudos em animais têm demonstrado que a resistência/susceptibilidade à infecção pelo *T. cruzi* é, pelo menos em parte, determinada nos estágios iniciais da infecção, antes do desenvolvimento de resposta imune adquirida (TRISCHMANN *et al*., 1986; DUTHIE *et al*., 2005). Além disso, o efeito combinatório das diferentes vias de sinalização desencadeadas pelo *T. cruzi*, em células não imune e células da imunidade inata, pode ter uma importante conseqüência em diferentes aspectos da infecção, tais como parasitismo, tropismo e a patogênese da doença de Chagas. Neste contexto, propõe-se que a resposta imune inata atue de duas formas durante os estágios iniciais da infecção pelo *T. cruzi*. Enquanto seus mecanismos efetores atuam na redução e controle da replicação do parasita nos tecidos, outros mecanismos propiciam a geração de resposta imune adquirida menos patológica, especialmente durante a fase crônica da infecção (VITELLI-AVELAR *et al*., 2005).

Durante a fase crônica da infecção chagásica, as duas subpopulações de linfócitos T – $CD4^+$ e $CD8^+$ – apresentam importância central quanto à capacidade de controlar a infecção pelo *T. cruzi*. O linfócito T $CD4^+$, com sua habilidade em produzir elevados níveis de IFN-γ

auxilia na destruição de formas intracelulares do parasita, durante a fase crônica da doença (TARLETON *et al*., 1996). Estudos utilizando células mononucleares de sangue periférico constataram, em pacientes com a forma clínica indeterminada, uma predominância de linfócitos T $CD4^+$, enquanto que pacientes com sintomas cardíacos exibiam fenótipos predominantemente de células T $CD8^+$ ativadas, sugerindo uma possível participação dessas células em mecanismos imunopatológicos (CUÑA *et al*., 1995). Esta hipótese é reforçada por estudos utilizando imunohistoquímica em tecido cardíaco, obtidos em autópsia de pacientes com cardiopatia chagásica grave, nos quais as lesões inflamatórias eram principalmente caracterizadas por linfócitos T $CD8^+$, muitos dos quais produzem granzimas. Além disso, observou-se expressão aumentada de moléculas do MHC classe I (HLA-ABC) nas células miocárdicas de pacientes chagásicos com cardiopatia crônica, reforçando que elas podem representar alvos de linfócitos para a citotoxicidade mediada por linfócitos T $CD8^+$ (REIS *et al*., 1993).

Vários estudos têm demonstrado que as diferentes manifestações clínicas estão associadas com uma distinta e complexa interação parasita/hospedeiro envolvendo diretamente o sistema imune. De fato, é bem aceito que a ausência de patologia na doença de Chagas está associada com a habilidade do indivíduo em controlar a resposta imune desencadeada no controle do parasitismo, o qual pode contribuir para os danos inflamatórios, característicos da doença (BRENER *et al*., 1997). Esses danos podem ser mais intensos na ausência de mecanismos reguladores. Tem sido descrito que existem tipos de células reguladoras, entretanto a população celular mais estudada são as células T $CD4^+CD25^{HIGH}$ reguladoras (BLUESTONE *et al*., 2003; BELKAID *et al*., 2005). As células T $CD4^+CD25^{HIGH}$ podem exercer sua função reguladora inibindo a atividade citotóxica das células T $CD8^+$ através da modulação de IL-10 ou pelo contato direto com a célula (TRZONKOWSKI *et al*., 2004; O'GARRA *et al*., 2004; GROSSMAN *et al*., 2004). Neste

contexto, estudos mais recentes vêm demonstrando a participação de outras populações celulares com funções imunomoduladores no sangue periférico de indivíduos chagásicos. Vitelli-Avelar *et al*. (2005) observaram um aumento no percentual de células $CD4^{+}CD25^{HIGH}$ exclusivamente na forma clínica indeterminada.

Considerando a complexibilidade da resposta imune, Bahia-Oliveira *et al*. (1998) demonstraram que o perfil de citocinas produzido pelos pacientes chagásicos pode estar relacionado à gravidade da doença, já que pacientes portadores da forma clínica indeterminada, apresentam níveis elevados de IL-10, quando comparados com pacientes cardiopatas, cuja principal citocina produzida é o IFN-γ. Gomes *et al*. (2003), ao estudarem o papel funcional dos leucócitos do sangue periférico de pacientes infectados com o *T. cruzi* estimulados "in vitro", constataram que a principal fonte de IFN-γ em cardiopatas são os linfócitos T $CD4^{+}$, enquanto, em pacientes portadores da forma indeterminada, os monócitos são responsáveis pela produção de elevados níveis de IL-10, favorecendo a regulação da resposta imune e o controle da morbidade da doença. É possível que células T reguladoras estejam envolvidas nesse processo, uma vez que possuem habilidade em inibir a síntese de IFN-γ, o que poderia explicar os baixos níveis de IFN-γ produzidos por células mononucleares do sangue periférico de indivíduos portadores da forma indeterminada da doença de Chagas (GOMES *et al*., 2003; VITELLI-AVELAR *et al*., 2005). Estes achados claramente reforçam a hipótese de que mecanismos imunomoduladores sejam importantes no controle do processo inflamatório desencadeado durante a infecção chagásica, uma vez que, as células $CD4^{+}CD25^{HIGH}$ estão associadas com a redução na atividade citotóxica e na produção de IFN-γ pelas células NK e linfócitos T $CD8^{+}$ (TRZONKOWSKI *et al*., 2004). A perda dos mecanismos imunoreguladores nas formas clínicas mais graves pode contribuir para um desequilíbrio da resposta imune que levaria à potente atividade inflamatória e conseqüente dano tecidual. Futuros estudos funcionais para a caracterização das populações

imunoreguladoras serão de grande valia para o entendimento dos complexos mecanismos imunológicos que permeiam a doença de Chagas.

1.5. Tratamento etiológico da doença de Chagas

A hipótese da participação ativa de mecanismos autoimunes na patogênese da doença de Chagas foi sustentada por vários anos (CUNHA-NETO *et al*., 2006; MARIN-NETO *et al*., 2007). Entretanto, com o surgimento de técnicas de detecção do parasita mais apuradas, estudos recentes têm revelado uma associação da persistência do *T. cruzi* nas lesões crônicas com a evolução clínica da doença de Chagas (ANEZ et al, 1999; TARLETON *et al*., 1994; BRITTO *et al*., 2001). Neste contexto, o tratamento etiológico da doença de Chagas com drogas tripanomicidas, que poderiam eliminar o parasita, exerceria efeito benéfico na evolução da infecção. Todavia, a eficácia do tratamento na fase crônica ainda não está bem estabelecida (ANDRADE *et al*.,1988; BRITTO *et al*., 2001, VIOTTI & VIGLIANO, 2007).

As drogas nifurtimox (Lampit®, Bayer) e benzonidazol (Rochagan®, Roche) atuam sobre as formas tripomastigotas sanguíneas e amastigotas. A primeira, depois de amplamente utilizadas no Brasil e países latino-americanos, foi retirada do mercado farmacêutico brasileiro, permanecendo apenas o benzonidazol (CANÇADO *et al*., 2000, COURA & DE CASTRO, 2002).

O benzonidazol é um N-benzil- 2 nitro- 1 imidazol acetamida que interfere diretamente com a síntese de macromoléculas pelo *T. cruzi* e vários componente celulares, tais como DNA, lipídios e proteínas (MARR & DOCAMPO, 1986). Deve ser administrado durante 30-60 dias, na dosagem de 5 – 7mg/Kg. Apesar da sua atividade tripanossomicida, o benzonidazol pode causar varias reações colaterais caracterizadas por dermatites, depressões da medula óssea e polineuropatia periférica (MEDRANO-MERCADO *et al*., 2000; CANÇADO *et al*., 2000).

Atualmente a administração do benzonidazol é recomendada apenas para os casos de infecção aguda da doença, infecção congênita e crianças com sorologia positiva. O tratamento específico para a forma crônica indeterminada e forma cardíaca inicial, por instituições de pesquisa, foi aconselhado pelos participantes do Simpósio sobre Tratamento Específico da Doença de Chagas, realizado na II Reunião Anual de Pesquisa Aplicada em Doença de Chagas em 1985, a fim de possibilitar melhor avaliação da atividade de cura. Em fevereiro de 1996, a Câmara Técnica reunida em Brasília, sob o patrocínio do Programa de Controle da Doença de Chagas da FNS/MS, preocupada com o abandono da medicação específica pelos médicos, em decorrência da baixa eficácia na fase crônica e pelos escassos trabalhos mostrando a influência do tratamento na evolução da doença reiterou estas recomendações.

Os pesquisadores concordam que os critérios de cura, na fase aguda, baseiam-se na negativação do xenodiagnóstico e/ou hemocultivo assim como das reações sorológicas, o que ocorre na maioria dos casos. Entretanto, os critérios de cura, na fase crônica, parecem ser mais complexos e constituem ainda matéria de investigação científica. Um dos principais desafios para a avaliação da eficácia terapêutica é o desenvolvimento de técnicas laboratoriais eficientes para serem utilizadas como ferramenta na verificação de cura. Utilizando a técnica de sorologia, Krettli e Brener (1982) propuseram que o soro de pacientes chagásicos crônicos apresenta dois tipos de anticorpos anti-parasita com diferentes atividades funcionais, anticorpos líticos e anticorpos de sorologia convencional. Os anticorpos líticos estão associados com resistência na infecção ativa e podem ser detectados pela lise mediada pelo complemento e imunofluorescência indireta, empregando formas tripomastigotas vivas (KRETTLI & BRENER 1982; MARTINS-FILHO 1995, 2002). Já os anticorpos de sorologia convencional não estão relacionados com resistência e nem se ligam às formas tripomastigotas vivas, mas são reativos contra antígenos solúveis ou formas epimastigotas fixadas (LUQUETTI *et al*., 2000). Com base nesses achados, estudos demonstraram que os

títulos dos anticorpos líticos tornam-se gradativamente negativos após o tratamento, enquanto que os anticorpos de sorologia convencional podem permanecer positivos durante décadas (GALVÃO *et al*., 1993; MARTINS-FILHO *et al*., 2002; VITELLI-AVELAR *et al*., 2007).

A inexistência de parâmetros seguros para se determinar a eficácia da terapêutica na fase crônica pode ser responsável pelos baixos índices de cura, quando se considera a negativação sorológica convencional. Estudo realizado por Cançado *et al*. (2002) detectou cura em 76% de pacientes na fase aguda e apenas em 8% na fase crônica, empregado como critério de cura a negativação da sorologia convencional. Viotti *et al.* (1994) acompanharam por oito anos, 200 pacientes cronicamente infectados pelo *T. cruzi*, sendo 130 tratados com benzonidazol e 70 não tratados. Eles relatam que os pacientes submetidos ao tratamento, independente da negativação sorológica e ou parasitológica, apresentaram poucas alterações nos eletrocardiogramas (4,2%) comparados com os pacientes não tratados (30%), e uma baixa incidência na evolução clínica da doença (2,1% versus 17%). Recentemente, Garcia *et al*. (2005) avaliaram o efeito tratamento em camundongos com cardiomiopatia chagásica. Eles constataram que os corações dos animais submetidos ao tratamento com benzonidazol tiveram queda no parasitismo e na miocardite quando comparado com os corações dos animais não tratados. Apesar do grupo de animais infectados, submetidos ou não ao tratamento, apresentarem alterações significativas em seus eletrocardiogramas em comparação com os animais não infectados, os animais não tratados tiveram maiores alterações em relação aos tratados. Além disso, foi observada correlação entre a queda na inflamação e o parasitismo do tecido cardíaco nos animais submetidos ao tratamento, reforçando a importância do parasita no desenvolvimento da cardiomiopatia chagásica. Neste contexto, é importante ressaltar, que os eventos imunológicos desencadeados pela presença do parasita são múltiplos e incluem diversos processos efetores e reguladores que resultam em um balanço entre resistência e patogênese.

O efeito do tratamento etiológico da doença de Chagas na resposta imune tem sido investigado em estudos nos quais células mononucleares do sangue periférico (CMSP) foram incubadas na presença de antígenos do *T. cruzi*. Os dados demonstram maior produção de IFN-γ pelas CMSP no grupo de pacientes curados quando comparado com o grupo de pacientes não curados (BAHIA-OLIVEIRA *et al*., 1998), mas os autores não podem determinar se os altos níveis de IFN-γ produzidos pelas CMSP dos pacientes curados é a causa ou a conseqüência da eliminação do parasita. Entretanto, evidências levam a especular que os elevados níveis de IFN-γ atuam de forma sinergética com o quimioterápico durante o tratamento. Vale ainda ressaltar que, apesar dos elevados níveis de IFN-γ estarem associados com cura, eles também estão relacionados com as formas clínicas mais graves da doença (GOMES *et al*., 2003). Diante dessas incertezas, é necessário que se realizem estudos mais detalhados que estabeleçam os efeitos da terapêutica específica no sistema imune e na evolução da doença de Chagas.

2. OBJETIVOS

2.1. Objetivo Geral

Com o intuito de contribuir para um maior entendimento do efeito do tratamento específico durante a infecção crônica recente pelo *T. cruzi* e oferecer suporte para estudos futuros no campo da pesquisa sobre o tratamento etiológico com benzonidazol na doença de Chagas, este trabalho teve como objetivo geral:

"Avaliar o perfil fenotípico e o padrão de citocinas leucocitárias de células do sangue periférico de crianças portadoras da forma crônica indeterminada recente da doença de Chagas antes e após o tratamento com benzonidazol".

2.2. Objetivo Específico

2.2.1. Estudar de forma descritivo-analítica o perfil imunofenotípico dos leucócitos do sangue periférico de indivíduos portadores da forma crônica indeterminada recente da doença de Chagas antes e após o tratamento com benzonidazol, através dos seguintes parâmetros:

a) Distribuição das populações e subpopulações linfocitárias;

b) Distribuição percentual de células T reguladoras ($CD4^{+}CD25^{HIGH}$) e de monócitos pró-inflamatórios ($CD14^{+}CD16^{+}HLA\text{-}DR^{++}$);

c) Distribuição percentual das populações e subpopulações de linfócitos T expressando moléculas de ativação e de adesão celular;

2.2.2. Avaliar o padrão de citocinas (IL-12, IFN-γ, TNF-α, IL-4 e IL-10) produzidas *in vitro*, por leucócitos do sangue periférico de indivíduos portadores da forma crônica indeterminada recente da doença de Chagas antes e após o tratamento etiológico com benzonidazol;

2.2.3. Correlacionar os marcadores de ativação celular com o perfil de citocinas citoplasmáticas de leucócitos do sangue periférico de indivíduos portadores da forma crônica indeterminada recente da doença de Chagas, antes e após o tratamento com benzonidazol.

3. PACIENTES, MATERIAL E MÉTODOS

3.1. Pacientes

Em 2003, realizou-se inquérito sorológico para doença de Chagas envolvendo crianças em idade escolar residentes nas cidades de Berilo e José Gonçalves de Minas, estado de Minas Gerais, empregando a técnica de ELISA a partir de eluato de sangue colhido em papel de filtro. Foram identificadas 38 crianças com resultados reativos. Posteriormente, esses resultados foram confirmados por ensaios de ELISA convencional, ELISA recombinante (Biomanguinhos/FIOCRUZ), imunofluorescência indireta (IFI) e hemaglutinação indireta (HAI) em soro de sangue periférico, considerando o critério da Organização Mundial da Saúde e do Ministério da Saúde do Brasil, que recomenda a positividade em pelo menos dois testes sorológicos de princípios diferentes, para o estabelecimento do diagnóstico da doença de Chagas. Dos 38 casos avaliados, apenas seis foram confirmados, sendo quatro do sexo masculino e dois do sexo feminino com idades entre 9 e 14 anos. Exames clínicos e físicos revelaram que as crianças eram assintomáticas com eletrocardiogramas e radiografias de tórax normais (Tabela 1). As crianças infectadas foram assim classificadas como portadoras da forma crônica indeterminada recente da doença de Chagas ("Early INDeterminate chronic phase" – E-IND).

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* ("Non-Infected" – NI).

Todos os procedimentos, incluindo os estudos eletrocardiográficos, radiológicos e a assistência médico-laboratorial dada aos pacientes, bem como o tratamento das crianças infectadas com benzonidazol (8mg/kg/peso por dia, durante 60 dias consecutivos) estiveram sob a responsabilidade da Dra. Marta de Lana e do Dr. João Carlos Pinto Dias. Acrescenta-se que todas as crianças realizaram exame parasitológico de fezes e, naquelas com parasitose intestinal, o tratamento específico foi instituído pelo menos 60 dias antes do estudo.

Para o estudo longitudinal, foram feitas duas coletas (antes e após um ano do término do tratamento) de amostras de 15 mL de sangue periférico coletadas em tubos Vacutainer®, contendo EDTA ou heparina sódica como anticoagulantes.

Após a coleta, as amostras de sangue foram encaminhadas para o Laboratório de Doença de Chagas do Centro de Pesquisa René Rachou, onde foi realizada análise fenotípica e avaliação do padrão de produção de citocinas intracitoplasmáticas dos leucócitos por citometria de fluxo.

Este estudo cumpriu as disposições da resolução 196/96, do Conselho Nacional de Saúde, que regulamenta a pesquisa envolvendo seres humanos, tendo sido aprovado pelo Comitê de Ética em Pesquisa da Fundação Oswaldo Cruz – FIOCUZ (Protocolo 11/2004 – Anexo). Os pais ou responsáveis da crianças foram esclarecidos quanto ao estudo e, aqueles que concordaram em participar, assinaram o Termo de Consentimento Livre e Esclarecido para Pacientes Voluntários (Anexo). A Figura 2 mostra de forma esquemática o desenho do estudo.

Tabela 1: Características demográficas e clínico laboratoriais das seis crianças soropositivas.

Dados	**Municípios**					
	Berilo			**José Gonçalves de Minas**		
Nº de Registro	#52	#1172	#1313	#701	#477	#499
Idade	9	12	13	11	13	14
Sexo	M	M	F	M	M	F
Avaliação Clínica e Física	Normal	Normal	Normal	Normal	Normal	Normal
Exame de Raio-X de tórax	Normal	Normal	Normal	Normal	Normal	Normal
Ecocardiograma	Normal	Normal	Normal	Alterado	Normal	Normal
Hemocultivo	Positiva	Positiva	Positiva	Positiva	Positiva	___

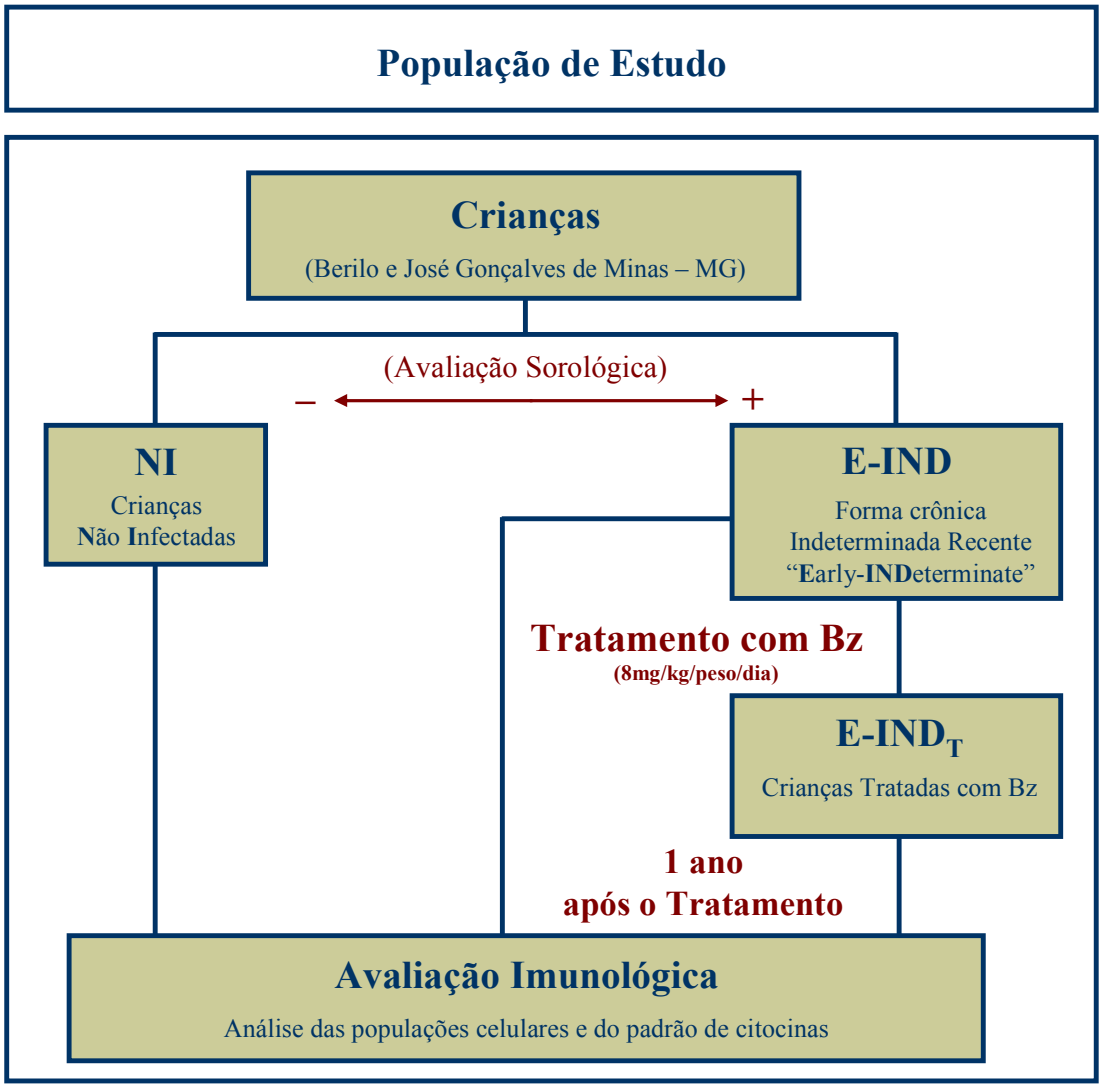

Figura 2: Desenho do estudo

3.2. Obtenção de extrato antigênico das formas epimastigotas do *T. cruzi* (EPI)

As formas epimastigotas do *T. cruzi* da cepa Y, criopreservadas em nitrogênio líquido no criobanco do laboratório de Doença de Chagas do Centro de Pesquisa René Rachou – CPqRR, Belo Horizonte/MG, foram descongeladas e cultivadas em frasco de cultura contendo 50 mL de meio de cultura LIT (Liver Infusion Tryptose), por 7 dias, em estufa B.O.D. (modelo 347) a temperatura de 28 ± 1ºC. Após atingirem a fase estacionária de crescimento, a suspensão de formas epimastigotas foi submetida ao teste de viabilidade pela técnica de coloração pelo "trypan blue". Após constatação da viabilidade celular e da ausência de agentes contaminantes, as formas epimastigotas foram recuperadas por centrifugação a 800×g, durante 10 minutos a 4ºC e submetidas a duas lavagens com tampão fosfato salino – PBS 0,015M, pH 7,4. Em seguida, os parasitas foram ressuspensos para 10^8 células/mL. Para obtenção do antígeno solúvel, os parasitas foram então submetidos a três processos de congelamento (-70ºC por 2 minutos) e descongelamento (37ºC por 10 minutos), intercalados com processos de sonicação de 30 segundos, utilizando-se um homogeneizador de tecido com pistão de teflon (Wirtis, DT). Posteriormente, o lisado de parasitas foi centrifugado a 37.000×g, durante 90 minutos a 4ºC. O sobrenadante foi coletado e dialisado em PBS, durante 72 horas a 4ºC e, em seguida, esterilizado por filtração em membrana de 0,22μm (Filter milex HA, E.U.A). A quantificação do conteúdo protéico resultante da preparação do antígeno solúvel da forma epimastigota do *T. cruzi* foi determinada pelo método de LOWRY *et al*. (1951). A preparação antigênica foi conservada a -70ºC em alíquotas de 250μg/mL, para uso posterior nos ensaios de avaliação do perfil de produção de citocinas após estimulação curta antígeno-específica *in vitro*.

3.3. Análise do fenótipo celular dos leucócitos do sangue periférico

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 50µL 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, 6,67g/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.

3.4. Avaliação do padrão de produção de citocinas citoplasmáticas após estimulação celular *in vitro* induzida por antígenos solúveis da forma epimastigota (EPI) do *T. cruzi*

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 epimastigotas do *T. cruzi* (EPI).

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 contendo 5% de CO_2 a 37^{o}C. Utiliza-se a BFA para inibir a secreção da citocina, mantendo-a no interior do complexo de Golgi.

A cultura estimulada com antígeno solúvel foi previamente incubada, por 1 hora em estufa contendo 5% de CO_2 a 37^{o}C, na presença de 100µL de EPI numa concentração final de 25µg/mL. Em seguida foram adicionados 10µL de BFA, numa concentração de 10 µg/mL e a cultura re-incubada por 4 horas em estufa contendo 5% de CO_2 a 37^{o}C.

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. Posteriormente ao tratamento com EDTA, as células 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 homogeneizar bem a suspensão celular, procedeu-se à identificação das populações celulares através da marcação de moléculas de superfície específicas. Para tanto, 400µL da suspensão

celular foram adicionados em 2 tubos contendo 3µL de anticorpo monoclonal de camundongo anti-CD4 TC ou anti-CD8 TC; ou 200µL em 3 tubos contendo 3µL de anticorpo monoclonal de camundongo anti-CD14 TC, 9µL de anti-CD16 TC ou 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 por 20 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, anti-CD16 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 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-IL-12, anti-IFN-γ, anti-TNF-α, anti-IL-4 e anti-IL-10 – Tabela 4) e 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 e estocadas a 4ºC ao abrigo da luz até a sua leitura no citômetro de fluxo dentro de 24 horas.

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

Anticorpos	Fluorocromo	Clone	Fenótipo Alvo no Estudo
Anti-CD3	FITC ou PE	UCHT1	Linfócitos T
Anti-CD4	FITC, PE ou TC	RPA-T4	Linfócitos T auxiliares
Anti-CD5	FITC	L17F12	Linfócitos B1
Anti-CD8	FITC ou TC	RPA-T8	Linfócitos T citotóxicos
Anti-CD14	TC	TüK4	Monócitos
Anti-CD16	FITC ou TC	3G8	Células NK, “Macrófagos-like”
Anti-CD18	FITC	YF118.3	Linfócito T ativado
Anti-CD19	PE ou TC	4G7, SJ25-C1	Linfócitos B
Anti-CD23	PE	M-L233	Subpopulações de Linfócito B
Anti-CD25	PE	3G10	Célula T reguladora
Anti-CD28	FITC	15E8	Linfócito T ativado
Anti-CD38	PE	AT13/5	Linfócito T ativado
Anti-CD54	PE	15.2	Linfócito T ativado
Anti-CD56	PE	B159	Células NK
Anti-CD62L	FITC	DREG-56	Linfócito T ativado
Anti-HLA-DR	PE	Tü36	LT ativados e Monóc. pró-inflamatórios

Tabela 3: Anticorpos monoclonais utilizados para identificação das subpopulações leucocitárias na metodologia de citocinas citoplasmáticas

Anticorpos	Fluorocromo	Fabricante	Clone	Fenótipo Alvo no Estudo
Anti-CD4	TC	Caltag	RPA-T4	Linfócitos T auxiliares
Anti-CD8	TC	Caltag	RPA-T8	Linfócitos T citotóxicos
Anti-CD14	TC	Caltag	TüK4	Monócitos
Anti-CD16	TC	Caltag	3G8	Células NK
Anti-CD19	TC	Caltag	SJ25-C1	Linfócitos B

Tabela 4: Anticorpos monoclonais utilizados para identificação de citocinas intracelulares em subpopulações leucocitárias.

Anticorpos	Fabricante	Clone	Concentração
Anti-TNF-α PE	Pharmingen	MAB11	0,25μg/mL
Anti-IL-12p40/p70 PE	Pharmingen	C11.5.14	0,25μg/mL
Anti-IFN-γ PE	Pharmingen	B27	0,25μg/mL
Anti-IL-4 PE	Pharmingen	MP4-25D2	0,25μg/mL
Anti-IL-10 PE	Pharmingen	JES3-9D7	0,25μg/mL

3.5. Estratégias de análises dos resultados

Os dados obtidos foram analisados utilizando diferentes estratégias, dependendo do fenótipo celular a ser analisado: análise convencional (SATHLER-AVELAR, 2003), análise de células NK (GADDY *et al*., 1997), análise de células NKT (DOHERTY *et al*., 1999), análise de monócitos pró-inflamatórios (BELGE *et al*., 2002), análise de células T reguladoras (BAECHER-ALLAN *et al*., 2001), análise combinada "gated" (SATHLER-AVELAR, 2003) e análise semiquantitativa (MARTINS-FILHO, 2000).

3.5.1. Análise convencional

A análise convencional foi realizada segundo estratégia proposta por Sathler-Avelar (2003). A Figura 3 ilustra a seqüência de passos para a análise convencional. Esse tipo de análise consistiu na seleção da população celular de interesse em gráficos de distribuição pontual de tamanho (FSC) *versus* granulosidade (SSC) (Figura 3A). Após a seleção da região de interesse (R1), a freqüência de subpopulações celulares fluorescentes, dentro de R1, foi obtida em gráficos bidimensionais de distribuição pontual de fluorescência, incluindo as modalidades *FL1 versus FL2, FL2 versus FL3 ou FL1 versus FL3* (Figura 3B).

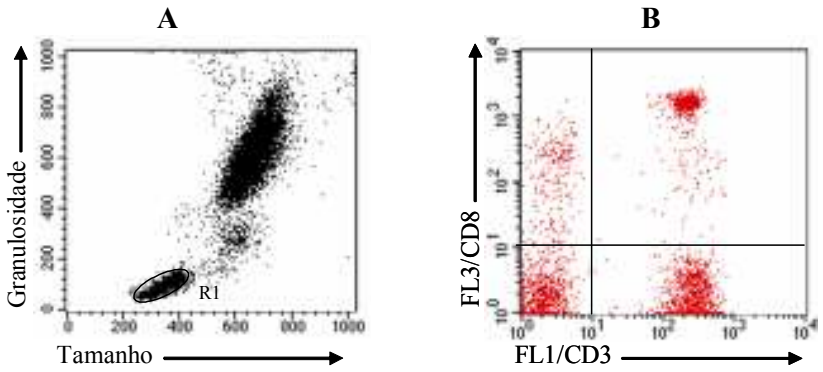

Figura 3: Análise convencional para a quantificação dos percentuais de populações celulares por citometria de fluxo. (A) Gráfico de distribuição pontual FSC *versus* SSC utilizado para a seleção da população de linfócitos – R1. (B) Gráfico de distribuição pontual FL1 *versus* FL2 utilizado para quantificar o percentual das populações celulares específicas em R1.

3.5.2. Análise de células NK

A análise de subpopulações de células $CD3^-CD16^+$ foi realizada segundo protocolo proposto por Gaddy *et al*. (1997). A Figura 4 ilustra a seqüência de procedimentos para a análise das subpopulações de células pré-NK ($CD3^-CD16^+CD56^-$), NK maduras ($CD3^-CD16^+CD56^+$) e NK ativadas ($CD3^-CD16^-CD56^+$). Esse tipo de análise consistiu na seleção da população celular de interesse em gráficos de distribuição pontual de tamanho (FSC) *versus* granulosidade (SSC) (Figura 4A). Após a escolha da região de interesse (R1), foram construídos gráficos de FL1/CD3 *versus* FL3/CD16, onde uma nova região R2 foi

determinada para a população $CD3^{-}CD16^{+}$ (Figura 4B). Posteriormente, gráficos de FL1/CD3 *versus* FL2/CD56 foram empregados, onde uma nova região (R3) foi determinada para a população $CD3^{-}CD56^{+}$ (Figura 4C). 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 interseção dos eventos presentes simultaneamente em G2 e R1. Posteriormente gráficos de FL3/CD16 *versus* FL2/CD56, contendo as células em G3, foram utilizados para quantificar os percentuais das populações $CD3^{-}CD16^{+}CD56^{-}$, $CD3^{-}CD16^{+}CD56^{+}$ e $CD3^{-}CD16^{-}CD56^{+}$ (Figura 4D).

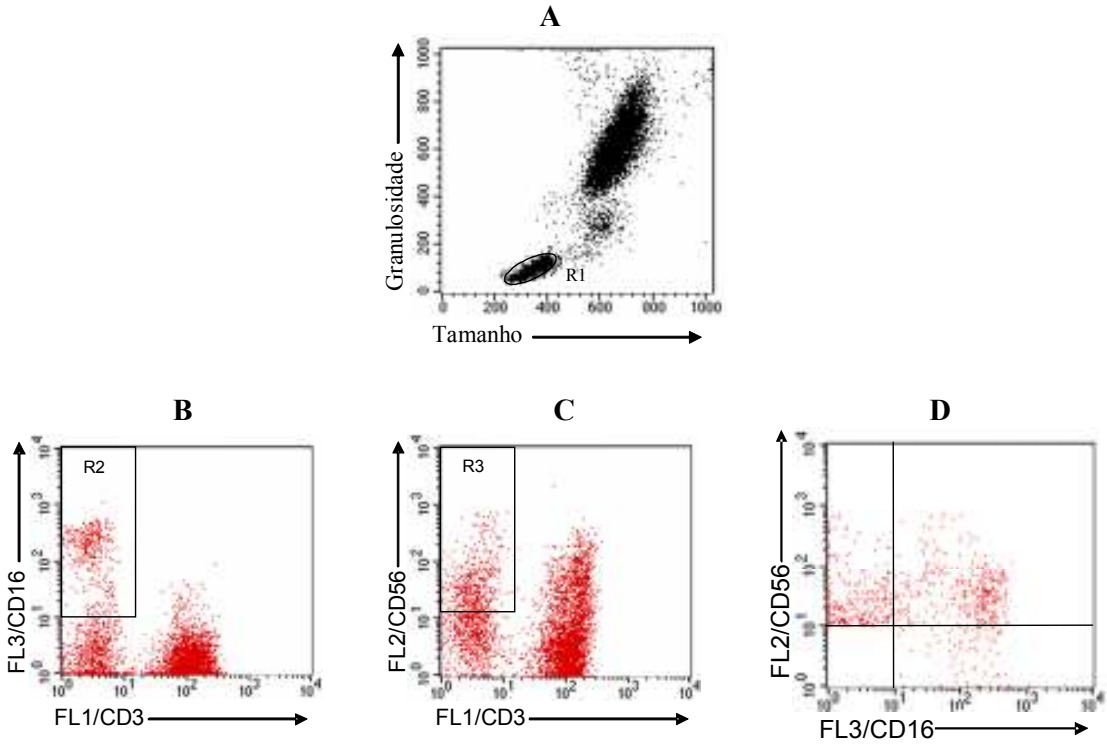

Figura 4: Análises dos percentuais das subpopulações de células NK: pré-NK ($CD3^{-}CD16^{+}CD56^{-}$) e NK madura ($CD3^{-}CD16^{+}CD56^{+}$) por citometria de fluxo. (A) Gráfico de distribuição pontual FSC *versus* SSC utilizado para a seleção da população de linfócitos – R1. (B) Gráfico de distribuição pontual FL1/CD3 *versus* FL3/CD16 para selecionar a população celular $CD3^{-}CD16^{+}$ (R2). (C) Gráfico de distribuição pontual FL1/CD3 *versus* FL2/CD56 para selecionar a população celular $CD3^{-}CD56^{+}$ (R3). (D) Gráfico de FL3/CD16 *versus* FL2/CD56 contendo as células em "G3=R1 "and" G2(R2+R3) para quantificar os percentuais das subpopulações de células NK.

3.5.3. Análise de célula NKT

A análise de células NKT foi feita segundo protocolo proposto por Doherty *et al*. (1999). A Figura 5 ilustra a seqüência de procedimentos para a análise de células NKT ($CD3^{+}CD16^{-/+}CD56^{-/+}$). Após a seleção da região de interesse (R1), em gráficos de distribuição pontual de tamanho (FSC) *versus* granulosidade (SSC) (Figura 5A), foram construídos gráficos de FL1/CD3 *versus* FL3/CD16, onde a região (R2) foi delimitada selecionando a população $CD3^{+}CD16^{-/+}$ (Figura 5B). 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 utilizadas para quantificar o percentual das subpopulações de células NKT (Figura 5C): NKT1 ($CD3^{+}CD16^{+}CD56^{-}$), NKT2 ($CD3^{+}CD16^{-}CD56^{+}$) e NKT3 ($CD3^{+}CD16^{+}CD56^{+}$).

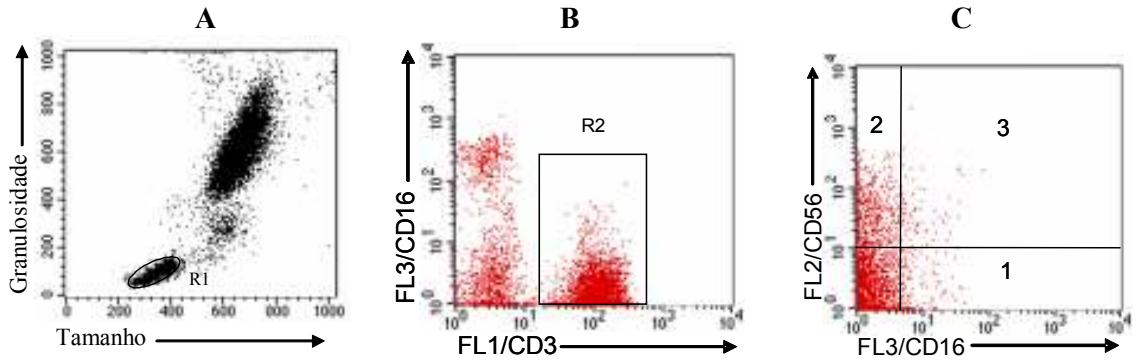

Figura 5: Análises do percentual das subpopulações de células NKT por citometria de fluxo. (A) Gráfico de distribuição pontual de tamanho (FSC) *versus* granulosidade (SSC) utilizado para a seleção da população de interesse, nesse caso linfócitos pequenos – R1. (B) Gráfico de distribuição pontual FL1/CD3 *versus* FL3/CD16 (R2) utilizado para selecionar a população celular $CD3^{+}$ - R2. (C) Gráfico de FL3/CD16 *versus* FL2/CD56, contendo as células confinadas em "G2=R1 and R2", para quantificar o percentual das subpopulações de células NKT ($CD3^{+}CD16^{-/+}CD56^{-/+}$).

