

Prednisolone and cyclosporine A: Effects on an experimental model of ancylostomiasis

Sílvia Regina Costa Dias^a, Ana Flávia Dias Vieira da Costa^a, Pedro Henrique Gazzinelli-Guimarães^a, Bruno Mendes Roatt^b, Kátia da Silva Fonseca^b, Nívia Carolina Nogueira de Paiva^b, Rodolfo Cordeiro Giunchetti^b, Cláudia Martins Carneiro^b, Ricardo Toshio Fujiwara^{c,d,e}, Éli da Mara Leite Rabelo^{a,*}

^a Laboratório de Parasitologia Molecular, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, CEP 31270-901, Belo Horizonte, Minas Gerais, Brazil

^b Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas, Universidade Federal de Ouro Preto, Brazil

^c Laboratório de Imunologia e Genômica de Parasitos, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, CEP 31270-901, Belo Horizonte, Minas Gerais, Brazil

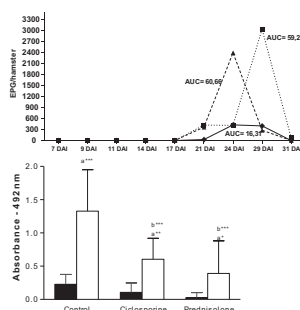
^d Laboratório de Imunologia Celular e Molecular, Instituto René Rachou, FIOCRUZ, Brazil

^e Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais (INCT-DT), Brazil

HIGHLIGHTS

- ▶ The infection by *Ancylostoma* causes peripheric immunosuppression in hamsters.
- ▶ Cyclosporine A does not interfere in the hamster's infection by *Ancylostoma*.
- ▶ Treatment with prednisolone affects the integrity of the intestinal mucosal.
- ▶ Inflammation of the intestinal mucosa does not favor the development of *Ancylostoma*.
- ▶ Prednisolone causes a decrease in egg laying of *Ancylostoma ceylanicum*.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 16 February 2012

Received in revised form 20 September 2012

Accepted 19 October 2012

Available online 7 November 2012

Keywords:

Ancylostoma ceylanicum

Cyclosporine

Glucocorticoids

Hamster

Immunosuppression

Hookworm

ABSTRACT

Corticosteroids and cyclosporine A (CsA) are important clinical immunosuppressive drugs used in the maintenance of organ transplants and in suppressing undesired autoimmune or allergic immune responses. To study the effect of CsA and prednisolone on the course of an *Ancylostoma ceylanicum* infection, hamsters were treated with commercially available prednisolone or CsA. For both drugs, half the recommended dose was sufficient to inhibit the proliferation of more than 70% of hamster lymph node cells. There was no difference in the recovery of adult worms; however, animals treated with prednisolone presented with low egg counts in the feces. Infection with *A. ceylanicum* resulted in an increase in specific antibodies against adult worm antigens, but hamsters treated with either drug presented with lower IgG titers. We observed that *A. ceylanicum* infection caused peripheral cellular immune suppression, which is characterized by a reduction in the total white cell count, neutropenia and lymphopenia. We also observed a lymphoplasmacytic pattern and few eosinophils in the mucosal inflammatory infiltrate for all the animals. The animals treated with prednisolone showed changes in the architecture of the intestine, including the loss of the mucosa, intense congestion and inflammation. In spleen, we observed hyperplasia of white pulp in all infected animals; in addition, there was a loss of tissue

* Corresponding author. Fax: +55 31 34992970.

E-mail address: rabelo@icb.ufmg.br (E.M.L. Rabelo).

architecture in the animals treated with prednisolone. In conclusion, this work shows that an *A. ceylanicum* infection leads to acute peripheral cellular immune suppression in hamsters but not humoral immune suppression and that CsA treatment does not interfere with the process of infection. However, prednisolone treatment causes intestinal injury, what could hamper the parasite attachment to the intestinal wall, and as a result affects copulation and, consequently, decreases the number of eggs eliminated in the feces. Moreover, the possibility that the drug can also be exerting an effect on female fertility should be considered.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Hookworms infect an estimated 740 million people worldwide and can lead to severe clinical manifestations in their host (De Silva et al., 2003). This infection is often neglected because it mainly affects poor population worldwide who live on less than two dollars per day (Hotez et al., 2005; Hotez, 2008). Hookworm parasites are thought to be able to evade or suppress the host immune response because infected people have high worm burdens and can be rapidly reinfected in endemic areas (Albonico et al., 1995, 2003; Loukas et al., 2006; Loukas and Prociv, 2001; Quinnell et al., 1993).

Our understanding of the immune responses against hookworm infection is based on immunological studies in endemic areas, which provide important, although limited, data. These data are limited because of the lack of information in the patients' medical history. Experimental models with dogs infected with *Ancylostoma caninum* or hamsters (*Mesocricetus auratus*) infected with *A. ceylanicum* have also added to our understanding. The hamster model has been used as a hookworm infection model in pathology, immunology and vaccination studies because it reproduces human pathology better than other hookworms, such as *Necator americanus* and *A. duodenale* (Dondji et al., 2010; Fujiwara et al., 2006; Mendez et al., 2005).

Infective larvae (L_3) that are administered via an oral route in the hamster develop into adult worms in the small intestine (Bungiro et al., 2001) and elicit a mixed Th1/Th2 response (Mendez et al., 2005). A similar response has also been observed in human hookworm infections (Fujiwara et al., 2006).

Corticosteroids are drugs that are used to suppress undesired autoimmune or allergic immune responses and those induced by graft rejection (Cohn, 1997; Magee et al., 2002; Trowald-Wight et al., 1998). Glucocorticoids, such as prednisolone, act through intracellular receptors that are expressed in almost every cell of the body. The complex formed by corticosteroids and their intracellular receptor interferes with the synthesis or binding of transcription factors to the promoter region of specific genes (Kamiyama et al., 2008; Paliogianni et al., 1993; Scheinman et al., 1995). Furthermore, glucocorticoids hormones are inducers of apoptosis in lymphocytes and other cells found in the spleen and other tissues (Bamberger et al., 1996; Kamiyama et al., 2008; Lucin et al., 2009; Riccardi et al., 1999). At the same time, glucocorticoids stimulate the migration of apoptosis-resistant cells to the spleen (Luzicova and Efimova, 2009). The immunosuppressive effects of glucocorticoids result in the inhibition of B lymphocyte migration to the bone marrow and in the release of cytokines (IL-1, IL-2 and IFN- γ) by leukocytes and macrophages, which suppress the activity of T and B lymphocytes, the degradation of complement components and the blockage of immunoglobulin receptors (Luzicova and Efimova, 2009; Scheinman et al., 1995; Wilckens and de Rijk, 1997).

