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# 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, even block the immunological response *in vivo*. Therefore, the objectives of this study were: a) to evaluate treatment with monoclonal mice anti-CD28 (PV-1 cell line hybridoma), b) to evaluate the contribution of the synthesis and release of IL-4 and IL-5 when correlated with IgE and IgG1 levels and granuloma and fibrosis formation during schistosomiasis. Our results suggest that treatment with anti-CD28, on day 64 post-infection, favored a decrease in IL-4 and IL-5 production and decreased levels of IgG1 and IgE expression, as well as a reduction in the collagen deposition resulting from fibrosis.



## 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**.** This disease is caused by a parasitic trematode of the Schistosoma genus, species belonging to the Schistosomatidae mansoni family. Its diagnosis is based on parasitological, clinical and laboratory tests. [2] Eggs, once retained in the tissues, begin to release antigens, which cause an inflammatory reaction called granulomatous reaction, fibrosis or granuloma. [2] The granuloma consists of an organized entity in which several migrant cells of the immune system, adhesion proteins, extracellular matrix components, growth factor and angiogenesis are found, forming a spherical structure that surrounds each egg individually. Granuloma formation is related to immune response by T cells against *Schistosoma mansoni* soluble egg antigens (SEA) deposited. Granuloma formation in schistosomiasis is dependent on CD4+ T cells, T lymphocytes (TCR αβ) sensitized with SEA antigens. 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 the 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 APC with a CD28 molecules, a T cell receptor [3]. After activation, 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 *S. mansoni*– infected mice model in order to study the interaction of the immune system and the role of Th1 and Th2 cells for immune protection, disease progression and establishment of the granuloma. [6] The natural progression of the disease generates a Th2-type immune response induced by SEA of *S. mansoni*. This disease stage 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 thus increasing the morbidity and mortality 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 IgG1 and IgE antibodies. [9] Therefore, the objectives of this study were to evaluate the contribution of the synthesis and release of IL-4 and IL-5 after treatment with hamster monoclonal anti-CD28 (PV-1 cell line hybridoma), correlating IL-4 and IL-5 release with IgE and IgG1 levels and also correlate all these with 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

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Free (SPF) mice. All animals were subjected to parasitological, immunological and histopathological examination on day 64th post-infection.

**Anti-CD28 antibody and mice treatment**

Hamster anti-CD28 antibody (PV-1) was purified from culture supernatant. Antibody was prepared using a bioreactor spinner. Mice were treated with hamster anti-CD28 whole antibody (100µg/kg/mice) i.p. on day 7, 21 and 47 post parasite infection.

**Experimental groups**

Group I: Control group (untreated and uninfected).
Group II: Treated group with total hamster (Ab) IgG and uninfected.
Group III: Infection control group (intradermic infection with 50 cercariae) *S. mansoni* (LE-BH-MG strain).
Group IV: Infected mice (intradermic infection with 50 cercariae) *S. mansoni* (LE-BH-MG strain) and treated with anti-CD28 (100 ug/Kg/mice).
Group V: Infected mice (intradermic infection with 50 cercariae) *S. mansoni* (LE-BH-MG strain) and treated with Praziquantel (PZQ) (500mg/Kg/mice).

**ELISA assay**

**IL-4 and IL-5**

ELISA were performed according to manufacturer (BD) test instructions using serum samples, with purified mAbs as capture Abs and biotinylated mAbs as developing Abs, followed by incubation with streptavidin-alkaline phosphatase and substrate. 96-well plates were read in a spectrophotometer (Microquant-Sellex, Inc 450nm) and data was analysed 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 performed using serum samples, with standardization of IgG1 (5 mg/ml total protein antigen of adult *S. mansoni* worm and sample dilution of 1:64) and of IgE (10 mg/ml of total protein antigen of adult *S. mansoni* worm and sample dilution of 1:4). 96-well micro plates were used and coated with 10 or 5 mg/mL of total protein antigen of *S. mansoni* (diluted using 0.1 M carbonate buffer pH 9,6 and applying 100 µl/well). The wells were blocked for 1 h with PBS-1% BSA kept at room temperature and then incubated for 2 h with sera from mice. Subsequently biotin-conjugated secundary Ab was added. After 1 hour the plate was washed three times using PBS containing 0.05% Tween-20, then a 1:200 streptavidin dilution was added. After 30 minutes 100 uL of the substrate, a 1:1 mixture of $H_2O_2$ and tetramethylbenzidine (TMB) (BD-opt lot. 39814) were added to each well followed by the blocking of the reaction, with 50 µl/well of 1M $H_2SO_4$. The absorbance reading was made at 450 nm on an ELISA reader (Microquant-Sellex, Inc.).

