Expression of the costimulatory molecules CD80, C86 and MHC II in Eosinophil, during the peak of eosinophilia in the syndrome larvas migrans

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ABSTRACT

High levels of eosinophils are important in several diseases such as allergy and parasitic infections. Eosinophilia has been associated with parasitic diseases mainly when the parasite invades the tissues and promotes damage at mucosal surfaces. In this paper, we evaluated the expression of the costimulatory molecules MHC II, CD80 and CD86 during the peak of eosinophilia to investigate if eosinophils have a phenotype potential to act as APC. To this end, we used syndrome of visceral larva migran (VLMS) model, a parasitic disease caused by *Toxocara canis*, one of the most frequent helminthes in dog puppies. Our results showed increased expression of CD80 and MHC II. In general, the enhanced expression of CD80 and MHC II and lowest CD86 expression may be correlated with cytokine production, since there was also an increase in IL- 4 and IL- 5. The increase in CD80 expression suggests the activation of the antigen specific immune response of *T. canis* occurs efficiently. Thus, we suggest a potential role of eosinophils to act as APC in VLMS experimental model.

Introduction

Eosinophils are hematopoietic cells originated from bone marrow. Its regulation and proliferation are monitored by growth factors such as GM-CSF (granulocyte-macrophage colony-stimulating factor) and interleukins IL-3 and IL-5, responsible for the differentiation and proliferation of eosinophils [1,2,3]. These products delay the eosinophils apoptosis in inflammatory site, increasing its survival rate for long periods [4,5]. Eosinophils remain for only 48 hours at the inflammation site until they die and are removed by macrophages.

One of the major functions of eosinophils is defense against helminths. They stimulate the release of IL-4 and IL-5 by Th2 cells, which for turn promote the IgE production and activation of eosinophils, favoring the secretion of enzymatic components [6]. Antigen presenting cells (APC) are able to ingest, process and present antigens. Dendritic cells (DC), macrophages, B lymphocytes and possibly eosinophils are APC [7]. However, these cells have different immune functions [8]. The clinical and experimental research has shown that eosinophils may to function as APC, being able to process and presenting a variety of antigens [9]. CD80 (B7-1) and CD86 (B7-2) are the most well costimulatory molecules characterized, and predominantly expressed on the APC surface [10,11]. Although CD80 and CD86 molecules have similar functions, the expression and the affinity them by receptors present in T cells (CD28 and CTLA-4) are different [12]. The activation and differentiation of T cells depends on two major signs. First, the contact between APC and T cells with recognition of peptide MHC complex and second,

an interaction of molecules on APC surface and cytokines production [13]. Experiments using eosinophils from upper airway of mice showed the capacity of eosinophils to process inhaled antigens, to migrate to lymph nodes and the express MHC, CD80 and CD86 (B7) molecules [9]. This feature allows us to suggest that these cells are able to present antigens as well as induce the TCD4 proliferation.

Intestinal nematodes cause some of the most prevalent parasitic infections in humans. *Toxocara canis* is an intestinal parasite of dogs, beyond to cause visceral larva migrans syndrome (VLMS) in humans. In rodents and humans, the larvae do not complete the migration through the lungs and into the intestines. Ingestion of embryonated *T. canis* eggs and your eclosion in small intestine release a larvae resulting in VLMS. The larvae migrate to other tissues may cause systemic inflammation due to reaction against excretory/secretory products released by the larvae [14].

In general, helminths parasites infect vertebrate hosts and promote a typical Th2-type inflammatory response marked by systemic eosinophilia. There is little evidence that eosinophils are capable of eliciting an immune response to parasite antigens. It has been demonstrated that eosinophils activated by GM-CSF are able to act as specific APC to a T-cell clone derived from mice infected with the cestode *Mesocestoides corti* [15]. In addition, eosinophils recovered from infection mice with *Brugia malayi* express high levels of MHC class II molecules [16].

This ex vivo study used mice infected with *T. canis* as model to investigate if eosinophils have a phenotype potential to act as APC.