Cyclosporine A (CsA) is an important clinical immunosuppressant that is used in the maintenance of organ transplants and the treatment of autoimmune diseases (Brazis et al., 2006; Dzik et al., 2002). The pharmacological effect of CsA depends on its binding to cyclophilin, its cytoplasmic receptor. This binding results in the following: (i) specific inhibition and reversible activation of several cell types, including T cells, eosinophils and mast

cells (Akhavan and Rudikoff, 2003; Triggiani et al., 1989; Robson, 2003); (ii) inhibition of the release of mediators such as histamine (Garcia et al., 1998) but not prostaglandins (Brazis et al., 2006); and (iii) apoptosis of T cell lines (Huss et al., 1995). Furthermore, CsA inhibits the production of cytokines that are normally induced upon T cell activation, especially IL-2, which is considered the main effect of the drug (Dzik et al., 2002; Fruman et al., 1992; Kahan, 1989). In addition, CsA inhibits the synthesis of nitric oxide (a pro-inflammatory molecule) in macrophages, hepatocytes and other cells (Farghali et al., 2002; Trajkovic, 2001).

In parasitic infections, CsA acts as an immunosuppressant and can either worsen the infection or delay the elimination of parasites (Bell et al., 1996). Indeed, evidence from the literature demonstrates that the *in vivo* anti-parasitic effect of the CsA can worsen certain infections (e.g., *Hymenolepis diminuta* and *Echinococcus multilocularis*), and relieve the condition of others (e.g., schistosomes, *H. microstoma* and *E. granulosus*) (Chappell and Wastling, 1992). However, it is not possible to predict when the administration of CsA to infected laboratory animals will result in an anti-parasitic or an immunomodulatory treatment because the effect seems to be independent of the taxonomic group and the location of the parasite in the host (Bell et al., 1996). Thus, the current study examined the effect of CsA and prednisolone during the course of *A. ceylanicum* infection in hamsters. Therefore, we evaluated the parasitological and immunopathological aspects of infection.

2. Materials and methods

2.1. Experimental design

Hamsters (*Mesocricetus auratus*), female, 4–6 weeks old, were housed in the Animal Facility at the Department of Parasitology, UFMG. Water and commercial diet were provided *ad libitum*. Animals at the facility are routinely treated with one dose 2 mg/kg Ivomec® (Ivomec® - Merial, Brazil) via gavage, 15 days prior *A. ceylanicum* infection.

Animals were divided into four treatment groups, with ten animals/each, according to the treatment received: (i) negative – untreated and uninfected; (ii) control – untreated and infected (see below); (iii) CsA – treated with CsA and infected; and (iv) Pd – treated with prednisolone and infected.

For infection, 50 L_3 of *A. ceylanicum* were inoculated via oral gavage into the upper digestive tract of the hamsters. Seven days after inoculation (DAI), feces were collected direct from cages at every three days to count eggs using a McMaster chamber (Gordon and Whitlock, 1939). Individual determination of eggs per gram of feces (EPG) was not performed due to the small volume of stool obtained from each animal. Therefore, statistical analyses are not presented to these data. To prevent the spontaneous elimination of adult worms, the animals were euthanatized at 31 DAI with an anesthetic overdose (150 mg/kg Thiopental®, Cristália, Brazil).

After euthanasia, part of the spleen was removed and placed in 10% formaldehyde–PBS solution (pH 7.2) for histopathological analysis. The small intestine was removed and opened in a Petri dish containing PBS, and adult parasites were recovered from the

intestinal mucosa. The worms were counted, sexed and frozen at -80°C for future antigen preparation, which was used in the immunological assays. After recovering the adults, a fragment of the bowel (about 10 cm from the pylorus) was fixed in hard cardboard and immersed in a 10% formaldehyde buffer solution (pH 7.2) for histopathological analysis.

All procedures followed the recommendations of the Ethics Committee on Animal Experimentation of Universidade Federal de Minas Gerais (CETEA/UFGM), project number 088/09.

2.2. Drug administration

Two commercially available drugs, prednisolone (Prelone[®], 3 mg, Aché) and CsA (Sandimun Neoral[®], 100 mg, Novartis), were administered via oral route at doses of 10 mg/kg (0.6 mg/hamster). The animals were treated for three consecutive days before the infection and, thereafter, with the purpose of minimizing toxic side effects (like renal dysfunction, hypertension, animal stress or life-threatening), the treatment was applied every other day until the end of the experiment.

2.3. Cell proliferation assay

Cell proliferation was assessed using an MTT assay as previously described (Chow et al., 2000). Briefly, 160 μL aliquots of a mesenteric lymph nodes cell suspension (1×10^6 cells) obtained from an uninfected hamster in RPMI 1640 medium (Invitrogen, USA), which was supplemented with 2% antibiotic/antimycotic solution (Invitrogen, USA), 1.6% L-glutamine (Synth, Brazil) and 10% heat-inactivated fetal calf serum (Cutlab, Brazil), were cultured in a flat-bottom 96-well plate with 20 μL of both drugs at varying concentrations of 20 μL of a 50 $\mu\text{g}/\text{mL}$ phytohemagglutinin (PHA) solution (Sigma Chemical Co. – St Louis, MO, USA) for 24 h in an atmosphere of 5% CO_2 at 37°C . Twenty microliters of a MTT solution (5 mg/mL) were added to each well. The cells and the plates were incubated for an additional 4 h. After incubation, the supernatants were carefully removed from the wells, and 100 μL DMSO (Merck, Germany) was added and thoroughly mixed. The optical density at 570 nm was determined on an ELISA plate reader. Cell proliferation was expressed as a percentage of the absorbance of the PHA-treated control cells after subtracting the absorbance from the appropriate blanks.

2.4. Larvae and adult crude antigen preparation

Crude antigen preparations were obtained by mechanically macerating the adult worms in a tissue grinder (Tissue Grinder, Fisher Scientific, USA), and L_3 extracts were prepared using sonication (Ultrasonic homogenizer, 40 cycles of 30 s). Both extracts were centrifuged to separate debris (20,800g, 10 min, 4°C). The protein concentration of the supernatants was measured using a BCA kit (BCA kit, Pierce, USA), as recommended by the manufacturer. The supernatant aliquots were stored at -80°C until future use.

2.5. ELISA assay

Humoral immunity was determined by measuring the response against L_3 and adult *A. ceylanicum* crude antigen. Anti-*A. ceylanicum* IgG concentration in plasma was measured by ELISA. Briefly, plates (BD Falcon, USA) were coated with 1 $\mu\text{g}/\text{mL}$ of antigen in carbonate buffer (0.05 M NaHCO_3 , pH 9.6) for 18 h at 4°C . For blocking, the plate was incubated for 2 h at 25°C in a PBS-0.05% Tween 20 (PBST) solution containing 2% casein (Molico[®] – Nestlé). Sera were analyzed individually diluted 1:100 and incubated overnight at 4°C . The secondary antibody (Biotinylated anti-Armenian and Syrian hamster IgG – BD Pharmingen), diluted 1:5000, was

incubated for 2 h at 25°C . Streptavidin (R&D Systems, USA), diluted 1:200 in 0.05% PBST, was then added and the plate was incubated for 20 min. The assay was developed with O-phenylenediamine dihydrochloride – OPD[®] (Sigma Aldrich, USA) for 30 min in the dark. The reaction was stopped by adding 50 μL of 2 N H_2SO_4 to each well of the plate. The plate was read at 492 nm on a spectrophotometer (VersaMax Microplate Reader, Molecular Devices Inc., USA), using the SoftMax Pro Software. After each incubation step (except after the incubation with OPD[®]), the plate was washed five times with 0.05% PBST.

