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Time- and concentration-dependent expression of immune and barrier genes in the RTgutGC fish intestinal model following immune stimulation

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ABSTRACT

The fish intestine comprises an important environment-organism interface that is vital to fish growth, health and pathogen defense. Yet, knowledge about the physiology and defense mechanisms toward environmental stressors, such as bacterial or viral cues, is limited and depends largely on *in vivo* experiments with fish. On this background, we here explore the immune competence of a recently established *in vitro* intestinal barrier model based on the rainbow trout (*Oncorhynchus mykiss*) intestinal epithelial cell line, RTgutGC. We demonstrate that the RTgutGC cell barrier reacts to two immune stimuli, the bacterial lipopolysaccharide (LPS) from *Escherichia coli* and the viral Poly(I:C), by regulating the mRNA abundance of selected genes in a partly time- and concentration dependent manner. The immune stimuli activated the Myd88-and Ticam-dependent signalling cascades, which resulted in downstream activation of pro-inflammatory cytokines and interferon, comparable to the regulatory patterns known from *in vivo*. Stimuli exposure furthermore influenced the regulation of epithelial barrier markers and resulted in slightly impaired barrier functionality after long-term exposure to LPS. Collectively, we provide proof of the usefulness of this unique cell culture model to further gain basic understanding of the fish innate immune system and to apply it in various fields, such as fish feed development and fish health in aquaculture or the evaluation of immuno-toxicity of chemical contaminants.

1. Introduction

The fish intestinal epithelium represents an organism interface that constantly interacts with the environment. The major physiological functions of the fish intestinal epithelium are nutrient uptake, osmoregulation and protection from infectious diseases and environmental stressors [1,2]. Here, the intestinal epithelium acts as both a physical and an immunological barrier against external threats. The connectivity between the intestinal epithelial cells contributes to the formation of a selective physical barrier against pathogens and toxicants, as does a mucus layer covering the cells. The capability of the intestinal epithelium to activate the immune system to efficiently clear infections [3] is the basis for the immunological barrier property. At the same time, the intestine hosts a vast number of commensal bacteria that are beneficial for fish health and play a pivotal role for the physical barrier as well. Thus, to assure proper protection, the intestinal epithelium requires the ability to reliably differentiate and combat pathogens.

As part of the innate immune system, the intestinal epithelial cells express pattern recognition receptors (PRR). PPRs identify pathogens by their pathogen-associated molecular patterns (PAMPs). In fish, the majority of research on the PRRs has focused on the toll-like-receptors (TLRs), which are crucial for the activation of the innate and the adaptive immune system [4]. Up to date, 20 fish TLRs have been identified with partly conserved functionality between mammals and fish, in addition to some fish specific TLRs [5]. All TLRs contain extracellular leucine-rich domains for ligand recognition and a cyto-

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plasmic toll/IL-1R (TIR) domain for signalling. Signalling starts with the recruitment of TIR-domain-containing adaptor proteins, such as myeloid differentiating factor 88 (MyD88) or TIR-containing adaptor molecule 1 (Ticam1), also called TIR-containing adaptor inducing interferon-β (TRIF) [6]. Recruiting different adaptors, and thus forming different downstream signalling proteins, provides for the diversified biological responses induced by TLRs [4].

Among different PAMPs, Polyinosinic:polycytidylic acid (Poly(I:C)) and lipopolysaccharide (LPS) are the model molecular patterns to mimic viral and bacterial infection, respectively. Poly(I:C), as a synthetic analogue of double-stranded RNA, has been shown to exert its biological effect by interacting with the toll-like receptor 3 (TLR3) [7–9]. TLR3 is located intracellularly within the endosomal compartment [10] and has been identified in a number of teleost species, including rainbow trout (*Oncorhynchus mykiss*) [8]. TLR3 signalling has been shown to directly interact with Ticam [7]. Downstream, the antiviral response is activated by interferon (IFN) regulatory transcription factors (IRF) 3 or 7, which translocate to the nucleus and bind to specific promoter elements of the type I IFN initiating transcription [7]. Further, pro-inflammatory transcription factors, such as nuclear factor-κB (NFκB), and their downstream responses, such as cytokines and chemokines, are activated in an Myd88-independent manner [7].

