Antibody and cytokine – production during granulomatous response in *Schistosoma mansoni* - infected mice: role of exposure and treatment with anti-CD28

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**ABSTRACT**

Schistosomiasis mansoni is among the neglected diseases that occur in Brazil, being endemic in 76 countries and territories spread across Africa, Asia and the Americas. The granulomatous process in schistosomiasis is dependent on CD4 + and requires recruitment and accumulation of inflammatory cells at the site of eggs deposition. Schistosomal fibrosis is the result of a granulomatous reaction developed in response to antigens released by eggs of *Schistosoma mansoni* that are retained in the portal veins of smaller caliber. Manipulation of the interaction between B7 antigen molecules presenting cells (APC) and T cell receptors CD28/CTLA4 modulate and, in some circumstances, block the immunological response *in vivo*. Therefore, the objective of this study was to evaluate treatment with monoclonal anti-CD28 (PV-1 cell line hybridoma), the contribution of the synthesis and release of IL-4 and IL-5 correlated with levels of IgE and IgG1 and the granuloma formation and fibrosis during schistosomiasis. Our results suggests that treatment with anti-CD28, in the 64th day post-infection, favored a decrease in IL-4 and IL-5 and decreased levels of IgG1 and IgE, as well as less collagen deposition resulting from fibrosis.

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**Introduction**

Schistosomiasis is among the neglected diseases that occur in Brazil. In this context, schistosomiasis appears as one of the most prevalent parasitic infections worldwide, being endemic in 76 countries and territories spread across Africa, Asia and the Americas [1]. This represents a major public health problem, affecting 240 million individuals worldwide. In clinical medicine, schistosomiasis presents an etiological cause and a biological model of transmission. This disease is caused by a parasitic trematode of the Schistosoma genus, species belonging to the Schistosomatidae mansoni family. Its diagnosis is made based on parasitological, clinical and laboratory tests. [2] The eggs, once retained in the tissues, begin to release antigens, which causes an inflammatory reaction called a granulomatous reaction, fibrosis or granuloma. [2] The granuloma consists of an organized entity in which are found several migratory cells of the immune system, adhesion proteins, extracellular matrix components, growth factor and angiogenesis, forming a spherical structure that surrounds each egg individually. Granuloma formation is related to the response by T cells against SEA eggs deposited. Granuloma formation in schistosomiasis is dependent on CD4 + T cells, T lymphocytes (TCR αβ) sensitized with antigens of parasite eggs (SEA). The T cell activation requires at least two independent signals. The first is given by the binding of the complex peptide-major histocompatibility complex (MHC) to T-cell receptor TCR, and the second by a co stimulatory signal, emitted by the binding of B7 molecules B7-1 (CD80) and B7-2 (CD86), present on antigen presenting cells (APC) with its receptor on T cells, the CD28 molecule [3]. After activation, the T cells begin to display an additional receiver, homologous to CD28, called CTLA-4 (CD152) [4]. This molecule binds to B-7, sending an inhibitory signal to the activated T cells. Thus, CTLA-4 binding to B7 molecules limits the proliferative response of activated T cells. [5] Several studies use the S. mansoni – infected mice model in order to study the interaction of the immune system and the role of Th1 and Th2 cells for protection, disease progression and establishment of the granuloma. [6] The natural progression of the disease generates Th2-type immune response induced by egg antigens of S. mansoni. This phase of the disease is characterized by high levels of Th2 cytokines such as IL-4, IL-5, IL-13 and IL-10 [7]. Although the early Th2 response seems to have a crucial role in modulating the acute inflammatory response, a prolonged Th2 response contributes to the development of liver fibrosis and increases the morbidity of the disease [8]. As a consequence of the Th2 response, it has been shown that IL-4 and IL-5 can influence the selection of classes and subclasses of antibodies of IgG1 and IgE. [9] Therefore, the objective of this study was to evaluate treatment with monoclonal anti-CD28 (PV-1 cell line hybridoma), the contribution of the synthesis and release of IL-4 and IL-5 correlated with levels of IgE and IgG1 and the granuloma formation and fibrosis during schistosomiasis.

