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Responsiveness of isolated salmonid GALT leucocytes to PAMPs and recombinant cytokines

Attaya, A.¹, Wang, T.¹, Zou, J.¹, Herath, T.^{2§}, Adams, A.², Secombes, C.J.^{1*} & Yoon, S.^{1*}

¹Scottish Fish Immunology Research Centre, Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK.

²Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, UK.

*Joint corresponding author.

§Present address: Department of Animal Production, Welfare and Veterinary Sciences, Harper Adams University, Newport, Shropshire, TF10 8NB, UK.

Abstract

Increased knowledge of the immune response of the intestine, a physiologically critical organ involved in absorption, secretion and homeostasis in a non-sterile environment, is needed to better understand the mechanisms involved in the induction of long-lasting immunity and, subsequently, the development of efficacious gastrointestinal immunization approaches. To this end, analysis of isolated gut cells will give an insight into the cell types present and their immune capability. Hence, in this study we first optimised a method for salmonid gut leucocyte isolation and characterised the cells on the basis of their expression of a range of selected cell markers associated with T & B cells and dendritic cells. The GALT leucocytes were then stimulated with a variety of PAMPs, recombinant cytokines and PHA, as a means to help characterise the diversity of the immune repertoire present in such cells. The stimulants tested were designed to examine the nature of the antibacterial, antiviral and T cell type responses in the cells (at the transcript level) using a panel of genes relevant to innate and adaptive immunity. The results showed distinct responses to the stimulants, with a clear delineation seen between the stimulant used (eg viral or bacterial PAMP) and the pathway elicited. The changes in the expression patterns of the immune genes in these cells indicates that the salmonid intestine contains a good repertoire of competent immune cells able to respond to different pathogen types. Such information may aid the development of efficient priming by oral vaccination in salmonids.

Introduction

Despite marked progress in our understanding of the teleost immune system over the last decade, the gut immune response is still not well-understood. From an immune perspective the teleost gut is structurally and morphologically different from the mammalian gut, in that it lacks

lymph nodes, Peyer's patches, M cells and IgA secreting lymphocytes, but it does possess gut-associated lymphoid tissue (GALT) that consists of individual and aggregated cells scattered in the lamina propria (lamina propria leucocytes, LPL) and within the epithelia (intraepithelial leucocytes, IEL) (Doggett and Harris 1987, Buddington et al. 1997, Gomez et al. 2013). These cells are diverse and different to those in other immune tissues, such as the spleen and head kidney (Buddington et al. 1997, Rombout et al. 1998b), and include lymphocytes, macrophages, eosinophilic & neutrophilic granulocytes, and antigen sampling cells that resemble immature M cells (Temkin and McMillan 1986, Rombout et al. 1989, Rombout et al. 1993, Dalmo et al. 1997; Fuglem et al., 2010). Most studies have used transcript analysis to define the responses occurring in the gut post infection or oral vaccination (Kai et al, 2014, Martin et al. 2016, Beck et al, 2017). A few have used immunohistochemistry, as with the identification and characterisation of IgT+ B cells that increase in number during parasite infection (Zhang et al. 2010, Parra et al. 2016). Further study of these leucocytes and their bioactivities, the molecules they express and secrete, and the way immune responses are established are needed to enable a better understanding of the immune response in the gut (Gomez et al. 2013, Salinas 2015). Such information will help drive the development of effective oral vaccination strategies, in terms of efficacy, affordability, mass administration and safety.

One approach to study GALT cell immune responsiveness is to isolate the cells and determine their functionality following *in vivo* or *in vitro* stimulation. There are a few reports on isolated IEL and LPL from fish intestine, using methods modified from protocols for isolating such cells from the mammalian GALT (Lundqvist et al. 1992, Di Sabatino et al. 2001). For example, in rainbow trout IEL have been isolated and shown to exhibit phagocytosis and reactive oxygen species (ROS) production (Davidson et al. 1991; Clerton et al. 2001), cytotoxicity against a murine thymoma (EL4) cell line (McMillan & Secombes, 1997) and to express a range of T cell markers (Bernard et al., 2006). TCR β transcripts in trout IEL were highly diverse and showed marked changes in the TCR β repertoire after viral infection. In seabream, isolated GALT cells were also shown to undergo ROS production, and could be separated into three subpopulations by flow cytometry (FSC/SSC) analysis (Salinas et al. 2007). Rather few (<10%) B cells are reported in isolated GALT cells (Rombout et al. 1993; Zhang et al. 2010), which in rainbow trout have similar numbers of IgT+ and IgM+ populations (~54% vs ~46%) in cells from unstimulated fish (Zhang et al. 2010). Following vaccination specific antibody secreting cells can be detected in these isolated cells, with the kinetics varying dependent upon the route of delivery used (Davidson et al. 1993). Whilst such studies give clues to the function of the cells present, clearly there is still much to be learnt about GALT cell responses in fish.