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Material And Methods

Animals

Female Balb/c mice SPF (Specific Pathogens Free), weighing between 15 and 18 grams, were obtained from the animal facilities of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil. These animals were maintained under standard laboratory conditions throughout the experiments at the Department of Morphology and Pathology, Federal University of São Carlos (DMP-UFSCar) with free access to potable water and standard commercial diet. The experimental delineation this work was based upon the recommendations of the Animal Experimentation Ethical Principles, adopted by the Brazilian Society of Laboratory Animals Science (SBCAL) and was submitted and approved by the Ethics Commission in Animal Experimentation (CEEA) of the Federal University of São Carlos, No. 056/2009

Infection with *Toxocara canis*

Pregnant female worms were recovered from infected dogs and the eggs were obtained from the worms' uterus. Then, the eggs were washed and incubated in 2% formalin at 37°C to allow their progress to the infectious stage. Previously, the eggs *T. canis* were washed with 0,9% NaCl. Mice were infected by intragastric route with 0.5 ml of saline containing 500 embryonated eggs of *T. canis* [17].

Evaluation of Eosinophils influx in body fluids

At 18 days after infection, the animals were euthanized with a lethal dose of Tionembutal (North Chicago, Illinois USA), and blood samples were obtained by cardiac puncture. Lavage Peritoneal Cavity (LPC) cells were collected after the injection of 3ml PBS (Phosphate-Buffered Saline) containing 0.5% sodium citrate followed by aspiration. To collect the bronchoalveolar lavage fluid (BALF), a polyethylene cannula was introduced into the trachea and 2ml of PBS 0.5% sodium citrate was injected. Cells of BALF were recovered by aspiration. This procedure was performed twice to obtain greater numbers of cells. The count of the total number of leukocytes in the blood, PC and BALF was done in a Neubauer chamber. Absolute counts were obtained from the slides prepared by cytospin (SEROCITO MOD. 2400 FANEM) and stained with Rosenfeld. The plasma was stored at -20°C.

Flow cytometry

Expression of CD80, CD86 and MHC II molecules were determined through a flow cytometry immunostaining protocol using fluorescein isothiocyanate: (1) I-Ab (FITC), (2) MHC II (rat IgG2b), and (3) phycoerythrin - labelled anti-mouse CD80 (Armenian hamster IgG) with FITC-labelled anti-mouse CD86 (mouse IgG1) and blocking antibody ("Fc Block"). To isotype controls were used: (1) rat IgG2b, (2) irrelevant antibody (specifics unknown) labeled with FITC and PE, respectively. All antibodies were obtained from PharMingen (San Diego, CA, USA) and used according to manufacturer's instructions. Molecules expression analysis (CD80, CD86 and MHC II) was performed by flow cytometry in the device BD FACSCanto - Flow Cytometry, in Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil. Results were expressed in histogram and represent the average fluorescence intensity, which reflects the expression of MHC II, CD80 and CD86 on cell surface. Cells were primarily selected based on the size, as determined by the forward scatter (FSC). The granularity was determined by the side scatter (SSC) of their distribution patterns. The average fluorescence intensity the molecules were measured and the cell population were selected to represent granularity and complexity to lymphocytes or eosinophils. After euthanasia of mice, in time pre-established, lymphocytes and eosinophils were collected from the LPC and whole blood, being made "pool" of three animals. For this, we added 5mL of lysis buffer (8.02g NH4Cl, NaHCO3 0.84g, 0.370g EDTA) in each tube (15mL) and kept in the dark for 5 minutes on ice. Blood and PC cells were centrifuged, resuspended in 1mL of PBS-2% FBS (FACS buffer) and prepared a slurry of 1 x 107 cells/ml. Then, it was pipetted 100 µl of each sample (1 x 10^6 cells) in appropriate tube and incubated with blocking antibody nonspecific binding ("Fc block") in dark for 40 minutes. Subsequently, cells were incubated with antibodies labeled with fluorescent compounds for 30 minutes at 4°C. Immediately, washed with FACS buffer and resuspended in 300µL of fixation (1% formaldehyde-PBS).

Immunoenzymatic assay – ELISA

IL-4, IL-5 and IL13

Commercially available enzyme-linked immunosorbent assay antibodies were used to measure IL-4, IL-5 and IL-13 according to manufacturer's instructions (BDOptEIA™). Briefly, the assay was made in plasma and BALF, using purified mAbs as capture Abs and biotinylated mAbs as secundary Abs, followed by incubation with streptavidin-alkaline phosphatase and substrate. Plates were read in a 96-well spectrophotometer (Microquant-Sellex, Inc 450nm) and data were analyzed using software by comparison against a standard curve, which was generated using known concentrations of recombinant cytokines.