**Material And Methods**

**Animals**

This research was approved by the Animal Experimentation of São Carlos Federal University – UFSCar (Process N. 066/2009). Age-matched, female BALB/c Specific Pathogen
Free (SPF) mice. All animals were subjected to parasitological, serological and histopathological examination 64 days after infection from the start of the experiment. All samples were coded and stored.

**Anti-CD28 antibody and treatment of mice**

Hamster anti-CD28 (PV-1) was purified from the culture supernatant. The antibody was prepared using a bioreactor spinner. The mice were treated with anti-CD28 whole antibody (100 µg/kg/mice) i.p. for three doses 7th, 21th and 47th days post infection.

The mice divided into five groups:

- **Group I**: control group untreated and uninfected.
- **Group II**: control treated group with total hamster (Ab) IgG and uninfected.
- **Group III**: Infection control group (50 cercariae, infection performed by intraderm), *S. mansoni* (LE - BH-MG, strain).
- **Group IV**: Infected mice (50 cercariae, infection performed by intraderm) and treated with anti-CD28 (100 µg/kg/mice).
- **Group V**: Infected mice (50 cercariae, infection performed by intraderm) and treated with Praziquantel (PZQ) (500 mg/kg/mice).

**Immunosorbent assay - ELISA**

- **IL-4 and IL-5**

ELISA were performed according to the instructions of the manufacturer (BD) test was made in sample serological (plasma), using purified mAbs as capture Abs and biotinylated mAbs as developing Abs, followed by incubation with streptavidin-alkaline phosphatase substrate. Plates were read in a 96-well spectrophotometer (Microquant-Sellex, Inc 450 nm) and data were analyzed using software by comparison against a standard curve, which was generated using recombinant cytokines at known concentrations.

**IgG1 and IgE**

ELISA (Kit BD) test was made in sample serological (plasma), with standardization of (5 mg/mL total protein antigen of adult worm and dilution sample of 1:64) to IgG1 and to IgE (10 mg/mL of total protein antigen of adult worm and sample dilution 1:4). Were used 96-well micro plates and coated with 10 or 5 mg/mL of total protein antigen of *S. mansoni* (diluted 0.1 M carbonate buffer pH 9.6 them and applied 100 µl/well). The wells were blocked for 1 h with PBS-1% BSA, and then the plates were kept at ambient temperature and then incubated for 2 h with sera from mice. Subsequently added biotin-conjugated secondary Ab. After the duration of 1 h, the plate was washed with three cycles of PBS containing 0.05% Tween-20, then added the streptavidin dilution 1:200 enzyme. After 30 minutes, and 100 µL of the substrate, a 1:1 mixture of H2O2 and tetramethylbenzidine (TMB) (BD-opt lot. 39814), were added, followed by the blocking of the reaction, with 50 µL/well of 1M H2SO4. The absorbance reading was made at a wavelength of 450 nm in the ELISA reader (Microquant-Sellex, Inc.).

**Histological and Photomicrographies**

The livers were removed from mice at the 64 th day post infection, and then, these tissues were fixed in phosphate buffer containing 10% formalin during 24 hours, and were then dehydrated in 70% alcohol and xylene clarification and subsequently included in paraffin blocks, sectioned in slices of 5 µm, to be arranged in slides and incubated at 58 - 60 °C for fixation. Then, the slices were washed in xylene, in order to remove the excess of paraffin and rehydrated with decreasing concentrations of alcohol (80% of the Absolute AOS). Two slides were produced, the first stained with H.E. (hematoxylin / eosin), for a qualitative assessment of granuloma periovular cells, and the second was stained with M.T. (Masson trichrome), for an assessment of hepatic fibrosis. Both were analyzed by optical microscopy. Slides were analysed and photographed with the aid of a microscope (Nikon) containing an adapted camera (SONY - Cybershot DSC - H55) with a magnification of 100x and 400x.

