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Title: Which Th pathway is involved during late stage amoebic gill disease?

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Abstract: Amoebic gill disease (AGD) is an emerging disease in North European Atlantic salmon (Salmo salar Linnaeus 1758) aquaculture caused by the amoeba Paramoeba perurans. The host immune response to AGD infection is still not well understood despite past attempts to investigate host-pathogen interactions. With the significant increase in our knowledge of cytokine genes potentially involved in Th responses in recent years, we examined their involvement in this disease using Atlantic salmon post-smolts sampled 3 weeks after exposure to either 500 or 5000 cells/l P. perurans. Gene expression analysis of cytokines potentially involved in the different Th pathways was performed on the first gill arch including the interbranchial lymphoid tissue (ILT). Th1, Th17 and Treg pathways were found to be significantly down regulated, mainly in samples from fish given the higher dose. In contrast, the Th2 pathway was found to be significantly up regulated by both infection doses. Correlation analysis of the gene expression data and the P. perurans load, assessed by real time RT-PCR of the 18S rRNA, was also performed. In humans, Th2 driven responses are characterized by the production of IgE, which in the majority of worm infections results in the generation of a Th2-mediated response and directs the immune system away from a Th1 inflammatory response. The present results seen during late stage AGD suggest that either an immune evasion strategy, similar to the responses driven by helminthic parasites to avoid cell-mediated killing mechanisms, or an allergic reaction caused by the parasite is occurring.

8th May, 2015

**Ms. Ottavia Benedicenti**

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Editors of Fish and Shellfish Immunology,

Prof Chris Secombes, University of Aberdeen, Aberdeen, UK

Prof Ikuo Hirono, Tokyo University of Marine Science and Technology, Tokyo, Japan

Dear Editors,

Please find attached our paper entitled "Which Th pathway is involved during late stage amoebic gill disease?" that we are submitting for publication in your journal Fish and Shellfish Immunology. We hope the referees will find it suitable for publication in this journal.

Looking forward to hearing from you in due course.

Yours sincerely,

Ottavia Benedicenti

University of Aberdeen

**Reviewer #1:** This manuscript is interesting for the immune responses to amoeba infection in Atlantic salmon. It is almost acceptable, however, there are only several minor comments to be improved.

1. "Figure legends" (Page 15) should be located after "References".

**This has been done.**

2. In Figure legends, "mean + SEM" should be "mean ± SEM".

**The graphs show only the positive standard error and not both positive and negative standard errors, and for this reason we believe the figure legends are correct.**

3. Figure 9, the alphabets and arrows should be more clarified (making bigger).

**They have been made bigger, in terms of the letters, arrows, scale bars and the image itself.**

**Reviewer #2:** Dear authors

I find your paper important, thorough and very well written. The parasite/host interaction is of great importance and knowledge on pathways and immune evasion strategies are very valuable. You have presented your data in an orderly and statistically solid way and I only have minor comments:

1. Please write all abbreviations in full the first time you mention them.

**Corrected.**

2. Please rewrite the sentence from line 253 - 257 on page 9.

**The sentence has been changed.**

3. Remember that the latin name (P. perurans) should not be in italics when it is mentioned in a headline, which is in italics.

**This has been corrected in the headline 3.3.**

4. You should go through your MS and add commas to improve reading of the text.

**More been added to improve the reading of the text.**

5. You write that Treg cells produce IL-10 and TGF-beta, which inhibit immune responses. You find that the Treg pathway is downregulated, which means that immune responses are upregulated. Does this include all immune responses? And if it does can you elaborate on this? I can't see how this would fit with an immune evasion strategy.

**TGF-β is involved in the inhibition of T cell proliferation and macrophage activation, and promotes the development of Th17 cells and tissue repair after a local immune and inflammatory reaction. IL-10 inhibits the production of IL-12, suppressing the secretion of IFN-γ and subsequent activation of innate and adaptive cell-mediated responses. Down regulation of the Th17 pathway could be explained by the suppression of TGF- β1B expression and both down regulation of TGF- β1B and IL-10 could be related to an excessive inflammatory reaction at the level of the gills caused by the presence of the amoeba. Furthermore, the balance between the Th2 cells and Treg cells is important during allergic diseases. For example, during human allergic disease, effector Th2 cells produce IL-4, IL-5, IL-9, and IL-13 and these cytokines induce the production of allergen-specific IgE by B cells, development and recruitment of eosinophils, with a production of mucus. Treg cells directly or indirectly suppress effector cells of allergic inflammation, such as mast cells, basophils, and eosinophils, and contribute to remodelling in asthma and atopic dermatitis. Increased levels of IL-10 and TGF-β produced by Treg cells can suppress IgE production and they can be important to control the allergic inflammation. A down regulation of IL-10 and TGF-β, as seen in this study, might be a limiting factor for the development of a healthy immune response in the case of an allergic reaction to the *P. perurans* occurring during AGD. Further comment on these points has been added to the discussion.**

6. I think you should change the maximum on your Y-axis to a lower number on some of your figures (3, 4, 5, 7). That would increase the visual understanding of the data.

**The Y-axis have been lowered for figures 3, 4, 5, 6, 7, as suggested.**

7. Can you change figure 8 so that it is visually apparent that the value is higher in the high dose? (not a must)

**The Y-axis is now in the opposite orientation, starting with high Cp value from the bottom of the axis and going to a low Cp value. In this graph it is now apparent visually that the value is higher in the higher dose.**

8. Figure 9 is too small. I can't see what you want to show me.

**The figure has been made it bigger, in terms of the letters, arrows, scale bars and the image itself.**

9. It would be very interesting to look into the role of IgT.

**Indeed, and this suggestion will be taken into account for future investigations once an anti-IgT serum has been sourced.**

I wish you good luck.

**Thank you very much.**

**Reviewer #3:** This paper describes the response of a range of genes associated with the Th pathways associated with infection of *Paramoeba perurans* in Atlantic salmon. It makes a valuable contribution to knowledge on the immune response to this important pathogen. The main issues I raise relate to clarity of certain areas which would assist the reader understanding the importance of the observations.

1. Line 114 . Centrifugation should be in 'g' not rpm.

**The values have been converted to 'g' as follows. 6000 rpm = 2200 x g based on the formula that G = 1.12 x radius (mm) x (rpm/1000)$^2$**

2. Line 228. Whilst the histopathological section a valuable contribution and the use of score between 0-5 is appropriate, the criteria used to define each stage in this score would be useful.

**The histological gill score was assessed according to Adams and Nowak (2001). The number of lesions occurring dorsally, medially and ventrally in the gill filaments were noted, along with size of lesion and the degree of pathological severity and pathological changes. This has been added to the methods.**

3. Table 2. The authors correlate the amoeba load with Cp values of the genes of interest. This is a valuable contribution but relies on the use of 18rRNA as a measure of intensity of infection of amoeba. Has the authors' supportive evidence that this parameter reflects proportionally parasite load.

**Yes, a linear relationship ($R^2$=0.9995) between the log of 18S rRNA copies derived from plasmid DNA containing a partial *N. perurans* 18S RNA gene and DNA extracted from increasing numbers of *N. perurans* trophozoites was demonstrated by Bridle et al. (2010). This has been added to the methods.**

4. Fig 3 Th1 pathway IFN and Fig. 6 Th2 pathway IL4, show large SEM both associated the lower trophozoites. Are there any possible causes for the large variation in the data within these categories?

**Larger variation among individuals, as seen by the standard error bars, was found mainly when using the lower infection dose, suggesting a higher variability in infection/responsiveness with the administration of a lower dose of trophozoites. This point has been added to the discussion.**

5. Fig 9. Scale bars difficult to see.

**The letters, arrows, scale bars and image itself have been made bigger.**

## Highlights

- This report describes the characterisation of T helper responses to AGD;
- Gene expression of cytokines have been screened in the ILT of Atlantic salmon;
- Cytokines related to Th1, Th17 and Treg pathways were found down-regulated;
- Expression analysis revealed a high expression of IL-4/13A and IL-4/13B1;
- A mechanism of immune evasion or allergic response might be elicited.

# Which Th pathway is involved during late stage amoebic gill disease?

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## Abstract

Amoebic gill disease (AGD) is an emerging disease in North European Atlantic salmon (*Salmo salar* Linnaeus 1758) aquaculture caused by the amoeba *Paramoeba perurans*. The host immune response to AGD infection is still not well understood despite past attempts to investigate host-pathogen interactions. With the significant increase in our knowledge of cytokine genes potentially involved in Th responses in recent years, we examined their involvement in this disease using Atlantic salmon post-smolts sampled 3 weeks after exposure to either 500 or 5000 cells/l *P. perurans*. Gene expression analysis of cytokines potentially involved in the different Th pathways was performed on the first gill arch including the interbranchial lymphoid tissue (ILT). Th1, Th17 and Treg pathways were found to be significantly down regulated, mainly in samples from fish given the higher dose. In contrast, the Th2 pathway was found to be significantly up regulated by both infection doses. Correlation analysis of the gene expression data and the *P. perurans* load, assessed by real time RT-PCR of the 18S rRNA, was also performed. In humans, Th2 driven responses are characterized by the production of IgE, which in the majority of worm infections results in the generation of a Th2-mediated response and directs the immune system away from a Th1 inflammatory response. The present results seen during late stage AGD suggest that either an immune evasion strategy, similar to the responses driven by helminthic parasites to avoid cell-mediated killing mechanisms, or an allergic reaction caused by the parasite is occurring.

