Benznidazole: Hero or villain of cellular immune response in chronic Chagas disease patients?

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ABSTRACT

Although the treatment of chronic Chagas disease (CCD) patients with Benznidazole (Bz) is still controversial, its use may prevent or delay the progression of the disease to the most severe forms. One of the main factors that can influence the effectiveness of the treatment is the possible cooperation between drug effect and the host immune response. Herein, we evaluated the immune response of peripheral blood mononuclear cells (PBMCs) infected with *Trypanosoma cruzi* and submitted to Bz treatment. Blood samples of CCD patients (n = 7) and non-infected individuals (n = 6) were drawn to obtain PBMCs. After cell culture, the supernatants were harvested and stored, and the cell analyzed by flow cytometer. The results showed that Bz positively regulated the molecular process of cell activation (CD80) and antigen presentation (HLA-DR), increased phagocytosis receptor and macrophage activation (CD64), and did not induce an exacerbated immune response. In conclusion, these results highlight the relevance of using Bz that, despite not being a true hero, it is also not a villain, as it presents a wide range of pharmacological/immunological response interactions, important for the immune balance in the clinical progression of CCD.

1. Introduction

Chagas disease (CD) is recognized by World Health Organization as one of 17 neglected tropical diseases, which affects about 6–7 million people in the world with less than 1% of the infected individuals receiving etiological treatment (Barrett and Croft, 2012; World Health Organization, 2015). In Brazil, the only available drug for CD therapy is Benznidazole (Bz), which efficacy is dependent on the disease phase, treatment period and the dose, age and geographical origin of the patient, and immunological profile (Coura and Castro, 2002). Evidence of attenuation in the course of myocarditis in animals infected with *T. cruzi*, as well as observational studies of individuals who showed negative serological tests and prevention of clinical and electrocardiographic changes, ratify the role of etiological treatment in controlling the course of the disease (Viotti et al., 1994; Cançado, 2002; Marin-Neto et al., 2008, 2009). However, studies evaluating patient post-treatment verified the failure of the medication to eliminate the parasite in the chronic phase, without interfering with disease progression (Lauria-Pires et al., 2000; Duffy et al., 2009; Aguiar et al., 2011).

Despite the controversies regarding Bz therapy, its use in cooperation with immune response can be an important factor in determining the treatment effectiveness (Albareda and Laucella, 2015; Cutrullis et al., 2011). Immunological markers are potential indicators of treatment response. Understanding these molecules associated with the treatment provides tools to monitor and predict the onset of treatment success

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allowing the development of new therapeutic approaches (Albareda and Laucella, 2015). Studies on the immune response of CD patients have shown that the host specific immune response to the parasite persistence in the tissues influences the clinical evolution of the chronic phase of the disease (Teixeira et al., 2002), which could be of great importance in association with the etiological treatment (Olivieri et al., 2002; Albareda and Laucella, 2015; Cutrullis et al., 2011). However, little is known about the real impact of Bz on the host immune response, and whether this immune response potentiates the action of this drug or not, or vice versa.

In view of these evidences, the present work aimed to evaluate the immune response of CCD patients submitted to Bz treatment. Understanding the effects of Bz on the immune response is of great importance for the development of new therapeutic strategies, enhancing or improving its effectiveness. In addition, we believe this work contributes to increase the knowledge about the events resulting from the etiological treatment with this drug, including the mechanisms involved in the parasite/host interaction that are associated with the antiparasitic drug.

2. Material and methods

2.1. Parasites

Parasites (trypomastigotes forms - Y strain) were distributed in tubes with incomplete RPMI 1640 medium (Sigma™) and cetrifuged at $400 \times g$ for 10 min at 22 °C. The pellet was ressuspended in RPMI 1640 medium (Sigma™) and 2% Fetal Bovine Serum (FBS). The cell suspension was incubated for 24 h, at 37 °C and 5% CO_2. Non-internalized parasites were removed and incubated for 7 days in RPMI 1640 medium (Sigma™) and 10% FBS.