In the present study we have examined further the methods used to obtain salmonid GALT cells, to obtain high yields of viable cells for experimentation, in a time and cost-effective manner. We first characterised the cells obtained in terms of their expression of T-cell, B-cell and dendritic cell (DC) markers, and then studied their responsiveness to a range of pathogen-associated molecular patterns (PAMPs), cytokines and phytohaemagglutinin, to examine whether it is possible to detect antibacterial, antiviral and T cell responses in such cells. The results revealed clear responses to the different stimulants, with differential effects apparent in the target gene expression profiles dependent upon the stimulus.

Materials and methods

1. Fish

Rainbow trout (*Oncorhynchus mykiss*), average weight 250 g, were used for optimizing the protocol for isolating gut leucocytes. The protocol deemed optimal was then applied to isolate gut leucocytes from Atlantic salmon (*Salmo salar*) parr, average weight 15 g, to investigate their responses post stimulation *in vitro*. Fish were maintained in 1m-diameter fibreglass tanks with recirculating freshwater at 14°C and fed twice a day with a commercial diet (2% body weight). Fish were starved for 48 h prior to use in order to evacuate the gut contents before collecting the gut tissue. The experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

2. Gut marker gene analysis

To verify whether particular gut regions were more suitable for GALT cell isolation, a marker gene analysis was undertaken initially. Rainbow trout were killed and the oesophagus, stomach, pyloric caeca, midgut and hindgut were collected, homogenised in TRI reagent (Sigma) and the TRI lysate stored at -80°C until RNA extraction. The genes studied included cell surface markers for T-lymphocytes (CD4-1, CD8 α , TCR α , and TCR γ), B-lymphocytes (membrane (m)IgM, secreted (s)IgM, mIgT and sIgT) and dendritic cells (CD83, CD208, CD209 and MHCII β), since these are key for adaptive immune responses.

3. Optimizing salmonid gut leucocyte isolation

3.1.1. Isolation protocols

Initially, the protocol described by Salinas et al. (2007) for isolation of gilthead seabream (*Sparus aurata*) gut leucocytes was employed to isolate rainbow trout gut leucocytes and was named protocol-1. Later this protocol was modified and named protocol-2, and was in turn modified and named protocol-3. Protocol-1 was carried out as follows: rainbow trout were bled, then the gut from the pyloric caeca to the anus collected from 5 fish and placed into cold PBS. All the connective tissue was removed and any remaining gut contents rinsed off. The guts were opened longitudinally and cut into 1cm long segments, put in 50 ml tubes containing 15-20 ml of a predigestion solution (0.145 mg/ml DTT + 0.37 mg/ml EDTA in Ca²⁺ & Mg²⁺ free HBSS, Sigma) and shaken in an orbital shaker at 50 rpm for 20 min. The supernatants were filtered through 100 μ m nylon mesh strainers (Greiner) to get the first suspension (S1) which was kept at 20°C. Tissue fragments were washed with washing media (0.05 mg DNase I /ml Ca²⁺ & Mg²⁺ free HBSS) containing 5% foetal bovine serum (FBS, Sigma) to remove any remaining DTT and then shaken in 50 ml tubes containing 15-20 ml of digestion solution (0.37 mg collagenase IV /ml washing medium) in an orbital shaker at 50 rpm for 60 min. Supernatants were filtered through 100 μ m nylon mesh strainers, and the obtained suspension was added to S1 to get S2, and washed twice in RPMI-1640 culture medium (Sigma) containing 100 units /ml penicillin & 100 μ g /ml streptomycin (P/S, Gibco) and 10% FBS. The cells were then counted and adjusted to 10⁷ cells /ml. Nylon wool columns (10 ml syringes packed with 0.5 g nylon wool fibre, Dutscher), were loaded with culture medium for 1 h prior to adding 5 ml S2/column. The columns were incubated for 1 h and then washed twice with culture medium to collect the purified cells, which were washed twice with culture medium, counted and adjusted to 10⁷ cells /ml. The cell suspension was then carefully layered over a discontinuous Percoll gradient with two densities (75% and 25%) and centrifuged at 4°C for 30 min at 400 g. Cells in the intermediate density (ID) and high density (HD) bands were collected and washed twice with the culture medium. Protocol-2 was carried out as for protocol-1 but did not include passing S2 through a nylon wool column. Protocol-3 was essentially the same as protocol-2