2.6. Blood cell count

Cellular parameters were assessed on the day of infection and on the day of sacrifice, 1 mL of blood was collected from the retro orbital plexus to perform a complete blood count (Veterinary Hematology Analyzer BC2800, Quibasa, Brazil) and to obtain plasma. Reference values for hamsters were obtained from Mitruka and Rawnsley (1981) and Gad and Hess (2007).

2.7. Histopathological analysis

Spleen and small intestine samples fixed with 10% neutral buffered formalin at the time of sacrifice were embedded in paraffin. The sections (5 μm thick) were stained with hematoxylin and eosin (H&E) for a qualitative analysis of histological changes. The parameters analyzed for the small intestine included inflammation, loss of villi and/or mucosa and an increase in the number of goblet cells. In the spleen, the levels of white pulp hypertrophy and inflammatory infiltrate were evaluated in the capsule and trabecula.

2.8. Statistical analysis

The results were expressed individually (each point) with the arithmetic mean (black line) or as the mean $\pm$ standard deviation (SD). The Grubbs test was used to detect outliers, which were removed from the sample. The effects for each treatment/infection were tested using ANOVA, with 5% level of significance ($p < 0.05$), using the statistical package Graph Pad Prism 5.0.

3. Results

3.1. Hamsters immunosuppression

The results show that cells from the mesenteric lymph nodes stimulated with PHA exhibited a wide variation in cell proliferation when the cells were incubated with different doses of prednisolone ($p > 0.05$). For all the doses tested, cell proliferation was less than that of the cells incubated without drug ($p < 0.01$) (Fig. 1A). In this trial, 0.3 mg of prednisolone, the half of the dose administered to hamsters, was sufficient to inhibit more than 70% of proliferation compared to the group stimulated with only PHA ($p < 0.001$).

Only high doses ($\geq 30 \mu\text{g}/\text{mL}$) of CsA (Fig. 1B) were able to inhibit more than 75% the proliferation of mesenteric lymph node cells from hamsters when compared to stimulated cells without the drug ($p < 0.001$).

3.2. Adult worm's recovery

Fig. 2A shows the number of adult *A. ceylanicum* worms that were recovered from the small intestine of each individual hamster. There was no statistical difference between groups ($p > 0.05$); however, compared to the control group (mean = 16.4), more worms were recovered from the animals treated with CsA (mean = 21.7), whereas fewer worms were recovered from animals

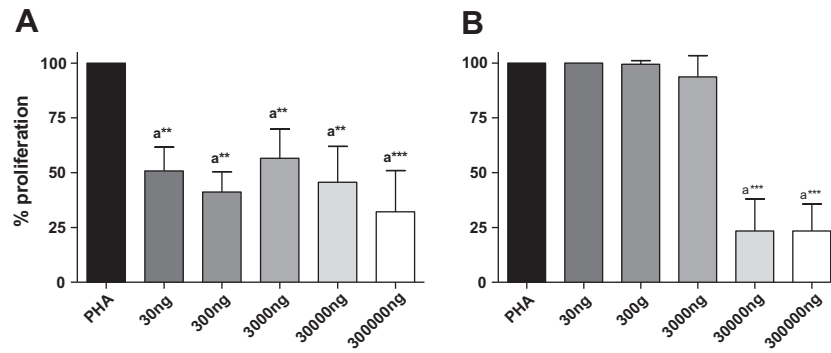


Fig. 1. Proliferation of mesenteric lymph node cells by MTT assay. Cells from uninfected hamsters were cultured and stimulated with PHA and incubated with prednisolone (A) or cyclosporine A (B) at different concentrations for 24 h in an atmosphere of 5% CO₂ at 37 °C. MTT solution was added and the plates were incubated for 4 h. The optical density at 570 nm was determined and the cell proliferation was expressed as a percentage of the absorbance of the PHA-treated control cells after subtracting the absorbance from the appropriate blanks. Data are shown as the mean + SD. *Statistically significant compared to the culture stimulated with only PHA. **($p < 0.01$), ***($p < 0.001$).

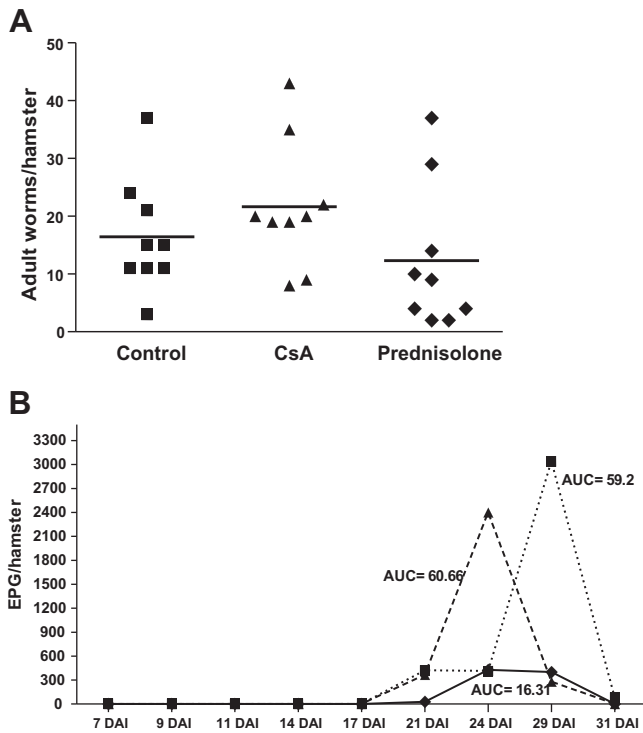


Fig. 2. (A) Recovery of adult *Ancylostoma ceylanicum* worms from individual hamsters sacrificed at 30 DAI. (B) Elimination of eggs per gram of feces (EPG) determined from feces collected direct from cages at every three days (starting at 7 DAI) and eggs number counted using a McMaster chamber. Groups of animals untreated and treated with cyclosporine A or prednisolone and infected with 50 L₃ larvae by oral route. Symbols correspond to: ■ control group (untreated and infected); ▲ hamsters treated with cyclosporine A and infected; ◆ hamsters treated with prednisolone and infected. The bars indicate the average value of the group.

treated with prednisolone (mean = 12.3). There was a wide variation for all the groups in the number of parasites recovered from each individual. The male:female proportion was similar in all infected groups.

3.3. Egg counts

The prepatent period for all the groups was 21 DAI. We observed an earlier egg elimination peak in animals treated with CsA compared to the control group (Fig. 2B), and the area under the curve (AUC) indicates that these animals eliminated more eggs

than the control group. Animals treated with prednisolone did not have an egg elimination peak; instead, they maintained low eggs-per-gram (EPG) counts throughout the observation period.

