Seric and hepatic NTPDase and 5′ nucleotidase activities of rats experimentally infected by *Fasciola hepatica*

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SUMMARY

The enzymatic activities of NTPDase and 5′nucleotidase are important to regulate the concentration of adenine nucleotides, known molecules involved in many physiological functions. Therefore, the objective of this study was to evaluate the activity of NTPDase and 5′nucleotidase in serum and liver tissue of rats infected by *Fasciola hepatica*. Rats were divided into two groups: uninfected control and infected. NTPDase activity for adenosine triphosphate (ATP) and ADP substrates in the liver was higher compared with the control group at 15 days post-infection (PI), while seric activity was lower. In addition, seric and hepatic samples did not show changes for 5′nucleotidase activity at this time. On the other hand, either NTPDase or 5′nucleotidase activities in liver homogenate and serum were higher at 87 days PI. Early in the infection, low NTPDase activity maintains an increase of ATP in the bloodstream in order to activate host immune response, while in hepatic tissue it decreases extracellular ATP to maintain a low inflammatory response in the tissue. As stated, higher NTPDase and 5′nucleotidase activities 87 days after infection in serum and tissue, probably results on an increased concentration of adenosine molecule which stimulates a Th2 immune response. Thus, it is possible to conclude that *F. hepatica* infections lead to different levels of nucleotide degradation when considering the two stages of infection studied, which influences the inflammatory and pathological processes developed by the purinergic system.

Key words: ATP, adenosine, Fasciolosis, NTPDase, 5'nucleotidase.

INTRODUCTION

Fasciolosis is a zoonotic disease caused by the helminth parasite *Fasciola hepatica* that damages host's liver tissue (Mas-Coma *et al*. 2005; Flynn *et al*. 2010). In response to infection, the host shows a Th2 immune response characterized by inhibition of T lymphocytes and decreased pro-inflammatory cytokines production (Mendes *et al*. 2013). As a consequence, the host immune response prevents further damage to its own tissues, but on the other hand, it also helps parasite survival. Research has shown that helminth helps to induce host immune response by the release of immunomodulatory mediators (Guasconi *et al*. 2015).

The purinergic system has many important functions in the body, such as regulation of immune responses, vasodilation and platelet aggregation (Burnstock and Verkhratsky, 2010). These events occur due to the several interactions of purine nucleotides with purinoreceptors present in the plasma membrane of many cells. Extracellular purine nucleotide adenosine triphosphate (ATP) can interact with purinoreceptors type P2, triggering cell proliferation, vasodilation and secretion of pro-inflammatory cytokines (Bours *et al*. 2006). Moreover, nucleoside adenosine (Ado) interacts with purinoreceptors type P1 and produces a protective cellular environment, inducing the production of anti-inflammatory cytokines and vasoconstriction (Borowiec *et al*. 2006).

The importance of nucleotides is associated with the essential function of the enzymatic system that degrades nucleotides and promotes its suitable concentration extracellularly, being found on the plasma membrane of cells or on its soluble form in the serum and throughout interstitial tissues. The nucleotide enzymatic regulation initiates with NTPDase that hydrolyses ATP into ADP and AMP. Degradation continues with the 5′nucleotidase activity that hydrolyses AMP into Ado. Several authors have demonstrated the important role of ectonucleotidases in numerous physiological functions and diseases (Zimmermman, 2000), as well as the role of purine molecules, ectonucleotidases and purinoreceptors in some liver cells (Fausther *et al*. 2011).

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The parasitosis caused by *F. hepatica* is still not fully understood, especially its ability to survive and immunomodulate host's immune responses. Thus, this study has the objective to determine the enzymatic activity of NTPDase and 5′nucleotidase, known modulators of ATP, ADP, AMP and Ado in serum and liver of rats experimentally infected by *F. hepatica*.