except that individual fish guts were used as independent samples rather than pooling from 5 fish, and the tissue fragments were shaken in the digestion solution twice for 60 min rather than once. This protocol was also used to isolate GALT leucocytes from Atlantic salmon.

3.2. Leucocyte viability

Isolated GALT leucocytes were distributed into 12-well plates at 2×10^6 cells /well and incubated at 20°C for 4 h and 24 h, at which times they were counted using a Neubauer chamber and 0.5% trypan blue. The viabilities were determined by comparison to the respective time 0 h controls.

3.3. Marker gene analysis

Isolated GALT leucocytes (2×10^6 cells /well) were incubated at 20°C and harvested at 4 h and 24 h by centrifugation at 400 g for 10 min at 4°C. The supernatant was discarded and TRI reagent added, with pipetting up and down several times. The TRI lysate was stored at -80°C until RNA extraction for marker gene analysis (see below). The genes studied were the same as those used for the initial gene expression analysis of the different gut regions.

3.4. Stimulation by LPS

Isolated GALT leucocytes obtained using each protocol, cultured as above (ie 2×10^6 cells /well, 20°C), were stimulated with 50 µg /ml lipopolysaccharide from *Pseudomonas aeruginosa* (LPS, Sigma) for 4 h and 24 h, then harvested, homogenised in TRI reagent and stored at -80°C until RNA extraction. In this case the expression of three key pro-inflammatory genes were studied, namely IL-1β1, IL-6 and TNF-α2.

4. Atlantic salmon GALT leucocyte analysis

Purified Atlantic salmon GALT leucocytes obtained using protocol-3, were suspended in RPMI-1640 containing P/S and 10% FBS, then plated at 2×10^6 cells /well into 12 well plates and cultured at 20°C. The cells, from individual fish, were then stimulated with 10 µg /ml phytohaemagglutinin from *Phaseolus vulgaris* (PHA, Sigma), 50 µg /ml LPS, 100 µg /ml polyinosinic acid: polycytidylic acid (poly I:C, Sigma), 100 ng /ml recombinant flagellin from *Yersinia ruckeri* (rYRF, Wangkahart et al. 2016), 25 ng /ml IL-1β1 (Hong et al. 2001), 100 ng /ml IL-2B (Wang, unpublished), 100 ng /ml IL-6 (Costa et al. 2011), 200 ng /ml IL-21 (Wang et al. 2011) and 20 ng /ml IFN-α2/IFN2 (Zou et al. 2007). All doses used were previously shown to be optimal for trout cells. Untreated cells were included as controls. The GALT cells were incubated for 4 h and 24 h then harvested, homogenised in TRI reagent and stored at -80°C until RNA extraction.

5. RNA extraction, cDNA synthesis and qPCR

Total RNA extraction, cDNA synthesis and real-time PCR (qPCR) analysis of gene expression were as described by Wang et al. (2011, 2014). Tissue samples (or cells) were homogenised in TRI reagent using a Qiagen Tissue Lyser II and stored at -80°C before further processing. The Tri lysates were thawed at room temperature and total RNA prepared as per the manufacturer's instructions. Using Oligo (dT)28VN (Eurofins), dNTPs (Thermo Scientific) and RevertAid Reverse Transcriptase, RNA was reverse transcribed to cDNA under the following conditions:

42°C for 60 min, 45°C for 30 min, 50°C for 30 min and 90°C for 5 min. The resultant cDNAs were diluted in TE- buffer (pH 8.0) (Sigma) and duplicate real-time qPCR reactions were run in a light Cycler 480 machine (Roche) using SYBR green (Sigma). The amplification conditions were as follow: 1 cycle of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 62-66 °C for 30 s and extension at 72°C for 30 s, followed by melting curve analysis between 75°C and 95°C. The primer sets used in this study are shown in Table 1, and were designed with at least one primer across an intron.