**Histological and Photomicrographies**

Livers were removed from mice on day 64 post parasite infection, and then, these tissues were fixed in PBS containing 10% formalin during 24 hours followed by dehydratation with 70% alcohol and clarification with xylene and subsequently inclusion in paraffin blocks, that were posteriorly sectioned in slices of 5 µm each, to be arranged in slides and incubated at 58-60 ° C for fixation. Then, the slices were washed using xylene, in order to remove the excess of paraffin and rehydrated with decreasing concentrations of alcohol (80% of the absolute ethanol). For each liver sample, two slides were produced, the first was stained with hematoxylin/eosin (HE), for a qualitative assessment of granuloma peri-ovular cells, and the second was stained with Masson trichrome (MT), for hepatic fibrosis evaluation. Both were analysed by optical microscopy. Slides were analysed and photographed with the aid of a microscope (Nikon) containing an adapted camera (SONY – Cybershot DSC - H55) with amplifications of 100x and 400x.

**Statistical Analysis**

Results were expressed as mean±SEM. Results obtained in different experiments were analysed 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 mice sera in all experimental groups (infected with *S. mansoni* or not infected, treated with hamster anti-CD28 antibody or not treated, treated with total hamster IgG and PZQ treated). On day 64 post parasite infection, interleukin concentration (pg/ml) on the different experimental groups was determined (Figure 1). Infected groups treated with hamster anti-CD28 and PZQ showed changes in the concentration of IL-4 and IL-5 when compared with the control group. The infected group treated with hamster anti-CD28 showed a statistically significant decrease on IL-4 and IL-5 when compared with the infected group, and the infected group treated with PZQ showed a significant increase on IL-4 and a significant decrease on IL-5 when compared with the infected only group.

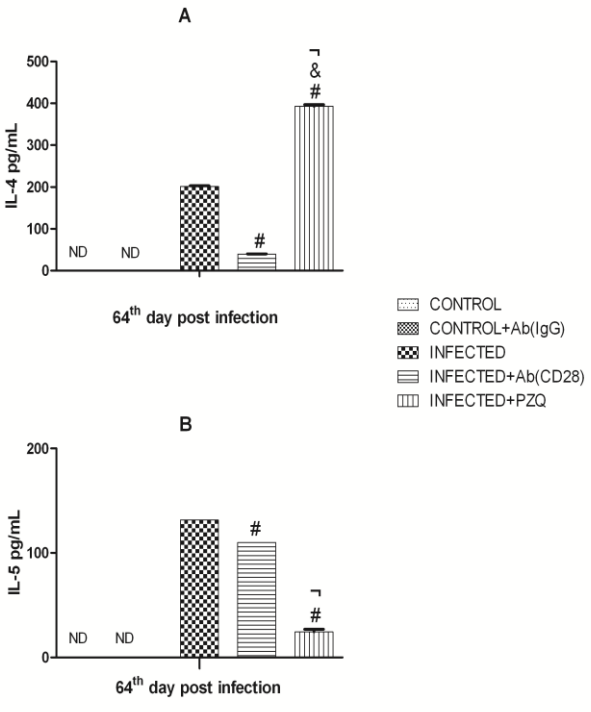


**Figure 1**. Evaluation of IL-4 (A) and IL-5 (B) on day 64 post parasite infection in sera. IL-4 pg/mL in sera, IL-5 pg/mL in sera. Data represent the mean±SEM (n=10 animals) of two

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 hamster 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 *S. mansoni* adult worms present in sera from animals, either infected or not with *S. mansoni* and either treated or not with total hamster (Ab) IgG, antibody (mAb) anti-CD28 or PZQ on day 64 post parasite infection (Figure 2). The sera of the control group (uninfected and untreated) was used as negative control (non-specific links), thus determining the cutting OD. The infected groups treated with hamster anti-CD28 or PZQ showed a significant decrease in the levels of IgG1 when compared with the infected group on day 64 post-parasite infection (Figure 2A). Moreover, IgE expression levels also showed a significant decrease in infected groups treated with hamster anti-CD28 or PZQ, when compared with the untreated infected group on day 64 post parasite infection (Figure 2B).