3.5.4. Análise de monócitos pró-inflamatórios

A análise de monócitos pró-inflamatórios ($CD14^{+}CD16^{+}HLA\text{-}DR^{++}$) foi feita segundo protocolo proposto por Belge *et al.* (2002). A Figura 6 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, determinadas através de gráficos de distribuição pontual de FL3/CD14 *versus* granulosidade (SSC) (Figura 6A), foram construídos gráficos de FL3/CD14 *versus* FL1/CD16, onde uma região (R2) foi determinada para a seleção da população $CD14^{+}CD16^{+}$ (Figura 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, gráficos de FL3/CD14 *versus* FL2/HLA-DR, contendo as células em G2, foram utilizados para quantificar o percentual de células $CD14^{+}CD16^{+}HLA\text{-}DR^{++}$ (Figura 6C).

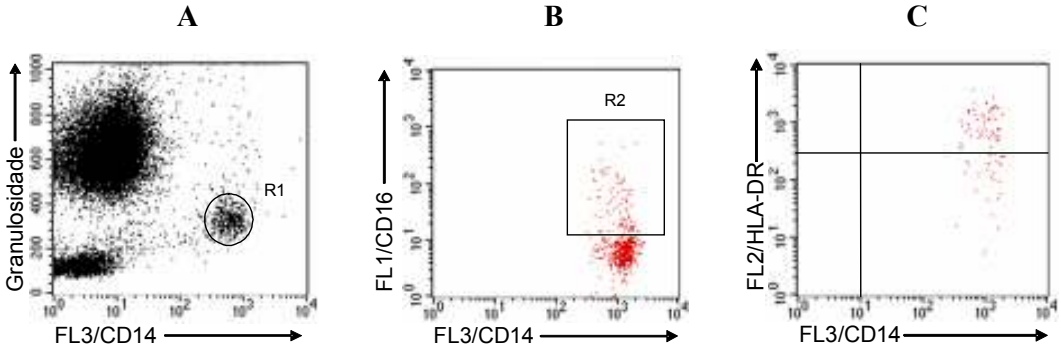

Figura 6: Análises dos percentuais de monócitos pró-inflamatórios ($CD14^{+}CD16^{+}HLA\text{-}DR^{++}$) por citometria de fluxo. (A) Gráfico de distribuição pontual FL3/CD14 *versus* granulosidade (SSC) utilizado para a seleção da população de monócitos – R1. (B) Gráfico de distribuição pontual FL3/CD14 *versus* FL1/CD16 (R2) para selecionar a população $CD14^{+}CD16^{+}$ - R2. (C) Gráfico FL3/CD14 *versus* FL2/HLA-DR, contendo as células em "G2=R1and R2", para quantificar o percentual de monócitos pró-inflamatórios.

3.5.5. Análise de células T reguladoras

A análise de células T reguladoras foi realizada segundo protocolo proposto por Baecher-Allan *et al.* (2001). A Figura 7 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), em gráficos de distribuição pontual de tamanho (FSC) *versus* granulosidade (SSC) (Figura 7A), foram construídos gráficos de FL1/CD4 *versus* FL2/CD25, 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 em R4 representa o valor percentual de células T reguladoras na população de linfócitos totais (Figura 7B).

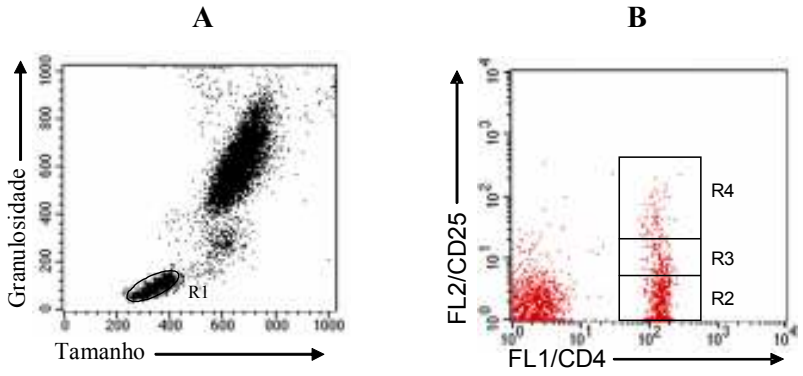

Figura 7: Análises dos percentuais de células T reguladoras ($CD4^+CD25^{HIGH}$) por citometria de fluxo. (A) Gráfico de distribuição pontual FSC *versus* SSC para a seleção de linfócitos–R1. (B) Gráfico de distribuição pontual FL1/CD4 *versus* FL2/CD25 para quantificar o percentual de células T reguladoras.

3.5.6. Análise combinada "gated"

A análise combinada "gated" foi realizada segundo protocolo proposto por Sathler-Avelar *et al.* (2003). A Figura 8 ilustra a seqüência de procedimentos para a análise de subpopulações de células T ativadas ($CD4^+HLA\text{-}DR^+/CD4^+$ e $CD8^+HLA\text{-}DR^+/CD8^+$) e subpopulações de linfócitos B ($CD19^+CD5^+/CD19^+$ e $CD19^+CD23^+/CD19^+$). Após a seleção da região de interesse (R1), em gráficos de distribuição pontual de tamanho (FSC) *versus* granulosidade (SSC) (Figura 8A), foi estabelecida uma nova região de interesse (R2), selecionada como subpopulação celular dentro de R1, empregando histogramas unidimensionais de fluorescência (Figura 8B). O próximo passo consistiu na combinação das duas regiões selecionadas (R1 "and" R2). O resultado final do percentual da subpopulação celular fluorescente foi identificado em histograma unidimensional de fluorescência – M1 (Figura 8C).

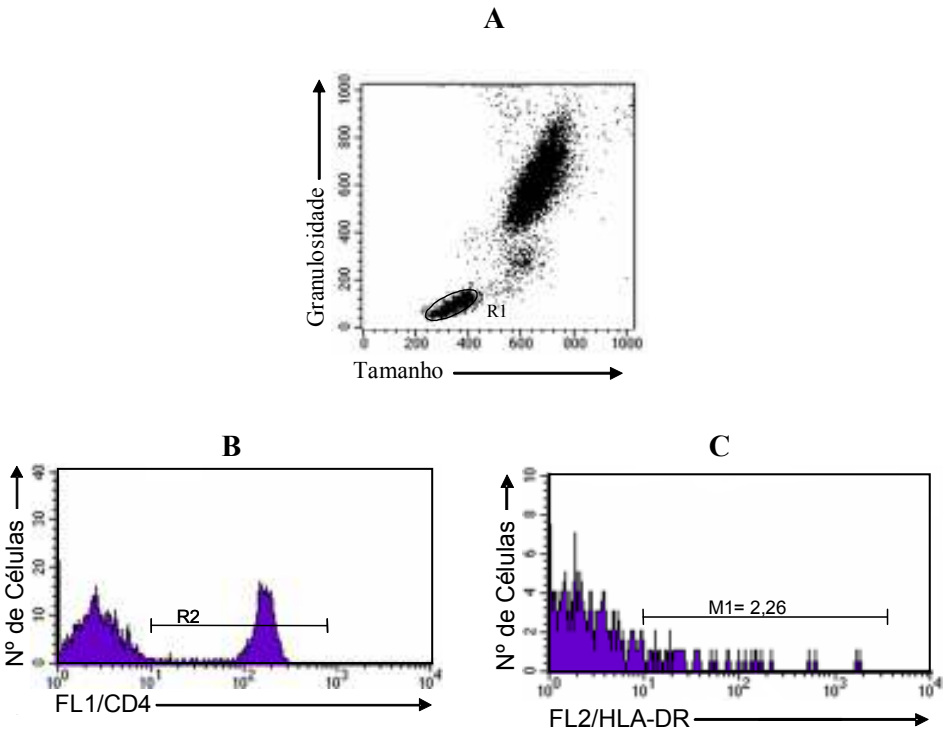

Figura 8: Seqüência de procedimentos utilizados para a análise do percentual da subpopulação de linfócito T ativado ($CD4^+HLA\text{-}DR^+/CD4^+$) por citometria de fluxo. (A) Gráfico de distribuição pontual FSC *versus* SSC utilizado para a seleção da população de interesse, nesse caso linfócitos pequenos – 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.

3.5.7. Análise da produção de citocinas por linfócitos

A análise da produção de citocinas pelas células NK, linfócitos T e B foi feita através da análise convencional. Em gráficos de distribuição pontual de tamanho (FSC) versus granulosidade (SSC) foi estabelecida a população celular de interesse (R1) (Figura 9A), no caso linfócitos. Após a seleção da região de interesse, a freqüência de subpopulações celulares produtoras de citocinas, dentro de R1, foi obtida em gráficos bidimensionais de distribuição pontual de fluorescência FL3 *versus* FL2 (Figura 9B).

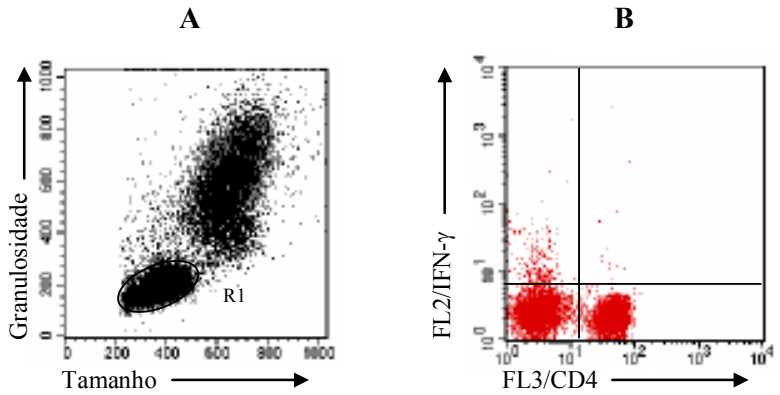

Figura 9: Análise da produção de citocinas citoplasmáticas por células NK, linfócitos T e B. (A) Gráfico de distribuição pontual FSC *versus* SSC utilizado para a seleção da população de linfócitos – R1. (B) Gráfico de distribuição pontual FL3 *versus* FL2 utilizado para quantificar o percentual de células produtoras de citocinas em R1.

3.5.8. Análise da produção de citocinas por 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 10A). 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 (Figura 10B).

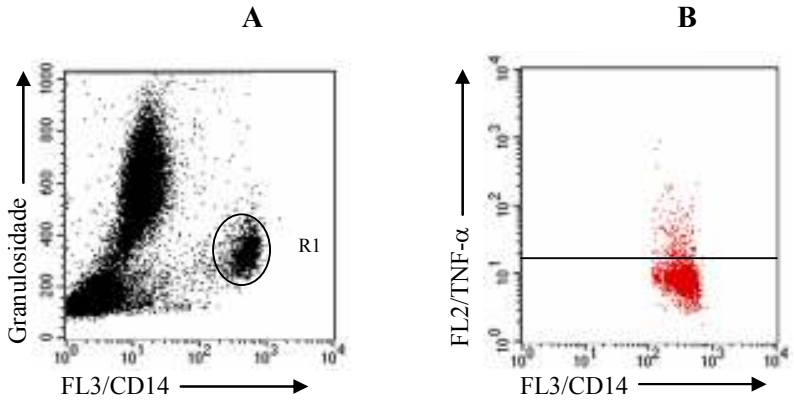

Figura 10: Análise da produção de citocinas citoplasmáticas por monócitos (A). Gráfico de distribuição pontual FL3/anti-CD14 TC *versus* SSC utilizado para a seleção da população de monócitos – R1. (B) Gráfico de distribuição pontual FL3 *versus* FL2 utilizado para quantificar o percentual de células produtoras de citocinas em R1.

3.6. Análises Estatísticas

As análises estatísticas 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, sendo empregados testes t não pareado ou pareado, usando o programa estatístico Prisma (versão 3.0). As demais amostras foram consideradas não paramétricas e os testes empregados foram Mann-Whitney para dados pareados ou Wilcoxon para os não pareados. As diferenças foram consideradas significativas quando o $p<0,05$.

4. RESULTADOS

4.1. Análise do perfil fenotípico leucocitário de crianças portadoras da forma crônica indeterminada recente da doença de Chagas antes e após tratamento com benzonidazol

4.1.1. Distribuição das populações leucocítárias

a) Distribuição percentual das populações de linfócitos T, B e células NK

Para avaliar o percentual de linfócitos T, B e células NK na população de linfócitos totais circulantes de crianças com a forma crônica indeterminada recente da doença de Chagas, antes e após o tratamento etiológico com benzonidazol e de crianças não infectadas, foram realizados ensaios de dupla marcação e utilizada a estratégia de análise convencional, como descrito em Material e Métodos**.**

Os resultados do percentual de linfócitos T ($CD3^+$) na população de linfócitos totais circulantes estão representados na Figura 11A. A análise dos dados mostrou que o grupo E-IND apresenta percentual de células T significativamente menor ($p<0,05$) em relação ao grupo NI (NI = 76,5±6,5%; E-IND = 67,1±7,5%). Nossos dados demonstram ainda queda significativa no percentual de células T no grupo E-IND_T em relação aos grupos E-IND e NI (E-IND_T = 56,2±7,2%).

Na Figura 11B estão representados os resultados do percentual de linfócitos B ($CD19^+$) na população de linfócitos totais circulantes. A análise dos resultados mostrou que o grupo E-IND_T apresenta percentual de linfócitos B $CD19^+$ significativamente maior ($p<0,05$) em relação ao grupo NI. A análise entre os grupos de indivíduos infectados antes e após o tratamento com Bz não mostrou diferença significativa (NI = 13,1±3,5%; E-IND = 15,8±3,1%; E-IND_T = 20,5±7,5%).

A Figura 11C mostra o resultado do percentual de células NK totais ($CD3^-CD16^{-/+}CD56^{-/+}$) na população de linfócitos totais circulantes. A análise dos resultados revelou que apenas o grupo E-IND_T apresenta valor percentual de células NK totais significativamente

maior ($p<0,05$) em relação aos grupos NI e E-IND (NI = 7,8±1,6%; E-IND = 11,1±7,4%; E-IND_T = 22,9±10,6%).

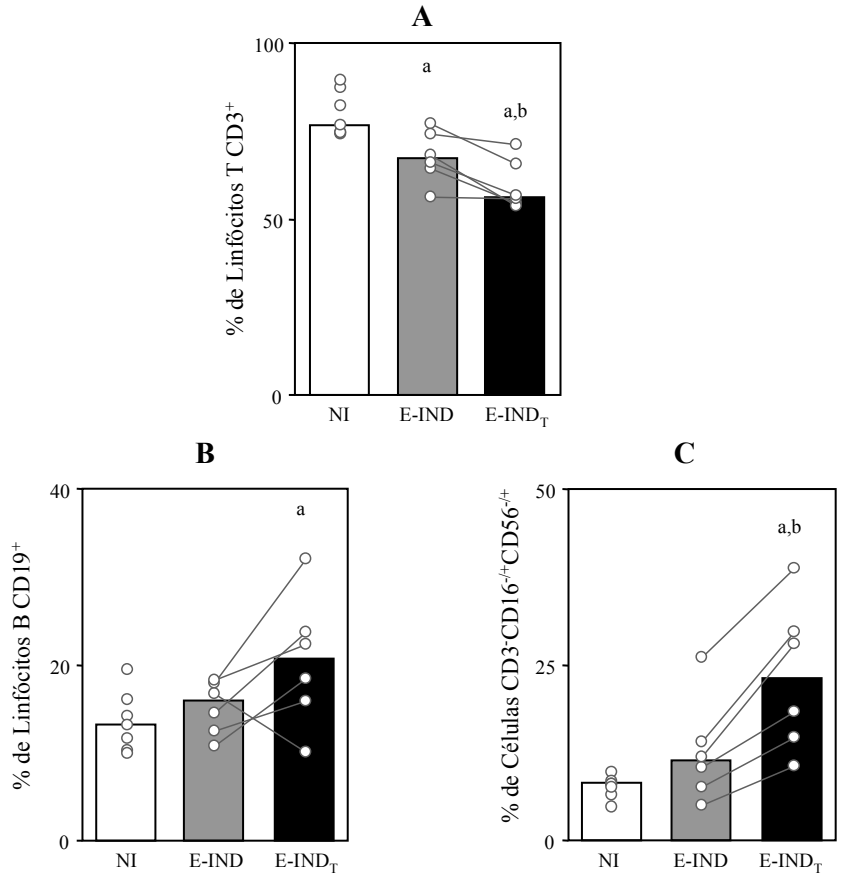

Figura 11: Análise das células T (A), B (B) e NK (C) do sangue periférico de crianças infectadas, antes (E-IND ▭) e após o tratamento com benzonidazol (E-IND_T ■); e de indivíduos não infectados (NI □). As populações celulares foram identificadas utilizando anticorpos monoclonais anti-CD3/FITC (linfócitos T), anti-CD19/TC (linfócitos B) e anti-CD16/TC e ou anti-CD56/PE (células NK), como descrito em Material e Métodos. Os resultados estão expressos como dispersão dos valores individuais e mediana dos percentuais para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente.

b) Distribuição percentual das subpopulações de linfócitos T

Para avaliar o efeito do tratamento no percentual das subpopulações de linfócitos T do sangue periférico foram realizados ensaios de dupla marcação e estratégia convencional para identificar os linfócitos T auxiliares ($CD3^+CD4^+$) e T citotóxicos ($CD3^+CD8^+$)**.**

A Figura 12 mostra os resultados do percentual de linfócitos T $CD4^+$ e T $CD8^+$ na população de linfócitos totais do sangue periférico de crianças infectadas e não infectadas. A análise dos resultados não demonstrou nenhuma diferença significativa ($p>0,05$) na população de linfócitos T $CD4^+$ (NI = 43,6±5,9%; E-IND = 40,8±8,0%; E-IND_T = 36,6±6,2%) (Figura 12A). A análise do percentual dos linfócitos T $CD8^+$ revelou percentual significativamente menor ($p<0,05$) nas crianças infectadas antes e após o tratamento com Bz quando comparada com a das crianças não infectadas. (NI = 30,8±7,7%; E-IND = 22,9±3,4%; E-IND_T = 18,7±5,5%). A análise entre os grupos de crianças infectadas não revelou diferença significativa na freqüência de linfócitos T $CD8^+$ no grupo E-IND_T em relação ao grupo E-IND (Figura 12B).

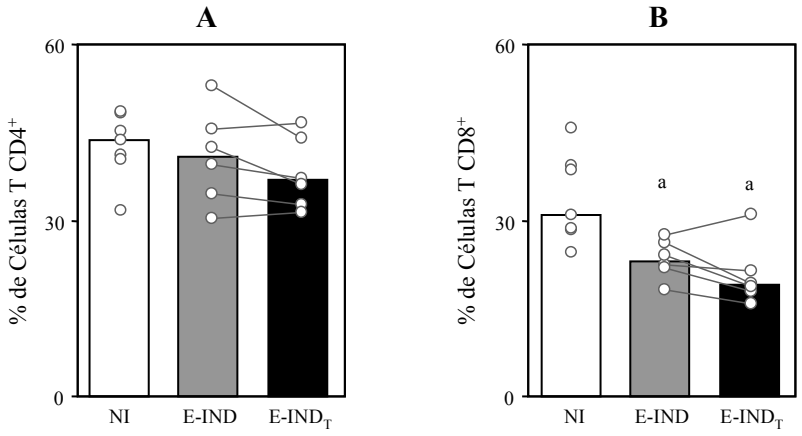

Figura 12: Análise do percentual de células T $CD4^+$ (A) e T $CD8^+$ (B) do sangue periférico de crianças infectadas, antes (E-IND ▭) e após o tratamento com benzonidazol (E-IND_T ■); e de indivíduos não infectados (NI ▭). As populações celulares foram identificadas utilizando anticorpos monoclonais anti-CD4/FITC (linfócitos T auxiliares) e anti-CD8/TC (linfócitos T citotóxicos), como descrito em Material e Métodos. Os resultados estão expressos como dispersão dos valores individuais e mediana dos percentuais para cada grupo avaliado. A diferença significativa ($p<0,05$) está representada pela letra "a" em comparação com o grupo NI.

Para avaliar a fração de subpopulações de células NKT ($CD3^+CD16^{-/+}56^{-/+}$) dentro da população de linfócitos $CD3^+$ do sangue periférico foram realizados ensaios de tripla marcação e utilizada a estratégia de análise para as células NKT, segundo proposto por Doherty *et al*. (1997), como descrito em Material e Métodos.

A Figura 13A mostra os resultados da fração de células NKT1 ($CD3^+CD16^+CD56^-$) na população de linfócitos $CD3^+$ do sangue periférico. A análise dos dados revelou um aumento no percentual de células NKT1 no grupo E-IND_T quando comparado com o grupo E-IND. Nenhuma diferença foi encontrada entre o grupo de crianças infectadas, antes e após o tratamento, e o grupo de crianças não infectadas (NI = 2,4±0,9%; E-IND = 1,0±1,2%; E-IND_T = 2,9±2,1%).

A Figura 13B mostra os resultados da fração de células NKT2 ($CD3^+CD16^-CD56^+$) na população de linfócitos $CD3^+$ circulantes. A análise dos dados revelou redução estatisticamente significativa ($p<0,05$) do percentual de células NKT2 nas crianças infectadas antes e após o tratamento quando comparado com o das crianças não infectadas. (NI = 16,1±13,7%; E-IND = 3,5±0,9%; E-IND_T = 4,2±2,3%). A análise entre os grupos de crianças infectadas não revelou nenhuma diferença estatisticamente significativa.

A Figura 13C mostra os resultados da fração de células NKT3 ($CD3^+CD16^+CD56^+$) na população de linfócitos $CD3^+$ circulantes. A análise do percentual de células NKT3 mostrou aumento estatisticamente significativo ($p<0,05$) apenas no grupo E-IND em relação ao grupo E-IND_T (NI = 1,2±0,9%; E-IND = 2,4±2,6%; E-IND_T = 0,4±0,8%).

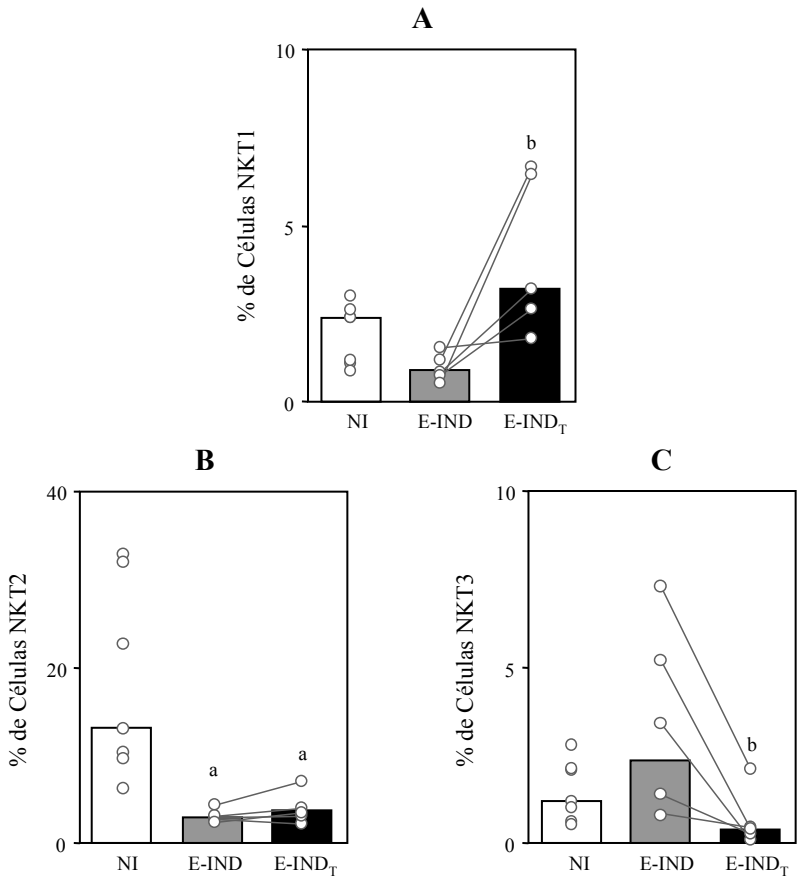

Figura 13: Percentual de células NKT1 ($CD3^+CD16^+CD56^-$) (A), NKT2 ($CD3^+CD16^-CD56^+$) (B) e NKT3 ($CD3^+CD16^+CD56^+$) (C) em linfócitos $CD3^+$ do sangue periférico de crianças infectadas, antes (E-IND ▒) e após o tratamento com benzonidazol (E-IND$_T$ ■); e de indivíduos não infectados (NI □). As populações celulares foram identificadas em ensaios de tripla marcação utilizando anticorpos monoclonais anti-CD3/FITC, anti-CD56/PE e anti-CD16/TC, como descrito em Material e Métodos. Os resultados estão expressos como dispersão dos valores individuais e mediana dos percentuais para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente.

c) Distribuição percentual das subpopulações de linfócitos B

As subpopulações de linfócitos B circulantes foram identificadas em ensaios de dupla marcação utilizando anticorpos monoclonais anti-CD19/TC e anti-CD5/FITC, para identificar as células B convencionais ($CD19^+CD5^-$) e as células B1 ($CD19^+CD5^+$) na população de linfócitos totais. Para a análise das subpopulações celulares foi utilizada a estratégia combinada "*gated*", como descrito em Material e Métodos.

Os resultados do percentual de células B convencionais e linfócitos B1 na população de linfócitos totais circulantes estão representados na Figura 14A e 14B, respectivamente. A análise da fração de células B convencionais (NI = 8,2±3,6%; E-IND = 12,9±4,0%; E-IND_T = 15,4±6,4%) e linfócitos B1 (NI = 3,5±2,1%; E-IND = 2,6±4,2%; E-IND_T = 5,3±4,5%) revelou um aumento estatisticamente significativo no grupo E-IND_T quando comparado com o grupo E-IND (Figura 15A e 14B).

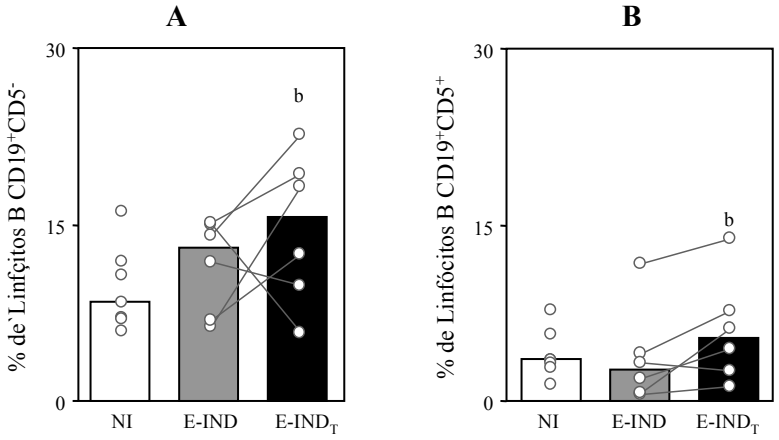

Figura 14: Análise do percentual de subpopulações de linfócitos B na população de linfócitos totais do sangue periférico de crianças infectadas, antes (E-IND ▭) e após o tratamento com benzonidazol (E-IND_T ■); e de indivíduos não infectados (NI □). As populações celulares foram identificadas em ensaios de dupla marcação utilizando anticorpos monoclonais anti-CD19/TC e anti-CD5/FITC para identificar as células $CD19^+CD5^-$ (A) e linfócitos B1 $CD19^+CD5^+$ (B) dentro da população de linfócitos totais, como descrito em Material e Métodos Os resultados estão expressos como dispersão dos valores individuais e mediana dos percentuais para cada grupo avaliado. A diferença significativa ($p<0,05$) está representada pela letra "b" em comparação com o grupo E-IND.

d) Distribuição percentual das subpopulações de linfócitos NK

Para quantificar os percentuais das subpopulações de células NK (Pré-NK – $CD3^-CD16^+CD56^-$, NK maduras – $CD3^-CD16^+CD56^+$ e NK ativadas – $CD3^-CD16^-CD56^+$) na população de células NK do sangue periférico foram realizados ensaios de tripla marcação e utilizada a estratégia de análise para as células NK, segundo proposto por Gaddy *et al.* (1997), como descrito em Material e Métodos. Os resultados da fração das células pré-NK, NK maduras e NK ativadas na população das células NK do sangue periférico estão representados na Figura 15.

A análise do percentual das células Pré-NK revelou freqüência significativamente maior ($p<0,05$) nas crianças infectadas antes e após o tratamento com Bz quando comparada com a das crianças não infectadas (NI = 9,4±3,2%; E-IND = 20,4±16,2%; $E\text{-}IND_T$ = 38,1±13,9%). A análise no grupo de crianças infectadas, antes e após o tratamento com Bz, revelou aumento significativa na freqüência de células pré-NK no grupo $E\text{-}IND_T$ em relação ao grupo E-IND (Figura 15A). Entretanto, a análise do percentual de células NK maduras não revelou diferenças estatisticamente significativas entre os grupos avaliados (NI = 54,8±8,9%; E-IND = 49,1±14,2%; $E\text{-}IND_T$ = 49,8±15,7%) (Figura 15B). Com relação à fração de células NK ativadas, foi observada queda significativa ($p<0,05$) no grupo $E\text{-}IND_T$ em relação ao grupo E-IND e NI (NI = 30,8±8,7%; E-IND = 24,0±11,8%; $E\text{-}IND_T$ = 9,2±3,1%) (Figura 15C).

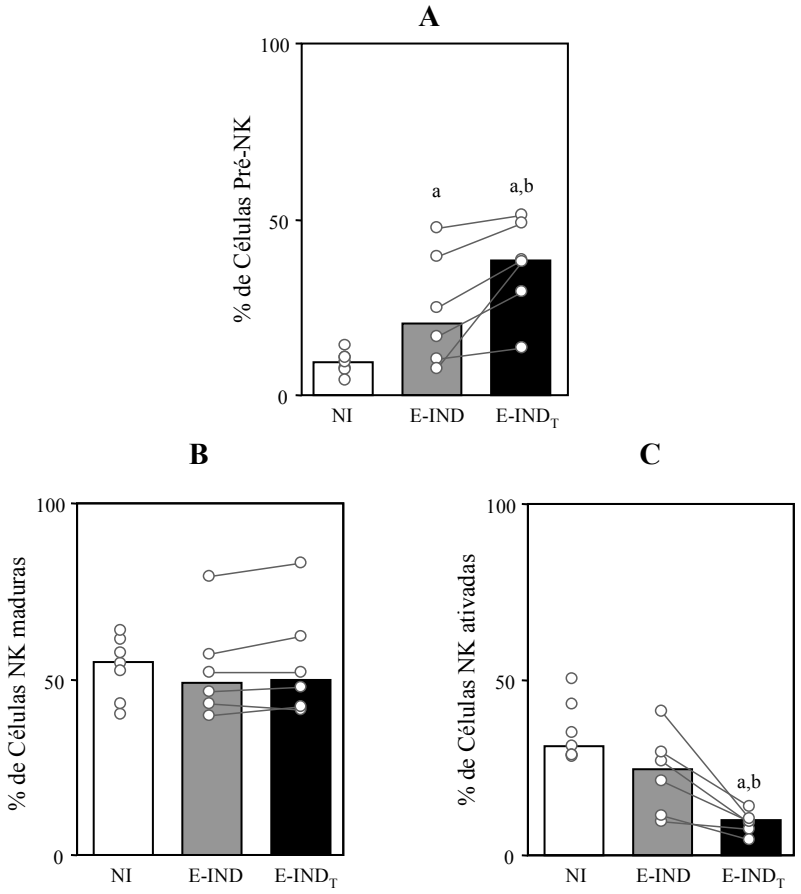

Figura 15: Análise do percentual de células pré-NK (A), NK maduras (B) e NK ativadas (C) do sangue periférico de crianças infectadas, antes (E-IND ▭) e após o tratamento com benzonidazol (E-IND$_T$ ■); e de indivíduos não infectados (NI □). As populações celulares foram identificadas em ensaios de tripla marcação utilizando anticorpos monoclonais anti-CD3/FITC, anti-CD56/PE e anti-CD16/TC, para identificar as células pré-NK ($CD3^-CD16^+CD56^-$), NK maduras ($CD3^-CD16^+CD56^+$) e NK ativadas ($CD3^-CD16^-CD56^+$), dentro da população de células $CD3^-CD16^{-/+}CD56^{-/+}$, como descrito em Material e Métodos. Os resultados estão expressos como dispersão dos valores individuais e mediana dos percentuais para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente.

4.1.2. Estudo da freqüência de populações e subpopulações de linfócitos ativados

a) Análise da freqüência de subpopulações leucocitárias $CD69^+$

Para avaliar o percentual de células $CD69^+$ nas subpopulações leucocitárias do sangue periférico foram realizados ensaios de dupla marcação e utilizada a estratégia de análise convencional, como descrito em Material e Métodos**.**

Os percentuais de células NK $CD16^+CD69^+$, linfócitos B $CD19^+CD69^+$, linfócitos T $CD3^+CD69^+$ e suas subpopulações ($CD4^+CD69^+$ e $CD8^+CD69^+$) na população de linfócitos totais estão mostrados na Figura 16. A análise dos dados mostrou queda estatisticamente significativa ($p<0,05$), com exceção das células $CD4^+CD69^+$, no percentual de todas as células $CD69^+$ avaliadas no grupo E-IND em relação ao grupo NI. A análise dos resultados, no grupo $E\text{-}IND_T$, ao contrário do grupo E-IND, mostrou um aumento estatisticamente significativo no percentual de todas as populações celulares $CD69^+$, aqui avaliadas, em relação aos grupos E-IND e NI.

b) Análise da freqüência de linfócitos T $HLA\text{-}DR^+$

Para avaliar o percentual de células ativadas ($HLA\text{-}DR^+$) nas subpopulações de linfócitos T $CD4^+$ e $CD8^+$ do sangue periférico foram realizados ensaios de dupla marcação e utilizada a estratégia de análise combinada "gated", como descrito em Material e Métodos**.**

Os percentuais de linfócitos T $CD4^+HLA\text{-}DR^+$ e T $CD8^+HLA\text{-}DR^+$ na população de linfócitos T $CD4^+$ e T $CD8^+$, respectivamente, estão mostrados na Figura 17. A análise dos dados revelou que o grupo E-IND apresenta percentual de células T $CD4^+HLA\text{-}DR^+$ significativamente menor ($p<0,05$) em relação ao grupo NI. Entretanto, a análise entre os grupos de crianças infectadas revelou aumento significativo no percentual de células $CD4^+HLA\text{-}DR^+$ no grupo $E\text{-}IND_T$ em relação ao grupo E-IND (Figura 17A). Com relação ao percentual de linfócitos T $CD8^+HLA\text{-}DR^+$, foi observado um aumento significativo no grupo $E\text{-}IND_T$ em relação aos grupos E-IND e NI (Figura 17B).

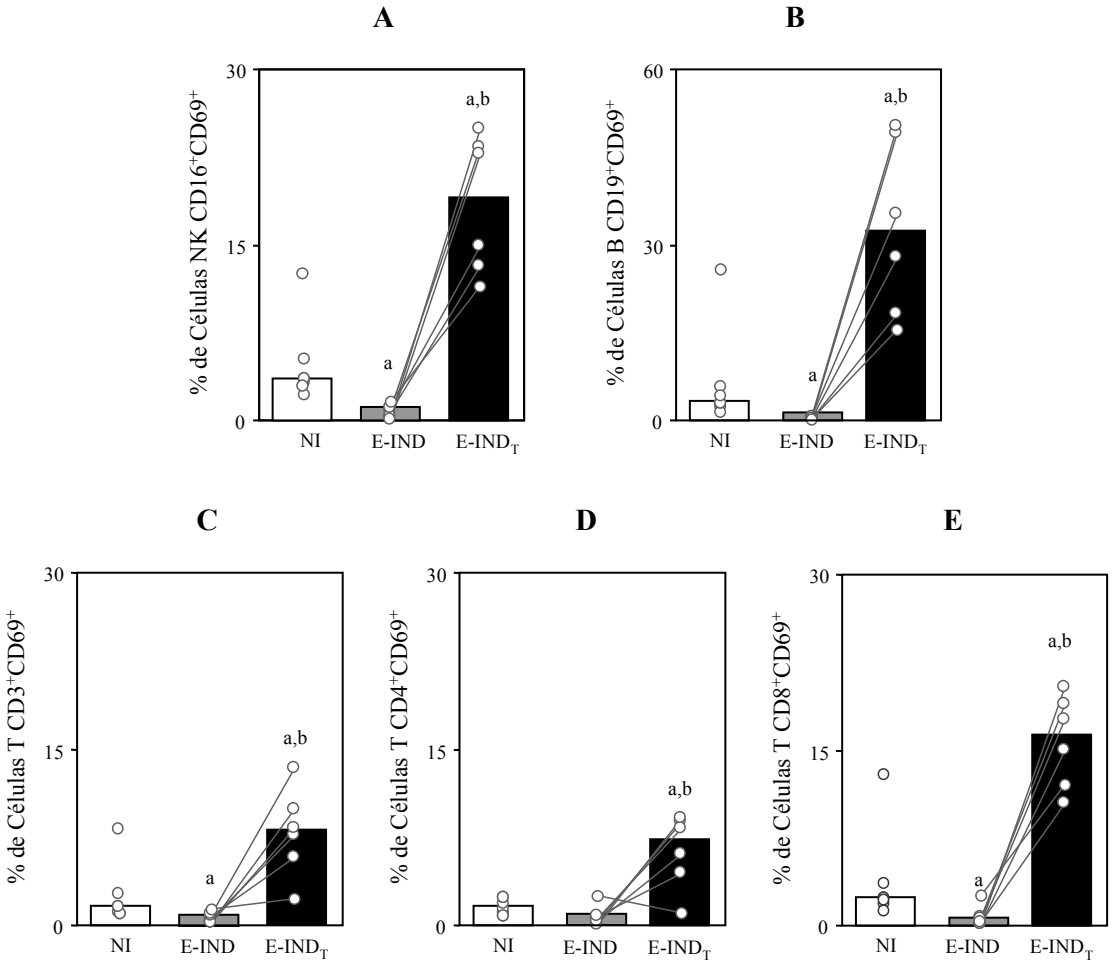

Figura 16: Análise da expressão da molécula CD69 nas populações de linfócitos do sangue periférico de crianças infectadas, antes (E-IND ▒) e após o tratamento com benzonidazol (E-IND$_T$ ■); e de indivíduos não infectados (NI □). As populações celulares foram avaliadas empregando ensaios de dupla marcação como descrito em Material e Métodos. Os resultados estão expressos como dispersão dos valores individuais e mediana dos percentuais para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente.