3.4. IgG response

We determined the humoral response by measuring IgG antibody titers, which recognize *A. ceylanicum* L₃ and adult worm crude antigens, in plasma taken from animals on the day of infection and the day of sacrifice. At sacrifice, the control group had higher antibody titers against adult worm crude antigens, whereas the CsA and prednisolone groups had lower levels of circulating IgG ($p < 0.001$) (Fig. 3A). All animals had minimal antibody titers against L₃ crude antigens on the day of infection and the day of sacrifice (data not shown).

3.5. Hematological parameters

3.5.1. Red and white blood cells

Infected animals showed a decrease in the total erythrocyte count compared to the negative animals (uninfected) ($p < 0.001$), but the counts still remained within normal physiological limits (Fig. 3B). All infected groups showed a decrease in the number of circulating leukocytes, especially the control ($p < 0.05$) and the prednisolone treated animals ($p < 0.05$), in which the values were below the normal physiological limits (Fig. 3C).

3.5.2. WBC differential count

Control and prednisolone treated animals showed a decrease in the number of circulating lymphocytes, whereas there were no differences in the negative group ($p > 0.05$). However, animals that were treated with prednisolone had a greater variability in the number of lymphocytes compared to the other groups (Fig. 3D).

We observed reduced numbers of neutrophils in peripheral circulation in all the infected animals compared to hamsters of the negative group ($p < 0.05$) (Fig. 3E). Moreover, the score profiles are more homogeneous for all the infected animals compared to the negative group.

Eosinophil counts for the negative animals indicate that most of the animals presented with a higher degree of eosinophilia prior to infection, in which values were well above normal physiological levels (Fig. 3F). However, this trend was reversed after the treatment and infection, in which the number of eosinophils decreased, especially in the control and CsA groups ($p < 0.05$).

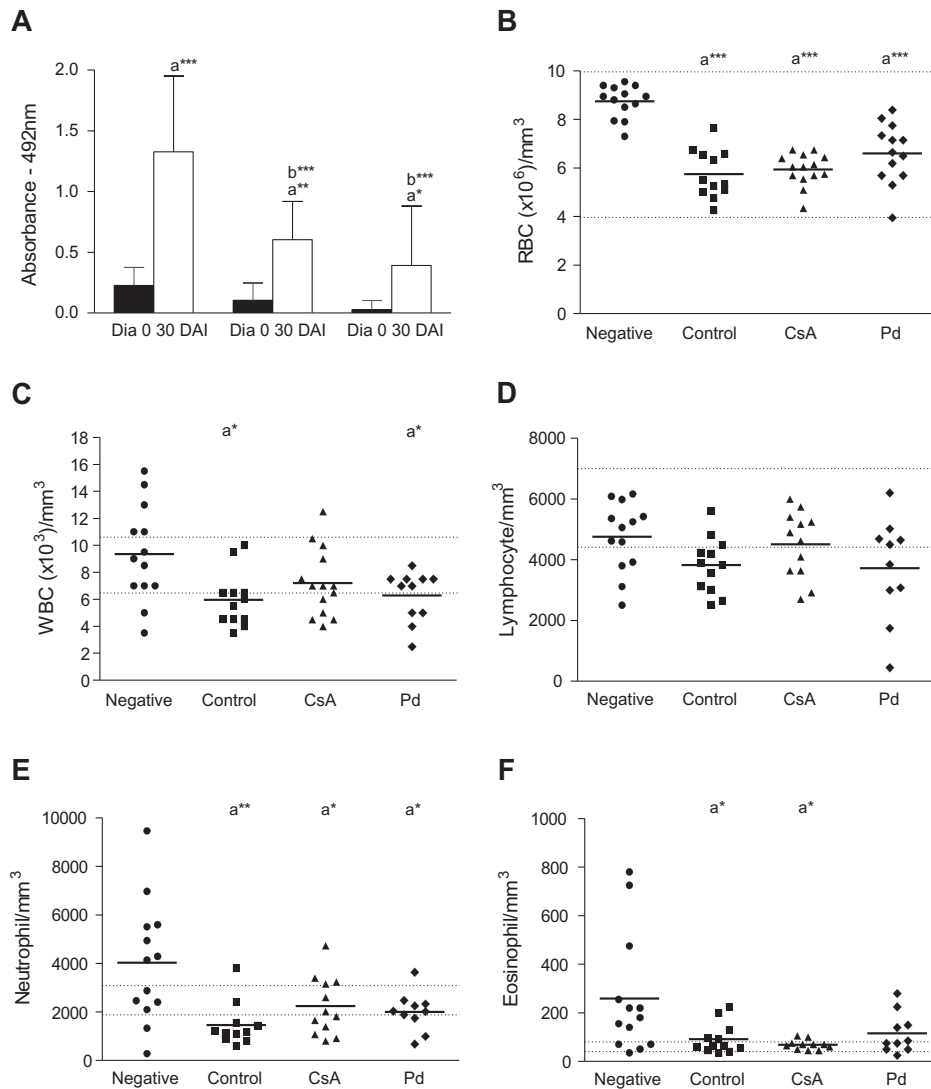


Fig. 3. (A) IgG antibody response – Anti-*A. ceylanicum* IgG levels measured by ELISA. Plasma of untreated hamsters and hamsters treated with cyclosporine A or prednisolone and infected with 50 L_3 larvae by oral route were analyzed individually (1:100). Black bars indicate the values obtained upon inoculation, and the white bars indicate the values obtained at sacrifice. ^aStatistically significant compared to day 0 (infection). ^bStatistically significant compared to the control group. Data is shown as the mean + SD. Hematological parameters on the day of infection and on the day of sacrifice: (B) total red and (C) total white blood cells count per individual per cubic millimeter of blood. Differential counting of leukocytes are representing in D–F: (D) lymphocytes, (E) total neutrophils and (F) eosinophils per individual per cubic millimeter of blood. ^aStatistically significant compared to the negative group. Symbols correspond to: ● negative group (untreated and uninfected). At sacrifice: ■ control group (untreated and infected), ▲ infected hamsters treated with cyclosporine A, ◆ infected hamsters treated with prednisolone. The dashed lines indicate the normal physiological values for the species. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.6. Histopathological analysis

Inflammation was not present in the intestinal muscular layer for any of the infected groups. A lymphoplasmacytic infiltration was observed in the small intestine of infected animals, and few eosinophils were observed in the mucosal inflammatory infiltrate, which is suggestive of chronic inflammation (Fig. 4). In control group, 40% of the animals showed a loss of intestinal epithelium, while maintaining the framework of the villi (Fig. 5). *A. ceylanicum* infection led to an increase in goblet cells (Fig. 4E and H), except for the hamsters treated with prednisolone (Fig. 4K). With the prednisolone treatment, the animals showed changes in the architecture of the intestine, including the loss of mucosa and intense congestion and inflammation (Figs. 4J,K and 5). In other groups, there was only a decrease of the height of the villus (Figs. 4D,G and 5).

