Comparative Biochemistry and Physiology, Part C 260 (2022) 109402

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Participation of ecto-5′-nucleotidase in the inflammatory response in an adult zebrafish (*Danio rerio*) model

Luiza Reali Nazario [a,1], Jéssica Streb de Sousa [a], Francielle Schroeder de Moraes Silveira [a], Kesiane Mayra Costa [b], Giovanna Medeiros Tavares de Oliveira [b], Maurício Reis Bogo [b], Rosane Souza da Silva [a,*]

[a] Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Avenida Ipiranga, 6681, Caixa Postal 1429, 90619-900 Porto Alegre, RS, Brazil

[b] Laboratório de Biologia Genômica e Molecular, Escola de Ciências da Saúde e Vida, PUCRS, Caixa Postal 1429, 90619-900 Porto Alegre, RS, Brazil

ARTICLE INFO

Edited by: Dr. Martin Grosell

Keywords:
Adenosine
CD75
Endotoxin
Lipopolysaccharide
Purinergic system

ABSTRACT

The ecto-5′-nucleotidase is an important source of adenosine in the extracellular medium. Adenosine modulation appears early in evolution and performs several biological functions, including a role as an anti-inflammatory molecule. Here, we evaluate the activity and mRNA expression of ecto-5′-nucleotidase in response to lipopolysaccharide (LPS) using zebrafish as a model. Adult zebrafish were injected with LPS (10 μg/g). White blood cell differential counts, inflammatory markers, and ecto-5′-nucleotidase activity and expression in the encephalon, kidney, heart, and intestine were evaluated at 2, 12, and 24 h post-injection (hpi). At 2 hpi of LPS, an increase in neutrophils and monocytes in peripheral blood was observed, which was accompanied by increased *tnf-α* expression in the heart, kidney, and encephalon, and increased *cox-2* expression in the intestine and kidney. At 12 hpi, monocytes remained elevated in the peripheral blood, while *tnf-α* expression was also increased in the intestine. At 24 hpi, the white blood cell differential count no longer differed from that of the control, whereas *tnf-α* expression remained elevated in the encephalon but reduced in the kidney compared with the controls. AMP hydrolysis in LPS-treated animals was increased in the heart at 24 hpi [72 %; $p = 0.029$] without affecting ecto-5′-nucleotidase gene expression. These data indicate that, in most tissues studied, inflammation does not affect ecto-5′-nucleotidase activity, whereas in the heart, a delayed increase in ecto-5′-nucleotidase activity could be related to tissue repair.

1. Introduction

Lipopolysaccharide (LPS) is an amphiphilic molecule from membranes of gram-negative bacteria composed of a unique moiety of lipid A that acts as the main factor to produce LPS toxicity and the large polysaccharide chain responsible for adaptive immunity (Kotani and Takada, 1990; Hoshino et al., 1999; Tuin et al., 2006; Lu et al., 2008; Kabanov and Prokhorenko, 2010). As an endotoxin, LPS activates the production of several inflammatory mediators such as interleukins and tumor necrosis factor-α (TNF-α) through an evolutionary-conserved mechanism on toll-like receptor 4 (TLR4) (Lu et al., 2008).

The zebrafish has become an excellent model for studying pathways involved in the physiopathology of inflammatory diseases and an important platform for seeking new therapies to treat these conditions (Morales Fénero et al., 2016; Belo et al., 2021). Fish present sophisticated lines of defense against invading pathogens and parasites, such as mechanical and antimicrobial barriers provided by the mucus membrane layer of the gills, skin, and intestines, as well as by innate immune cells, such as macrophages and neutrophils (Jovanović and Palić, 2012). Despite the differences and uncertainties surrounding the zebrafish's mechanism of action and tolerance to LPS, this fish exhibits one or more orthologs of toll-like receptors (TLRs), interleukin receptors, and associated adaptor proteins containing toll-interleukin receptor domains (TIR), which share a common ancestor with their human counterparts (Jault et al., 2004; Meijer et al., 2004; Novoa et al., 2009; Yang et al., 2014). LPS toxicity in zebrafish leads to an increase in *il-1β*, *tnf-α*, *cox-2*,

* Corresponding author.
E-mail address: rosane.silva@pucrs.br (R.S. da Silva).
[1] Present address: Department of Nuclear Medicine and Molecular Imaging, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713GZ Groningen, The Netherlands.

https://doi.org/10.1016/j.cbpc.2022.109402
Received 1 April 2022; Received in revised form 20 June 2022; Accepted 26 June 2022
Available online 30 June 2022

inos, and *il-6* expression levels, resulting in the tissue injury and death of the animals (Ruyra et al., 2014; Yang et al., 2014; Jakhar et al., 2018).

The purinergic system appears early in evolution and is operated by ATP- and adenosine-related molecules, nucleotide- and nucleoside-metabolizing enzymes, specific receptors, and membrane transporters (Verkhratsky and Burnstock, 2014). The nucleoside adenosine performs several biological functions, including an anti-inflammatory role (Di Virgilio and Vuerich, 2015). Adenosine is involved in cell–cell interactions, secretion of cytokines and chemokines, removal of intracellular pathogens, generation of reactive oxygen species (ROS), and chemotaxis to promote tissue protection and repair (Haskó et al., 2008; Junger, 2011; Ramakers et al., 2012). While the nucleotide ATP acts as an indicator of cellular damage by acting on specific receptors called P2 purinoreceptors, its hydrolysis in the extracellular medium generates adenosine (Burnstock, 1976; Zimmermann and Braun, 1999). Data from studies with humans and rodents indicate that adenosine levels increase dramatically in the extracellular space during the inflammatory state (Fredholm, 2007; Ramakers et al., 2012; Antonioli et al., 2014). Adenosine levels in the extracellular space are controlled by equilibrative nucleoside transporters (ENT1 and ENT2), concentrative nucleoside transporters (CNTs), adenosine transport coupled to Na^+ gradient formation, and the activity of ecto-5′-nucleotidase and adenosine deaminase (Yegutkin, 2014). The increased level of adenosine allows it to act as an innate immunomodulatory molecule, mainly through its specific receptors, A_{2A} and A_{2B}. According to research, the anti-inflammatory response of adenosine in mammals is primarily manifested by inhibiting immune cell recruitment and changing cytokine production to prevent a higher level of tissue damage and a persistent state of inflammation (Haskó et al., 2008; Fujimoto et al., 2012; Antonioli et al., 2013; Ouyang et al., 2013). There is a fine-tuning adjustment between the proinflammatory mechanisms exerted by ATP, the anti-inflammatory action exerted by adenosine, and the chemotactic gradient caused by these adenine derivatives (Antonioli et al., 2014). This gradient is under control of purinergic enzymes, transporters, and receptors (Haskó et al., 2008; Ramakers et al., 2012).