2.2. Study population

Seven patients with CCD (INFEC group) were selected from the Ambulatory of Chagas Disease and Heart Failure of the Cardiology Emergency Facility of Pernambuco (PROCAPE) of the University Hospital Oswaldo Cruz (HUOC), University of Pernambuco (UPE), Recife – PE, Brazil. Inclusion criteria for INFEC group were: I – Seropositive for *T. cruzi;* II – Clinical exams characterizing clinical form according to the II Brazilian Consensus on Chagas Disease (Dias et al., 2016); III – Treatment-naïve; IV – No digestive complains such as dysphagia and/or constipation.

The INFEC group were classified as: indeterminate (n = 4) (no clinical symptoms or electrocardiographic findings) and cardiac (n = 3) (presence of electrocardiographic findings or mild echocardiographic disorders but without ventricular dysfunction).

Six non-infected patients (CN group) were assessed for control group. The inclusion criteria for CN group were: live in non-endemic regions; no history of blood transfusion; seronegative for *T. cruzi* antibodies. Two immunoenzymatic tests with different antigenic preparations were used for the confirmation of the chagasic infection: the first is a mixture of total extracts of *T. cruzi* adsorbed to the microtiter plate (Chagas test ELISA III) from Bioschile Ingenieria Genetica S. A.™ and the second uses Ag-Recs adsorbed to the plate (Imuno-ELISA Chagas) from Wama Diagnóstica™.

The individuals included in the study signed an informed consent form (ICF), which was previously approved by the Ethics Committee of the Institute Aggeu Magalhães-Fiocruz (IAM / Fiocruz) (CAEE: 07511612.2.0000.5190).

2.3. Blood samples

From each participant were collected 35 mL of blood in sodium heparin tubes (Vacutainer™) to obtain PBMCs. In addition, 5 mL of blood were collected in dry tubes to obtain serology confirmation of *T. cruzi.*

2.4. PBMC isolation

PBMC were isolated by density gradient centrifugation with Ficoll-Hypaque PLUSTM (Amersham Biosciences) according to Souza et al. (2004). PBMCs concentration was 10^6 cells/mL, however, for the assessment of monocytes, the used concentration was 2.5×10^4 monocytes/mL, based on the number of circulating monocytes.

2.5. PBMC/Trypomastigote/Benznidazole culture

PBMC were cultured in 48-well polystyrene plates with trypomastigotes and Bz as follows: well 1 - only adherent and non-adherent PBMC (C); well 2 - PBMC + trypomastigotes (C + T); well 3 - PBMC + trypomastigotes + Bz (C + T + Bz) and well 4 – PBMC + Bz (C + Bz). For cell adhesion, the cells were incubated for 1 h and the plate was shaken at 30 min intervals to avoid non-adherent cells precipitation. The supernatant containing the non-adherent cells was removed and deposited in previously identified conical bottom polypropylene tubes (BD FalconTM; 15 mL). Trypomastigotes were added at a proportion of 4:1 trypomastigotes/monocyte and incubated for 2 h. After the incubation, non-internalized parasites were removed and non-adherent cells were added to the wells and with Bz (1 μg/mL). The co-cultures were incubated for 24 h and 5 days (d).

2.6. Immunophenotyping

The cell suspension of PBMC was deposited in a polystyrene tube (BD Systems™), followed by washing with PBS-Wash (400 × g for 5 min). The surface monoclonal antibodies (anti-CD14 conjugated to FITC, 1.5 μl; anti-CD64 conjugated to PE, 5.0 μl; anti-CD16a conjugated to PE-CY7, 1.5 μl; anti-CD11b conjugated to APC, 1.5 μl; anti-CD32 conjugated to APC, 10.0 μl; anti-CD35 conjugated to PE, 10.0 μl; anti-HLA-DR conjugated to PerCP, 1.5 μl; anti-CD80 conjugated to PE, 1.5 μl and anti-CD86 conjugated to PE, 1.5 μl), were added to the tubes and incubated for 30 min at room temperature and protected from light. After incubation, the cells were washed with PBS-Wash (400 × g for 5 min). The cells were fixed with Cytofix solution (BD ™ Systems) and after washed with PBS-Wash (400 × g for 5 min), the cells were maintained at 4° C until analysis by flow cytometer FACScalibur (Beckton Dickson, USA).