IgG1 and IgE

ELISA test (BDOptEIA™) was made in plasma and BALF. To IgG1 was standardized at 5mg/ml of total protein antigen *T.canis* and diluted 1:64. To IgE was used 10mg/ml of total protein antigen *T. canis* and diluted 1:4. Were used 96-well microplates and coated with 10 or 5mg/mL of total protein antigen of *T. canis* (diluted 0.1M carbonate buffer pH 9,6 and applied 100µl/well). The wells were blocked with PBS-1% BSA for 1 hour at room temperature. Then, the microplates were incubated with plasma or BALF from mice for 2 hours. Subsequently, we added biotin-conjugated secondary Ab and incubated for 1 hour. Streptavidin enzyme diluted 1:200 was added and incubated for 30 minutes. 100uL of the substrate (H_2O_2 and tetramethylbenzidine 1:1) were added, followed by the reaction blocking with 50µl/well of 1M H_2SO_4. The plate was washed 3 times with PBS containing 0.05% Tween-20 between each step. The absorbance reading was made at a wavelength of 450nm in the ELISA reader (Microquant-Sellex, Inc.).

Statistical analysis

The results were expressed as mean ± SEM. The results obtained in different experiments were analyzed by ANOVA. Statistical analysis was performed using the PRISM-(TWO-ANOVA) (San Diego, California, USA). The level of significance was 5%.

Results

Recruitment of eosinophils

During *T. canis* infection, an inflammatory reaction intense is characterized by a rise in total number of eosinophils in the blood, LPC and BALF. Time-dependent eosinophilia was typically identified in Blood, LPC and BALF (Figure 1). Furthermore, at the 18 day after infection, we observed eosinophilia in the all body fluids from infected mice (Figure1 A, B and C).
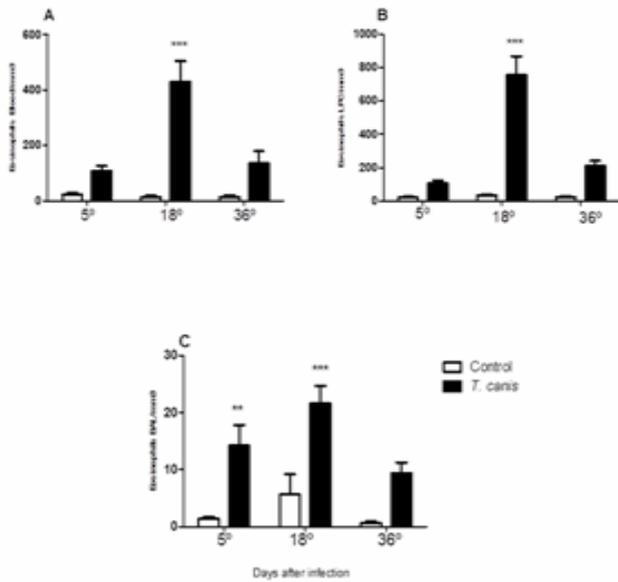
Figure 1: Total number of eosinophils in blood (A), LPC (B) BALF (C), with 5,18 and 36 days after infection. The data represent the mean +/- SEM (n =8 animals) of two independent experiments. P<0,01, *** P<0,001 represents significant differences using TWO – ANOVA test
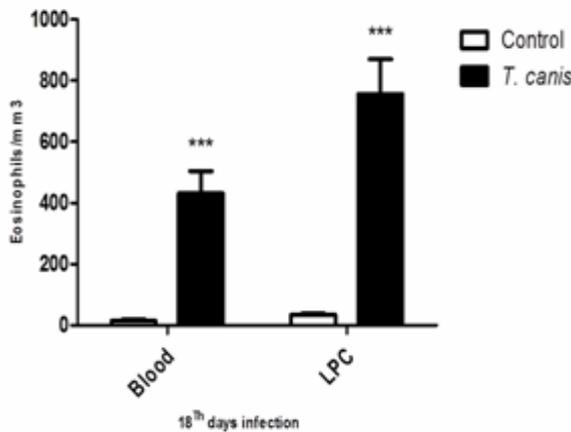