Elongation factor-1 α (EF-1 α) was the house-keeping gene used as the endogenous control to normalize gene expression. The transcript levels were calculated using the light Cycler 480 integrated software. Initially leucocyte marker gene relative expression was calculated as arbitrary units by dividing the marker transcript mean concentration by the respective EF-1 α transcript mean concentration. In the case of stimulated GALT cells, the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was then used to express the immune gene expression as a fold change relative to the unstimulated control cells. In each experiment 4 independent replicates were analysed (tissues/cells from 4 individual fish or 4 independent pools of cells) and the data expressed as means and standard error of the mean (SEM).

6. Data and statistical analyses

Expression data were analysed statistically using the IBM SPSS Statistics package 24.0 (SPSS Inc., Chicago, Illinois). One-way analysis of variance (ANOVA) followed by the Tukey post hoc test was used to determine the level of significance between protocols in terms of leucocyte yield and viability. The independent samples T test was used to determine the level of significance between treated and untreated (control) cell samples. $P < 0.05$ was deemed significant.

Results

1. Gut marker gene analysis

Gene expression analysis showed detectable expression levels of the lymphocyte (T-cell, B-cell) and dendritic cell markers in the different gut regions (Fig. 1). However, generally the expression levels were lower in the oesophagus and stomach relative to the other gut regions. The expression levels in the pyloric caeca, midgut and hindgut were relatively similar. An exception was the Ig levels that tended to increase posteriorly along the gut from the pyloric caeca to the hind gut. On the basis of these results the oesophagus and stomach were considered unsuitable for GALT cell isolation but there was no clear advantage to using only the pyloric caeca, mid gut or hind gut. Hence the whole gut posterior of the stomach was taken for cell isolation.

2. Optimizing gut leucocyte isolation

The average cell yield per fish obtained by each protocol differed and ranged from 4.2×10^6 cells /fish, 6.4×10^6 cells /fish and 12.0×10^6 cells /fish for protocol-1, -2 & -3, respectively. The cell yield using protocol-2 was significantly higher than that of protocol-1, and the yield from protocol-3 was, in turn, significantly higher than that of protocol-2 (Fig. 2a). No significant differences between the protocols were found in the viability of the cells obtained but there was a significant fall in viability from 4 h to 24 h in culture, from ~93% to ~53% (Fig. 2b).

Analysis of the T-cell, B-cell and dendritic cell markers in the cells isolated by the three protocols showed that all of the genes were expressed at 4 h and 24 h post-isolation (Fig. 3). The expression patterns, however, differed to some extent between the protocols and in comparison to the expression patterns in the gut tissue (Fig. 1). MHCII β and IgM were relatively highly expressed in cells isolated by all 3 protocols, as was CD83 using protocol-2 and -3 (Fig. 3a-c). Of the T cell markers, CD4-1 was relatively low, whilst IgT was low compared to the IgM transcript levels. The expression patterns did not vary significantly between the sampling times (4 h vs 24 h) using protocol-1 and -2, but there was a decrease of the T cell markers in particular at 24 h using protocol-3, and to a lesser extent with protocol-2.

Stimulating GALT leucocytes isolated by the different protocols with LPS resulted in a significant increase in expression of IL-1 β , IL-6 and TNF- α (Fig. 4). Generally the increases occurred at both time points, with the exception of TNF- α at 4 h in cells isolated by protocol-1 (Fig. 4c) and IL-6 at 4 h in cells isolated by protocol-3. The increases were mostly similar at both time points, especially for protocol-3. However, up-regulation of IL-1 β was higher at 4 h vs 24 h in protocol-2 isolated cells, whilst the increases in TNF- α were higher at 24 h vs 4 h in cells isolated by protocol-1 and -2.

