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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-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.
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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-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,

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 $600 \times g$ 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-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-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-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
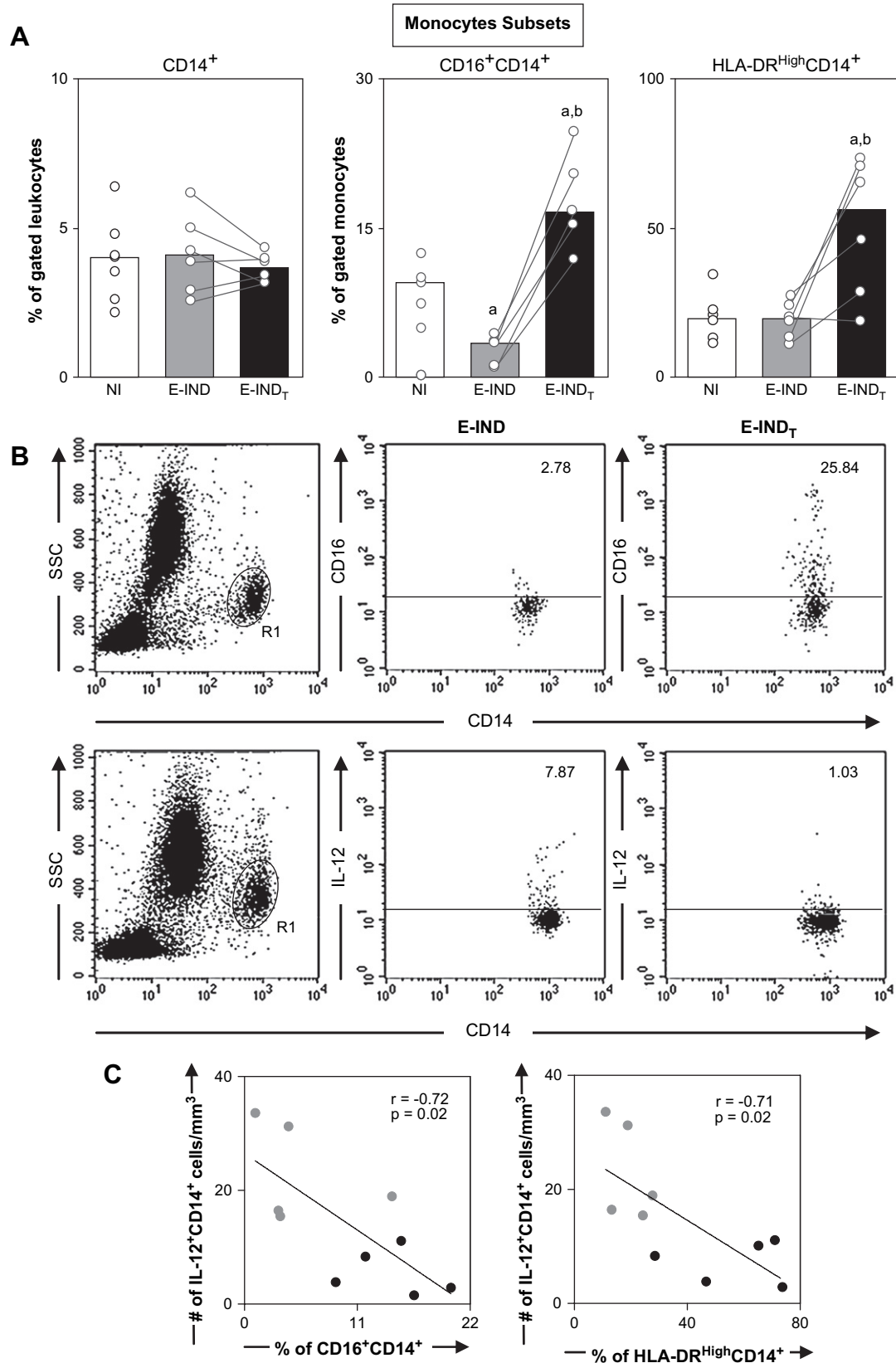
Fig. 1. (A) Analysis of monocyte subsets in the peripheral blood from non-infected children (NI ─☐) and early indeterminate *T. cruzi* infected patients prior to the Bz-treatment (E-IND ─▨) and one year after it (E-IND$_T$ ─■). Phenotypic studies were performed by a triple-labeling protocol using anti-CD16 FITC, anti-HLA-DR PE, and anti-CD14 TC to identify total monocytes CD14$^+$, macrophage-like monocytes CD16$^+$CD14$^+$, and pro-inflammatory monocytes HLA-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-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-12$^+$ CD14$^+$ cells after Bz-treatment.