Unlike the negative group (Fig. 4C), all the infected animals showed hyperplasia of white pulp in the spleen (Fig. 4F,I,L). In

the animals treated with prednisolone, there was a loss of tissue architecture (Fig. 4L).

4. Discussion

Although ancylostomiasis affects millions of people in different health and medical conditions worldwide, with exception of anti-helminthic drugs, there is an evident lack of data showing the effect of drugs in the course of hookworm infection in both, human and experimental models of infections. In this work, the study of the effect of two immunosuppressive drugs (CsA and prednisolone) with different action mechanisms administered in experimental dose of 10 mg/kg has contributed to elucidate some aspects of the hookworm biology. *In vitro*, we observed a reduction in cell proliferation for both drugs. However, despite the extrapolation to the *ex vivo* be complex, studies have shown that the living

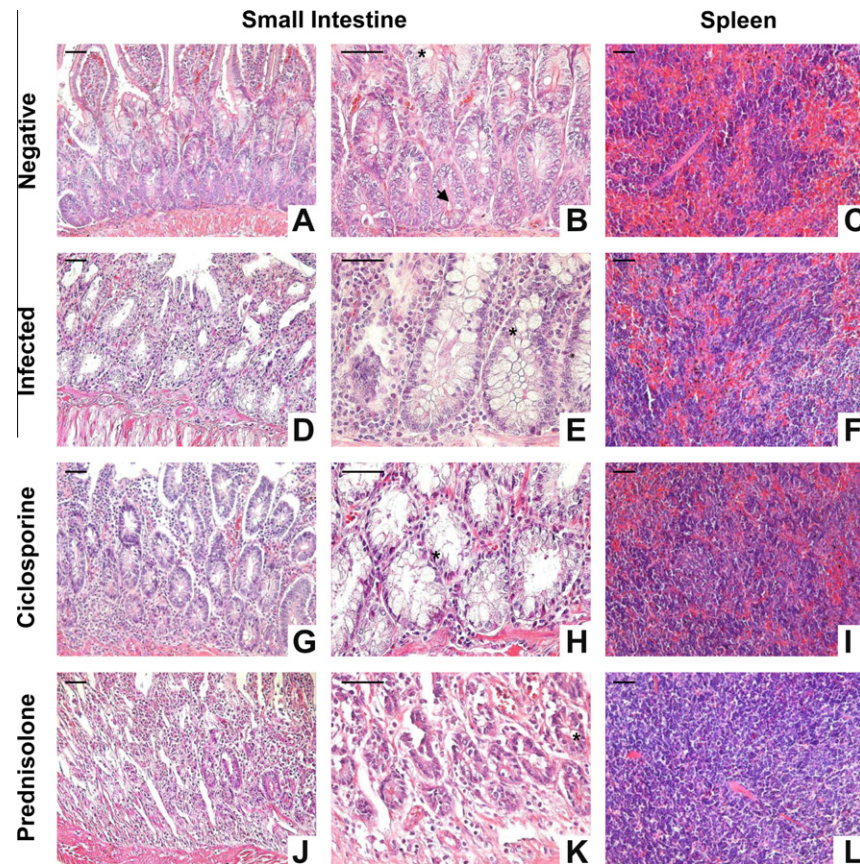


Fig. 4. Small intestine and spleen histopathological analysis. Photomicrographs of small intestine sections (A, B, D, E, G, H, J, K) and spleen sections (C, F, I, L). Negative group (untreated and uninfected) (A, B and C). Control group (untreated and infected) (D, E and F). Infected hamsters treated with cyclosporine A (G, H and I). Infected hamsters treated with prednisolone (J, K and L). Hematoxylin-eosin staining. Bar = 50 μ m.

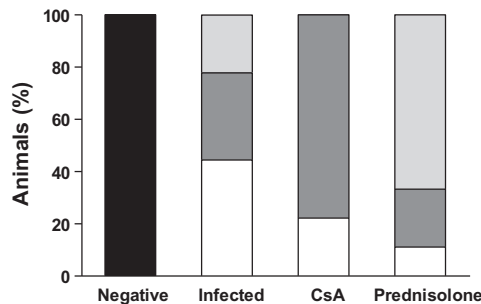


Fig. 5. Frequency of progressive lesions incidence in the small intestine wall of infected and uninfected hamsters that were left untreated or treated with cyclosporine A or prednisolone. Small intestine of uninfected and infected hamsters treated with cyclosporine A or prednisolone were classified based on hematoxylin-eosin sections: intact epithelium (black bar), loss of epithelium (white bar), loss of mucosa (light gray bar) or loss of villi (dark gray bar).

organism has additional factors to improve the response of lymphocytes to the inhibitory effects of the drug (Ferron and Jusko, 1998; Magee et al., 2002).

Studies have shown that the CsA and its metabolites can be detected in mouse tissues for more than two weeks after treatment (Kahan, 1985; Bout et al., 1985; Chappell et al., 1987; Bell et al., 1996). Steroids are available in several formulations. Moderately insoluble esters, including prednisolone acetate, release the active ingredient gradually over days or weeks (Cohn, 1997). In this work, we opted to administer the drugs every other day to minimize the toxic side effects, namely renal dysfunction and hypertension that result from prolonged treatment with CsA (Bell et al., 1996) and

effects that range from annoying (e.g., polyuria) to the life-threatening (e.g., opportunistic infection) for prednisolone (Cohn, 1997).

Although all groups have presented similar pre patent periods, animals treated with CsA showed an earlier egg elimination peak in comparison to the other groups. The highest average recovery of adult worms may have been responsible for the major amount of eggs released by this group during the experimental period. In contrast, animals treated with prednisolone eliminated very few eggs, although there was no difference between the numbers of adult worms, especially females, recovered for this group. In this case, we suggest that the drug may have interfered directly with female parasite oviposition; however, host immunity may influence the number of eggs shed by adult female nematodes (Strain et al., 2002) and the subsequent development of those eggs (Jorgensen et al., 1998).

A. ceylanicum infection leads to an increase in specific antibodies against adult worm antigens but not larva antigens. We suggest that the low recognition of antibodies against the larva form of the parasite is due to the short period of time during which the host organism comes into contact with their antigenic products (L_3) because larvae do not make the pulmonary migration path in an oral infection (Fujiwara et al., 2006; Garside and Behnke, 1989; Ghosh et al., 2006; Loukas and Prociw, 2001). As previously described by other authors, high levels of antibodies against adult worm antigens from *A. ceylanicum* were found for the infected and untreated groups in our study (Bungiro et al., 2001, 2008; Garside et al., 1989; Mendez et al., 2005). Greer et al. (2005), who evaluated the immune response of *Trichostrongylus columbriformes*-infected sheep that were treated with prednisolone, showed a lower antibody response in treated sheep compared to untreated sheep. Our results

are consistent with previously published results for these two immunosuppressive treatments, especially for prednisolone, in which the results for the immune responses, were statistically significant compared to the control group (infected and untreated).