LPS is an important component of the outer membrane of gram-negative bacteria. In mammals, TLR4 [9], together with essential adaptor proteins, such as myeloid differentiation protein 2 (MD-2), LPS-binding protein (LBD) and CD14 [11], are responsible for LPS detection and activation of the downstream signalling pathways. The downstream response activation occurs either via the Myd88-dependent pathway promoting the pro-inflammatory NfκB response or via the Ticam-dependent pathway, which, like TLR3, leads to the activation of the IRF3/7 and INF-mediated response [11,12]. Compared to mammals, fish have been shown to be less sensitive to LPS [13–15], which is likely due to a missing TLR4 and the complete genomic absence of the essential MD-2 and CD14 [16–18]. So far, only zebrafish (*Danio rerio*) and few other cyprinidae have been shown to possess the TLR4 gene [9,11,19]. Nevertheless, a number of biological effects following LPS exposure in fish have been reported [20] and LPS is primarily used in experimental studies to elicit a bacteria-triggered immune response. It has been discussed that impurities of LPS, e.g. peptidoglycans, could activate the TLR4 mediated response. However, more likely, other receptors, such as beta integrins, might be responsible for LPS detection in fish. Yet, these receptors are thought to be less sensitive compared to the mammalian TLR4 complex [11,13].

Besides activating the innate immunity, cytokines have been suspected to interact with the tight-junction complexes by rearrangements, downregulation or internalization of tight junction related proteins. This change in the tight junction has been shown to affect the barrier permeability of the intestinal epithelium and often leads to diseased intestinal states [21–23]. For fish, information regarding the putative cross-talk between the immune and the physical intestinal barrier is scarce [21]. So far, only one study using fish directly showed that IL-1β and IL-6 decrease physical barrier tightness while IFN strengthened barrier functionality [24].

Research on the responses of fish intestinal cells to environmental stressors and immune stimuli can provide valuable fundamental knowledge about fish immune mechanisms and potential application for fish health in aquaculture, animal protection and chemical risk assessment. Therefore, in order to advance mechanistic knowledge of PAMPs induced immune responses, we here used a fish intestinal barrier model, built on the epithelial rainbow trout cell line, RTgutGC [1,25]. By measuring mRNA abundance, we show that LPS and Poly (I:C) activate either the Myd88-or the Ticam-dependent signalling cascade alone as well as in mixture and lead to the downstream regulation of pro-inflammatory cytokines and interferon, along with a regulation of markers of the fish intestinal barrier.

2. Materials and methods

2.1. RTgutGC cell culture

Routine cell culture The rainbow trout intestinal epithelial cell line, RTgutGC, was originally isolated from the distal intestine of a female rainbow trout (*Oncorhynchus mykiss*) [25]. Cells were routinely cultured in Leibovitz L-15 medium (Invitrogen, Basel, Switzerland) supplemented with 5% fetal bovine serum (FBS; PAA, Basel, Switzerland) and 1% gentamycin (PAA, Basel, Switzerland) (complete medium, L-15/FBS). Cells were cultured in 75 cm^2 flasks (TPP, Transadingen, Switzerland) at 19 ± 1 °C and sub-cultured every 7–10 days.