2.7. Statistical analysis

Statistical analysis of PBMC profile data was performed using the PRISM 5.0 Windows® software (USA). To confirm the assumption of normality, the D'Agostino test was used. To compare the mean expression of surface molecules between the INFEC and CN groups, the Mann-Whitney test was used, since the sample did not meet the assumption of normality. The results between the experimental conditions were evaluated using the Wilcoxon test (for paired non-parametric samples). All statistical conclusions were taken at a significance level of 5%.

3. Results

3.1. Evaluation of the activation profile and antigen presentation of $CD14^+$ macrophages

The $CD14^+$ macrophages activation profile and antigens presentation were evaluated by the expression of HLA-DR, CD80, and CD86 surface molecules.

Neither significant differences were observed in the percentage of $CD14^+/HLA\text{-}DR^+$ cells when comparing the different culture conditions nor between 24 h and 5 days of culture in INFEC group (Fig. 1A and B). However, INFEC group showed an increased percentage of CD14 + HLA-DR + cells in the C + T + Bz condition compared to the CN group at 24 h (p = 0.0081) (Fig. 1A). The same phenomenon was observed at the 5d in the conditions C + T + Bz (p = 0.0087) and C + Bz (p = 0.0159)
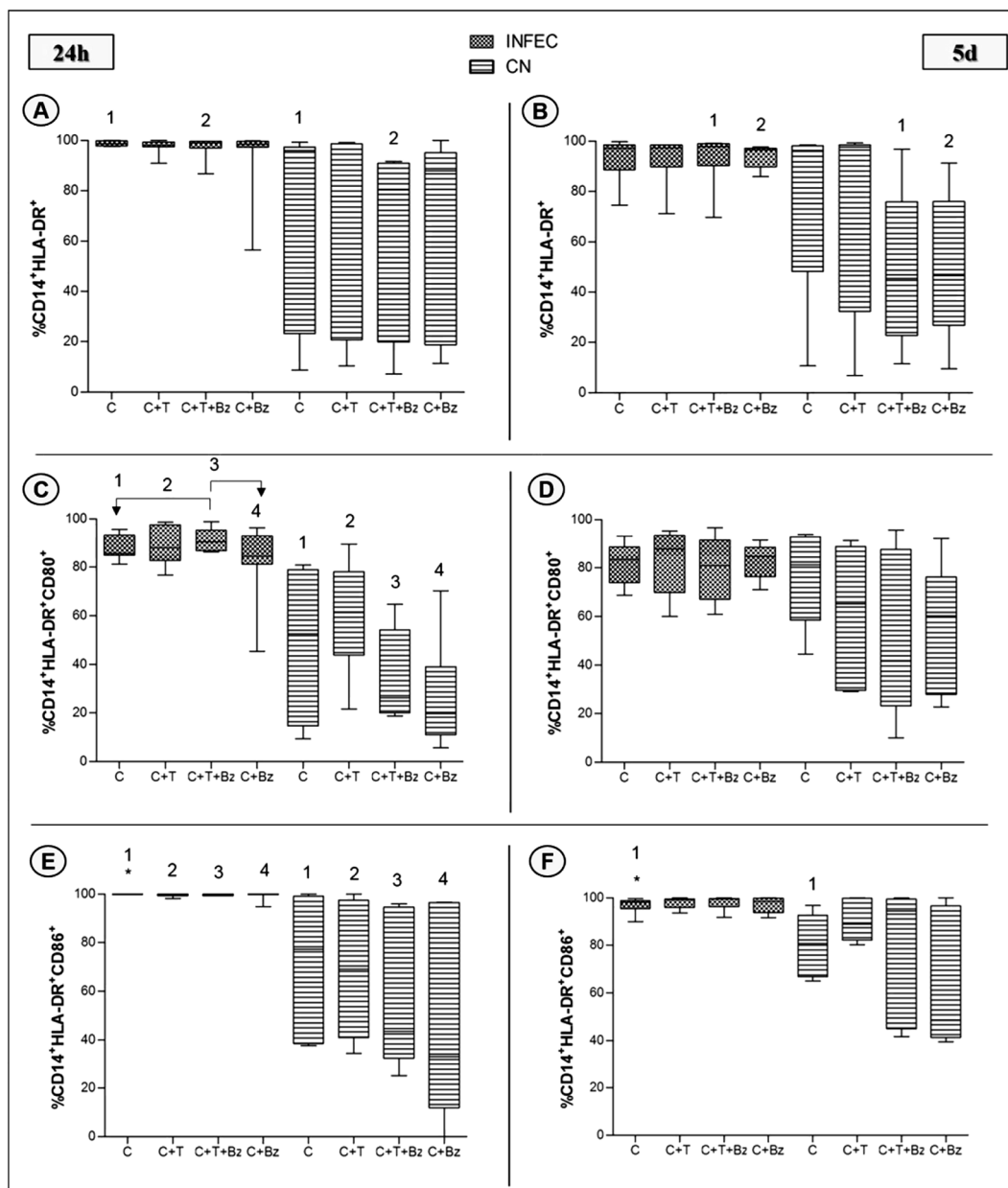
Fig. 1. Comparison of the percentage of CD14$^+$HLA-DR$^+$, CD14$^+$ HLA-DR$^+$CD80$^+$, CD14$^+$HLA-DR$^+$CD86$^+$ cells between the INFEC and the CN groups at 24 h and five day. Legend - INFEC - patients (n = 7); NC - uninfected individuals (n = 6). C – PBMC; C + T – PMBC and trypomastigotes; C + T + Bz – PBMC, trypomastigotes and Bz; C + Bz – PBMC and Bz. (A) % of CD14$^+$HLA-DR$^+$ in the INFEC and the CN group at 24 h; (B) % of CD14$^+$ HLA-DR$^+$ cells in the INFEC and the CN group at five day; (C) % of CD14$^+$ HLA-DR$^+$CD80$^+$ cells in the INFEC and the CN group at 24 h; (D) % of CD14$^+$HLA-DR$^+$CD80$^+$ cells in the INFEC and the CN group at five day time; (E) % of CD14$^+$ HLA-DR$^+$CD86$^+$ cells in INFEC and CN in 24 h; (F) % of CD14$^+$ HLA-DR$^+$CD86$^+$ in INFEC and CN at five day time. The horizontal bars represent the median and the vertical bars the lower and upper limit. Identical numbers above the bars indicate a statistical difference with p ≤ 0.05 between the INFEC versus the CN groups. Symbols (*) above the bars indicate statistical difference with p ≤ 0.05 among the INFEC group different culture conditions at 24 h versus five day.