**Keywords:** amoebic gill disease, Atlantic salmon, Th pathways, cytokines, interbranchial lymphoid tissue

## 1 Introduction

Amoebic gill disease (AGD) is an ectoparasitic infection that affects marine fish species farmed in sea net cages [1]. The causative agent of this disease in Atlantic salmon (*Salmo salar* Linnaues 1758) is *Neoparamoeba perurans* [2], an amphizoic amoeba that has successfully fulfilled the Koch's postulates [2, 3]. Recently, nuclear small subunit (SSU) rDNA phylogenetic analysis has shown that the genera *Neoparamoeba* and *Paramoeba* are phylogenetically inseparable and, therefore, *Neoparamoeba* can be used as a junior synonym of *Paramoeba* [4]. AGD has been reported from numerous countries worldwide: South Eastern Australia (Tasmania), Ireland, Japan, New Zealand, Portugal, Norway, USA, Chile, South Africa [5-11] and, since 2011, AGD has been an issue for Scottish Atlantic salmon farms, mainly in summer periods.

The host immune response to AGD infection in Atlantic salmon is still not well understood despite past attempts to investigate host-pathogen interactions. Early studies on transcriptional responses to AGD have shown no differences in the gill tissue expression of tumour necrosis factor (TNF)-α1, TNF-α2, interleukin (IL)-1β, inducible nitric oxide synthase (iNOS), and interferon (IFN)-γ mRNAs compared to tissue from healthy fish, during the early onset of the disease in Atlantic salmon [12]. With the progression of the disease, IL-1β mRNA level was found to be up regulated and lesion-restricted in numerous studies [1, 12-14]. Gene expression profiling using a 16K salmonid microarray has also been performed [15, 16]. In AGD-affected tissue, significant, coordinated down regulation of the major histocompatibility complex (MHC) class I (MHC-I) pathway-related genes occurred during the later stages of infection and appeared to be mediated by down regulation of interferon regulatory factor (IRF)-1, independent of type I interferon, IFN-γ and IRF-2 expression [16]. However, anterior gradient- (AG)-2, involved in inhibiting the tumour suppressor protein p53 and required for mucin (MUC) 2 post-transcriptional synthesis and secretion, was up regulated in AGD-affected gill tissue, while p53 tumour suppressor protein mRNA was concurrently down regulated in AGD lesions, suggesting a role for AG-2 and p53 in AGD pathogenesis [17]. MHC class $II^+$ cells, considered to be antigen-presenting cells and B cells, were found within gill lesions by immunohistochemistry and it was shown that these cells exhibited variable levels of expression [17].

A recent study showed that mRNA expression level of pro-inflammatory cytokines (IL-1β, TNF-α), cellular markers of cell-mediated immunity (T cell receptor (TCR)-α chain, cluster of differentiation (CD) 4, CD8, MHC-I, MHC-IIα), and antibody-mediated immunity (IgM,

IgT) is correlated with a classical inflammatory response in the gills of AGD-affected Atlantic salmon at 10 days post-infection [1]. Moreover, in the same study, it was found that mRNA expression levels of these genes within gill lesions were different to the mRNA expression levels of the same genes in parts of the gill without lesions during AGD, suggesting that there are differences in the transcriptional response to AGD between areas of the gill with lesions and without lesions.

In 2008, a novel lymphoid tissue called the interbranchial lymphoid tissue (ILT) was discovered in the gills of salmonids and the ILT together with leucocytes in the gill filaments constitute the gill associated lymphoid tissue (GIALT) [18-20]. This novel lymphoid tissue is located at the base of the caudal edge of the interbranchial septum between the gill filaments [18] and is visible as a greyish structure by the naked eye [19]. The ILT consists largely of T-cells embedded in a meshwork of epithelial cells [19] and a few B-cells [21]. Abundant MHC class $II^+$ cells are also detectable in the epithelium on the caudal rim of the interbranchial septum and in the epithelium covering the gill filaments [18]. Functional investigations of the ILT have been performed in fish infected with infectious salmon anaemia (ISA) virus and *P. perurans* [21-23]. In ISA virus infected fish, there is a small delayed increase in IgT transcripts [21] and a decrease in size of the ILT compared with healthy fish [22]. Fish affected by AGD show an increased length of the ILT 28 days post exposure in the dorsal area of the gill arch, with a peak of lymphocyte density 7 days post exposure [23].

Further investigation of the T and B cell responses in the ILT is needed to clarify the function of this novel lymphoid tissue during AGD infection in Atlantic salmon. With the increasing number of cytokine genes that are now known, many of which may have a role in adaptive immune responses, the aim of this study was to perform an analysis of T helper (Th) type responses to AGD. For this purpose, gene expression profiles of signature cytokines produced by Th subsets (Th1, Th2, Th17, regulatory T cells - Treg) were screened in the ILT of Atlantic salmon infected with amoeba at two different concentrations (500 and 5000 cells/l), to determine their potential role in host defences against this parasite.

## 2 Materials and Methods

### *2.1 Amoeba culture*

The amoebae were cultivated at 15 °C in small petri dishes containing a 5 ml underlay of malt yeast agar (MYA) (0.05 g malt extract, 0.05 g yeast extract, 10.00 g bacteriological agar, 500 ml of 35 ppt filtered seawater), with approximately 7 ml overlay of 35 ppt filtered

sterilized seawater. Stericup® Filter Units (© EMD Millipore Corporation, Billerica, MA, USA, 2014) with a 0.22 µm pore size were used to filter the seawater coming from the North Sea (ca. 35 ppt salinity). Cultures were maintained in a non-axenic environment containing different bacterial strains isolated with amoebae from gills during culture establishment.

### *2.2 In vivo challenge and sampling of ILT*

Amoebae were cultivated to reach a concentration of 500 cells/l and 5000 cells/l for the *in vivo* challenge. The neutral red (NR)-assay was used to determine cell viability. Briefly, 0.35 µl of neutral red (Sigma-Aldrich, Germany) was added to a 100 µl aliquot of amoeba cultures. After 30 min to allow NR uptake, amoebae were centrifuged at 2200 x g for 10 min, the supernatant was removed and amoebae resuspended in 100 µl of sterilized seawater (35 ppt). Counts were performed in triplicate in 96-well plates.

The experiment was designed to establish an AGD challenge with a type I error of 5% assuming a success rate of 70% (power analysis). Atlantic salmon were taken through smoltification in aquarium facilities at the Marine Scotland Science Marine Laboratory in Aberdeen, UK. Fish were held at 12 °C in full-strength seawater (ca. 35 ppt) and fed daily to 1% body weight using the Skretting Atlantic Smolt diet. Two groups of 5 fish (ca. 400 g) were exposed to 500 cells/l and 5000 cells/l in a total volume of 120 l of seawater (ca. 33 - 35 ppt) and held in this static volume, with aeration, for 4 h. The same procedure was applied for a negative control where 5 fish were exposed to the medium used for amoeba culture, which was filtered with a 3.0 µm pore size Cyclopore$^{TM}$ Track Etched Membrane (GE Healthcare, Whatman, UK), in order to separate out the amoebae but retain the culture bacteria. No signs of distress were observed in fish during this period. After 4 h, the water volume was increased to 350 l and exchanged in a flow through system at a rate of 3 l/min. Fish were fed daily to satiation. At 3 weeks post-exposure, fish were anaesthetised with 0.3 g/l of ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma-Aldrich, Germany) and killed. Tissue samples from the first gill arch were collected, primarily to include the interbranchial lymphoid tissue (ILT) avoiding the gill arch and the end of the gill filaments (Fig. 1). Gill samples were stored in RNA*later* (RNA*later*® Stabilization Solution, Ambion®) at - 80 °C for gene expression and *P. perurans* load analyses.

For histological analysis and assessment of the pathology associated with AGD, samples from the first gill arch were fixed in 10% buffered neutral formalin solution for a minimum of 24 h, washed in 100% ethanol (EtOH), and then stored in 70% EtOH until processing. Samples were then washed three times in 100% EtOH, in xylene (3 dips) and embedded in

paraffin wax. Sections (3 µm) were stained with haematoxylin and eosin (H&E stain) and scored (category 0 - 5) according to Adams and Nowak (2001) [24]. Briefly, the number of lesions occurring dorsally, medially and ventrally in the gill filaments were noted, along with size of lesion, the degree of pathological severity and pathological changes, with a score of 0 (none), 1 (light), 2 (mild), 3 (moderate), advanced (4), and 5 (severe).

### *2.3 RNA extraction and cDNA synthesis*

Total RNA isolation from the gill samples was conducted using TRIzol, following the manufacturer´s instructions (TRIzol® Reagent, Ambion®). Total RNA was dissolved in 50 – 60 µl diethylpyrocarbonate (DEPC)-treated water and concentration [ng/µl] determined on a NanoDrop ND-1000 Spectrophotometer (PEQLAB GmbH, Germany). To assess the sample quality, the A260/A280 and A260/A230 ratios were checked to ensure that the RNA had an A260/A280 ratio of ~2.0 and that the A260/A230 ratio was in the range of 1.8 – 2.2.

To guarantee constant and comparable amounts of RNA in the analyses, the concentration of treated RNA was set to approximately 1000 ng of total RNA per assay for the reverse transcription (RT), to reach a final concentration of approximately 50 ng/µl of input total RNA. The RNA was treated with gDNA Wipeout Buffer (QuantiTect Reverse Transcription Kit, Qiagen) to remove genomic DNA (gDNA) contamination and incubated for 2 min at 42 °C. Each RT was performed in a mix containing: 14 µl RNA previously treated to eliminate gDNA, 1 µl of reverse-transcription master mix (reverse transcriptase and RNase inhibitor), 4 µl of Quantiscript RT Buffer, 1 µl of RT Primer Mix optimized blend of oligo-dT and random primers dissolved in water (QuantiTect Reverse Transcription Kit, Qiagen). The mixture was incubated at 42 °C for 30 min and afterwards the enzyme was inactivated at 95 °C for 3 min. A negative cDNA control sample with DEPC-treated water (Invitrogen™, Carlsbad, USA) instead of reverse transcriptase was included to check for cDNA quality. The generated cDNA template was diluted 1:10 with DEPC-treated water and then stored at - 20 °C until real time RT-PCR analysis.