This work analyses peripheral cellular response in hamsters infected with *A. ceylanicum* where it was observed that infection causes a framework of peripheral cellular immune suppression, which is characterized by a reduction in total white cell count, neutropenia, lymphopenia and eosinopenia. Similar values were also seen for animals treated with CsA or prednisolone. Although there is little conclusive information about the immune response during experimental hookworm infection, our finding are consistent with other authors who have demonstrated that experimental infection models of human hookworm or other human intestinal worms are associated with impaired lymphocyte proliferation, impaired antigen processing and presentation and increased secretion of nitric oxide (Jenson et al., 2002; Dondji et al., 2008). Regarding eosinophils, Garside et al. (1989) also found physiologically elevated counts in uninfected hamsters, and the literature shows that hookworm infection stimulates migration of these and other peripheral blood leukocytes for cellular infiltration that is established at the site where the parasite feeds, especially during the maturation of adult worms in the intestine (Carroll and Grove, 1984; Croese and Speare, 2006). However, inflammatory foci with abundant eosinophils were not detected in intestinal sections. Recent reports have suggested that helminthic worms secrete specific molecules to interfere with components of the eosinophil-mediated tissue inflammatory response, which leads to the down regulation of this response during helminth infection (Maizels et al., 2004; Loukas and Prociv, 2001; Geiger et al., 2011). This secretion represents one mechanism by which the parasitic worms evade the host immune response. For example, some hookworm metalloproteases cleave eotaxin, which is derived from eosinophils, and block its chemotactic effects *in vivo* and *in vitro* (Culley et al., 2000; Shin et al., 2009). Moreover, rodents are known to be susceptible to glucocorticoids and, therefore, have classic components of a “stress leukogram”, which is characterized by mature neutrophilia (not observed in this work), lymphopenia via lymphocyte lysis and eosinopenia (Cohn, 1997). However, *A. ceylanicum* infection appears to have masked any adverse effects of the treatment, given that there were no differences in hematological results between treated and untreated animals.

The lymphoplasmohistiocytic profile of the intestinal mucosa, which we found in all infected animals, is consistent with data from the literature that have shown high levels of secreted antibodies in the feces of infected hamsters, especially IgA (Bienenstock, 1970; Bungiro et al., 2008; Haakenstad and Coe, 1971). Alkazmi et al. (2006) showed that hamsters infected with *A. ceylanicum* have changes in cell proliferation and marked alterations in the architecture of the mucosa of the small intestine. The authors also emphasize that these changes are dependent on the presence/absence of worms, the density of worms (intensity of infection) and the duration of infection and, furthermore, that the normal structure of the intestine is restored after the adult parasites are eliminated (Alkazmi et al., 2006). Several studies have shown that hookworms have the ability to create a site of ‘immune privilege’ around themselves in the intestinal mucosa (Chow et al., 2000; Loukas and Prociv, 2001; Croese et al., 2006; Croese and Speare, 2006). According to some authors, this strategy could induce the formation of an infiltrate of activated leukocytes, including eosinophils, at the site of parasite colonization, which would lead to apoptosis, reduced inflammation and, consequently, an asymptomatic infection (Chow et al., 2000; Loukas and Prociv, 2001; Croese et al., 2006). The intestinal sections of animals treated with prednisolone are characterized by a change in the architecture of the organ, with intense loss of mucosa and inflammation

and congestion, whereas the other two groups were characterized by mild inflammation. These histopathological results agree with our parasitological findings, suggesting that the prednisolone treatment may cause the mucosa degradation creating an unfavorable environment and forcing the worms to change their location frequently, what leads to a negative nutritional balance to the worms, decreasing their sizes and making the chances of mating more difficult and, consequently, decreasing the number of eggs eliminated in the feces (Croese et al., 2006; Alkazmi et al., 2006; Bungiro et al., 2008).

We evaluated the histology of the spleen from infected animals, and the influence treatments had on the organ. Immune stimulation, characterized by hyperplasia of white pulp, was observed in infected animals. Studies have shown that the spleens of animals that have been infected with hookworms for thirty days are approximately three times larger than those of uninfected mice (Garside et al., 1989; Dondji et al., 2008). Conversely, other studies have shown that spleen and mesenteric lymph node cells from *A. ceylanicum*-infected animals shown minimal or a lack of proliferation in response to hookworm antigen (Garside et al., 1989; Geiger et al., 2004, 2007; Dondji et al., 2008; Fujiwara et al., 2009), which is consistent with our results indicating blood cell immunosuppression in the infected group.

5. Conclusions

We conclude that an *A. ceylanicum* infection leads to acute peripheral cellular immune suppression in hamsters but not humoral suppression and that CsA treatment, in the dose of 10 mg/kg, does not interfere with the process of infection. However, prednisolone acetate treatment, at the dose of 10 mg/kg, causes injury to the intestine of the host and leads to decreased maintenance of the parasite on the intestinal wall, which affects the worm development and, consequently, decreases the number of eggs eliminated in the feces.

Acknowledgments

We would to thanks Mr. Carlos Manoel, for the biotery cares. This work received financial support from PRPq/UFGM, CNPq, FAP-EMIG (project number CBB-APQ-0280-4.01/07) and CAPES institutions.

References

- Akhavan, A., Rudikoff, D., 2003. The treatment of atopic dermatitis with systemic immunosuppressive agents. *Clinics of Dermatology* 21, 225–240.
- Albonico, M., Smith, P.G., Ercole, E., 1995. Rate of reinfection with intestinal nematodes after treatment of children with mebendazole or albendazole in a highly endemic area. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 89, 538–541.
- Albonico, M., Bickle, Q., Ramsan, M., Montresor, A., Savioli, L., Taylor, M., 2003. Efficacy of mebendazole and levamisole alone or in combination against intestinal nematode infections after repeated targeted mebendazole treatment in Zanzibar. *Bulletin of World Health Organization* 81, 343–352.
- Alkazmi, L.M., Dehlawi, M.S., Behnke, J.M., 2006. The effect of the hookworm *Ancylostoma ceylanicum* on the mucosal architecture of the small intestine in hamsters. *Journal of Helminthology* 80, 397–407.
- Bamberger, C.M., Schulte, H.M., Chrousos, G.P., 1996. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocrine Reviews* 17, 245–261.
- Bell, A., Roberts, H.C., Chappell, L.H., 1996. The antiparasite effects of cyclosporine A: possible drug targets and clinical applications. *General Pharmacology* 27, 963–971.
- Bienenstock, J., 1970. The significance of secretory immunoglobulins. *Canadian Medicine Association Journal* 103, 39–43.
- Bout, D., Desl  e, D., Capron, A., 1985. Antischistosomal effect of cyclosporine A: cure and prevention of mouse and rat schistosomiasis *mansoni*. *Infection and Immunity* 52, 823–827.
- Brazis, P., Barandica, L., Garc  a, F., Clough, G.F., Church, M.K., Puigdemont, A., 2006. Dermal microdialysis in the dog: in vivo assessment of the effect of cyclosporine