(Fig. 1B), confirming the action of Bz in increase the number these cells in INFEC group.

The percentage of CD14$^+$HLA-DR$^+$CD80$^+$ cells were increased in the condition C + T + Bz and C + Bz (p = 0, 0156) compared to C (p = 0.0156) and at 24 h in INFEC group (Fig. 1C). When the INFEC and the CN groups were compared, CD14$^+$HLA-DR$^+$CD80$^+$ cells level was a significantly higher in INFEC group in all experimental culture conditions at 24 h incubation (C/C - p = 0.0012; C + T/C + T - p = 0.0140; C + T + Bz / C + T + Bz - p = 0.012; C + Bz / C + Bz - p = 0.0023) (Fig. 1C).

When assessing the percentage of CD14$^+$HLA-DR$^+$CD86$^+$ cells in the INFEC group no statistically significant differences between different treatment conditions and time were observed (Fig. 1E and F). On the

other hand, when the level of CD14$^+$HLA-DR$^+$CD86$^+$ expressing cells in the INFEC group and the CN group at 24 h were compared, a higher percentage of this cell population was found in the INFEC group independent on the experimental conditions (C / C - p = 0.0162; C + T / C + T - p = 0.0370; C + T + BZ / C + T + Bz - p = 0.0033; C + Bz / C + Bz - p = 0.0079) (Fig. 1E). At day five of incubation, no statistically significant differences were observed between the INFEC and the CN groups.