Overall, the three protocols gave similar cell viabilities and the isolated cells had similar marker gene profiles. Also, cells isolated by all three protocols could respond to LPS stimulation by induction of pro-inflammatory genes. However, the increase in cell yield using protocol-3 allowed the analysis of GALT leucocytes from individual fish, and in addition was high enough to allow relatively small fish to be used, meaning that freshwater stage salmon as well as trout could be studied. Hence protocol-3 (Suppl. Fig. 1) was selected for further experiments to look at salmon GALT leucocyte responsiveness to a wider range of stimulants.

3. Atlantic salmon GALT leucocyte analysis

3.1. Marker gene analysis

Initially the GALT cells isolated from Atlantic salmon parr using protocol-3 were checked for their expression of T-cell, B-cell and dendritic cell marker genes at 4 h and 24 h in culture. As seen in Figure 5, the profiles were similar to the trout cells. CD83 and MHCII β were relatively highly expressed, as was TCR α . mIgM was again higher than mIgT, but sIgM/sIgT and CD4-1 /CD8 α were more equal in transcript level. No major differences were seen between the 4 h and 24 h samplings.

3.2. Bacterial PAMP and pro-inflammatory stimulation

LPS was a potent stimulator of pro-inflammatory cytokine expression in the GALT leucocytes, with IL-1 β , IL-6, IL-8 and TNF- α all increasing, especially at 24 h post-stimulation (Fig. 6). The antimicrobial peptide CATH-2 was also highly induced (Fig. 6). Smaller effects were seen on interferon (type I and II) and IRF3 (Figs. 6&7) gene expression, and with Th17-type cytokine (IL-17A/F and IL-22, Fig. 7) gene expression at 24 h. Curiously a small down-regulation of IL-4/13B was seen at 4 h. Bacterial flagellin (YRF) was also a good stimulator of IL-8 expression and IL-22 expression, and to a lesser extent on IL-1 β , IL-6, TNF- α , IFN- γ and CATH-2 transcript levels. Curiously it had no impact on GALT cell IL-1 β expression. rIL-1 β itself was able to induce IL-8 (4 h and 24 h) and CATH-2 (24 h) but had no impact on the other genes studied. Similarly rIL-6 was only able to impact CATH-2 expression, at 24 h post-stimulation.

3.3. Viral PAMP and interferon stimulation

Poly I:C was a potent inducer of type I and II interferons, and IRF3, as expected (Figs. 6&7), with marked increases seen at 24 h. However, it also induced to a lesser extent IL-2, IL-17A/F and IL-22 expression at 24 h (Fig. 7). Recombinant type I interferon was also a good inducer of IRF3 and IFN- γ expression, at 4 h and 24 h, and had a small effect on itself, IL-2 and IL-22 transcript levels at 24 h post-stimulation.

3.4. PHA and T cell cytokine stimulation

PHA was a strong inducer of cytokines potentially associated with adaptive immunity, including IL-2, IL-4/13B, IL-17A/F, IFN- γ and TNF- α , at 4 h and 24 h post-stimulation (Figs. 6&7). It also had a small impact on IL-1 β and IL-22 expression levels at 4 h post-stimulation (Figs. 6&7), and type I and IRF3 expression levels at 24 h post-stimulation (Figs. 6&7). rIL-2 stimulation had relatively few effects, and increased IL-22 expression at 4 h post-stimulation (Fig. 7), and itself and IRF3 at 24 h post-stimulation (Figs. 6&7). Lastly, rIL-21 similar to PHA induced cytokines of adaptive immunity, including IL-4/13B, IL-22 and IFN- γ at 4h and 24 h post-stimulation (Fig. 7), and IL-17A/F at 24 h (Fig. 7). It also induced a small increase of IL-6, TNF- α , type I interferon and IRF3 at 24 h (Figs. 6&7), but had no effect on IL-1 β , IL-2 or IL-8 expression.

Discussion

Fish GALT is structurally and physiologically different from mammalian GALT (Salinas 2015), and the composition of the immune cells present are considered distinct compared to those in other immune tissues such as the head kidney, spleen, skin and gills (Rombout et al. 1998, 2011). Such facts underline the necessity for a better basic understanding of the gut immune system in fish. In the present study we used an *in vitro* approach to examine the responses of isolated GALT leucocytes to a variety of PAMPs and cytokines as a means to help characterise the diversity of the immune repertoire present in such cells.

