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Is the adaptive immune response in murine *Trypanosoma cruzi* infection influenced by zinc supplementation?

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ABSTRACT

Chagas disease afflicts 7 to 8 million people worldwide and congenital Chagas' disease usually leads to changes in the maternal environment, culminating in fetal adaptations. Several articles have described the importance of micronutrients on pregnancy, which is sensitive to infections. In *Trypanosoma cruzi* endemic regions, the Chagas disease is aggravated by the lack of micronutrients in an average diet, to which pregnant women are more susceptible. The aim of this study was to evaluate distinct T cells phenotypes and intracellular cytokines by flow cytometry in pregnant Wistar rats under zinc therapy during experimental Chagas' disease. Twenty female Wistar rats were infected with 1×10^5 blood trypomastigotes (Y strain) and 30 days after infection the animals were mated and grouped: pregnant infected (PI–$n = 5$), pregnant infected/zinc supplied (PIZ–$n = 5$), pregnant control (PC–n = 5), control/zinc supplied (PCZ–n = 5). Zinc supplementation: 20 mg of zinc/Kg/day (gavage) for 18 days followed by euthanasia. The immune parameters showed: decreased percentages of $CD62L^{low}CD44^{high}$ surface marker for infected and treated group (PIZ) when compared to PI ($p < 0.05$). Concerning to T regulatory cells (Treg cells), a significantly lower percentage of splenic Treg cells was found in the infected and treated group (PIZ) as compared to the PI group ($p < 0.05$). The expression of the co-stimulatory molecule $CD28^+$ displayed a significant reduced percentage in $TCD8^+$ for infected and zinc treated group (PIZ) as compared to (PI). The percentages of $CD4^+/CD11a^+$ T cells subsets were lower on PIZ as compared to PI. Concerning to $CD45RA^+$ (B lymphocytes) analysis, infected pregnant and treated group (PIZ) showed a significant decrease in CD45RA percentage when compared to (PI) ($p < 0.05$). The intracellular cytokine profiles for $TCD4^+$ and $TCD8^+$ producing IL-4 and IFN-γ revealed that zinc treated and untreated infected pregnant group (PI and PIZ) displayed increased cytokines concentrations as compared to zinc treated and untreated pregnant controls (PC and PCZ). Our data revealed the involvement of zinc as a signaling molecule in the modulation of the inflammatory process and immune response which occurs during pregnancy of *T. cruzi* infected rats. Zinc acted in a dual fashion, modulating the host's immune response in a way to protect the organism against the deleterious effects of the infection and an overwhelming pro-inflammatory response during pregnancy.

1. Introduction

Chagas disease is a potentially life-threatening illness caused by the protozoan parasite *Trypanosoma cruzi*. About 6 million to 7 million people worldwide are estimated to be infected with *T. cruzi*. In Latin America vector-borne transmission is the main way of infection where humans come in contact with faeces or urine of triatomine bugs (Steverding, 2014). The importance of micronutrients in human health is unquestionable and zinc (Zn) is an essential trace element with a wide range of actions such as antioxidant activity, cellular metabolism and as a component of proteins involved in cell structures. It also plays an important role in immune function, protein and DNA synthesis, cell division, energy metabolism and growth (Bonaventura et al., 2015; Yasuno et al., 2011). The immunomodulatory role of zinc during pregnancy in the acute phase of Chagas' disease has been demonstrated by our group (da Costa et al., 2013). Although pregnancy is a normal physiological state, the maternal environment depends on an adequate nutrition, and is closely related to an appropriate fetal growth and development (King, 2006). Maternal nutritional deficiency is associated with poor pregnancy outcomes, including low birth weight, fetal

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growth restriction and prematurity (Black et al., 2008; Christian, 2010).

(Beach et al., 1982) described that marginal zinc deficiency in mice during gestation exerted both short- and long-term detrimental effects in their offspring, with reduced lymphoid organ size and immunoglobulin concentrations. (Dardenne, 2002) studied zinc-deficient mice and showed that several immuno-deficiencies observed at birth persisted through adulthood, even when offspring were fed with a zinc-adequate diet. Depending on the different sub-populations of T lymphocytes a distinct cytokine profile is present leading to a successful pregnancy or abortion. T helper type 1 lymphocytes (Th1) produce interleukin-2 (IL-2), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), which is a characteristic pro-inflammatory response, enhancing host's immunity against alloantigen. T helper type 2 lymphocytes (Th2) cells produce IL-3, IL-4 and IL-10, promoting the production of antibodies with anti-cytotoxic activity (Carlier et al., 2011). Transmitting chagasic mothers have a higher parasite load, reduced ability to produce IFN-γ, IL-2 and TNF-α by T cells, although they are able to synthesize enhanced levels of IL-10. On the other hand, non-transmitting chagasic mothers show high monocyte activation and enhanced concentrations of circulating TNF-α (Alonso-Vega et al., 2005; Carlier, 2005).

During pregnancy, several factors, including regulatory T cells, are involved in the physiological changes responsible for maternal-fetal tolerance (Leber et al., 2010). The importance of cytotoxic $CD8^+$ T cells (CTLs) in host defense against *T. cruzi* has already been described (Padilla et al., 2009). Several studies using different experimental models for Chagas' disease described that these cells also play an essential role enhancing effectors mechanisms of the immune response such as activating phagocytes, cytotoxic $CD8^+$ T lymphocytes and the production of cytokines (Martin and Tarleton, 2005).