MATERIALS AND METHODS

Fasciola hepatica isolation and inoculum preparation

Fasciola hepatica eggs were obtained from one sheep experimentally infected by the Weybridge strain. Feces were collected weekly using a 60-mesh sieve and parasite eggs were collected with a 400-mesh sieve. These eggs were maintained at 24 °C for 10 days, and mass hatching was induced by light exposure. Using a 24-well plate (Corning, USA), snails (*Pseudosuccinea columella*) with approximately 5–6 mm of shell lengths were infected by 3 miracidia using 3 mL of water/well. At day 40 post-infection (PI), metacercariae were collected and stored at 4 °C for 60 days. Previous to use, viability of metacercariae was established at 98%. Inoculum was composed of 20 viable metacercariae kept at room temperature by 24 h prior to oral infection.

Animals

Thirty female rats with 70 days of age and 250 g (±31) of body weight were kept in cages housed in an experimental room with controlled temperature and humidity (22 °C; 60%). They were fed with commercial feed and received water *ad libitum*. All animals had a period of 30 days for acclimatization and were clinically healthy in the beginning of experiment (day 0). The procedure was approved by the Animal Welfare Committee of the Instituto Federal Catarinense (IFC), number 001/2013.

Animals were divided into two groups: control and infected, where the control group was composed of 10 healthy (uninfected) rats. Twenty animals from infected group were experimentally infected orally with 20 metacercariae of *F. hepatica*.

Sampling

At 15 and 87 days PI, animals were anesthetized with isoflurane for blood sampling (2 mL each^{-1}) as follows: 15 days PI, control group ($n = 5$) and infected group ($n = 10$); and 87 days PI control group ($n = 5$) and infected group ($n = 10$). Blood samples were stored into tubes without anticoagulant, serum was obtained after centrifugation at 3500 ***g*** for 10 min, and stored at −20 °C for the NTPDase and 5′ nucleotidase assays. A fragment of liver was removed, weighed and homogenized with Tris–HCl 50 mM with 4 mM EDTA (to exclude possible interference of endogenous divalent cations). Each homogenate was centrifuged at 2200 ***g*** for 10 min with the supernatant collected and frozen at −20 °C until analyses.

Protein determination

Protein concentration of samples was determined by the Coomassie Brilliant Blue method according to Bradford (1976), using bovine serum albumin as the standard.

Seric NTPDase and 5'nucleotidase activities

NTPDase and 5'nucleotidase activities in serum samples were determined as previously described by Oses *et al*. (2004). The reaction mixture for the NTPDase activity contained 3 mM of ATP or ADP as substrate and 112·5 mM Tris–HCl (pH 8·0). The reaction mixture for 5'nucleotidase was composed of 3 mM of AMP as substrate and 100 mM Tris–HCl (pH 7·5). The reaction mixtures were incubated with approximately 1·0 mg of homogenized protein at 37 °C for 40 min on a final volume of 0·2 mL. The reaction was stopped by the addition of 0·2 mL of 10% trichloroacetic acid (TCA). All samples were centrifuged at 5000 ***g*** for 5 min to eliminated precipitated protein and the supernatant was used for the colorimetric assay. The samples were chilled on ice and the amount of released inorganic phosphate (Pi) was measured by the method of Chan *et al*. (1986). In order to correct non-enzymatic hydrolysis, control samples were used by adding the homogenate after the reaction was stopped with TCA. Enzyme activities were expressed as nanomoles of Pi released per min per milligram of protein (nM of Pi min^{-1} mg^{-1} protein).

Hepatic NTPDase and 5'nucleotidase activities

NTPDase and 5'nucleotidase activities in liver homogenized were determined using a modification of the method described by Rosemberg *et al*. (2010). First, liver homogenates were centrifuged at 2500 rpm for 10 min to remove possible impurities. The reaction mixture for the NTPDase activity contained 1 mM of ATP or ADP as substrate, 5 mM CaCl2 and 50 mM Tris–HCl (pH 8·0). For 5'nucleotidase, the reaction mixture contained 10 mM of AMP as substrate, 5 mM $MgCl_2$ and 50 mM Tris–HCl (pH 7·5). The reaction mixtures were incubated with approximately 1·0 mg of homogenized protein at 37 °C for 30 min in a final volume of 0·2 mL. The reaction was stopped by the addition of 0·2 mL of 10% TCA. The samples were chilled on ice and the amount of Pi liberated was measured by the method of Chan *et al*. (1986). In order to correct non-enzymatic hydrolysis, samples were

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used by adding the homogenate after the reaction was stopped with TCA. Enzymatic activities were expressed as nM of Pi min^{-1} mg^{-1} protein.