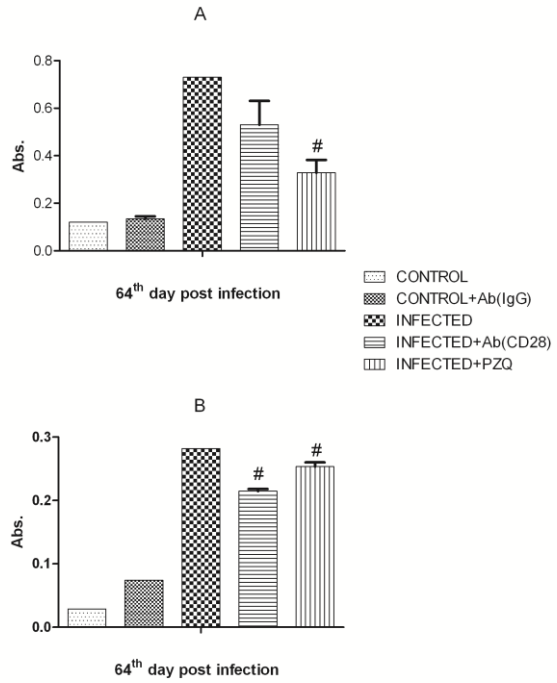


**Figure 2**. Levels of *S. mansoni* antibody present in sera on day 64 post parasite infection. Levels of IgG1 (A) and IgE (B) in sera. Data represent the mean±SEM (n=10 animals) of two independent experiments. #$p<0.05$ represents significant difference between the results obtained from the infected/treated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

**Liver histology on day 64 post parasite infection**

Figures 3 and 4 represent photomicrographies of liver histology sections where liver granulomas from infected groups of animals either treated or not can be observe. On Figure 3, HE stained slides can be seen and on Figure 4 we can see slides stained with MT on day 64 post parasite infection. The slides were stained with HE in order to observe the granuloma cellular characteristics and with MT with the objective of observing fibrosis. In the control group (untreated and uninfected) and the group treated with total hamster IgG, the liver is preserved in the absence of either granulomas or eggs (Figures 3A and 3B respectively); in experimental groups of infected animals, either treated or untreated, (infected, infected treated with hamster anti-CD28 and infected treated with PZQ), a formation of peri-ovular liver granuloma with mixed cell infiltrate (macrophages, eosinophils, neutrophils and lymphocytes) was observed (Figures 3C, 3D and 3E respectively). On Figure 4, due to MT staining the presence of collagen deposition (stained in blue) in the liver of infected animals, either treated or not (infected untreated, infected treated with hamster anti-CD28 and infected treated with PZQ) can be observed. It was observed the presence of collagen deposition in the granulomatous areas, especially on the peri-ovular region, which corresponds to fibrosis (Figures 4C, 4D and 4E, respectively). In the infected and treated with hamster anti-CD28 group and in the infected and treated with PZQ group (Figures 4D and 4E), there is also an apparently more discrete collagen deposition in relation to the infected group untreated (Figure 4C).

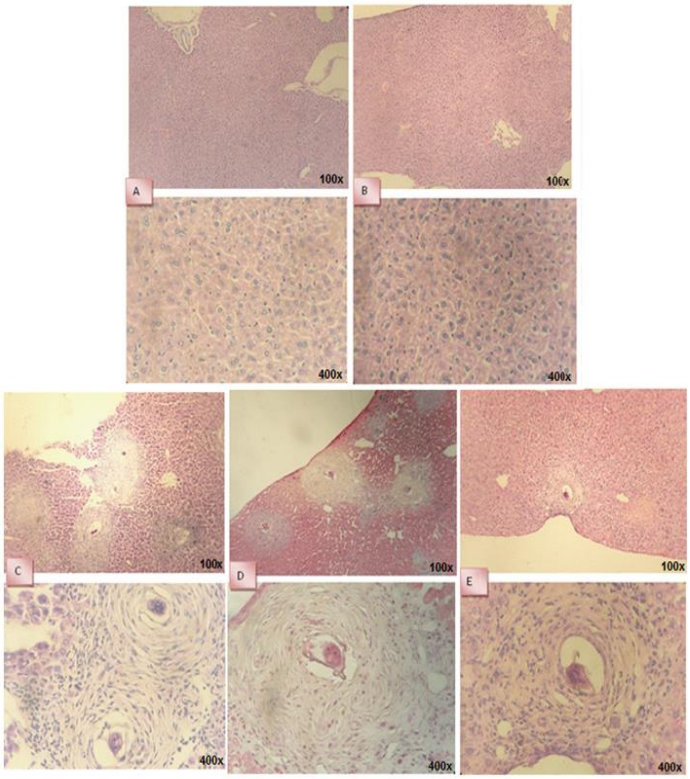


**Figure 3**. Photomicrography of liver histology sections from infected mice with *S. mansoni*, either treated or untreated. Morphological analysis of histological sections of liver on day 64 post parasite infection on animals on different experimental groups: control (A), control+(Ab) IgG (B), infected (C), infected+hamster anti-CD28 (D) and infected+PZQ (E) Staining: HE; Amplification: 100x and 400x.