3.2. Evaluation of the phagocytic and inflammatory profile of CD14$^+$CD16a$^+$ macrophages

To analyze the inflammatory profile of CD14$^+$ macrophages, the surface expression of CD16a, CD11b, CD64 and CD32 molecules was

evaluated. Initially, it was found that the level of cells expressing CD14$^+$CD16a$^+$ in the INFEC group was increased in C + T condition at 24 h when compared to C (p = 0.0469) (Fig. 2A). When the two INFEC times of culture, were compared a significant decrease in these cells at day five was observed in the presence of *T. cruzi* (C + T / C + T - p = 0.0313; C + T + Bz / C + T + Bz - p = 0.0313). In addition, there was an increase in the percentage of CD14$^+$ CD16a$^+$ cells in C + T at 24 h of culture (p = 0.0221) in the INFEC group compared to the same condition in the CN group.

At day five, there was a reversal of the phenomenon, with a significant decrease in the percentage of CD14 + CD16a + cells in the presence of *T. cruzi* compared to other conditions at the same time (C / C + T - p = 0.0313; C / C + T + Bz - p = 0.0313; C + Bz / C + T - p = 0.0313; C + Bz / C + T + Bz - p = 0.0313) (Fig. 2B). This result shows that the presence of the parasites is determinant to modulate the frequency of in these cell/this cell phenotipic type. Therefore, we conclude that Bz has no influence on the levels of CD14 + CD16a + cells.

The percentage of CD14$^+$CD16a$^+$CD11b$^+$ cells in INFEC showed decreased levels in C + T compared conditions C (p = 0.0313) and C + Bz (p = 0.0313) groups at day five of incubation (Fig. 3A). This result shows that Bz has no influence on the expression distribution of this molecule in CD14$^+$CD16a$^+$ cells.

Regarding the percentage of CD14$^+$CD16a$^+$CD32$^+$ cells in INFEC group, there was a decrease in their levels at day five of culture after exposure to *T. cruzi* compared C + Bz (C + Bz / C + T - p = 0.0313; C + T + Bz / C + Bz - p = 0.0313) groups (Fig. 3D). It was also observed that comparing the INFEC groups in the two times of cultivation, there was a significant decrease in the frequency of this cell at day five (C + T / C + T - p = 0.0313; C + T + Bz / C + T + Bz - p = 0.0313) (Fig. 3C and D). When the INFEC and the CN cells were compared with C + T + Bz group, at day five of cultivation there was a decrease in the levels of CD14$^+$CD16a$^+$CD32$^+$ cells in INFEC (p = 0.0381).

INFEC CD14$^+$CD16a$^+$CD64$^+$ cells were significantly higher in the C + T condition compared to C (p = 0.0313) (Fig. 3E), at day five of cultivation, suggesting that *T. cruzi* positively modulated the expression of this cell. When comparing the percentage of these cells between INFEC and CN groups, it was observed that the cells were significantly higher in INFEC group independent on both experimental condition and incubation time (24 h: C / C - p = 0.0012; C + T / C + T - p = 0.0034; C + T + Bz / C + T + Bz - p = 0.0012; C + Bz / C + Bz - p = 0.0012; 5d: C + T / C + T - p = 0.0209; C + T + Bz / C + T + Bz - p = 0.0139; C + Bz / C

+ Bz - p = 0.0095) (Fig. 3E and F).

4. Discussion

The presence of the parasite associated with the immune response can cause tissue damage due to an exacerbated inflammatory response (Dutra and Gollob, 2008). And the question is: Bz is a hero or villain in this process? Understanding the role of Bz in the triad host/parasite/drug is extremely important for the improvement of current etiological treatments, as well as for the development of new treatments and medical procedures (Pérez-Antón et al., 2018, 2020; Llaguno et al., 2009). As an important cell of the immune response, the macrophage displays different response patterns depending on the stimulus received and may present an expression profile of receptors activation and antigen presentation (Freeman et al., 1993a, 1993b), as well as a profile of inflammatory and phagocytic receptors (Ziegler-Heitbrock, 1996; Belge et al., 2002).