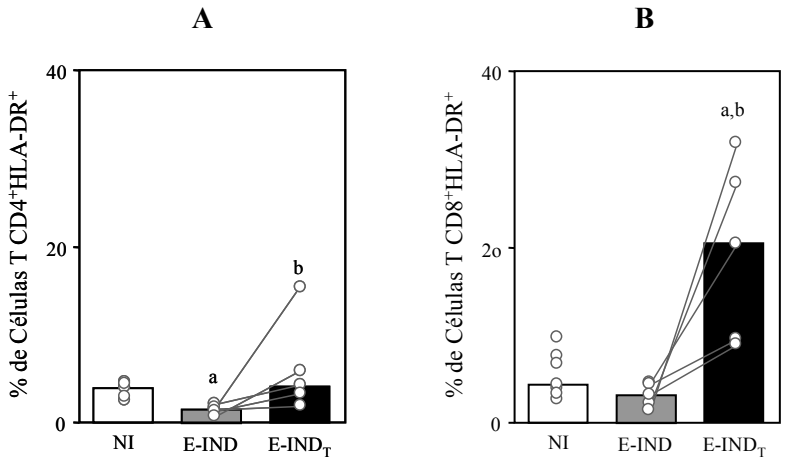

Figura 17: Análise da expressão da molécula HLA-DR nas subpopulações de linfócitos T do sangue periférico de crianças infectadas, antes (E-IND ▒) e após o tratamento com benzonidazol (E-IND$_T$ ■); e de indivíduos não infectados (NI □). As populações celulares foram avaliadas empregando ensaios de dupla marcação como descrito em Material e Métodos. Os resultados estão expressos como dispersão dos valores individuais e mediana dos percentuais para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente.

c) Análise da expressão da molécula CD28 e CD38 em linfócitos T

Para avaliar o percentual de linfócitos T $CD4^+$ e $CD8^+$ co-expressando os marcadores CD28 e CD38 no sangue periférico foram realizados ensaios de dupla marcação e utilizada a estratégia de análise combinada "gated", como descrito em Material e Métodos.

Os percentuais de linfócitos T $CD4^+CD28^+$ e T $CD8^+CD28^+$ na população de linfócitos T $CD4^+$ e T $CD8^+$, respectivamente, estão mostrados na Tabela 5. A análise dos resultados não identificou diferença significativa no percentual de linfócitos T $CD4^+CD28^+$ entre os grupos avaliados. Entretanto, foi observado uma queda significativa no percentual de linfócitos T $CD8^+CD28^+$ no grupo E-IND$_T$ em relação ao grupo E-IND. Nenhuma diferença foi observada entre os grupos de crianças chagásicas e o de indivíduos não infectados.

A análise da co-expressão da molécula CD38 nas subpopulações de linfócitos T revelou que o grupo E-IND apresenta redução estatisticamente significativa ($p<0,05$) na população de linfócitos T $CD4^+CD38^+$ em relação ao grupo NI. A análise da expressão da

molécula CD38 em linfócitos T $CD8^+$ não demonstrou alterações significativas entre os grupos avaliados (Tabela 5).

Tabela 5: Análise das moléculas de co-ativação em subpopulações de linfócitos T

Fenótipo Celular	Grupos		
	NI (n = 7)	**E-IND (n = 6)**	**E-IND$_T$ (n = 6)**
$CD4^+CD28^+/CD4^+$	95,22±5,11	97,91±0,83	96,44±3,49
$CD8^+CD28^+/CD8^+$	51,97±14,73	68,20±11,25	40,10±8,64 [b]
$CD4^+CD38^+/CD4^+$	64,51±17,41	45,40±10,21 [a]	54,91±8,95
$CD8^+CD38^+/CD8^+$	55,99±8,24	54,94±9,89	59,44±5,71

As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente.

d) Análise da freqüência de linfócitos B ativados ($CD19^+CD23^+$)

A subpopulação de linfócitos B ativados no sangue periférico foi identificada em ensaios de dupla marcação utilizando anticorpos monoclonais anti-CD19/TC e anti-CD23/PE, para identificar as células $CD19^+CD23^+$ na população de linfócitos $CD19^+$. Para a análise da subpopulação celular foi utilizada a estratégia combinada "*gated*", como descrito em Material e Métodos.

Os resultados do percentual de linfócitos B ativados ($CD19^+CD23^+$) estão representados na Figura 18. A análise dos dados mostrou que o grupo E-IND apresenta percentual de células B $CD19^+CD23^+$ significativamente maior ($p<0,05$) em relação ao grupo NI. Nossos dados demonstram ainda aumento significativo no percentual de células B ativadas no grupo E-IND_T em relação aos grupos E-IND e NI.

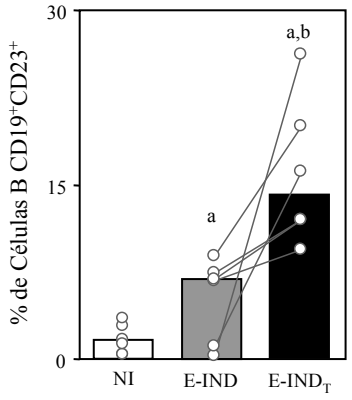

Figura 18: Análise do percentual de linfócitos B ativados ($CD19^+CD23^+$) do sangue periférico de crianças infectadas, antes (E-IND ▭) e após o tratamento com benzonidazol (E-IND_T ■); e de indivíduos não infectados (NI ▭). A população celular foi identificada em ensaios de dupla marcação utilizando anticorpos monoclonais anti-CD19/TC e anti-CD23/PE como descrito em Material e Métodos. Os resultados estão expressos como dispersão dos valores individuais e mediana dos percentuais para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente.

4.1.3. Análise da freqüência de subpopulações de linfócitos T co-expressando moléculas de adesão celular (CD62L, CD18 e CD54)

Para avaliar o percentual de células $CD62L^+$, $CD18^+$ e $CD54^+$ nas subpopulações de linfócitos T $CD4^+$ e $CD8^+$ no sangue periférico foram realizados ensaios de dupla marcação e utilizada a estratégia de análise convencional, como descrito em Material e Métodos.

A análise da fração de linfócitos T $CD4^+CD62L^+$ na população de linfócitos T $CD4^+$ demonstrou queda significativa no grupo E-IND em comparação com o grupo NI (Tabela 6). Já, a análise da fração de linfócitos T $CD8^+CD62L^+$ na população de linfócitos T $CD8^+$ mostrou redução estatisticamente significativa ($p<0,05$) no grupo E-IND_T em relação aos grupos E-IND e NI (Tabela 6). Com relação ao percentual de linfócitos T $CD4^+CD18^+$ e T $CD8^+CD18^+$ na população de linfócitos T $CD4^+$ e T $CD8^+$, respectivamente, a análise dos dados não revelou diferenças significativas entre os grupos avaliados (Tabela 6).

A análise do percentual de linfócitos T $CD4^+CD54^+/CD4^+$ não revelou diferenças significativas entre os grupos avaliados (Tabela 6). Entretanto, foi observado um aumento significativo na fração de linfócitos T $CD8^+CD54^+/CD8^+$ no grupo E-IND ($p<0,05$) quando comparado com o grupo NI. Após o tratamento o percentual de linfócitos T $CD8^+CD54^+/CD8^+$ ($E\text{-}IND_T$) apresentou uma queda em relação às crianças não infectadas (Tabela 6).

Tabela 6: Análise dos marcadores relacionados com migração de células T

Fenótipo Celular	**Grupos**		
	NI (n = 7)	**E-IND (n = 6)**	**$E\text{-}IND_T$ (n = 6)**
$CD4^+CD62L^+/CD4^+$	83,18±10,34	64,06±7,36 [a]	69,20±7,93
$CD8^+CD62L^+/CD8^+$	52,18±13,04	48,30±12,03	35,50±9,55 [a,b]
$CD4^+CD18^+/CD4^+$	23,55±9,69	25,77±12,69	22,11±8,54
$CD8^+CD18^+/CD8^+$	65,48±14,49	55,56±13,10	59,82±16,76
$CD4^+CD54^+/CD4^+$	4,63±10,01	0,73±4,86	2,47±2,87
$CD8^+CD54^+/CD8^+$	33,27±17,17	71,77±15,03 [a]	15,89±5,46 [b]

As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente.

4.1.4. Análise da freqüência de células T reguladoras ($CD4^+CD25^{HIGH}$) em leucócitos do sangue periférico

Para avaliar o percentual de células T reguladoras $CD4^+CD25^{HIGH}$ na população de linfócitos totais do sangue periférico foram realizados ensaios de dupla marcação e a estratégia de análise empregada foi a proposta por Baecher-Allan *et al*. (2001), como descrita em Material e Métodos.

Os resultados apresentados na Figura 19 mostram o valor percentual de linfócitos T $CD4^{+}CD25^{HIGH}$ na população de linfócitos totais do sangue periférico de crianças na fase crônica recente da doença de Chagas, antes e após o tratamento etiológico com benzonidazol, e de crianças não infectadas. A análise dos dados mostrou que as crianças chagásicas, antes e após o tratamento, apresentam percentuais menores de células T reguladores em relação às crianças não infectadas. A análise dos dados entre os grupos E-IND e $E\text{-}IND_T$ não revelou diferença estatisticamente significativa (NI = 4,5±3,5%; E-IND = 1,8±0,7%; $E\text{-}IND_T$ = 1,8±0,3%).

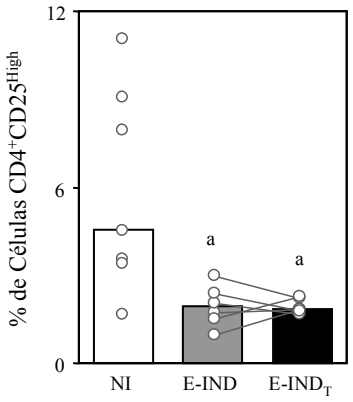

Figura 19: Análise do percentual de células T reguladoras $CD4^{+}CD25^{HIGH}$ circulantes do sangue periférico de crianças infectadas, antes (E-IND ▒) e após o tratamento com benzonidazol ($E\text{-}IND_T$ ■); e de indivíduos não infectados (NI □).As populações celulares foram identificadas em ensaios de dupla marcação utilizando anticorpos monoclonais anti-CD4/FITC e anti-CD25/PE, como descrito em Material e Métodos. Os resultados estão expressos como dispersão dos valores individuais e mediana dos percentuais para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" em comparação com os grupos NI.

4.1.5. Análise da freqüência de monócitos pró-inflamatórios em leucócitos do sangue periférico

Para avaliar o percentual de monócitos pró-inflamatórios ($CD14^{+}CD16^{+}HLA\text{-}DR^{High}$) na população de monócitos $CD14^{+}CD16^{+}$ do sangue periférico foram realizados ensaios de tripla marcação e utilizada a estratégia de análise proposta por Belge *et al.* (2002).

A figura 20A mostra os resultados dos percentuais de monócitos "macrófagos like" ($CD14^+CD16^+$) na população de monócitos do sangue periférico. A análise dos dados revelou uma quenda significatica no grupo E-IND em comparação com o grupo NI. Já o grupo E-IND_T apresentou um aumento em relação aos grupos NI e E-IND.

A Figura 20B mostra os resultados dos percentuais de monócitos $CD14^+CD16^+$HLA-DR^{High} do sangue periférico de crianças na fase crônica recente da doença de Chagas, antes e após o tratamento etiológico com benzonidazol, e de crianças não infectadas. A análise dos resultados revelou aumento estatisticamente significativo ($p<0,05$) do percentual de monócitos pró-inflamatórios no grupo E-IND em relação ao grupo NI. A análise entre os grupos de crianças infectadas, antes e após o tratamento, revelou redução no percentual dos monócitos pró-inflamatórios após o tratamento com Bz (NI = 67,7±15,0%; E-IND = 92,3±10,4%; E-IND_T = 51,3±29,2%). Entretanto, análises adicionais revelaram aumento no percentual de monócitos $CD14^+$HLA-DR^{High} no grupo E-INDT em aos grupos E-IND e NI (Figura 20C).

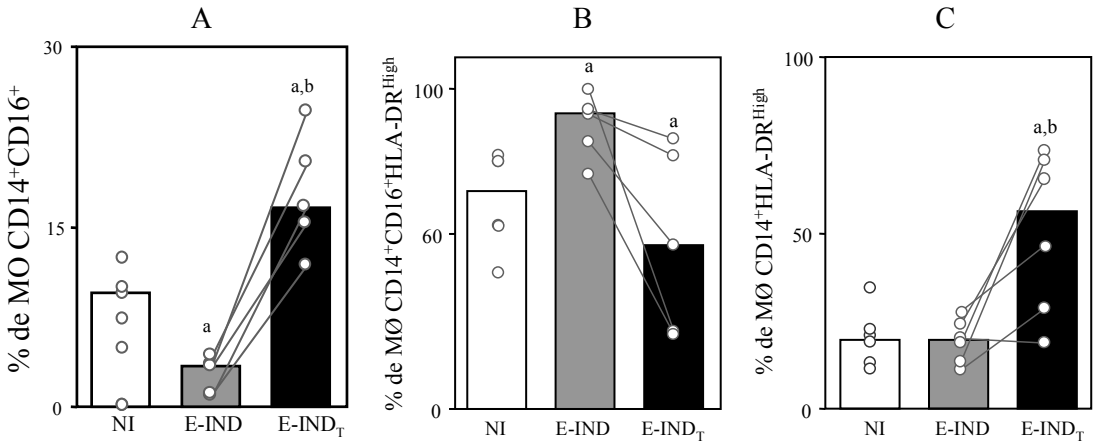

Figura 20: Percentual de monócitos $CD14^+CD16^+$ (A), $CD14^+CD16^+$HLA-DR^{high} (B) e $CD14^+$HLA-DR^{high} (C) circulantes do sangue periférico de crianças infectadas, antes (E-IND ▭) e após o tratamento com Bz (E-IND_T ▬); e de indivíduos não infectados (NI ▭). Os resultados estão expressos como dispersão dos valores individuais e mediana dos percentuais para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" em comparação com os grupos NI.

4.2. Análise do padrão de citocinas produzidas por leucócitos do sangue periférico de indivíduos portadores da forma crônica indeterminada recente da doença de Chagas antes e após o tratamento com benzonidazol

Apesar dos vários estudos focalizando o papel da resposta imune durante os estágios iniciais da infecção chagásica em modelos experimentais, investigações neste campo, durante a fase crônica indeterminada recente em humanos, ainda são limitadas. Para avaliar o papel da resposta imune durante a fase crônica recente da doença de Chagas em humanos, antes e após o tratamento com Bz, determinamos i*n vitro* o perfil de citocinas inflamatórias/anti-inflamatórias citoplasmáticas em leucócitos circulantes. Esse tipo de abordagem permitiu identificar o padrão de citocinas produzidas, bem como, a fonte produtora das mesmas. Para tanto, foram realizadas primeiramente, culturas na presença de Brefeldina A e na ausência de estímulo exógeno, objetivando caracterizar o perfil de citocinas *ex-vivo* (Cultura Controle). Em paralelo foram realizadas culturas estimuladas com antígenos solúveis das formas epimastigotas do *T. cruzi* – EPI, com o intuito de verificar a influência de antígenos do parasita na indução da resposta imune (Cultura estimulada).

É importante resaltar que os dados referentes à análise do padrão de citocinas produzidas por leucócitos do sangue periférico serão apresentados em valores absolutos, uma vez que estes resultados foram semelhantes aos dados percentuais. Para a obtenção dos valores absolutos foram utilizados os dados leucométricos (Anexo).

4.2.1. Perfil citocinas produzidas pelas células da resposta imune inata

a) Expressão de citocinas por monócitos

Os resultados apresentados na Figura 21 mostram os valores absolutos de monócitos TNF-α^+, IL-12^+ e IL-10^+ do sangue periférico. Nossos dados demonstram, na cultura controle, um aumento no número de monócitos IL-12^+ no grupo E-IND quando comparado ao grupo NI. Entretanto, no grupo E-IND_T o número de monócitos TNF-α^+ e IL-

12^+ diminui quando comparado com os grupos NI e E-IND, respectivamente (Figura 21A). A análise dos dados, referentes à cultura estimulada com EPI, não revelou nenhuma diferença estatisticamente significativa no número de monócitos citocinas$^+$ no grupo E-IND quando comparado com o grupo NI, apesar de se observar um aumento no número de monócitos TNF-α^+. Entretanto, a análise dos dados revelou uma redução nos valores absolutos de monócitos TNF-α^+ no grupo E-IND$_T$ em relação os demais grupos avaliados, na presença de EPI (Figura 21B). Além disso, o estimulo com EPI foi capaz de aumentar a produção de todas as citocinas avaliadas em monócitos do grupo E-IND$_T$ quando comparado com a cultura controle (Figura 21B).

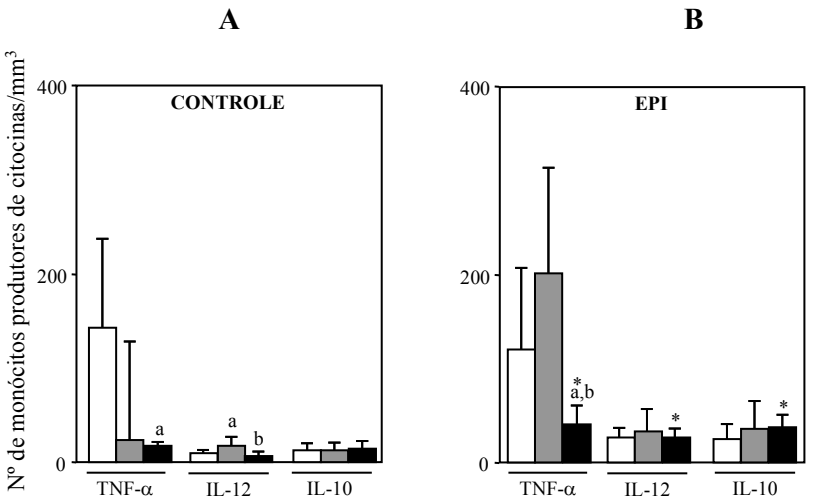

Figura 21: Análise da expressão de citocinas por monócitos circulantes do sangue periférico de crianças infectadas antes (E-IND ▒) e após o tratamento com Bz (E-IND$_T$ ■); e de indivíduos não infectados (NI □), após cultura rápida com Brefeldina A, na ausência (A) ou presença de EPI (B), como descrito em Material e Métodos. Os resultados estão expressos como mediana ± desvio padrão dos valores absolutos de monócitos TNF-α^+, IL12$^+$ e IL-10$^+$ para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente. As diferenças estatísticas entre as culturas controle e estimulada, dentro do mesmo grupo, estão representadas pelo *.

b) Expressão de citocinas pelas células NK

Os resultados apresentados na Figura 22 mostram os valores absolutos de células NK IFN-α^+, TNF-α^+ e IL-4^+ do sangue periférico. Nossos dados não demonstram, na cultura controle, alterações significativas no número absoluto de células NK expressando citocinas no grupo E-IND quando comparado ao grupo NI. Entretanto, no grupo E-IND_T, a análise dos dados mostrou um aumento estatisticamente significativo no número de células NK IFN-α^+, TNF-α^+ e IL-4^+ quando comparado com os demais grupos avaliados (Figura 22A). A análise dos dados, referentes à cultura estimulada com EPI, revelou um aumento no número absoluto de células NK IFN-γ^+, TNF-α^+ e IL-4^+ no grupo E-IND em relação ao grupo NI. Além disso, o estimulo com EPI foi capaz de aumentar a expressão de todas as citocinas avaliadas nas células NK do grupo E-IND em comparação com a cultura controle. Para o grupo E-IND_T, os dados mostram um aumento na expressão de todas as citocinas, por nós avaliadas, nas células NK, quando comparado com os grupos NI e E-IND. Diferente do grupo E-IND, a presença do antígeno induziu um aumento apenas no número absoluto de células NK IFN-γ^+ no grupo E-IND_T (Figura 22B).

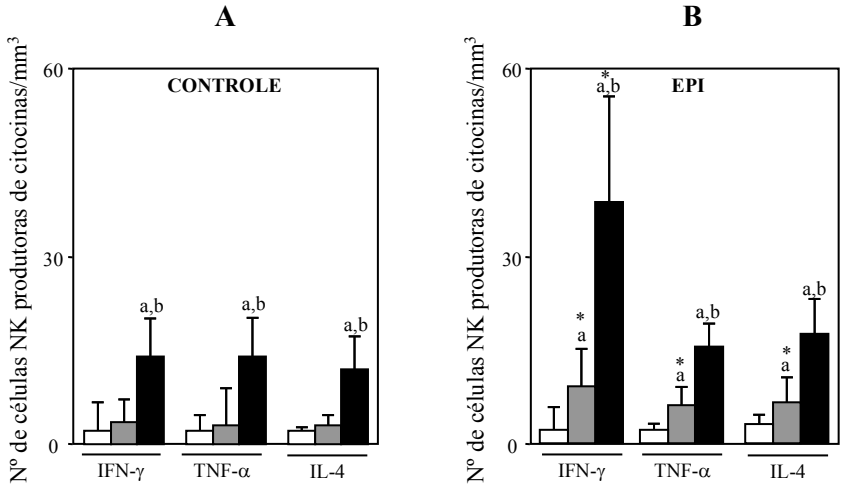

Figura 22: Análise da expressão de citocinas por células NK circulantes do sangue periférico de crianças infectadas antes (E-IND ▒) e após o tratamento com Bz (E-IND_T ■) e de indivíduos não infectados (NI □), após cultura rápida com Brefeldina A, na ausência (A) ou presença de EPI (B), como descrito em Material e Métodos. Os resultados estão expressos como mediana ± desvio padrão

dos valores absolutos de células NK IFN-γ^+, TNF-α^+ e IL4$^+$ para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente. As diferenças estatísticas entre as culturas controle e estimulada, dentro do mesmo grupo, estão representadas pelo *.

4.2.2. Perfil citocinas produzidas pelas células da resposta imune adaptativa

a) Expressão de citocinas por linfócitos T CD4$^+$

Os resultados apresentados na Figura 23 mostram os valores absolutos de linfócitos T CD4$^+$ IL-12$^+$, IFN-γ^+, TNF-α^+, IL4$^+$ e IL10$^+$ do sangue periférico. Nossos dados não demonstram, na cultura controle, alterações significativas no número absoluto de células T CD4$^+$ expressando citocinas no grupo E-IND quando comparado ao grupo NI. Entretanto, no grupo E-IND$_T$ o número de linfócitos CD4$^+$ IFN-γ^+ mostrou-se aumentado quando comparado com o grupo E-IND. Surpreendentemente, neste grupo, o número de linfócitos T CD4$^+$IL10$^+$ aumentou cerca de 9 a 20 vezes em relação ao grupos NI e E-IND, respectivamente (Figura 23A). A análise dos dados, referentes à cultura estimulada com EPI, revelou que a presença do antígeno foi capaz de aumentar o número de linfócitos T CD4$^+$ IFN-γ^+, TNF-α^+ e IL-10$^+$ do grupo E-IND em comparação com a cultura controle. Para o grupo E-IND$_T$, os dados mostram um aumento no número absoluto dos linfócitos T CD4$^+$ TNF-α^+ e IL-4$^+$ quando comparado com o grupo NI. Além disso, assim como na cultura controle, o número de linfócitos T CD4$^+$IL-10$^+$, na presença do antígeno, permanece muito elevado em relação aos demais grupos avaliados (Figura 23B).

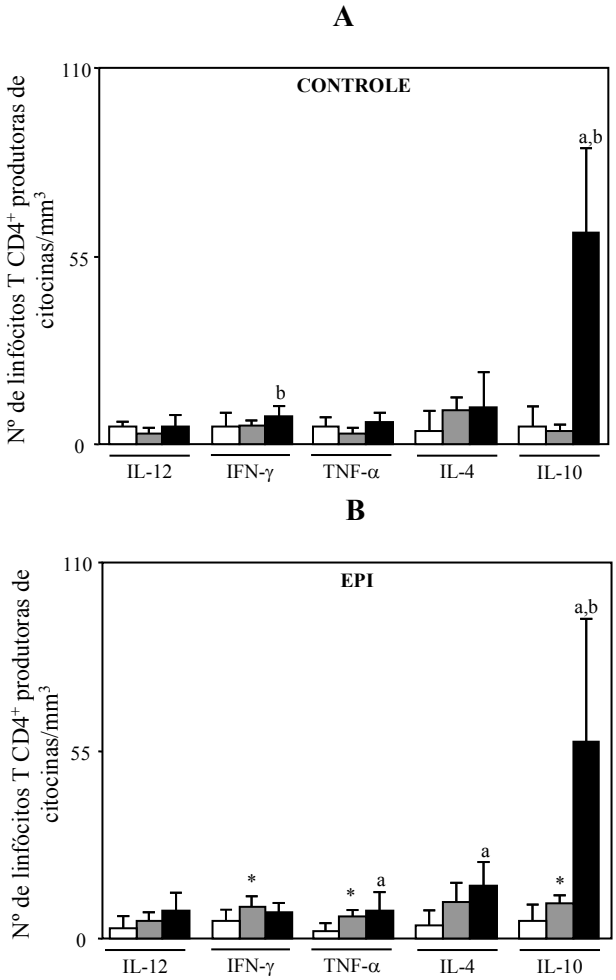

Figura 23: Análise da expressão de citocinas por linfócitos T $CD4^+$ circulantes do sangue periférico de crianças infectadas antes (E-IND ▒) e após o tratamento com Bz ($E\text{-}IND_T$ ■), e de indivíduos não infectados (NI □), após cultura rápida com Brefeldina A, na ausência (A) ou presença de EPI (B), como descrito em Material e Métodos. Os resultados estão expressos como mediana ± desvio padrão dos valores absolutos de linfócitos T $CD4^+$ $IL\text{-}12^+$, $IFN\text{-}\gamma^+$, $TNF\text{-}\alpha^+$, $IL4^+$ e $IL10^+$ para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI-1 e E-IND, respectivamente. As diferenças estatísticas entre as culturas controle e estimulada, dentro do mesmo grupo, estão representadas pelo *.

b) Expressão de citocinas por linfócitos T $CD8^+$

Os resultados apresentados na Figura 24 mostram os valores absolutos de linfócitos T $CD8^+$ IL-12^+, IFN-γ^+, TNF-α^+, $IL4^+$ e $IL10^+$ do sangue periférico. Nossos dados não demonstram, na cultura controle, alterações significativas no número absoluto de células T $CD8^+$ expressando citocinas no grupo E-IND quando comparado ao grupo NI. Entretanto, no grupo E-IND_T os números de linfócitos $CD8^+$ IL-12^+, IFN-γ^+, TNF-α^+, $IL4^+$ e $IL10^+$ mostraram-se extremamente elevados quando comparado com os demais grupos avaliados (Figura 24A). A análise dos dados, referentes à cultura estimulada com EPI, revelou um perfil semelhante ao da cultura controle, quando a comparação foi feita entre os grupos. Além disso, o estimulo com EPI foi capaz de aumentar a produção apenas do número de linfócitos $CD8^+$IFN-γ^+ do grupo E-IND_T quando comparado com a cultura controle (Figura 24B).

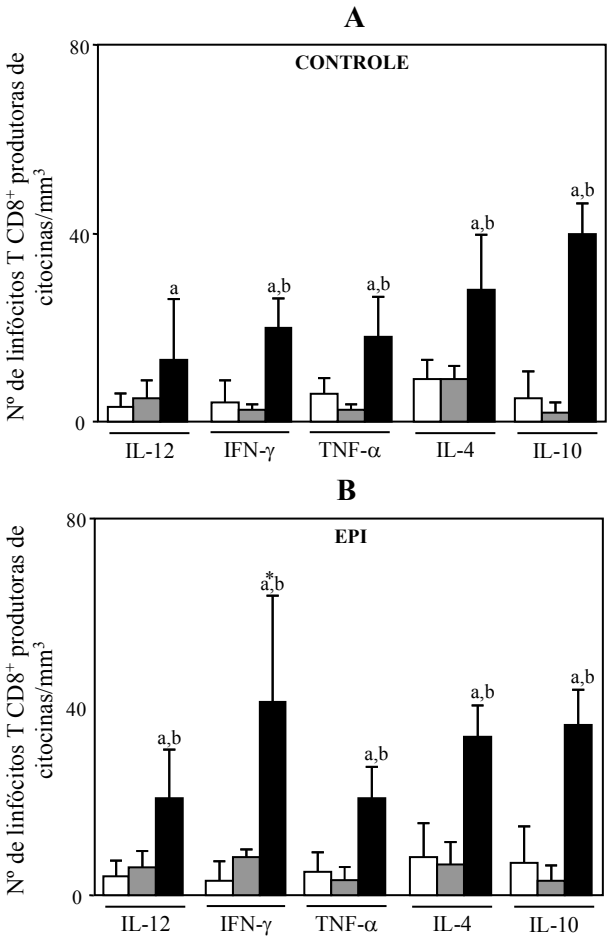

Figura 24: Análise da expressão de citocinas por linfócitos T $CD8^+$ circulantes do sangue periférico de crianças infectadas antes (E-IND ▒) e após o tratamento com Bz ($E\text{-}IND_T$ ■), e de indivíduos não infectados (NI □), após cultura rápida com Brefeldina A, na ausência (A) ou presença de EPI (B), como descrito em Material e Métodos. Os resultados estão expressos como mediana ± desvio padrão dos valores absolutos de linfócitos T $CD4^+$ $IL\text{-}12^+$, $IFN\text{-}\gamma^+$, $TNF\text{-}\alpha^+$, $IL4^+$ e $IL10^+$ para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI-1 e E-IND, respectivamente. As diferenças estatísticas entre as culturas controle e estimulada, dentro do mesmo grupo, estão representadas pelo *.

c) Expressão de citocinas por linfócitos B CD19$^+$

Os resultados apresentados na Figura 25 mostram os valores absolutos de linfócitos B CD19$^+$ TNF-α^+, IL4$^+$ e IL10$^+$ do sangue periférico. Nossos dados não demonstram, na cultura controle, alterações significativas no número absoluto de células B CD19$^+$ TNF-α^+, IL4$^+$ e IL10$^+$ no grupo E-IND quando comparado ao grupo NI. Entretanto, no grupo E-IND$_T$ os números de linfócitos CD19$^+$ TNF-α^+, IL4$^+$ e IL10$^+$ mostraram-se elevados quando comparado com os demais grupos avaliados (Figura 25A). A análise dos dados, referentes à cultura estimulada com EPI, revelou um perfil semelhante ao observado na cultura controle (Figura 25B).

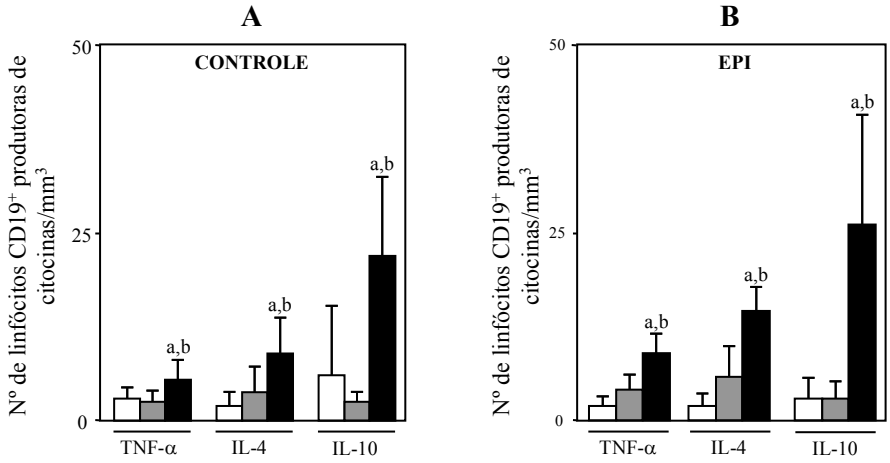

Figura 25: Análise da expressão de citocinas por linfócitos B CD19$^+$ circulantes do sangue periférico de crianças infectadas antes (E-IND ▒) e após o tratamento com Bz (E-IND$_T$ ■), e de indivíduos não infectados (NI □), após cultura rápida com Brefeldina A, na ausência (A) ou presença de EPI (B), como descrito em Material e Métodos. Os resultados estão expressos como mediana ± desvio padrão dos valores absolutos de linfócitos T CD19$^+$ TNF-α^+, IL4$^+$ e IL10$^+$ para cada grupo avaliado. As diferenças significativas ($p<0,05$) estão representadas pelas letras "a" e "b" em comparação com os grupos NI e E-IND, respectivamente.

4.3. Análise da correlação entre os marcadores de ativação celular e o padrão de citocinas produzidas por leucócitos do sangue periférico de indivíduos portadores da forma crônica indeterminada recente da doença de Chagas antes e após o tratamento com benzonidazol

Uma vez que estudos têm demonstrado que monócitos "macrófagos-like" ($CD14^+CD16^+$) e monócitos pro-inflamatórios ($CD14^+HLA\text{-}DR^{High}$) expressam citocinas pró-inflamatórias (BELGE *et al*., 2002; DAYYANI *et al*., 2003) e estas células estão aumentadas após o tratamento com benzonidazol, realizamos análises de correlação entre as células $CD14^+$ produtoras de citocinas inflamatórias (IL-12 ou TNF-α) e o percentual de monócitos "macrófagos-like" ou monócitos pró-inflamatórios. As análises de correlação, de forma interessante, revelaram uma correlação negativa entre monócitos "macrófagos-like" e monócitos produtores de IL-12 (Figura 26). Resultado semelhante foi observado para a correlação entre monócitos pro-inflamatório e monócitos produtores de IL-12 (Figura 26). Nenhuma correlação foi observada entre os monócitos $CD14^+CD16^+$ e $CD14^+HLA\text{-}DR^{High}$ com os monócitos $CD14^+TNF\text{-}\alpha^+$ (dados não mostrados).

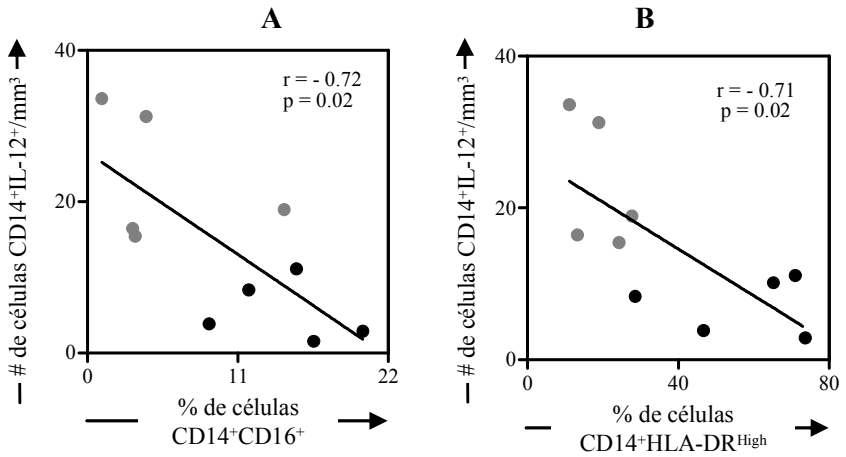

Figura 26: Análise da correlação entre monócitos "macrófagos-like" e monócitos $CD14^+IL12^+$ (A) e monócitos pro-inflatório e monócitos $CD14^+IL\text{-}12^+$ (B) do sangue periférico de crianças infectadas antes (E-IND ●) e após o tratamento com Bz ($E\text{-}IND_T$ ●).

As células NK apresentam um papel importante durante a infecção chagásica, elas secretam uma grande quantidade de IFN-γ favorecendo uma resposta do tipo 1, essencial para o controle do parasita durante os estágios iniciais da infecção chagásica (SILVA *et al.,* 1992; TORRICO *et al.,* 1991). Entretanto, uma resposta do tipo 1 exacerbada pode leva a processos deletérios durante a fase crônica da doença. Nossos resultados demonstraram um aumento no percentual de células NK ativadas ($CD16^+CD69^+$) no sangue periférico. Em uma análise superficial, esse aumento na ativação das células NK poderia favorecer o desenvolvimento de danos teciduais relacionados com a elevada produção de IFN-γ. Nesse contexto, avaliamos se o aumento no percentual de células NK ativadas estaria relacionado com o produção de IFN-γ e de IL-4 por células NK. Análise de correlação revelou uma correlação positiva entre o aumento de células NK totais e um perfil misto de citocinas, aqui representado pelos elevados números de células $CD16^+IFN\text{-}\gamma^+$ e $CD16^+IL\text{-}4^+/mm^3$ (Figura 27). O mesmo resultado foi observado para análise da correlação entre as células NK ativadas ($CD16^+CD69^+$) e número de células $CD16^+IFN\text{-}\gamma^+$ e $CD16^+IL\text{-}4^+/mm^3$ (Figura 27).

Estudos recentes, em nosso laboratório demonstraram que as células T possuem um papel importante tanto nos estágios iniciais quanto nos estágios mais avançados da fase crônica da doença de Chagas (SATHLER-AVELAR *et al*., 2003; VITELLI-AVELAR *et al*., 2005). Dutra *et al*. (1994) demonstraram que independentemente da forma clínica da infecção chagásica os pacientes apresentaram uma elevada freqüência de linfócitos T ativados. Reis *et al*. (1993) ao avaliar o infiltrado inflamatório do miocárdio, de pacientes portadores da forma crônica cardíaca, constataram associação entre linfócitos T citotóxico ativados e o acometimento do órgão. Nossos resultados revelaram um aumento na ativação de células T após o tratamento com Bz. Entretanto, análises de correlação revelaram uma correlação positiva entre aumento no percentual de células T $CD4^+$ ativadas ($CD4^+CD69^+$ ou $CD4^+$HLA-

DR$^+$) e o aumento no número absoluto de linfócitos T CD4$^+$IL-10$^+$ (Figura 28). Já para as células T CD8$^+$ ativadas (CD8$^+$CD69$^+$ ou CD8$^+$HLA-DR$^+$), uma correlação positiva foi observada com perfil misto de citocinas, representado pelo aumento no número absoluto de linfócitos T CD8$^+$IFN-γ^+ e CD8$^+$IL-10$^+$ (Figura 29).