Before isolating GALT leucocytes we examined whether differences existed in different gut regions in terms of the expression of a variety of marker genes typical of T-lymphocytes (CD4-1, CD8 α , TCR α and TCR γ), B-lymphocytes (sIgM, mIgM, sIgT and mIgT) and dendritic cells (CD83, CD208, CD209 and MHCII β), since this could influence whether to focus on a particular area for cell isolation. That differences may be apparent come from studies in trout showing that IgM⁺ B-cells are highly recruited to the pyloric caeca after oral stimulation (Ballesteros et al. 2013), that IgT⁺ B-cells are particularly abundant in the midgut/hind gut (Ballesteros et al. 2014), and that antigen uptake is most prominent in the second segment of the midgut (Georgopoulou et al. 1998, Fuglem et al. 2010). Expression levels of the marker genes were relatively low in oesophagus and stomach, especially the latter, in agreement with past immunohistochemical studies of intestinal B cells and sites of antigen uptake (Fuglem et al. 2010, Ballesteros et al. 2014). However, in the pyloric caeca, midgut and hindgut higher expression levels were seen that were relatively similar, showing rainbow trout has a good repertoire of the immune cells along the intestine. Nevertheless, there was a trend for increasing Ig transcript level from the pyloric caeca to the hindgut, similar to results seen in sea bass with IgM⁺ B cells (Abelli et al., 1997). On the basis of these results the pyloric caeca, mid gut and hind gut were considered suitable for GALT cell isolation and the whole gut posterior from the stomach was taken for this purpose.

Initially we used the method of Salinas et al. (2007) for isolation of gilthead seabream (*Sparus aurata*) GALT leucocytes. This approach (designated protocol-1) gave a yield of 4.2×10^6 cells /fish, which was relatively low, requiring pooling of gut tissue from multiple fish to allow *in vitro* experimentation. Nevertheless, the cells had relatively good viability, expressed the marker genes as seen in the gut regions from which they were derived, and were responsive to LPS in terms of up-regulation of the pro-inflammatory genes IL-1 β , IL-6 and TNF- α . However, the marker gene analysis showed there were relatively low levels of IgT and CD4-1 transcripts in the isolated cells, relative to IgM and CD8/TCR expression levels. This may reflect fewer IgT B cells (Zhang et al. 2010, 2011) and Th cells in the unstimulated gut, although it is known that CD4-1 can also be expressed by myeloid cells in fish (Takizawa et al. 2016, Dee et al. 2016). Since protocol-1 used nylon wool columns that could impact on yield and cell activity/gene expression, as seen in mice (Wohler and Barnum 2009, Goodyear et al. 2014), this step was excluded in protocol-2. This significantly increased the cell yield, to 6.4×10^6 cells /fish, with no effect on cell viability or LPS responsiveness, but did result in a higher relative expression of some dendritic cell markers. Since the ultimate aim was to get enough cells from a single fish for experimentation *in vitro*, to avoid the potential for reciprocal immune reactions between the cell populations that could give rise to altered gene expression, protocol-2 was modified further by incorporation of a second enzymatic digestion (protocol-3). This again led to a significant increase in cell yield, which went to 12.0×10^6 cells /fish. The viability, expression profiles and responsiveness to LPS of these cells were similar to those from protocol-2, and hence this method was considered a time- and cost-effective means to isolate GALT leucocytes from salmonids. This protocol should be suitable for other fish species but the length of enzymatic digestion may need to be optimised further.

Protocol-3 was next used to isolate GALT leucocytes from Atlantic salmon. The cells were once again analysed for marker gene expression, which revealed similar expression profiles to the isolated cells from rainbow trout and little impact of culture time (4 h vs 24 h), and were considered suitable for the stimulation studies. The stimulants tested were designed to examine the nature of the antibacterial, antiviral and T cell type responses in the GALT leucocytes (at the transcript level) using a panel of cytokines relevant to innate and adaptive immunity, the antimicrobial peptide (AMP) cathelicidin-2 (CATH-2) and interferon regulatory factor (IRF) 3. Since multiple paralogues of such genes can exist in salmonid fish (Lien et al. 2016, Pasquier et al. 2017), a particular paralogue was selected where necessary.