By analyzing the action of Bz on these different response patterns, we verified the induction of increased expression of activation molecules (HLA-DR) and antigen presentation (CD80) in the INFEC group. This result suggests that Bz can help to induce a more efficient immune response through signaling mechanisms by binding co-stimulatory molecules to the CD28 receptor.

However, Magalhães et al. (2015), state that the initial increase in the frequency expression of CD14$^+$HLA-DR$^+$ cell may be due to the early activation of this molecule after stimulation. In addition, they claimed that monocytes infected with the Y or Colombiana strains of *T. cruzi* showed an increase in the expression of HLA-DR molecule compared to uninfected cells after 15h culture. Soares et al. (2016), showed that CD14$^+$HLA-DR$^+$CD80$^+$ patient cells cultured for 24 h were increased after stimulation with specific *T. cruzi* antigens. Therefore, Bz can potentiate the early activation of these receptors, as it can be confirmed by comparing the INFEC versus the CN group.

Regarding the inflammatory profile of CD14$^+$ macrophages, some authors believe in a general hypothesis that the etiological treatment contributes to the reduction of parasitism and the reorganization of the host immune response, leading to a balanced inflammatory response, which would be crucial for the control of morbidity of CD (Garcia et al., 2005; Sathler-Avelar et al., 2006, 2008).