A suitable animal model should be able to exhibit the face, the construct, and the prediction validities of a specific condition to be modeled. While zebrafish exhibit all the major components of a purinergic system, which means enzymes such as ectonucleotidases (Senger et al., 2004; Rosemberg et al., 2007; Rosemberg et al., 2010) and purinergic receptors (Boehmler et al., 2009; Capiotti et al., 2011), there is a paucity of literature using zebrafish to investigate the purinergic system in an inflammatory context (Leite et al., 2013; Cruz et al., 2019). Studies of zebrafish larvae have explored the effects of radiotherapy on the purinergic system to identify an adjustment by reducing AMP hydrolysis and in adenosine receptor A_{2B}-type expression (Cruz et al., 2019). Also, in a model of copper-induced inflammation, zebrafish larvae had diminished AMP hydrolysis and deamination, showing the adjustment in adenosine levels during the early stages of inflammation (Leite et al., 2013).

Considering the contribution of zebrafish to the study of inflammatory diseases (Zanandrea et al., 2020) and the participation of adenosine in the tissue response to inflammation, this study aims to evaluate whether the enzyme ecto-5′-nucleotidase is a key point for adjusting adenosine levels during the induction of an inflammatory response by LPS and its resolution using an adult zebrafish model, through evaluating its activity and expression in a tissue-specific manner.

2. Methods

2.1. Chemicals

LPS (phenol extracted from *Escherichia coli* serotype O111:B4, premium quality), AMP, Tetramizole, Trizma base, EDTA, EGTA, sodium citrate, Coomassie blue, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.2. Animals

All animals used were from the local breeding at Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. Wild-type adult zebrafish (*Danio rerio*; 6–18 months; Tübingen background; 3–5 cm) of both sexes were used. Animals were housed in groups of 20 (5 animals/L) in thermostatically controlled (28 ± 2 °C) tanks. The water used was of reverse-osmosis quality reconstituted with marine salt (Cristalsea, Marinemix) at 0.4 parts per thousand and kept under constant chemical and mechanical filtration and aeration (7.20 mg $O_2 \cdot L^{-1}$) in a recirculating system. Fish were maintained under a 14/10 h day/night photoperiod cycle, fed three times a day with commercial flakes (TetraMin, NC, USA), and supplemented with live brine shrimp twice a day. Adult zebrafish were euthanized by a stunning blow to the head followed by pithing of the cervical area for enzyme activity assay and by overdose of anesthetic (0.5 $g \cdot L^{-1}$ tricaine solution) for gene expression. The procedures were executed in compliance with the ARRIVE guidelines. All protocols were approved by the Institutional Animal Care Committee (14/00417–CEUA PUCRS) and followed Brazilian legislation. The use and maintenance of zebrafish complied with the "Guide for the Care and Use of Laboratory Animals" published by the United States National Institutes of Health.

2.3. Inflammation model

To induce inflammation in zebrafish, we used a model established in the literature (Gonçalves et al., 2012). Before intraperitoneal injection of LPS, the animals were anesthetized with 0.1 $g \cdot L^{-1}$ tricaine solution (MS-222, MERK, Darmstadt, Germany) until they lost posture (Menezes et al., 2014). The fish were injected with 10 μL of an LPS solution in saline (10 μg/g body weight) and placed in a tank (5 animals/L). A saline injection (0.9 $g \cdot L^{-1}$) was used in the control animals. The animals were euthanized 2, 12, and 24 h post-injection (hpi), and their organs were removed using microdissection tools under a stereomicroscope. During the dissection, the organs were constantly rinsed with cold saline.

2.4. Hematologic parameters

Six to seven animals from each group (control and 2, 12, and 24 hpi of LPS) were used to evaluate hematologic parameters. Animals were anesthetized with tricaine solution (0.1 $g \cdot L^{-1}$), and their blood was collected from the dorsal aorta. Immediately after collection, a small drop of blood was taken for smear evaluation using May-Grunewald-Giemsa staining (Costa et al., 2014). The fractions of differential cell types (lymphocytes, monocytes, neutrophils, eosinophils, basophils, and immature cells) were estimated under a 40× objective lens (Olympus® CH30 model) by counting 100 cells per sample. Representative pictures were captured by a Zeiss® AxioImager M2 light microscope (Carl Zeiss, Gottingen, Germany). Cell types were identified according to the literature (Menke et al., 2011).

2.5. Preparation of cellular fraction for ecto-5′-nucleotidase assay

Five to eight pools of 10 kidneys, 10 hearts, 2 intestines, and 5 brains were collected from each of the saline-injected and LPS-treated experimental groups. The whole organ samples were homogenized by tissue type in 60 volumes (*v/w*) of chilled Tris-citrate buffer (50 mM Tris-citrate, 2 mM EDTA, and 2 mM EGTA, pH 7.4 adjusted with citric acid) for ecto-5′-nucleotidase assays (Rico et al., 2003; Senger et al., 2004). Organ sample homogenates were centrifuged at 800 ×*g* for 10 min, and the supernatant fraction was subsequently centrifuged for 25 min at 40,000 ×*g*. The pellet of membrane preparations was frozen in liquid nitrogen, thawed, and re-suspended in the same buffer as

described earlier. This freeze–thaw–wash procedure was used to ensure the lysis of vesicle or organelle membranes. After the lysis procedure, the membranes of organ samples from adult zebrafish underwent an additional centrifugation at 40,000 ×*g* for 20 min. The final pellet was resuspended and used for biochemical analyses. All cellular fractions were maintained at 2–4 °C throughout preparation and used immediately for enzyme assays. Protein concentration was measured by the Bradford assay with bovine serum albumin as a protein standard (Bradford, 1976).