Gross and histopathology exam

Animals were euthanized by isoflurane overdose followed by decapitation. At the necropsy, tissue samples were collected from the left and right hepatic lobes, fixed in 10% buffered formalin, and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin (H&E) for histopathological examination. Liver sections were evaluated in a blind way and lesions were described and scored as follows: absent, mild, moderate and severe.

Statistical analysis

The data showed a normal distribution (Shapiro–Wilk test) and therefore all comparisons between groups were performed by one-way analysis of variance followed by the student test. Differences between groups were considered significant when $P < 0{\cdot}05$. Data were expressed as mean ± standard error of the mean (S.E.M.).

RESULTS

Disease clinical course and histopathology

Infected rats did not show clinical signs of the disease during the study. In all infected animals, the right hepatic lobe was the more severe affected. At day 15 PI, liver from infected animals showed focal extensive or multifocal necrosis associated with mild to moderate inflammatory infiltrate of lymphoplasmacytic cells, macrophages and giant cells. In some samples, there were migrating larvae surrounded by moderate inflammatory infiltrate of eosinophils often attached to the larva cuticle (Fig. 1A and B).

At day 87 PI, the main changes observed in the hepatic tissue were proliferation of biliary ducts and connective fibrous tissue, moderate to severe inflammatory infiltrate of lymphoplasmacytic cells, and some specimens of adult *F. hepatica*. Furthermore, there were multifocal haemorrhages and hemosiderosis (Fig. 1C). Animals from the uninfected group did not show histopathological changes in liver samples.

Seric NTPDase and 5'nucleotidase activities

ATP and ADP hydrolysis by NTPDase in serum samples were showed in Fig. 2A. The activity of NTPDase for ATP and ADP was decreased on day 15 PI, i.e. a reduction of 42% for ATP and 46% for ADP when compared with the control group. After 87 days PI, the infected group showed an increase of 85% in the NTPDase activity for ATP and 39% for ADP when compared with the control group. Activity of the 5'nucleotidase in serum was showed in Fig. 3A. The result of 5'nucleotidase for the infected group at day 15 PI did not show significant difference between the control groups. 5'nucleotidase activity of the infected group at day 87 PI showed an increase of 129% when compared with the control group.

Hepatic NTPDase and 5'nucleotidase activities

Results of NTPDase activity in liver homogenate with ATP and ADP as substrate were showed in Fig. 2B. Hepatic NTPDase activity (ATP and ADP) had an increase of 364% for ATP and 228% for ADP on day 15 PI for the infected group, as well as 136% for ATP and 171% for ADP on day 87 PI for the infected group when compared with the control group. Results for the 5'nucleotidase activity were showed in Fig. 3B. Infected group did not show significant difference between the control groups, but on day 87 PI this group showed an increase of 68% when compared with the control group.

DISCUSSION

After 15 days PI, animals infected by *F. hepatica* went through the acute phase of infection, where the parasite had already crossed the liver tissue to stay in the bile ducts (Nyindo and Lukambagire, 2015). Tissue damage caused by the parasite stimulates the release of ATP by the cells, thus signals the entire tissue to initiate an inflammatory response (Corriden and Insel, 2010). However, in hepatic samples there was an increase in ATP degradation by NTPDase and no changes were observed in 5′ nucleotidase activity, which is the enzyme responsible for the degradation of AMP. In addition, AMP forming adenosine can be deaminated by adenosine deaminase, an enzyme that showed reduced activity in the liver of rats infected by *F. hepatica* (Baldissera *et al.* 2015). According to the literature, this occurs probably because the adenosine levels can be increased in the extracellular space interacting with purinoreceptors P1 or be reuptaken by adenosine transporters channels in cells of the host or by the parasite. With the knowledge that high concentrations of ATP can be toxic to cells, we have hypothesized that the increase the NTPDase can be cytotoxic under high concentrations of ATP, which can lead to lysis of host cells after purinoreceptors interactions (Ralevic and Burnstock, 1998). Other hypothesis, is that after a triggered uncontrolled inflammation response, the body would control the concentration of the nucleotide in the parasited tissue, modulating NTPDase enzymatic activity and thereby reducing the concentration of ATP.