Finalmente, a análise da correlação das células B ativadas (CD19$^+$CD69$^+$) com o número absolutos das células CD19$^+$TNF-α^+ e CD19$^+$IL-4$^+$ revelou uma correlação positiva entre as células B ativadas e um padrão misto de citocinas (TNF-α e IL-4) (Figura 30).

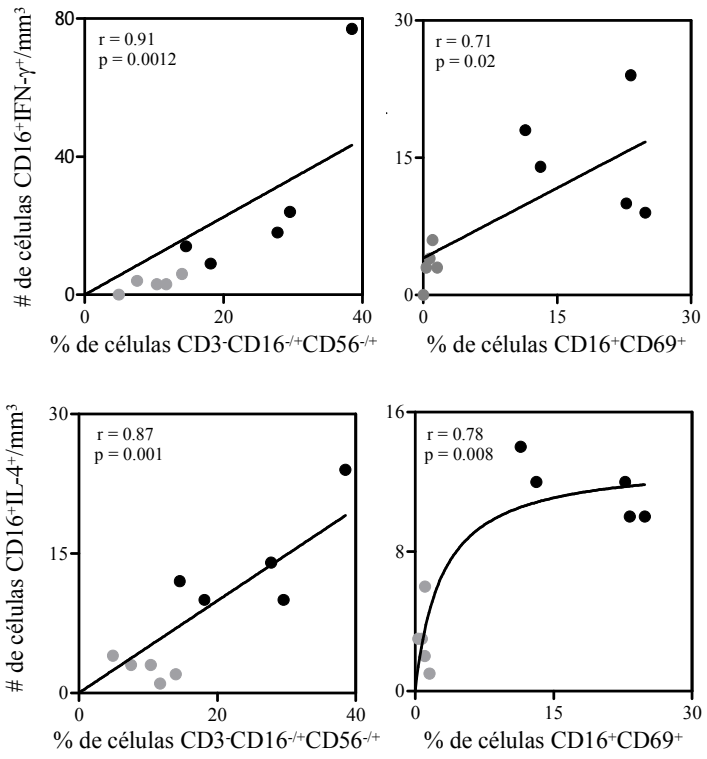

Figura 27: Análise da correlação entre células NK total e o número absoluto de células CD16+ produtoras de citocinas – IFN-γ^+ e IL-4 – e entre as células NK ativadas (CD16$^+$CD69$^+$) e o número absoluto de células CD16$^+$ produtoras de citocinas – IFN-γ e IL-4 – do sangue periférico de crianças infectadas antes (E-IND ●) e após o tratamento com Bz (E-IND$_T$ ●).

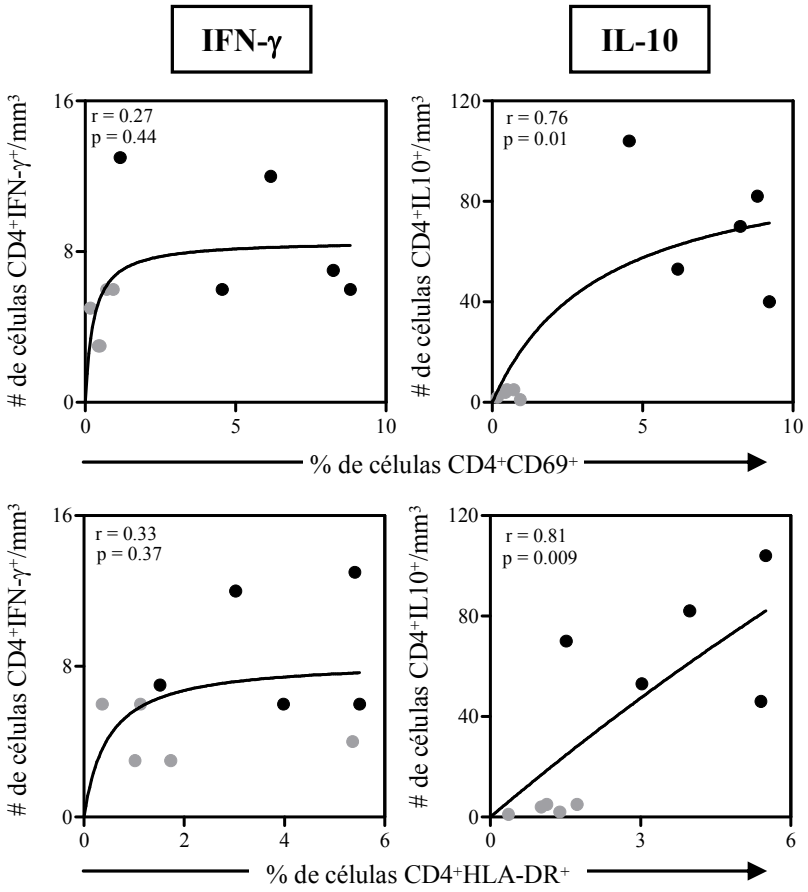

Figura 28: Análise da correlação entre células T $CD4^+$ ativadas ($CD4^+CD69^+$ ou $CD4^+HLA\text{-}DR^+$) e o número absoluto de células T $CD4^+$ produtoras de citocinas – IFN-γ e IL-10 – do sangue periférico de crianças infectadas antes (E-IND ●) e após o tratamento com Bz ($E\text{-}IND_T$ ●).

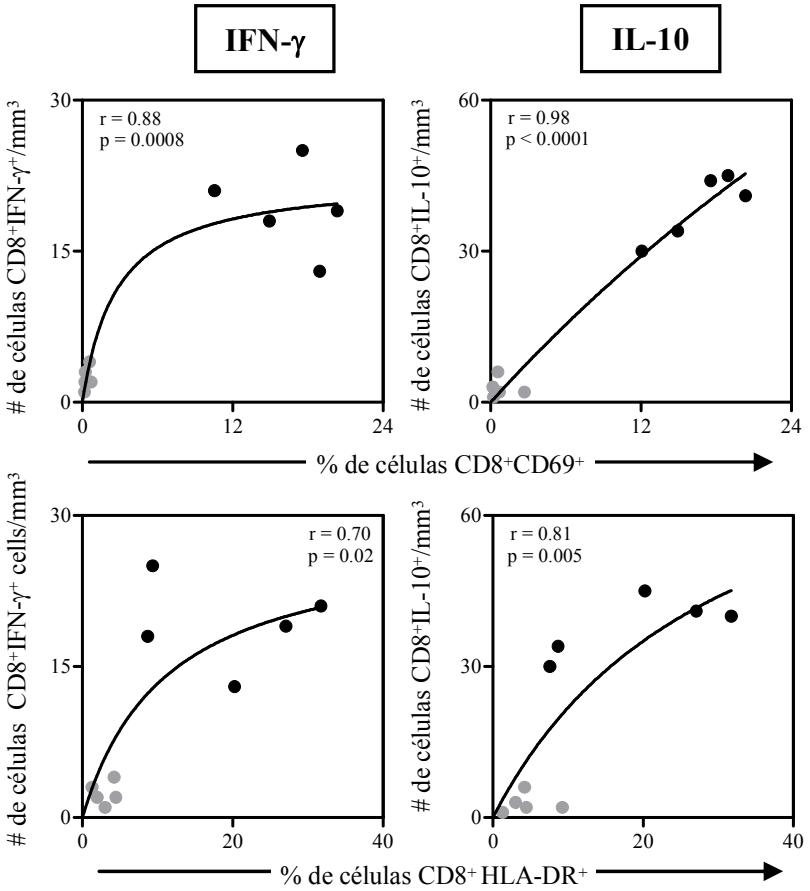

Figura 29: Análise da correlação entre células T $CD8^+$ ativadas ($CD8^+CD69^+$ ou $CD8^+HLA\text{-}DR^+$) e o número absoluto de células T $CD8^+$ produtoras de citocinas – IFN-γ e IL-10 – do sangue periférico de crianças infectadas antes (E-IND ●) e após o tratamento com Bz ($E\text{-}IND_T$ ●).

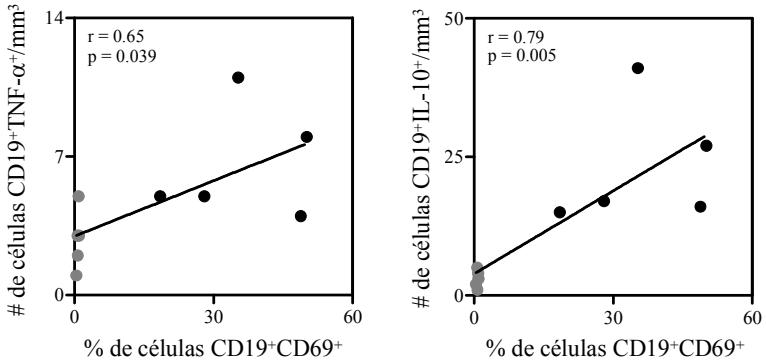

Figura 30: Análise da correlação entre células B $CD69^+CD19^+$ ativadas e o número absoluto de células B $CD19^+$ produtoras de citocinas – TNF-α e IL-10 – do sangue periférico de crianças infectadas antes (E-IND ●) e após o tratamento com Bz ($E\text{-}IND_T$ ●).

5. DISCUSSÃO

5.1. Aspectos imunológicos em indivíduos portadores da forma crônica indeterminada recente da doença de Chagas

O entendimento do papel da resposta imune contra o *T. cruzi* e os mecanismos promotores de dano tecidual na doença de Chagas têm sido os maiores desafios desde a descoberta de seu agente etiológico. Diversos componentes dos compartimentos celular e humoral, incluindo a imunidade inata e a adaptativa, são recrutados durante a infecção pelo *T. cruzi*. Considerando a intensa estimulação imune e o processo inflamatório desencadeados durante a infecção aguda pelo *T. cruzi*, é possível que eventos imunológicos precoces determinem não apenas a imunopatologia da doença mas também atuem como instrumentos para o clareamento da infecção, seqüestrando o agente etiológico para o sítio intracelular (HONTEBERYRIE-JOSKOWICZ *et al.,* 1991; SAMUDIO *et al.,* 1998; MARINHO *et al.,* 1999). Contudo, os estudos que abordam o papel da resposta imune durante os estágios iniciais da infecção pelo *T. cruzi* estão, em sua maioria, restritos a modelos experimentais, sendo os mecanismos da ativação do sistema imune durante este período pouco investigados em humanos. Desta forma, este trabalho objetivou desenvolver um estudo descritivo-analítico detalhado do fenótipo de células mononucleares do sangue periférico de crianças infectadas pelo *T. cruzi* e do padrão de citocinas intracitoplasmáticas, a fim de delinear características da resposta imune durante os estágios iniciais da doença de Chagas.

No presente estudo, demonstrou-se que a fase crônica indeterminada recente está associada com alterações fenotípicas relevantes em células envolvidas na imunidade inata e adaptativa celular, sugestivas do envolvimento destas populações celulares nos eventos imunológicos de ativação e modulação na infecção crônica recente pelo *T. cruzi*.

Nossos achados revelaram baixa freqüência de células T $CD3^+$, devido principalmente à queda no percentual de linfócitos T $CD8^+$, na ausência do fenótipo de ativação de linfócitos T, tais como expressão de CD69 e HLA-DR. Entretanto, níveis maiores na freqüência de

linfócitos B ativados foram observados nas crianças infectadas, assemelhando-se a um estado de "*imunomodulação*" previamente proposto na infecção pelo *T.* cruzi em modelos experimentais ou em células humanas *in vitro* (TARLETON, 1988a,b; ARGIBAY *et al*. 2002). Esses dados estão de acordo com nossos relatos prévios decorrentes da caracterização fenotípica de leucócitos do sangue periférico de crianças bolivianas portadoras da infecção recente pelo *T. cruzi* (SATHLER-AVELAR *et al*., 2003). Esse estado imunológico misto de ativação e modulação pode ser explicado, em parte, pela ação de diferentes moléculas presentes na superfície do parasita que induzem ativação de células B com secreção de imunoglobulinas não específicas, assim como supressão de linfócitos T (BENTO *et al*., 1996; BRENER & GAZZINELLI *et al*., 1997; GAO *et al*., 2002). Consistente com esta hipótese, alguns estudos demonstraram que moléculas ancoradas via glicosil-fosfatidil inositol (GPI) na membrana do *T. cruzi*, tais como trans-sialidase e mucinas, são capazes de desempenhar funções distintas, incluindo indução da ativação de linfócitos B e supressão na ativação de linfócitos T, com produção de imunoglobulinas não específicas para o *T. cruzi* (BILATE *et al.,* 2000; ZUNIGA *et al.,* 2000; ACOSTA-SERRANO *et al*., 2001; ARGIBAY *et al*., 2002; GAO *et al.,* 2002; DOS REIS *et al*., 2005). Tem sido sugerido que a resposta humoral desencadeada durante a infecção recente pelo *T. cruzi,* caracterizada pela intensa produção de imunoglobulinas inespecíficas, possa representar um dos elos da considerada "*imunossupressão*" favorecendo o crescimento do parasita, a instalação da doença e o desenvolvimento da imunopatologia (D'IMPERIO-LIMA *et al.,* 1985; SPINELLA *et al.,* 1992).

As células B não possuem apenas um papel central na produção e amplificação da resposta imune humoral. Elas também têm uma função importante como células apresentadoras de antígenos favorecendo uma resposta imune mediada por células T (MAMULA & JANEWAY, 1993). Embora, as células dendríticas e macrófagos promovam

diferenciação das células T para o fenótipo Th1, a apresentação de antígeno pelas células B induz a anergia dos linfócitos T, favorecendo o desenvolvimento do fenótipo Th2 anti-inflamatório (ADORINE *et al*., 1997; CROFT *et al*., 1997; RODRIGUEZ *et al*., 2007). Zuniga *et al*. (2001) reportaram que a lectina denominada Galectina-1 (Gal-1), secretada por células B ativadas, pode induzir apoptose em células T e diminuir a síntese de IFN-γ. Esses autores demonstraram que as células B ativadas de camundongos infectados pelo *T. cruzi* secretam elevados níveis de Gal-1, podendo exercer vários efeitos imunomoduladores. Esses resultados são consistentes com nossos achados, em que as crianças infectadas apresentam aumento na população de linfócitos B ativados, na ausência de fenótipos de ativação em células T.

A importância das células NK na resistência da fase aguda da doença de Chagas é demonstrada por estudos em que a neutralização de IL-12 ou IFN-γ, citocinas essenciais para ativação das células NK, assim como a depleção das mesmas, tornam os animais mais susceptíveis à infecção pelo *T. cruzi* (ALIBERTI *et al*., 1996; CARDILLO *et al*., 1996). Assim, além da função citotóxica das células NK já bem estabelecida, acredita-se que essas células possuam um papel importante na secreção de IFN-γ antes do desenvolvimento de resposta imune mediada pelas células T na infecção pelo *T. cruzi*.

Um aspecto importante relacionado às células NK, durante infecção experimental pelo *T. cruzi*, consiste no favorecimento da ativação de células B (DE ARRUDA HINDIS *et al.,* 2001). Neste contexto, a ativação de células B, observada durante a fase crônica indeterminada recente da infecção, via NK, poderia ser um evento importante para manter a resposta humoral. Blanca *et al.* (2001), ao estudarem a interação *in vitro* de linfócitos B e células NK, constataram que a ativação policlonal de células B é dependente da interação de CD40, presente na superfície de células NK, com o seu ligante CD40L na superfície de linfócitos B. Esses dados sugerem que a interação entre essas moléculas apresenta uma

importante função na ativação de células B mediada por NK independente de linfócitos T, reforçando nossa hipótese. A expressão da molécula CD40L em células B ativadas, como sugerido por Blanca *et al.* (2001), é fundamental na interação com células NK devido à sua contribuição na co-estimulação de células B, que, em combinação com fatores autócrinos dos linfócitos B, promove a diferenciação dessas células em plasmócitos secretores de imunoglobulinas. Neste estudo, não tivemos a oportunidade de avaliar a expressão de CD40 e CD40L por linfócitos. Estudos futuros poderiam fornecer informações adicionais permitindo o esclarecimento desses eventos. A ativação de linfócitos B via NK é capaz de promover a síntese de imunoglobulinas tanto por células B de memória quanto por linfócitos B1. Na doença de Chagas, a ativação policlonal e secreção de anticorpos não específicos, que levam à hipergamaglobulinemia, são fenômenos freqüentes que poderiam favorecer a evasão do *T. cruzi* (MINOPRIO *et al.,* 1986; MINOPRIO *et al.,* 1989).

Um achado interessante em nosso estudo foi a expansão de uma linhagem distinta de células NK, apresentando o fenótipo $CD3^-CD16^+CD56^-$. Convencionalmente, células NK humanas são definidas como sendo linfócitos $CD3^-CD16^+$ e/ou $CD56^+$ (LANIER *et al.,* 1986). A maioria das células NK do sangue periférico humano apresenta o fenótipo $CD3^-CD16^+CD56^+$ (GADDY *et al.*, 1997). Pouco é conhecido sobre as propriedades das células NK $CD3^-CD16^+CD56^-$. Gaddy *et al*. (1997) sugerem que células $CD3^-CD16^+CD56^-$ são funcionalmente e fenotipicamente imaturas, capazes de maturação, sendo consideradas como linfócitos pré-NK, possíveis precursores de células NK $CD3^-CD16^+CD56^+$ maduras (GADDY *et al.*, 1997). Esses autores, ao estudarem a ontogenia de células NK utilizando sangue de cordão umbilical, demonstraram a existência de duas subpopulações de células NK, sendo as células $CD3^-CD16^+CD56^-$ de baixa atividade lítica e alta capacidade proliferativa. Essa nova população celular, na presença de IL-2, é capaz de originar o fenótipo $CD3^-CD16^+CD56^+$ e, quando na presença de IL-12, pode transformar-se tanto em $CD3^-CD16^+CD56^+$ quanto $CD16^-$

$CD56^{+}$. No contexto da infecção pelo *T. cruzi*, as células pré-NK ($CD3^{-}CD16^{+}CD56^{-}$), presentes durante ativação da resposta imune inata, na fase crônica indeterminada recente da doença de Chagas, poderiam influenciar nos eventos imunológicos na fase crônica tardia da infecção. Estes dados são consistentes com a hipótese de que antígenos derivados de *T. cruzi* são hábeis para ativar células NK, antes do desenvolvimento da imunidade mediada por células T como sugerido por BRENER *et al.* (1997). O mecanismo proposto para a ativação de células NK, durante os estágios iniciais da infecção em modelos experimentais, sugere que as formas tripomastigotas e amastigotas do *T. cruzi* induzem a síntese de IL-12 por macrófagos que, em associação com TNF-α, promovem a produção de IFN-γ por NK (ALIBERTI *et al.,* 1996; BRENER *et al.,* 1997; GAZZINELLI *et al.,*1997). Em contrapartida, os monócitos/macrófagos são eficientemente ativados por IFN-γ derivado das células NK, aumentando a síntese de óxido nítrico e controlando a replicação do parasita durante os estágios iniciais da infecção pelo *T. cruzi* (SILVA *et al*., 1992; CAMARGO *et al*., 1997; CAMPOS *et al*., 2001; MICHAILOWSKY *et al*., 2001). Neste contexto, nossos dados sugerem que a expansão das células pré-NK poderia estar relacionada a um mecanismo importante na ativação de monócitos/macrófagos durante a fase crônica indeterminada recente da doença de Chagas. Corraborando com essa hipótese, nossos dados mostram que apesar dos baixos percentuais de monócitos macrófagos-like, há aumento na freqüência de monócitos pró-inflamatório ($CD14^{+}CD16^{+}$HLA-DR^{High}) no sangue periférico das crianças infectadas. Belge *et al*. (2002) propuseram a existência de duas subpopulações de monócitos em humanos, as quais podem ser distinguidas como monócitos clássicos ou monócitos pró-inflamatórios, baseado na intensidade de expressão do marcador HLA-DR. Monócitos pró-inflamatórios apresentam uma alta expressão do receptor "Toll-like-2" (TLR-2) e alta produção de TNF-α e IL-12 (BELGE et al., 2002). É interessante observar que os nossos dados referentes à análise de citocinas intracitoplasmáticas produzidas por monócitos, no

contexto *ex-vivo*, demonstraram aumento no número absoluto de monócitos $CD14^{+}IL\text{-}12^{+}$ no grupo E-IND quando comparado com o grupo NI.

Este perfil celular inflamatório nos levou a hipotetizar que as células NK podem assumir um perfil de citocinas do tipo 1. Apesar do perfil de citocinas produzidas pelas células $CD16^{+}$ do sangue periférico das crianças portadoras da doença de Chagas, no contexto *ex-vivo*, não apresentar alterações em relação às crianças não infectadas, as células $CD16^{+}$, quando estimuladas com antígeno da forma epimastigota do *T. cruzi*, assumem um perfil do tipo 1 modulado na produção de citocinas, aqui representado pelo aumento no número absoluto das células $CD16^{+}IFN\text{-}\gamma^{+}$, $TNF\text{-}\alpha^{+}$ e $IL\text{-}4^{+}$. Recentemente, estudos também têm demonstrado que as células NKT do fígado de camundongos cronicamente infectados com o *T. cruzi* produzem altos níveis de IFN-γ e IL-4 (DUTHIE *et al*., 2002). Esses achados sugerem que as células $CD16^{+}$ (células NK ou células NKT) possam proteger os indivíduos contra o *T. cruzi*, não somente na fase aguda, mas também na fase crônica inicial da doença de Chagas, pela produção de perfil misto de citocinas (pró-inflamatório e anti-inflamatório) que favoreceria a geração de mecanismos efetivos na eliminação do parasita na ausência de efeitos imunopatológicos.

Os mecanismos imunopatológicos têm sido foco de muitos trabalhos na doença de Chagas, sugerindo que uma resposta inflamatória exacerbada possa favorecer o desenvolvimento das formas clínicas mais graves da doença (GOMES *et al*., 2003; GOLGHER & GAZZINELLI, 2004). Neste contexto, é importante ressaltar que uma forte ativação das células NK assim como de monócitos pró-inflamatório poderia induzir danos teciduais e progressão da forma indeterminada para as formas cardíacas e ou digestiva (REIS *et al*., 1993; REIS *et al*., 2001). Assim, o estabelecimento de mecanismo imunoregulador, seja por células reguladoras ou por citocinas moduladoras, seria importante no controle da atividade do sistema imune, prevenindo os efeitos deletérios da estimulação excessiva.

Estudos recentes sugeriram que as células NKT e células T reguladoras ($CD4^+CD25^{HIGH}$) apresentam importante função na manutenção da homeostase do sistema imune (WACK *et al*., 1997; DOHERTY *et al*., 1999; BAECHER-ALLAN *et al*., 2001; GOLDFREY & KRONENBERG, 2004; MURRAY *et al*., 2004). As células NKT são particularmente abundantes no fígado humano, representando um terço de todas as células $CD3^+$ hepáticas, mas no sangue periférico elas totalizam somente 2% das células T (NORRIS *et al*., 1999). Tem sido proposto que as células NKT desempenham um importante papel na eliminação de células autólogas no fígado, órgão responsável pela eliminação de células T ativadas via apoptose. Huang *et al.,* (1994) relataram que após ativação, as células T $CD8^+$ circulantes são direcionadas para o fígado, onde sofrem apoptose. Estudos recentes, avaliando o papel das células NKT durante a infecção recente pelo *T. cruzi* em modelo experimental, revelaram a presença de duas subpopulações com funções diferenciadas, denominadas células NKT invariantes e NKT variantes. Os camundongos deficientes de ambas subpopulações desenvolvem aumento no número de células mononucleares no baço e no fígado, na produção de anticorpos anti-*T. cruzi* e do infiltrado inflamatório no tecido muscular cardíaco. Já os animais deficientes apenas da subpopulação de células NKT invariante desenvolvem um robusto processo inflamatório no baço, fígado e fibras musculares esqueléticas, caracterizado pela presença de células NK, dendríticas, linfócitos B e T. Essas células inflamatórias expressam marcadores de ativação, produzem mais IFN-γ, TNF-α, NO e menos anticorpos anti-*T. cruzi*. Assim, estes dados sugerem que as células NKT variantes estariam relacionadas com o aumento do processo inflamatório, enquanto que as células NKT invariantes possuiriam um papel imunoregulador, importante durante a infecção pelo *T. cruzi* (DUTHIE *et al*., 2002, 2005).

Ainda no contexto da imunorregulação, Trzonkowsky *et al*. (2004) revelaram que a produção de IFN-γ, perforina, assim como a atividade citotóxica das células NK e células T

$CD8^+$ são reduzidas na presença de células T reguladoras. Nossos resultados demonstraram um percentual baixo de células NKT e células T reguladoras nas crianças infectadas, consistente com os níveis elevados de células NK e monócitos pró-inflamatórios. Esses dados sugerem que a inabilidade em mudar a resposta imune em direção a um perfil fenotípico caracterizado por um aumento no percentual de células reguladoras possa contribuir para desenvolvimento de danos teciduais no coração e no trato digestório durante o curso da doença de Chagas (VITELLI-AVELAR *et al.*, 2006).

Os valores baixos de células NKT e células T reguladoras levantam o questionamento de por que alterações fenotípicas relacionadas com a ativação de células T não foram observadas no sangue periférico das crianças portadoras da doença de Chagas. Acreditamos 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, não detectável no sangue periférico, considerando relatos prévios que mostram a presença de células T, principalmente linfócitos T $CD8^+$, no infiltrado inflamatório do tecido cardíaco de indivíduos portadores da doença de Chagas (FUENMAYOUR *et al.*, 2005). Estudos realizados em modelo experimental murino para a infecção pelo *T. cruzi* demonstraram que o aparecimento no coração de células T produtoras de citocinas está correlacionado com o aumento local da expressão de moléculas de adesão celular como LFA-I (CD18) o que seria consistente com a migração de linfócitos $CD54^+$ para este sítio. Neste 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. Nossos achados de uma expansão no percentual de linfócitos T $CD8^+$ expressando a molécula CD54, refletem um aumento no potencial migratório de células T citotóxicas para o foco inflamatório com a finalidade de controlar o parasitismo tecidual. Esses achados reforçam a hipótese previamente mencionada de que a ativação de linfócitos T

poderia ser um evento compartimentalizado, restrito aos tecidos-alvo do parasita, como o miocárdio. Além disso, é importante ressaltar que a ausência de fenótipos de ativação na população de células T do sangue periférico poderia representar um fenômeno importante no controle de uma atividade sistêmica durante a infecção pelo *T. cruzi*. Isto pode ser confirmado pela análise da produção de citocinas intracitoplasmáticas, no contexto *ex-vivo*, nas populações de linfócitos T e B. Nossos resultados revelaram que as crianças infectadas apresentaram um perfil de citocinas semelhante ao das crianças não infectadas. Entretanto, na presença do antígeno, simulando um possível contato dessas células com o parasita no foco inflamatório, os linfócitos T e B assumem um padrão misto na produção de citocinas, importante para a eliminação do parasita na ausência de mecanismos imunopatológicos (SAMUDIO *et al*., 1998).

Durante a fase crônica da doença, observa-se, em camundongos experimentalmente infectados pelo *T. cruzi*, aumento no número de células T $CD8^+$ no sangue, fígado e cavidade peritonial (GRISOTTO *et al*., 2001) e predomínio desse tipo de celular entre os linfócitos presentes no baço (MARINHO *et al*., 1999) e no coração desses animais (dos Santos *et al*., 2001). A importância das células T $CD8^+$ na eliminação dos parasitas intracelulares foi demonstrada *in vivo*, através de um modelo experimental, onde a depleção prolongada desse tipo de célula, durante a fase crônica da infecção, causou um aumento no número de ninhos de parasitas no tecido cardíaco (TARLETON *et al*., 1994). Por outro lado, Marinho *et al*. (1999) observaram correlação positiva entre o número de linfócitos T $CD8^+$ no baço e a gravidade da doença em camundongos cronicamente infectados, reforçando a idéia de que linfócitos T $CD8^+$ desempenham papel importante na fase crônica da infecção chagásica. Estes dados, em conjunto, mostram que as células T $CD8^+$, enquanto importantes no controle do parasitismo, também parecem desempenhar papel determinante na destruição tecidual. Embora estudos avaliando a freqüência de linfócitos T no sangue periférico de pacientes

portadores da doença de Chagas e de indivíduos não infectados não tenham mostrado qualquer diferença percentual nas células T $CD4^+$ e $CD8^+$ (DUTRA *et al*., 1994), análises imunohistoquímicas de lesões cardíacas de necropsias de pacientes chagásicos crônicos revelaram predomínio de células $CD8^+$ no infiltrado inflamatório associado (HIGUCHI *et al*., 1993, REIS *et al*., 1993a). A alta freqüência de expressão de granzima-A nas lesões (REIS *et al*., 1993a), bem como o aumento da expressão de antígenos HLA-ABC nos cardiomiócitos (REIS *et al*., 1993b), sugerem o envolvimento de mecanismos imunes citotóxicos na miocardite chagásica crônica humana com participação efetiva de linfócitos T $CD8^+$.

Recentemente, Vitelli-Avelar *et al*. (2005) demonstraram um novo panorama que aponta perfis celulares distintos para as diferentes formas clínicas da doença de Chagas. Esses achados são, sem dúvida, de grande importância para o entendimento da resposta imune celular desencadeada durante a fase crônica da infecção pelo *T. cruzi*. Esses autores demonstraram pela primeira vez a presença de uma elevada freqüência de células T reguladoras e células NKT no sangue periférico de indivíduos portadores da forma clínica indeterminada da doença de Chagas. Já as formas clínicas mais graves apresentaram aumento no percentual de células $CD8^+$ ativadas. Uma análise comparativa entre esses resultados e os observados no grupo E-IND sugere que a expansão de monócitos pró-inflamatórios assim como valores elevados de células pré-NK, em um microambiente deficiente de células NKT e células T reguladoras, representam um importante perfil imunológico que contribuiria para o controle da replicação do parasita durante a fase crônica inicial. Entretanto, a persistência deste perfil, paralelo ao estabelecimento de uma resposta com o predomínio de células T $CD8^+$ ativadas, poderia levar, durante a fase crônica tardia, a danos teciduais que são associados com as formas mais graves (VITELLI-AVELAR *et al.*, 2006).

5.2. Efeito do tratamento específico com benzonidazol sobre o sistema imune de crianças infectadas pelo *T. cruzi*

Uma vez estabelecido o perfil imunofenotípico durante a fase crônica indeterminada recente da doença de Chagas, nosso próximo passo foi avaliar o efeito do tratamento específico com benzonidazol sobre o sistema imune de crianças infectadas pelo *T. cruzi*.

Atualmente, o tratamento específico para a doença de Chagas, no Brasil, é restrito a uma única droga, o benzonidazol, cuja administração é recomendada apenas para os casos de infecção aguda da doença, infecção congênita e crianças com sorologia positiva, que apresentam maior probabilidade de cura parasitológica (DE ANDRADE *et al*., 1996). Já o tratamento específico para a forma crônica indeterminada tardia e forma cardíaca inicial, com percentual de cura reduzido, é sugerido apenas por instituições de pesquisa, a fim de possibilitar melhor avaliação da atividade de cura.

Nas últimas décadas, a literatura acumulou evidências de que o tratamento etiológico da doença de Chagas, além de eliminar o *T. cruzi*, possui impacto benéfico para o quadro clínico do paciente e pode também afetar a natureza da resposta imune (ANDRADE *et al*., 1991; GOMES *et al*., 2003; VIOTTI *et al*., 1994; GONTIJO *et al*., 1999; GARCIA *et al*., 2005; SATHLER-AVELAR *et al*., 2006). Apesar de sua habilidade em adiar ou prevenir a progressão clínica da doença em humanos e modelos experimentais (De ANDRADE *et al*., 1996, SOSA-ESTANI *et al*., 1998; GARCIA *et al*., 2005), ainda são poucos os trabalhos que avaliam o efeito do tratamento com benzonidazol na resposta imune de pacientes infectados pelo *T. cruzi*. Garcia *et al.* (2005), ao avaliarem o efeito do tratamento com benzonidazol em animais cronicamente infectados, demonstraram redução das alterações cardíacas, mesmo na ausência da eliminação completa do parasita. Estes resultados são reforçados pelos achados de Viotti *et al*. (1994). Estes autores, ao avaliarem o efeito do tratamento com benzonidazol em

pacientes cronicamente infectados pelo *T. cruzi*, demonstraram menor evolução clínica para a forma cardíaca da doença, independente da cura parasitológica.

Por outro lado, tem sido postulado que o benzonidazol por si parece não ser responsável pelas mudanças observadas durante o tratamento etiológico, uma vez que pacientes com falha terapêutica, demonstrada pela presença de parasita circulante, não apresentam alterações significativas no perfil imunológico (DUTRA *et al*., 1996). Esses autores, demonstraram que na ausência de parasitas circulantes, altos níveis de células T ativadas podem ser observadas no sangue periférico de pacientes que foram submetidos ao tratamento com benzonidazol a pelo menos 5 anos. Outro estudo revelou clara tendência à queda no percentual de células T ativadas após 6 meses do término do tratamento com benzonidazol (GONTIJO *et al*., 1999). Esses dados em conjunto sugerem que o tratamento com benzonidazol pode levar a mudanças seletivas no perfil imunológico tempo dependente, as quais podem também diferir se a intervenção terapêutica for realizada durante a fase aguda ou crônica da doença.

Na doença de Chagas, a análise do sistema imunológico antes e após o tratamento é essencial para o entendimento dos mecanismos de ação da droga, bem como o desenvolvimento racional de novos agente tripanossomatídeos (De CASTRO, 1993; COURA & De CASTRO, 2002). Nas últimas décadas, estudos têm acumulado evidências que correlacionam a resposta imune e o efeito quimioterápico (URBINA, 1999). Estudos recentes sugerem que a ativação do sistema imune aumenta a eficácia do tratamento com benzonidazol durante a infecção pelo *T. cruzi* em modelos experimentais (MICHAILOWSKY *et al*., 1998). Com o intuito de elucidar um pouco mais esta questão e contribuir para o entendimento do impacto do tratamento no sistema imune, nosso trabalho desenvolveu um estudo longitudinal para avaliar o efeito do benzonidazol no padrão de citocinas intracitoplasmáticas e no fenótipo dos leucócitos do sangue periférico de crianças infectadas pelo *T. cruzi*.

Nossos dados demonstraram que apesar de baixa ativação global no sistema imune durante a fase crônica indeterminada recente da doença de Chagas, grande ativação das células da imunidade inata e adaptativa pode ser observada no sangue periférico das crianças submetidas ao tratamento com benzonidazol. Esses resultados estão de acordo com os achados de Dutra *et al*. (1996) que demonstram um aumento no percentual de células T e B ativadas no sangue periférico de pacientes portadores da doença de Chagas por mais de 5 anos após o término do tratamento etiológico com benzonidazol. Além disso, Bahia-Oliveira *et al*. (2000) mostraram alta resposta proliferativa das células mononucleares do sangue periférico, quando estimuladas com antígenos do *T. cruzi*, de pacientes tratados curados, sugerindo a presença de células T de memória. Neste contexto, sugere-se que a memória imunológica após a cura parasitologica possa ser favorecida pela presença de antígenos do *T. cruzi*, mas não o parasita intacto, na superfície de esplenócitos do centro germinativo e no foco inflamatório do miocárdio de camundongos submetidos ao tratamento etiológico (ANDRADE *et al*., 1991). Esses resultados em conjunto nos levaram a hipotetizar que a grande liberação de antígenos do parasita durante o tratamento possa induzir o perfil imunológico ativado com conseqüente síntese de citocinas, que poderiam ser mantidas por antígenos parasitários residuais e ou interação idiotípica. Além disso, estes resultados estão de acordo com aqueles reportados por Olivieri *et al.* (2002) demonstrando que o tratamento com benzonidazol leva a um aumento no número de linfócitos nos órgãos linfóides de camundongos infectados pelo *T. cruzi*, principalmente devido à expansão de células T $CD8^+$. A migração seletiva das células T $CD8^+$ para os órgãos linfóides poderia explicar os baixos números de linfócitos T $CD8^+$ observados no grupo $E\text{-}IND_T$. Entretanto, nossos resultados revelaram queda no percentual de linfócitos T $CD8^+$ expressando moléculas de adesão celular ($CD8^+CD62L^+$ e $CD8^+CD54^+$) no sangue periférico das crianças após o tratamento com benzonidazol.

Laucela *et al*. (1996) demonstraram que níveis elevados de moléculas de adesão celular estão associados com a gravidade da doença de Chagas durante a fase crônica. Estes autores também demonstraram queda na expressão de moléculas de adesão 48 meses após o termino do tratamento. Com base nesses resultados, nós hipotetizamos que a resposta inflamatória após o tratamento possa também ser controlada pela regulação das moléculas de adesão nas células T $CD8^+$. Essa hipótese pode ser confirmada pelos resultados de dois grupos de pesquisas que observam correlação direta entre o tratamento com benzonidazol e redução no número de infiltrados inflamatórios, com conseqüente redução no depósito de componentes da matrix extracelular no coração de camundongos infectados pelo *T. cruzi* (GARCIA *et al*., 2005; ANDRADE *et al*., 2007). Assim, a associação entre moléculas de adesão e a gravidade da doença (LAUCELLA *et al*., 1996), e o fato de o percentual de linfócitos T $CD8^+CD62L^+$ e $CD8^+CD54^+$ diminuir após o tratamento, sugerem que a redução na expressão das moléculas de adesão pelas células T $CD8^+$ possa ser um indicador para um prognóstico favorável na evolução clínica dos pacientes submetidos ao tratamento etiológico.