Figure 2: Relationship between the total number of eosinophils present in blood and LPC at the 18th day post infection
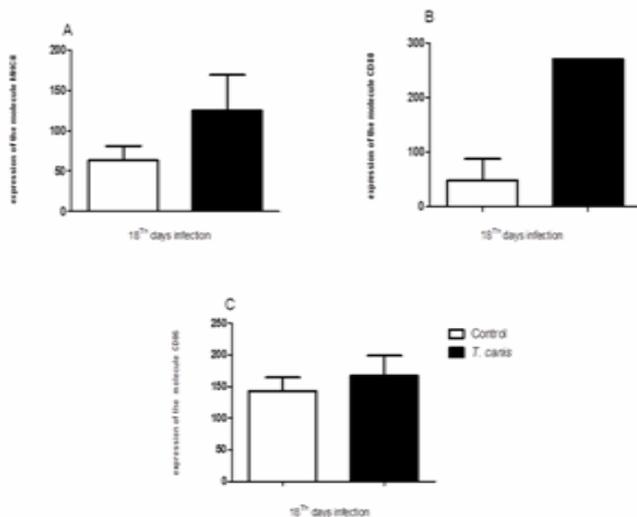

Figure 3: The average fluorescence intensity of molecules (CD80, CD86 and MHC II) in blood eosinophils. Control group is represented in white and the Control group in black

Expression profile of CD80, CD86 and MHCII molecules in blood eosinophils

We examined the expression of CD80, CD86 and MHCII molecules involved in eosinophils migration. At the 18 day post-infection, the blood eosinophils expression was analyzed by flow cytometry (Figure 3 A, B and C). Representative single-color Dot Blot illustrating the regulation of CD80, CD86 and MHC II in infected group compared with the control group (Figure 4).
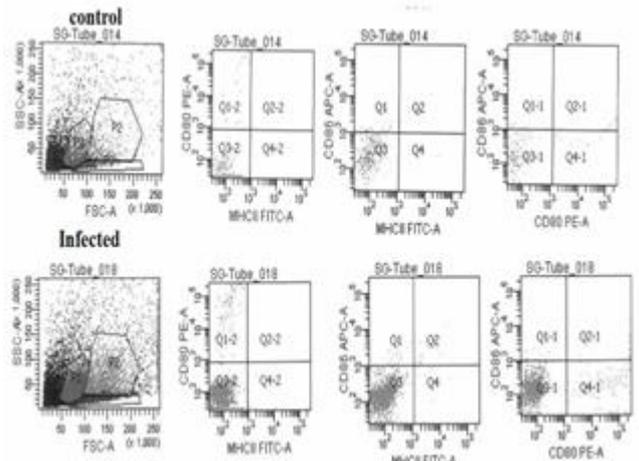

Figure 4: Single-colour Dot Blot showing CD80, CD86 and MHC II expression in the peripheral blood eosinophils at the 18th day post-infection. Data are expressed as mean ± standard deviation of the average fluorescence intensity (PE, APC and FITC) realized by flow cytometry analysis.

IL-4, IL-5 and IL-13 expression in plasma and BALF

IL-4, IL-5 and IL-13 levels were determined in plasma and BALF of mice, infected or not with *T. canis* on the days 5, 18 and 36 post-infection. The interleukin values are shown in figure 5. Infected group showed changes in the concentration of IL-4, IL-5 and IL-13. We observed a significant decrease of IL-4 and IL-13 (Figure 5 B and C) and also a decrease of IL-5 on day 18° in infected group (Figure 5 A). In BALF, there was significant decrease of IL-5 on day 5° (Figure 6 A).
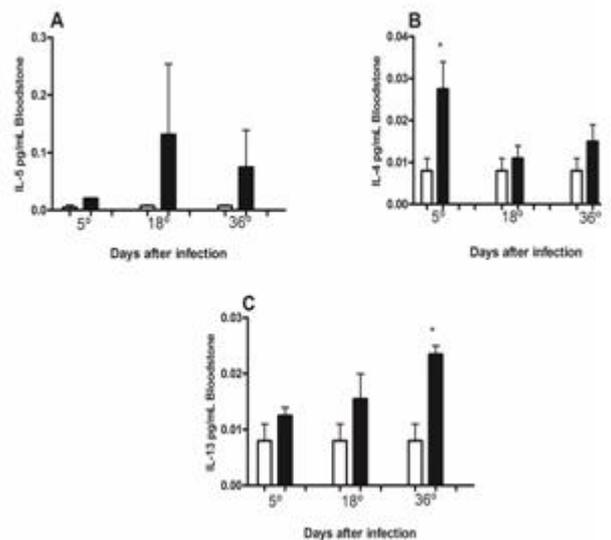