### *2.4 Real time RT-PCR*

Real time RT-PCR was carried out using a LightCycler® 480 (Roche Applied Science) in a 20 µl reaction using SYBR® Green I Nucleic Acid Gel Stain (Invitrogen™, Carlsbad, USA) and IMMOLASE™ DNA Polymerase (Bioline, UK). 4 µl cDNA (corresponding to 20 ng of input total RNA) were used in each reaction to maintain data integrity for gene expression comparisons. The real time analysis program consisted of 1 cycle of denaturation (95 °C for

10 min), 40 cycles of amplification (95 °C for 30 s, 63 °C for 30 s, 72 °C for 20 s, 84 °C for 5 s), followed by 95 °C for 5 s and 75 °C for 1 min. Program profiles differed for annealing temperature and time for elongation. At least one of each real time RT-PCR primer pair was designed to cross an exon-intron boundary to avoid amplification of gDNA (Table 1). Primer efficiency was tested using 10 fold serial dilutions of cDNA [25] from pooled RNA samples and calculated by the 'LightCycler® 480 software version 1.5.1.62' (Roche Applied Science) as $E = 10^{(-1/s)}$, where s is the slope generated from the Log dilution of cDNA plotted against Cp (cycle number of crossing point). A sample of genomic DNA from muscle of Atlantic salmon parr was used in each real time RT-PCR assay to confirm that no gDNA was amplified.

Each quantification was carried out in duplicate and a sample containing only DEPC-treated water instead of cDNA was used as a negative control. The expression level of the gene of interest (GOI) relative to that of the reference gene elongation factor-1α (EF-1α) was calculated by the 'Relative expression software tool' (REST©) [25, 26]. Cytokines potentially involved in adaptive immunity and released by Th cells, and arginase as a marker of alternatively activated macrophages, were studied.

### *2.5 Correlation analyses between amoeba load and GOIs*

*P. perurans* load in each sample was determined by analysing the amoeba 18S rRNA by real time RT-PCR, as it was reported previously that there is a linear relationship between the log of 18S rRNA copies derived from plasmid DNA containing a partial *N. perurans* 18S RNA gene and DNA extracted from increasing numbers of *N. perurans* trophozoites [27]. For *P. perurans* load the same cDNA samples were used as for the gene expression analysis. The association between the *P. perurans* load (18S rRNA) Cp values at two different concentrations was tested by a linear regression model (R software, software 3.0.1). Normality of the GOI and *P. perurans* load Cp values was tested with a Shapiro-Wilk's W-Test (R software, software 3.0.1), which found that the Cp data were not normally distributed ($p > 0.05$, $n = 5$) both for the GOI and the *P. perurans* load. Therefore, to test the correlation between the *P. perurans* load and gene expression Cp values, a non-parametric test (Spearman's rank correlation coefficient) was applied (GraphPad Prism® Software, version 5.04) (Table 2).

### *2.6 Immunohistochemistry*

Gill tissues from the first gill arch were snap frozen in liquid nitrogen and stored at - 80 °C. They were then embedded in Tissue-Tek® optimum cutting temperature (O.C.T.) compound (VWR Chemicals) and sectioned (10 µm) using a cryostat (Leica CM1850 UV; Leica Microsystems). The sections were mounted onto SuperFrost® UltraPlus (Menzel-Gläser) positively charged glass slides and dried overnight at room temperature. Slides were then stored at - 80 °C until antibody staining. The slides were thawed and the tissue fixed in 4% phosphate buffered formaldehyde for 15 min, and then washed with tap water. Staining was performed using a Leica Bond-Max autostainer (Leica Microsystems), using the Bond Polymer Refine Detection system. The main steps used in the detection system are: 1) incubation with hydrogen peroxide for 5 min; 2) application of the specific primary antibody for 15 min; 3) a post antibody treatment that prepares the tissue for penetration of the subsequent polymer reagent for 8 min; 4) incubation with the polymer reagent, which included the polymeric horseradish peroxidase (HRP) – secondary antibody conjugates that recognize both mouse and rabbit immunoglobulins - for 8 min; 5) 3,3'-diaminobenzidine (DAB) for 10 min; 6) haematoxylin counterstaining that allows the detection of cell nuclei for 5 min. After the staining, samples were washed with tap water for 10 s, then incubated with 70% EtOH for 30 s, 100% EtOH for 30 s, and then xylene (3 dips). Slides were finally covered using a Coverslip Leica CV5030 (Leica Microsystems). Gill tissues from negative control fish and fish in the two treatments (three fish each) were used for immunohistochemistry using an anti - salmonid IgM (4c10) monoclonal antibody (mAb), diluted 1:15 [28]. Consecutive sections from the same fish were used for H&E staining and for a negative control, with a different primary mAb against respiratory syncytial virus - clone 4.15 [29].

### *2.7 Statistical analysis*

A Kruskal-Wallis one-way analysis of variance was used as a non-parametric test to compare the terminal pathology following infection with the two concentrations of trophozoites (500 cells/l and 5000 cells/l), and Dunn’s Multiple Comparison Test was applied to compare differences in the sum of ranks (GraphPad Prism® Software, version 5.04). A pairwise fixed reallocation randomization test (software REST©) was used to compare gene expression analysis. This method has the advantage of making no distributional assumption about the

data, is more flexible than non-parametric tests, does not suffer from a reduction in power relative to parametric tests, and is based on randomization tests [26].

## 3 Results

### *3.1 Histopathology*

Pathological changes in the gills (e.g. hyperplasia) were scored as 0 - 5 for each fish, according to the degree of severity. The negative control (gills from fish exposed to culture media containing culture bacteria) had a gill score of 1 across all five fish. A Kruskal-Wallis one-way analysis of variance was used to compare the difference in the sum of ranks among all the treatments. Medians between the negative control (culture media) and gills, from fish exposed to the higher concentration of trophozoites (5000 cells/l), differed significantly with a p-value of 0.0031 ($n = 5$), based on a Gaussian approximation (Fig. 2). Comparisons between the negative control and gills from fish exposed to the lower concentration of trophozoites (500 cells/l), and between gills from fish given the two concentrations of *P. perurans* trophozoites (500 cells/l and 5000 cells/l), did not reveal any significant differences ($p > 0.05$, $n = 5$).

### *3.2 Gene expression analysis*

Some of the signature cytokines associated with Th1 and Th17 effector cells were found to be significantly down regulated in the gene expression analysis, mostly in samples from fish given the higher concentration of amoebae (5000 cells/l) (Figs. 3 and 4). For example, IFN-γ ($p < 0.05$, $n = 5$) and one of the isoforms of TNF-α (TNF-α3, $p < 0.001$, $n = 5$; [30]), related to the Th1 effector cells, and IL-17A/F1b ($p < 0.01$, $n = 5$) [31, 32], IL-17D ($p < 0.001$, $n = 5$) and IL-22 ($p < 0.01$, $n = 5$) [33] associated with Th17 effector cells, were all significantly down regulated in comparison to the controls. Transforming growth factor (TGF)-β1 and IL-10, regulatory cytokines highly produced by Treg cells, also showed a significant down regulation in comparison to the controls, for both isoforms, in the case of IL-10 in gills from fish given the higher concentration of trophozoites (IL-10A with a p-value < 0.01, IL-10B with a p-value < 0.05, $n = 5$) [34]. Only one isoform of TGF-β1 (TGF-β1B, [35]) was significantly down regulated, but at both concentrations of trophozoites (500 cells/l with a p-value < 0.01, and 5000 cells/l with a p-value < 0.001, $n = 5$) (Fig. 5). In the case of the two isoforms of IL-4/13 (IL-4/13A, IL-4/13B1) [36, 37], that are potentially involved in Th2 effector cell responses, both were found to be significantly up regulated ($p < 0.001$, $n = 5$) in

comparison to the control in gills from fish given the higher concentration of trophozoites, with IL-4/13A also affected in gills exposed to the lower concentration (Fig. 6). Lastly, gene expression analysis of the enzyme arginase, induced by macrophages activated by Th2-type cytokines during the so-called 'alternative macrophage activation' (alternative M2 activation), was studied and revealed a significant down regulation ($p < 0.01$, $n = 5$) in gills exposed to the higher concentration of trophozoites in comparison to gills exposed to the culture media (Fig. 7).

### *3.3 Correlation analyses between* P. perurans *load and GOIs*

A linear regression analysis was performed to estimate the relationship between the *P. perurans* load (18S rRNA) Cp values in gills exposed to the two different initial concentrations of the amoeba (500 cells/l and 5000 cells/l). As expected, it was found that the *P. perurans* load 18S rRNA (Cp values) was significantly different ($p < 0.01$, $n = 5$) between the gills from fish exposed to the two doses, being significantly higher at the higher dose (Fig. 8). The constancy of variance and normality of residuals were checked for the validation of the analysis with model-checking plots (data not shown).

Spearman's rank correlation coefficient was used to test the correlation between the Cp values of GOI and the Cp values of *P. perurans* load in the gill samples (Table 2). A negative correlation coefficient was found for all the genes except for the two IL-4/13 isoforms and IL-17A/F2a that showed a fold change higher than the fold change of the negative control (culture media) gill samples. The strongest and most significant relationships were seen with the negative correlations for IFN-γ, IL-17A/F1b, IL-22, and TGF-β1B (Table 2).