- A on cutaneous histamine and prostaglandin D2 release. *Veterinary Dermatology* 17, 169–174.
- Bungiro Jr., R.D., Greene, J., Kruglov, E., Capello, M., 2001. Mitigation of hookworm disease by immunization with soluble extracts of *Ancylostoma ceylanicum*. *Journal of Infectious Disease* 183, 1380–1387.
- Bungiro Jr., R.D., Sun, T., Harrison, L.M., Shoemaker, C.B., Capello, M., 2008. Mucosal antibody response in experimental hookworm infection. *Parasite Immunology* 30, 293–303.
- Carroll, S.M., Grove, D.I., 1984. Parasitological, hematologic, and immunologic responses in acute and chronic infections of dogs with *Ancylostoma ceylanicum*: a model of human hookworm infection. *Journal of Infectious Disease* 150, 284–294.
- Chappell, L.H., Thomson, A.W., Barker, G.C., Smith, S.W., 1987. Dosage, timing, and route of administration of cyclosporin A and nonimmunosuppressive derivatives of dihydrocyclosporin A and cyclosporin C against *Schistosoma mansoni* in vivo and in vitro. *Antimicrobial Agents and Chemotherapy* 31, 1567–1571.
- Chappell, L.H., Wastling, J.M., 1992. Cyclosporin A: antiparasite drug, modulator of the host–parasite relationship and immunosuppressant. *Parasitology* 105, 25–40.
- Chow, S.C., Brown, A., Pritchard, D.I., 2000. The human hookworm pathogen *Necator americanus* induces apoptosis in T lymphocytes. *Parasite Immunology* 22, 21–29.
- Cohn, L.A., 1997. Glucocorticosteroids as immunosuppressive agents. *Seminars in Veterinary Medicine & Surgery (Small Animal)* 12, 150–156.
- Croese, J., Speare, R., 2006. Intestinal allergy expels hookworms: seeing is believing. *Trends in Parasitology* 22, 547–550.
- Croese, J., Wood, M.J., Melrose, W., Speare, R., 2006. Allergy controls the population density of *Necator americanus* in the small intestine. *Gastroenterology* 131, 402–409.
- Culley, F.J., Brown, A., Conroy, D.M., Sabroe, I., Pritchard, D.I., Williams, T.J., 2000. Eotaxin is specifically cleaved by hookworm metalloproteases preventing its action in vitro and in vivo. *Journal of Immunology* 165, 6447–6453.
- De Silva, N.R., Brooker, S., Hotez, P.J., Montresor, A., Engels, D., Savioli, L., 2003. Soil transmitted helminth infections: updating the global picture. *Trends in Parasitology* 19, 547–551.
- Dondji, B., Bungiro, R.D., Harrison, L.M., Vermeire, J.J., Bifulco, C., McMahon-Pratt, D., Capello, M., 2008. Role for nitric oxide in hookworm-associated immune suppression. *Infection and Immunity* 76, 2560–2567.
- Dondji, B., Sun, T., Bungiro, R.D., Vermeire, J.J., Harrison, L.M., Bifulco, C., Capello, M., 2010. CD4 T cells mediate mucosal and systemic immune responses to experimental hookworm infection. *Parasite Immunology* 32, 406–413.
- Dzik, J.M., Zieliński, Z., Gołos, B., Jagielska, E., Wrancik, M., Walajtyś-Rode, E., 2002. Effect of cyclosporin A on immunological response in lungs of guinea pigs infected with *Trichinella spiralis*. *Acta Biochimica Polonica* 49, 233–247.
- Farghali, H., Canová, N., Gaier, N., Lincová, D., Kmonicková, E., Strestíková, P., Masek, K., 2002. Inhibition of endotoxemia-induced nitric oxide synthase expression by cyclosporin A enhances hepatocyte injury in rats: amelioration by NO donors. *International Immunopharmacology* 2, 117–127.
- Ferron, G.M., Jusko, W.J., 1998. Species- and gender-related differences in cyclosporine/prednisolone/sirolimus interactions in whole blood lymphocyte proliferation assays. *Journal of Pharmacology and Experimental Therapeutics* 286, 191–200.
- Fruman, D.A., Klee, C.B., Bierer, B.E., Burakoff, S.J., 1992. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. *Proceedings of the National Academy of Sciences of the United States of America* 89, 3686–3690.
- Fujiwara, R.T., Geiger, S.M., Bethony, J., Mendez, S., 2006. Comparative immunology of human and animal models of hookworm infection. *Parasite Immunology* 28, 285–293.
- Fujiwara, R.T., Cançado, G.G., Freitas, P.A., Santiago, H.C., Massara, C.L., Dos Santos Carvalho, O., Corrêa-Oliveira, R., Geiger, S.M., Bethony, J., 2009. *Necator americanus* infection: a possible cause of altered dendritic cell differentiation and eosinophil profile in chronically infected individuals. *PLoS Neglected Tropical Diseases* 3, 399–408.
- Gad, S.C., Hess, F.G., 2007. The hamster. In: Gad, S.C. (Ed.), *Animal Models in Toxicology*. CRC Press, USA, p. 297.
- Garcia, C., Michelon, T., Barros, V., Mota, D., Uhlmann, A., Randon, R., Ramalho, H., Abbad Filho, M., 1998. Cyclosporine in the treatment of steroid-dependent and steroid-resistant idiopathic nephrotic syndrome in children. *Transplantation Proceedings* 30, 4156–4157.
- Garside, P., Behnke, J.M., 1989. *Ancylostoma ceylanicum* in the hamster: observations on the host–parasite relationship during primary infection. *Parasitology* 98, 283–289.
- Garside, P., Behnke, J.M., Rose, R.A., 1989. The immune response of male DSN hamsters to a primary infection with *Ancylostoma ceylanicum*. *Journal of Helminthology* 63, 251–260.
- Geiger, S.M., Massara, C.L., Bethony, J., Soboslay, P.T., Corrêa-Oliveira, R., 2004. Cellular responses and cytokine production in post-treatment hookworm patients from an endemic area in Brazil. *Clinical and Experimental Immunology* 136, 334–340.
- Geiger, S.M., Caldas, I.R., Mc Glone, B.E., Campi-Azevedo, A.C., De Oliveira, L.M., Brooker, S., Diemert, D., Corrêa-Oliveira, R., Bethony, J., 2007. Stage-specific immune responses in human *Necator americanus* infection. *Parasite Immunology* 29, 347–358.
- Geiger, S.M., Fujiwara, R.T., Freitas, P.A., Massara, C.L., Carvalho, O.S., Corrêa-Oliveira, R., Bethony, J.M., 2011. Excretory-secretory products from hookworm L3 and adult worms suppress proinflammatory cytokines in infected individuals. *Journal of Parasitology Research* 2011, 512154, Epub 2011 Jun 9.
- Ghosh, K., Wu, W., Antoine, A.D., Botazzi, M.H., Valenzuela, J.G., Hotez, P.J., Mendez, S., 2006. The impact of concurrent and treated *Ancylostoma ceylanicum* hookworm infections on the immunogenicity of a recombinant hookworm vaccine in hamsters. *Journal of Infectious Disease* 193, 155–162.