Cultivation on permeable supports For exposure to model bacterial and viral stimuli, RTgutGC monolayers were cultured on 24-well (for cell viability) and 6-well (for gene expression) permeable filter supports in *trans*-well inserts according to optimized protocols that yield a polarized epithelial monolayer within three weeks of culture [1]. Briefly, prior to cell seeding, permeable filter supports with PET-filter membranes (0.4 μm pore size, Greiner-bio-one, Frickenhausen, Germany) were coated with 50 μL (24-well) or 300 μL (6-well) of 30 μg/mL fibronectin (Roche, Mannheim, Germany) in sterile PBS (Invitrogen, Basel, Switzerland) for 1 h at room temperature and 100 rpm horizontal shaking. Fibronectin solution was discarded and supports were washed twice with sterile PBS. The cells were seeded at a density of 5.8×10^4 cells/cm^2 in 0.3 mL (24-well) or 3.5 mL (6-well) of L-15/FBS. The basolateral compartment was filled with 1 mL (24-well) or 4 mL (6-well) of L-15/FBS. The cells were cultured for three weeks at 19 ± 1 °C with weekly medium changes of the apical and basolateral solution. For background detection, cell-free permeable supports were filled with L-15/FBS and treated identically.

2.2. Exposure to model viral and bacterial immune-modulating stimuli

Experiments to determine mRNA abundance of selected genes were conducted with non-toxic concentrations of LPS (*Escherichia coli* O111:B4, Sigma-Aldrich, Germany, < 3% contamination with e.g. proteins) and Poly(I:C) (Sigma-Aldrich, Germany). To derive non-toxic concentrations, the cell viability of RTgutGC cells following exposure to both immune stimuli in either L-15/ex or mucosal saline (MuS) was evaluated as described in the supplemental information (SI Fig. 1). L-15/ex is derived from L-15 but contains only the salts, galactose and pyruvate. It was selected because it mimics the conditions in the intestinal lumen of fish living in the freshwater environment [1]. MuS has less sodium and more magnesium and calcium than L-15/ex [1,26]. It was selected because it mimics the intestinal lumen conditions of fish that migrate to seawater [1].

Time- and concentration-dependency of gene expression The time- and concentration dependency of gene expression after immune stimulation was investigated using the RTgutGC cells seeded in 6-well plate *trans*-well inserts. After three weeks of cultivation, RTgutGC cell monolayers were exposed to different concentrations or different exposure times of LPS or Poly(I:C) singly and in combination. To initiate each experiment, the transepithelial electrical resistance (TEER) was assessed as a quality measure of monolayer formation using an epithelial tissue EVOM (Epithelial Volt/Ohm Meter) Voltohmmeter equipped with an Endohm-24SNAP chamber (World Precision Instruments, Berlin, Germany) as previously described [1,27,28]. To adjust the monolayer to the exposure conditions, the apical medium was replaced with 3.5 mL of the exposure medium, L-15/ex, and the basolateral medium with 4 mL of L-15/FBS and incubated at 19 ± 1 °C for 30 min.

To evaluate the time-dependent gene expression response following immune stimulations, cells from three *trans*-well inserts were exposed in the apical chamber to either 50 mg/L of LPS or Poly(I:C) in L-15/ex or to L-15/ex alone for 1, 6, 16, 24 h at 19 ± 1 °C. 50 mg/L was chosen as a non-toxic concentration based on cell viability assessment (SI

Fig. 1) and to yield an intermediate gene expression based on pre-experiments conducted in 6-well plates (data not shown). In these pre-experiments, the highest change in gene expression was observed after 6 h while the change in expression after 1 h of exposure was very low compared to a medium control. Based on this, 1 h of exposure was chosen for normalization as described below (section data treatment and statistical evaluation). For both immune stimuli, the same concentration was chosen to be able to compare both exposures. The basolateral chamber was filled with 4 mL of L-15 without FBS. Each experiment was independently carried out three times with cells from different passages. However, the L-15/ex control was only added in the 2nd and 3rd biological replicate.

To study the effect of different concentrations, the RTgutGC monolayers were exposed to a range of non-toxic concentrations selected from the cell viability experiments: 0, 10, 50, 100 mg/L of, respectively, LPS or Poly(I:C), for 6 h. For evaluation of the effect of a mixed stimulation, the monolayers were exposed for 6 h to L-15/ex containing either 10 mg/L Poly(I:C) or 10 mg/L LPS or a mixture (i.e. 2 × 10 mg/L) of Poly(I:C) and LPS.

