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Etiological treatment of Chagas disease patients with benznidazole lead to a sustained pro-inflammatory profile counterbalanced by modulatory events

A.C. Campi-Azevedo [a], J.A.S. Gomes [a,b,c,d,*], A. Teixeira-Carvalho [a], D. Silveira-Lemos [a], D.M. Vitelli-Avelar [a], R. Sathler-Avelar [a], V. Peruhype-Magalhães [a], S.R. Béla [a], K.F. Silvestre [c], M.A. Batista [c], N.C.C. Schachnik [a], R. Correa-Oliveira [b,e], S.M. Eloi-Santos [a], O.A. Martins-Filho [a]

[a] *Laboratório de Biomarcadores de Diagnóstico e Monitoração, Centro de Pesquisas René Rachou, Fiocruz, Belo Horizonte, Brasil*
[b] *Laboratório de Imunologia Celular e Molecular, Centro de Pesquisas René Rachou, Fiocruz, Belo Horizonte, Brasil*
[c] *Laboratório de Biologia das Interações Celulares, Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brasil*
[d] *Programa de Pós- graduação em Medicina Tropical e Infectologia, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Brasil*
[e] *Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais – INCT-DT, Brasil*

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ABSTRACT

In the present study, we characterized the phagocytic capacity, cytokine profile along with the FCγ-R and TLR expression in leukocytes from Chagas disease patients (indeterminate-IND and cardiac-CARD) before and one-year after Bz-treatment (IND_T and $CARD_T$). A down-regulation of IL-17, IFN-γ and IL-10 synthesis by neutrophils was observed in $CARD_T$. The Bz-treatment did not impact on the expression of phagocytosis-related surface molecules or monocyte-derived cytokine profile in IND_T. Although $CARD_T$ showed unaltered monocyte-phagocytic capacity, up-regulated expression of Fcγ-RI/III and TLR-4 may be related to their ability to produce IL-10 and TGF-β. Down-regulation of lymphocyte-derived cytokine was observed in IND_T whereas up-regulated cytokine profile was observed for lymphocytes in $CARD_T$. Analysis of cytokine network revealed that IND displayed a multifaceted cytokine response characterized by strong connecting axes involving pro-inflammatory/regulatory phagocytes and lymphocytes. On the other hand, CARD presented a modest cytokine network. The Bz-treatment leads to distinct cytokine network: decreasing the links in IND_T, with a pivotal role of $IL\text{-}10^+$ monocytes and expanding the connections in $CARD_T$. Our findings highlighted that the Bz-treatment contributes to an overall immunomodulation in IND_T and induces a broad change of immunological response in $CARD_T$, eliciting an intricate phenotypic/functional network compatible with beneficial and protective immunological events.

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Introduction

Chagas disease is a neglected disease caused by the protozoan *Trypanosoma cruzi*. It is estimated that there are 16–18 million infected people in Latin America, with more than 300,000 new cases every year (Schofield et al. 2006; Moncayo and Silveira 2009). After infection a short-term acute phase with patent parasitemia is followed by a lifelong chronic phase with scarce circulating parasites that lead to the development of symptomatic chronic cardiomyopathy and/or digestive symptoms in approximately 30% of the Chagas disease patients (Marin-Neto et al. 2007).

The specific treatment of Chagas disease is based on the use of the nitroderivative benznidazole (*N*-benzyl-2-nitroimidazole acetamide – Bz), which is known to reduce parasitism during the acute and early in the chronic infection (Fragata-Filho et al. 1995; de Andrade et al. 1996). In this context, specific chemotherapy is recommended for the treatment of Chagas disease applying the general assumption that the earlier the specific treatment is

Abbreviations: Bz-treatment, benznidazole treatment; IND_T, benznidazole-treated indeterminate patients; $CARD_T$, benznidazole-treated cardiac patients.

* Corresponding author at: Departamento de Morfologia, Instituto de Ciências Biológicas, UFMG, Avenida Antônio Carlos, 6627 – Pampulha, Belo Horizonte, Minas Gerais 31270-010, Brasil. Tel.: +55 31 3409 3003.
E-mail address: juliana@icb.ufmg.br (J.A.S. Gomes).

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initiated the greater are the chances of parasitological cure and consequently significant clinical benefits to the host (de Andrade et al. 1996). The effect of treatment during the chronic phase is more controversial, although there are reports in post-treatment follow-up studies showing that individuals treated with Bz and evaluated decades after the initial infection demonstrated significant protection from progression of heart pathology due to Chagas disease (Viotti et al. 2006; Fabbro et al. 2007). However, despite the low cure rates observed in most patients submitted to treatment during the chronic disease, several studies have suggested that the Bz-treatment should be still recommended at late stages of Chagas disease to prevent disease progression, regardless of lack of complete parasite clearance (Viotti et al. 2006; Garcia et al. 2005; Sosa-Estani and Segura 2006).

It has been proposed that a relevant factor potentially influencing parasite clearance as well as the morbidity control following the treatment for Chagas disease is the cooperative action between the drug effects and the host immune response (Michailowsky et al. 1998; Rassi et al. 1999; Sathler-Avelar et al. 2006; Sathler-Avelar et al. 2008; Sathler-Avelar et al. 2006, 2008, 2012). In fact, there is a general hypothesis that etiological treatment contributes to reduce the parasitism and rearrange the host immune response, leading to a balanced inflammatory response that is crucial to control Chagas disease morbidity (Viotti et al. 2006; Garcia et al. 2005; Sathler-Avelar et al. 2006; Sathler-Avelar et al. 2006, 2008; Viotti et al. 1994).

Despite the large number of studies focusing on the immune response following *T. cruzi* infection, there are relatively few studies on the impact of Bz-treatment on this response. We have previously shown that early after the end of the Bz-treatment, the NK-cells and $CD8^+$ T-lymphocytes are important sources of IFN-γ and that IL-10 produced by $CD4^+$ T-cells and that B lymphocytes are putative key element on the modulation of the immune response and control of tissue damage induced by the pro-inflammatory response (Sathler-Avelar et al. 2006; Sathler-Avelar et al. 2006, 2008). Moreover, in the IND patients, Bz therapy induced a change on cytokine patterns of peripheral blood monocytes, NK-cells and $CD8^+$ T-cells toward a long-lasting pro-inflammatory/modulated profile that could be important to the maintenance of a non-deleterious immunological microenvironment (Sathler-Avelar et al. 2012).

In this present work, we have explored novel aspects of human immune response following the etiological treatment of patients in the chronic phase of Chagas disease, presenting IND or CARD clinical forms. We have evaluated the phagocytic capacity, cytokine profile (IL-17, IFN-γ, IL-4 and IL-10) and cell surface molecules expression (FCγ-R and TLR) of circulating leukocytes before and one-year after Bz-treatment after *in vitro* stimulation with live *T. cruzi* organisms. Our findings support the hypothesis that the Bz-treatment causes a change in the host immune response leading to an immunomodulatory profile in IND and a broader change in the in CARD, eliciting a complex phenotypic/functional network compatible with beneficial and protective immunological events.