### *3.4 Immunohistochemistry*

In order to examine possible downstream effects of the up regulation of IL-4/13, the presence of immunoglobulin-positive lymphocytes in gill tissue during the AGD infection was studied using a mAb to salmonid IgM (4c10) to detect $IgM^+$ B cells by immunohistochemistry. $IgM^+$ cells were not found in the hyperplastic lesion associated with AGD infection, and were detected only in association with putative blood capillaries. H&E stained slides confirmed the existence of the blood capillaries within the gill sections. Negative controls, using a different primary antibody, on a consecutive gill section for each fish, did not show a positive reaction, confirming the positive results seen using the anti-IgM mAb.

## 4 Discussion

An investigation of the immune response elicited in the interbranchial lymphoid tissue (ILT) can increase our understanding of the role of this tissue in adaptive immunity during late stage AGD infection. The ILT consists largely of T-cells embedded in a meshwork of epithelial cells [19], therefore different cytokines, potentially involved in the development of and secreted by different effector T cells of the $CD4^+$ lineage, have been screened by gene expression analysis in this study.

Initially, following infection of fish with two different exposure concentrations of amoebae (500 cells/l and 5000 cells/l) for a period of 3 weeks, we assessed the pathological changes in the gills (gill score) and *P. perurans* load (18S rRNA) for each fish in the experiment. Histopathological changes in gill score showed significant differences between gills from the negative control (culture media) group and fish exposed to the higher concentration of trophozoites, whereas analysis of the *P. perurans* load by real time RT-PCR (18S rRNA) found a significant difference between Cp values of gill samples from fish exposed to an initial concentration of 500 cells/l versus 5000 cells/l (Fig. 8). The apparent lack of a precise association between gill pathology (gill score) and *P. perurans* load may indicate heterogeneity of both methods across different regions of the first gill arch, similar to that seen for the transcriptional response to AGD infection in gill tissue with lesions and without lesions [1]. To test the correlation between the Cp values of the GOI studied and the Cp values for *P. perurans* load in the gill samples, Spearman's rank correlation coefficient was used. Significant relationships were only seen with the negative correlations for IFN-γ, IL-17A/F1b, IL-22 and TGF-β1B suggesting that results in gene expression profiles and the presence of *P. perurans* in infected gills are not always directly correlated (Table 2).

Gene expression profiles of signature cytokines produced by Th subsets (Th1, Th2, Th17, Treg) were screened in the ILT of Atlantic salmon exposed to the two different concentrations of amoebae, to assess their potential role in host defences against this parasite.

The main function of Th1 cells is to activate macrophages and destroy intracellular microbes following phagocytosis. The principal signature cytokine of Th1 cells is IFN-γ although TNF is also released after activation of Th1 cells. In this study, gene expression analysis showed that IFN-γ and one of the isoforms of TNF-α (TNF-α3) were significantly down regulated in comparison to gill tissue from the negative control when the samples were from fish exposed to the higher concentration of trophozoites (5000 cells/l). Two other salmon isoforms of TNF-α (TNF-α1, TNF-α2) were not significantly down regulated, similar to previous findings

at an early stage of AGD infection in Atlantic salmon [12]. Differences in the expression of the TNF-α isoforms suggest that TNF-α3 may have some unique functions and expression characteristics, as was also seen in a previous study where a differential expression and modulation of these paralogues was hypothesized as a subfunctionalisation that allows a fine-tuned gene regulation [30]. Th17 cells secrete IL-17 and IL-22 cytokines that mainly recruit neutrophils to the site of infection. IL-17 is a key cytokine produced by Th17 cells which is involved in the inflammatory and neutrophil response, and IL-22 is a IL-10 cytokine family member involved in different aspects of the immune response including its production by activated T cells with a role in downstream production of antimicrobial peptides and a promotion of barrier function and tissue repair at the level of epithelial cells. The expression of IL-17A/F1b, IL-17D and IL-22 were found to be significantly down regulated in comparison to the negative control also in gills from fish exposed to the higher concentration of trophozoites.

Treg cells produce IL-10 and TGF-β that inhibit immune responses. Among the different and diverse roles that these cytokines have in the immune system, TGF-β is involved in the inhibition of T cell proliferation and macrophage activation, and promotes the development of Th17 cells and tissue repair after a local immune and inflammatory reaction. IL-10 inhibits the production of IL-12, suppressing the secretion of IFN-γ and subsequent activation of innate and adaptive cell-mediated responses. Only one isoform of the two known salmonid TGF-β1 genes (TGF-β1B) was found to be significantly down regulated in comparison to the negative control, at both concentrations of trophozoites, but both isoforms of IL-10 were found to be significantly down regulated in gills from fish exposed to the higher concentration of trophozoites. That TGF-β1B is more responsive to a range of stimulants has been seen previously, as in trout head kidney (HK) macrophages exposed to bacterial and viral pathogen associated molecular patterns (PAMPs), proinflammatory cytokines, mitogens and pathway activators where highly elevated levels of TGF-β1B are seen but TGF-β1A is unchanged [35]. Down regulation of the Th17 pathway could be potentially explained by the suppression of TGF-β1B expression. Both down regulation of TGF- β1B and IL-10 could be related to an imbalance of the immune system resulting in a severe inflammatory reaction in the gills, caused by the presence of the amoeba, which limits tissue repair mechanisms [38]. Similar imbalances have been described during chronic inflammatory conditions in mammals [38].

Overall, cytokines related to the Th1, Th17 and Treg pathways were found to be significantly down regulated, mostly in gill samples from fish exposed to the higher concentration of

amoebae (Figs. 3-5). In contrast, IL-4/13A and IL-4/13B1, believed to be related to the Th2 pathway, were found to be significantly up regulated (Fig. 6). More variation in gene expression among individuals (as seen by the standard error bars) was found at the lower infection dose, suggesting a higher variability in infection/responsiveness when using lower doses of trophozoites. Th2-mediated immune responses are induced by extracellular parasites [39] and the secretion of IL-4 can be involved either in inducing an alternative pathway of macrophage activation or in the production of antibodies. In order to test the potential effects of the up regulation of IL-4/13, gene expression analysis of the enzyme arginase that is induced in macrophages by alternative (M2) activation and immunohistochemistry (IHC) analysis that investigated the role of immunoglobulin-positive lymphocytes in AGD affected gill tissue were carried out. Gene expression analysis of arginase revealed a significant down regulation in comparison to the negative control in gill tissues from fish expose to the higher concentration of trophozoites (Fig. 7). This suggests that the up regulation of IL-4/13 is not related to a downstream activation of macrophages. The IHC analysis revealed that IgM positive cells were not found in hyperplastic lesions associated with AGD infection (Fig. 9), and were only detected in blood vessels/capillaries in the sections. Hence there was no evidence for proliferation of IgM positive cells or a local IgM humoral response to the parasite where the pathology is present, although we cannot exclude a role for IgT at the present time. Similar findings in gene expression analysis have been found in skin of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) infected with the parasitic flagellate *Ichthyobodo necator* (Henneguy, 1883), where the up regulation of IL-4/13A and IL-10 genes were found as well as the transcription factor GATA3 that is connected to the proliferation of B cells. It was suggested that a partial shift towards a Th2 response was occurring with the *I. necator* infection [40]. Interestingly, *P. perurans* possesses one or more intracellular perinuclear bodies, known as ‘parasomes’ [41]. These ‘parasomes’ are eukaryotic endosymbionts that are described as *Perkinsela amoebae* (Hollande, 1980) - like organisms and seem to be related phylogenetically to flagellated, parasitic marine protozoans (i.e. *Ichthyobodo* spp.) [2, 41, 42]. Thus, the similar findings in relation to the up regulation of the IL-4/13A in skin of rainbow trout infected with *I. necator* and the two IL-4/13 isoforms shown in this study may reflect the phylogenetic relatedness of *I. necator* to the eukaryotic endosymbionts present in *P. perurans*, perhaps suggesting that the eukaryotic endosymbionts might have a role in the pathogenicity seen during AGD.

In humans, Th2-driven responses are characterized by the activation of humoral immunity and the production of IgE, and in the majority of worm infections this results in the

generation of a Th2-mediated response that directs the immune system away from a Th1 inflammatory response [39]. Furthermore, it is known that the balance between the Th2 cells and Treg cells is important during allergic diseases. During human allergic disease, effector Th2 cells produce IL-4, IL-5, IL-9, and IL-13 and these cytokines induce the production of allergen-specific IgE by B cells, development and recruitment of eosinophils, and production of mucus [43]. Treg cells directly or indirectly suppress effector cells of allergic inflammation, such as mast cells, basophils, and eosinophils, and contribute to remodelling in asthma and atopic dermatitis [43]. Increased levels of IL-10 and TGF-β produced by Treg cells can suppress IgE production and can be important for control of allergic inflammation. Down regulation of IL-10 and TGF-β, as seen in this study, might be a limiting factor for the development of a healthy immune response in the case of an allergic reaction to *P. perurans* occurring during AGD infection. However, in the present study, the up regulation of IL-4/13 was not associated with either up regulation of arginase or an increase in IgM positive cells in the hyperplastic lesions, and it may either represent an immune evasion strategy used by the parasite to avoid cell-mediated killing mechanisms, or an allergic reaction caused by the parasite itself.

In conclusion, signature cytokines related to Th1, Th17 and Treg pathways were found to be significantly down regulated, mainly in gill samples from fish exposed to the higher dose of amoebae. In contrast, IL-4/13 expression, potentially involved in the Th2 pathway, was found to be significantly up regulated in gills from both trophozoite exposed groups. The lack of evidence for activation of the alternative macrophage pathway and the absence of IgM positive cells in the hyperplastic lesions associated with AGD infection may suggest that either an immune evasion strategy, similar to the responses driven by helminthic parasites to avoid cell-mediated killing mechanisms, or an allergic mechanisms caused by the parasite, is occurring in AGD infected fish.