Selection of immune and barrier relevant genes The genes of interest were selected to represent different organisational levels from the upstream activation of receptors and the corresponding signalling and response molecules to the downstream triggering of specific and general responses (Fig. 1, Panel A, Table 1). Further, genes were selected such that they represent an immune activation on the viral and bacterial signalling pathway. For viral signalling molecules, the following genes were chosen: *tlr3*, *ticam*, *irf3* and total *ifn1*. Genes chosen for bacterial stimulation were: *tril*, *myd88*, *nfκb* and *il8*. In addition, the pro-inflammatory cytokines, *tnfα*, *il6* and *il1β* were selected (see Table 1).

The barrier relevant genes were decided to represent three main characteristics of intestinal epithelial cells (Fig. 1, Panel B). The first characteristics are the cell-cell connections accomplished by tight junctions, which were represented by the tight junction protein Zonula Occludens (*zo-1*). The second characteristics are the apical surface enlargements of intestinal epithelial cells, i.e. the villi structures forming the brush border membrane. These were exemplified by villin, which is an actin-binding protein that has been shown to bundle, nucleate, cap and disrupt actin in a Ca^{2+}-dependent manner [29]. The third considered characteristics of intestinal epithelial cells is the secretion of a mucus layer, represented by the intestinal mucin (*i-muc*). Expression for all genes was assessed in RTgutGC cell samples. In addition, specificity of primers was tested in isolated rainbow trout tissue.

2.3. Determining mRNA abundance of selected genes

RNA isolation – RTgutGC cells After exposure, the medium was removed and 0.4 mL of RTL lysis buffer (Qiagen, Germany) was added for 30 s. The cell lysate was transferred to a 2 mL Eppendorf tube and RNA isolation was performed with the RNeasy Plus Mini Kit including genomic DNA eliminator columns (Qiagen, Germany) using a QIAcube robot (Qiagen, Germany). RNA concentration and purity was measured using a Nanodrop Spectrophotometer (NanoDrop®, ND-1000, ThermoFischer, USA). RNA integrity was evaluated by 1% agarose gel electrophoresis.

RNA isolation – Rainbow trout tissue Whole intestinal tissue from rainbow trout, kept as described previously [32], were thawed on ice and cut into small pieces. Since the obtained amount of tissue might overload the RNeasy Kit columns, RNA was extracted using Trizol-chloroform extraction. Tissues pieces were transferred into 2 mL Eppendorf tubes containing 1 mL of Trizol (Invitrogen, USA). The tissue was homogenized using metal beats and the Qiagen Tissue Lyser II (3 × 1 min, frequency s^{-1}) (Qiagen, Germany). After the extraction, an equal amount of RTL buffer was added and RNA isolation was performed as described for the cell samples.

cDNA synthesis RNA was diluted to 100 ng/μL in nuclease-free water (Promega, Switzerland) and used for cDNA synthesis with the PrimeScript RT reagents using oligo dT primers (Takara, France) performed with BioRad temperature cycler (BioRad Laboratories DNA Engine Dyad TM, USA).

Primer design qPCR primers were designed based on assembled nucleotide sequences from NCBI Genebank using the OligoAnalyzer 3.1 and PrimerQuest from IDT – integrated DNA technologies (https://eu.idtdna.com/calc/analyzer) (Table 1). Primer oligomers were synthetized by Microsynth (Balgach, Switzerland).