Patients, materials and methods

Study population

All individuals who agreed to participate in this study were identified and clinically evaluated at the Referral Outpatient Center for Chagas Disease of the Hospital das Clínicas of the Federal University of Minas Gerais (UFMG) or at the HEMOMINAS Blood Bank Centre, Belo Horizonte, Minas Gerais, Brazil. Serology for Chagas disease was carried out by indirect immunofluorescence, ELISA or indirect hemagglutination and patients were considered positive when at least two tests presented positive results. In this cross-sectional study, we collected blood samples from 24 Chagas disease patients in chronic phase before and one year after the end of etiological treatment with benznidazole (*N*-benzyl-2-nitro-1-imidazol acetamide – Bz). A group of non-infected individuals was included as a control. According to their clinical records, the chronic Chagas disease patients were divided into four categories. Subjects presenting positive serology with no clinical manifestations of the disease classified as asymptomatic indeterminate patients (IND; $n = 5$; mean age $= 42.5 \pm 5.5$ years, ranging from 35 to 52 years). Subjects with cardiac dysfunction, presenting dilated cardiomyopathy diagnosed by a detailed clinical examination, including eletrocardiography, 24-h Holter examination and chest X-ray were included as cardiac patients (CARD; $n = 6$; mean age $= 44.0 \pm 8.7$ years, ranging from 33 to 56 years). Bz-treated Chagas disease patients were re-evaluated one year after the end of etiological treatment and referred as Bz-treated IND (IND_T; $n = 5$; mean age $= 51.4 \pm 7.41$ years, ranging from 38 to 60 years) or Bz-treated CARD ($CARD_T$; $n = 8$; mean age $= 54.4 \pm 5.46$ years, ranging from 48 to 61 years). Bz treatment was carried out with daily doses of 5 mg/kg of body weight, twice a day for 60 days, according to the Brazilian Ministry of Health regulations (Ministério da Saúde 2005).

The control group was composed of blood donors, seronegative adults referred as non-infected individuals (NI; $n = 7$, mean age $= 29.0 \pm 9.7$ years, ranging from 18 to 47 years).

Informed written consent was obtained from all participants. This work complied with resolution number 466/2012 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

Forty milliliters of heparinized (Becton Dickinson, CA, USA) whole peripheral blood were collect from each participant and used for *in vitro* phagocytic capacity assay, surface/intracellular immunostaining following the phagocytosis protocol as well as intracellular nitric oxide assessment performed by flow cytometry.

T. cruzi live organisms and soluble antigen preparations

T. cruzi 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, NY, USA) at 28 °C in a BOD incubator (REVCO, Asheville, USA.). *T. cruzi* epimastigote organisms were harvested during the log growth phase and transferred to a 50 mL polypropylene tube (Falcon, Becton Dickinson, CA, USA). Parasite suspension was submitted to vortex shaking to disrupt epimastigotes rosettes. Differential centrifugation at $100 \times g$ for 10 min at room temperature was performed to guarantee the exclusion of parasite rosettes in the pellet. The tubes were maintained at 28 °C for 30 min to increase the parasite recovery in the supernatant. Subsequently, the supernatant was transferred to another 50 mL polypropylene tube and live *T. cruzi* washed three times with 15 mM phosphate buffered saline (PBS) supplemented with 10% FBS at $1000 \times g$ for 15 min at 4 °C and the volume adjusted to 1×10^8/mL and stored at 28 °C in a BOD incubator until use in the phagocytosis protocol.

Soluble *T. cruzi* antigen preparation was obtained from *T. cruzi* epimastigote harvested during the log growth phase in LIT medium, washed three times in cold PBS, followed by disruption through repeated freezing at −70 °C and thawing at 37 °C and homogenization at 4–6 °C in a Potter–Elvejem centrifuge (Vir Tis – Precise, USA) at 20,000 rpm five times for 60 s, with 30 s intervals at 4–6 °C. The lysate was subsequently centrifuged at $40{,}000 \times g$ for 60 min on ice. The clear supernatant was dialyzed for 24 h at 4 °C against PBS,

filter sterilized on 0.2 μm membranes, assayed for protein concentration, aliquoted, and stored at −20 °C until use for the nitric oxide protocol.

T. cruzi FITC-labeling procedure

Live *T. cruzi* were stained with fluorescein isothiocyanate-FITC (Sigma, MO, USA), at final concentration of 200 μg/mL at 37 °C for 30 min in a 5% CO_2 humidified incubator, as previously described by Gomes et al. (2012). After incubation, live parasites were washed three times with PBS and resuspended in PBS-10% FBS, adjusted to 1×10^8/mL and stored at 4 °C until use for the phagocytosis protocol. Aliquots of FITC-labeled parasites were run in the flow cytometer to evaluate the efficiency of FITC staining procedure. The ideal FITC labeled parasite staining would lead to a single peak around 10^2 and 10^3 log intervals in FL1/FITC histogram. Additionally, the FITC-labeled live *T. cruzi* were monitored by optical microscopy for motility and by trypan blue staining for viability control. For the phagocytosis protocols the live parasites were stored at 28 °C in a BOD incubator until use.

Short-term in vitro phagocytosis protocol with FITC-labeled live T. cruzi

Heparinized peripheral blood was centrifuged at $1200 \times g$ for 10 min at room temperature and final leukocyte suspension adjusted to 1×10^7 cells/mL. Short-term *in vitro* incubation was performed in the presence of FITC-labeled live *T. cruzi*. Briefly, aliquots of 3.5 mL of whole blood leukocyte suspension at 1×10^7 cells/mL were transferred to 14 mL polypropylene tubes containing 3.5 mL of RPMI, followed by the addition of 175 μL of FITC-labeled live *T. cruzi* at the concentration of 1×10^8 parasites/mL. The tubes were incubated under slow motion in orbital shaker for 2 h at 37 °C in a 5% CO_2 humidified incubator. After incubation, each tube received 20 μL of BFA (Sigma, MO, USA) at 10 μg/mL and re-incubated for 4 h at 37 °C in a 5% CO_2 humidified incubator. Following, the cultures were treated with 220 μL of EDTA at 20 mM and maintained at room temperature for 15 min prior immunophenotypic staining for cell surface markers and intracellular cytokine analysis. Additionally, confocal microscopy analysis of live *T. cruzi* phagocytizing monocytes was monitored.

Analysis of phagocytic capacity and intracytoplasmic cytokines

The short-term *in vitro* cultures was washed with 6 mL of FACS buffer (PBS supplemented with 0.5% bovine serum albumin-BSA and 0.1% sodium azide (Sigma, MO, USA) by centrifugation at $600 \times g$ for 7 min at room temperature and resuspended in 5 mL of FACS buffer.

For the phagocytic capacity analysis, aliquots of 150 μL were transferred to polystyrene tubes and incubated for 30 min at room temperature with 2 μL of TriColor (TC)-labeled anti-CD14 mAbs (Serotec, NC, USA). Following incubation, the red blood cells were lysed by the addition of 3 mL of FACS lysing solution (Becton Dickinson, CA, USA) for 10 min, and the cells washed with 2 mL of PBS by centrifugation at $600 \times g$ for 7 min at room temperature. The cells were fixed with 200 μL of FACS FIX solution (10 g/L of paraphormaldehyde, 10.2 g/L of sodium cacodilate, 6.63 g/L of sodium chloride, pH 7.2-Sigma, MO, USA) and stored at 4 °C for flow cytometric acquisition. Fluorescence quenching technique with crystal violet staining adapted from Van Amersfoort and Van Strijp (1994) was used to discriminate membrane associated from intracytoplasmic *T. cruzi* organisms (data not shown).