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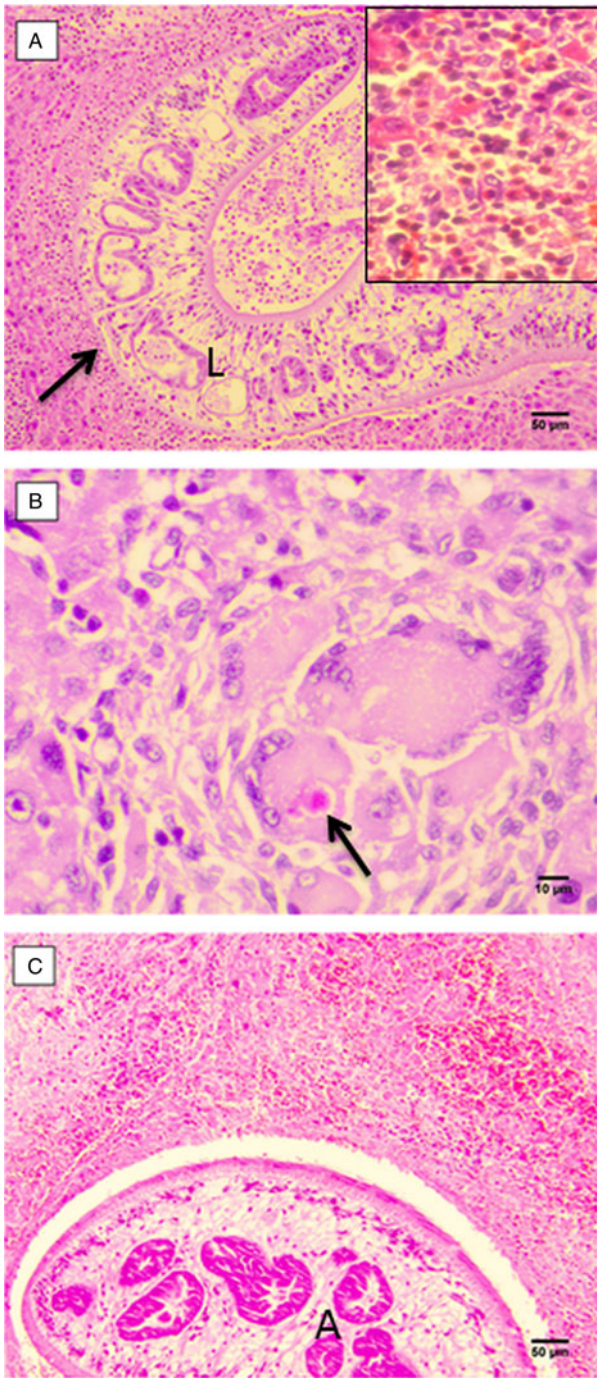

Fig. 1. Liver of rats infected by *Fasciola hepatica* at days 15 and 87 PI, H&E. (A) Migrating larva surrounded by moderate inflammatory infiltrate of eosinophils often attached to the larva cuticle, as well as macrophages and lymphocytes at day 15 PI. (B) Multinucleated giant cells and moderate lymphoplasmacytic inflammatory cell infiltrate. Inside one giant cell phagocyted eosinophilic material can be seen at day 15 PI. (C) Adult *F. hepatica* surrounded by moderate multifocal haemorrhage and hepatocyte necrosis at day 87 PI.