Quantitative real-time PCR (qPCR) Quantitative real-time PCR (qPCR) was performed using 384 well plates prepared by the QIAgility pipetting robot (Qiagen, Germany) on the LightCycler® 480 (Roche, Germany) using GoTaq®qPCRs Master Mix (Promega, Switzerland). Samples contained 7.5 μL SYBR green master mix, 0.5 μL forward and reverse primer (from 10 μM stock concentration), 3 μL of 1:5 diluted cDNA template and 3.5 μL nuclease free H_20. Cycling parameters were as follows: 95 °C (5 min), 55 cycles of 95 °C (15 s), 56 °C (15 s) and 72 °C (20 s). A melting curve analysis was performed at the end of each run (95 °C for 5 s, 65 °C for 60 s, 0.22 °C per s increases up to 97 °C). For each target gene a standard curve to determine the amplification efficiency (AE) with five 1 : 5 dilutions and a water and reverse

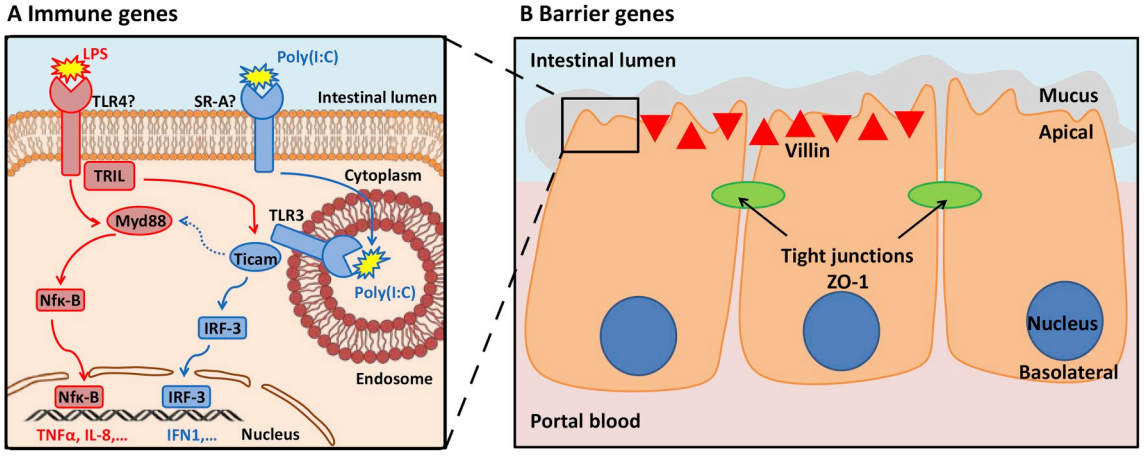

Fig. 1. Schematic representation of selected barrier- and immune-related genes
Genes were selected to represent two immune signalling pathways: the bacterial (= LPS, in red) and viral (= Poly(I:C), in blue) from the receptor to the downstream response elements (Panel A). Genes representing the barrier properties of the RTgutGC monolayer were selected to cover the cell-cell connection via tight junctions (ZO-1), the apical microvilli formation (villin) and the secretion of a mucus layer on the apical side (i-muc) (Panel B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Gene and primer information used for qPCR

Gene name, abbreviation, accession number, forward (F) and reverse (R) primer sequences, the product size and the amplification efficiency (AE) are shown. The AE for each gene was calculated as the mean ± SD over all replicates and experiments conducted. For the primer design of some genes, several sequences were extracted from the Genbank database and assembled. For these cases, all used accession numbers are shown. For *tril*, *i-muc*, *nfκb* and *ticam* only predicted nucleotide sequences were available. Based on these, partial sequences were confirmed by sanger sequencing performed by Synergene Biotech GmbH (Schlieren, Switzerland) and later used for qPCR primer design.