Figure 5: Expression of IL-5 (A), IL-4 (B) and IL-13 (C) in plasma with 5, 18 and 36 days after infection. The data represent the mean +/- SEM (n =8 animals) of two independent experiments using TWO-ANOVA test. P<0,01, *** P<0,001
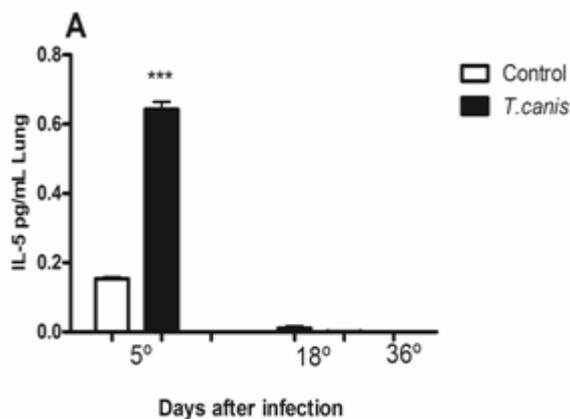
Figure 6: IL-5 expression in BALF (A) with 5, 18 and 36 days after infection. The data represent the mean +/- SEM (n =8 animals) of two independent experiments. P<0,01, *** P<0,001 represents significant differences using TWO-ANOVA test
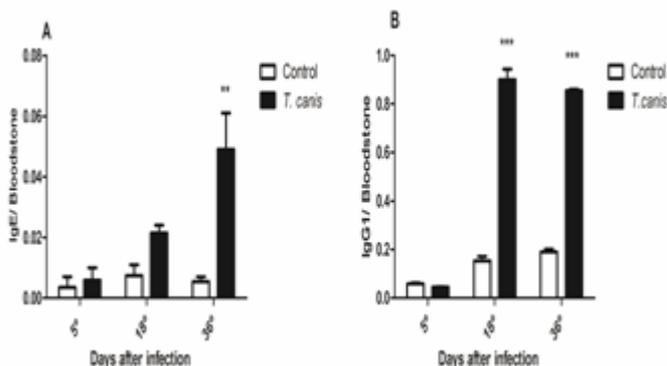

Figure 7: IgE (A) and IgG1 (B) levels in plasma with 5, 18 and 36 days after infection. The data represent the mean +/- SEM (n =8 animals) of two independent experiments. P<0,01, *** P<0,001 represents significant differences using TWO –ANOVA test
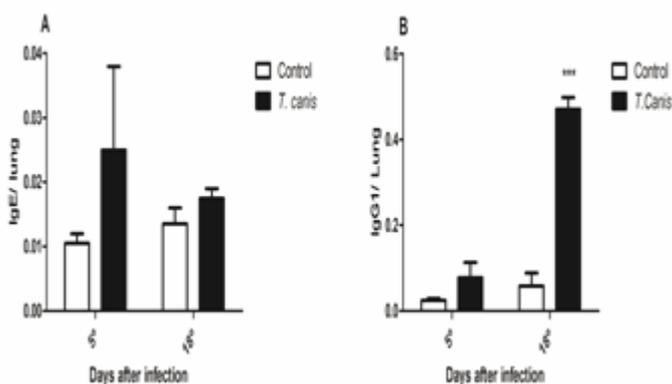

Figure 8: IgE (A) and IgG1 (B) levels in BALF with 5 and 18 days after infection. The data represent the mean +/- SEM (n =8 animals) of two independent experiments. P<0,01, *** P<0,001 represents significant differences using TWO – ANOVA test.

Expression of IgG1 and IgE levels in plasma and BALF

IgG1 and IgE reactive to antigens of *T. canis* present in plasma and BALF of the all groups are shown in Figure 7 and 8. The plasma of infected group showed a significant decrease in the levels of IgG1 in 18th and 36th day post infection (Figure 7B). Moreover, IgE levels in the infected group were significantly lower than control group at the 36º day post-infection (Figure 7A). In BALF, the infected group showed a

significant decrease in IgG1 levels at the 18º day post-infection (Figure 8B). IgE levels were lower in the group infected at the 5º day post-infection (Figure 8A).