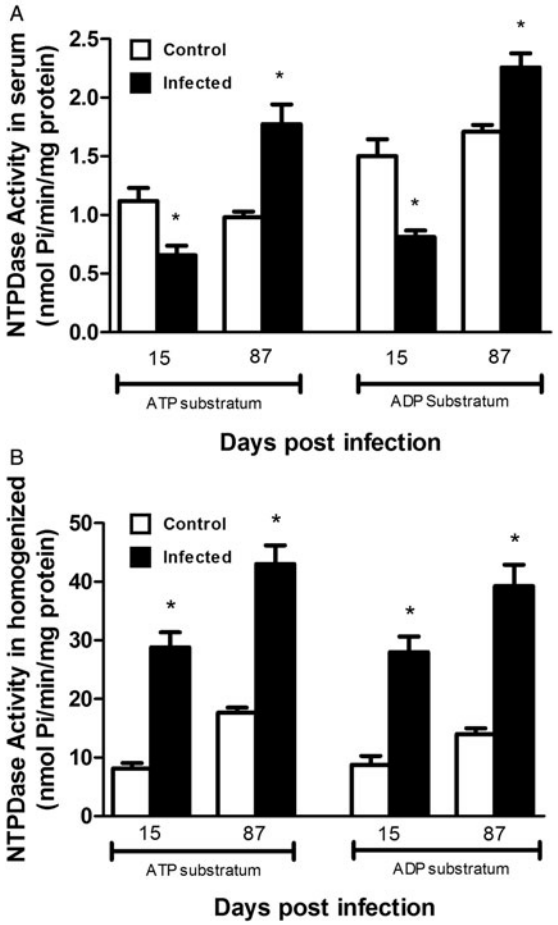

Fig. 2. NTPDase activity to ATP e ADP substrate in serum (A) and liver homogenate (B) of *Fasciola hepatica* infected rats compared with uninfected (control) on days 15 and 87 PI. Asterisks indicate statistical difference ($P < 0{\cdot}05$) between infected and uninfected groups.

Serum samples of rats after 15 days of *F. hepatica* infection showed a decrease in the hydrolyses of ATP and ADP nucleotides by NTPDase enzyme, and no changes in the nucleotide AMP degradation by the enzyme 5′nucleotidase were observed. These results point to an increase in the concentration of ATP in the bloodstream during the acute phase of parasitism by *F. hepatica*. Gressler *et al.* (2014) showed in his work that lambs infected by *Haemonchus contortus* showed an increase of ATP concentrations during the acute phase of the disease. This fact supports the hypothesis that modulation in NTPDase and 5′nucleotidase enzymatic activities caused by infection in the acute phase causes an increase in ATP concentrations. It is known that ATP is a major pro-inflammatory agent by vasodilating, platelet-activating and stimulating the production of reactive species and thereby stimulating an increase in the inflammatory response (Bours *et al.* 2006). Besides the action of degrading enzymes, the concentration of ATP can increase due to the release of ATP by damaged cells (Trautmann, 2009). After 15 days of infection, the body is in the acute phase of infection, and it searches through numerous ways to activate inflammation in order to remove the invading pathogen.

The response to parasitism in the chronic phase of the infection showed an increase in the activity of

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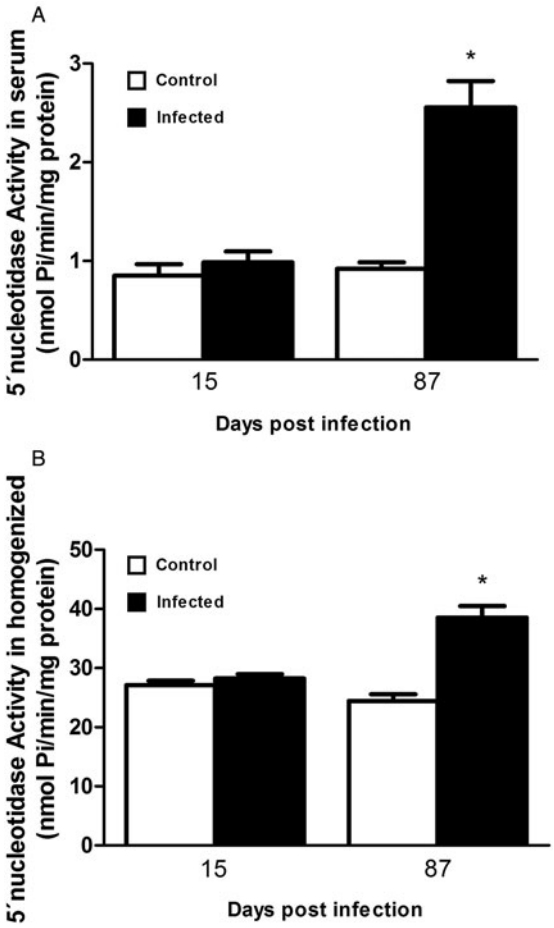

Fig. 3. 5'nucleotidase activity to AMP substrate in serum (A) and liver tissue homogenized (B) of *Fasciola hepatica* infected rats compared with uninfected (control) on days 15 and 87 PI. Asterisk (*) indicates statistical difference ($P < 0{\cdot}05$ between infected and uninfected groups.