**Statistical Analysis**

The results were expressed as mean ± SEM. The results obtained in different experiments were analyzed using the program PRISM, version 5 - Graph Pad (2005) (San Diego, California, USA) by testing non-parametric One-way ANOVA (one-way analysis of variance), post-test using the Tukey method (compare all pairs of columns). Statistical significance was set at p values <0.05.

**Results**

**IL-4 and IL-5**

Concentrations of IL-4 and IL-5 were determined in plasma of mice, infected with *S. mansoni* or not and treated with anti-CD28 antibody or not, total IgG of hamsters and PZQ. On Day 64 post infection, the values in pg/mL of interleukin of different groups (Fig. 1). Infected groups treated with anti-CD28 and PZQ showed changes in the concentration of IL-4 and IL-5. The infected group treated with anti-CD28 showed a significant decrease of IL-4 and IL-5 when compared with the infected group, and the infected and treated with PZQ group showed a significant increase of IL-4 and a significant decrease in IL-5 when compared with the infected only group.

![Figure 1](image-url)

Figure 1. Evaluation of IL-4 (A) and IL-5 (B) in the 64th day post infection in plasma. IL-4 pg/mL in plasma, IL-5 pg/mL in plasma. The data represent the mean ± SEM (n = 10 animals) of two independent experiments. # p <0.05 represents significant difference between the results obtained from the infected / treated groups, compared with untreated and infected group. ¬ p <0.05 represents significant difference between the results obtained from the infected group / PZQ-treated group compared with the infected / treated with anti-CD28 group, using the nonparametric one-way ANOVA test Tukey’s. ND (not detected).
IgG1 and IgE

IgG1 and IgE reactive to antigens of adult worms present in the plasma of animals, infected or not with S. mansoni and treated or not with total hamster (Ab) IgG, antibody (mAb) anti-CD28 and PZQ on day 64 post infection (Fig.2). The plasma of the control group (uninfected and untreated) was used as negative control (non-specific links), thus determining the cutting O.D.. The infected groups treated with anti-CD28 and PZQ showed a significant decrease in the levels of IgG1 when compared to the infected group in the 64 th day post infection (Fig. 2A). Moreover, IgE levels showed too a significant decrease was observed in infected groups treated with anti-CD28 and PZQ, when compared to the infected group at the 64 th day post infection (Fig. 2B).

Figure 2. Levels of antibody S. mansoni present in plasma at the 64 th day post infection. Levels of IgG1 (A) and IgE (B) in plasma. The data represent the mean ± SEM (n = 10 animals) of two independent experiments. # p <0.05 represents significant difference between the results obtained from the infected / treated groups, when compared with untreated and infected group, using the nonparametric one-way ANOVA test Tukey’s

Liver histology in the 64 th day post infection

In Figures 3 and 4, it can be observed the liver granuloma in infected groups of animals, treated or not. In Figure 3, stained slides with H.E. can be seen and in figure 4 we can see the slides stained with Masson's trichrome in the 64 th day after infection. Being that the slides were stained with H.E. in order to observe the cellular characteristics and with Trichrome Masson in order to observe of fibrosis. In the control group and the control treated with total hamster IgG, the liver is preserved in the absence of either granuloma or eggs (Fig. 3A and B respectively); in groups of infected animals, treated or not, (infected, infected treated with anti-CD28 and infected treated with PZQ), it was observed the formation of periovular liver granuloma, with mixed cell infiltrate (macrophages, eosinophils, neutrophils, lymphocytes) (Fig. 3C, 3D and 3E respectively). In

Figure 3. Photomicrography of liver sections from infected mice with S. mansoni, treated or not. Morphological analysis of histological sections of liver in the 64 th day post infection in animals in groups: control (A), control + (Ab) IgG (B), infected (C), infected + anti-CD28 (D) and infected + PZQ (E) Staining: HE Magnification: 100x and 400x.