2.6. Determination of ecto-5′-nucleotidase activity

The optimum protein concentration and incubation time to evaluate AMP hydrolysis from the kidney, heart, and intestine were determined according to time and protein curves, while for the encephalon we followed previously described data (Rico et al., 2003). To standardize the optimal conditions for AMP hydrolysis by membrane-bound enzymes from the kidney, heart, and intestine of adult zebrafish, we tested different incubation times (Supplementary Fig. S1) and total protein concentrations (Supplementary Fig. S2). In order to ensure optimal conditions for AMP hydrolysis for each organ analyzed, we chose an incubation time of 30 min for all organs, 3 μg of protein for the heart and kidney, and 5 μg for the intestine, since these conditions are in the linear phase of the reaction. The conditions described above were used in the following experiments. Membranes from organs (1–7 μg protein) were added to the reaction mixture containing 50 mM Tris-HCl (pH 7.2) and 5 mM $MgCl_2$ in a final volume of 200 μL. Samples were pre-incubated for 10 min at 37 °C before starting the reaction with the addition of substrate (AMP) to a final concentration of 1 mM. The reaction was stopped after 5, 10, 30, and 60 min, with the addition of 200 μL of trichloroacetic acid at a final concentration of 5 %. The samples were chilled on ice for 10 min, and 1 mL of a colorimetric reagent composed of 2.3 % polyvinyl alcohol, 5.7 % ammonium molybdate, and 0.08 % malachite green was added in order to determine the inorganic phosphate released (Pi) (Chan et al., 1986). The quantification of Pi released was determined spectrophotometrically at 630 nm, and the specific activity was expressed as nmol of $Pi{\cdot}min^{-1}{\cdot}mg^{-1}$ of protein. To discount the non-enzymatic hydrolysis of AMP, controls were used where the biological preparation denatured by trichloroacetic acid (denatured sample) was added. To avoid the participation of alkaline phosphatase in the AMP hydrolysis detected from the kidney, intestine, and heart, we tested the *in vitro* effect of Tetramizole, an inhibitor of alkaline phosphatase. Tetramizole (1 mM) was added to the medium during the pre-incubation (Rico et al., 2003). The use of Tetramizole did not affect AMP hydrolysis from the intestine, kidney, and heart (Supplementary Table 1), thus excluding the possibility that alkaline phosphatase affected the results. All enzyme assays were performed in triplicate for each sample. We performed independent experiments, and each experimental group had five to eight samples.

2.7. Gene expression analysis by real-time PCR

Four pools of 10 kidneys, 10 hearts, 2 intestines, and 5 encephala were collected to check for *tnf-α* and *cox-2* expression. Each analysis was performed in quadruplicate. The expression of *nt5e* (ecto-5′-nucleotidase) was evaluated in heart samples following the same protocol. Total RNA was isolated with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Total RNA was quantified by spectrophotometry, and the cDNA was synthesized with ImProm-IITM Reverse Transcription System (Promega, Madison, WI, USA) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were carried out in a volume of 25 μL using 12.5 μL of diluted cDNA (1:50), 100 μM dNTP, 1× PCR buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen), 200 nM each of reverse and forward primers (Table 1) (Tang et al., 2007; Leite et al., 2013), and a final concentration of 0.2× SYBR® Green I (Invitrogen). The PCR cycling conditions included an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing, and 15 s at 72 °C for extension. At the end of the cycling protocol, a melting-curve analysis was included, and the fluorescence was measured between 60 and 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection software v.2.0.5 (Applied Biosystems, CA, USA). The efficiency per sample was calculated using LinRegPCR 11.0 software (http://LinRegPCR.nl; Academic Medical Center, Amsterdam, the Netherlands) and the stability of the reference genes *ef1α* and *rlp13α* (M-value), and the optimal number of reference genes according to the pairwise variation (V) were analyzed using GeNorm 3.5 software (http://medgen.ugent.be/genorm/; Ghent University, Ghent, Belgium). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method (Nery et al., 2014). Molecular data are presented as fold change relative to the control and the standard deviation of the treated group.

2.8. Statistical analysis

Data were checked for normality using the Shapiro–Wilk test and expressed as mean ± standard deviation per treatment (saline *vs.* LPS). After that, all data were analyzed by Student's *t*-test or one-way ANOVA, where appropriate. Statistically significant differences between groups were considered when $p < 0.05$. Correlation between inflammatory markers and ecto-5′-nucleotidase activity was checked using Pearson coefficients (two-tailed). For enzyme assays, five to eight samples (AMP hydrolysis analysis) from each group were tested, and for gene expression, four independent experiments were performed. The white blood cell differentiation counts were obtained in three to eight independent experiments. The statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Inflammatory response to LPS

3.1.1. White blood cell differential counting

To demonstrate that the inflammatory response could be observed in peripheral blood, we performed differential counting of white blood cells (Fig. 1). White blood cell differential counts were recorded in animals from the control group (Fig. 1A) and from the 2, 12, and 24 h after LPS injection groups (Fig. 1B-D, respectively). We observed the presence of reactive neutrophils (Fig. 1b), monocytes (Fig. 1c), and lymphocytes (Fig. 1d). The results showed an increase in neutrophils [751 %; $p = 0.0023$] and monocytes [419 %; $p = 0.0227$] and a decrease in lymphocytes [36 %; $p = 0.0024$] at 2 hpi of LPS in comparison with the control group (Fig. 2a). An increase in monocytes [414 %; $p < 0.0001$] and a decrease in lymphocytes [36 %; $p < 0.0001$] were also observed at 12 hpi of LPS. The differential counting of white blood cells at 24 hpi did not show any statistically significant difference between the control and the LPS groups (Fig. 2b). Although we did not find eosinophils in the control group, in the LPS-treated (2 hpi) group, we registered <1 % of this type of cell (data not shown).

3.1.2. Inflammatory markers

To determine the intensity of the response in each organ sample, we analyzed the gene expression of the inflammatory markers *cox-2* and *tnf-α* at 2, 12, and 24 hpi of LPS (Fig. 3). The results showed increased expression of *tnf-α* in the heart [$p = 0.031$], kidney [$p < 0.0001$], and encephalon [$p = 0.022$] at 2 hpi of LPS (Fig. 3a). At 12 hpi of LPS, *tnf-α* expression returned to control values in the heart, kidney, and encephalon, while in the intestine an increase in *tnf-α* expression was registered [$p = 0.026$]. The transcript levels of *tnf-α* at 24 hpi of LPS remained similar to those of the control in the heart, intestine, kidney,

Table 1
Primer sequences for RT-qPCR experiments.