NTPDase and 5′nucleotidase in serum and liver homogenate. In this case, the NTPDase enzyme degrades ATP and ADP, and AMP is degraded by the resulting 5′nucleotidase forming adenosine. The nucleoside adenosine is an important endogenous anti-inflammatory, vasoconstrictor and platelet activation acting as an inhibitor of a protective cellular environment (Barankiewicz *et al*. 1988). As a result of enzymatic modulation, high concentrations of adenosine roam the bloodstream signalling the body to reduce the inflammatory response caused by acute phase. Lambs infected by the helminth parasite *H. contortus* presented in the chronic phase of infection an increase in adenosine concentrations in the bloodstream, maintaining that high adenosine levels present are due to an offsetting effect on inflammation, which reduces the cellular damage and inflammation (Gressler *et al*. 2014). All of these results lead to the assumption that the post-acute phase shows high levels of adenosine, which leads to a cell protective environment confirmed by the presence of necrosis in liver tissue histology.

Several studies in the chronic phase of the disease have shown that the parasite located in the gallbladder is capable of producing a range of compounds able to modulate the activity of many immune cells (lymphocytes and macrophages), and alter cytokine production which leads to inhibition of cell response, and development of a humoral immune response (Th2) (Guasconi *et al*. 2015). Associated with this, increase in adenosine concentration found in infected rats by *F. hepatica* (Baldissera *et al*. 2015) also results on immunomodulatory effects and possibly contributes to the production of the Th2 response seen in the chronic phase. Besides the interaction with purinoreceptors P1, the formed adenosine can be captured by alert channel nucleoside transporters present on the membrane of both host cells as well as the parasite itself (Junger, 2011).

Our results show that *F. hepatica* is able to modulate the activity of host enzymes differently depending on each tissue involved. First, after 15 days of infection, it was observed a decreased in NTPDase activity in serum, but on the contrary, there was an increase in the enzyme activity in the liver tissue. Probably, this regulation is related to increase the levels of seric ATP at the onset of infection, which acted as damage-associated molecular patterns (DAMP) and activated the immune system of the host. However, on tissue the enzymatic activity is increased to hydrolyse the high concentrations of ATP released from damaged cells by infection, being the parasite benefited since ATP is a promoter of immune defence against invading organisms. Moreover, in the chronic phase of infection, it was observed an increase on NTPDase and 5′nucleotidase activities in serum and tissue samples likely to produce high concentrations of adenosine, which promotes cell protection environment due to tissue damage caused by the infection, and also assists the formation of Th2 immune response. The activities found in this study may be associated with the protection of the organism, but can also be related to the permanence of the parasite in the host tissue. Thus, the purinergic system might be a possible target for new drugs and treatments for the zoonotic disease caused by *F. hepatica*.

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REFERENCES

Baldissera, M. D., Mendes, R. E. and Da Silva, A. S. (2015). Activity of cholinesterases, pyruvate kinase and adenosine deaminase in rats experimentally infected by *Fasciola hepatica*: influences of these enzymes on

https://doi.org/10.1017/S0031182015001882 Published online by Cambridge University Press

inflammatory response and pathological findings. *Pathology Research and Practice* **211**, 871–876.

Barankiewicz, J., Dosch, H. M. and Cohen, A. (1988). Extracellular nucleotide catabolism in human B and T lymphocytes. The source of adenosine production. *Journal of Biological Chemistry* **263**, 7094–7098.

Borowiec, A., Lechward, K., Tkacz-Stachowska, K. and Skladanowski, A. C. (2006). Adenosine as a metabolic regulator of tissue function: production of adenosine by cytoplasmic 5'-nucleotidases. *Acta Biochimica Polonica* **53**, 269–278.