For intracytoplasmic cytokine analysis, aliquots of 500 μL of cells were transferred to three separate polystyrene tubes and incubated for 30 min at room temperature with 5 μL of TC-labeled anti-CD14 mAbs. Following incubation, the red blood cells were lysed, washed and the cell suspension fixed with FACS lysing solution for 10 min and permeabilized by the addition of 3 mL of Perm buffer (FACS buffer supplemented with 0.5% saponin-Sigma, MO, USA) for 10 min at room temperature. The leukocytes were resuspended in 250 μL of Perm buffer and 30 mL aliquots were transferred to U-shaped 96 well plates for intracellular staining by incubation with 20 μL of PE-labeled anti-cytokine mAbs: anti-IL-12p40/p70 (clone C11.5), anti-TNF-α (clone MAb11), anti-IL-10 (clone JES3-19F1), TGF-β (clone TB21), anti-IL17 (clone SCPL1362), anti-IFN-γ (clone 4S.B3) and anti-IL-4 (clone BVD4-1D11) (Becton Dickinson, CA, USA). TC and PE-labeled isotypic controls were included in each batch of experiments. After two wash steps, the cells were fixed with FACS FIX solution and stored at 4 °C for flow cytometric acquisition. Data were acquired 5000 $CD14^+$ events for each sample. Cell Quest™ software was used for the flow cytometric acquisition using a FACScalibur cytometer (Becton Dickinson, CA, USA). The analysis of neutrophils was performed by establishing a specific scatter gate (SSC) using the combination of anti-cell surface antigens and laser SSC to discriminate, and the neutrophils were gated as SSC^{high} (200–800)$CD14^{intermediate+}$. The analysis of monocytes was performed by using SSC *versus* FL-1/anti-CD14-FITC dot plots to select the monocytes as $SSC^{low}CD14^{high+}$ cells. Moreover, analysis of total lymphocytes was performed by gating the cells according to the forward scatter (FSC) × SSC properties as $FSC^{low}SSC^{low+}$ cells.

Phenotypic analysis of circulating monocytes

Short-term *in vitro* incubation of heparinized whole blood samples, performed in the absence exogenous stimuli was carried out under the same conditions described above and used to characterize the Fc-gamma receptors (CD16, CD32 and CD64), complement receptor CD35 and TLR-2 and TLR-4 expression by monocytes. For cell surface marker analysis, aliquots of 150 μL were transferred to polystyrene tubes and incubated for 30 min at room temperature with 2 μL of TC-labeled anti-CD14 mAbs and phycoerythrin (PE)-labeled mAbs specific for cell surface markers, including anti-Fc-γ receptors (anti-CD16, clone 3G8; anti-CD32, clone 3D3; and anti-CD64, clone 10.1); Complement receptor (anti-CD35, clone E11); Toll-like receptors (anti-TLR-2, clone TL2.1; and anti-TLR-4, clone HTA123); activation markers (anti-MHC-I, clone DX17; anti-MHC-II, clone L243) and co-stimulatory molecules (anti-CD80, clone L307.4; anti-CD11c, clone B-ly6 and anti-CD86, clone 2331 (FUN-1) all purchased from Becton Dickinson. Following incubation, the red blood cells were lysed, the cell suspension washed and fixed with FACS FIX solution and stored at 4 °C for flow cytometric acquisition.

Nitric oxide protocol following short-term in vitro incubation of whole blood samples with soluble T. cruzi antigens

The intracellular nitric oxide assay was performed using plasma-free whole blood samples as previously reported by Schachnik et al. (2009). Plasma-free whole blood was resuspended to 1×10^7 leukocytes/mL in PBS 2%BSA. Fifty microliters of leukocytes were pre-incubated with *T. cruzi* soluble antigen preparation at final concentration of 25 μg/mL at 37 °C for 2 h in a 5% CO_2 humidified incubator. Following, samples were incubated in the presence of 4,5-diaminofluorescein-diacetate (DAF-2DA) (Sigma, MO, USA) (final concentrations of 2.0 μM) at 37 °C for 180 min in a 5% CO_2 humidified incubator. The samples were labeled on ice bath for 20 min in the presence of 1 μL of TC-labeled anti-CD14 mAbs (clone M5E2). The cells were lysed, washed, the supernatant discarded and the cell pellet gently resuspended with 200 μL of PBS for immediate flow cytometer acquisition. A total of 50,000 events/tube were acquired using the CELLQuest™ software. Negative and Positive controls were performed by pre-incubation of leukocytes samples for 60 min without or with NO inducer (LPS 10 μg/mL, Sigma, USA) or 10 min pre-incubation with NO inhibitors (Nω-nitro-L-arginine

methyl ester 10 mM and aminoguanidine 10 mM-Sigma, Mo, USA) at 37 °C in a 5% CO_2 humidified incubator (data not shown).

Cytokine network analysis

Frequency of low and high cytokine-producers

The analysis of “low” and “high” cytokine-producers was performed as previously described by Luiza-Silva et al. (2011). Briefly, the whole data set of cytokine+ cells from IND, CARD, IND_T, $CARD_T$ and NI were first taken together to calculate the global median value for each cytokine+ leukocyte subpopulations (neutrophils, monocytes and lymphocytes). The global median values were used as the cut-off to categorize each subject as “low” or “high” cytokine-producers as they present levels of cytokine+ cells below or above the global cut-off medium. Following categorization of each individual sample, the frequency of “low” and “high” producers was calculated for each clinical group (IND, CARD, IND_T, $CARD_T$ and NI). According with the predominance of “low” or “high” producers (>50% of subjects) the group was finally referred as “LOW” or “HIGH” cytokine producers.

Biomarkers networks assembling

Biomarkers networks were assembled to assess the association between the cytokine+ leukocyte subpopulations (neutrophils, monocytes and lymphocytes), for each clinical group. Significant correlations representing the interactivity between biomarkers tested were compiled using the open source software, Cytoscape (version 2.8) as previously reported by Shannon et al. (2003). The biomarkers networks were constructed using circle layouts with each biomarker represented by specific gray-scale globular nodes (NI = ○ , ◌ ; IND/IND_T = ● , ◍ and CARD/$CARD_T$ = ● , ◉) to highlight predominant “LOW” (◌ , ◍ , ◉) and “HIGH” (= ○ , ● , ●) producers. Connecting edges display underscore moderate (—) and strong (▬) as proposed by Taylor (1990).

Statistical analysis

Differences between groups were first evaluated by Minitab software (Minitab Inc., PA, USA) to test three hypotheses: independence, normality and variance of data sets. All data sets showed parametric distributions and were first compared by One-way analysis of variance (ANOVA) followed by Tukey post-test. Cytokine correlations used to construct the biomarkers networks were generated by Pearson correlation analysis. Significance was always considered at $p \leq 0.05$ and highlighted by connecting lines for differences between IND *versus* IND_T or CARD *versus* CARD $_T$ and by * means for differences in comparison to NI. The correlation index (r) was used to categorize the correlation strength as low ($r > 0.35$), moderate ($0.36 > r < 0.67$) and strong ($r > 0.68$) as proposed by Taylor (1990). The Graphpad prism 5.00 software (San Diego, USA) was used for all statistical analysis and graph arts.

Results

Bz-treatment promotes a down-regulation of IL-17, IFN-γ and IL-10 synthesis by neutrophils in $CARD_T$

Aiming to determine the impact of Bz-treatment on the functional features of circulating phagocytic cells from chronic Chagas diseases patients, we characterized the phagocytic capacity and the intracytoplasmic cytokine pattern of circulating neutrophils following short-term *in vitro* cultures of whole blood samples in the presence of fluorescent live *T. cruzi*. Our data demonstrated that the neutrophils from Bz-treatment IND and CARD patients (IND_T and $CARD_T$) displayed similar *T. cruzi* phagocytic capacity as compared to non-Bz-treatment individuals (IND and CARD) and non-infected individuals (Fig. 1A). However, the higher levels of IL-17+, IFN-γ+ and IL-10+ neutrophils observed in CARD when compared to NI were observed to be down-regulated in $CARD_T$, returning to baseline pattern of that observed in NI as well as IND and IND_T. No differences were observed in the expression of IL-4 amongst the groups evaluated (Fig. 1B).