Um ponto preocupante em nossos achados é a possibilidade deste grande número de células ativadas resultarem em efeitos adversos aos pacientes submetidos ao tratamento, levando a uma resposta pró-inflamatória e danos teciduais. Com o objetivo de esclarecer esta questão, este trabalho também determinou pela primeira vez o impacto do tratamento etiológico no padrão de citocinas intracitoplasmáticas dos leucócitos do sangue periférico de crianças submetidas ao tratamento.

Nossos resultados demonstraram que o tratamento com benzonidazol durante a fase crônica indeterminada recente da doença de Chagas induz aumento no percentual de monócitos "macrófagos-like" e monócitos pró-inflamatórios. Uma vez que estudos demonstram aumento na síntese de citocinas pró-inflamatórias por monócitos "macrófagos-like" e monócitos pro-inflamatórios (BELGE *et al*., 2002; DAYYANI *et al*., 2003) e estas

células estão aumentadas após o tratamento com benzonidazol, nós realizamos análises de correlação entre as células $CD14^+$ produtoras de citocinas inflamatórias (IL-12 ou TNF-α) e o percentual de monócitos "macrófagos-like" ou monócitos pró-inflamatórios. As análises de correlação, de forma interessante, não revelaram uma correlação positiva entre estas células e a produção de citocinas inflamatórias por monócitos ($CD14^+TNF\text{-}\alpha^+$ e $CD14^+IL\text{-}12^+$). Inicialmente, estes dados parecem controversos, entretanto é possível que algum mecanismo imunoregulador possa estar controlando a síntese de citocinas pelos monócitos após o tratamento com benzonidazol. É possível que o benzonidazol possa modular a síntese dessas citocinas pelos monócitos, uma vez que estudos recentes reportaram um efeito inibitório do benzonidazol na síntese de IL-12 e TNF-α por monócitos estimulados por LPS (PASCUTTI *et al*., 2004). Outra possibilidade seria ação de citocinas moduladoras sintetizadas por outras fontes celulares, uma vez que a via imuno-reguladora autócrina parece não estar presente no controle da população de monócitos.

Em humanos, foi proposto que a alta produção de IFN-γ possa favorecer a eficácia terapêutica (BAHIA-OLIVEIRA *et al*., 1998; BAHIA-OLIVEIRA *et al*., 2000). Nossos dados revelaram que o tratamento com benzonidazol desviou a síntese de citocinas pelas células NK na cultura controle para um padrão de citocinas do tipo 1 modulado (IFN-γ, TNF-α e IL-4), fato não observado na avaliação antes do tratamento. Já, na presença de estimulação antígeno-específica, além do perfil do tipo 1 modulado, as células NK demonstraram aumento na produção de IFN-γ. Resultados semelhantes foram demonstrados por Peruhype-Magalhães *et al*. (2005) para pacientes curados de leishmaniose visceral, cuja resposta imune inata pôde ser eficientemente restabelecida após estimulação com antígenos do parasita. Como já apresentado anteriormente, estudos revelaram que antígenos da forma tripomastigota do *T. cruzi* são hábeis em estimular a síntese de IFN-γ pelas células NK (BRENER & GAZZINELLI, 1997). Nesse contexto, análise adicional de correlação revelou que o aumento

na freqüência de células NK totais e NK ativadas estão correlacionadas positivamente com o padrão de citocinas do tipo 1 modulado ($CD16^+IFN\text{-}\gamma^+$ e $CD16^+IL\text{-}4^+$). Nossos dados da imunidade inata em conjunto sugerem que a expansão na população de monócitos pró-inflamatórios e das células NK ativadas pode representar um mecanismo efetivo na eliminação do parasita durante o tratamento com benzonidazol, uma vez que macrófagos podem ser eficientemente ativados pelas células NK ativadas produtoras de IFN-γ, aumentando a produção de óxido nítrico e controlando a replicação do parasita (BRENER & GAZZINELLI *et al*., 1997; CHADRASEKAR *et al*., 1998; 2000; BRODSKYN *et al*., 2002). Além disso, a produção de IL-4, uma citocina do tipo 2, pelas célula NK pode ser relevante no controle da síntese de IL-12 pelo monócitos, direcionando uma via moduladora (BAHIA-OLIVEIRA *et al*., 1998; CORREA-OLIVEIRA *et al*., 1999; SATHLER-AVELAR *et al*., 2006).

No compartimento da resposta imune adaptativa, nós observamos um padrão distinto na produção de citocinas pelos linfócitos T $CD4^+$, T $CD8^+$ e células B $CD19^+$ no grupo E-IND_T. Um perfil do tipo 2 foi observado para os linfócitos T $CD4^+$, representado pelo aumento notável na síntese de IL-10 tanto na cultura controle quanto na presença do antígeno solúvel do parasita. Para a população de células B $CD19^+$, foi observado um padrão na produção de citocinas semelhante ao tipo 2, caracterizado pelo predomínio na síntese da citocina IL-10 acompanhado pelo pequeno aumento na produção de IL-4 e TNF-α no grupo E-IND_T. De forma diferente ao padrão de citocinas dos linfócitos T $CD4^+$ e células B $CD19^+$, um perfil do tipo 1 modulado típico foi observado para os linfócitos T citotóxicos na presença do antígeno do *T. cruzi*, com uma expressão considerável de IFN-γ pelas células T $CD8^+$.

Análises adicionais de correlação da produção de citocinas por essas populações com os fenótipos de ativação demonstraram um perfil imunológico do tipo 1 modulado dentro das

populações de linfócitos T e B, semelhante ao observado para a imunidade inata. Especificamente, essas análises demonstraram que os linfócitos T $CD4^+$ ativados produzem exclusivamente IL-10. Já as células T $CD8^+$ e B $CD19^+$ ativadas estão correlacionadas positivamente com a síntese IFN-γ e TNF-α, respectivamente, e a expressão de IL-10, representando padrão misto de citocinas.

Esses dados validam e reforçam mais uma vez o papel do IFN-γ como citocina essencial para o sucesso terapêutico, e acrescenta mais detalhes a esta hipótese, descrevendo as células NK $CD16^+$ e os linfócitos T $CD8^+$ como as principais fontes dessa citocina durante o tratamento da doença de Chagas. Além disso, nossos dados também indicam a citocina IL-10 produzida pelo linfócitos T, principalmente linfócitos T $CD4^+$, assim como linfócitos B, como um elemento chave para a ausência de danos teciduais secundários inerentes ao intenso processo inflamatório desencadeado durante o tratamento etiológico.

Finalmente, é interessante mencionar que o padrão de citocinas observado para a imunidade inata e adaptativa do grupo $E\text{-}IND_T$ foi muito semelhante ao encontrado para os leucócitos do grupo E-IND após estimulação com o antígeno do parasita. Essa observação sugere que a grande quantidade de antígenos liberada durante o tratamento, hábil para ativar a síntese de citocinas *in vivo*, pode ser reproduzida artificialmente *in vitro* pela estimulação, com antígenos do *T. cruzi*. Considerando a dependência do sistema imunológico para o sucesso terapêutico na doença de Chagas, esta estratégia pode representar um ferramenta adicional para predizer o tipo de resposta imune que o paciente possa desenvolver durante o tratamento com benzonidazol.

Nós acreditamos que a grande quantidade de antígenos do parasita, liberada pelas células do hospedeiro infectadas durante o tratamento etiológico, provavelmente medeia uma forte ativação dos linfócitos T $CD4^+$ e $CD8^+$ que não causam danos teciduais devido à habilidade dos antígenos do *T. cruzi* em promover um perfil modulado. Assim, nosso trabalho

suporta a idéia de que a presença de leucócitos ativados no sangue periférico não é um fator limitante para a indicação do tratamento etiológico, uma vez que a administração do benzonidazol pode direcionar a síntese de citocinas pelos leucócitos para um padrão misto pró/anti-inflamatório, benéfico ao paciente.

6. CONCLUSÃO

A análise do perfil fenotípico e do padrão de citocinas leucocitárias das crianças infectadas pelo *T. cruzi* antes do tratamento permitiu identificar alterações que reforçam a hipóteses da participação da resposta imune inata e da imunidade humoral nos eventos iniciais da fase crônica da doença de Chagas, na ausência de sinais fenotípicos relativos à ativação efetiva da resposta adaptativa mediada por linfócitos T e na secreção de citocinas. Além disso, nossos dados contribuem para a hipótese de que a imunidade mediada por células T, durante a forma clínica indeterminada recente, possa representar um fenômeno restrito ao compartimento cardíaco e linfonodos, não detectado no sangue periférico. Com relação ao efeito do tratamento na forma crônica indeterminada recente da doença de Chagas, nossos resultados demonstraram que o padrão do tipo 1 modulado observado após o tratamento reforçam o papel das citocinas IFN-γ, produzido pelas células NK e linfócitos T $CD8^+$, e IL-10, pelas células B e linfócitos T $CD4^+$ e T $CD8^+$ como um elemento chave na eliminação do parasito na ausência de danos teciduais inerente ao intenso processo inflamatório desencadeado durante o tratamento etiológico. Além disso, nossos achados demonstraram, pela primeira vez, que a presença de leucócitos ativados no sangue periférico não é um fator limitante para a recomendação do tratamento etiológico, uma vez que a administração do benzonidazol pode direcionar a síntese de citocinas pelos leucócitos para um padrão do tipo 1 modulado, benéfico ao paciente.

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7. PERSPECTIVAS

- ✓ Estudar de forma descritivo-analítica, no contexto *"ex-vivo"*, o impacto do tratamento etiológico sobre o perfil imunofenotípico de leucócitos do sangue periférico de pacientes adultos portadores da forma crônica indeterminada da doença de Chagas;

- ✓ Avaliar o impacto do tratamento com benzonidazol sobre aspectos fenotípico-funcionais de células da imunidade inata do sangue periférico de pacientes adultos portadores da forma crônica indeterminada da doença de Chagas, após estimulação antigênica;

- ✓ Estudar o impacto do tratamento etiológico no padrão de citocinas (IL-12, IFN-γ, TNF-α, IL-4 e IL-10) produzidas *in vitro*, por leucócitos do sangue periférico de pacientes adultos portadores da forma crônica indeterminada da doença de Chagas, antes e após o tratamento com benzonidazol.

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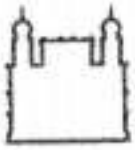
ANEXOS

Tabela: Leucometria do sangue periférico dos pacientes portadores da forma clínica indeterminada recente da doença de Chagas antes (E-IND) e após o tratamento com benzonidazol (E-IND$_T$) e de indivíduos não infectados (NI)

Parâmetros Hematológicos	**Grupos avaliados**		
	NI	**E-IND**	**E-IND$_T$**
Leucócitos totais (x 10^3/mm^3)	6.993±1.823	6.660±1.266	6.413±1.777
Granulócitos (x 10^3/mm^3)	3.941±1.717	3.278±1.069	3.423±1.032
Monócitos (x 10^3/mm^3)	584±524	445±162	503±286
Linfócitos (x 10^3/mm^3)	1.967±715	2.245±460	2.241±698



Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

Centro de Pesquisas René Rachou
Comitê de Ética em Pesquisa em Seres Humanos

PARECER Nº 06/2002 – CEPSH- CPqRR

Protocolo nº: 07/2002

Projeto de Pesquisa: Doença de Chagas nos municípios de Berilo e José Gonçalves de Minas, MG: epidemiologia, terapêutica e controle

Pesquisador Responsável: Marta de Lana

Instituição: Universidade Federal de Ouro Preto

Data de entrada no CEP: 28 de maio de 2002

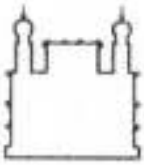
Ao se proceder à análise do protocolo em questão, após consideradas as respostas as pendências anteriores, constatou-se que as informações enviadas atendem aos aspectos fundamentais da Resolução CNS 196/96, sobre Diretrizes e Normas Regulamentadoras de Pesquisa envolvendo Seres Humanos.

Diante do exposto, o Comitê de Ética em Pesquisa em Seres Humanos do Centro de Pesquisas René Rachou / FIOCRUZ, de acordo com as atribuições da Resolução 196/96, manifesta-se pela aprovação do projeto de pesquisa proposto.

Situação: **PROJETO APROVADO**

Belo Horizonte, 22 de outubro de 2002

Alvaro José Romanha
Coordenador do CEPSH- CPqRR



Ministério da Saúde

FIOCRUZ

Fundação Oswaldo Cruz

Centro de Pesquisa René Rachou

Comitê de Ética

PARECER Nº 11/2004 – CEPSH- CPqRR

Protocolo CEPSH/CPqRR nº: 012a/2004

Projeto de Pesquisa: "Doença de Chagas nos municípios de Berilo e José Gonçalves de Minas, MG: epidemiologia, terapêutica e controle"

Aditivo 1: Acrescentar objetivos ao projeto:
Objetivos:

1- Avaliar o perfil imunológico dos pacientes portadores de estágios recentes da doença de Chagas com ênfase no padrão de síntese de citocinas intracitoplasmáticas por leucócitos do sangue periférico, antes e após terapêutica específica;
2- Empregar a Reação em Cadeia da Polimerase – PCR como critério de cura pós-terapêutica da doença de Chagas

Pesquisador Responsável: Marta de Lana

Instituição: Centro de Pesquisa René Rachou / FIOCRUZ

Data de entrada no CEP: 1 de junho de 2004

Ao se proceder à análise do protocolo em questão, constatou-se que a adição dos objetivos ao projeto atende aos aspectos fundamentais da Resolução CNS 196/96, sobre Diretrizes e Normas Regulamentadoras de Pesquisa envolvendo Seres Humanos.

Diante do exposto, o Comitê de Ética em Pesquisa em Seres Humanos do Centro de Pesquisas René Rachou / FIOCRUZ, de acordo com as atribuições da Resolução 196/96, manifesta-se pela aprovação do projeto de pesquisa proposto.

Extensão do projeto: **APROVADO**

Belo Horizonte, 16 de junho de 2004.

Álvaro José Romanha
Coordenador do CEPSH- CPqRR

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Artigos Relacionados com a Tese

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2006.03123.x

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 km². 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 km² 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-DR$^+$ and CD4$^+$ CD38$^+$ T cells with no changes in CD8$^+$ HLA-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

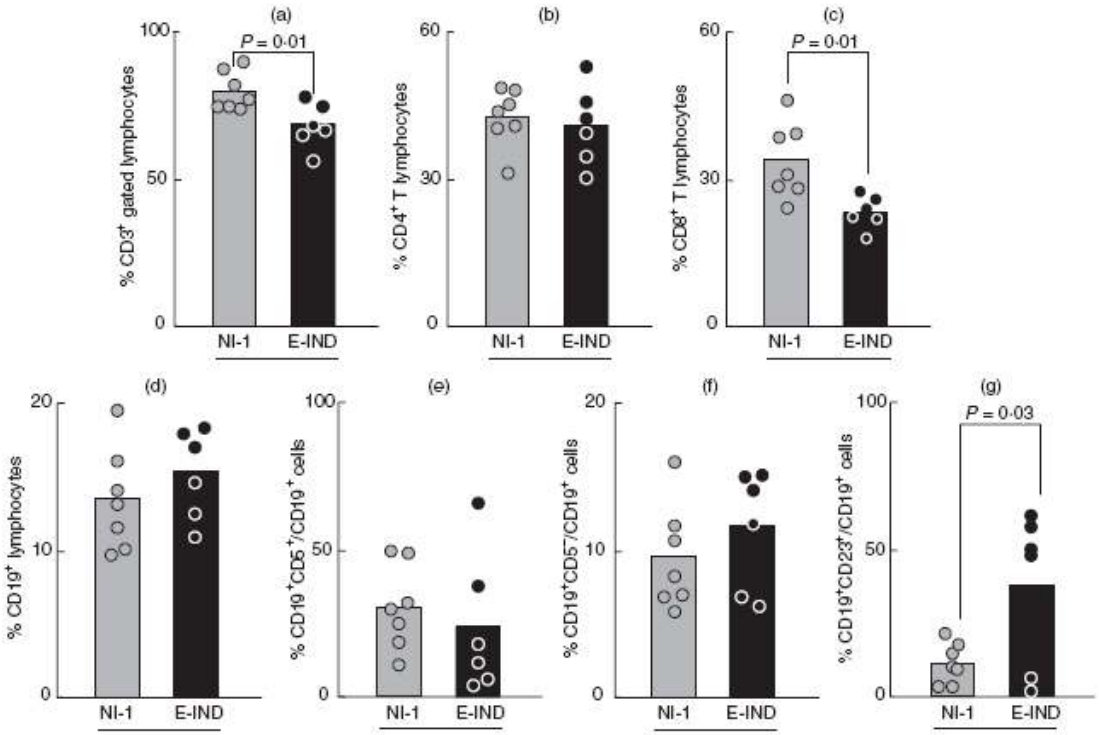

Fig. 1. Analysis of T and B cell subsets in the peripheral blood of early indeterminate *Trypanosoma cruzi* infected children (E-IND, ●) and non-infected children (NI-1, ○). Phenotypic studies were performed by a double-labelling protocol using anti-CD3 fluorescein isothiocyanate (FITC) and anti-CD4 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 $CD14^+$ $CD16^+$ $HLA\text{-}DR^{++}$ 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*	NI-1	E-IND	NI-1	E-IND
$HLA\text{-}DR^+$	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*

*The results are expressed as proportion within a given T cell subset, e.g. ratio of $CD4^+$ $HLA\text{-}DR^+$ 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.

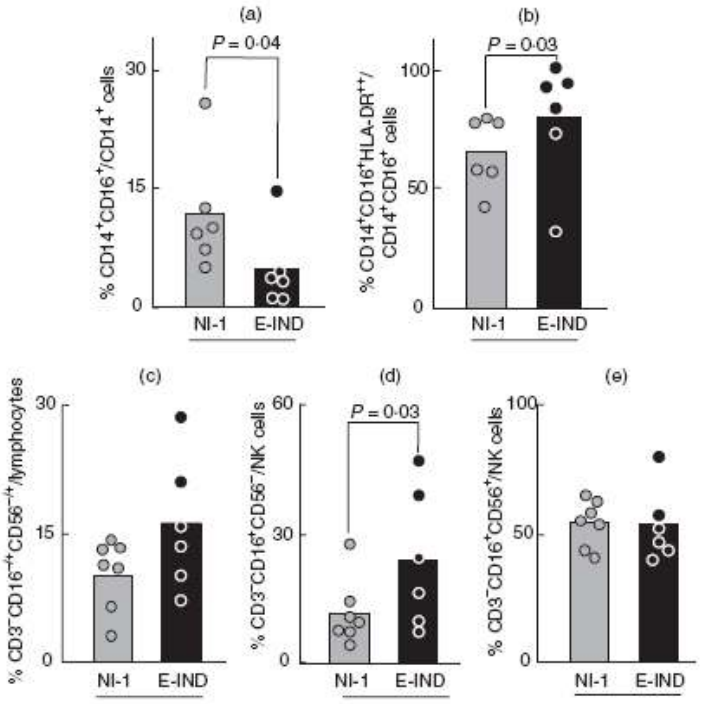

Fig. 2. Analysis of monocyte and natural killer (NK) cell subsets in the peripheral blood of early indeterminate *Trypanosoma cruzi*-infected early indeterminate (E-IND, ●) children and non-infected children (NI-1, ○). Monocyte subpopulation analysis was performed by a triple-labelling platform using 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 $HLA\text{-}DR^+$ monocytes and proinflammatory $HLA\text{-}DR^{++}$ monocytes [19]. Quantification of the $CD14^+$ $CD16^+$ $HLA\text{-}DR^{++}$ 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 $CD14^+$ $CD16^+$ $HLA\text{-}DR^{++}$ 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

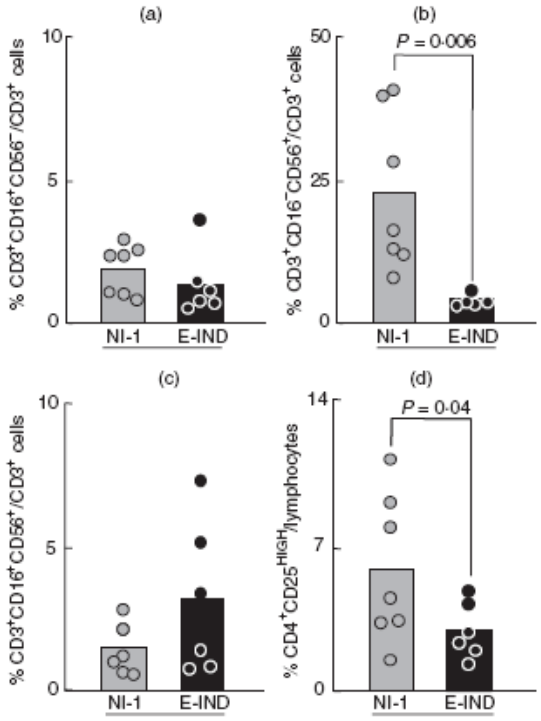

Fig. 3. Analysis of regulatory T cells [natural killer (NK) T and $CD4^+CD25^{HIGH}$] in the peripheral blood of early indeterminate *Trypanosoma cruzi*-infected children (E-IND, ●) and non-infected children (NI-1, ○). NKT phenotypic studies were performed by a triple-labelling protocol using anti-CD3 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

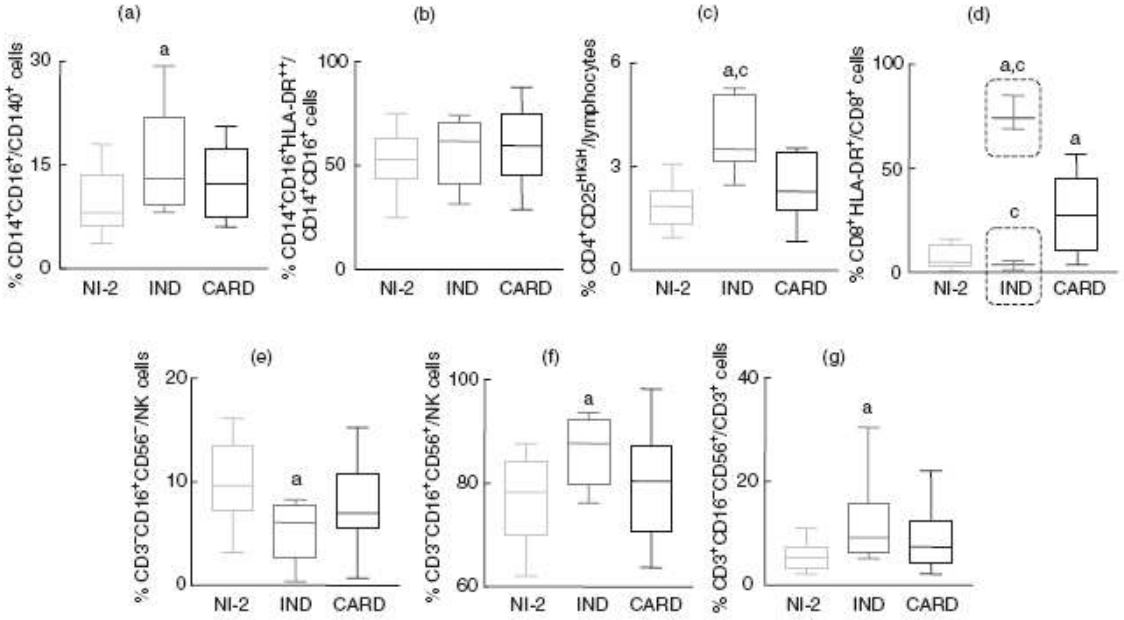

Fig. 4. Analysis of major discriminatory immunophenotypes among individuals with indeterminate disease (IND, ▭) or cardiac disease (CARD, ▭) and uninfected adults (NI-2, ▭). Phenotypic studies were performed using a double or triple-labelling protocol to identify macrophage-like $CD14^+$ $CD16^+$ cells (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 $P < 0.05$.

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

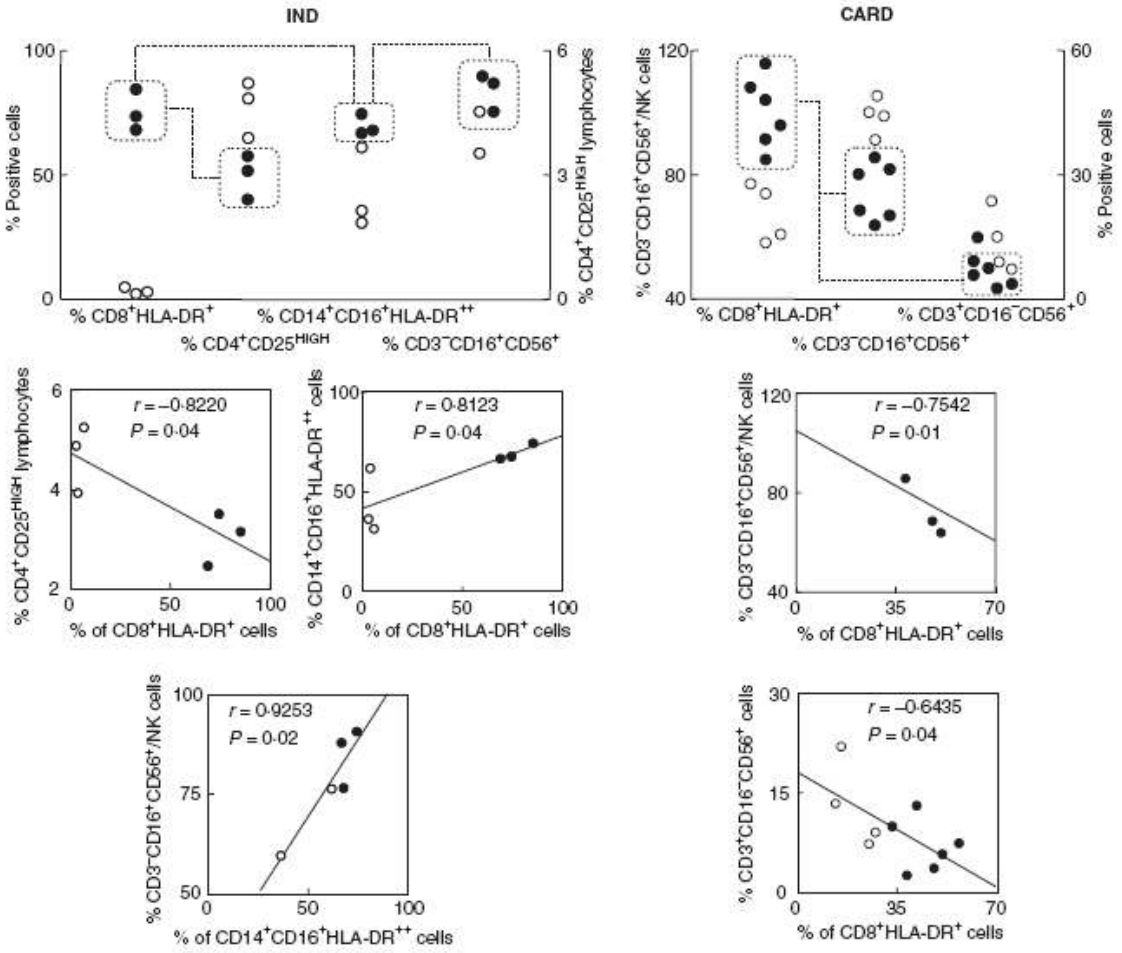

Fig. 5. Analysis of major discriminatory immunophenotypes of indeterminate (IND, left panels) and cardiac patients (CARD, right panels). Analysis of individual data from IND demonstrates an association (dotted rectangles and lines) between high frequency of $CD8^+$ HLA-DR^+ cells in IND (○) with low 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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Benznidazole Treatment during Early-indeterminate Chagas' Disease Shifted the Cytokine Expression by Innate and Adaptive Immunity Cells toward a Type 1-modulated Immune Profile

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Abstract

Trypanosoma cruzi-infected children was treated with benznidazole (Bz) during the early-indeterminate disease (E-IND) and the cytokine pattern of innate and adaptive immune compartments were evaluated prior to the treatment and 1 year after it. At first, we observed that the *ex vivo* cytokine profile of circulating leukocytes from E-IND ($n = 6$) resembled the one observed for healthy schoolchildren ($n = 7$). Additionally, *in vitro* stimulation with *T. cruzi* antigens drove the E-IND cytokine pattern toward a mixed immune profile with higher levels of IFN-γ^+, TNF-α^+ and IL-4$^+$ NK cells, increased numbers of IFN-γ^+, TNF-α^+ and IL-10$^+$ CD4$^+$ T cells in addition to enhanced frequency of TNF-α^+/IL-4$^+$ CD19$^+$ lymphocytes. Interestingly, upon *T. cruzi* antigen *in vitro* stimulation, E-IND CD8$^+$ lymphocytes displayed a selective enhancement of IFN-γ expression, accounting for a global type 1-modulated cytokine microenvironment. A shift toward a type 1-modulated profile was also the hallmark of Bz-treated children (E-IND$_{Tr}$). In this context, despite the mixed overall *ex vivo* cytokine profile observed for NK and CD8$^+$ T cells, increased ability of these leukocytes to produce IFN-γ in response to *T. cruzi* antigens was reported. Most noteworthy was the IL-10 production evidenced at T lymphocytes, mainly CD4$^+$ cells, as well as B lymphocytes, both *ex vivo* and upon antigen stimulation. Together, these findings gave evidence that NK cells and CD8$^+$ T lymphocytes are the major sources of IFN-γ, a pivotal cytokine for successful therapeutic response in human Chagas' disease. Moreover, our data have also brought additional information, pointing out IL-10 production by CD4$^+$ cells and B lymphocytes, as the putative key element for parasite clearance in the absence of deleterious tissue damage.

Introduction

Infection of susceptible mammalian hosts with intracellular protozoa *Trypanosoma cruzi* leads to chronic progressive disease, which may cause deleterious inflammatory infiltrations into specific target tissue [1]. This illness, known as American Trypanosomiasis or Chagas' disease, affects nearly 20 million people in Central and South America [2].

The clinical course of Chagas' disease comprises two distinct stages with intrinsic parasitological and immunological features, named acute and chronic phases of the infection. In endemic areas, the great majority of the patients are identified during long-lasting chronic disease, as they remain clinically asymptomatic for many years, characterizing the late-indeterminate clinical form of the disease [3]. Acute clinical features are not frequently observed in endemic areas with most acute cases being

usually non-apparent leading to an asymptomatic stage known as early-indeterminate disease (E-IND).

The mechanisms underlying the early-indeterminate Chagas' disease are not yet fully understood. However, there is a general consensus that the host immune response plays a pivotal role in controlling the pathogenesis through protective events that may control the tissue damage [4].

We had previously dedicated special attention to characterize the frequency of major and minor peripheral blood leukocyte subsets during early-indeterminate Chagas' disease. We have reported an expansion of pre-NK cells ($CD16^+CD56^-$) associated with enhanced frequency of activated B cells in the absence of phenotypic features of T-cell activation at peripheral blood of early-indeterminate children [5].

Good insight into the role of cell-mediated immunity associated with the pathogenesis control of Chagas' disease has been provided from experimental models for *T. cruzi* infection. It has become clear that cytokines are integral components of the complex intercellular system required to mount and control disease morbidity [6, 7]. However, little is known about the cytokine profile during early human infection with *T. cruzi*.

Samudio *et al.* [8] have suggested that although the selective induction of a type 1-cytokine pattern (IFN- with minimal IL-4 production) is the hallmark of acute symptomatic Chagas' disease, the establishment of a type 0 immune profile (IFN- and IL-4) was associated with asymptomatic disease. The apparently paradoxical role for IFN-γ in human Chagas' disease has been discussed by Bahia-Oliveira *et al.* [9]. It has been proposed that the presence of an immune response with high levels of IFN-γ may favour the effectiveness of the treatment or influence the development of chronic cardiac form of the disease in the absence of chemotherapy. In agreement with this hypothesis, it has been postulated that higher ability to produce IFN-γ may be associated with the therapeutic effectiveness during acute human Chagas' disease [10]. Regardless of these insights, no longitudinal investigation has been carried out to address this issue as well as the most important cell sources of cytokines during early stages of human Chagas' disease neither the impact of the aetiological treatment with Benznidazole (Bz) on this cytokine profile was determined.

The main goal of the current investigation was to evaluate, in a longitudinal study, the cytokine profile at whole blood leukocytes, freshly isolated from early-indeterminate *T. cruzi*-infected children, prior and 1 year after the end of aetiological treatment. Herein, we showed evidences that the *ex vivo* cytokine profile at circulating leukocytes from E-IND resembled the one observed for healthy schoolchildren. A shift toward a type 1-modulated profile was also the hallmark of Bz-treatment, with NK cells and $CD8^+$ T lymphocytes representing the major sources of IFN-γ and $CD4^+$ cells and $CD19^+$ lymphocytes supplying the IL-10 production as the key element for parasite clearance in the absence of deleterious tissue damage.

Patients, materials and methods

Study area. Berilo and José Gonçalves de Minas are located at Jequitinhonha Valley, north-east of Minas Gerais State, Brazil, extending over 970 km^2 and are referred as one of the most important former endemic area for Chagas' disease in Brazil. Together, these two municipalities have 17,632 habitants with 78.43% of these individuals living in rural areas and having their economy based on agriculture and cattle-ranching activities (ibge.cidades@, available at: http://www.ibge.gov.br/cidadesat, search for Berilo and José Gonçalves de Minas). Accessed on 4 January 2006.

Study population. The patients included in this investigation consisted of thirteen schoolchildren (9–14 years old, mean 12) from Berilo and José Gonçalves de Minas, classified into two groups named early-indeterminate Chagas' disease (E-IND) and non-infected controls (NI). The E-IND group consisted of six schoolchildren (four boys and two girls) with positive serology for anti-*T. cruzi* performed by ELISA, EIE-Rec-ELISA (Biomanguinhos/FIOCRUZ), Indirect immunofluorescence (IIF) and IHA tests, as recommended by the Brazilian Health Ministry and World Health Organization criteria [2]. All chagasic children displayed at least two positive serological tests, with distinct principles. 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-rays (Table 1). Haemoculture, performed as described by Luz [11] was positive in all but one (5/6) seropositive children, generally within the first month of blood cultivation in Liver Infusion Tryptose medium (LIT). Chagasic children were treated with benznidazole (Rochagan®; Roche, SP, Brazil) following the protocol recommended by the Brazilian Health Ministry, consisting of 8 mg/kg/day for 60 consecutive days. Following aetiological treatment, all children were longitudinally re-evaluated 1 year after the end of Bz-treatment ($E\text{-}IND_T$).

Seven non-infected schoolchildren were included as a control group. The NI group consisted of age-matched schoolchildren with negative serology for anti-*T. cruzi* IgG immunodiagnosis (ELISA and IIF). The seronegative cases included one boy and six girls, with ages ranging from 9 to 14 years (mean 12.4).

Informed written consent was obtained from all through their parents or legal guardians. This work fulfilled the resolution number 196/1996 from the Brazilian National Health Council for research involving humans

Table 1 Demographic, clinical and laboratorial features of six *Trypanosoma cruzi* seropositive children from Jequitinhonha Valley, Brazil.

	Municipality					
	Berilo			José Gonçalves de Minas		
	Register number					
Major features	52	1172	1313	701	477	499
Age (years)	9	12	13	11	13	14
Sex	Male	Male	Female	Male	Male	Female
Clinical and physical examination	Normal	Normal	Normal	Normal	Normal	Normal
Thoracic X-ray	Normal	Normal	Normal	Normal	Normal	Normal
ECG	Normal	Normal	Normal	Enlarged Pri	Normal	Normal
Haemoculture	Positive	Positive	Positive	Positive	Positive	NP

NP, not performed; Pri = Pr interval.

and was approved by the Ethical Committee at Centro de Pesquisas René Rachou (CPqRR/FIOCRUZ – protocol no. 11/2004).

Trypanosoma cruzi antigen preparation. Soluble Epimastigote Antigen (EPI) was prepared from a stationary phase Y strain *T. cruzi* epimastigotes grown in LIT-medium. After the third or fourth *in vitro* passage, epimastigotes were harvested, washed in 15 mM phosphate-buffered saline (PBS), pH 7.4, and resuspended to 10^8 cells/ml in 15 mM PBS, pH 7.4. The suspension was rapidly frozen at −70 °C and thawed at 37 °C three times, with a sonication procedure between each step. The crude lysate was centrifuged (37,000 *g*) for 90 min and the supernatant collected, dialysed overnight against 15 mM PBS, pH 7.4, sterilized by filtration through a 0.22-μm pore membrane (Filter millex, Milipore Products Division, Billerica, MA, USA) and stored at −70 °C until use. The protein content was assayed by the method of Lowry *et al.* [12].

In vitro short-term culture of whole blood leukocytes. Peripheral blood samples from 13 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 named: the control culture, in the absence of exogenous stimuli, to reproduce the *ex vivo* immunological status (control) and the EPI-stimulated culture (EPI), to mimic the *in vivo* challenge with *T. cruzi* antigen.

The control culture consisted of 500 μl of whole blood incubated in the presence of 500 μl of RPMI-1640 (GIBCO, Grand Island, NY, USA) plus Brefeldin A (BFA) (Sigma, St Louis, MO, USA), at a final concentration of 10 μg/ml, performed in 14 ml polypropylene tubes (Falcon®, BD Pharmingen). The Culture was maintained for 4 h at 37 °C in a 5% CO_2 humidified incubator.

Antigen-specific stimulation was performed by a previous challenge of 500 μl of whole blood with EPI antigens at a final concentration of 25 μg/ml for 1 h at 37 °C in a 5% CO_2 humidified incubator, followed by incubation with BFA for an additional period of 4 h.

A positive control culture was also performed to evaluate the sample viability. For this purpose, a whole blood sample was respectively treated with 500 μl of RPMI-1640 plus Phorbol 12-Myristate 13-Acetate-PMA (Sigma) at a final concentration of 25 ng/ml, ionomycin (Sigma) at 1 μg/ml and BFA. The positive one was maintained for 4 h at 37 °C in a 5% CO_2 humidified incubator. Cytokine patterns observed in the positive control culture-confirmed viability of the sample, as demonstrated by high levels of IFN-γ^+ and TNF-α^+ cells (data not shown).