Classification	Genes			Primer sequence 5′-3′	Product size [bp]	AE mean ± SD
	Name	Abbr.	Genbank accession No.			
Receptor	Toll-like receptor 3	*tlr3*	DQ459470.1	F: CTCATCCTCAGCCCTATGT R: GCTTGAACGGAGAGGTATTC	109	1.93 ± 0.08
	Toll-like receptor 4 interactor[i]	*tril*[i]	based on sequencing	F: GAGGTGAGGAAGGAGATAGAA R: CTACTCCCAATTTCCCTATGC	89	2.06 ± 0.33
Adaptor proteins	Myeloid differentiation primary response 88	*myd88*	AJ878918.1 NM_001124421.1	F: GATGCCTTCATCTGCTACTG R: CAAACACACACAGCTTCAAC	100	1.94 ± 0.08
	TIR-domain containing adaptor inducing IFN	*ticam/trif*	based on sequencing	F: GCTAACCATCTGGCTGAAA R: CACGGTACACTCTTGGAAAG	90	1.83 ± 0.08
Response/regulating element	Interferon regulatory factor 3	*irf3*	NM_001257262.1 HF565492.1	F: AAGCTCACTTCAGGGTTTC R: CAGAAGCGGTTGTGTAAGT	116	1.96 ± 0.09
	Nuclear factor kappa-light-chain enhancer	*nfκb*	based on sequencing	F: CACAGCACAGTTCAGTAACC R: TTGCCTCCTCTTCTCATCTC	97	1.87 ± 0.10
Specific response	Total interferon 1[ii]	*ifn1*[ii]	FJ184371.1 AY788890.1	F: CTTGAGCGCAGAATACCTT R: TCCTCAAACTCAGCATCATC	120	1.91 ± 0.07
	Interleukin 8 or C-X-C motif ligand 8	*il8*	NM_001140710.2 NM_001124362.1	F: ATTGAGACGGAAAGCAGAC R: CTCAGAGTGGCAATGATCTC	101	1.94 ± 0.07
General response	Interleukin 6	*il6*	NM_001124657.1 FR715329.1	F: GTTCTGGGTGAGGTGTCTA R: GGTGTCAACCAGGAAGTTAC	93	1.89 ± 0.08
	Tumor necrosis factor α	*tnfα*	AJ277604.2 AJ401377.1	F: GTGATGCTGAGTCCGAAAT R: GTCTCAGTCCACAGTTTGTC	97	2.04 ± 0.14
	Interleukin 1 β	*il1β*	AJ223954.1	F: AGTGCTGTGGAAGAACATATAG R: CAGTGACGCTACACTCATAC	106	1.90 ± 0.06
Barrier	Zonula Occludens	*zo-1*	XM_021607172.1	F: GCTGTTCCTCCTAGACCTT R: TCACCCACATCTGACTCTAC	99	1.92 ± 0.08
	Intestinal mucin[iii]	*i-muc*[iii]	based on sequencing	F: TCAACACATTCTCTGACACC R: GGCAGTTACTGTACCAAGTC	96	1.82 ± 0.14
	Villin	*villin*	XM_021579239.1	F: AGAGCGGTCTGAGTCTTT R: GCACCATCATTCACCATCT	93	1.73 ± 0.77
Reference genes[iv]	Elongation factor 1	*ef1*	NM_001124339	F: TGCCCCTGGACACAGAGATT R: CCCACACCACCAGCAACAA	90	1.90 ± 0.13
	18s	*18s*	AF308735	F: TGGAGCCTGCGGCTTAATTT R: ATGCCGGAGTTTCGTTCGTT	170	1.57 ± 0.08

[i] In rainbow trout and other salmonids such as Atlantic salmon, only predicted sequences of the TLR4 interactor with leucine rich repeats (*tril*), but not of TLR4 itself, could be identified.

[ii] Interferon 1 primer were designed to cover the short and the long form of IFN-1 and is therefore referred to as total *ifn1*.

[iii] Intestinal i-muc (*i-muc*) was selected as mucus marker based on available sequences from sea trout (*Salmo trutta trutta*) and predicted sequences from rainbow trout. *I-muc* was first annotated as *trans*-membrane mucin [30] but later as well discussed to belong to the family of gel-forming mucins [31].

[iv] Two reference genes were tested to assure stability of expression independent of the treatment. For final calculations, only *ef1* was used because since mRNA abundance was close to the target genes and a better amplification efficiency was achieved with the applied qPCR settings.

transcription controls to detect genomic DNA were performed in technical triplicates. The mean AE ± SD for all genes can be found in Table 1.