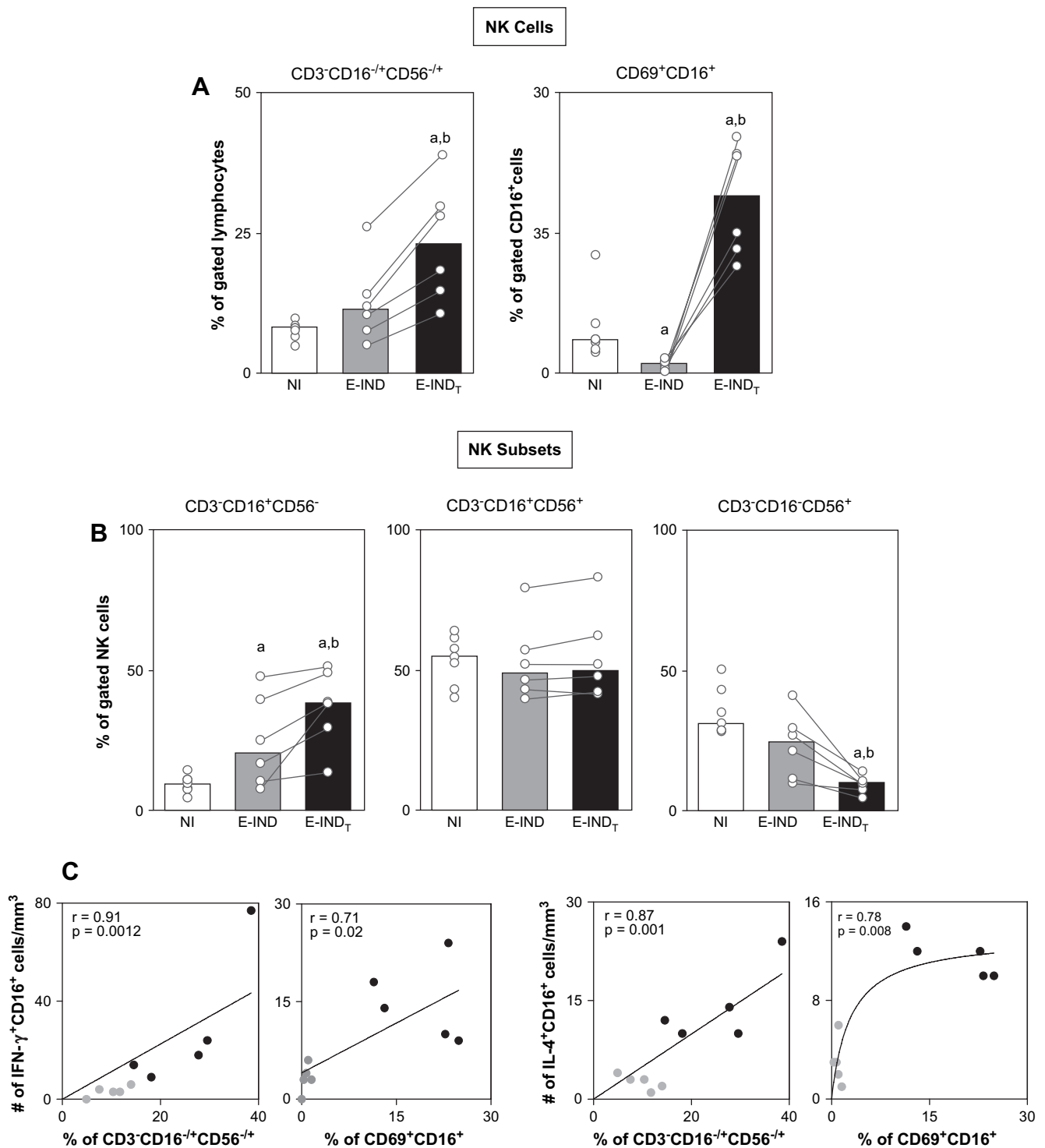
Fig. 2. (A) Analysis of total $CD3^-CD16^{-/+}CD56^{-/+}$ NK cells, $CD69^+CD16^+$ NK cells and (B) its subsets in the peripheral blood from non-infected children (NI — ☐) and early indeterminate *T. cruzi*-infected patients prior to the Bz-treatment (E-IND — ▨) and one year after it (E-IND$_T$ — ■). 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.