Immunostaining for cell surface markers and intracellular cytokines. At the end of incubation periods, all cultures were treated with EDTA (Sigma) at a final concentration of 2 mM for 15 min, at room temperature. EDTA-treated whole blood cultures were washed once with FACS buffer (PBS 0.5% of bovine serum albumin, BSA; Sigma, pH 7.4), by centrifugation at 600 *g* for 7 min at room temperature, and resuspended to half original volume with FACS buffer. Samples of 400 μl of cell suspension from three cultures were dispensed into two 5-ml polystyrene tubes (Falcon®), one containing 10 μl of diluted anti-CD4 (clone S3.5) labelled with Tricolor dye (TC) and another with anti-CD8-TC (clone M-L233) monoclonal antibodies (MoAb). Samples of 200 μl aliquots from the three cultures were dispensed into three 5 ml polystyrene tubes containing 10 μl of diluted anti-CD14-TC (clone TüK4), anti-CD16-TC (clone 3G8) and anti-CD19-TC (clone 4G7) MoAb. The anti-human cell surface marker TC-labelled MoAb were purchased from Caltag Laboratories (Burlingame, CA, USA) and used at 0.5 μg/ml (final concentration). TC-labelled isotypic control was included in each batch of experiments.

After incubation for 30 min at room temperature in the dark, cell surface-stained samples were treated with 2 ml of FACS Lysing/fix Solution (BD Pharmingen), immediately vortexed and re-incubated for an additional period of 10 min. After lysing/fixation procedure,

membrane-stained leukocytes were permeabilized for other 10 min with 2 ml of FACS perm-buffer (FACS buffer supplemented with 0.5% of saponin, Sigma), washed once with 3 ml of FACS buffer and resuspended into 200 μl of FACS perm-buffer. Fixed/permeabilized membrane-stained leukocytes suspensions were distributed in 30-μl aliquots on 96 wells U-bottomed microtiter plates (Falcon®) and incubated, for 30 min at room temperature, in the dark, in the presence of 20 μl of diluted PE-labelled anti-cytokine MoAb including anti-IL-12p40/p70 (clone C11-5.14), anti-IFN-γ (clone B27), anti-TNF-α (clone MAB11), anti-IL-4 (clone MP4-25D2), or anti-IL-10 (clone JES3-9D7), all purchased from BD Pharmingen and used at a final concentration of 25 μg/ml. PE-labelled isotypic control was included in each batch of experiments.

After intracytoplasmatic staining, the cells were washed once with FACS perm-buffer, followed by one wash step with FACS buffer and fixed in FACS FIX Solution (10 g/l paraformaldehyde, 10.2 g/l sodium cacodilate, 6.63 g/l sodium chloride, pH 7.2, all from Sigma).

Flow cytometry acquisition and analysis. Flow cytometric acquisition was performed using a FACScalibur® flow cytometer (BD Pharmingen) considering a total of 30,000 events per tube. CELLQuest® software (Franklin Lakes, NJ, USA) provided by the manufacturer was used for data acquisition and analysis.

To analyse the cytokine profile of distinct leukocyte subsets, several specific gating strategies were applied. Identification of NK cells ($CD16^+$), T lymphocyte subsets ($CD4^+$ and $CD8^+$ cells) and B cells ($CD19^+$) was performed by first establishing a scattering gate on lymphocyte population, using FSC versus SSC dot plots, followed by quantification of cytokine expressing cells on FL3 versus FL2 dot plots. Cytokine positive lymphocyte subsets were identified into the upper-right quadrant on dual colour graphs with FL3 representing the anti-cell surface marker TC-labelled MoAb and FL2 corresponding to the anti-cytokine PE-labelled MoAb.

A specific gating strategy to analyse the cytokine pattern of monocytes ($SSC^{low}CD14^{high+}$ cells) was carried out using FL3/anti-CD14-TC versus SSC dot plot. After monocyte gating, the frequency of cytokine-positive cells was identified into the upright quadrant on dual colour graphs with FL3 representing the anti-CD14 TC-labelled MoAb and FL2 corresponding to the anti-cytokine PE-labelled MoAbs.

The percentage of cells expressing cytokine was further multiplied by the absolute counts of specific leukocyte populations, derived from routine haematological analysis, to obtain the absolute number of cytokine-positive leukocyte subpopulations (cells/mm^3 of peripheral blood), as previously described by Peruhype-Magalhães *et al.* [13].

Statistical analysis. Differences between groups were first evaluated by Minitab sofrware (release 13.20) (State College, PA, USA) to test three hypotheses: independence, normality and variance of data sets. The data sets showing three true hypotheses were then considered parametric and were further compared by the unpaired *t*-test (NI versus E-IND) or the paired *t*-test (E-IND versus $E\text{-}IND_T$) using Graphpad prism software 3.0 (San Diego, CA, USA). Non-parametric data was analysed by Mann–Whitney (NI versus E-IND) or Wilcoxon-matched pairs test (E-IND versus $E\text{-}IND_T$) also using Graphpad prism 3.0. Significance was defined at $P < 0.05$.

Results

Cytokine profile of innate immunity during the early-indeterminate Chagas' disease

Levels of both pro- and anti-inflammatory cytokine-expressing circulating monocytes and NK cells are presented in Fig. 1. Data analysis demonstrated a slight increase of $IL\text{-}12^+$ monocytes, despite equivalent numbers

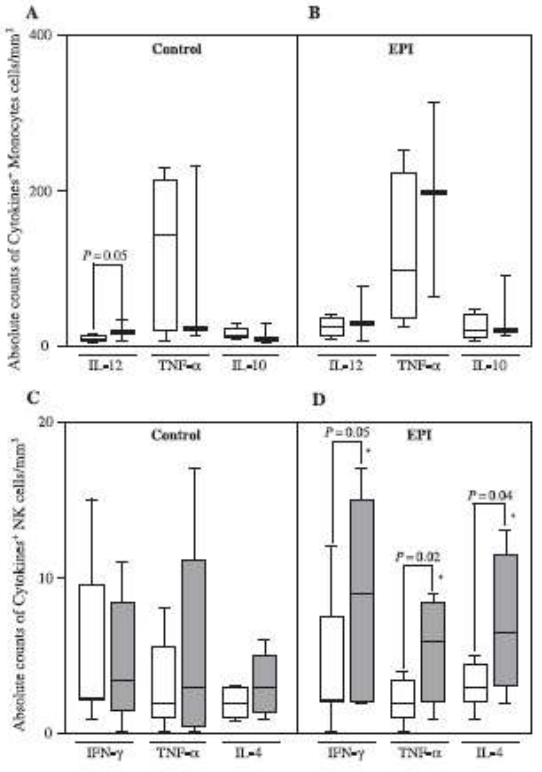

Figure 1 Intracellular cytokine profile of monocytes (A and B) and NK cells (C and D) in peripheral blood from NI (□) and E-IND (■), at *ex vivo* (control culture) and after short-term *in vitro* stimulation of whole-blood samples with *T. cruzi* soluble antigen (EPI). The results are expressed in box-plot format highlighting the gap of 50% of data set measurement. Significant differences are identified by dotted connecting lines in comparison to NI and E-IND at $P < 0.05$.

of TNF-α^+, IL-10$^+$ monocytes as well as IFN-γ^+, TNF-α^+ and IL-4$^+$ NK cells were observed for E-IND when compared with NI (Fig. 1A and C). Together, these findings revealed that the *ex vivo* innate immune compartment at the peripheral blood of the early-indeterminate chagasic patients resembled one of healthy uninfected children.

Cytokine profile of adaptive immunity during early-indeterminate Chagas' disease

Levels of both pro- and anti-inflammatory cytokine-expressing cells were also evaluated on cells from the adaptive immune compartment under controlled conditions, implementing the *ex vivo* profile (Fig. 2). Data analysis demonstrated that despite a decrease of CD8$^+$TNF-α^+ lymphocytes, a comparable number of IFN-γ^+, TNF-α^+ and IL-10$^+$ cells were observed within the adaptive immune compartment from E-IND when compared with NI (Fig. 2A, C and E). Together, these findings pointed out that, likewise the innate immune compartment, the cytokine patterns of adaptive immunity at the peripheral blood of early-indeterminate chagasic patients are equivalent to that observed for healthy schoolchildren.

Changes on the cytokine profile of innate and adaptive immunity induced by *in vitro* stimulation with *T. cruzi* antigens

In a search to identify the impact of *T. cruzi* antigen on the immunological response triggered during early-indeterminate disease, we have further investigated the changes on the intracellular cytokine induced by short-term *in vitro* stimulation with epimastigote antigens (EPI). We have hence quantified pro- and anti-inflammatory cytokines in peripheral blood leukocyte subsets related to innate (Fig. 1B and D) and adaptive immunity (Fig. 2B, D and F). Data analysis demonstrated that, in the presence of EPI antigens, cytokines-expressing monocytes from E-IND resemble the basal level observed for NI (Fig. 1B). On the other hand, we have observed that incubation with *T. cruzi* antigens triggered an enhancement of IFN-γ^+, TNF-α^+ and IL-4$^+$ NK cells in E-IND when compared with NI (Fig. 1D). Together, these data pointed out that *in vitro* stimulation with *T. cruzi* antigens drove the E-IND cytokine pattern of innate immunity toward a mixed immune profile.

Analysis of the adaptive immune compartment in E-IND revealed that EPI stimulation induced two distinct cytokine patterns. A mixed type-1/type 2 profile was observed at T-helper lymphocytes and B cells as demonstrated by increased levels of IFN-γ^+, TNF-α^+ and IL-10$^+$ CD4$^+$ lymphocytes and TNF-α^+ and IL-4$^+$ CD19$^+$ cells respectively (Fig. 2B and F). Interestingly, a distinct cytokine pattern was observed for T-cytotoxic

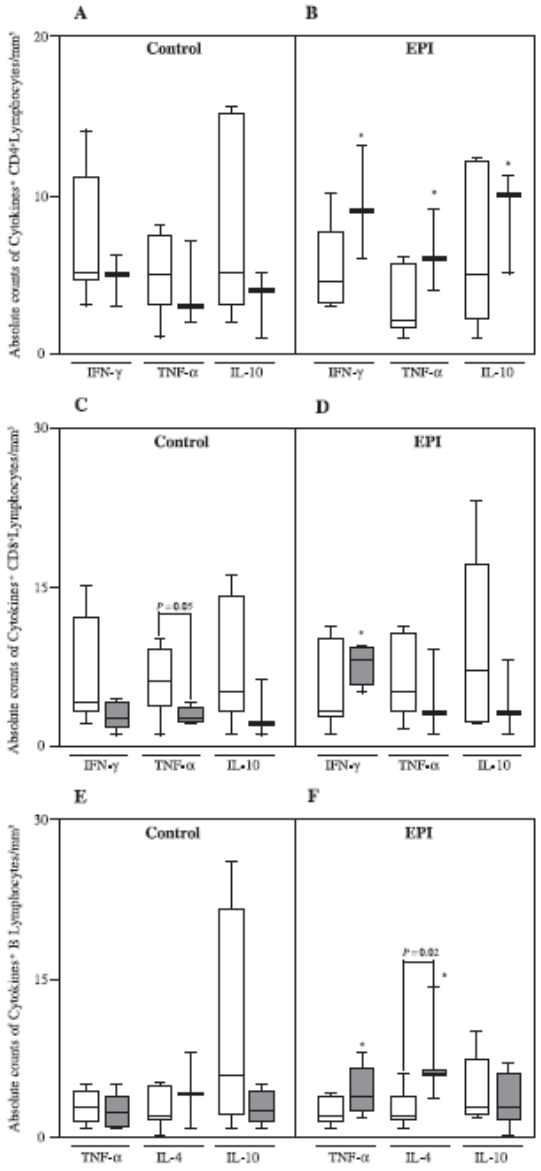

Figure 2 Intracellular cytokine profile of lymphocyte subsets in peripheral blood from NI (□) and E-IND (■), at *ex vivo* (control culture) and after short-term *in vitro* stimulation of whole-blood samples with *T. cruzi* soluble antigen (EPI). The results are expressed in box-plot format highlighting the gap of 50% of data set measurement. Significant differences are identified by dotted connecting lines in comparison to NI and E-IND or * in comparison to CONTROL and EPI cultures at $P < 0.05$.

lymphocytes, with EPI stimulation being able to drive a selective increase in the number of CD8$^+$IFN-γ^+ lymphocytes when compared with control cultures (Fig. 2D). It is important to notice that no impact of EPI stimulation

was documented for the adaptive compartment in NI. Together, these results showed that *T. cruzi* antigen drives a typical type 1-modulated specific immune response in early-indeterminate chagasic patients.

Impact of aetiological treatment on the cytokine pattern of the innate immune compartment

The levels of both pro- and anti-inflammatory cytokine-expressing cells in the innate immune compartment in NI, E-IND and E-IND$_T$, regarding circulating monocytes and NK cells are presented in Fig. 3. Data analysis showed that the *ex vivo* number of TNF-α^+ monocytes was similar in E-IND$_T$ in comparison with E-IND, being lower in contrast to NI (Fig. 3A). Upon EPI stimulation, monocytes from E-IND$_T$ showed increased frequency of cytokine-positive events in comparison with *ex vivo* data. However, in spite of the increased cytokine expression, the number of CD14$^+$TNF-α^+ monocytes persisted modulated in E-IND$_T$, at this time in comparison with both NI and E-IND (Fig. 3B). On the other hand, the aetiological treatment led to up-regulation of pro- and anti-inflammatory cytokines-expressing NK cells. Data analysis showed increased numbers of CD16$^+$IFN-γ, TNF-$\alpha^+$and IL-4$^+$ NK cells in E-IND$_T$ in comparison with NI and E-IND, in both *ex vivo* and EPI stimulated cultures (Fig. 3C and D).

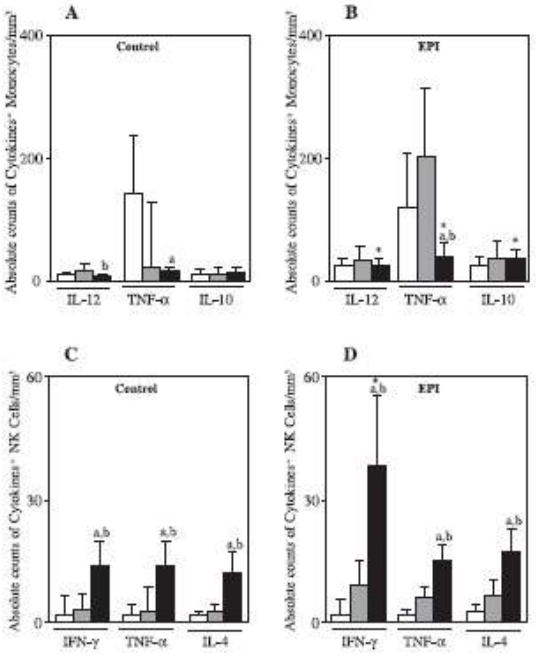

Figure 3 Impact of etiological treatment of intracellular cytokines pattern of CD4+ (A and B) and CD8$^+$ (C and D) lymphocytes in peripheral blood from NI (□), E-IND (▩) and E-IND$_T$ (■) individuals, at *ex vivo* (control culture) and after short-term in vitro stimulation of whole-blood samples with *T. cruzi* soluble antigen (EPI). The results are expressed in median ± SD. Significant differences are identified by letters "a" and "b" in comparison to NI and E-IND, respectively. The * shows statistic differences between CONTROL and EPI cultures at $P < 0.05$.

The ability of NK cells to produce IFN-γ in response to *T. cruzi* antigens in E-IND$_T$ was outstanding. Despite the mixed overall cytokine profile still observed for the innate immunity leukocytes after Bz-chemotherapy, the aetiological treatment was accompanied by increased ability of NK cells to produce IFN-γ in response to *T. cruzi* antigens (Fig. 3D). These data point out that Bz aetiological treatment during the early-indeterminate Chagas disease shift innate immunity toward a type 1-modulated immune profile.

Impact of aetiological treatment on the cytokine pattern of the adaptive immune compartment

Analysis of the adaptive immune compartment in E-IND$_T$ revealed distinct cytokine patterns in CD4$^+$, CD8$^+$ and CD19$^+$ cells.

A typical type 2 profile was observed for T-helper cells. Analysis of *ex vivo* CD4$^+$ T cells showed that Bz-eetiological treatment led to slight increase of IFN-γ^+ synthesis when compared with NI (Fig. 4A). However, there was a marked increment on IL-10 synthesis by CD4$^+$ T cells from E-IND$_T$ when compared with E-IND and NI (Fig. 4A). Upon EPI stimulation, besides a slight increase on the frequency of TNF-α^+ and IL-4$^+$ from E-IND$_T$ in comparison with NI, a similar pattern of higher IL-10 synthesis was observed at CD4$^+$ T cells (Fig. 4B).

Differently the cytokine pattern observed for T-helper cells, a typical type 1-modulated profile was observed for cytotoxic T cells. Despite the broad-spectrum of cytokine-expressing cells within CD8$^+$ T lymphocytes observed in E-IND$_T$, here represented by increased levels of IL-12$^+$, IFN-γ^+, TNF-α^+, IL-4$^+$ and IL-10$^+$ CD8$^+$ T cells (Fig. 4C), upon *T. cruzi* antigen stimulation, a striking expression of IFN-γ was observed (Fig. 4D).

Analysis of B lymphocytes revealed type 2-like profile both *ex vivo* and upon EPI *in vitro* stimulation, characterized by predominant IL-10 synthesis in E-IND$_T$ when compared with NI and E-IND. Slighter increases on IL-4$^+$ and minor enhancement of TNF-α^+ cells was also observed (Fig. 5A and B).

Together, these findings gave evidences that Bz-treatment led to a type 1-modulated immune profile characterized by important IFN-γ production by NK and CD8$^+$ T cells besides outstanding IL-10 production throughout CD4$^+$ T cells and B lymphocytes. Representative dot plots illustrating these findings are presented in Fig. 6.

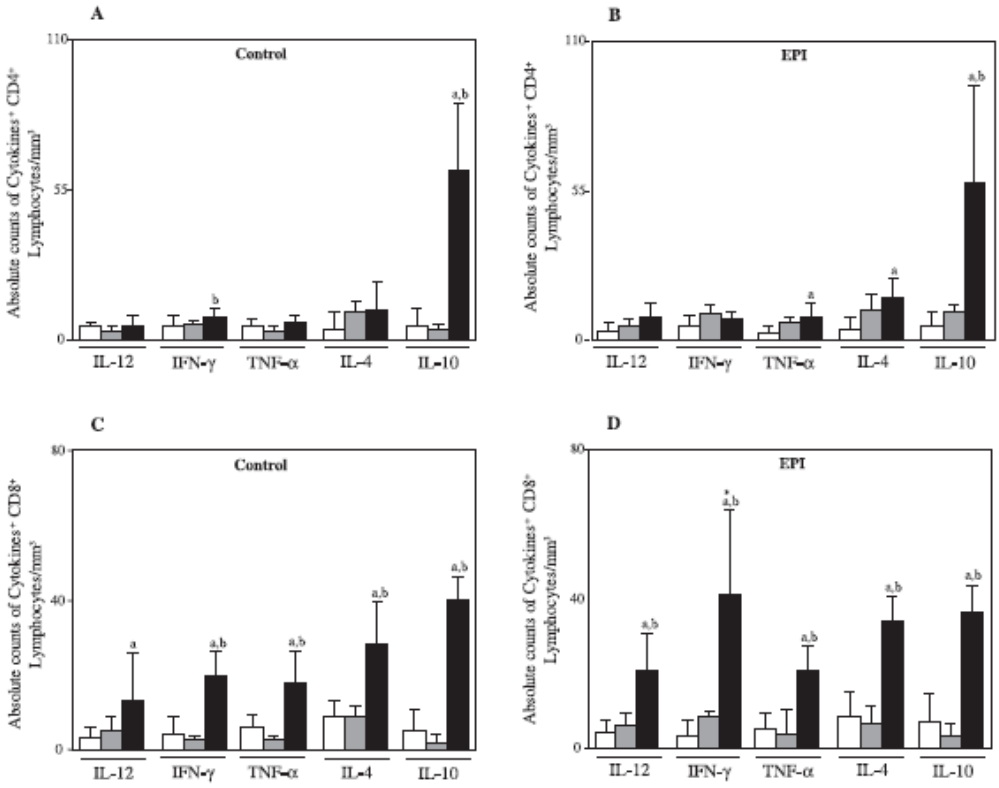

Figure 4 Impact of etiological treatment of intracellular cytokines pattern of CD4+ (A and B) and CD8+ (C and D) lymphocytes in peripheral blood from NI (□), E-IND (▩) and E-IND$_T$ (■) individuals, at *ex vivo* (control culture) and after short-term *in vitro* stimulation of whole-blood samples with *T. cruzi* soluble antigen (EPI). The results are expressed in median ± SD. Significant differences are identified by letters "a" and "b" in comparison to NI and E-IND, respectively. The * shows statistic differences between CONTROL and EPI cultures at $P < 0.05$.

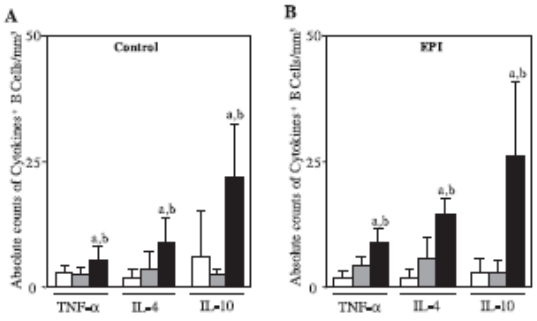

Figure 5 Impact of etiological treatment of intracellular cytokines pattern of CD19+ lymphocytes (A and B) in peripheral blood from NI (□), E-IND (▩) and E-IND$_T$ (■) individuals, at *ex vivo* (control culture) and after short-term *in vitro* stimulation of whole-blood samples with *T. cruzi* soluble antigen (EPI). The results are expressed in median ± SD. Significant differences are identified by letters "a" and "b" in comparison to NI and E-IND, respectively. The * shows statistic differences between CONTROL and EPI cultures at $P < 0.05$.

Discussion

The immune response triggered during early-indeterminate human Chagas' disease is not yet fully understood, despite considerable experimental evidences of its relevance for the development/control of pathogenesis throughout multiple innate and adaptive immunological mechanisms [4]. Data from early experimental *T. cruzi* infection, have pointed out that monocytes/macrophages-mediated trypanosomicidal event throughout nitric oxid, further enhanced by an up-regulation of TNF-α synthesis with monocyte/macrophage-derived IL-12 playing a central role in stimulating the NK-cell activity [4].

According to these observations, herein we have documented at an *ex vivo* investigation, a higher frequency of IL-12+ monocytes in E-IND children when compared with NI. This inflammatory cellular immune profile would suggest that NK cells might assume a type 1 cytokine pattern. Despite the *ex vivo* cytokine profile at circulating leukocytes from E-IND resembled the one

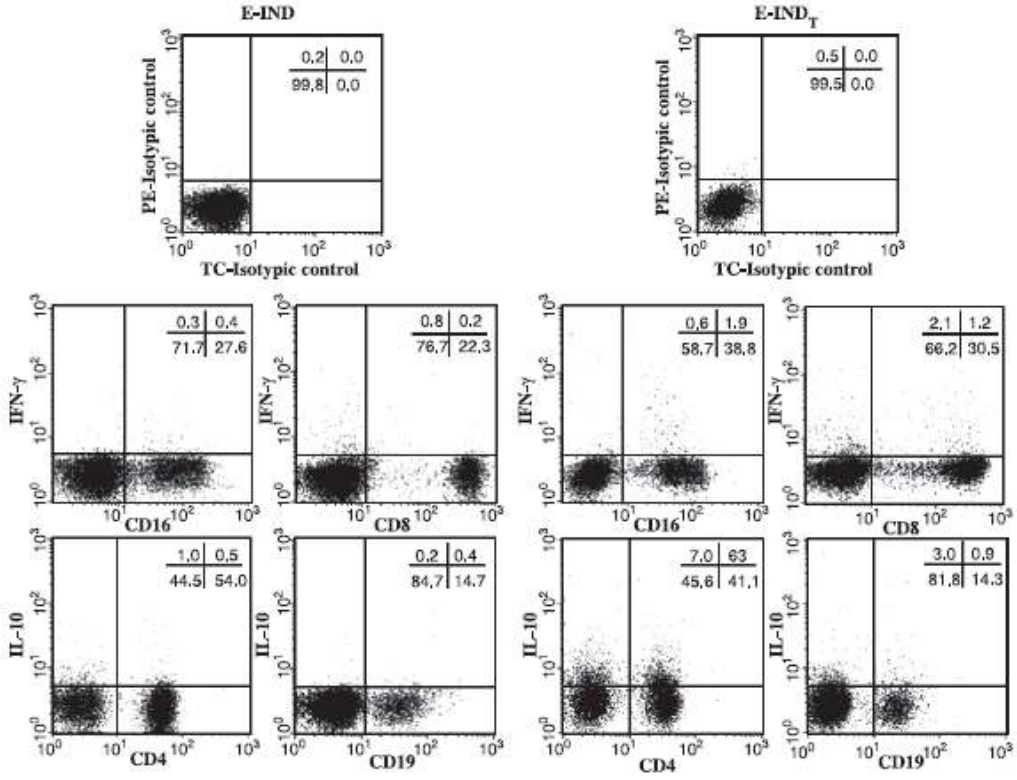

Figure 6 Representative dot plots illustrating the higher frequency of IFNγ$^+$ within CD16$^+$ NK-cells and CD8$^+$ T-lymphocytes besides enhanced levels of IL-10$^+$ within CD4$^+$ T-cells and B-lymphocytes from E-IND$_T$ (right panels) as compared to E-IND (left panels) after short-term *in vitro* stimulation of whole-blood samples with *T. cruzi* soluble antigen (EPI). Dot plots illustrating the isotypic controls are also included.

observed in healthy schoolchildren, the E-IND cytokine pattern displays a shift toward a mixed immune profile with higher levels of IFN-γ^+, TNF-α^+ and IL-4$^+$ NK cells upon *in vitro* stimulation with *T. cruzi* antigens.

Beside the ability of NK cells to produce IFN-γ in response to *T. cruzi* antigens [14–16], it has been also observed that NKT cells derived from livers of *T. cruzi* chronically infected mice are able to produce high levels of IFN-γ and IL-4 [17]. Our data demonstrated that, *in vitro* stimulation with *T. cruzi* antigens drove the E-IND cytokine pattern toward a mixed immune profile with higher levels of IFN-γ^+, TNF-α^+ and IL-4$^+$ CD16$^+$ lymphocytes. These findings may suggest that CD16$^+$ cells (NK and NKT cells) could provide protection against *T. cruzi* not only in the acute phase but also in the early-indeterminate Chagas' disease, producing a mixed pro- and anti-inflammatory cytokine profile favouring the generation of effective and non-deleterious inflammatory mechanisms.

Additional *in vitro* stimulation with *T. cruzi* antigens drove the E-IND cytokine pattern toward increased numbers of IFN-γ^+, TNF-α^+ and IL-10$^+$ CD4$^+$ T cells in addition to an enhanced frequency of TNF-α^+ and IL-4$^+$ CD19$^+$ lymphocytes, illustrating the mixed nature of the cytokine microenvironment. Interestingly, at this time, upon *T. cruzi* antigen stimulation, E-IND CD8$^+$ lymphocytes showed a striking enhancement of IFN-γ expression, compared with NI, which counted to a confident global type 1-modulated cytokine microenvironment.

Our data are in agreement with previous reports from Samudio *et al.* [8] that suggested a type-1/type 2-mixed cytokine pattern *T. cruzi*-infected children in Paraguay and the establishment of a type 0 (expression of both IFN-γ and IL-4), herein named type 1-modulated, in association with asymptomatic early Chagas' disease, important for the development of an effective non-deleterious adaptive immune response.

Besides establishing the cytokine profile for E-IND, the present study also determined in a pioneer investigation the impact of Bz-treatment on the cytokine pattern of circulating leukocytes during the early-indeterminate Chagas' disease. In the last decades, the literature has accumulated evidences concerning the immune dependence of chemotherapy and more recent studies regarding anti-*T. cruzi* chemotherapy efficacy in experimental

models have considered the host immune response [18, 19]. In humans, it has been proposed that the maintenance of an immune response with high levels of IFN-γ production may favour the effectiveness of chemotherapy [9, 10]. To further focus this issue, we have performed a longitudinal follow-up investigation to evaluate the impact of aetiological treatment on the cytokine profile of *T. cruzi*-infected children. Our data point out that the Bz aetiological treatment during the early-indeterminate Chagas' disease shift innate immunity toward a type 1-modulated immune profile as demonstrated by the outstanding ability of NK cells to produce IFN-γ in response to *T. cruzi* antigens in E-IND$_T$, despite the mixed overall cytokine profile still observed for the innate immunity leukocytes after Bz-chemotherapy. Likewise demonstrated by Peruhype-Magalhães *et al.* [13] for patients cured of visceral leishmaniasis, we have observed that after chemotherapy, early-indeterminate children remain with an effective innate immune response that could be efficiently recalled upon antigenic stimulation. The production of IFN-γ by NK cells has been extensively reported as a fundamental element to the establishment of effective immune response against *T. cruzi* infection [4].

Distinct cytokine profile were observed for the adaptive immunity compartment in E-IND$_T$ at CD4$^+$, CD8$^+$ and CD19$^+$ cells. A typical type 2 profile was observed for T-helper cells, illustrated by the outstanding increment on IL-10 synthesis by T lymphocytes from E-IND$_T$, mainly CD4$^+$ cells, both *ex vivo* and after EPI stimulation. A type-2-like profile was also observed for B lymphocytes, both *ex vivo* and upon EPI *in vitro* stimulation, characterized by predominant IL-10 synthesis besides slighter increases on IL-4$^+$ and minor enhancement of TNF-α^+ cells in E-IND$_T$. Unlikely the cytokine pattern observed for T-helper cells and B lymphocytes, a typical type 1-modulated profile was observed for cytotoxic T cells upon *T. cruzi* antigen stimulation, with a striking expression of IFN-γ by CD8$^+$ T cells.

Together, our findings validate and re-enforce previous reports suggesting the role of IFN-γ as a pivotal cytokine for successful therapeutic intervention in human Chagas' disease. Furthermore, we have added more details to this hypothesis, describing NK cells and CD8$^+$ T lymphocytes as the major sources of IFN-γ during the Chagas' disease treatment. Moreover, we have also brought in additional information to this puzzling phenomenon, pointing out the IL-10, T lymphocytes, mainly CD4$^+$ cells, as well as B lymphocytes, as a key element to allow parasite clearance in the absence of deleterious tissue damage during chemotherapy. In this context, the spontaneous secretion of IL-10 by CD8$^+$ cells may also represent an effective event of modulatory impact mediated by type 2 cytotoxic T cells, usually named Tc-2 cells [20–22]. Recently, it has also been demonstrated that cytokine-induced IL-10-secreting CD8$^+$ T cells represent a phenotypically distinct suppressor T-cell lineage [23].

It was interesting to notice that the cytokine pattern observed for E-IND$_T$ innate and adaptive immunity was very similar to that documented for E-IND leukocytes upon *T. cruzi* antigen stimulation. Together this observation suggests that the massive antigen release triggered by Bz-treatment, which is able to activate cytokine synthesis *in vivo*, can be artificially reproduced *in vitro* upon *T. cruzi* antigenic stimulation of E-IND leukocytes. Considering the cumulative evidences of immunological dependency of therapeutic success in Chagas' disease, this strategy may represent a useful tool to predict the immunological response that a given patient would develop during Bz-treatment intervention, allowing to forecast the therapeutic outcome.

Our group is focusing this issue further to establish whether a type 1-modulated immune response is also observed during Bz-treatment applied to chronic indeterminate patients. We hypothesize that those indeterminate patients named high IFN-γ producers are more prone to successfully exhibit response to Bz-chemotherapy. Moreover, considering that patients bearing cardiac Chagas' disease are nearly all IFN-γ producers, it is reasonable to suppose that the Bz-treatment prescribed to these patients should lead to an effective parasite clearance. However, the chemotherapeutical intervention should be carried out with caution, as the lower levels of type 2 cytokines observed for most cardiac patients could count to unexpected side effects.

Acknowledgment

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Original article

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

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Abstract

Pro-inflammatory immune response is usually associated with Chagas disease pathogenesis, but is also relevant to treatment effectiveness. Cross-sectional studies have suggested that this activated state may persist for years after therapeutic intervention. However, short-term longitudinal investigation has suggested that the Benznidazole treatment (Bz-treatment) leads to decreased immunological activation. In order to elucidate this issue, we performed a longitudinal study to evaluate the immunological status following Bz-treatment during early indeterminate Chagas disease. Our results demonstrated that Bz-treatment led to higher activation status of circulating monocytes but was negatively associated with the number of $IL\text{-}12^{+}CD14^{+}$ cells. Moreover, Bz-treatment triggered a high frequency of circulating $CD3^{-}CD16^{+}CD56^{-}$ NK cells, in addition to elevated activation status associated with a type 1-modulated cytokine pattern. Bz-treatment induced substantial T and B-cell activation status associated with an overall IL-10 modulated type 1 cytokine profile. In summary, these findings provide new information regarding immune activation status following the etiological treatment of Chagas disease. These results suggest that in addition to the increased number of activated leukocytes in the peripheral blood, Bz-treatment may also involve a qualitative change in their functional capacity that drives their activation state toward a modulated cytokine profile. These changes may account for the benefits of etiological treatment of Chagas disease.

Keywords: Chagas disease; Benznidazole; Immune response; Cytokines; Leukocytes subsets

1. Introduction

Trypanosoma cruzi is the etiological agent of American Trypanosomiasis or Chagas disease, which affects 16–18 million people in South and Central America [1]. Chagas disease is a long-lasting infection with a short acute phase, which is usually clinically non-apparent, that progresses to a lifelong chronic phase characterized by distinct clinical forms known as indeterminate, cardiac and digestive [1].

Specific chemotherapy is recommended for the treatment of Chagas disease applying the general assumption that the earlier the specific treatment is initiated the greater the chance of parasitological cure [2]. At present, Chagas disease chemotherapy in Brazil has been restricted to the use of Benznidazole, which is recommended for the treatment of acute,

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congenital and the initial stage of the indeterminate form, known as early-indeterminate disease (E-IND), usually seen in children and adolescents [2,3]. An effective treatment might lead to parasite clearance and prevent the progression of infection to disease, heart-related pathology and its complications [4–7].

It has been suggested that parasite clearance following chemotherapy in the chronic phase may contribute to better clinical outcome [4,5,8]. Indeed, Andrade et al. [8] have demonstrated regression of myocardial inflammatory lesions following parasite clearance archived by etiological treatment in mice.

Despite the well known role of the host immune response in the pathogenesis of Chagas disease, little has been reported about the impact of Bz-treatment on this response. It has been demonstrated that after specific treatment cured patients produce high levels of IFN-γ [9]. Because an exacerbated production of IFN-γ may favor the development of a strong pro-inflammatory response, which is mainly observed in patients with cardiac disease [10], it is possible that a fine balance of pro- and anti-inflammatory cytokines could be the major key in controlling Chagas disease morbidity following treatment [11]. We have previously reported that Bz-treatment leads to a type 1-modulated immune profile, with IL-10 as the putative key element for controlling the deleterious tissue damage that eventually might occur due to the IFN-γ-mediated pro-inflammatory response observed during Bz-treatment [11]. These findings suggest that in addition to the direct role in blocking parasite growth *in vivo*, Bz-treatment appears to affect host immune regulation [12]. Together, these parasitological and immunological hypotheses have brought new perspectives to clinical investigations and have stimulated studies to establish the effect of specific anti-parasite therapy on the immune state and the evolution of Chagas disease.

In spite of these insights, little research has been carried out to address the impact of etiological Bz-treatment in the host immune response during early indeterminate Chagas disease. Therefore, our goal in this study was to evaluate in a longitudinal investigation, the impact of Bz-treatment on the *ex vivo* phenotypic profile of peripheral blood leukocytes in association with their cytokine pattern during early indeterminate *T. cruzi* infection, prior to, and one year after the end of the Bz-therapeutic intervention.

2. Patients, materials and methods

2.1. Study population

The patients included in this investigation consisted of thirteen schoolchildren from Berilo and José Gonçalves de Minas. The early-indeterminate Chagas disease group (E-IND) consisted of six schoolchildren (9–14 years old), with antibodies to *T. cruzi* as detected by serology performed as recommended by the World Health Organization criteria [1]. The clinical and physical examination revealed that all children were asymptomatic, with normal conventional electrocardiograms and unaltered thoracic X-rays. Haemoculture was positive in all but one (5/6) of the seropositive children, generally within the first month of blood cultivation in Liver Infusion Tryptose medium (LIT). Chagasic children were treated with benznidazole (Rochagan®; Roche, SP, Brazil) following the protocol recommended by the Brazilian Health Ministry [3], consisting of 8 mg/kg per day for 60 consecutive days. Following etiological treatment, all children were re-evaluated one year after the end of Bz-treatment ($E\text{-}IND_T$).

The non-infected control group (NI) consisted of seven age-matched schoolchildren with negative serology for anti-*T. cruzi* (9–14 years old).

Informed written consent was obtained from all through their parents or legal guardians. This work fulfilled resolution number 196/1996 from the Brazilian National Health Council for research involving humans.

2.2. Immune-staining for cell surface markers of peripheral blood

White blood cell phenotypes were analyzed following an immunofluorescence procedure recommended by Becton-Dickinson (Mountain View, CA, USA), modified as follows: 100 μl peripheral blood, collected using Vacutainer tubes with EDTA as anticoagulant, was mixed with 5 μl undiluted monoclonal antibodies (mAbs) specific for several cell surface markers labeled with, fluorescein isotiocyanate-FITC, phycoerithrin-PE or tricolor dye-TC, all purchased from Becton Dickinson (San Diego, CA, USA) or Caltag (Burlingame, CA, USA), including anti-CD3-FITC and PE, clone UCHT1; anti-CD4-FITC and PE, clone RPA-T4; anti-CD5-FITC, clone L17F12; anti-CD8-FITC and TC, clone RPA-T8; anti-CD14-TC, clone TüK4; anti-CD16-FITC and TC, clone 3G8; anti-CD18-FITC, clone YF118.3; anti-CD19-FITC and TC, clone 4G7; anti-CD23-PE, clone M-L233; anti-CD28-FITC, clone 15E8; anti-CD54-PE, clone 15.2; anti-CD56-PE, clone B159; anti-CD62L-FITC, clone DREG-56; anti-CD69-FITC, clone FN50 and anti-HLA-DR-PE, clone TÜ36. Following incubation in the dark for 30 min at room temperature, erythrocytes were lysed in FACS Lysing Solution (Becton Dickinson Biosciences Pharmigen, San Diego, CA, USA). The remaining cells were then washed in phosphate-buffered saline containing 0.01% sodium azide. Cell preparations were fixed in 200 μl of FACS-FIX Solution (10 g/l paraformaldehyde, 1% sodium-cacodylate, 6.65 g/l sodium-chloride, 0.01% sodium azide). Data acquisition was performed with a Becton-Dickinson FACScalibur instrument. CELLQuest™ software provided by the manufacturer was used for data acquisition and analysis.