The CD16a receptor, that is associated with phagocytosis and antibody-dependent cell cytotoxicity (Abbas, Lichtman and Pillai,
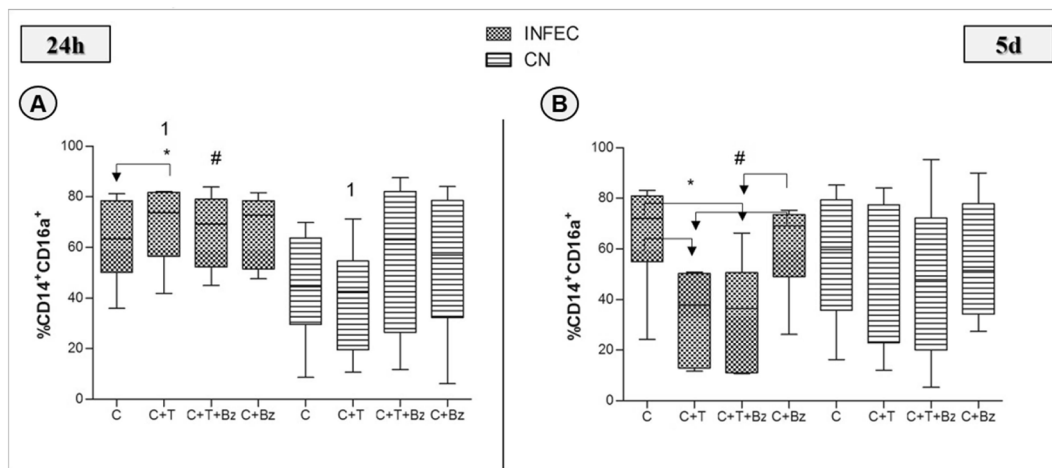
Fig. 2. Comparison of the percentage of CD14$^+$CD16a$^+$ cells between INFEC and CN groups at 24 h and five day of culture. Legend - INFEC - patients (n = 7); NC - uninfected individuals (n = 6). C – PBMC; C + T - PBMC and trypomastigotes; C + T + Bz – PBMC, trypomastigotes and Bz; C + Bz – PBMC and Bz. (A) % of CD14$^+$CD16a$^+$ cells in INFEC and CN groups at 24 h of culture and (B)% of CD14$^+$CD16a$^+$ cells in INFEC and CN groups at day five of culture. The horizontal bars represent the median and the vertical bars represent the lower and upper limit. Symbols (*, #) above the bars indicate statistical difference with p ≤ 0.05, between INFEC conditions at 24 h versus day five of culture. Identical numbers above the bars indicate a statistical difference with p ≤ 0.05 between the INFEC versus CN groups. Arrows indicate significant differences with p ≤ 0.05 for comparisons among culture conditions during the same time in INFEC group.
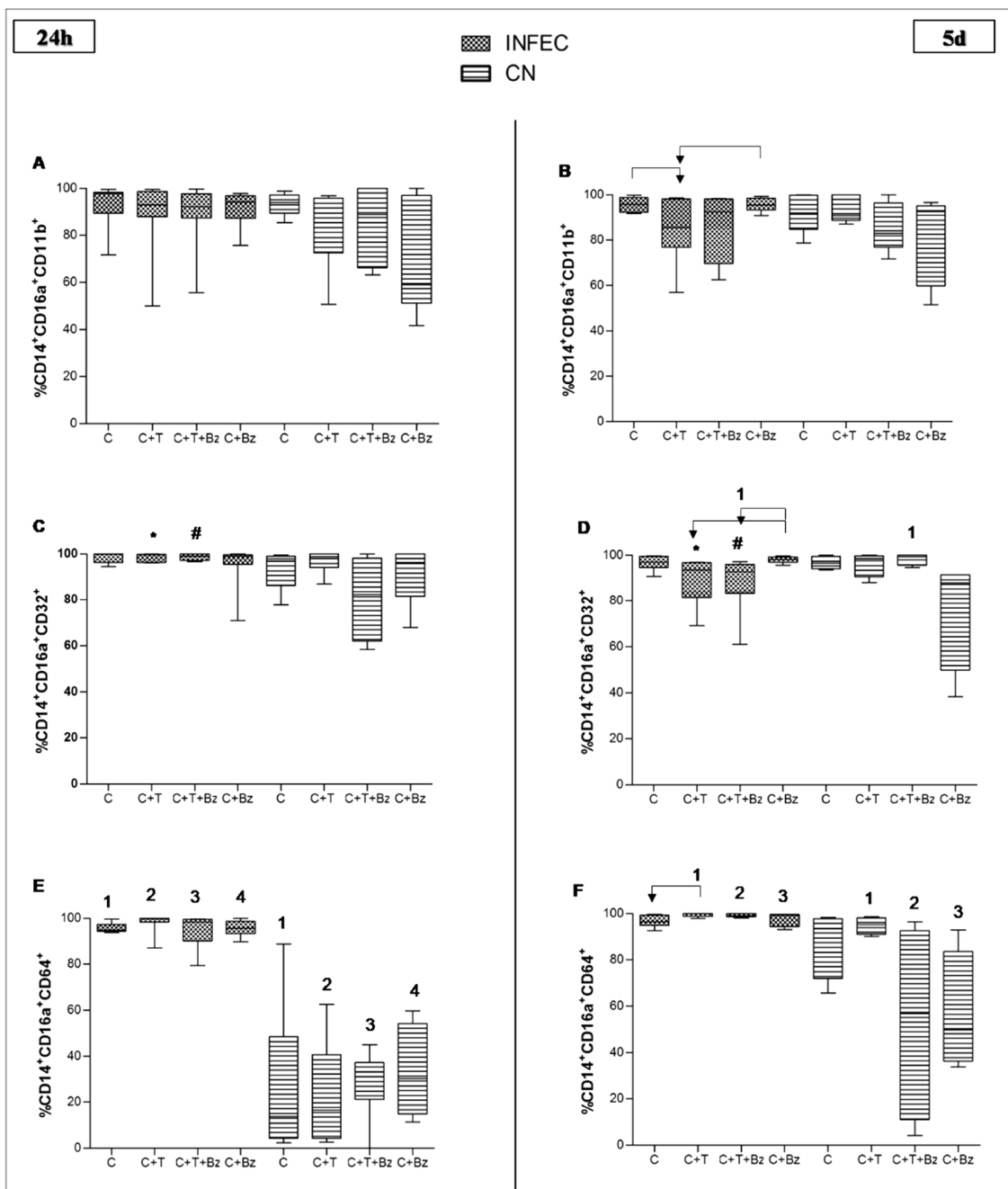
Fig. 3. Comparison of the percentage CD14$^+$CD16a$^+$CD11b$^+$, CD14 CD16a$^+$CD32$^+$, and CD14$^+$CD16a$^+$CD64$^+$ of cells between the INFEC and the CN groups at 24 h and at day five of culture. Legend - INFEC - patients (n = 7); NC - uninfected individuals (n = 6). C – PBMC; C + T – PBMC and trypomastigotes; C + T + Bz – PBMC, trypomastigotes and Bz; C + Bz – PBMC and Bz. (A) % of CD14$^+$CD16a$^+$CD11b$^+$ cells in INFEC and CN groups at 24 h of culture; (B) % of CD14$^+$CD16a$^+$CD11b$^+$ cells in INFEC and CN groups at day five of culture. (C) % of CD14 CD16a$^+$CD32$^+$ cells in INFEC and CN groups in 24 h of culture; (D) % of CD14 CD16a$^+$CD32$^+$ cells in INFEC and CN groups at day five of culture; (E) % of CD14$^+$CD16a$^+$CD64$^+$ cells in INFEC and CN groups at 24 h of culture; (F) % of CD14$^+$CD16a$^+$CD64$^+$ cells in INFEC and CN groups at day five of culture. The horizontal bars represent the median and the vertical bars the lower and upper limit. Symbols (*) above the bars indicate statistical difference with p ≤ 0.05, between INFEC conditions at 24 h versus five day. Arrows indicate significant differences with p ≤ 0.05 for comparisons between culture conditions during the same time in INFEC.