**Discussion**

The process of initiating and maintaining granulomatous immune responses is induce by *S. mansoni* infection and requires recruitment and accumulation of inflammatory cells around the deposited eggs mostly in the liver and intestine of the host [10]. Thus, soon after sexual maturation of the parasites starts and subsequently oviposition also starts, egg antigens-reactive (SEA)-lymphocytes proliferate in the spleen [11] and the SEA-reactive lymphoblasts are recruited to the liver

granuloma [12]. Granuloma formation around *S. mansoni* eggs is the central event in the development of the pathology associated with both acute and chronic schistosomiasis infection, although it has been shown that antigens derived from other stages of the parasite are involved in both pathology development as well as resistance to infection by *S. mansoni* [13]. However granuloma modulation was demonstrated to be highly dependent on T cells. In our study, we observed that there was egg deposition in liver tissue and subsequently peri-ovular granulomatous response on day 64 post parasite infection in the experimental groups: infected and untreated, infected and treated with hamster anti-CD28 and infected and treated with PZQ (Figure 3C, 3D and 3E), but there was no statistical difference in number and volume and cellular composition of granulomas between the different experimental groups (Data not shown), but it is possible to observe a less intense cellular response in the infected group treated with hamster anti-CD28 when compared to the infected untreated group (Figure 3E).

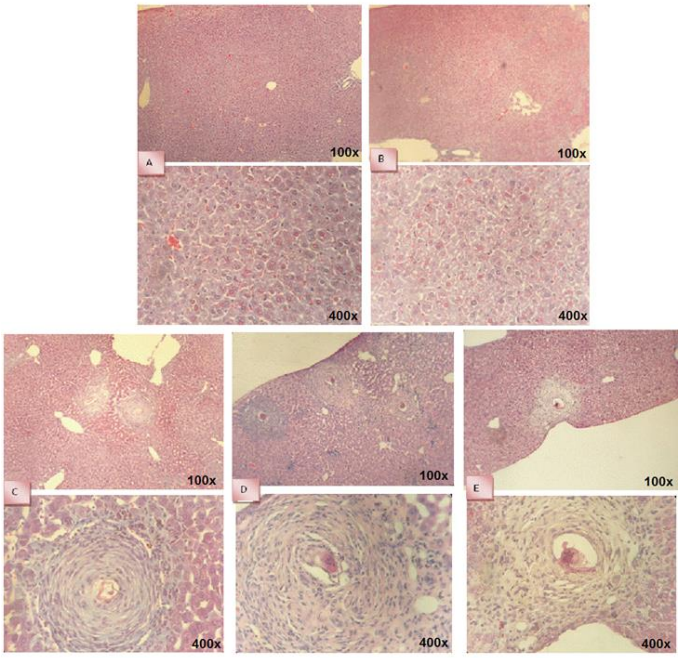


**Figure 4**. Photomicrography of liver histology sections from infected mice with *S. mansoni*, either treated or untreated. Morphological analysis of histological sections of liver on day 64 post parasite infection in animals in different experimental groups: control (A), control+(Ab) IgG (B), infected (C), infected+hamster anti-CD28 (D) and infected+PZQ (E)
Staining: MT. Amplification: 400x and 100x.

At the beginning of oviposition, and with the deposition of eggs in the tissues, cytokine production is predominantly directed to a Th2 response. Thus, there is an increased in the production of IL-4, IL-5 and IL-10. The role of IL-4 has been extensively explored in schistosomiasis. 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 the liver [14, 15]. On our study on day 64 post parasite infection there was a significant decrease in IL-4 in the group treated with hamster anti-CD28, when compared to the infected untreated group (Figure 1A), suggesting that treatment with hamster anti-CD28 can negatively modulate IL-4, favouring the contribution to the reduction or inhibition formation of fibrosis in schistosomiasis. These data corroborate the literature, as we notice a decrease in collagen deposition, resulting from fibrosis in this group (infected and treated with hamster anti-CD28) (Figure 4D) [18]. Silva and col. (2004) [18] 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 hamster anti-CD28 (Figure 2A) showed a low level of this Ig isotype on day 64 post parasite infection, which corroborates with the low levels of IL-4 on day 64 post parasite infection (Figure 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 to *Schistosoma* sp. infection. 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]. On this study, infected animals that were treated with hamster anti-CD28 showed a significant decrease in levels of IL-5 (Figure 1B) contributing to the fostering in the decrease expression of IgE in response to antigens of adult *S. mansoni* worms on day 64 post parasite infection (Figure 2B).

## Conclusion

As conclusion from our work we strongly suggest that *S. mansoni* mice infection treatment with anti-CD28 antibody on day 64 post parasite infection favours a decrease on IL-4 and IL-5 expression, also providing a high decrease in the levels of expression of IgG1 as well as a decrease on the collagen deposition resulting from fibrosis thus modulating the decrease in protective response by IgE.

## Conflict Of Interest

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

## Acknowledgments

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