Bz-treatment down-regulates the monocyte-phagocytic capacity in IND, but does not impact their NO synthesis or the cytokine profile

The phagocytic capacity, the nitric oxide (NO) synthesis and the cytokine pattern of circulating monocyte were investigated in IND_T when compared to IND and NI. Our findings show that monocytes from IND_T patients display a down-regulated phagocytic capacity as compared to IND and NI (Fig. 2A). However, no changes on the levels of pro-inflammatory (IL-12 and TNF-α) and regulatory (IL-10 and TGF-β) cytokines were observed in IND_T when compared to all other groups evaluated (Fig. 2B). No changes were observed in the nitric oxide (NO) synthesis by monocytes amongst the groups evaluated (Fig. 2C). Analysis of FC-γR and TLR-2 by monocytes did not show any differences between, IND, IND_T and NI (Fig. 3A and B). Despite the lower expression of complement receptor CD35 observed in IND, the Bz-treatment restored this biomarker expression to a baseline profile observed in NI. The analysis of TLR-4 demonstrated Bz-treatment was not able to alter the down regulated expression of this cell surface marker since IND and IND_T presented lower levels of expression when compared to NI (Fig. 3C).

$CARD_T$ show an up-regulated expression of Fcγ-RI/III and TLR-4 supporting their ability to produce IL-10 and TGF-β

Our results demonstrated that monocytes from $CARD_T$ presented a baseline phagocytic capacity as observed in NI, CARD and IND. However, an overall up-regulated profile to produce regulatory cytokines (IL-10 and TGF-β) was observed in monocytes from $CARD_T$ as compared to CARD and NI. No changes on the levels of pro-inflammatory cytokine (IL-12 and TNF-α) was observed amongst the groups evaluated (Fig. 2C).

We have also investigated the expression of Fc-γ receptors, complement receptor (CD35) and Toll-like receptors (TLR-2 and TLR-4) by monocytes in CARD and $CARD_T$. Our data showed that monocytes from $CARD_T$ presented higher expression of CD16, CD64 (Fig. 3A) and TLR-4 (Fig. 3C) when compared to CARD and NI. No changes on the expression of TLR-2 by monocytes was observed between, CARD, $CARD_T$ and NI (Fig. 3B).

An overall down-regulation of lymphocyte-derived cytokine was observed in IND_T whereas up-regulated/balanced cytokine was the profile hallmark of lymphocytes from $CARD_T$

Aiming to evaluate the impact of Bz-treatment in the cytokine profile of circulating lymphocytes we have further characterized the intracytoplasmic expression of IL-17, IFN-γ, IL-4 and IL-10 in peripheral blood lymphocytes from IND, IND_T, CARD and $CARD_T$ individuals, following short-term *in vitro* live *T. cruzi* stimuli using NI as a control group. Data analysis demonstrated that IND_T presented a significant global lower frequency of lymphocytes-derived cytokines, whereas $CARD_T$ showed overall up-regulation of the cytokines evaluated, with a simultaneous gain of IL-17, IFN-γ, IL-4 and IL-10 supporting the establishment of a balanced lymphocytes-mediated microenvironment in these individuals (Table 1).

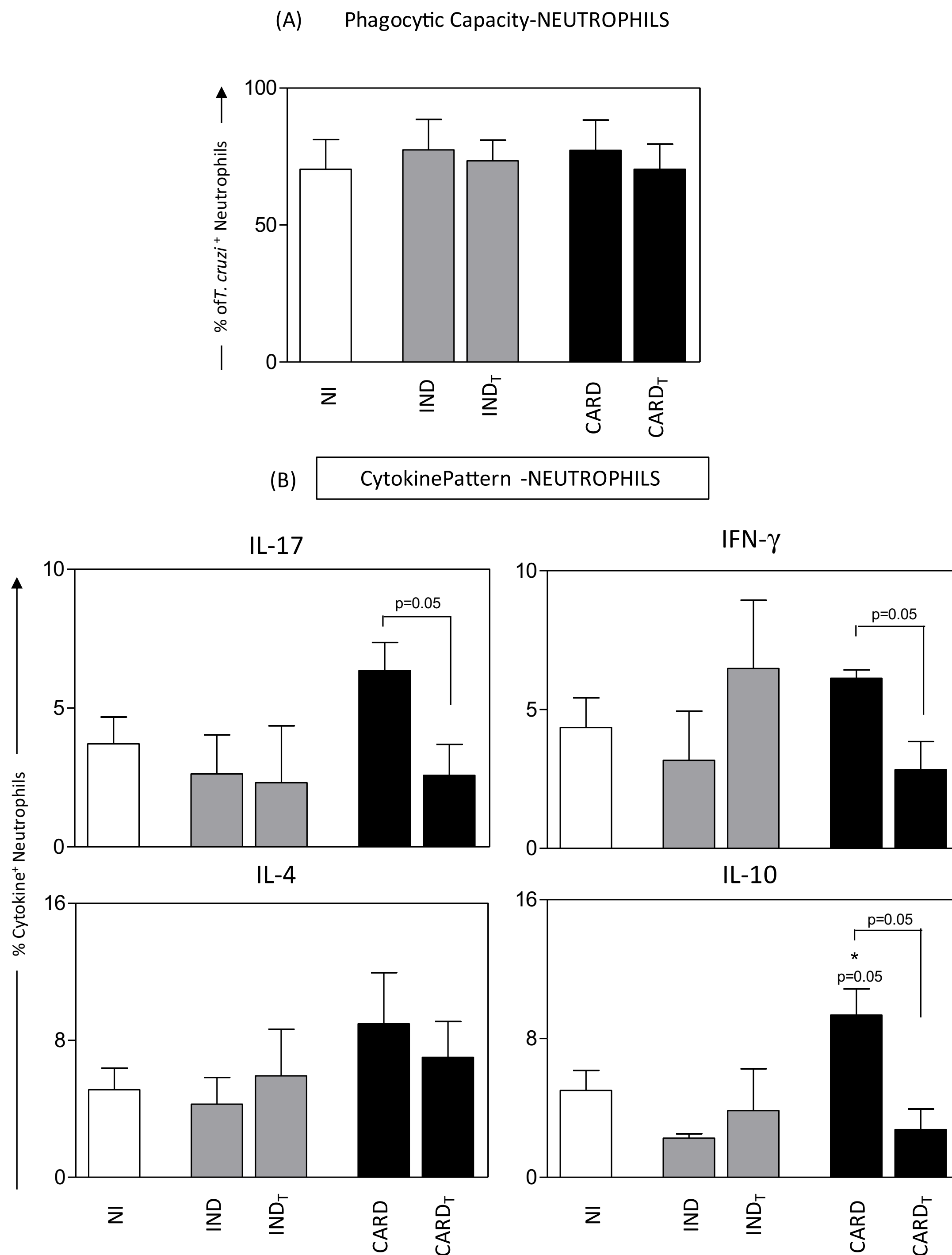

Fig. 1. Impact of benznidazole (Bz) treatment on functional features of peripheral blood NEUTROPHILS from patients presenting distinct clinical forms of Chagas disease. Flow cytometric approaches were performed to determine the phagocytic capacity (A) and IL-17, IFN-γ, IL-4 and IL-10 cytokine pattern (B) of circulating neutrophils from indeterminate and cardiac Chagas disease patients before and one year after the end of the etiological treatment (IND and IND$_T$ = ; CARD and CARD$_T$ =) as compared to non-infected individuals (NI =). Whole blood samples were incubated with fluorescent live *T. cruzi* organisms and the results expressed as mean percentage ± standard error (SE) of *T. cruzi*+ neutrophils and cytokine+ neutrophils, respectively. Significance was considered at $p \leq 0.05$ and highlighted by connecting lines for differences between IND *IND$_T$ or CARD versus* CARD$_T$ and by * means for differences in comparison to NI.