Gene		Sequences (5′-3′)	GenBank accession number	REF
ef1alpha	F	CTGGAGGCCAGCTCAAACAT	ENSDART00000023156	38
	R	ATCAAGAAGAGTAGTACCGCTAGCATTAC		
rpl13alpha	F	TCTGGAGGACTGTAAGAGGTATGC	NM_212784	38
	R	AGACGCACAATCTTGAGAGCAG		
cox-2	F	AACTAGGATTCCAAGACGCAGCATC	ENSDART00000093609	39
	R	AAATAAGAATGATGGCCGGAAGG		
tnf-α	F	AGGAACAAGTGCTTATGAGCCATGC	NM_212859	39
	R	AAATGGAAGGCAGCGCCGAG		
nt5e	F	TGGACGGAGGAGACGGATTCACC	BC055243.1	39
	R	GGAGCTGCTGAACTGGAAGCGTC		

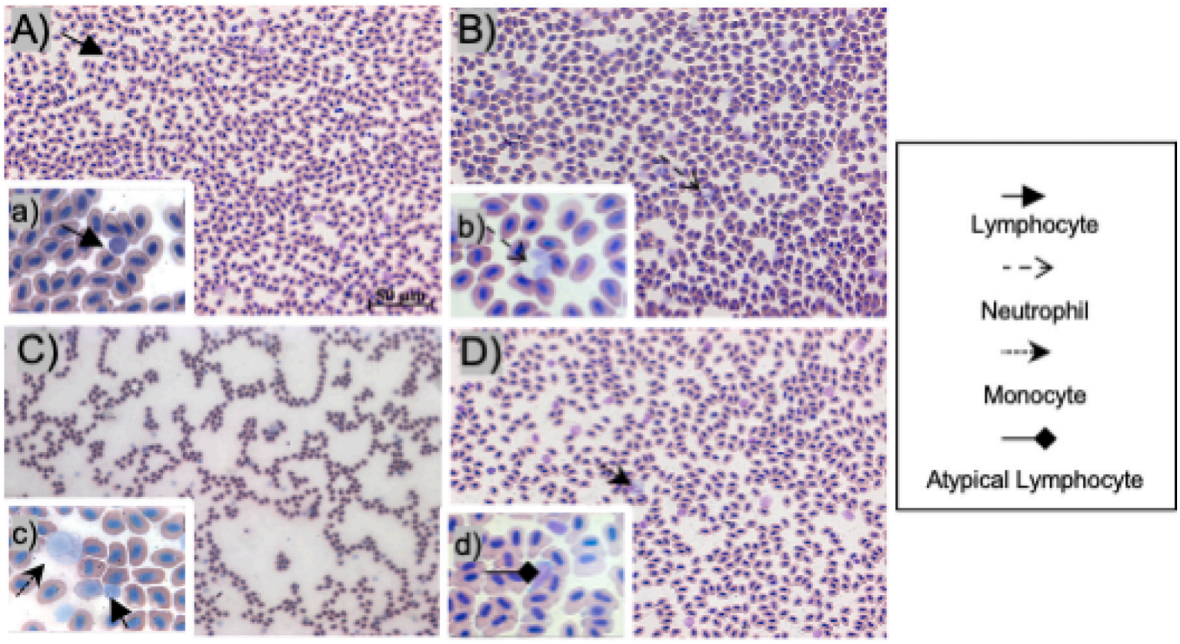

Fig. 1. Hematologic analysis of control and LPS-treated animals. A) Control group, B) 2 hpi group, C) 12 hpi group, and D) 24 hpi group. Representative pictures were captured under original 10× (A-D) objective and 1000× objective (a-d). Arrows indicate representative monocytes, neutrophils, and lymphocytes.

and encephalon (Fig. 3a). The mRNA levels of *cox-2* were increased only in the kidney [$p = 0.035$] and intestine [$p = 0.0437$] at 2 hpi of LPS (Fig. 3b).

3.2. Enzyme activity and mRNA levels of ecto-5′-nucleotidase in adult zebrafish following the induction of inflammation

Ecto-5′-nucleotidase activity in the zebrafish membrane fraction from the intestine, kidney, encephalon, and heart were determined at 2, 12, and 24 h after acute injection of LPS. The exposure to LPS altered AMP hydrolysis in the heart only at 24 hpi of LPS, reaching enzyme activity values 78 % higher than the control condition [$p = 0.029$] (Fig. 4b). While AMP hydrolysis increased in the heart, there was no correlation with inflammatory marker expression [*tnf-α* and ecto-5′-nucleotidase: R squared 0.07; $p = 0.11$; *cox-2* and ecto-5′-nucleotidase: R squared 0.01; $p = 0.567$]. AMP hydrolysis from the intestine, encephalon, and kidney was insensitive to LPS exposure at all time points studied [$p > 0.05$] (Fig. 4acd, respectively).

To investigate the mRNA levels of *nt5e* in the heart, we performed real-time PCR analysis. We found a decrease in *nt5e* expression at 12 hpi of LPS [$p = 0.028$] (Fig. 5), while at 2 and 24 hpi of LPS, the mRNA levels of *nt5e* did not significantly change.

4. Discussion

In the current study, we exposed adult zebrafish to LPS to induce inflammation, which produced distinctive effects on ecto-5′-nucleotidase activity from the heart, intestine, kidney, and encephalon. Although there were changes in white blood cell counts and increased expression of inflammatory markers in the heart, kidney, encephalon, and intestine, the ecto-5′-nucleotidase activity was altered only in the heart.

In the kidney, the strongest effect of LPS was observed at 2 hpi for *tnf-α* and *cox-2* gene expression, compared with the control. After that, the expression of inflammatory markers reached control levels in the kidney. In samples of intestine, *tnf-α* expression increased at 12 hpi and returned to basal levels at 24 hpi, while gene expression of *cox-2* increased at 2 hpi and returned to control levels at 12 hpi. Since *cox-2* plays a crucial role in the defense and repair of the gastrointestinal mucosa (Wallace and Devchand, 2005; Hobbs et al., 2011), its increase corresponds with its important role as a primary barrier to microorganism pathogens and its contribution to endotoxin resistance. In the encephalon, the increase of *tnf-α* gene expression occurred at 2 hpi of LPS. In mammals, the stimulation of cytokines like *tnf-α* by LPS induces an increase in blood–brain barrier (BBB) permeability, thereby producing inflammatory responses in neural tissue that can induce chronicity (Abbott, 2000; Mallard, 2012). Here, *tnf-α* gene expression in the encephalon reached control levels already at 12 hpi. In the heart, a slight