Bours, M., Swennen, E., Di Virgilio, F., Cronstein, B. N. and Dagnelie, P. C. (2006). Adenosine 5′triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacology and Therapeutics* **112**, 358–404.

Bradford, M. M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72**, 248–254.

Burnstock, G. and Verkhratsky, A. (2010). Long-term (trophic) purinergic signaling: purinoceptors control cell proliferation, differentiation and death. *Cell Death Disease* **1**, e9.

Chan, K. M., Delfert, D. and Junger, K. D. (1986). A direct colorimetric assay for the Ca^{2+} – ATPase activity. *Analytical Biochemistry* **157**, 375–380.

Corriden, R. and Insel, P. A. (2010). Basal release of ATP: an autocrine–paracrine mechanism for cell regulation. *Science Signaling* **3**, re1.

Fausther, M., Lecka, J., Soliman, E., Kauffestein, G., Pelletier, J., Sheung, N., Dranoff, J. A. and Sévigny, J. (2011). Coexpression of ecto-5=–nucleotidase/CD73 with specific NTPDases differentially regulates adenosine formation in the rat liver. *American Journal Physiology Gastrointestinal Liver Physiology* **302**, G447–G459.

Flynn, R. J., Mulcahy, G. and Elsheikha, H. M. (2010). Coordinating innate and adaptive immunity in *Fasciola hepatica* infection: implications for control. *Veterinary Parasitology* **169**, 235–240.

Gressler, L. T., Da Silva, A. S., Oliveira, C. B., Schafer, A. S., Aires, A. R., Rocha, J. F. X., Tonin, A. A., Schirmbeck, G. H., Casali, E. A., Lopes, S. T. A., Leal, M. L. R. and Monteiro, S. G. (2014). Experimental infection by *Haemonchus contortus* in lambs: influence of disease on purine levels in serum. *Parasitology* **141**, 898–903.

Guasconi, L., Chiappelo, L. S. and Masih, D. T. (2015). *Fasciola hepatica* excretory–secretory products induce CD^{4+}T cell anergy via selective up-regulation of PD-L2 expression on macrophages in a Dectin-1 dependent way. *Immunobiology* **220**, 934–939.

Junger, W. G. (2011). Immune cell regulation by autocrine purinergic signalling. *Nature Reviews* **11**, 201.

Mas-Coma, S., Bargues, M. D. and Valero, M. A. (2005). Fascioliasis and other plant-borne trematode zoonoses. *International Journal for Parasitology* **35**, 1255–1278.

Mendes, E. A., Mendes, T. A. O., Santos, S. L., Menezes-Souza, D., Bartholomeu, D. C., Martins, I. V. F., Silva, L. M. and Lima, W. S. L. (2013). Expression of IL-4, IL-10 and IFN- in the liver tissue of cattle that are naturally infected with *Fasciola hepatica*. *Veterinary Parasitology* **195**, 177–182.

Nyindo, M. and Lukambagire, A. (2015). Fascioliasis: an ongoing zoonotic trematode infection. *BioMed Research International* **2015**, 786195.

Oses, J. P., Cardoso, C. M., Germano, R. A., Kirst, I. B., Rucker, B., Furstenau, C. R., Wink, M. R., Bonan, C. D., Battastini, A. M. and Sarkis, J. J. (2004). Soluble NTPDase: an additional system of nucleotide hydrolysis in rat blood serum. *Life Sciences* **74**, 3275–3284.

Ralevic, V. and Burnstock, G. (1998). Receptors for purines and pyrimidines. *Pharmacology Review* **50**, 413–492.

Rosemberg, D. B., Rico, E. P., Langoni, A. S., Spinelli, J. T., Pereira, T. C., Dias, R. D., Souza, D. O., Bonan, C. D. and Bogo, M. R. (2010). NTPDase family in zebrafish: nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart. Comparative Biochemistry and Physiology *B* **155**, 230–240.

Trautmann, A. (2009). Extracellular ATP in the immune system: more than just a "danger signal". *Science Signaling* **2**, e6.

Zimmermman, H. (2000). Extracellular metabolism of ATP and others nucleotides. *Naunyn-Schmiedeberg's Archives of Pharmacology* **362**, 299–309.

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