Treatment with Bz induces distinct changes in the Biomarkers networks of IND and CARD patients

The use of comprehensive approaches to explore the relationships between groups of leukocyte-derived cytokines likely to be related to disease morbidity as well as immunological rearrangements triggered by the Bz-treatment may contribute to better understand the complex connectivity among several mediators involved in the immune response of Chagas disease patients. In this study, we have applied an exploratory biomarkers network model to evaluate the relationship between pro-inflammatory/regulatory cytokines and their cell sources. The circle structure of the networks using specific gray-scale globular nodes was chosen to highlight the total number of possible edges between all pairs of

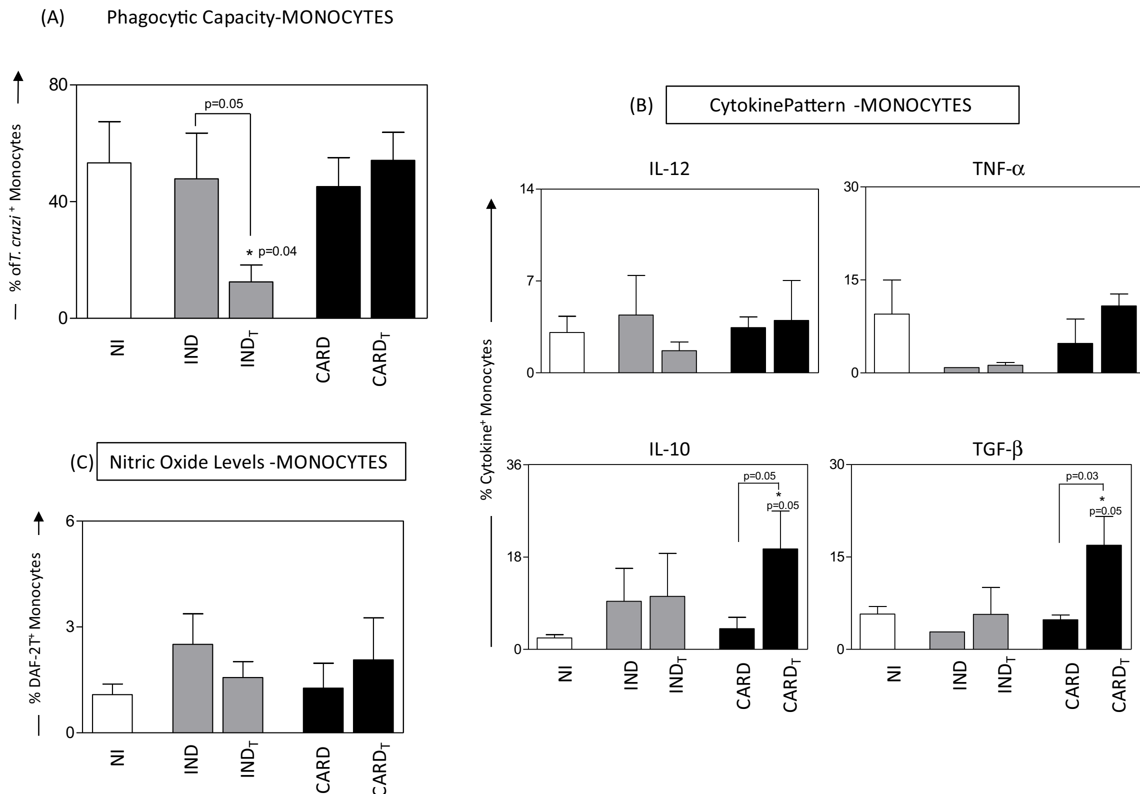

Fig. 2. Impact of benznidazole (Bz) treatment on functional features of peripheral blood MONOCYTES from patients presenting distinct clinical forms of Chagas disease. Flow cytometric approaches were performed to determine the phagocytic capacity (A), intracytoplasmic levels of nitric oxide (B) and IL-12, TNF-α, IL-10 and TGF-β cytokine pattern (C) of circulating monocytes from indeterminate and cardiac Chagas disease patients before and one year after the end of the etiological treatment (IND and IND_T = ▭ ; CARD and $CARD_T$ = ▬) as compared to non-infected individuals (NI = ▭). Whole blood samples were incubated with fluorescent live *T. cruzi* organisms and the results expressed as mean percentage ± standard error (SE) of *T. cruzi*$^+$ monocytes, DAF-2T$^+$ and cytokine$^+$ monocytes, respectively. Significance was considered at $p \leq 0.05$ and highlighted by connecting lines for differences between IND *versus* IND_T or CARD *versus* $CARD_T$ and by * means for differences in comparison to NI.

nodes observed for each clinical group. The edges density was normalized to identify moderate and strong correlations indexes (Fig. 4).

In the group of non-infected individuals (NI), it was evident that NEU-derived cytokines represent the most relevant pole of HIGH producers ( ), whereas the LYM-derived cytokines stand for the major pole of LOW producers ( ). Interestingly, the NEU ↔ LYM axes together with TGF-β$^+$MON and IL-12$^+$MON presented relatively strong connectivity (Fig. 4).

In the group of patients with indeterminate Chagas disease (IND) there is a shift of HIGH producers from NEU ( ) toward LYM ( ). Moreover, it its remarkable the presence of an overall multifaceted network with more and stronger connectivity amongst the NEU ↔ LYM axes along with strengthening of TGF-β$^+$MON and IL-12$^+$MON connection when compared to NI (Fig. 4).

In contrast, the patterns of connections between host biomarkers seen in the group of patients with cardiac Chagas disease were dramatically diverse than that observed in IND. The NEU axes represent a pole of HIGH cytokine producers ( ) whereas IL-10$^+$MON and IL-10$^+$LYM represent relevant LOW producers node ( ). In general, the CARD displayed a substantial loss in the number of significant interactions in the biomarkers network with great impact on the NEU ↔ LYM-derived IL-17 axis with the weakening of TGF-β$^+$MON and IL-12$^+$MON connection as compared to NI and IND (Fig. 4).

Upon Bz-treatment, besides the fact that the IND_T group become LOW producer of most cytokines ( ), substantial loss in the number of significant interactions between biomarkers, but preserve the IL-10$^+$NEU ↔ LYM edges along with the strengthening of NEU ↔ MON axis (Fig. 4).

Table 1
Cytokine pattern of lymphocytes after *in vitro* incubation of whole blood samples with live *T. cruzi* organisms.

Group	Frequency of cytokine$^+$ lymphocytes[a]			
	IL-17$^+$	IFN-γ$^+$	IL-4$^+$	IL-10$^+$
NI	8.8 ± 3.1	7.6 ± 4.5	9.3 ± 2.8	6.1 ± 2.7
IND	*14.1 ± 9.9*	*30.7 ± 25.0*	*15.2 ± 13.8*	*21.2 ± 21.0*
IND_T	10.5 ± 8.1	4.2 ± 3.1	5.2 ± 1.4	5.5 ± 1.8
CARD	6.8 ± 5.5	6.1 ± 3.2	5.3 ± 2.3	6.4 ± 4.3
$CARD_T$	*13.2 ± 6.2*	*16.9 ± 6.8*	*12.5 ± 6.1*	*9.8 ± 4.0*

[a] Data are presented in per thousand (‰) of cytokine$^+$ lymphocytes. Statistical differences ($p \leq 0.05$) are highlighted in italic format to underscore the similarity between $CARD_T$ and IND in comparison to the other groups. NI = non-infected individuals; IND = untreated indeterminate clinical form; IND_T = Bz-treated indeterminate clinical form; CARD = untreated cardiac clinical form; $CARD_T$ = Bz-treated cardiac clinical form.