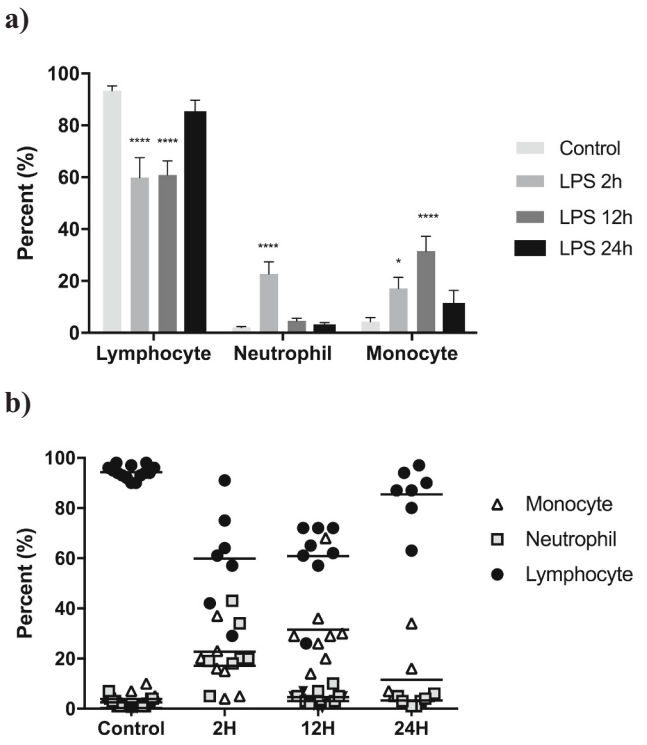

Fig. 2. Effect of LPS treatment at 2, 12, and 24 h after injection on white blood cell differential counts (lymphocytes, neutrophils, and monocytes). Data represent mean ± SD (%) of six to seven independent experiments. A total of 100 cells were counted for each animal. 2a) Cell counting by cell type. 2b) Distribution of white blood cells per treatment group. Data were analyzed by Student's *t*-test. $P < 0.05$ was considered significant. The * represents a significant difference from the control group, as * $p < 0.05$, **** $p < 0.0001$.

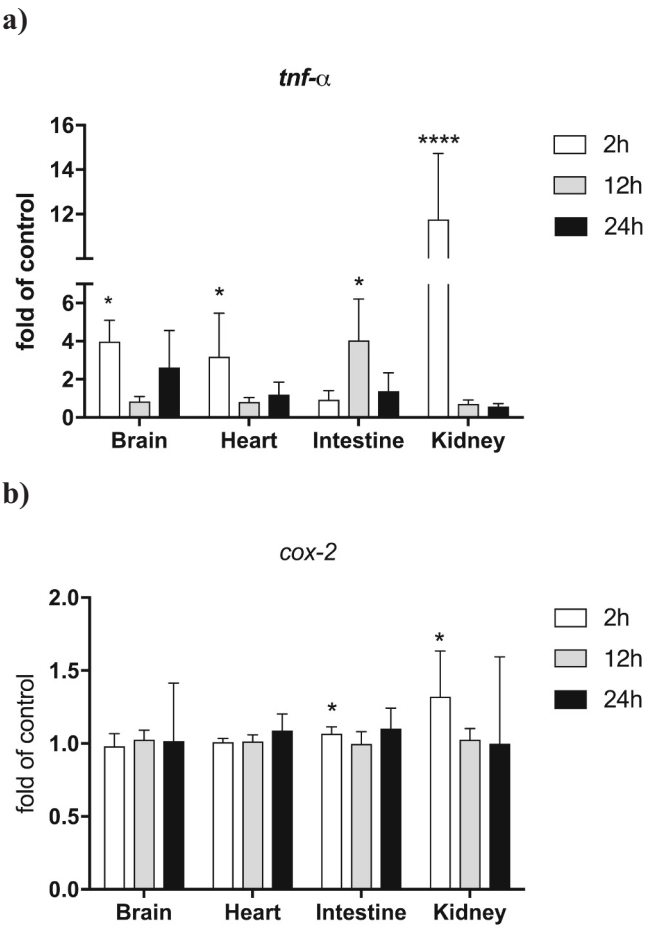

Fig. 3. Effect of LPS treatment at 2, 12, and 24 h after injection on 3a) *tnf-α* and 3b) *cox-2* gene expression from the encephalon, heart, intestine, and kidney of adult zebrafish. Data (mean ± SD) from the LPS-treated group were represented as fold change relative to the control from four independent experiments performed in quadruplicate. Data were analyzed by Student's *t*-test. $P < 0.05$ was considered as significant. The * represents a significant difference from the control group, as * $p < 0.05$ and *** $p < 0.001$.

but statistically significant increase (2.5-fold relative to the control) in *tnf-α* gene expression occurred at the acute phase (2 hpi) that vanished after 12 hpi, while no effects on *cox-2* expression were registered. Endocardial endothelial cells of fish can actively take up circulating macromolecules to promote elimination of waste molecules, and this could contribute to early anti-inflammatory responses in the heart (Seternes et al., 2002). In all tissues, the expression of both inflammatory markers diminished at 24 hpi of LPS. Together with the normalization of white blood cell counts, this is an indication of inflammation resolution. This temporal profile can be related to the fact that *tnf-α* promotes chemotaxis of inflammatory cells and increases phagocytosis (Ming et al., 1987; Zou et al., 2003), which could help fight against inflammation.

In the context of inflammation, the increase of adenosine levels has been indicated as an important strategy for tissue protection and repair in human experimental research and *in vitro* studies (Ramakers et al., 2012). The mechanism to control adenosine levels in humans appears to act mainly by adjusting ecto-5′-nucleotidase, adenosine deaminase, and adenosine kinase activity and expression (Ramakers et al., 2012). From the literature, the use of whole zebrafish larvae identified a general adjustment of adenosine metabolism enzymes and adenosine receptors through copper-induced inflammation (Leite et al., 2013) and gamma radiation (Cruz et al., 2019). Here, a tissue-specific analysis in adult zebrafish indicated that the ecto-5′-nucleotidase activity was altered only in heart samples at 24 hpi, when the inflammatory markers were already diminished. In fact, the literature indicates that the cardiovascular system is a key player in the survival of individuals affected by sepsis (Merx and Weber, 2007). Our results could be related to how tissues response to the inflammation to promote repair, since an increase in the ecto-5′-nucleotidase could provoke the increased adenosine levels important for vasodilation, angiogenesis, and neovascularization (Drury and Szent-Györgyi, 1929; Fredholm, 2007). This increase in ecto-5′-nucleotidase activity occurred at 24 hpi with no change in gene expression. While a direct correlation could be expected, several studies have demonstrated that peak protein expression does not always coincide with ecto-5′-nucleotidase activity (Banjac et al., 2001; Nedeljkovic et al., 2005; Stanojević et al., 2011). However, the allosteric and stoichiometric effects should not be excluded; since ecto-5′-nucleotidase is strongly affected by H^+, inorganic phosphate, Mg^{2+}, ADP, and ATP (Heyliger et al., 1981), such effects could contribute to the increased ecto-5′-nucleotidase in heart samples after LPS exposure. Moreover, ecto-5′-nucleotidases play other roles in addition to those related to purine metabolism (Heyliger et al., 1981).