**Ethics statement:** All handling of fish was conducted in accordance with the Animals (Scientific Procedures) Act 1986 and all proposed experiments were first subject to detailed statistical review to ensure that a minimum number of fish was used, which would allow statistically meaningful results to be obtained.

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437

## References


[1] Pennacchi Y, Leef MJ, Crosbie PBB, Nowak BF, Bridle AR. Evidence of immune and inflammatory processes in the gills of AGD-affected Atlantic salmon, *Salmo salar* L. Fish & Shellfish Immunology 2014 2;36(2):563-70.

[2] Young ND, Crosbie PBB, Adams MB, Nowak BF, Morrison RN. *Neoparamoeba perurans* n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar*). International Journal for Parasitology 2007;37(13):1469-81.

[3] Crosbie PBB, Bridle AR, Cadoret K, Nowak BF. In vitro cultured *Neoparamoeba perurans* causes amoebic gill disease in Atlantic salmon and fulfils Koch's postulates. International Journal for Parasitology 2012;42(5):511-5.

[4] Feehan CJ, Johnson-Mackinnon J, Scheibling RE, Lauzon-Guay J-, Simpson AGB. Validating the identity of *Paramoeba invadens*, the causative agent of recurrent mass mortality of sea urchins in Nova Scotia, Canada. Diseases of Aquatic Organisms 2013;103(3):209-27.

[5] Santos MJ, Cavaleiro F, Campos P, Sousa A, Teixeira F, Martins M. Impact of amoeba and scuticociliatidia infections on the aquaculture European sea bass (*Dicentrarchus labrax* L.) in Portugal. Veterinary Parasitology 2010;171(1-2):15-21.

[6] Crosbie PBB, Ogawa K, Nakano D, Nowak BF. Amoebic gill disease in hatchery-reared ayu, *Plecoglossus altivelis* (Temminck & Schlegel), in Japan is caused by *Neoparamoeba perurans*. Journal of Fish Diseases 2010;33(5):455-8.

[7] Bermingham ML, Mulcahy MF. Environmental risk factors associated with amoebic gill disease in cultured salmon, *Salmo salar* L., smolts in Ireland. Journal of Fish Diseases 2004 OCT 2004;27(10):555-71.

[8] Young ND, Dyková I, Snekvik K, Nowak BF, Morrison RN. *Neoparamoeba perurans* is a cosmopolitan aetiological agent of amoebic gill disease. Diseases of Aquatic Organisms 2008;78(3):217-23.

[9] Steinum T, Kvellestad A, Rønneberg LB, Nilsen H, Asheim A, Fjell K, Nygård SMR, Olsen AB, Dale OB. First cases of amoebic gill disease (AGD) in Norwegian seawater farmed Atlantic salmon, *Salmo salar* L., and phylogeny of the causative amoeba using 18S cDNA sequences. Journal of Fish Diseases 2008;31(3):205-14.

[10] Bustos PA, Young ND, Rozas MA, Bohle HM, Ildefonso RS, Morrison RN, Nowak BF. Amoebic gill disease (AGD) in Atlantic salmon (*Salmo salar*) farmed in Chile. Aquaculture 2011;310(3-4):281-8.

[11] Mouton A, Crosbie P, Cadoret K, Nowak B. First record of amoebic gill disease caused by *Neoparamoeba perurans* in South Africa. Journal of Fish Diseases 2013;37:407-9.

[12] Morrison RN, Zou J, Secombes CJ, Scapigliati G, Adams MB, Nowak BF. Molecular cloning and expression analysis of tumour necrosis factor-α in amoebic gill disease

(AGD)-affected Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology 2007;23(5):1015-31.

[13] Bridle AR, Morrison RN, Cupit Cunningham PM, Nowak BF. Quantitation of immune response gene expression and cellular localisation of interleukin-1ß mRNA in Atlantic salmon, *Salmo salar* L., affected by amoebic gill disease (AGD). Veterinary Immunology and Immunopathology 2006;114(1-2):121-34.

[14] Morrison RN, Young ND, Nowak BF. Description of an Atlantic salmon (*Salmo salar* L.) type II interleukin-1 receptor cDNA and analysis of interleukin-1 receptor expression in amoebic gill disease-affected fish. Fish and Shellfish Immunology 2012;32(6):1185-90.

[15] Morrison RN, Cooper GA, Koop BF, Rise ML, Bridle AR, Adams MB, Nowak BF. Transcriptome profiling the gills of amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.): A role for tumor suppressor p53 in AGD pathogenesis? Physiological Genomics 2006;26(1):15-34.

[16] Young ND, Cooper GA, Nowak BF, Koop BF, Morrison RN. Coordinated down-regulation of the antigen processing machinery in the gills of amoebic gill disease-affected Atlantic salmon (*Salmo salar* L.). Molecular Immunology 2008;45(9):2581-97.

[17] Morrison RN, Koppang EO, Hordvik I, Nowak BF. MHC class $II^+$ cells in the gills of Atlantic salmon (*Salmo salar* L.) affected by amoebic gill disease. Veterinary Immunology and Immunopathology 2006;109(3-4):297-303.

[18] Haugarvoll E, Bjerkås I, Nowak BF, Hordvik I, Koppang EO. Identification and characterization of a novel intraepithelial lymphoid tissue in the gills of Atlantic salmon. Journal of Anatomy 2008;213(2):202-9.

[19] Koppang EO, Fischer U, Moore L, Tranulis MA, Dijkstra JM, Köllner B, Aune L, Jirillo E, Hordvik I. Salmonid T cells assemble in the thymus, spleen and in novel interbranchial lymphoid tissue. Journal of Anatomy 2010;217(6):728-39.

[20] Salinas I, Zhang Y-, Sunyer JO. Mucosal immunoglobulins and B cells of teleost fish. Developmental and Comparative Immunology 2011;35(12):1346-65.

[21] Austbø L, Aas IB, König M, Weli SC, Syed M, Falk K, Koppang EO. Transcriptional response of immune genes in gills and the interbranchial lymphoid tissue of Atlantic salmon challenged with infectious salmon anaemia virus. Developmental & Comparative Immunology 2014 7;45(1):107-14.

[22] Aas IB, Austbø L, König M, Syed M, Falk K, Hordvik I, Koppang EO. Transcriptional characterization of the T cell population within the salmonid interbranchial lymphoid tissue. Journal of Immunology 2014;193(7):3463-9.

[23] Norte Dos Santos CC, Adams MB, Leef MJ, Nowak BF. Changes in the interbranchial lymphoid tissue of Atlantic salmon (*Salmo salar*) affected by amoebic gill disease. Fish & Shellfish Immunology 2014 2014-Dec;41(2):600-7.

[24] Adams MB, Nowak BF. Distribution and structure of lesions in the gills of Atlantic salmon, *Salmo salar* L., affected with amoebic gill disease. Journal of Fish Diseases 2001;24(9):535-42.

[25] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 2001 MAY 1 2001;29(9):e45.

[26] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 2002;30(9).

[27] Bridle AR, Crosbie PBB, Cadoret K, Nowak BF. Rapid detection and quantification of *Neoparamoeba perurans* in the marine environment. Aquaculture 2010;309(1-4):56-61.

[28] Thuvander A, Fossum C, Lorenzen N. Monoclonal antibodies to salmonid immunoglobulin: Characterization and applicability in immunoassays. Developmental and Comparative Immunology 1990;14(4):415-23.

[29] Gimenez HB, Cash P, Melvin WT. Monoclonal antibodies to human respiratory syncytial virus and their use in comparison of different virus isolates. Journal of General Virology 1984;65(5):963-71.

[30] Hong S, Li R, Xu Q, Secombes CJ, Wang T. Two types of TNF-α exist in teleost fish: Phylogeny, expression, and bioactivity analysis of type-II TNF-α3 in rainbow trout *Oncorhynchus mykiss*. Journal of Immunology 2013;191(12):5959-72.

[31] Monte MM, Wang T, Holland JW, Zou J, Secombes CJ. Cloning and characterization of rainbow trout interleukin-17A/F2 (IL-17A/F2) and IL-17 receptor a: Expression during infection and bioactivity of recombinant IL-17A/F2. Infection and Immunity 2013;81(1):340-53.

[32] Wang T, Jiang Y, Wang A, Husain M, Xu Q, Secombes CJ. Identification of the salmonid IL-17A/F1a/b, IL-17A/F2b, IL-17A/F3 and IL-17N genes and analysis of their expression following in vitro stimulation and infection. Immunogenetics 2015;in press.

[33] Monte MM, Zou J, Wang T, Carrington A, Secombes CJ. Cloning, expression analysis and bioactivity studies of rainbow trout (*Oncorhynchus mykiss*) interleukin-22. Cytokine 2011;55(1):62-73.

[34] Harun NO, Wang T, Secombes CJ. Gene expression profiling in naïve and vaccinated rainbow trout after *Yersinia ruckeri* infection: Insights into the mechanisms of protection seen in vaccinated fish. Vaccine 2011;29(26):4388-99.

[35] Maehr T, Costa MM, González Vecino JL, Wadsworth S, Martin SAM, Wang T, Secombes CJ. Transforming growth factor-β1b: A second TGF-β1 paralogue in the rainbow trout (*Oncorhynchus mykiss*) that has a lower constitutive expression but is more responsive to immune stimulation. Fish and Shellfish Immunology 2013;34(2):420-32.

550 [36] Takizawa F, Koppang EO, Ohtani M, Nakanishi T, Hashimoto K, Fischer U, Dijkstra
551 JM. Constitutive high expression of interleukin-4/13A and GATA-3 in gill and skin of
552 salmonid fishes suggests that these tissues form Th2-skewed immune environments.
553 Molecular Immunology 2011 7;48(12–13):1360-8.