2.3. Analysis of intracellular cytokines in leukocytes after in vitro short-term culture of whole blood

For each blood sample collected using Vacutainer tubes with sodium heparin as anticoagulant, short-term *in vitro* cultures of whole blood were performed to reproduce the *ex vivo* immunological status. Whole blood (500 μl) was incubated in the presence of 500 μl of RPMI-1640 (GIBCO, Grand Island,

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NY, USA) plus Brefeldin A (BFA) (Sigma, St. Louis, MO, USA), at a final concentration of 10 μg/ml, in 14 ml polypropylene tubes (Falcon®, BD Pharmingen). The culture was maintained for 4 h at 37 °C in a 5% CO_2 humidified incubator.

At the end of incubation, all cultures were treated with EDTA (Sigma) at a final concentration of 2 mM for 15 min at room temperature. EDTA-treated whole blood cultures were washed once with FACS buffer (PBS with 0.5% of bovine serum albumin, BSA, pH 7.4, Sigma), by centrifugation at 600 × *g* for 7 min at room temperature, and resuspended to half the original volume with FACS buffer. Samples of cell suspension (200 μl) from cultures were incubated with 10 μl diluted TC-labeled anti-cell surface marker mAbs purchased from Caltag (Burlingame, CA, USA) including anti-CD4, clone RPA-T4; anti-CD8, clone RPA-T8; anti-CD14, clone TüK4; anti-CD16, clone 3G8 and anti-CD19, clone 4G7. After incubation for 30 min at room temperature in the dark, cell surface-stained samples were treated with 2 ml of FACS Lysing/fix Solution (BD Pharmingen), immediately vortexed and re-incubated for an additional 10 min. After the lysing/fixation procedure, membrane-stained leukocytes were permeabilized for another 10 min with 2 ml of FACS perm-buffer (FACS buffer supplemented with 0.5% saponin, Sigma), washed and resuspended into 200 μl FACS perm-buffer. Fixed/permeabilized membrane-stained leukocyte suspensions were distributed in 30 μl aliquots in 96-well U-bottomed microtiter plates and incubated for 30 min at room temperature in the dark in the presence of 20 μl diluted PE-labeled anti-cytokine mAbs purchased from BD-Pharmingen (San Diego, CA, USA) including anti- IL-12p40/p70, clone C11.5; anti-IFN-γ, clone 4S.B3; anti-TNF-α, clone MAb11; anti-IL-4, clone BVD4-1D11 or anti-IL-10, clone JES3-19F1). TC and PE-labeled isotypic control was included in each batch of experiments. After intracytoplasmatic staining, the cells were washed once with FACS perm-buffer, followed by one wash step with FACS buffer and were fixed in FACS FIX Solution.

Flow cytometric analyses were performed using a FACScalibur® flow cytometer (BD Pharmingen, San Diego, CA, USA), which acquired a total of 30,000 events per tube.

2.4. Statistical analysis

Comparative analysis was performed by unpaired *t* test (NI versus E-IND or E-IND_T) or paired *t* test (E-IND versus E-IND_T) using Prism software (version 4.03). Correlation analysis was performed by Pearson's test. Significance was defined in all cases at $p < 0.05$.

3. Results

3.1. Higher activation status of circulating monocytes exhibiting negative association with IL-12$^-$ producing CD14$^+$ cells following Chagas disease Bz-treatment

In order to verify whether etiological treatment induces variations in the percentage of monocyte subsets, including macrophage-like ($CD16^+CD14^+$) and pro-inflammatory monocytes ($HLA\text{-}DR^{High}CD14^+$), we evaluated CD16 and HLA-DR expression on the surface of circulating $CD14^+$ cells. Despite the fact that the frequency of $CD14^+$ monocytes was similar in all groups (Fig. 1A), the percentage of macrophage-like and pro-inflammatory monocytes (Fig. 1A) was significantly higher in the E-IND_T group than in both E-IND and NI groups.

We observed a negative correlation between the frequency of $CD16^+CD14^+$ and $HLA\text{-}DR^{High}CD14^+$ with IL-12^+CD14^+ monocytes (Fig. 1C). Additionally, no correlation was found between these monocyte subsets and the level of TNF-α^+CD14^+ monocytes (data not shown). Representative flow cytometry charts illustrate the enhanced frequency of macrophage-like cells as well as lower IL-12^+CD14^+ monocytes in the E-IND_T group as compared to the E-IND group (Fig. 1B).

3.2. Bz-treatment during early indeterminate Chagas disease led to a high frequency of circulating total $CD3^-CD16^{-/+}CD56^{-/+}$ NK cells that mainly exhibit the $CD3^-CD16^+CD56^-$ phenotype as well as elevated percentage of $CD69^+CD16^+$ NK cells associated with a type 1-modulated cytokine pattern

Three major NK cell subsets have been previously evaluated in Chagas disease [13], including $CD3^-CD16^+CD56^-$, $CD3^-CD16^+CD56^+$ and $CD3^-CD16^-CD56^+$ cells. Our findings showed a higher percentage of total $CD3^-CD16^{-/+}CD56^{-/+}$ NK cells in the E-IND_T group than in the E-IND and NI groups (Fig. 2A, left panel), mainly due to the increased percentages of the $CD3^-CD16^+CD56^-$ subset (Fig. 2B). No significant differences were observed in the mean percentages of $CD3^-CD16^+CD56^+$ subset (Fig. 2B) in the E-IND_T group as compared to the E-IND and NI groups.

Despite the slight decrease in $CD3^-CD16^-CD56^+$ (Fig. 2B), the frequency of $CD16^+$ cells expressing the early activation marker $CD69^+CD16^+$ was considerably increased in the E-IND_T group compared to the E-IND and NI groups (Fig. 2A).

Additional analyses demonstrated a positive correlation between the percentage of total $CD3^-CD16^{-/+}CD56^{-/+}$ NK cells as well as $CD69^+CD16^+$ NK cells and the absolute number of both IFN-γ^+ and IL-4^+ NK-cells (Fig. 2C).

3.3. Bz-treatment led to substantial T-cell activation status, but was associated with an overall type 1 highly modulated T-cell-derived cytokine profile

Despite several reports of higher T-cell activation during chronic Chagas disease [14–16], we have previously demonstrated that E-IND is characterized by a T-cell-independent immunity with no changes in the frequency of circulating $HLA\text{-}DR^+$T-cells [17]. Because HLA-DR is commonly referred to as a late-stage activation cell surface marker, we have further focused the T-cell activation issue by characterizing the expression of an early activation marker, CD69, aiming to evaluate the T-cell activation profile before and after the Bz-treatment. CD69 is an early membrane receptor expressed

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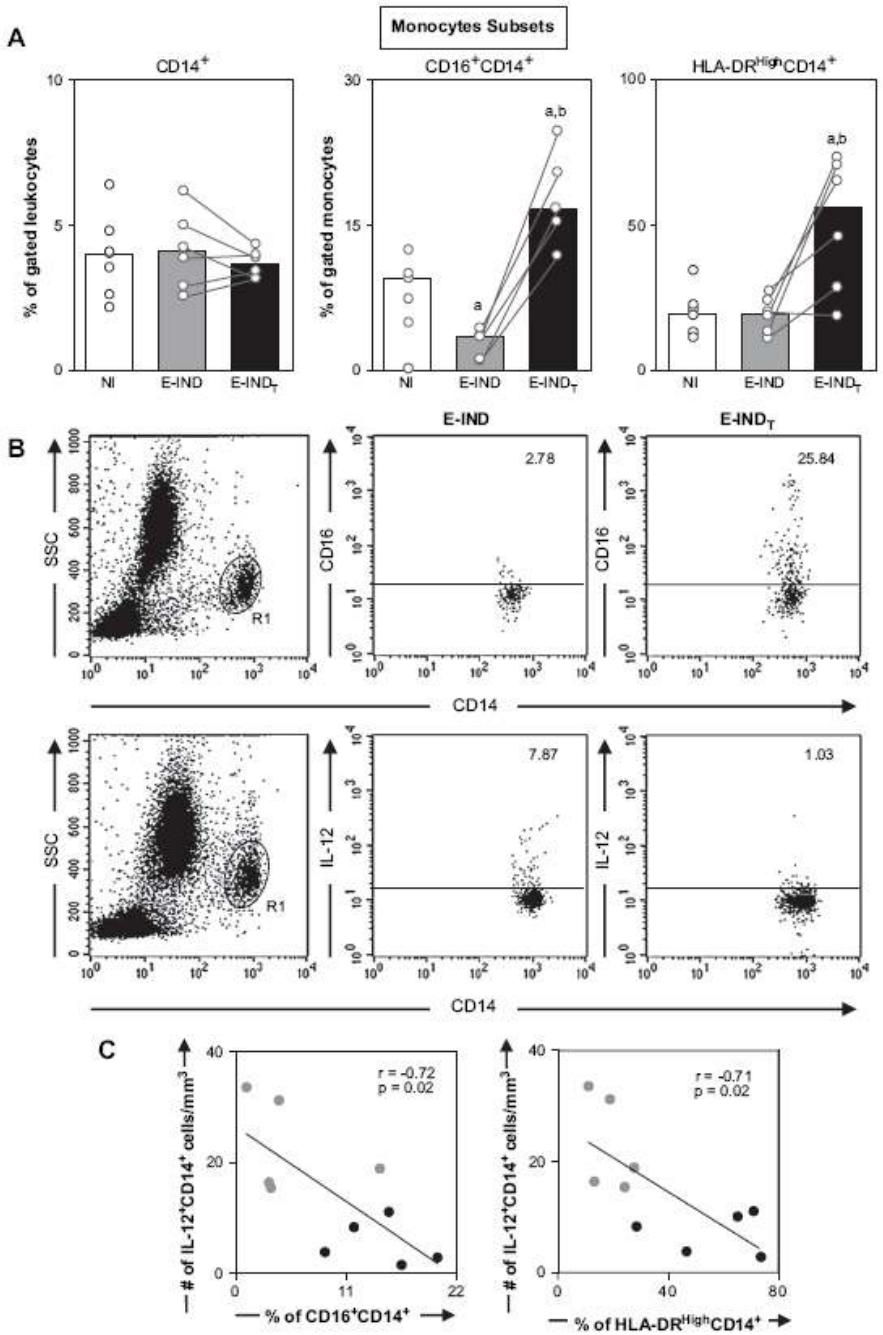

Fig. 1. (A) Analysis of monocyte subsets in the peripheral blood from non-infected children (NI – ▭) and early indeterminate *T. cruzi* infected patients prior to the Bz-treatment (E-IND – ▭) and one year after it (E-IND$_T$ – ■). Phenotypic studies were performed by a triple-labeling protocol using anti-CD16 FITC, anti-HLA-DR PE, and anti-CD14 TC to identify total monocytes $CD14^+$, macrophage-like monocytes $CD16^+CD14^+$, and pro-inflammatory monocytes $HLA\text{-}DR^{High}CD14^+$. The results are expressed as scattering of individual values and median percentage. Significant differences at $p < 0.05$ are identified by letters "a" and "b" in comparison to NI and E-IND groups, respectively. (B) Representative dot plots illustrating the higher frequency of $CD16^+CD14^+$ monocytes and a lower frequency of $IL\text{-}12^+CD14^+$ monocytes in the E-IND$_T$ group compared to the E-IND group. Quadrant statistics were used for data analysis, and the results are expressed as the percentage of positive cells within the selected gate. (C) Correlation analyses showed the negative correlation between macrophage-like or pro-inflammatory monocytes and $IL\text{-}12^+$ $CD14^+$ cells after Bz-treatment.

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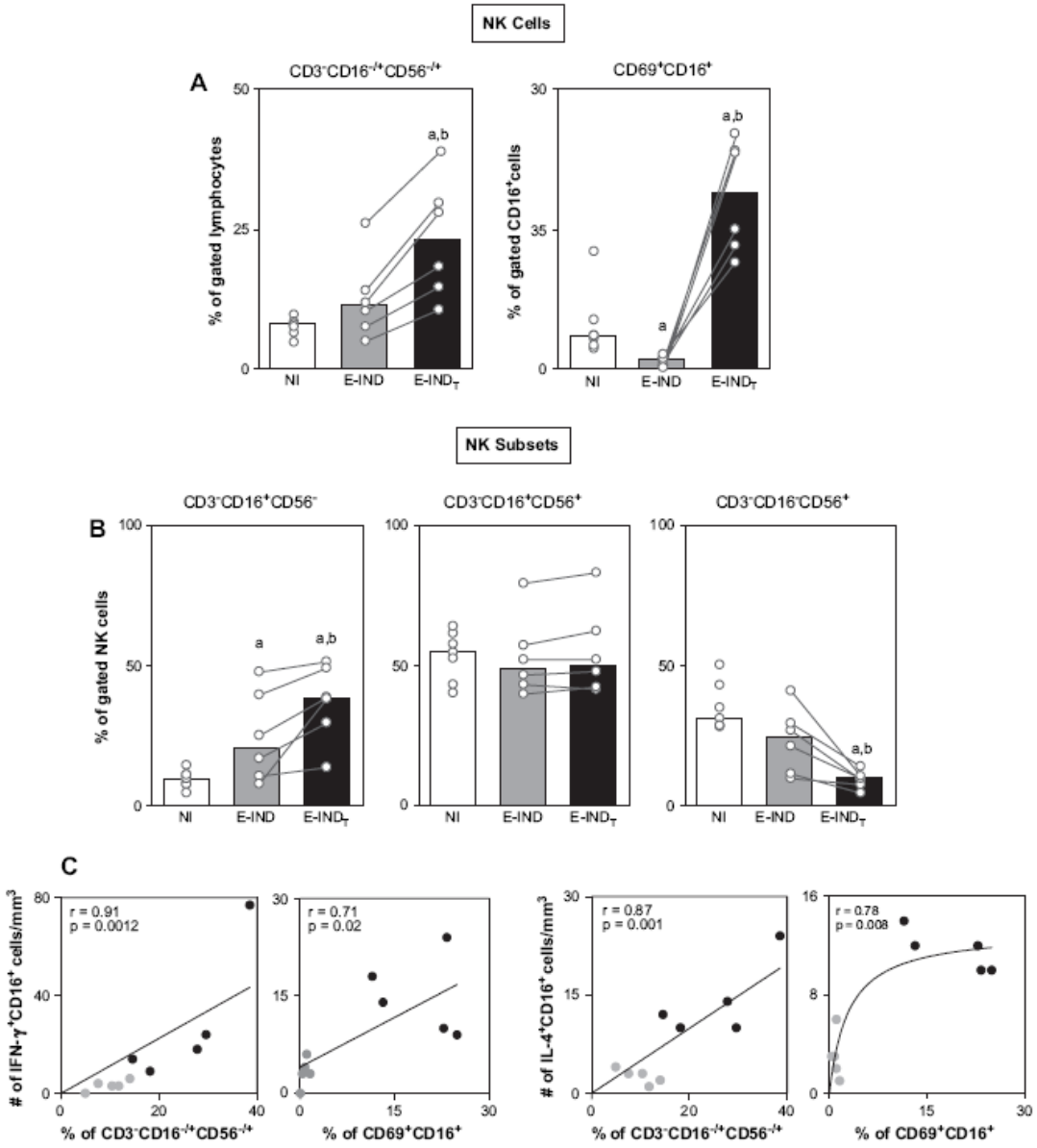

Fig. 2. (A) Analysis of total $CD3^-CD16^{-/+}CD56^{-/+}$ NK cells, $CD69^+CD16^+$ NK cells and (B) its subsets in the peripheral blood from non-infected children (NI – □) and early indeterminate *T. cruzi*-infected patients prior to the Bz-treatment (E-IND – ▒) and one year after it ($E\text{-}IND_T$ – ■). Phenotypic studies were performed by a triple-labeling protocol using anti-CD3 FITC, anti-CD56 PE, and anti-CD16 TC to identify $CD3^-CD16^{-/+}CD56^{-/+}$, $CD3^-CD16^+CD56^-$, $CD3^-CD16^+CD56^+$, and $CD3^-CD16^-CD56^+$ within total NK cells. Double-labeling protocol was performed to quantify the percentage of $CD69^+CD16^+$ cells. The results are expressed as scattering of individual values and median percentage. Significant differences at $p < 0.05$ are identified by letters "a" and "b" in comparison to the NI and E-IND groups, respectively. (C) Correlation analyses showed a positive correlation between $CD3^-CD16^{-/+}CD56^{-/+}$ NK cells or $CD69^+CD16^+$ NK cells with IFN-γ^+ $CD16^+$ and IL-4^+ $CD16^+$ cells after Bz-treatment.

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upon lymphocyte activation, not detected in resting cells. Although CD69 have been considered a transient cell surface molecule expressed following lymphocyte activation, it has been demonstrated that CD69 can be persistently expressed *in vivo* by T-cells under certain conditions characterized by chronic inflammation [18]. Moreover, despite CD69 have been considered a typical activation markers associated with pro-inflammatory function, recent studies have also indicated that this receptor may act as a regulatory molecule, dow-regulation the immune response through the production of pleiotropic cytokines [19]. Herein, we have evaluated the expression of CD69 immediately after blood collection, referred as "*ex vivo*" expression in the absence of in vitro stimulation with antigen or mitogen in culture. Therefore, the results reflect the activation *in vivo* status of circulation lymphocytes.

Despite the lower percentage of HLA-DR$^+$ CD4$^+$T-cells and the lower percentage of circulating CD69$^+$CD8$^+$T-cells observed in the E-IND group as compared with NI, which supports our previous hypothesis that E-IND is characterized by a T-cell independent immunity our results reveal, for the first time, an overall high activation status in both innate and adaptive immune response following Bz-treatment during early indeterminate Chagas disease. Interestingly, increased percentages of HLA-DR$^+$ as well as CD69$^+$ cells within both T-cell subsets can be observed in the E-IND$_T$ group (Fig. 3A) in comparison to the E-IND group.Moreover, decreased percentage of CD62L$^+$CD8$^+$T-cells was observed in the E-IND$_T$ group in comparison to the E-IND group (Table 1). Increased levels of activated T-cells following Bz-treatment of chronic Chagas disease have been already described [15].

We have further characterized this highly activated status by associating the T-cell activation phenotypes with their cytokine secretion pattern. Our data demonstrated a positive correlation between activated CD4$^+$T-cells (both CD69$^+$ and HLA-DR$^+$) and the number of IL-10$^+$CD4$^+$T-cells (Fig. 3C, left panel). A positive correlation was also observed between activated CD8$^+$T-cells (both CD69$^+$ and HLA-DR$^+$) and the absolute number of IL-10$^+$CD8$^+$ cells. Additionally, a positive correlation between activated CD8$^+$T-cells was observed with the IFN-γ^+CD8$^+$ cells.

Further analysis between have demonstrated positive correlation between activated CD8$^+$T-cells and the absolute number of IL-4$^+$CD8$^+$ cells ($r = 0.84$, $p = 0.002$ for CD69$^+$CD8$^+$ cells and $r = 0.79$, $p = 0.005$ for HLA-DR$^+$CD8$^+$ cells). Additional correlation analysis between cytokine producing cells revealed positive correlation between IFN-γ^+CD8$^+$T-cells and IL-10-producing T-cells ($r = 0.86$, $p = 0.005$ for CD4$^+$ and $r = 0.92$, $p = 0.0003$ for CD8$^+$T-cells) as well as between IFN-γ^+CD8$^+$T-cells and IL-4$^+$CD8$^+$ cells ($r = 0.84$, $p = 0.003$).

In summary, our data suggest that Bz-treatment led to substantial T-cell activation status associated with an overall type 1 highly modulated T-cell-derived cytokine profile (Fig. 3C, right panel). Representative flow cytometry charts are provided to illustrate the type 1-modulated cytokine pattern synthesized by T-cell subsets after Bz-treatment (Fig. 3B).

3.4. B-lymphocytes displayed an activated profile associated with a mixed type 1/type 2 cytokine pattern following Bz-treatment of early indeterminate Chagas disease

In order to further characterize the impact of Bz-treatment in the adaptive immunity, we have quantified the frequency of circulating B-cells and their subsets, as well as their activation status. The analysis on the B-cell compartment revealed that members of the E-IND$_T$ group have increased percentages of circulating B-lymphocytes (Fig. 4A), owing to increased percentages of both B1 (CD5$^+$CD19$^+$) and B2 (CD5$^-$CD19$^+$) lymphocyte subsets (Fig. 4A, middle and right panel). Analysis of B-cell activation status revealed a markedly higher percentage of activated CD69$^+$CD19$^+$ and CD23$^+$CD19$^+$B-cells in the E-IND$_T$ group compared with the E-IND and NI groups (Fig. 4B).

Additional analyses demonstrated a positive correlation between activated CD69$^+$CD19$^+$B-lymphocytes and the number of both TNF-α^+ and IL-10$^+$CD19$^+$B-cells (Fig. 4C). As was later demonstrated for CD4$^+$T-cells, a positive correlation was observed between IFN-γ^+CD8$^+$T-cells and IL-10$^+$CD19$^+$B-lymphocytes (data not shown), suggesting, again, an important role of IL-10 synthesis by other cells in the control of inflammatory CD8$^+$T-cell activity.

4. Discussion

The etiological treatment of Chagas disease has a beneficial impact on clinical status besides the parasite clearance, and may also affect the nature of the immune response of treated hosts [4,5,8–12,20]. Despite the ability of Bz-treatment to postpone or prevent clinical progression in human and experimental models [2,5,7], there are still few studies focusing on the immune response following Bz-treatment for Chagas disease.

It has been proposed that Bz-treatment is able to affect the host immunity profile and contributes to reduce the clinical symptoms of Chagas disease, regardless of the parasitological cure. Indeed, the Bz-treatment in chronic experimental infection prevents cardiomyopathy, despite the lack of complete parasite eradication [5], and long-term follow-up studies of Bz-treatment of human chronic infection demonstrated lower clinical progression to heart disease despite parasitological cure [4].

On the other hand, it has been postulated that etiological treatment *per se* does not seem to be responsible for the changes, since therapeutic failure, demonstrated by the presence of circulating parasites, is not accompanied by significant alterations in immunological profile [15]. Indeed, Dutra et al. [15] have demonstrated that even in the absence of circulating parasites, high levels of activated T-cells could be observed in peripheral blood of treated patients at least 5 years after the end of chemotherapy. Other studies have shown a clear tendency of decreased mean and individual percentages of activated T-cells as early as 6 months after completing Bz-treatment [20]. Additionally, it has been demonstrated that

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Cell phenotype %	Groups		
	NI (n=7)	E-IND (n=6)	E-IND$_T$ (n=6)
CD44	42.5±5.9	40.8±5.9	37.9±6.2
$CD69^+CD4^+$	1.5±0.7	0.9±0.9	**6.4±3.1**[a,b]
$HLA\text{-}DR^+CD4^+$	3,4±0.9	**1.9±2.0**[a]	**3.9±1.7**[b]
CD83	3.7±7.7	**23.2±3.4**[a]	**20.4±5.5**[a]
$CD69^+CD8^+$	3.8±4.1	**0.8±0.9**[a]	**15.7±3.9**[a,b]
$HLA\text{-}DR^+CD8^+$	5.3±2.6	4.0±2.9	**17.5±10.4**[a,b]

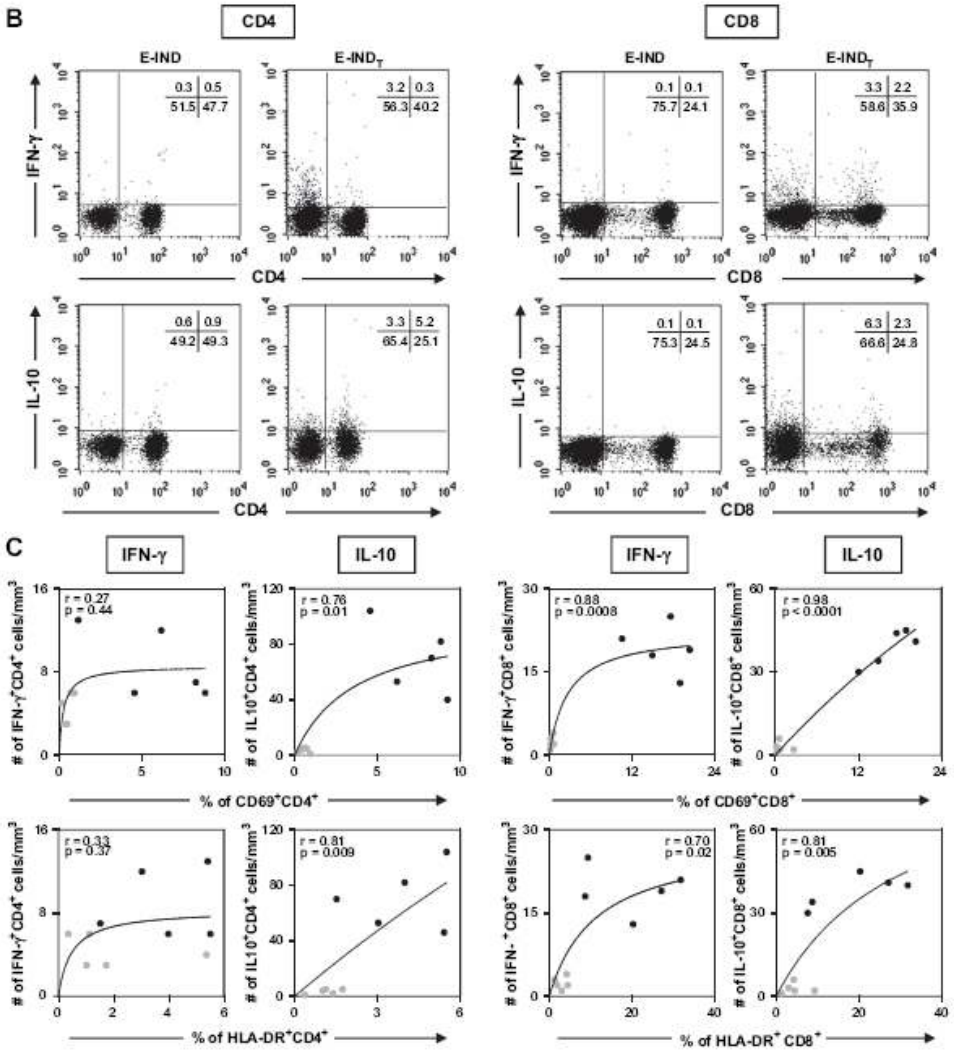

Fig. 3. (A) Activation status of T-lymphocytes subsets ($CD4^+$ and $CD8^+$) in the peripheral blood from non-infected children (NI) and early indeterminate *T. cruzi*-infected patients prior to the Bz-treatment (E-IND – ▭) and one year after it (E-IND$_T$ – ■). A double-labeling protocol was performed to identify percentage of $CD69^+$ and $HLA\text{-}DR^+$ in T-cell subsets. The results are expressed as scattering of individual values and median percentage. Significant differences at $p < 0.05$ are identified by letters "a" and "b" in comparison to the NI and E-IND groups, respectively. (B) Representative dot plots illustrating the increase on IL-10 synthesis by $CD4^+$T cells and a type 1-modulated immune profile by $CD8^+$T-cells in the E-IND$_T$ group compared to the E-IND group. Quadrant statistics were used for data analysis, and the results are expressed as the percentage of positive cells within the selected gate. (C) Correlation analyses showed the positive correlation between activated $CD4^+$T-lymphocytes subset and anti-inflammatory cytokine pattern ($IL\text{-}10^+CD4^+$) after Bz-treatment; however, a positive correlation was observed between activated $CD8^+$T-lymphocytes subset and a mixed cytokine pattern ($IFN\text{-}\gamma^+CD8^+$ and $IL\text{-}10^+CD8^+$) in the E-IND$_T$ group.

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Table 1
Frequency of adhesion molecule expression by peripheral blood $CD4^+$ and $CD8^+$T-cell subsets from early-indeterminate *T. cruzi* infected children, before and after Bz-treatment, and non-infected children

Phenotype*	Groups		
	NI ($n = 7$)	E-IND ($n = 6$)	E-IND$_T$ ($n = 6$)
$CD62L^+CD4^+/CD4^+$	83.18 ± 10.34	64.06 ± 7.36	69.20 ± 7.93
$CD62L^+CD8^+/CD8^+$	52.18 ± 13.04	48.30 ± 12.03	$35.50 \pm 9.55^{a,b}$
$CD18^+CD4^+/CD4^+$	23.55 ± 9.69	25.77 ± 12.69	22.11 ± 8.54
$CD18^+CD8^+/CD8^+$	65.48 ± 14.49	55.56 ± 13.10	59.82 ± 16.76
$CD54^+CD4^+/CD4^+$	4.63 ± 10.01	0.73 ± 4.86	2.47 ± 2.87
$CD54^+CD8^+/CD8^+$	33.27 ± 17.17	71.77 ± 15.03^a	15.89 ± 5.46^b

*The results are expressed as proportion within a give leukocyte subsets, e.g., ration of $CD4^+CD62L^+$ within $CD4^+$ population, allowing the normalization of data when percentage of a given subset may differ. NI = non-infected children, E-IND = early-indeterminate *T. cruzi* infected children before treatment and E-IND$_T$ = early-indeterminate *T. cruzi* infected children after treatment. Significant differences at $p < 0.05$ are identified by letters "a" and "b" in comparison to NI and E-IND, respectively.

Bz has a selective impact in host immune response as demonstrated by its ability to deregulate cytokine and nitric oxide synthesis [21]. It is possible that Bz-treatment may lead to selective time-dependent changes in the immunologic profile, which may also differ if therapeutic intervention is performed during acute or chronic disease.

In Chagas disease, analysis of immunity pre- and post-treatment is essential for both an understanding of the mechanisms of benznidazole action and the rational development of new trypanocidal agents [11,22,23]. In the last decades, the literature has accumulated evidence that correlates immune response and chemotherapy efficacy [24]. Recent studies suggest that activation of the immune system enhances Bz-treatment efficacy during murine *T. cruzi* infection [24]. To further highlight this issue, we have performed a longitudinal follow-up investigation to evaluate the effect of etiological treatment on circulating leukocyte phenotypes of *T. cruzi*-infected children and its correlation with cytokine pattern.

Our data demonstrated that despite an overall low immune activation observed during ongoing early indeterminate Chagas disease, an elevated activation of innate and adaptive immunity was observed in peripheral blood of Bz-treated children. These findings are in agreement with those from Dutra et al. [15], demonstrating increased percentages of activated T- and B-cells in peripheral blood of chagasic patients for at least 5 years after etiological specific treatment, independent of its success. Furthermore, Bahia-Oliveira et al. [9] showed higher rates of proliferative responses of peripheral blood cells against parasite antigens seen in treated and cured patients, suggesting the presence of long-term memory T-cells. In this regard, immunological memory after parasitological cure was suggested to be sustained by the presence of *T. cruzi* antigens, but not intact parasites, at the surface of germinal center splenocytes and in heart inflammatory foci in benzonidazole-treated mice [8]. These results are in agreement with those reported by Olivieri et al. [12] demonstrating that Bz-treatment leads to increased levels of lymphocyte expansion in peripheral lymphoid organs of *T. cruzi*-infected mice, mainly due to the expansion of effector and memory $CD8^+$T-cell subsets. The selective migration of $CD8^+$T-cells to lymphoid organs may explain the lower number of $CD8^+$T-cells in addition to decreased frequency of $CD62L^+CD8^+$ and $CD54^+CD8^+$ cell counts in peripheral blood of members of the E-IND$_T$ group. We hypothesize that the massive antigen release triggered by Bz-treatment could lead to enhanced immune activation with consequent cytokine synthesis, which can be maintained by residual parasite antigens and/or idiotypic interactions.

One point of concern is the possibility that this large number of activated cells may result in adverse outcome for treated patients, leading to a pro-inflammatory immune response and progressive tissue damage. In response, we have further characterized the *ex vivo* intracellular cytokine pattern of these circulating activated cells.

Our data indicate that Bz-treatment during early-indeterminate Chagas disease induced higher levels of macrophage-like ($CD16^+CD14^+$) and pro-inflammatory monocytes ($HLA\text{-}DR^{High}CD14^+$). Despite the higher percentage of circulating macrophage-like and pro-inflammatory monocytes, we did not observe a positive correlation between activated monocytes and pro-inflammatory cytokines synthesis by $CD14^+$ cells ($TNF\text{-}\alpha^+CD14^+$ and $IL\text{-}12^+CD14^+$). While these data may sound controversial, as the pro-inflammatory monocytes have been pointed out to be an important source of TNF-α and IL-12 [25,26], it is possible that some immunomodulatory mechanism controls the cytokine synthesis of these cells after Bz-treatment. We do not yet fully understand the down-regulation of monocyte IL-12 production. It is possible that Bz-treatment could modulate the cytokine synthesis by monocytes, since recent studies have reported the inhibitory effects of benznidazole on TNF-α and IL-12 cytokine synthesis by LPS-stimulated murine macrophage [21]. Another possibility is that the synthesis of modulatory cytokines from another cell source may underlie this phenomenon, since the autocrine immunomodulatory IL-10 loop seems not to be present following Bz-treatment as demonstrated by Sathler-Avelar et al. [11].

Additionally, our data revealed that the increased frequency of $CD69^+CD16^+$ NK cells was correlated with a mixed cytokine pattern, here represented by increased levels of both $IFN\text{-}\gamma^+$ and $IL\text{-}4^+$ NK cells. Studies have reported that trypomastigote antigens are able to stimulate IFN-γ synthesis by NK cells [27]. Together, our data suggest that the expansion of pro-inflammatory monocytes as well as $CD69^+CD16^+$ NK cells might represent effective anti-*T. cruzi* mechanisms after Bz-treatment, since macrophages can be efficiently activated by NK-derived IFN-γ, which invokes nitric oxide production and controls parasite replication [27]. In this context, the NK-derived IL-4 may be relevant to control the IL-12 synthesis by monocytes or drive a modulatory pathway that ultimately allows the presence of the monocyte and NK effector mechanism in the absence of the harmful immune response that would result in tissue damage. These findings corroborate a previous report [11], suggesting that a type 1-modulated cytokine pattern is important to successful

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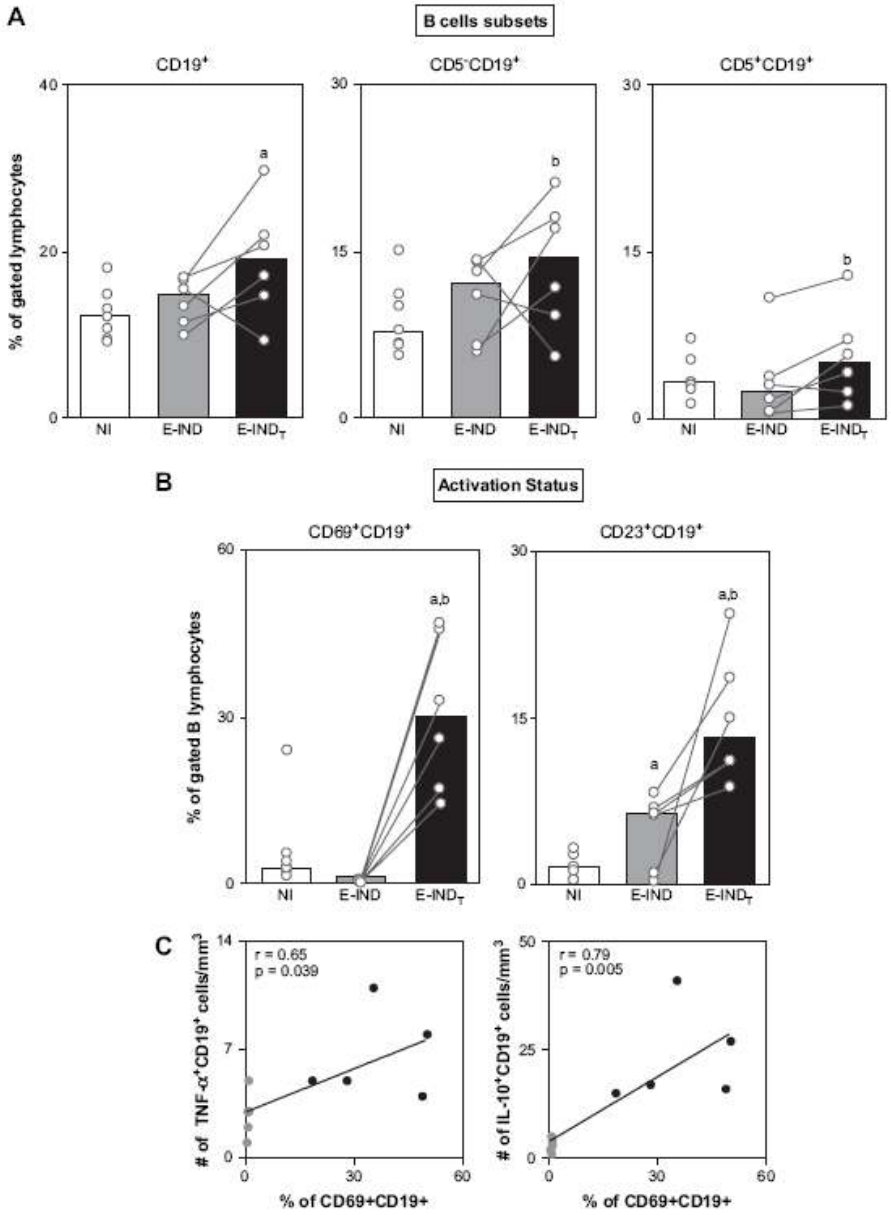

Fig. 4. (A) Percentage of B-cells ($CD19^+$), B-cell subsets ($CD5^-CD19^+$ and $CD5^+CD19^+$) and (B) activation status ($CD69^+CD19^+$ and $C23^+CD19^+$) in the peripheral blood from non-infected children (NI – ▭) and early indeterminate *T. cruzi*-infected patients prior to the Bz-treatment (E-IND – ▭) and 1 year after it (E-IND$_T$ – ■). To identify these cell populations phenotypic studies were performed using a triple-labeling protocol with anti-CD5 FITC, anti-CD69 or CD23 PE, and anti-CD19 TC. The results are expressed as scattering of individual values and median percentage. Significant differences at $p < 0.05$ are identified by letters "a" and "b" in comparison to the NI and E-IND groups, respectively. (C) Correlation analyses showed the positive correlation between activated $CD19^+$B-cells ($CD69^+CD19^+$) and a mixed cytokine pattern ($TNF\text{-}\alpha^+CD19^+$ and $IL\text{-}10^+CD19^+$) after Bz-treatment.