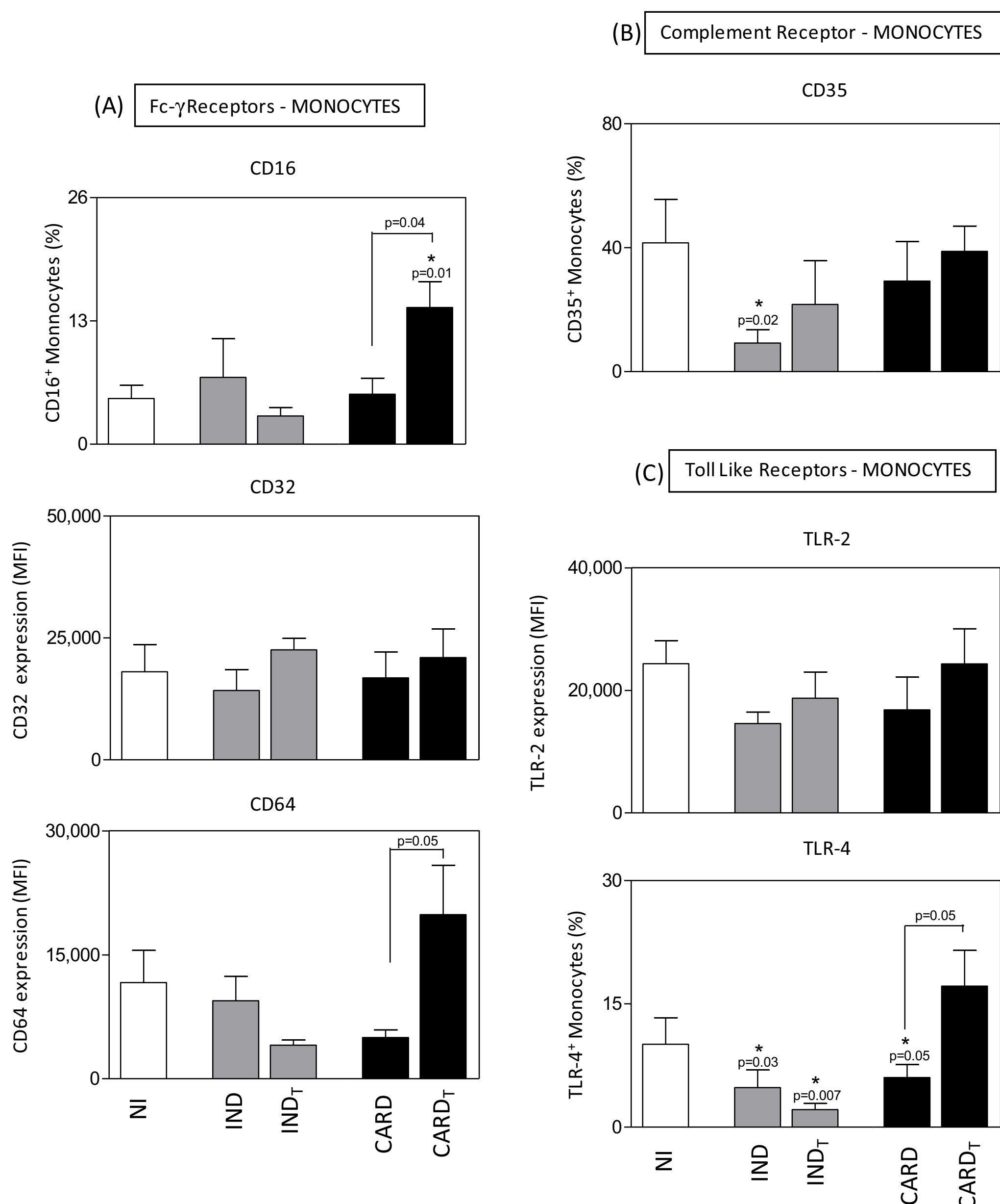

Fig. 3. Impact of benznidazole (Bz) treatment on phenotypic features of peripheral blood MONOCYTES from patients presenting distinct clinical forms of Chagas disease. Flow cytometric approaches were performed to determine the expression of FC-γR – CD16, CD32 and CD64 (A), complement receptor CD35 (B) and TLR-2 and TLR-4 (C) of circulating monocytes from indeterminate and cardiac Chagas disease patients before and one year after the end of the etiological treatment (IND and IND_T = ; CARD and $CARD_T$ =) as compared to non-infected individuals (NI =). Whole blood samples were incubated in the absence of exogenous stimuli and the results expressed as mean percentage ± standard error (SE) of CD16⁺, CD35⁺ and TLR-4⁺ monocytes and average ± SE of mean fluorescence intensity of CD32, CD64 and TLR-2 expression by monocytes. Significance was considered at $p \leq 0.05$ and highlighted by connecting lines for differences between CARD *versus* $CARD_T$ and by * means for differences in comparison to NI.

In Bz-treated cardiac patients it is clear that the NEU axis become a LOW cytokine producers pole ( ) whereas MON and LYM axes assume the HIGH producers mediators ( ). It the $CARD_T$ biomarker network, it was evident the strengthened of NEU ↔ LYM axis as well as the restoration of the TGF-β⁺MON and IL-12⁺MON connection as observed in NI, IND and IND_T (Fig. 4).

Bz-treatment shifted the baseline cytokine network of IND toward a pivotal role for IL-10⁺ monocytes in IND_T and enhances the connections of CARD toward an integrated/balanced cytokine microenvironment observed in $CARD_T$

Extensive research has been done to identify the most relevant biomarkers associated with Chagas disease morbidity and also to recognize those with significant contributions to the understanding of the impact of Bz-treatment on patients presenting distinct clinical forms of the chronic disease. We have selected a set of biomarkers axes of pro-inflammatory/regulatory cytokines and their cell sources that are closely related to Chagas disease clinical forms and those selectively observed in Bz-treatment.

First, we have determined the "Chagas disease related changes in the cytokine network" by selecting those axes observed in IND and CARD but not in the NI group (Fig. 5). The diagram presented in Fig. 5A demonstrated that IND presents a complex and interrelated cytokine network where the pro-inflammatory IFN-γ⁺LYM and regulatory IL-10⁺LYM poles present several links with pro-inflammatory/regulatory NEU and MON. On the other hand, in