554 [37] Wang T, Secombes CJ. Molecular and functional characterisation of immune genes in
555 salmonids. 2013;Acc. No. HG794522.

556 [38] Asadullah K, Sterry W, Volk HD. Interleukin-10 therapy - review of a new approach.
557 Pharmacological Reviews 2003;55(2):241-69.

558 [39] Williams AE. Immunology: Mucosal and body surface defences. First ed. Oxford, OX4
559 2DQ,UK: John Wiley & Sons, Ltd.; 2012.

560 [40] Chettri JK, Kuhn JA, Jaafar RM, Kania PW, Moller OS, Buchmann K. Epidermal
561 response of rainbow trout to *Ichthyobodo necator*: Immunohistochemical and gene
562 expression studies indicate a Th1-/Th2-like switch. Journal of Fish Diseases 2014
563 SEP;37(9):771-83.

564 [41] Dyková I, Fiala I, Pecková H. *Neoparamoeba* spp. and their eukaryotic endosymbionts
565 similar to *Perkinsela amoebae* (Hollande, 1980): Coevolution demonstrated by SSU
566 rRNA gene phylogenies. European Journal of Protistology 2008;44(4):269-77.

567 [42] Dyková I, Fiala I, Lom J, Lukeš J. *Perkinsiella amoebae*-like endosymbionts of
568 *Neoparamoeba* spp., relatives of the kinetoplastid *Ichthyobodo*. European Journal of
569 Protistology 2003;39(1):37-52.

570 [43] Akdis M, Blaser K, Akdis CA. T regulatory cells in allergy: Novel concepts in the
571 pathogenesis, prevention, and treatment of allergic diseases. Journal of Allergy and
572 Clinical Immunology 2005 11;116(5):961-8.

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## Figure legends

**Fig. 1.** Localization of the gill samples taken from the first gill arch for gene expression analysis, which included primarily the interbranchial lymphoid tissue (ILT) avoiding the gill arch and the end of the gill filaments (© Marine Scotland Science).

**Fig. 2.** Gill scores from 0 (no pathology) to 5 (greatest pathology) were used to assess the gill samples from the first gill arch. A Kruskal-Wallis one-way analysis of variance was used to compare the difference in the sum of ranks (GraphPad Prism® Software, version 5.04) between the three groups. Different letters indicate significantly differences, ** = $p < 0.01$, n = 5.

**Fig. 3.** Relative expression of Th1 pathway genes (mean + SEM) as determined using REST© 2009 (relative expression software tool). The fold change was calculated as the relative expression in comparison to gills from the negative control (culture media) fish, normalized to EF-1α. * = $p < 0.05$, *** = $p < 0.001$, n = 5.

**Fig. 4.** Relative expression of Th17 genes (mean + SEM) as determined using REST© 2009 (relative expression software tool). The fold change was calculated as the relative expression in comparison to gills from the negative control (culture media) fish, normalized to EF-1α. ** = $p < 0.01$, *** = $p < 0.001$, n = 5.

**Fig. 5.** Relative expression of Treg genes (mean + SEM) as determined using REST© 2009 (relative expression software tool). The fold change was calculated as the relative expression in comparison to gills from the negative control (culture media) fish, normalized to EF-1α. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n = 5.

**Fig. 6.** Relative expression of Th2 pathway genes (mean + SEM) as determined using REST© 2009 (relative expression software tool). The fold change was calculated as the relative expression in comparison to gills from the negative control (culture media) fish, normalized to EF-1α. *** = $p < 0.001$, n = 5.

**Fig. 7.** Relative expression of arginase (mean + SEM) as determined using REST© 2009 (relative expression software tool). The fold change was calculated as the relative expression in comparison to gills from the negative control (culture media) fish, normalized to EF-1α. ** = $p < 0.01$, n = 5.

**Fig. 8.** Box and whisker plots showing the distribution of the Cp values with the 5 and 95 percentiles. The relationship between the *P. perurans* load (18S rRNA) Cp values at the two different infection concentrations was tested with a linear regression model (** $p < 0.01$, $n = 5$). Note: higher Cp values correspond to a lower expression of the 18S rRNA in the sample; lower Cp values correspond to a higher expression of the 18S rRNA in the sample.

**Fig. 9.** A) H&E staining: arrow shows the location of a blood capillary; B) IHC staining with anti-IgM mAb: arrow shows the staining of antibody and $IgM^+$ B cells only in association with blood capillaries; C) IHC staining with a primary mAb to an unrelated antigen (negative control): arrow shows the position of the blood capillary; D) H&E staining: arrow shows the AGD pathology, with fusion of secondary lamellae; E) IHC staining with anti-IgM mAb: arrow shows the AGD pathology with no antibody staining; F) IHC staining with a primary mAb to an unrelated antigen (negative control): arrow shows the AGD pathology with fusion of the secondary lamellae.

## Tables

**Table 1.** Primer sequences used for gene expression analysis (real time RT-PCR).

| Gene | | Oligonucleotides (5' – 3') |
|---|---|---|
| **EF-1α** | Forward | CAAGGATATCCGTCGTGGCA |
| | Reverse | ACAGCGAAACGACCAAGAGG' |
| **IFN-γ** | Forward | GATGGGCTGGATGACTTTAGGATG |
| | Reverse | CCTCCGCTCACTGTCCTCAAA |
| **TNF-α1** | Forward | ACTGGCAACGATGCAGGACAA |
| | Reverse | GCGGTAAGATTAGGATTGTATTCACCCTCT |
| **TNF-α2** | Forward | ACTGGCAACGATGCAGGATGG |
| | Reverse | GCGGTAAGATTAGGATTGTATTCACCCTCT |
| **TNF-α3** | Forward | CACGGCAAGAAACAAGATCCCA |
| | Reverse | GATCCACTGGGGTTGTATTCACCTTCTA |
| **IL-4/13A** | Forward | ACCACCACAAAGTGCAAGGAGTTCT |
| | Reverse | CACCTGGTCTTGGCTCTTCACAAC |
| **IL-4/13B2** | Forward | GAGACTCATCTATTGCGTATGATCATCG |
| | Reverse | TGCAGTTGGTTGGATGAAACTTATTGTA |
| **IL-17A/F1b** | Forward | ACACACGGACCACACTCCAGAC |
| | Reverse | GCGTCGATCGTCATAGGTAGTGTTGTA |
| **IL-17A/F2a** | Forward | ACCCTGGACCTGGAAAACCAC |
| | Reverse | GCTGAAGTGTAGAGTACCACGACCTG |
| **IL-17A/F3** | Forward | CTGGTGCTGGGTCTGATCATGT |
| | Reverse | GGTCTCATCGTATGTGTCGCTGTATG |
| **IL-17C2** | Forward | CGAAACCGGTACCAGACAGATGT |
| | Reverse | CCTCCACGCTATCGATGCTGTA |
| **IL-17D** | Forward | AGAAATCCTCGAGCAGATGTTTGG |
| | Reverse | GGGTCGTGGGAGATCCTGTATG |
| **IL-22** | Forward | GAAGGAACACGGCTGTGCTATTAAAC |
| | Reverse | GATCTAGGCGTGCACACAGAAGTC |
| **TGF-β1A** | Forward | CTCACATTTTACTGATGTCACTTCCTGT |
| | Reverse | GGACAACTGCTCCACCTTGTG |
| **TGF-β1B** | Forward | CATGTCCATCCCCCAGAACT |
| | Reverse | GGACAACTGTTCCACCTTGTGTT |
| **IL-10A** | Forward | GGATTCTACACCACTTGAAGAGCCC |
| | Reverse | GTCGTTGTTGTTCTGTGTTCTGTTGT |
| **IL-10B** | Forward | GGGATTCTAGACCACATCAAGAGTCC |
| | Reverse | GGGATTCTAGACCACATCAAGAGTCC |
| **Arginase** | Forward | CATGTCCTACCTCATCCACGAGC |
| | Reverse | GATGGGCTTCTTCACCTTTGAGAA |

**Table 2.** Correlation coefficients between the Cp values of the gene of interest (GOI) and the Cp values of amoeba load (18S rRNA) in gill samples. Data were analysed using Spearman's rank order correlation (n = 10).

| Gene | Correlation coefficient | P-value |
|---|---|---|
| **IFN-γ** | - 0.6727 | 0.0390 |
| **TNF-α1** | - 0.4012 | 0.2475 |
| **TNF-α2** | - 0.1273 | 0.7330 |
| **TNF-α3** | - 0.4303 | 0.2182 |
| **IL-4/13A** | 0.4424 | 0.2044 |
| **IL-4/13B2** | 0.5758 | 0.0883 |
| **IL-17A/F1b** | - 0.6727 | 0.0390 |
| **IL-17A/F2a** | 0.5758 | 0.0883 |
| **IL-17A/F3** | - 0.0667 | 0.8651 |
| **IL-17C2** | - 0.5515 | 0.1049 |
| **IL-17D** | - 0.5879 | 0.0806 |
| **IL-22** | - 0.7697 | 0.0126 |
| **TGF-β1A** | - 0.0303 | 0.9460 |
| **TGF-β1B** | - 0.8667 | 0.0022 |
| **IL-10A** | - 0.2485 | 0.4918 |
| **IL-10B** | - 0.1394 | 0.7072 |
| **Arginase** | - 0.6242 | 0.0603 |