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therapeutic intervention in human Chagas disease, with IFN-γ produced by NK cells acting synergistically with the benznidazole, favoring the parasite clearance during Chagas disease treatment [11].

In the adaptive immune compartment, we have also observed that the activation status was closely related to an overall type 1-modulated immunological profile within circulating T- and B-lymphocytes. Specifically, we have pointed out that activated $CD4^+$T-cells exclusively produce IL-10, as a key element to the control of deleterious tissue damage triggered by exacerbated inflammatory immune response during chemotherapy. Moreover, the secretion of type 1 cytokines by $CD8^+$ T-cells (IFN-γ) and $CD19^+$B-cells (TNF-α) with simultaneous synthesis of IL-10 further illustrates the effective modulatory event following Bz-treatment.

Another cytokine that may also be involved in this modulatory phenomenon is IL-4. Increased level of IL-4^+T cells has already been reported following Bz-treatment exclusively produced by $CD8^+$T-cells [11]. Aiming to characterize whether IL-4-producing T-cells may contribute to modulate the increased levels of INF-γ produced by $CD8^+$T-cells we have further focus on additional correlation analysis between cytokine producing cells. Analysis between cytokine producing cells suggested that IL-10 from $CD4^+$ and $CD8^+$T-cells and IL-4 derived from $CD8^+$T-cells may represent putative regulatory events to overcome the higher levels of IFN-γ produced by $CD8^+$T-cells following the Bz-treatment, preventing deleterious implications of the increase in IFN-γ production, as increased levels of IFN-γ are usually associated with severe chronic Chagas disease [10].

We believe that the large amount of parasite antigens released by infected host cells following Bz-treatment probably mediates a strong activation of $CD4^+$ and $CD8^+$T lymphocytes, thereby preventing tissue lesions that arise from the type 1-modulated immune profile incited by these antigens. In fact, we have previously demonstrated that the addition of *T. cruzi* antigens to peripheral blood leukocytes from untreated infected children have a marked impact on their cytokine profile, leading to a pattern like that observed in treated individuals [11].

It is important to mention that the use of uninfected Bz-treated hosts is critical to confirm the hypothesis suggested by our findings. What is the effect of Bz on the immune system in the absence of parasite infection? Are the effects on the immune response truly a result of parasite reduction and the subsequent impact of this action on the immune response, or simply the drug working on the immune system directly? Since *T. cruzi* has been shown by others to generally down-regulation of the immune responses at various times during infection, perhaps by eliminating parasites this restriction is overcome, allowing the innate and adaptive immunity to progress at an optimal level. Therefore, the knowledge regarding effect of Bz-treatment in healthy hosts would help to know whether the impact of Bz-treatment observed here its direct impact on immunity or if it includes the pathogen in the resultant equation. However, it is important to keep in mind the limitations and conditions to work with human population considering the ethical restrictions. Indeed, the studies in experimental models should be an important alternative to answer to these queries. Even in experimental models it should be considered the fact that some models are not appropriate to mimicry the human infection since many event of the immune response does not reproduce the human disease. The administration of highly toxic compounds such as benznidazole into healthy children can not be performed nor justified for ethical reason. So the impact of Bz on immunological features of healthy individuals must be carried out in experimental models or even in vitro, using cells derived form healthy volunteers. It is possible that the drug causes a massive antigen release, but this would coincidentally be limiting the total amount of antigen available to the immune system by eliminating parasite replication and expansion.

Another important issue would be the measure of parasite antigen in serum under different treatment conditions. However, it is important to observe that a massive antigen realize may represent a compartimentalized phenomenon not detectable in the blood circulation. An important perspective that is under investigation by our group is the analysis of parasite-specific immune responses during treatment to assess the kinetic of changes in the immune response following Bz-treatment.

In summary, the major contribution of this investigation support the hypothesis that the presence of activated leukocytes in the peripheral blood is not sufficient to limit etiological treatment due to the findings that Bz-treatment alters the basal cytokine setting toward a mixed pro-/anti-inflammatory profile.

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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 $CD8^+HLA\text{-}DR^+$ 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 $CD8^+HLA\text{-}DR^+$ 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 $CD8^+HLA\text{-}DR^+$ 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 ($n=8$), 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, 24-h Holter examination and chest X-ray. Chagasic patients with gastrointestinal disease, DIG ($n=8$), 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-µl samples of peripheral blood, which had been collected in Vacutainer tubes containing EDTA (Becton Dickinson), were mixed in 12 × 75 mm tubes with 5 µl 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 µl 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).

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 $P < 0.05$.

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

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-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 ($n=12$)]	Indeterminate [IND ($n=8$)]	Cardiac [CARD ($n=13$)]	Digestive [DIG ($n=8$)]
Total T lymphocytes ($CD3^+$)	71.17 ± 8.12	63.28 ± 5.55†	62.56 ± 12.19†	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 ± 3.87	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 ± 11.29	55.72 ± 16.05

*The results are expressed as mean percentage ± SD.
†Significant 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

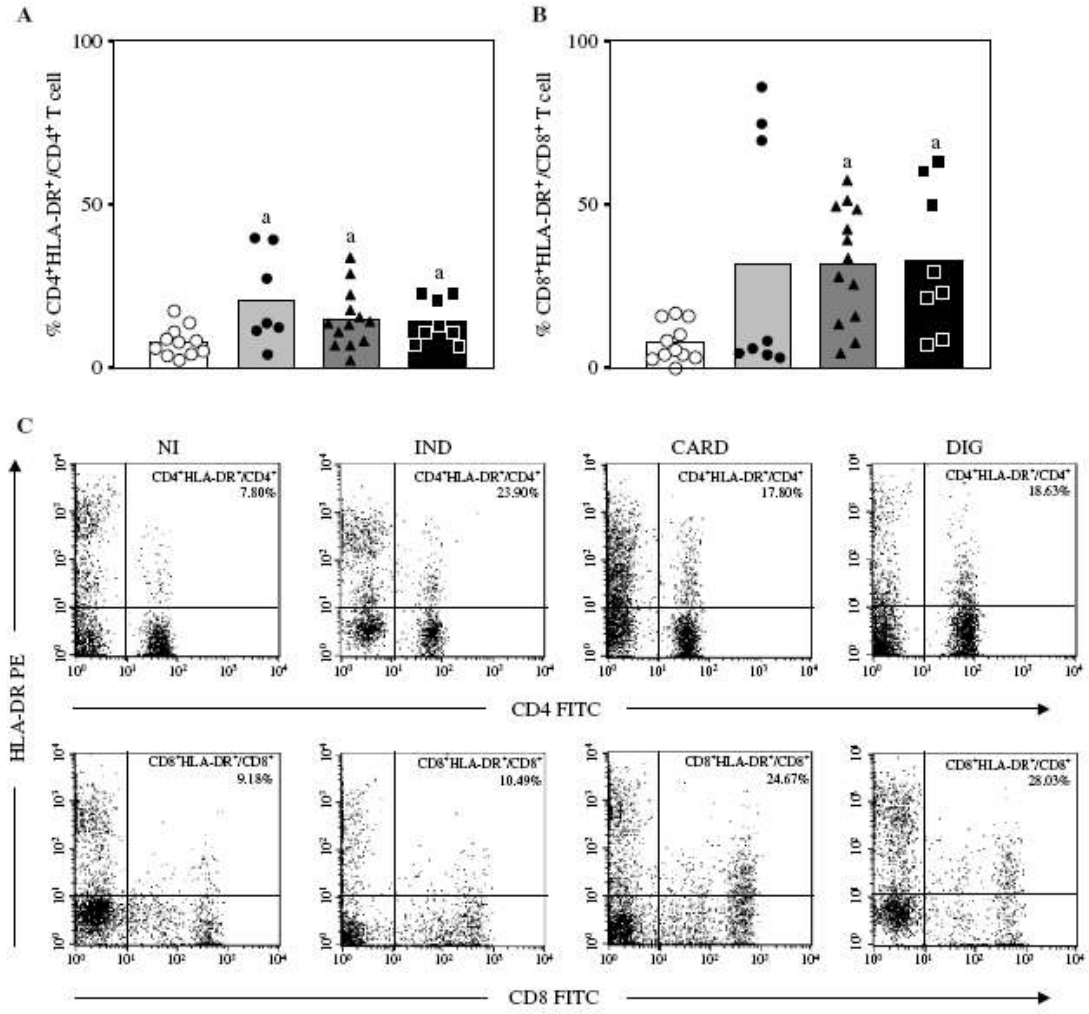

Figure 1 Analysis of activated $CD4^+$ and $CD8^+$ T lymphocytes in the peripheral blood of *Trypanosoma cruzi*-infected individuals including IND (●), CARD (▲) and DIG (■) and noninfected individuals, NI (○). Phenotypic studies were carried out using a double-labelling protocol involving anti-CD4 or anti-CD8 fluorescein isothiocyanate (FITC) and anti-HLA-DR phycoerythrin (PE) to identify (A) $CD4^+HLA\text{-}DR^+/CD4^+$ cells and (B) $CD8^+HLA\text{-}DR^+/CD8^+$ cells. The results are expressed as scattering of individual values and mean percentage of activated cells within $CD4^+$ and $CD8^+$ 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 $CD4^+HLA\text{-}DR^+/CD4^+$ in all chagasic individuals in comparison with NI individuals, and a restricted higher frequency of $CD8^+HLA\text{-}DR^+/CD8^+$ 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 $CD4^+$ cells with high CD25 expression, as proposed by Baecher-Allan *et al.* [33]. Our results showed an increased frequency of $CD4^+CD25^{high}$ 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

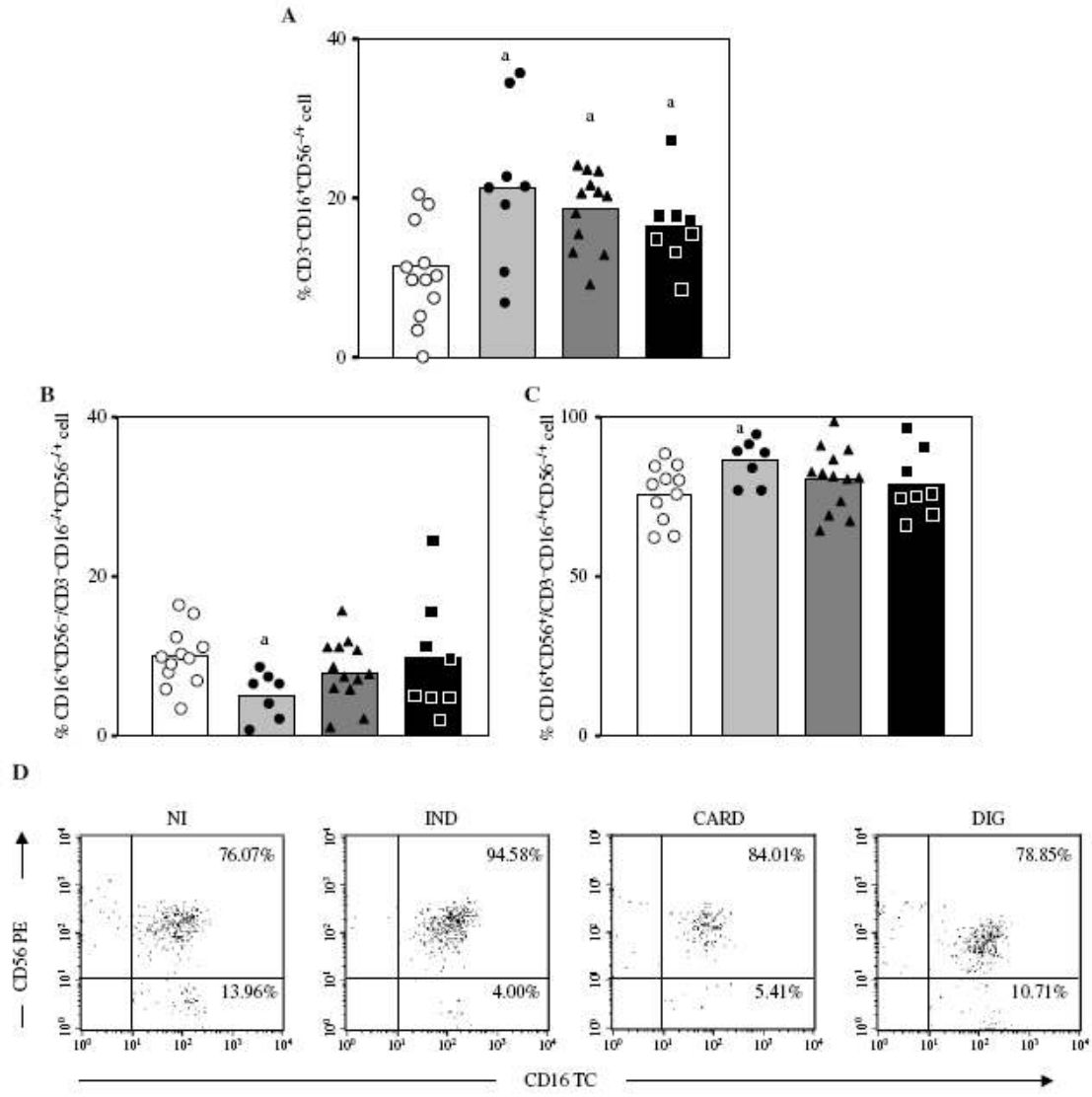

Figure 2 Analysis of total natural killer (NK) cell and subsets in the peripheral blood of *Trypanosoma cruzi*-infected individuals including IND (●), CARD (▲) and DIG (■) and noninfected individuals, NI (○). Phenotypic studies were carried out using a triple-labelling protocol anti-CD3 fluorescein isothiocyanate (FITC), anti-CD56 phycoerythrin (PE) and anti-CD16 tri-colour (TC) to identify (A) $CD3^-CD16^+CD56^{-/+}$ (NK cells), (B) $CD3^-CD16^+CD56^-$ (precursor NK cells) and (C) $CD3^-CD16^+CD56^+$ (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.

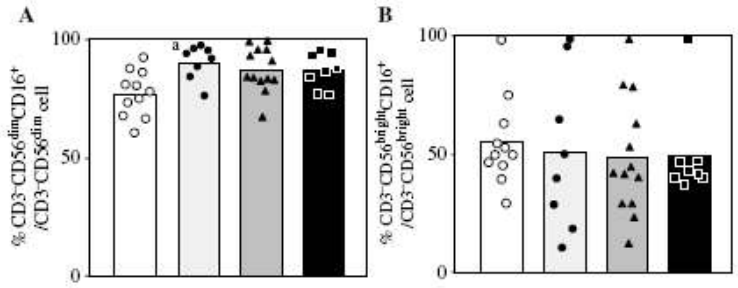

Figure 3 Analysis of natural killer (NK) cell subsets $CD3^-CD56^{dim}CD16^+$ and $CD3^-CD56^{bright}CD16^+$ within $CD3^-CD56^{dim}$ and $CD3^-CD56^{bright}$, respectively. Phenotypic studies were carried out using a triple-labelling protocol involving anti-CD3 fluorescein isothiocyanate (FITC), anti-CD56 phycoerythrin (PE) and anti-CD16 tri-colour (TC) to identify (A) $CD3^-CD56^{dim}CD16^+/CD3^-CD56^{dim}$ cells and (B) $CD3^-CD56^{bright}CD16^+/CD3^-CD56^{bright}$ 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 $CD3^-CD56^{dim}CD16^+/CD3^-CD56^{dim}$ 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 $CD3^+$ T lymphocytes in their peripheral blood despite no significant differences on $CD4^+$ and $CD8^+$ T-cell subsets and normal levels of $CD19^+$ 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 ($CD3^+$, $CD4^+$ and $CD8^+$) and B cells ($CD19^+$) observed in our DIG group was as documented in previous reports because lower absolute counts of $CD3^+$ T cells and $CD4^+$ 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 $CD4^+$ and $CD8^+$ 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 $CD4^+$HLA-DR^+ in all infected individuals; a higher frequency of activated $CD8^+$ 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 $CD8^+$HLA-DR^+ 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 $CD8^+$ T cells, within cardiac lesions of chagasic patients. Interestingly, a small number of IND patients also presented with a high frequency of $CD8^+$HLA-DR^+ 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 $CD8^+$ T cells are prone to develop cardiomyopathy sooner than those showing low frequencies of circulating $CD8^+$HLA-DR^+ 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 ($CD3^-CD16^+CD56^{-/+}$) can be found in the peripheral blood of all patients chronically infected with *T. cruzi*. However, only IND patients showed a higher percentage of $CD3^-CD16^+CD56^+$ and $CD3^-CD16^+CD56^{dim}$ NK cells. Human NK cells are generally defined as being membrane $CD3^-CD16^+CD56^{-/+}$ lymphocytes [41]. In humans,

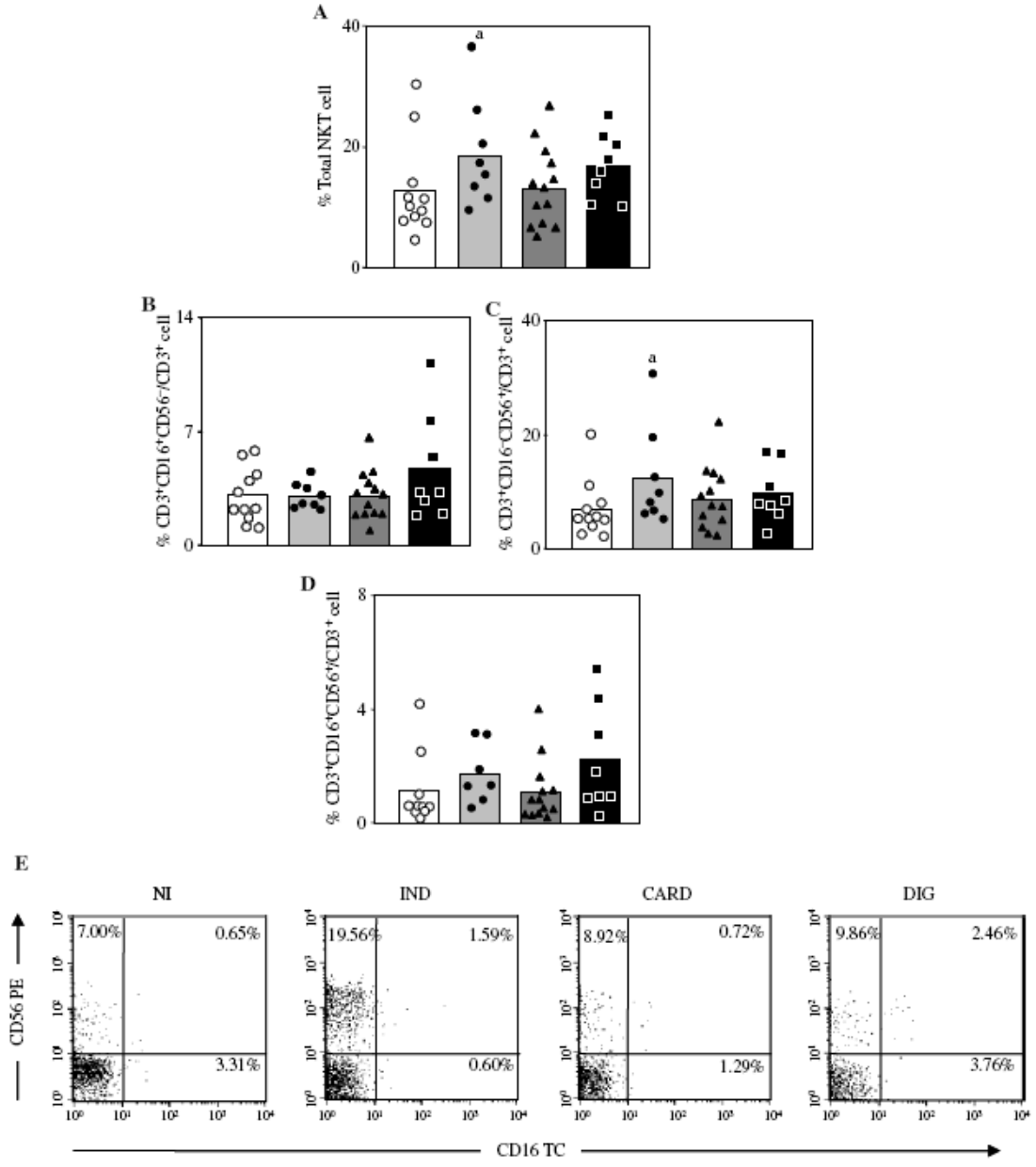

Figure 4 Analysis of total natural killer T (NKT) cells and subsets in the peripheral blood of *Trypanosoma cruzi*-infected individuals including IND (●), CARD (▲) and DIG (■) and noninfected individuals, NI (○). Phenotypic studies were carried out using a triple-labelling protocol involving anti-CD3 fluorescein isothiocyanate (FITC), anti-CD56 phycoerythrin (PE) and anti-CD16 tri-colour (TC) to identify (A) total NKT cells ($CD3^+CD16^{-/+}CD56^{-/+}$), (B) NKT1 cells ($CD3^+CD16^+CD56^-$), (C) NKT2 cells ($CD3^+CD16^-CD56^+$) and (D) NKT3 cells ($CD3^+CD16^+CD56^+$). The results are expressed as scattering of individual values and mean percentage of NKT cells within gated $CD3^+$ 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.

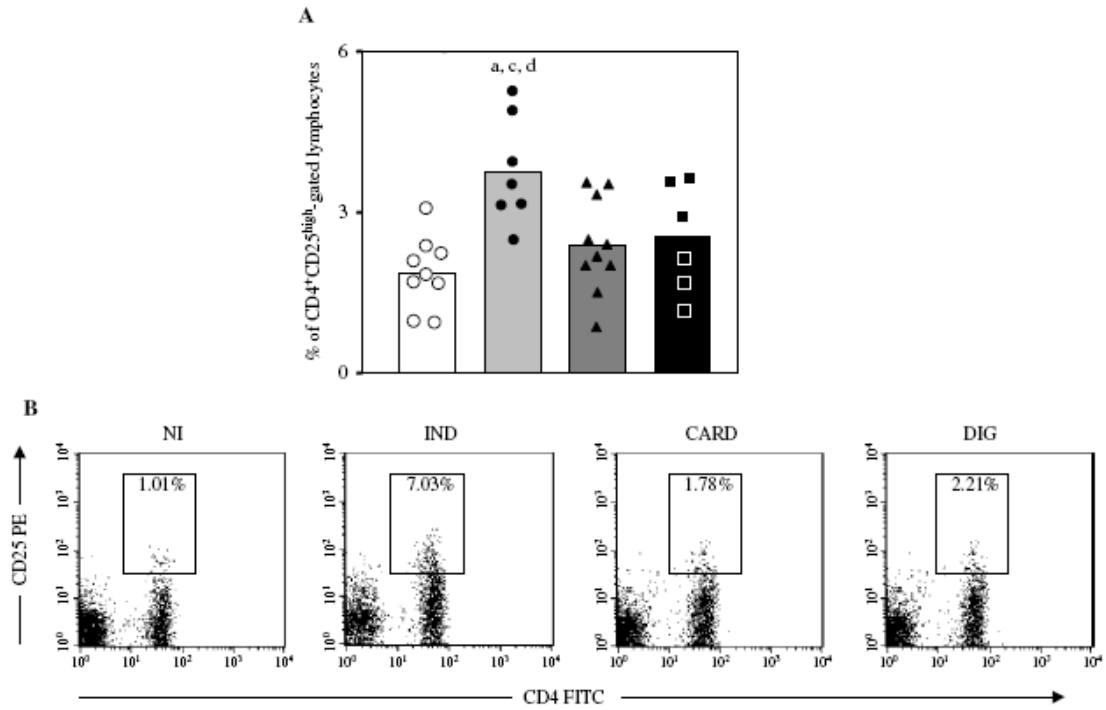

Figure 5 Analysis of $CD4^+CD25^{high}$ regulatory cells in the peripheral blood of *Trypanosoma cruzi*-infected individuals including IND (●), CARD (▲) and DIG (■) and noninfected individuals, NI (○). A double-labelling panel involving anti-CD4 fluorescein isothiocyanate (FITC) and anti-CD25 phycoerythrin (PE) was used to identify regulatory cells. The results are expressed as scattering of individual values and mean percentage of $CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ 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 $CD16^+CD56^+$ [31] and little is known about the properties of $CD16^+CD56^-$ NK cells. It has been suggested that those cells are functionally and phenotypically immature, capable of maturation and possible precursors to mature $CD16^+CD56^+$ NK cells [31]. Functional studies have demonstrated that $CD16^+CD56^-$ cells showed higher proliferative capacity, whereas $CD16^+CD56^+$ 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 $CD8^+HLA\text{-}DR^+$ 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 $CD8^+$ 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-γ 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-β [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-γ synthesis confirms previous observations of low levels of IFN-γ 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-α and IFN-γ 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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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.

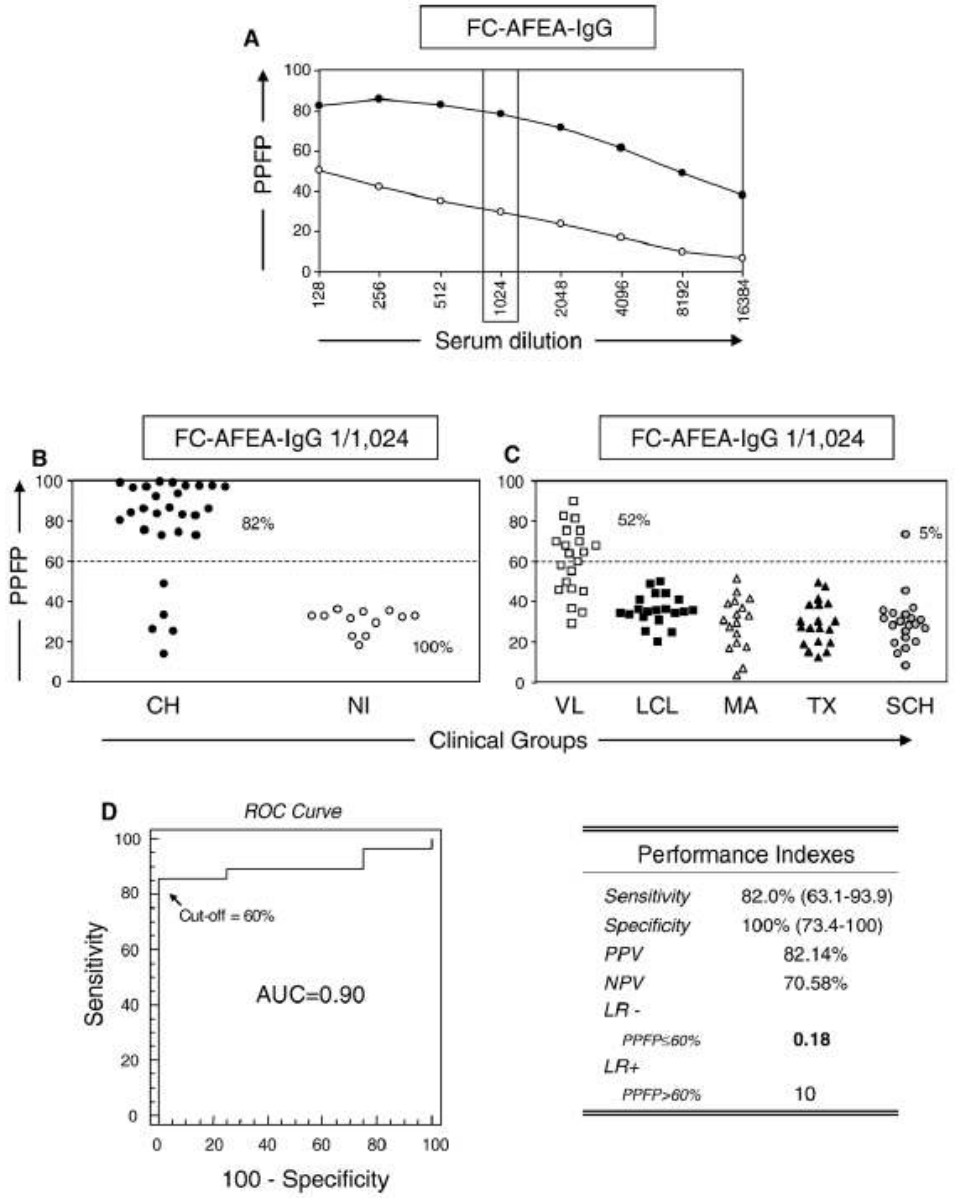

Fig. 1. Titration curve average of anti-fixed epimastigotes IgG reactivity (FC-AFEA-IgG) of serum samples from chagasic patients (CH ●, $n=28$) and non-infected individuals (NI ○, $n=12$). The continuous box marks the most promising serum dilution to discriminate CH from NI (A). FC-AFEA-IgG of individual 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% < PPFP ≤ 50%) and high positive (PPFP > 50%) 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), LR > 10 practically confirms disease diagnosis and LR < 0.1 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 > 60% 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

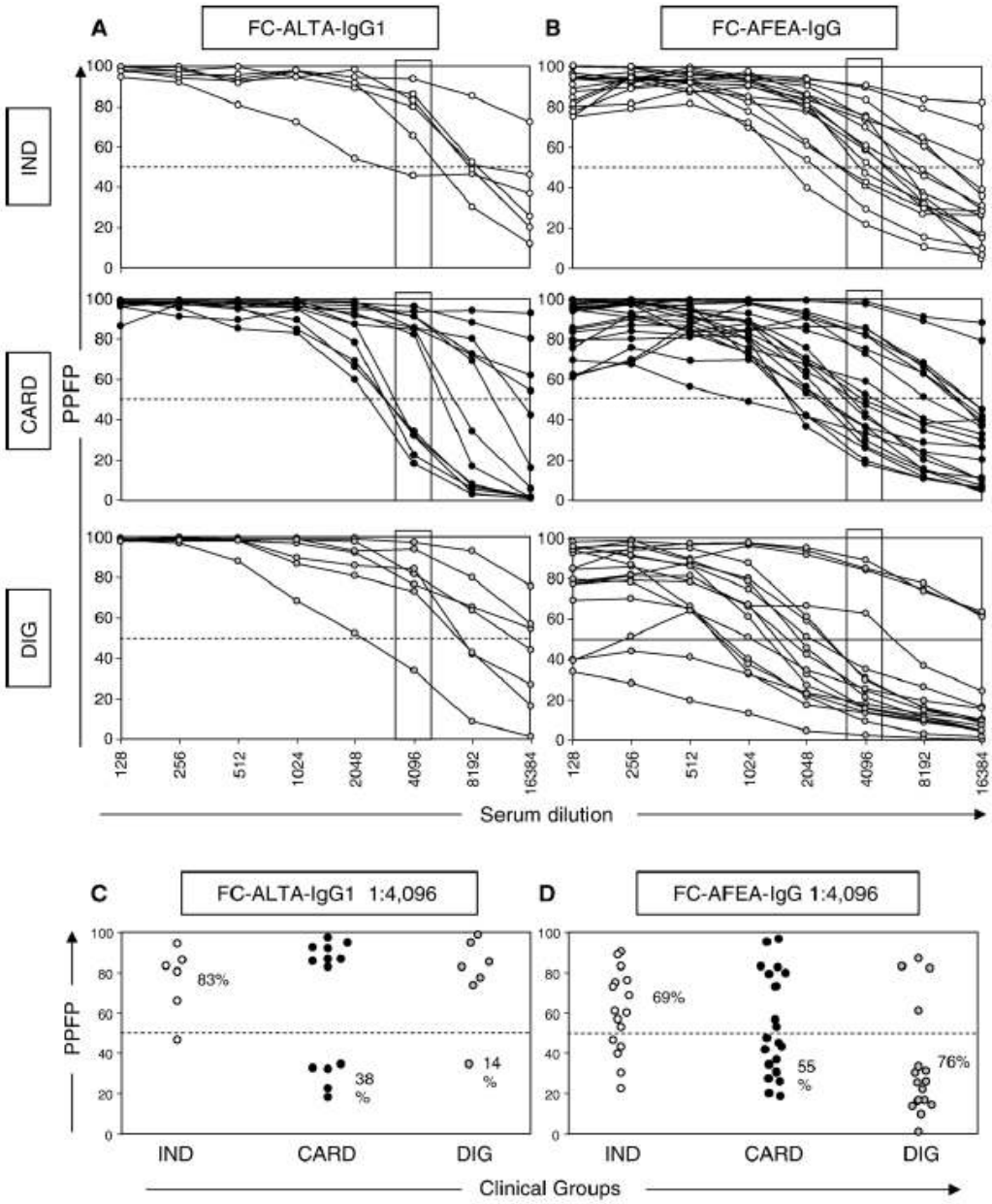

Fig. 2. Titration curve of FC-ALTA-IgG1 (A) and FC-AFEA-IgG (B) of individual serum samples from indeterminate (IND ○), cardiac (CARD ●) and digestive (DIG ◎) chagasic patients (subcohort 2 and subcohort 2+3, respectively). The results are expressed as PPFP. The dotted line represents the cut-off proposed 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% < PPFP ≤ 60%) and high positive (PPFP > 60%). 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

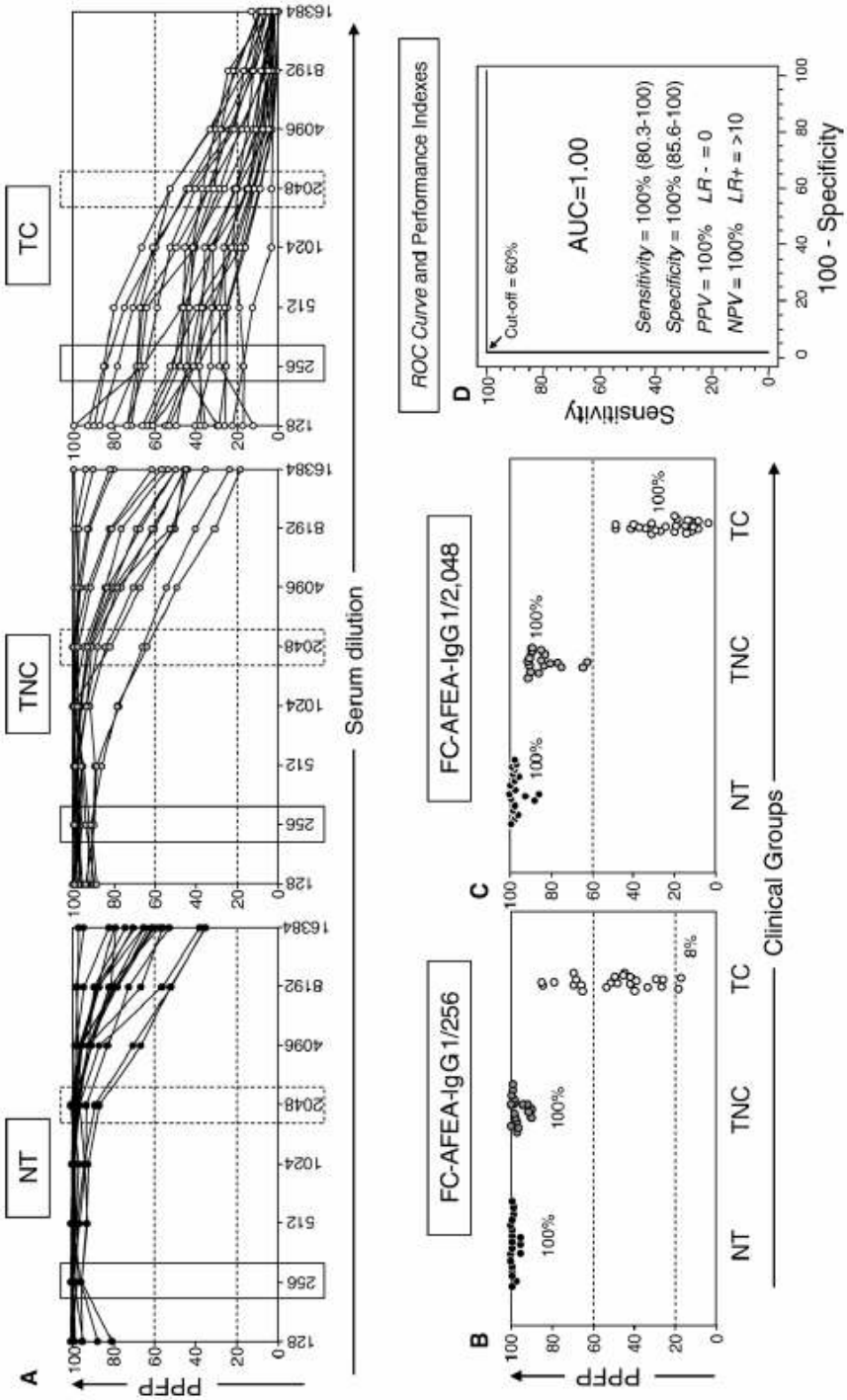

Fig. 3. Titration curve of anti-fixed epimastigote IgG reactivity (FC-AFEA-IgG) of individual serum samples from not-treated (NT ●, $n=19$), treated not-cured (TNC ○, $n=17$) and treated cured (TC ○, $n=24$) individuals (A). Continuous and dotted rectangles represented the two promising serum dilutions: 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%<PPFP ≤ 60%) and high positive (PPFP >60%) 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 > 20%), and 33% of them had high positive IgG reactivity (PPFP > 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+ > 10 practically confirms disease diagnosis and LR− < 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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