In this study, adult zebrafish demonstrated an early peripheral response to LPS, evidenced by the increased neutrophil and monocyte count and the altered expression of the proinflammatory molecule from 2 h after injection. During this inflammatory state, we observed an increased but delayed AMP hydrolysis in the heart, which calls our attention to the contribution of ecto-5′-nucleotidase activity to a possible adjustment in adenosine levels in this specific tissue. Furthers studies are needed to identify adenosine levels in the hearts of zebrafish treated with LPS so we can create a reliable picture of the role the purinergic system plays in studies of cardiac inflammation using zebrafish models.

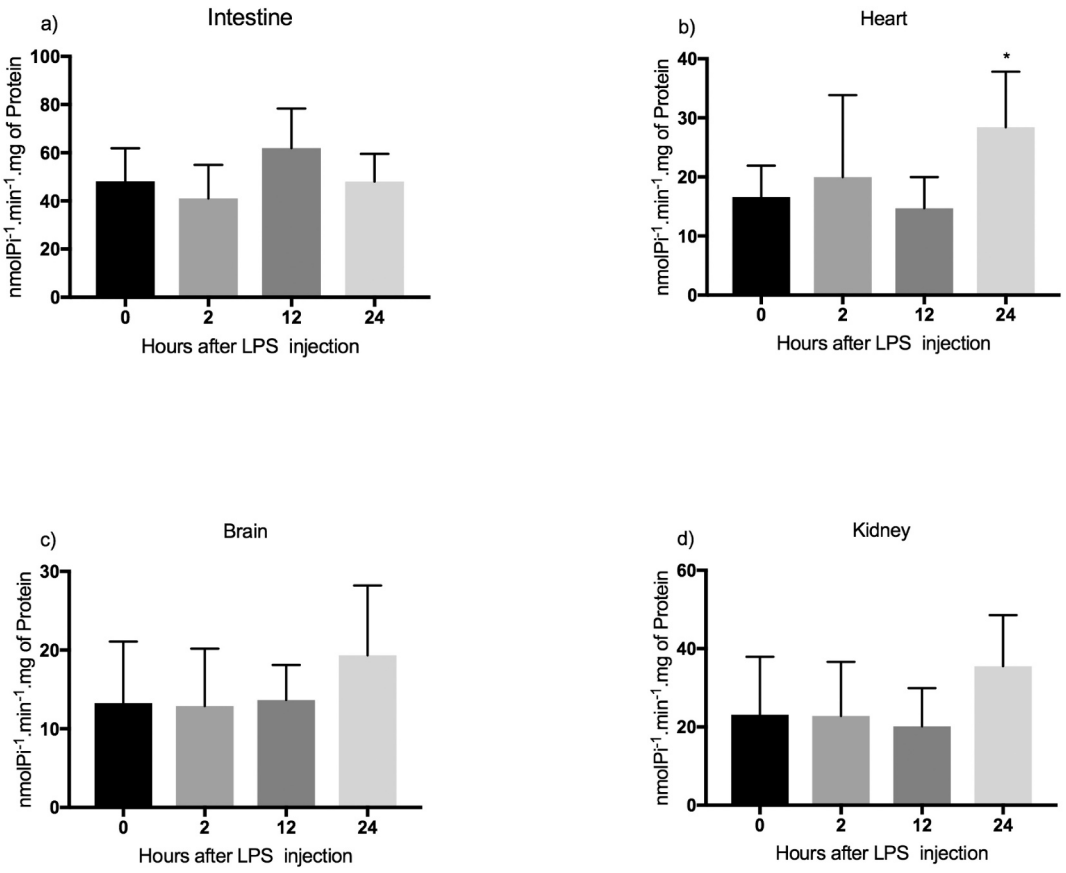

Fig. 4. Effect of LPS treatment at 2, 12, and 24 h after injection on AMP hydrolysis in different organs of adult zebrafish; 4a) intestine, 4b) heart, 4c) encephalon, and 4d) kidney. Data are expressed in nmol Pi·min^{-1}·mg^{-1} of protein (mean ± SD). Six to eight samples were analyzed in triplicate. Data were analyzed by one-way ANOVA. $P < 0.05$ was considered as significant. The * represents a significant difference from the control ($p < 0.05$).

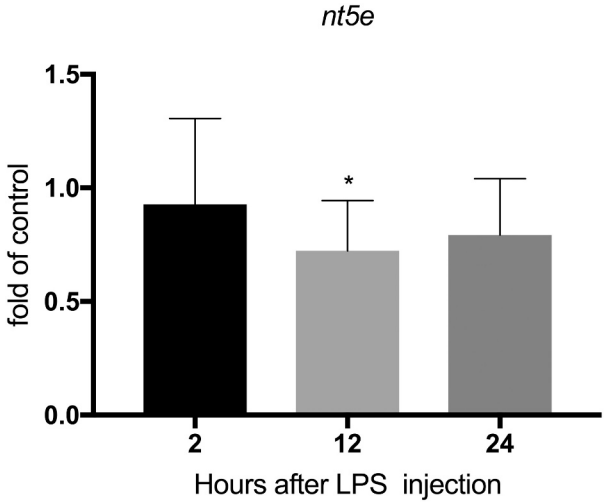

Fig. 5. Effect of LPS treatment at 2, 12, and 24 h after injection on *nt5e* gene expression from the heart of adult zebrafish. Data (mean ± SD) from the LPS-treated group are represented as fold change relative to the control from four independent experiments performed in quadruplicate. Data were analyzed by Student's *t*-test. $P < 0.05$ was considered as significant. The * represents a significant difference from the control group, as $p < 0.05$.

CRediT authorship contribution statement

LRN: conceptualization, validation, experimental approach, formal analysis, and writing the final draft; GMTO, FSM, JSS, and KMC: experimental approach, formal analysis of data, and review of the final draft. MRB: Funding acquisition, supervision, and review; RSS: conceptualization, validation, formal analysis, writing, original draft preparation, review, and funding acquisition.

Funding

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior – Brazil (CAPES) – Finance Code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS). Luiza Reali Nazario and Giovanna Medeiros T. de Oliveira were fellowship recipients of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Jéssica Streb de Sousa was a fellowship recipient of FAPERGS; and Francielle Schroeder de Moraes Silveira and Kesiane Costa were fellowship recipients of CNPq. Rosane Souza da Silva is a Research Career Awardee of the CNPq/Brazil (Proc: 301599/2016-5).

Declaration of competing interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgments

We thank Prof. Fernanda Bueno Morrone from PUCRS for supplying specific primers for RT-qPCR, and Prof. Erik de Vries from University of Groningen for his constructive critical reading.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2022.109402.