- Gordon, H.M., Whitlock, H.V., 1939. A new technique for counting nematode eggs in sheep faeces. *Journal of Scientific & Industrial Research* 12, 50–52.
- Greer, A.W., Stankiewicz, M., Jay, N.P., McNulty, R.W., Sykes, A.R., 2005. The effect of concurrent corticosteroid induced immuno-suppression and infection with the intestinal parasite *Trichostrongylus colubriformis* on food intake and utilization in both immunologically naïve and competent sheep. *Animal Science* 80, 89–99.
- Haakenstad, A.O., Coe, J.E., 1971. The immune response in the hamster. IV. Studies on IgA. *Journal of Immunology* 106, 1026–1034.
- Hotez, P.J., Bethony, J., Bottazzi, M.E., Brooker, S., Buss, P., 2005. Hookworm: “the great infection of mankind”. *PLoS Medicine* 2, 177–181.
- Hotez, P.J., 2008. Hookworm and poverty. *Annals of the New York Academy of Sciences* 1136, 38–44.
- Huss, R., Hoy, C.A., Ottinger, H., Grosse-Wilde, H., Deeg, H.J., 1995. Cyclosporine-induced apoptosis in CD4+ T lymphocytes and computer-simulated analysis: modeling a treatment scenario for HIV infection. *Research in Immunology* 146, 101–108.
- Jenson, J.S., O’Connor, R., Osborne, J., Devaney, E., 2002. Infection with *Brugia microfilariae* induces apoptosis of CD4(+) T lymphocytes: a mechanism of immune unresponsiveness in filariasis. *European Journal of Immunology* 32, 858–867.
- Jorgensen, L.T., Leathwick, D.M., Charleston, W.A., Godfrey, P.L., Vlassoff, A., Sutherland, I.A., 1998. Variation between hosts in the developmental success of the free-living stages of trichostrongyle infections of sheep. *International Journal for Parasitology* 28, 1347–1352.
- Kahan, B.D., 1985. Cyclosporine: the agent and its actions. *Transplantation Proceedings* 17, 5–18.
- Kahan, B.D., 1989. Cyclosporine. *The New England Journal of Medicine* 321, 1725–1738.
- Kamiyama, K., Matsuda, N., Yamamoto, S., Takano, K., Takano, Y., Yamazaki, H., Kageyama, S., Yokoo, H., Nagata, T., Hatakeyama, N., Tsukada, K., Hattori, Y., 2008. Modulation of glucocorticoid receptor expression, inflammation, and cell apoptosis in septic guinea pig lungs using methylprednisolone. *American Journal of Physiology – Lung Cellular and Molecular Physiology* 295, 998–1006.
- Loukas, A., Prociw, P., 2001. Immune responses in hookworm infections. *Clinical Microbiology Reviews* 14, 689–703.
- Loukas, A., Bethony, J., Brooker, S., Hotez, P., 2006. Hookworm vaccines: past, present, and future. *The Lancet Infectious Disease* 6, 733–741.
- Lucin, K.M., Sanders, V.M., Popovich, P.G., 2009. Stress hormones collaborate to induce lymphocyte apoptosis after high level spinal cord injury. *Journal of Neurochemistry* 110, 1409–1421.
- Luzicova, E.M., Efimova, O.A., 2009. Reaction of Bcl-2-positive splenic cells to glucocorticoids. *Bulletin of Experimental Biology and Medicine* 147, 257–261.
- Magee, M.H., Blum, R.A., Lates, C.D., Jusko, W.J., 2002. Pharmacokinetic/pharmacodynamic model for prednisolone inhibition of whole blood lymphocyte proliferation. *British Journal of Clinical Pharmacology* 53, 474–484.
- Maizels, R.M., Balic, A., Gomez-Escobar, N., Nair, M., Taylor, M.D., Allen, J.E., 2004. Helminth parasites—masters of regulation. *Immunological Reviews* 201, 89–116.
- Mendez, S., Valenzuela, J.G., Wu, W., Hotez, P.J., 2005. Host cytokine production, lymphoproliferation and antibody responses during the course of *Ancylostoma ceylanicum* infection in the Golden Syrian hamster. *Infection and Immunity* 73, 3402–3407.
- Mitruka, B.M., Rawnsley, H.M., 1981. *Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals and Normal Humans*, second ed. Masson Pub, New York.
- Paliogianni, F., Raptis, A., Ahuja, S.S., Najjar, S.M., Boumpas, D.T., 1993. Negative transcriptional regulation of human interleukin 2 (IL-2) gene by glucocorticoids through interference with nuclear transcription factors AP-1 and NF-AT. *The Journal of Clinical Investigation* 91, 1481–1489.
- Quinnell, R.J., Stater, A.F., Tighe, P., Walsh, E.A., Keymer, A.E., Pritchard, D.I., 1993. Reinfection with hookworm after chemotherapy in Papua New Guinea. *Parasitology* 106, 379–385.
- Riccardi, C., Cifone, M.G., Migliorati, G., 1999. Glucocorticoid hormone-induced modulation of gene expression and regulation of T-cell death: role of GTR and GILZ, two dexamethasone-induced genes. *Cell Death and Differentiation* 6, 1182–1189.
- Robson, D., 2003. Review of the properties and mechanisms of action of cyclosporine with an emphasis on dermatological therapy in dogs, cats and people. *Veterinary Records* 152, 768–772.
- Scheinman, R.I., Cogswell, P.C., Lofquist, A.K., Baldwin Jr., A.S., 1995. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science* 270, 283–286.
- Shin, M.H., Lee, Y.A., Min, D.Y., 2009. Eosinophil-mediated tissue inflammatory responses in helminth infection. *The Korean Journal of Parasitology* 47, 125–131.
- Strain, S.A., Bishop, S.C., Henderson, N.G., Kerr, A., McKellar, Q.A., Mitchell, S., Stear, M.J., 2002. The genetic control of IgA activity against *Teladorsagia circumcincta* and its association with parasite resistance in naturally infected sheep. *Parasitology* 124, 545–552.

- Trajkovic, V., 2001. Modulation of inducible nitric oxide synthase activation by immunosuppressive drugs. *Current Drug Metabolism* 2, 315–329.
- Triggiani, M., Cirillo, R., Lichtenstein, L.M., Marone, G., 1989. Inhibition of histamine and prostaglandin D2 release from human lung mast cells by ciclosporin A. *International Archives of Allergy & Applied Immunology* 88, 253–255.
- Trowald-Wight, G., Hakansson, L., Johannisson, A., Edqvist, L.E., 1998. The effect of prednisolone on canine neutrophil function: in vivo and in vitro studies. *Acta Veterinaria Scandinavica* 39, 201–213.
- Wilckens, T., De Rijk, R., 1997. Glucocorticoids and immune function: unknown dimensions and new frontiers. *Immunology Today* 18, 418–424.