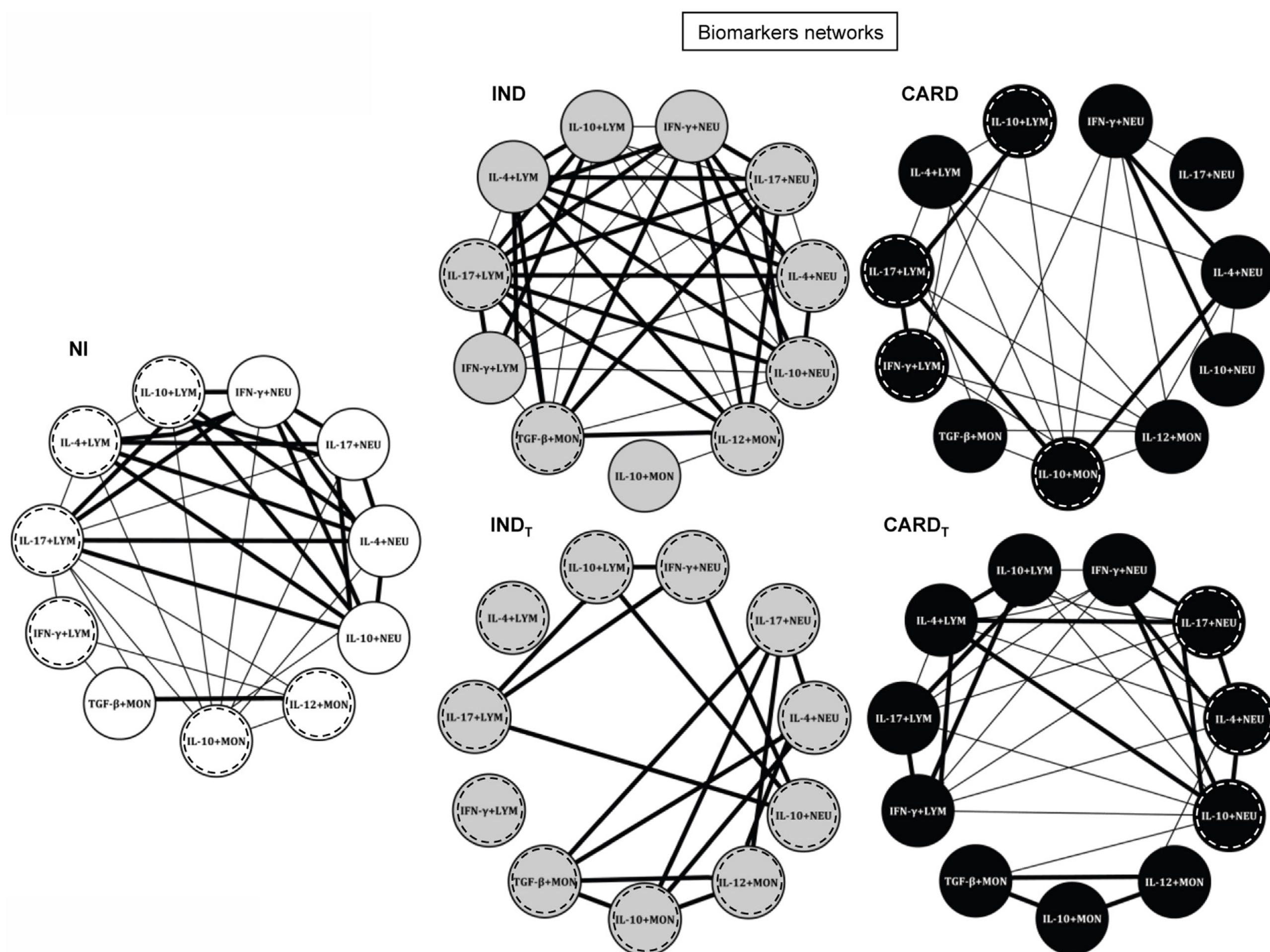

Fig. 4. Networks of leukocyte-derived cytokines associated with Chagas disease morbidity and the immunological rearranges triggered by the Bz-treatment. Biomarkers networks were assembled to assess the association between the cytokine+ leukocyte subpopulations (neutrophils, monocytes and lymphocytes), for each clinical group. Circle layouts underscore biomarkers by gray-scale globular nodes (NI = ◯, ◌; IND/IND_T = ●, ◌ and CARD/$CARD_T$ = ●, ◌) to highlight predominant "LOW" (◌, ◌, ◌) and "HIGH" (= ◯, ●, ●) producers. Significant correlations were compiled as previously reported by Shannon et al. (2003). Connecting edges display underscore moderate (—) and strong (━) as proposed by Taylor (1990).

CARD patients the pro-inflammatory IFN-γ^+LYM and regulatory IL-4^+LYM poles are set apart with no intrinsic association (Fig. 5B).

Network analysis is also a potential tool to identify the interactions between immunological mediators impacted by the Bz-treatment in order to point out beneficial aspects to the host immune response. As observed in Fig. 5C, although the number of significant interactions and the overall complexity of networks were systematically reduced in IND_T, these patients built relatively balanced cytokine connections mediated by NEU and MON with a pivotal role of IL-10^+MON.

Nevertheless, it was observed that in $CARD_T$ there was a clear expansion of connections toward the participation of NEU with the establishment of a complex, intricate cytokine network with pro-inflammatory IFN-γ^+LYM and regulatory IL-10^+LYM poles presenting several links with pro-inflammatory/regulatory NEU in a balanced lymphocyte-mediated microenvironment similar to that observed in IND (Fig. 5D).

Discussion

The etiological treatment of Chagas disease with benznidazole (Bz) has been shown to be effective for parasite clearance during acute infection with lower effectiveness during the chronic stage of the disease. Therefore, the use of Bz-treatment during long-lasting chronic Chagas disease is still controversial. Previous studies, supported by clinical non-randomized trials, have demonstrated that the Bz-treatment is associated with reduced disease progression and increased sero-conversion in Chagas disease patients presenting non-acute disease and no cardiac manifestations (Marin-Neto et al. 2009; Viotti et al. 2006). Supporting these findings, our group has demonstrated that Bz-treatment triggers changes on the host immune response leading to a relevant pro-inflammatory profile in NK-cells and CD8+ T-lymphocytes keeping the IL-10-modulated profile in IND form (Sathler-Avelar et al. 2006; Sathler-Avelar et al. 2006, 2008), re-inforcing the proposal of the beneficial impact of Bz-therapy for indeterminate Chagas disease patients. Currently, there is a large-scale randomized controlled study to provide evidences that support the recommendation of trypanocide therapy for the improvement of the clinical course of patients with chronic Chagas heart disease (Marin-Neto et al. 2008; Marin-Neto et al. 2008, 2009; Reyes and Vallejo 2005). Preliminary results have shown that Bz-therapy significantly reduces parasite burden, as assessed by polymerase chain reaction and also showed that treatment in the chronic phase is safe and well tolerated by Chagas disease population (Marin-Neto et al. 2009). A full-scale study to determine whether anti-trypanosomal therapy with Bz promotes relevant changes in the CARD immunological profile still remains to be performed.