References

Abbott, N.J., 2000. Inflammatory mediators and modulation of blood-brain barrier permeability. Cell. Mol. Neurobiol. 20, 131–147.

Antonioli, L., Blandizzi, C., Pacher, P., Haskó, G., 2013. Immunity, inflammation and cancer: a leading role for adenosine. Nat. Rev. Cancer 13, 842–857.

Antonioli, L., Csóka, B., Fornai, M., Colucci, R., Kókai, E., Blandizzi, C., Haskó, G., 2014. Adenosine and inflammation: what's new on the horizon? Drug Discov. Today 19, 1051–1068.

Banjac, A., Nedeljković, N., Horvat, A., Kanazir, D., Nikezić, G., 2001. Ontogenetic profile of ecto-ATPase activity in rat hippocampal and caudate nucleus synaptic plasma membrane fractions. Physiol. Res. 50, 411–417.

Belo, M.A.A., Oliveira, M.F., Oliveira, S.L., Aracati, M.F., Rodrigues, L.F., Costa, C.C., Conde, G., Gomes, J.M.M., Prata, M.N.L., Barra, A., Valverde, T.M., de Melo, D.C., Eto, S.F., Fernandes, D.C., Romero, M.G.M.C., Corrêa Júnior, J.D., Silva, J.O., Barros, A.L.B., Perez, A.C., Charlie-Silva, I., 2021. Zebrafish as a model to study inflammation: a tool for drug discovery. Biomed. Pharmacother. 144, 112310.

Boehmler, W., Petko, J., Woll, M., Frey, C., Thisse, B., Thisse, C., Canfield, V.A., Levenson, R., 2009. Identification of zebrafish A2 adenosine receptors and expression in developing embryos. Gene Expr. Patterns 9, 144–151.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burnstock, G., 1976. Purinergic receptors. J. Theor. Biol. 62, 491–503.

Capiotti, K.M., Menezes, F.P., Nazario, L.R., Pohlmann, J.B., de Oliveira, G.M.T., Fazenda, L., Bogo, M.R., Bonan, C.D., Da Silva, R.S., 2011. Early exposure to caffeine affects gene expression of adenosine receptors, DARPP-32 and BDNF without affecting sensibility and morphology of developing zebrafish (Danio rerio). Neurotoxicol. Teratol. 33, 680–685.

Chan, K.M., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca2+ -stimulated ATPase activity. Anal. Biochem. 157, 375–380.

Costa, K.M., Maciel, I.S., Kist, L.W., Campos, M.M., Bogo, M.R., 2014. Pharmacological inhibition of CXCR2 chemokine receptors modulates paraquat-induced intoxication in rats. PLoS One 9, e105740.

Cruz, F.F., Pereira, T.C.B., Altenhofen, S., da Costa, K.M., Bogo, M.R., Bonan, C.D., Morrone, F.B., 2019. Characterization of the adenosinergic system in a zebrafish embryo radiotherapy model. Comp Biochem Physiol C Toxicol Pharmacol 224, 108572.

Di Virgilio, F., Vuerich, M., 2015. Purinergic signaling in the immune system. Auton. Neurosci. 191, 117–123.

Drury, A.N., Szent-Györgyi, A., 1929. The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. J. Physiol. 68, 213–237.

Fredholm, B.B., 2007. Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ. 14, 1315–1323.

Fujimoto, Y., Nakatani, N., Kubo, T., Semi, Y., Yoshida, N., Nakajima, H., Iseri, T., Azuma, Y.T., Takeuchi, T., 2012. Adenosine and ATP affect LPS-induced cytokine production in canine macrophage cell line DH82 cells. J. Vet. Med. Sci. 74, 27–34.

Gonçalves, A.F., Páscoa, I., Neves, J.V., Coimbra, J., Vijayan, M.M., Rodrigues, P., Wilson, J.M., 2012. The inhibitory effect of environmental ammonia on Danio rerio LPS induced acute phase response. Dev. Comp. Immunol. 36, 279–288.

Haskó, G., Linden, J., Cronstein, B., Pacher, P., 2008. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat. Rev. Drug Discov. 7, 759–770.

Heyliger, C.E., Panagia, V., Dhalla, N.S., 1981. Effect of cyclic AMP phosphodiesterase inhibitors on cardiac sarcolemmal 5'-nucleotidase. J. Pharmacol. Exp. Ther. 217, 489–493.

Hobbs, S.S., Goettel, J.A., Liang, D., Yan, F., Edelblum, K.L., Frey, M.R., Mullane, M.T., Polk, D.B., 2011. TNF transactivation of EGFR stimulates cytoprotective COX-2 expression in gastrointestinal epithelial cells. Am. J. Physiol. Gastrointest. Liver Physiol. 301, G220–G229.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., Akira, S., 1999. Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the lps gene product. J. Immunol. 162, 3749–3752.

Jakhar, R., Sharma, C., Paul, S., Kang, S.C., 2018. Immunosuppressive potential of astemizole against LPS activated T cell proliferation and cytokine secretion in RAW macrophages, zebrafish larvae and mouse splenocytes by modulating MAPK signaling pathway. Int. Immunopharmacol. 65, 268–278.

Jault, C., Pichon, L., Chluba, J., 2004. Toll-like receptor gene family and TIR-domain adapters in Danio rerio. Mol. Immunol. 40, 759–771.

Jovanović, B., Palić, D., 2012. Immunotoxicology of non-functionalized engineered nanoparticles in aquatic organisms with special emphasis on fish–review of current knowledge, gap identification, and call for further research. Aquat. Toxicol. 118–119, 141–151.

Junger, W.G., 2011. Immune cell regulation by autocrine purinergic signalling. Nat. Rev. Immunol. 11, 201–212.

Kabanov, D.S., Prokhorenko, I.R., 2010. Structural analysis of lipopolysaccharides from gram-negative bacteria. Biochemistry (Mosc) 75, 383–404.

Kotani, S., Takada, H., 1990. Structural requirements of lipid a for endotoxicity and other biological activities–an overview. Adv. Exp. Med. Biol. 256, 13–43.

Leite, C.E., Maboni, L.E.O., Cruz, F.F., Rosemberg, D.B., Zimmermann, F.F., Pereira, T.C., Bogo, M.R., Bonan, C.D., Campos, M.M., Morrone, F.B., Battastini, A.M., 2013. Involvement of purinergic system in inflammation and toxicity induced by copper in zebrafish larvae. Toxicol. Appl. Pharmacol. 272, 681–689.