2012), did not change its expression after exposure to Bz. The presence of the parasite negatively modulated its expression in INFEC group cells, as observed under conditions C + T and C + T + Bz in both cultivation times.

Bz may modulate the expression of some of the phagocytic cells and macrophage activation. The expression of CD14$^+$CD16a$^+$CD64$^+$ was significantly increased in all culture conditions in INFEC group when compared to CN group at 24 h. A similar effect was observed at day five of cultivation. The presence of Bz during the infection process may be positively modulating the expression of this cell together with *T. cruzi*.

As for the expression of the CD11b molecule, an important receptor of the inflammatory response, the results showed that Bz did not induce changes in the expression of this molecule. On the other hand, in the presence of the parasite, this molecule shows a decrease in its expression when compared to other cultivation conditions.

The interaction between *T. cruzi* and phagocytes is an important event in the regulation of cell reactivity in CD. It has been shown that these cells can influence the differential clinical of patients with CD

([Kierszenbaum and Ramirez, 1990](#)). Therefore, although we observed the action of *T. cruzi* on these receptors, Bz can be an important factor in maintaining a balanced phagocytic response.

But the question is: Bz, hero or villain? Our results, in conjunction with the previous studies, suggest that treatment with Bz contributes to immunomodulation in patients with CD, but it does not induce extensive changes in the immune response. Therefore, the interactions observed between the immune response and Bz are compatible with a possible induction of an immune response balance, suggesting possible immunological protection. As a consequence, the Bz can be seen more as a hero than as a villain, being an important ally in the fight against CCD.

5. Conclusion

In view of the findings, we believe that the administration of Bz to chronic patients with CD can be beneficial, since the drug did not promote an exacerbated inflammatory profile. Thus, the results of the present study, together with the findings in the literature ([Pérez-Antón et al., 2018, 2020](#)) strengthen the hypothesis that Bz may prevent and/or delay the progression of CD to a more severe forms since the host immune response can directly influence clinical evolution of patients. Finally, prospective studies that assess the parasitic burden after the addition of Bz, together with an assessment of the action of this drug on immune response cells, are necessary to understand the mechanisms involved in the triad immune response/*T. cruzi*/Bz.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imbio.2020.152046.

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