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

A

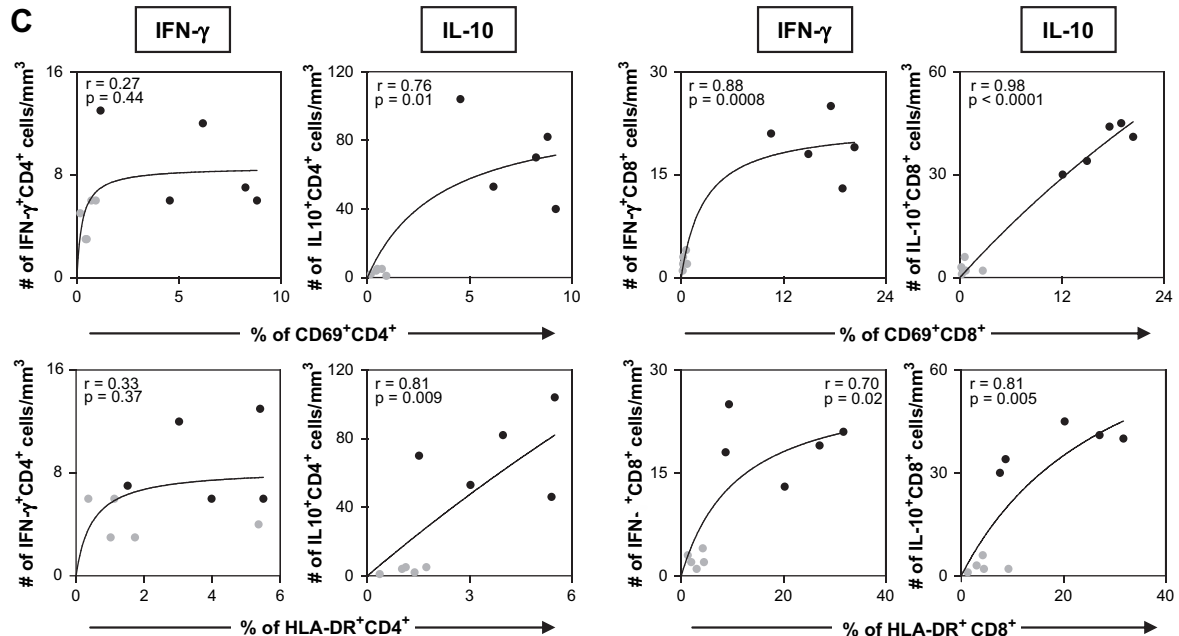| Cell phenotype %	Groups		
	NI (n=7)	E-IND (n=6)	E-IND$_T$ (n=6)
CD4	42.5±5.9	40.8±5.9	37.9±6.2
CD69$^+$CD4$^+$	1.5±0.7	0.9±0.9	**6.4±3.1**[a,b]
HLA-DR$^+$CD4$^+$	3,4±0.9	**1.9±2.0**[a]	**3.9±1.7**[b]
CD8	3.7±7.7	**23.2±3.4**[a]	**20.4±5.5**[a]
CD69$^+$CD8$^+$	3.8±4.1	**0.8±0.9**[a]	**15.7±3.9**[a,b]
HLA-DR$^+$ CD8$^+$	5.3±2.6	4.0±2.9	**17.5±10.4**[a,b]
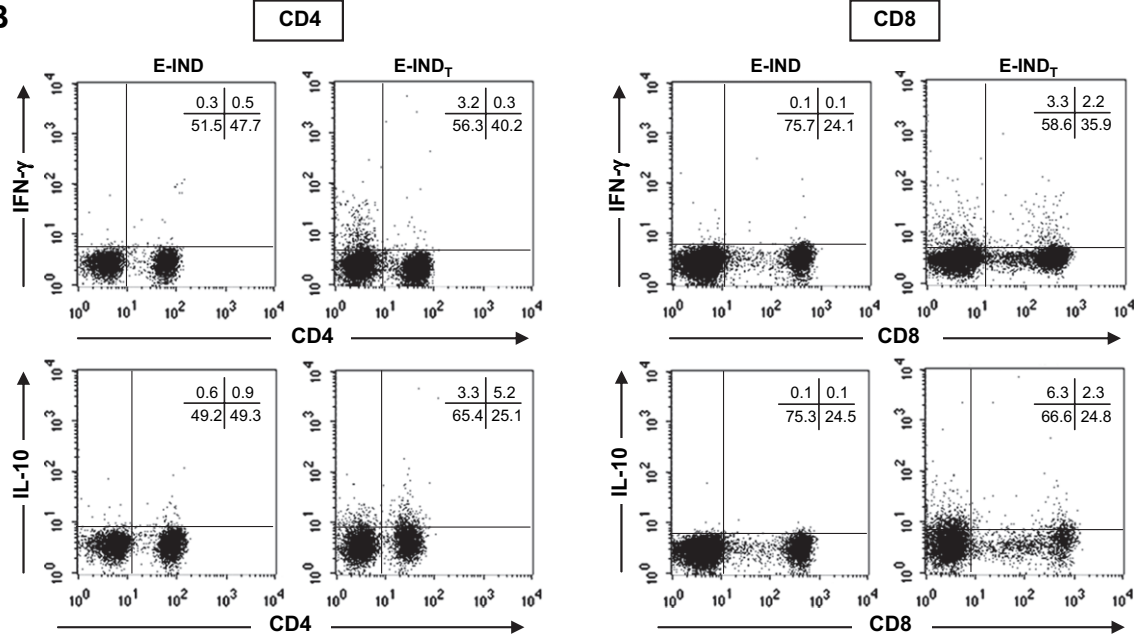
Fig. 3. (A) Activation status of T-lymphocytes subsets (CD4$^+$ and CD8$^+$) in the peripheral blood from non-infected children (NI) and early indeterminate *T. cruzi*-infected patients prior to the Bz-treatment (E-IND — ▢) and one year after it (E-IND$_T$ — ■). A double-labeling protocol was performed to identify percentage of CD69$^+$ and HLA-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-10$^+$CD4$^+$) after Bz-treatment; however, a positive correlation was observed between activated CD8$^+$T-lymphocytes subset and a mixed cytokine pattern (IFN-γ$^+$CD8$^+$ and IL-10$^+$CD8$^+$) in the E-IND$_T$ group.