Lu, Y.C., Yeh, W.C., Ohashi, P.S., 2008. LPS/TLR4 signal transduction pathway. Cytokine 42, 145–151.

Mallard, C., 2012. Innate immune regulation by toll-like receptors in the brain. ISRN Neurol. 2012, 701950.

Meijer, A.H., Gabby Krens, S.F., Medina Rodriguez, I.A., He, S., Bitter, W., Ewa Snaar-Jagalska, B., Spaink, H.P., 2004. Expression analysis of the toll-like receptor and TIR domain adaptor families of zebrafish. Mol. Immunol. 40, 773–783.

Menezes, F.P., Rico, E.P., Da Silva, R.S., 2014. Tolerance to seizure induced by kainic acid is produced in a specific period of zebrafish development. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 55, 109–112.

Menke, A.L., Spitsbergen, J.M., Wolterbeek, A.P., Woutersen, R.A., 2011. Normal anatomy and histology of the adult zebrafish. Toxicol. Pathol. 39, 759–775.

Merx, M.W., Weber, C., 2007. Sepsis and the heart. Circulation 116, 793–802.

Ming, W.J., Bersani, L., Mantovani, A., 1987. Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. J. Immunol. 138, 1469–1474.

Morales Fénero, C.I., Colombo Flores, A.A., Câmara, N.O., 2016. Inflammatory diseases modelling in zebrafish. World J. Exp. Med. 6, 9–20.

Nedeljkovic, N., Banjac, A., Horvat, A., Stojiljkovic, M., Nikezic, G., 2005. Developmental profile of NTPDase activity in synaptic plasma membranes isolated from rat cerebral cortex. Int. J. Dev. Neurosci. 23, 45–51.

Nery, L.R., Eltz, N.S., Martins, L., Guerim, L.D., Pereira, T.C., Bogo, M.R., Vianna, M.R., 2014. Sustained behavioral effects of lithium exposure during early development in zebrafish: involvement of the wnt-β-catenin signaling pathway. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 55, 101–108.

Novoa, B., Bowman, T.V., Zon, L., Figueras, A., 2009. LPS response and tolerance in the zebrafish (Danio rerio). Fish Shellfish Immunol. 26, 326–331.

Ouyang, X., Ghani, A., Malik, A., Wilder, T., Colegio, O.R., Flavell, R.A., Cronstein, B.N., Mehal, W.Z., 2013. Adenosine is required for sustained inflammasome activation via the A_2A receptor and the HIF-1α pathway. Nat. Commun. 4, 2909.

Ramakers, B.P., Wever, K.E., Kox, M., van den Broek, P.H., Mbuyi, F., Rongen, G., Masereeuw, R., van der Hoeven, J.G., Smits, P., Riksen, N.P., Pickkers, P., 2012. How systemic inflammation modulates adenosine metabolism and adenosine receptor expression in humans in vivo. Crit. Care Med. 40, 2609–2616.

Rico, E.P., Senger, M.R., Fauth, M.A.G., Dias, R.D., Bogo, M.R., Bonan, C.D., 2003. ATP and ADP hydrolysis in brain membranes of zebrafish (Danio rerio). Life Sci. 73, 2071–2082.

Rosemberg, D.B., Rico, E.P., Guidoti, M.R., Dias, R.D., Souza, D.O., Bonan, C.D., Bogo, M. R., 2007. Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (Danio rerio). Life Sci. 81, 1526–1534.

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

Ruyra, A., Cano-Sarabia, M., García-Valtanen, P., Yero, D., Gibert, I., Mackenzie, S.A., Estepa, A., Maspoch, D., Roher, N., 2014. Targeting and stimulation of the zebrafish (Danio rerio) innate immune system with LPS/dsRNA-loaded nanoliposomes. Vaccine 32, 3955–3962.

Senger, M.R., Rico, E.P., Dias, R.D., Bogo, M.R., Bonan, C.D., 2004. Ecto-5'-nucleotidase activity in brain membranes of zebrafish (Danio rerio). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 139, 203–207.

Seternes, T., Sørensen, K., Smedsrød, B., 2002. Scavenger endothelial cells of vertebrates: a nonperipheral leukocyte system for high-capacity elimination of waste macromolecules. Proc. Natl. Acad. Sci. U. S. A. 99, 7594–7597.

Stanojević, I., Bjelobaba, I., Nedeljković, N., Drakulić, D., Petrović, S., Stojiljković, M., Horvat, A., 2011. Ontogenetic profile of ecto-5'-nucleotidase in rat brain synaptic plasma membranes. Int. J. Dev. Neurosci. 29, 397–403.

Tang, R., Dodd, A., Lai, D., McNabb, W.C., Love, D.R., 2007. Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim. Biophys. Sin. Shanghai 39, 384–390.

Tuin, A., Huizinga-Van der Vlag, A., van Loenen-Weemaes, A.M., Meijer, D.K., Poelstra, K., 2006. On the role and fate of LPS-dephosphorylating activity in the rat liver. Am. J. Physiol. Gastrointest. Liver Physiol. 290, G377–G385.

Verkhratsky, A., Burnstock, G., 2014. Biology of purinergic signalling: its ancient evolutionary roots, its omnipresence and its multiple functional significance. Bioessays 36, 697–705.

Wallace, J.L., Devchand, P.R., 2005. Emerging roles for cyclooxygenase-2 in gastrointestinal mucosal defense. Br. J. Pharmacol. 145, 275–282.

Yang, L.L., Wang, G.Q., Yang, L.M., Huang, Z.B., Zhang, W.Q., Yu, L.Z., 2014. Endotoxin molecule lipopolysaccharide-induced zebrafish inflammation model: a novel screening method for anti-inflammatory drugs. Molecules 19, 2390–2409.

Yegutkin, G.G., 2014. Enzymes involved in metabolism of extracellular nucleotides and nucleosides: functional implications and measurement of activities. Crit. Rev. Biochem. Mol. Biol. 49, 473–497.

Zanandrea, R., Bonan, C.D., Campos, M.M., 2020. Zebrafish as a model for inflammation and drug discovery. Drug Discov. Today 25 (12), 2001–2011. https://doi.org/10.1016/j.drudis.2020.09.036.

Zimmermann, H., Braun, N., 1999. Ecto-nucleotidases–molecular structures, catalytic properties, and functional roles in the nervous system. Prog. Brain Res. 120, 371–385.

Zou, J., Peddie, S., Scapigliati, G., Zhang, Y., Bols, N.C., Ellis, A.E., Secombes, C.J., 2003. Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, Oncorhynchus mykiss. Dev. Comp. Immunol. 27, 813–822.