Discussion

Eosinophils have a protective role against helminths along the infection, being a very important cell in inflammatory response observed in helminths infections, displaying cytotoxicity against parasites [18]. We observed a significant increase of blood eosinophils in LPC and BALF during infection by *T. canis* (Figure1). Our results corroborate those of other authors in which were demonstrated in guinea pigs model [15], C57BL/6 mice model [19], BALB/c mice model [20] and humans [21]. Some authors have been demonstrated that, in course of infection (VLMS), the inflammatory reaction is characterized by increased levels of leukocytes in blood, BALF [20,15,] and LPC on day 18 after infection, independent of the experimental model [22,20,15]. Our results corroborate those of other authors in which we observed a significant increase in eosinophils in blood and LPC using BALB/c mice model (Figure 2) during VLMS experimental model. Eosinophils have been found in inflammation by *T. canis* and are well known and described, however, are poorly correlated with the activity of these costimulatory molecules expressed. The signal generated by binding between antigens and MHCII molecules is not sufficient to activate a immune response lymphocytic. A second signal provided by other cells is also required. An investigating the literature revealed few reports, but they suggest that eosinophils may function as antigen-presenting cells (APC) in helminth infections. In allergic diseases has been demonstrated possible increase in the eosinophils as APC [9]. These data sets provide evidence that eosinophils have the capacity to stimulate T-naive cells, suggesting they may function as true APC [23]. Papers have been showing that when eosinophils are stimulated with *Strongyloides* antigens *in vitro*, MHC II molecules are expressed and are able to stimulate IL-4 and IL-5 production by naive T cells, possibly. Our results demonstrate that eosinophils during infection by *T. canis* may present an APC phenotype (Figure3), possibly working like a T cell activator in this model [7]. Costimulatory molecule CD86 wasn´t expressed, whereas the CD80 and MHCII molecules were observed in mice cells. In general, the enhanced expression of CD80 and MHC II molecule and lower expression of CD86 molecules on the mice infected cells may be correlated with cytokine production, since there was also an increase in IL-5 and IL-4 production (Figure 5). Other authors have been demonstrated that CD80 and CD86 costimulatory molecules may to influence the cytokines profile produced during helminths infection [24, 25, 26]. CD80 appears to be more efficient than CD86 in T cells activation mainly due to a higher avidity for CD28. A main function of eosinophils is defense against helminths. They stimulate Th2 lymphocytes to produce IL-4 and IL-5 leading to an increase IgE levels and eosinophils activation, respectively. The IgE binding on the helminths surface gives rise to immunocomplex, essential to enzyme secretion and for release of granular components [4]. Otherwise, there would be an eosinophils accumulation due to recognition of allergen by DC. This will lead to IL-5 production, a important step to eosinophils maturation and differentiation. [6]. IL-13 acts in the regulation of immune response and is similar to IL-4 on IgE synthesis. The administration of IL-13 in immunized mice causes no mucus accumulation in eosinophilic lung. Neutralization of IL-13 reduces eosinophils migration and mucus production in the lungs of vaccinated and challenged mice with ovalbumin [27]. However, we found an increase in

the level of IL-13 at the 5th and 18th day after infection (Figure 5C), corroborating with eosinophilia in the same period. These findings are in agreement with other studies, which report the prevalence these cytokines in this model and specific pattern of Th2-type response typical in VLMS [27,28,29,30]. Analyzing the cytokines profile produced from the culture of spleen cells, we found a significant increase in IL-4 (Figure 5A) and an increase in expression of IL-5 and IL-13 (Figure 5B and 5C). However, it is clear that the increase in IL- 4 was significantly greater than other cytokines analyzed in the groups receiving stimulation by *T. canis*. Thus, we suggest a possible dependent modulation of IL-4 and IL-5 in induced inflammation model favored by antigens of larval *T. canis*. A possible agent of the increased levels of total IgE secretion would be secretory products of nematodes parasites, factors that stimulate the IL-4 production and increase IgE levels [29,30]. Our results demonstrated that plasma IgG1 levels increased at the 18 and 36 days after infection in the infected group (Figure 7). These data confirm previous findings which the immune response profile showed to be predominantly Th2. Thus, this study suggests that eosinophils are fundamental in determining of the immune response and helps directly in Th2 lymphocytes activation during VLMS.

Conclusion

We found that eosinophils were able to express coestimulatory molecules such as CD80, CD86 and MHC II. Ours results suggested that eosinophils play an important role on the syndrome of visceral larva migrans, wherein they protect the host and are responsible for much of the inflammatory response.

Conflict of interest

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

Acknowledgments

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