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 benznidazole-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-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-α^+CD14^+ and IL-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 downregulation 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-γ^+ and IL-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
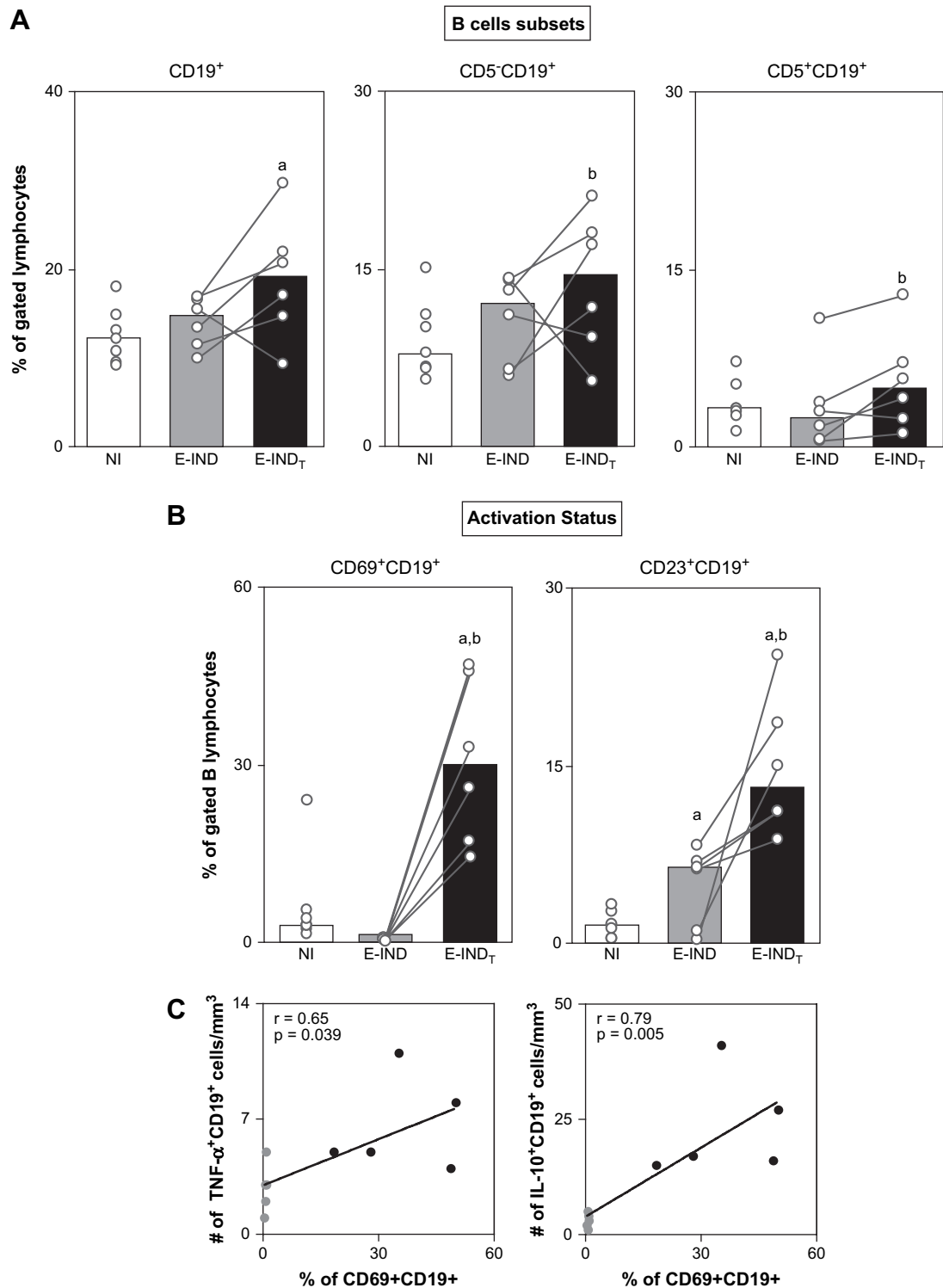
Fig. 4. (A) Percentage of B-cells (CD19^{+}), B-cell subsets (CD5^{−}CD19^{+} and CD5^{+}CD19^{+}) and (B) activation status (CD69^{+}CD19^{+} and C23^{+}CD19^{+}) in the peripheral blood from non-infected children (NI − ☐) and early indeterminate *T. cruzi*-infected patients prior to the Bz-treatment (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-α^{+}CD19^{+} and IL-10^{+}CD19^{+}) after Bz-treatment.

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 downregulation 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.

Acknowledgements

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