Figure

**Fig. 1:**

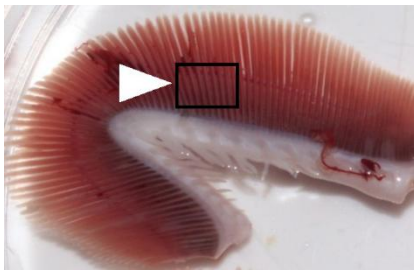

**Fig. 2:**

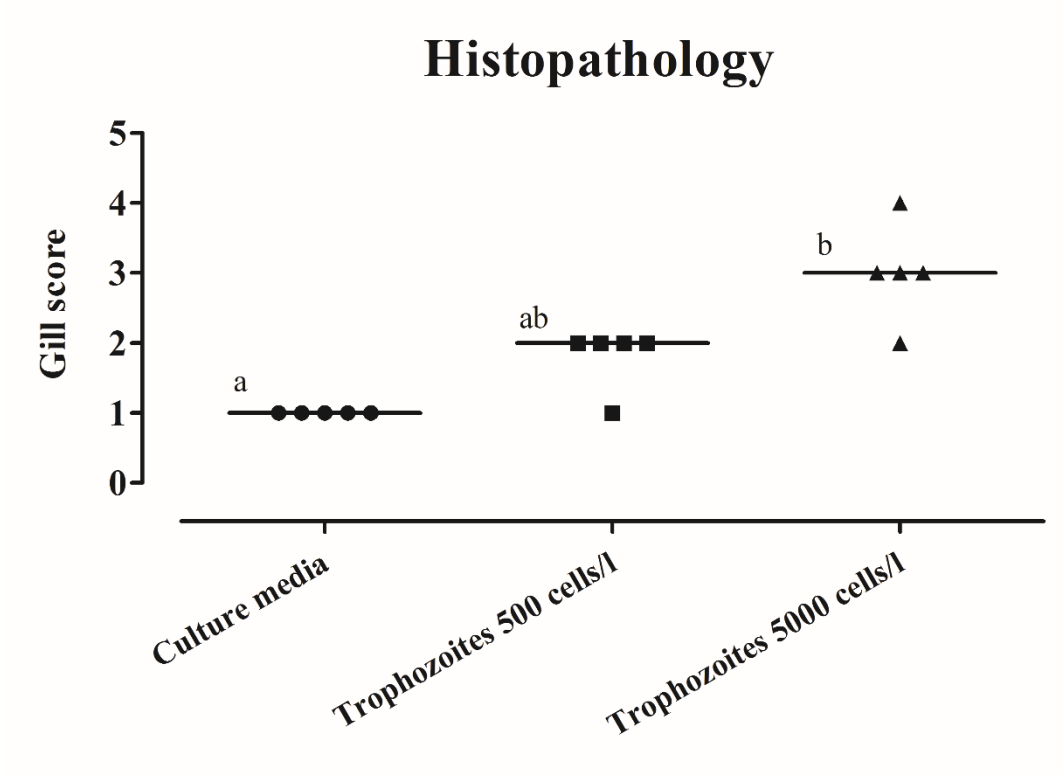

**Fig. 3:**

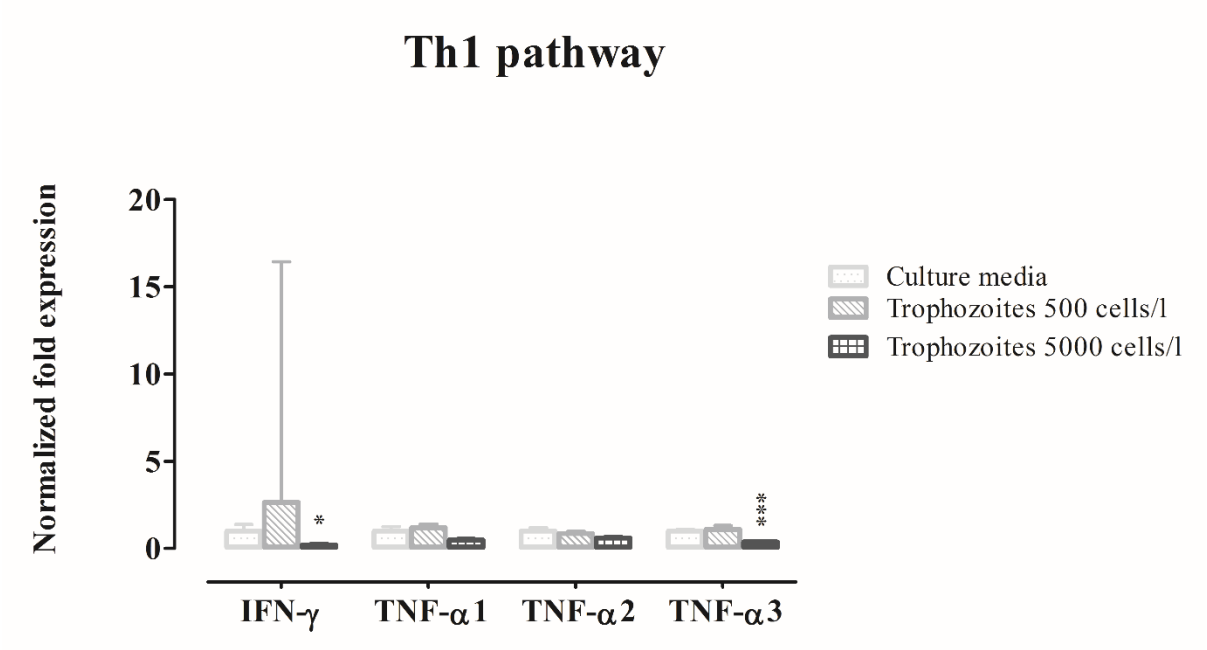
Th1 pathway
Normalized fold expression
20
15
10
5
0
*
***
IFN-γ
TNF-α1
TNF-α2
TNF-α3
Culture media
Trophozoites 500 cells/l
Trophozoites 5000 cells/l

**Fig. 4:**

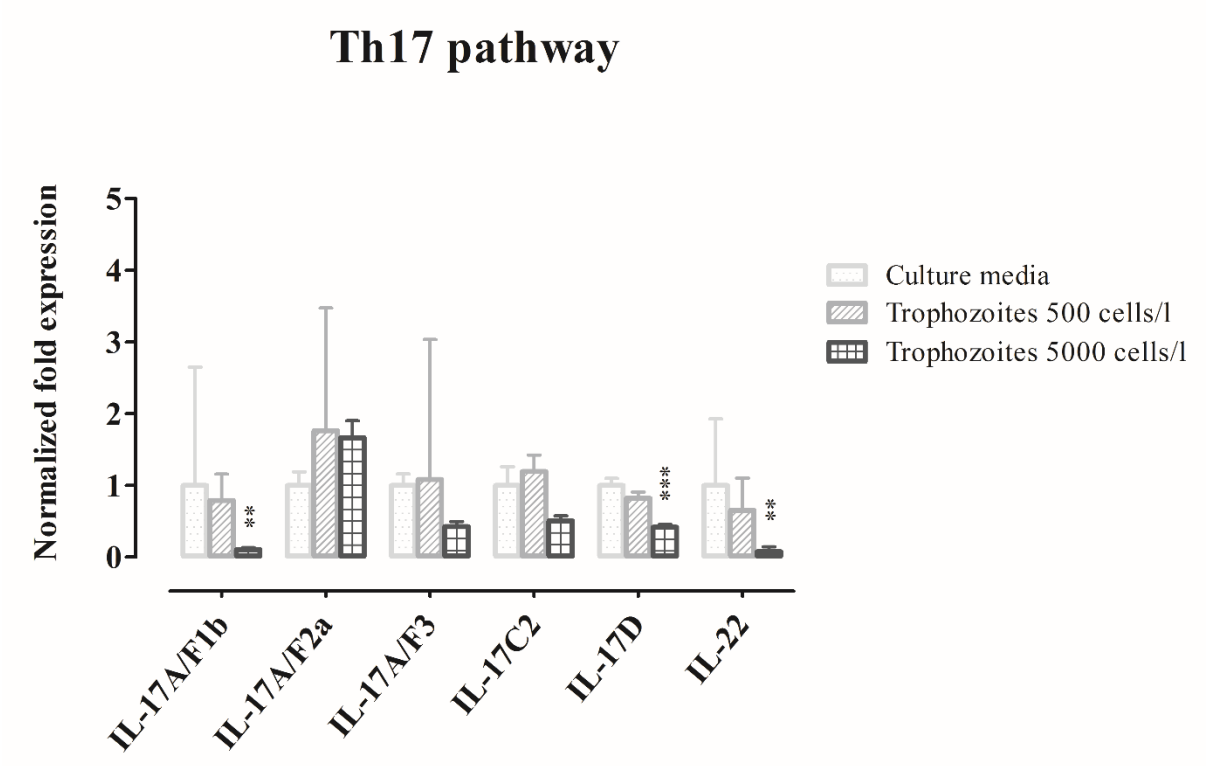
Th17 pathway
Normalized fold expression
5
4
3
2
1
0
**
***
**
IL-17A/F1b
IL-17A/F2a
IL-17A/F3
IL-17C2
IL-17D
IL-22
Culture media
Trophozoites 500 cells/l
Trophozoites 5000 cells/l