Clear responses to bacterial PAMPS (LPS, flagellin) were detected. IL-1 β , IL-6, IL-8 and TNF- α were all notably increased, as was CATH-2. These cytokines form part of the cytokine cascade typical of responses to Gram negative bacteria, in fish as in mammals (Hong et al. 2013), and reveal they are active in GALT leucocytes. They function to attract and activate phagocytes, to clear bacteria, and can induce expression of AMPs such as CATH-2 (Costa et al., 2011, Hong et al. 2013). Th17/ILC3-type cytokines (Yazdani et al. 2015) are also known to be effective inducers of anti-microbial defences, and the PAMPS were shown to induce both IL-17A/F (considered a forerunner of IL-17A and IL-17F, Wang et al. 2015) and IL-22 in the isolated GALT cells. rIL-22 has been shown to up-regulate a variety of AMPs (β -defensins and LEAPs) in trout (Monte et al. 2011), whilst in haddock marked upregulation of IL-22 occurs in gills following challenge of vaccinated fish (Corripio-Miyar 2009). CATH-2 is a potent AMP in salmonids, able to kill a range of fish bacterial pathogens as part of the innate immune response (Chang et al. 2006). It has been reported to be upregulated by IL-6 and flagellin (YRF) in a trout macrophage (RTS-11) cell line (Costa et al. 2011, Wangkahart et al. 2016), in keeping with the present findings. However, the effects of rIL-6 on CATH-2 expression were quite marginal, and indeed both pro-inflammatory cytokines tested (rIL-1 β and rIL-6) induced relatively few/small effects. Curiously the two cathelicidins present in trout are usually not co-

expressed upon stimulation (Costa et al. 2011, Wang et al. 2016) and it will be interesting to see if this is also the case in GALT leucocytes.

In terms of anti-viral responses, it was shown that poly I:C was a potent inducer of interferons (type I and II) and IRF3 in GALT leucocytes, demonstrating a functional signalling cascade in these cells. Poly I:C is a synthetic double stranded RNA and, as with viruses, is detected by TLR3, which is present in fish in addition to TLR22 that can also bind dsRNA (Sahoo et al. 2015). It serves to induce interferon production, with IRF3 a crucial transcription factor in regulating type I interferon expression (Robertsen 2018). Recombinant type I interferon was also a good inducer of IRF3 and IFN- γ expression, but had a relatively small positive feedback on itself. Neither stimulus impacted on the proinflammatory genes (IL-1 β , IL-6, IL-8 and TNF- α) or CATH-2, showing a clear delineation between these signalling pathways within the GALT leucocytes. Lastly, we examined the responses of possible T cells in the cell cultures, using PHA and two T-cell cytokines (IL-2 and IL-21). PHA was a strong inducer of cytokines of adaptive immunity, including IL-2, IL-4/13B, IL-17A/F, IFN- γ and TNF- α . This induction potentially reflects Th cell or ILC responses, and suggests that multiple subpopulations may be present. Interestingly, IL-4/13 exists as two genes in teleost fish (IL-4/13A, IL-4/13B), with IL-4/13A being more highly expressed constitutively but IL-4/13B is more inducible, as seen post vaccination and infection (Wang et al. 2016, Wangkahart et al. in press). Similar to PHA, rIL-21 was a good inducer of IL-4/13B, IL-17A/F, IL-22 and IFN- γ , and to a small extent TNF- α , however rIL-2 had very limited effects. Previous studies in trout have shown that rIL-21 is able to markedly upregulate IL-10, IL-22 and IFN- γ in head kidney cells, and can maintain the expression of T and B cell markers at a high level compared with control cultures without IL-21, suggesting it may be a survival factor for such cells (Wang et al. 2011).

In conclusion, the current study has optimized a protocol for isolation of GALT cells from salmonids, that gives good viability and yield. The cells expressed a wide range of T-cell, B-cell and dendritic cell markers. They were responsive to a panel of PAMPs, cytokines and PHA, and gave distinct expression profiles dependent upon the stimulus used. The gut leucocyte responses appear mostly comparable with known immune responses from other immune sites and reveal the adequacy of gut cells to elicit appropriate humoral and cellular pathways to combat immunological threats. Further studies are in progress to assess protective immune responses in the salmonid gut, associated with mucosal delivery of antigens, with a view to aid development of efficacious priming of protective responses by oral vaccination in salmonid aquaculture.