Zinc supplementation has a positive effect on immune system of male rats during Chagas disease chronic phase (Brazão et al., 2011). Since pregnancy displays a distinct immune response, we investigated the immunomodulatory role of this element during the chronic phase of *T. cruzi* infection in pregnant Wistar rats. The aims of this work were to evaluate the importance of zinc as a possible immunomodulatory element in order to improve the homeostasis during pregnancy as well as the rat's immune response against *T. cruzi*. For that the phenotypic profile of distinct T cell subsets involved in the adaptive immune response during pregnancy associated with zinc supplementation was evaluated.

2. Material and methods

2.1. Animals

Four-week old female Wistar rats (100 g) from Facility House of the São Paulo University Campus of Ribeirão Preto were used. Animals were randomized into the following groups: pregnant control (PC), pregnant control with zinc supplementation (PCZ), pregnant infected (PI), pregnant infected supplied with zinc (PIZ). The experimental protocols were approved by the local Ethics Committee (Protocol No 11.1.1210.53.2).

2.2. Experimental infection/pregnancy

Each group ($n = 5$) was inoculated intraperitoneally (i.p.) with 1×10^5 trypomastigote forms of the Y strain of *T. cruzi*. Thirty days post infection one male Wistar rat was introduced into each cage and was allowed to mate with the females. Pregnancy confirmation was made by the vaginal plug. The assays were performed on day 18 of gestation, corresponding to 48th day of *T. cruzi* infection (chronic phase).

2.3. Treatment schedule

Zinc-treated groups were orally administered with zinc sulfate (via gavage) (Sigma Chemical Co.MO, USA) dissolved in 0.1 ml of distilled water at a dose of 20 mg/kg body weight (da Costa et al., 2013), once a day, starting the day after the pregnancy confirmation until the end of the experiment.

2.4. Euthanasia

Each animal was anesthetized with 2.5% tribromoethanol (Sigma-Aldrich, USA) administered intraperitoneally (dose of 25 mg/kg/animal) and later euthanized by decapitation. Blood samples were collected to obtain plasma and serum.

2.5. Splenic and peritoneal cell suspension

After euthanasia, cells were harvested from peritoneal cavity and washed with RPMI 1640 medium (Sigma-Aldrich, USA) and resuspended (2×10^7 cells/ml). Spleens were aseptically removed and the splenic tissue was mechanically disaggregated by extrusion through a 100 μm nylon cell strainer (Falcon, USA) and washed in a hypotonic buffer (160 mM NH_4Cl, 10 mM Tris-HCl, pH = 7.4) for red blood cell lysis. Cells were homogenized in RPMI 1640 medium to produce a single cell suspension and the concentration was adjusted to 2×10^7 cells/ml. Splenic and peritoneal cell viability was assessed by 0.4% trypan blue solution (Sigma-Aldrich, USA) (da Costa et al., 2017).

2.6. Cell surface phenotype analysis

The cells (2×10^6 cells/well) were resuspended in staining buffer (BSA) (BD-Pharmingen, San Diego, USA) and dispensed into 12 × 75 mm round-bottomed polystyrene tubes (Falcon, USA) for cytofluorometric analysis. Following Fc receptor blocking (anti-CD32), cells were incubated with specific monoclonal antibodies for 30 min at 4 °C in the dark (da Costa et al., 2017). Fluorescence analysis was performed using a FACS Canto flow cytometer (BD Biosciences, California, USA) and the percentages of positive cells for each labeling were determined using the FACSDiva software. All conjugated monoclonal antibodies were obtained from BD Biosciences Pharmingen (CA, USA). Peritoneal cells phenotype and splenic T-cell subsets were identified according to the surface expression of the following CD markers: anti-$TCD3^+$ (APC), anti-$TCD4^+$ (PE-Cy7), anti-$TCD8^+$ (PerCP), anti-T-CD62L (PE), anti-TCD44 (FITC), anti-$TCD4^+$/$TCD28^+$ (PE-Cy7/PE) anti-$TCD8^+$/TCD28 (PerCP/PE), anti-$TCD4^+$/$CD11a^+$ (PE-Cy7/FITC), anti-$TCD8^+$/$CD11a^+$ (PerCP/FITC). For regulatory T cells analysis, membranes were marked with monoclonal antibodies anti-CD3 (FITC), anti-CD4 (PE-Cy7), and anti-CD25 (PE). Cells were also permeabilized and marked with an antibody to the forkhead box transcription factor (FoxP3) labeled with Alexa Fluor 647 (Biolegend, Inc. San Diego, USA).

2.7. CFSE labeling

The CFSE labeling was performed using Cell Trace CFSE Cell Proliferation kit, for flow cytometry (Molecular Probes, Life Technologies USA). Briefly, splenocytes cells were washed in warm phosphate-buffered saline (PBS) and labeled with CFSE (carboxyfluorescein succinimidyl ester). The samples were cultured at 2×10^6/ml cells in RPMI1640 culture medium with or without Concanavalin A 5 μg/ml (ConA, Sigma-Aldrich, USA) for 10 min at 37 °C. Dye was then quenched by washing in ice-cold RPMI-1640 medium (Sigma Aldrich, USA). Splenocytes were cultured in a humidified atmosphere of 5% CO_2 at 37 °C for 5 days. Cells were collected and incubated with monoclonal antibody, stained with anti-CD3-Allophycocyanin (APC) monoclonal antibody. The data were acquired by gating on $CD3^+$ T cell population, assessed by flow cytometry using FACSCan and FACSDiva software (BD