**Fig. 5:**

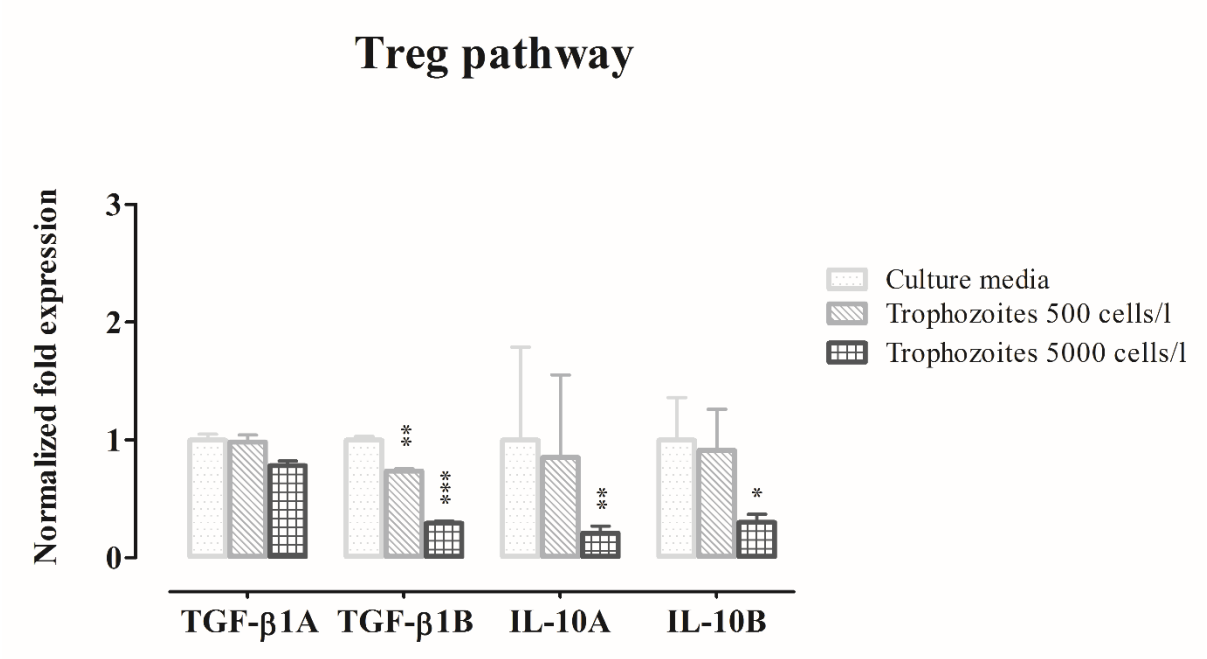
Treg pathway
Normalized fold expression
3
2
1
0
**
***
**
*
TGF-β1A
TGF-β1B
IL-10A
IL-10B
Culture media
Trophozoites 500 cells/l
Trophozoites 5000 cells/l

**Fig. 6:**

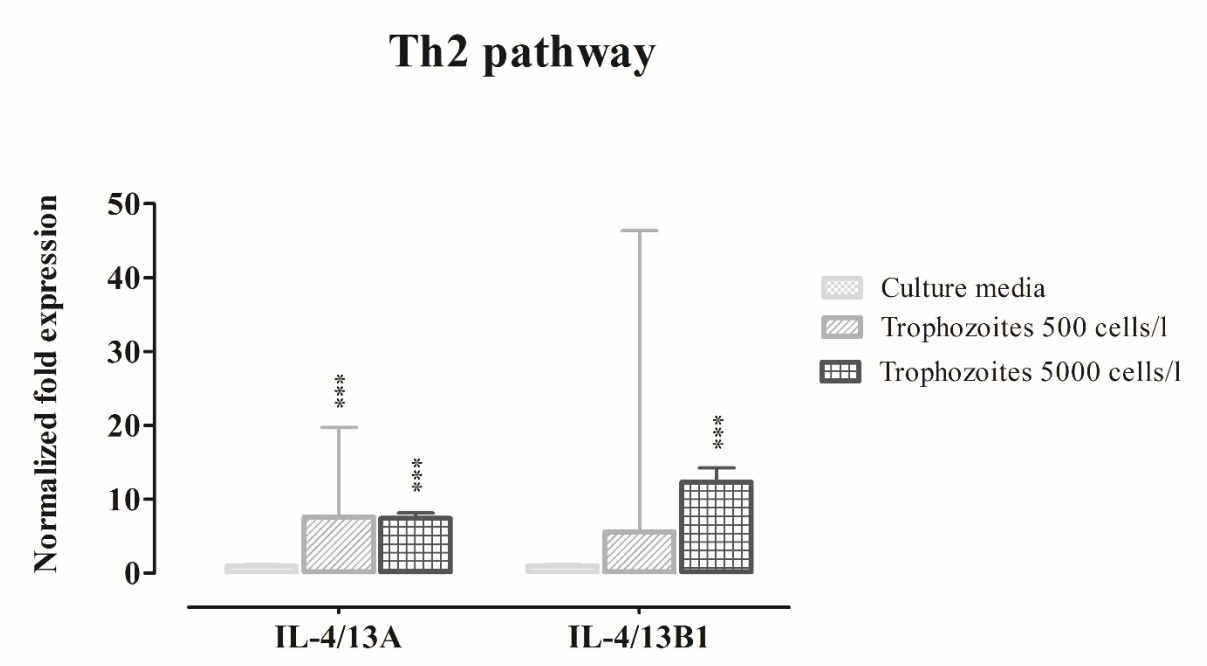
Th2 pathway
Normalized fold expression
50
40
30
20
10
0
***
***
***
IL-4/13A
IL-4/13B1
Culture media
Trophozoites 500 cells/l
Trophozoites 5000 cells/l

**Fig. 7:**

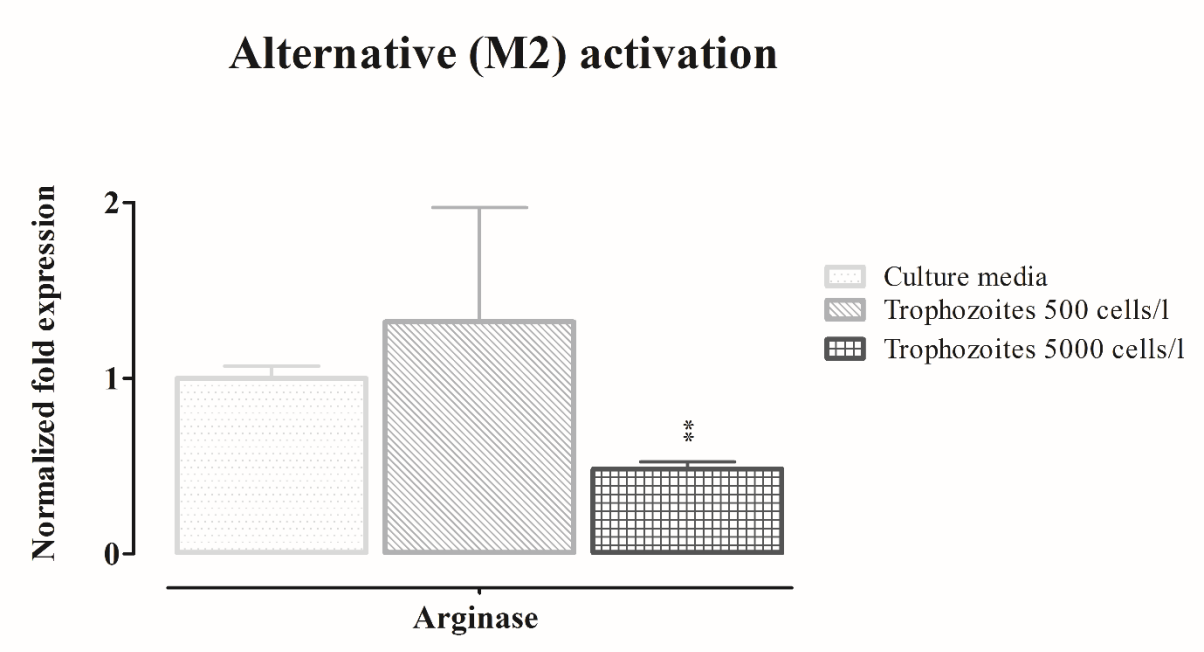
Alternative (M2) activation
Normalized fold expression
2
1
0
**
Arginase
Culture media
Trophozoites 500 cells/l
Trophozoites 5000 cells/l

**Fig. 8:**

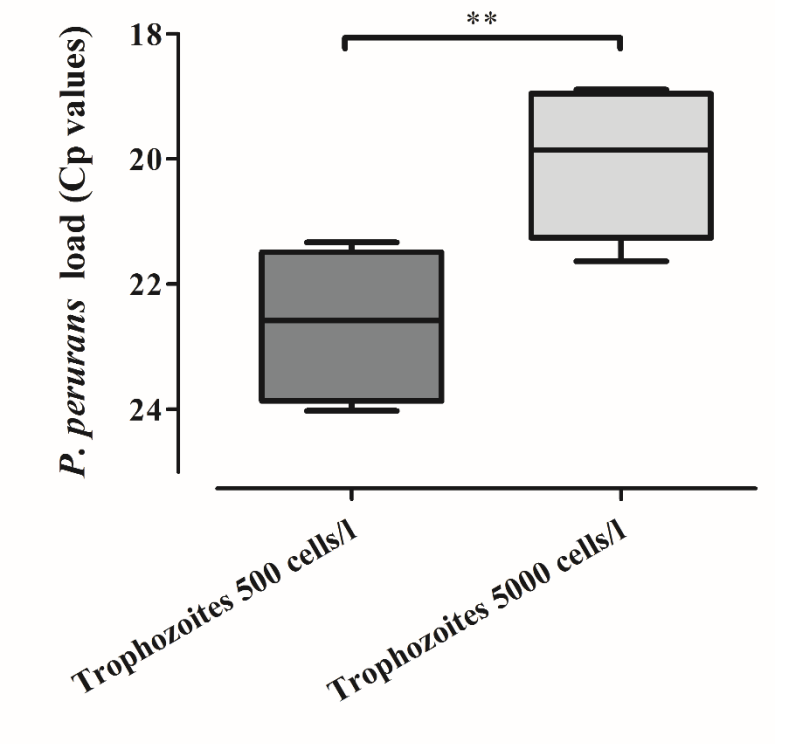
**
18
20
22
24
P. perurans load (Cp values)
Trophozoites 500 cells/l
Trophozoites 5000 cells/l

**Fig. 9:**