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Table & Figures legends

Table 1: qPCR primer sequences and GenBank accession numbers.

Fig. 1. The expression of marker genes for T-lymphocytes (CD4-1, CD8 α , TCR α and TCR γ), B-lymphocytes (sIgM, mIgM, sIgT and mIgT) and dendritic cells (CD83, CD208, CD209 and MHCII β), in rainbow trout oesophagus, stomach, pyloric caeca, midgut and hindgut. The marker gene expression was determined by qPCR and is presented as arbitrary units (after normalising to EF-1 α) relative to the oesophagus transcript level for each gene studied. Bars are means + SEM of four fish.

Fig. 2. (a) The average yield of GALT leucocytes isolated from rainbow trout gut by protocol-1, -2 and -3 and (b) their viability after incubation for 4 h and 24 h. Bars are means \pm SEM of four independent samples. Differences amongst the protocols were tested by One-way ANOVA followed by the Tukey post hoc test, with different letters indicating significant differences.

Fig. 3. The expression of marker genes for T-lymphocytes (CD4-1, CD8 α , TCR α and TCR γ), B-lymphocytes (sIgM, mIgM, sIgT and mIgT) and dendritic cells (CD83, CD208, CD209 and MHCII β), in primary cultures of rainbow trout GALT leucocytes isolated by (a) protocol-1, (b) protocol-2 and (c) protocol-3, and incubated for 4 h and 24 h. The marker gene expression was determined by qPCR, normalised to EF-1 α and presented relative to the CD4-1 transcript level at 4 h. Bars are means \pm SEM of four independent samples.

Fig. 4. Time-dependent induction of (a) IL-1 β 1, (b) IL-6A and (c) TNF- α 2 gene expressions by LPS in primary cultures of rainbow trout GALT leucocytes isolated by protocols-1, -2 and -3. The immune gene expression was determined by qPCR, normalized to EF-1 α and expressed as a mean fold change relative to the control cells. Bars are means \pm SEM of four independent samples. Differences between stimulated samples and controls are shown as * p <0.05, ** p <0.01 and *** p <0.001. Lines above the bars show significant differences between the two time points.

Fig. 5. The expression of marker genes for T-lymphocytes (CD4-1, CD8 α , TCR α and TCR γ), B-lymphocytes (sIgM, mIgM, sIgT and mIgT) and dendritic cells (CD83, CD208, CD209 and MHCII β), in GALT leucocyte cultures isolated from Atlantic salmon gut and incubated for 4 h and 24 h. The

marker gene expression was determined by qPCR, normalised to EF-1 α and presented relative to the CD4-1 transcript level at 4 h. Bars are means \pm SEM of GALT cells from 4 fish.

Fig. 6. Time-dependent induction of (a) IL-1 β 1, (b) IL-6A, (c) IL-8, (d) TNF- α 2, (e) CATH-2 and (f) IRF3 gene expression by PHA, LPS, poly I:C, YRF, IL-2B, IL-21, IFN- α 2, IL-1 β 1 and IL-6 in GALT leucocyte cultures isolated from Atlantic salmon gut. The immune gene expression was determined by qPCR, normalized to EF-1 α and expressed as a mean fold change relative to the control cells. Bars are means \pm SEM of GALT cells from 4 fish. Differences between stimulated samples and controls are shown as * p <0.05, ** p <0.01 and *** p <0.001.

Fig. 7. Time-dependent induction of (a) IFN- α , (b) IFN- γ 1, (c) IL-2A, (d) IL-4/13B1, (e) IL-17A/F-1 and (f) IL-22 gene expressions by PHA, LPS, poly I:C, YRF, IL-2B, IL-21, IFN- α 2, IL-1 β 1 and IL-6 in GALT leucocytes cultures isolated from Atlantic salmon gut. The immune gene expression was determined by qPCR, normalized to EF-1 α and expressed as a mean fold change relative to the control cells. Bars are means \pm SEM of GALT cells from 4 fish. Differences between stimulated samples and controls are shown as * p <0.05, ** p <0.01 and *** p <0.001.

Supplementary Fig. 1. Zigzag flow diagram of the optimised salmonid gut leucocyte isolation protocol.