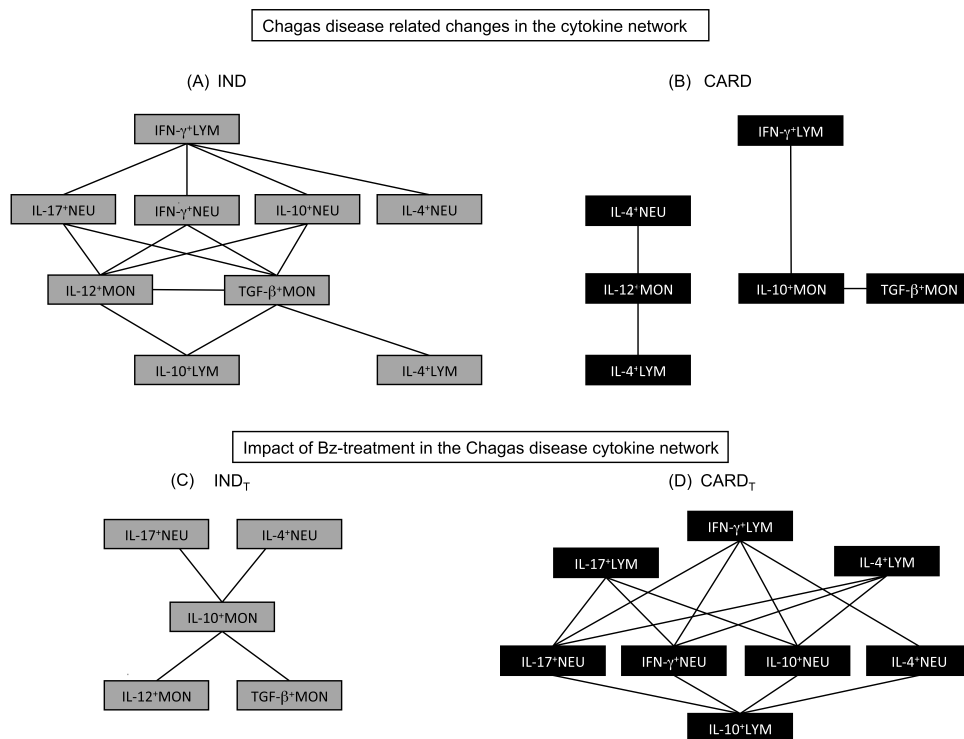

Fig. 5. Exploratory model of biomarkers network of candidate pro-inflammatory/regulatory cytokines and their cell sources in the context of the natural history of Chagas disease and upon Bz-treatment. Selected axes between pro-inflammatory/regulatory cytokines and their cell sources that are closely related to Chagas disease clinical forms IND (A) and CARD (B) as well as those selectively observed following Bz-treatment in IND_T (C) and $CARD_T$ (D) were assembled. This summary model underscores the complexity of the baseline cytokine network observed in IND, highlighting the dissimilarity with the two isolated sets without intrinsic association observed in CARD. The impact of Bz-treatment on the baseline patterns point out a pivotal role for IL-10$^+$ monocytes in IND_T and the enhanced connections of $CARD_T$ toward an integrated/balanced cytokine microenvironment.

In this work, we present an analysis of the impact of BZ-treatment on the functional and phenotypic features and cytokines networks of peripheral blood leukocytes from patients with the IND and CARD clinical forms of Chagas disease. We observed that Bz-therapy promoted a relevant shift in the phenotypic/functional features of host leading to an overall immunomodulated profile in IND and a rearrangement of the immunological network in CARD that could be relevant to the establishment of a beneficial/non-deleterious immunological microenvironment during long-lasting chronic *T. cruzi* infection.

In this context, our data demonstrated that after Bz-therapy an overall low immune activation was observed in individuals with the IND clinical form of Chagas disease that is reflected on the down-regulated phagocytic capacity, decrease of the FCγ-R (CD16, CD64) and TLR-4 expression in circulating leukocytes, lower levels of NO and most cytokines evaluated. It is possible that in these patients the Bz therapy leads to changes on neutrophils and macrophages phagocytosis, and therefore the expression of molecules involved on antigen presentation, cytokines and NO production, therefore contributing to prevent the disease progression. In fact, our findings are in agreement with this hypothesis and previous reports demonstrating the immunomodulatory effect of Bz treatment in experimental models (Bustamante et al. 2008; Piaggio et al. 2001; Revelli et al. 1999) and humans during the chronic phase (Sathler-Avelar et al. 2006; Sathler-Avelar et al. 2006, 2012).

We also evaluated, for the first time, the density and complexity of the network of interactions between cytokines and their cells source in association with the host immune responses and their relationship with Bz-therapy in the context of the disease clinical outcomes. We observed that IND displayed fine-tuned interactions involving the cytokine network that evidences a protective role of anti-inflammatory mediators through a modulatory effect on IFN-γ, emphasizing the relevance of these phagocytes on the establishment of a balanced cytokine microenvironment. These results strength our finds (Sathler-Avelar et al. 2006; Sathler-Avelar et al. 2006, 2012; Gomes et al. 2003; Sousa et al. 2014) and the other groups (Abel et al. 2001; Souza et al. 2007) showing the complex regulatory mechanism behind the control and/or the development of the pathology induced by *T. cruzi* infection. It also suggests that modulation of macrophages activity and of regulatory T cells producing IL-10 may maintain the balance between parasitism and tissue integrity in IND (Gomes et al. 2003; de Araújo et al. 2012). In addition it also suggests that the Th1 response observed in these patients may maintain parasitism under relative control. We observed that after Bz-therapy a substantial loss on the number of significant interactions between different markers occur, however, IL-10 production is preserved, suggesting that this molecule may be capable of mediating modulated protective immunity. This data reinforce our previous findings that IL-10 secretion may also represent an effective event of modulatory impact to allow parasite clearance in the absence of deleterious tissue damage during chemotherapy (Gomes et al. 2003; de Araújo et al. 2012). In fact, an association between lower expression of IL-10 and the worse heart function of Chagas disease cardiac form was demonstrated (Costa et al. 2009; Revelli et al. 1999). Although interesting, a role of a direct modulatory link between IL-10, IL-17, IL-4 and TGF-β with pro-inflammatory status in chagasic patients is yet unexplored mechanistically and deserves further investigation. In fact, it has been demonstrated that an exacerbated production of inflammatory cytokine such as IFN-γ and absence and/or reduced modulatory cytokines production may favor the cytolytic effects of leucocytes during Chagas disease increasing the chances of myocellular destruction of heart tissue (Gomes et al. 2003; Sousa et al. 2014; Souza et al. 2007). Extensive research has been done on the

identification of factors/biomarkers associated with development and/or control of cardiomyopathy, with significant contributions to the understanding of the disease pathogenesis.

We observed that Bz-treatment induces extensive modifications on the immunological profile of cardiac individuals. Despite no changes in the phagocytic capacity by neutrophils and monocytes, Bz-treatment leads to lower levels IFN-γ and high levels of IL-10 in $CARD_T$. The higher expression of CD16, CD64 and TLR-4 observed in $CARD_T$ may support the hypothesis that up-regulated expression of these molecules would be a relevant event in driving the ability of monocytes from $CARD_T$ to produce IL-10 and TGF-β. A major observation from the analysis of the cardiac group after Bz-therapy was that the overall complexity of the networks, as determined by the number of significant interactions, was similar to that observed in IND before treatment. These findings further support the hypothesis that there is a fine-tuning between all the mediators with major relevance to prevent the clinical progression of heart disease, as reported in other studies (Viotti et al. 2004; Viotti et al. 2004, 2006; Espinosa et al. 1991; Bestetti et al. 1994). In this context, Bz treatment not only may interrupt parasite replication but also induce new regulatory mechanisms, thus triggering a different host immune response (Laucella et al. 2009).

In Chagas disease, analysis of immunity pre- and post-treatment is essential for both understanding of the mechanisms of disease progression and the impact of benznidazole on the immune response. A better understanding of these factors will also aid on patient management as well as on the development of a rational for new trypanocidal agents (Dutra et al. 1996; Laucella et al. 2004; Bahia-Oliveira et al. 2000). In the last decades, the literature has accumulated evidence that correlates immune response and chemotherapy efficacy (Bonney 2014).

Conclusion

Our findings highlighted that besides contributing to an overall immunomodulation in IND, the Bz-treatment induced changes in the immunological profile of CARD, inducing a complex phenotypic/functional pattern compatible with beneficial and protective immunological events. These findings reinforce the usefulness of BZ treatment and encourage further studies to elucidate the potential use of these immune mediators as biomarkers of disease progression and prognosis.

Conflict of interest

The authors declare that are no competing interests.

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Ethical approval

Written informed consent was obtained from patients for tissues and project was approved by the Ethics Committee of the Centro de Pesquisas René Rachou Belo Horizonte, Minas Gerais, Brazil (CPqRR/FIOCRUZ protocol 11/2004).

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