Discussion

The initiation and maintenance of granulomatous responses are characteristics of S. mansoni infection and requires recruitment and accumulation of inflammatory cells around the deposited eggs, mainly in the liver and intestine [10]. Thus, soon after the start of sexual maturation of the parasites and subsequent oviposition, egg antigens-reactive (SEA) lymphocytes comes into proliferation in the spleen [11] and the SEA-reactive lymphoblasts are recruited to the liver granuloma [12]. Granuloma formation around eggs of S. mansoni is the central event in the development of the pathology associated to acute and chronic schistosomiasis infection, although it has been shown the participation of antigens derived from other stages of the parasite in both the development of pathology as well as resistance to infection by Schistosoma mansoni [13]. However was demonstrated as a reaction that is dependent mainly on T cells, named as modulation of the granuloma. In our study, we observed that there was the deposition of eggs in liver tissue and subsequent periovular granulomatous response on the 64th day post infection in the groups infected-only and infected and treated with anti-CD28 and PZQ (Fig. 3 C, D and E), but there were no statistical difference in the percentage of the volume of
granulomas, and numeric and volumetric density between the groups, but it is possible to observe a less intense cellular response in the infected group treated with anti-CD28 when compared to the infected-only group (Fig. 3E).

Figure 4. Photomicrography of liver sections from infected mce with S. mansoni, treated or not. Morphological analysis of histological sections of liver in the 64 th day post infection in animals in groups: control (A), control + (Ab) IgG (B), infected (C), infected + anti-CD28 (D) and infected + PZQ (E) staining: T. Masson. Magnification: 400x and 100x.

At the beginning of oviposition, and with the deposition of eggs in the tissues, the cytokines production is predominantly directed to the profile of the Th2 response. Thus, there is an increased production of IL-4, IL-5 and IL-10. In schistosomiasis the role of IL-4 has been extensively explored. Studies have shown that treatment with anti-IL-4 leads to a decrease in hepatic fibrosis with little interference in the size of granulomas in these organs [14,15]. We can observe in our study that in the 64 th day post-infection there was a significant decrease in IL-4 in the group treated with anti-CD28, when compared to the infected-only group (Fig. 1 A), suggesting that treatment with anti-CD28 can negatively modulate IL-4, favoring the contribution to the reduction or even be an important factor to inhibit the formation of fibrosis in schistosomiasis. These data corroborate the literature, as we notice a decrease in collagen deposition, resulting from fibrosis in this group (Fig. 4D). Silva and col. (2004) found that the antibody production in BALB/c mice infected with S. mansoni occurs with the predominance of IgG1 isotype. Other authors suggest that high levels of IgG1 are related to the chronic phase of infection and also with the synthesis of Th2 cytokines (IL-4, IL-5 and IL-13), which stimulate antibody production [16], although IL-4 [17] and IL-13 [18] seem to be fundamental in the production of IgG1 [19].

Regarding IgG1, the infected group treated with anti-CD28 (Fig. 2A) showed a low level of this isotype in the 64 th day post infection, which corroborates with the low levels of IL-4 at 64 th day post infection (Fig. 1 A). The interactions between cytokines and antibodies in response to the presence of S. mansoni demonstrate the importance of cellular and humoral responses in defining the mechanisms of resistance and susceptibility in front of infection. Thus, IL-5 positively modulates the effector functions of B cells proliferation and differentiation to immunoglobulins-secreting plasma cells [20], and the degree of resistance against infection depends, in part, on the protective function mediated by IgE [21]. In this study, infected animals that were treated with anti-CD28 showed a significant decrease in levels of IL-5 (Fig. 1B) contributing to the fostering in the decrease in the levels of IgE in response to antigens of adult worms in the 64 th day post infection (Fig. 2B).

Conclusion

We suggest that treatment with anti-CD28 in the 64 th day post infection favored a decrease in IL-4 and IL-5, provided the decrease in the levels of IgG1 as well as the lower collagen deposition resulting from fibrosis, and modulated the decrease in protective response by IgE, respectively.

Conflict Of Interest

The authors declare that they have no conflict of interests associated with this paper.

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