2.4. Data treatment and statistical evaluation

Raw fluorescence data were processed using R (version R-3.3.0) and R studio (version 0.99.902) using different packages available from CRAN and Bioconductor, such as ReadqPCR for data read in and chipPCR for data pre-processing and quantification cycle (Cq) determination. The code and an example for data input can be found on GitHub (https://github.com/UtoxEawag/RTgutGeneExpression). Samples with a cycle threshold above 37 were considered below the level of detection. Expression data for the target gene were normalized against the expression of the reference gene according to equation (1).

$$MNE = \frac{AE_{target}{}^{-Cq}}{AE_{reference}{}^{-Cq}} \quad (1)$$

where MNE stands for mean normalized expression, AE_{target} is the amplification efficiency for the respective target gene and $AE_{reference}$ the amplification efficiency for the reference gene and Cq stands for quantification cycle. Thereafter, the MNEs were normalized against the respective experimental control and plotted as log2 fold change on a linear scale. Experiments were the concentration dependency was evaluated were normalized against the L-15/ex control. Since the L-15/ex control was only introduced in the 2nd and 3rd replicate and, as determined in pre-experiments, expression at 1 h of exposure was comparatively low, time dependent data were all normalized against 1 h of exposure. In addition, genes were the basal expression was low, e.g. *tril*, *myd88*, *ticam*, *nfkb*, *villin*, *i-muc* and *zo-1* were normalized against a time point-specific L-15/ex control.

Gene expression data were analysed via two-way ANOVA (Table 2). Time- and concentration dependent data were followed by Tukey's post-hoc test to account for multiple comparisons. As significance level $p < 0.05$ was set. In addition, only gene expression changes where the mean fold change was ≥2 were considered as regulated by the treatment. Graphs were prepared using GraphPad Prism version 5.02 (Graphpad Software Inc., San Diego, CA). The heatmaps were created using R (version R-3.3.0) and R studio (version 0.99.902).

Table 2
P-values for two-way ANOVA and fold difference of the mixture induced expression compared to the sum of single stimuli.

The p-value was computed for a two-way ANOVA and asterisks denote statistically significant differences with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Fold difference was calculated as the ratio between the expression induced by the mixture exposure and the sum of expressions induced by the single stimuli.

Function	Genes	P-value			Fold difference
		LPS	Poly(I:C)	Interaction	
Receptor	*tril*	< 0.001***	0.364	0.073	0.70
	tlr3	0.173	< 0.001 ***	0.173	1.26
Adaptor	*myd88*	0.049 *	0.003 **	0.417	0.58
	ticam	0.141	0.040 *	0.061	1.24
Signalling	*nfκb*	< 0.001 ***	< 0.001 ***	0.119	0.72
	irf3	< 0.001 ***	< 0.001 ***	< 0.001 ***	1.08
Downstream response	*il8*	< 0.001 ***	< 0.001 ***	0.004 **	1.20
	ifn1	0.344	< 0.001 ***	0.615	1.05
	il6	< 0.001 ***	< 0.001 ***	< 0.001 ***	1.68
	tnfα	< 0.001 ***	< 0.001 ***	0.023 *	1.18
	il1β	< 0.001 ***	0.114	0.630	0.92
Barrier	*zo-1*	0.189	0.023 *	0.076	1.01
	i-muc	0.700	0.212	0.103	1.33
	villin	0.188	0.595	0.368	0.56

3. Results

3.1. Expression of immune relevant genes following immune stimulation

All exposure concentrations of LPS and Poly(I:C) were assured to be non-toxic to the cells by cell viability testing (SI Fig. 1). Based on these results, 0, 10, 50, 100 mg/L of either LPS or Poly(I:C) and a maximal exposure of 24 h were selected for the gene expression studies.

Basal expression levels in rainbow trout tissue and RTgutGC cells The qPCR primers were tested for their specificity in isolated whole intestinal rainbow trout tissue. For all tested genes, expression was detectable in the tissue as well as in the RTgutGC cell samples (SI Table 1). For some genes, expression compared to tissue was similar (*zo-1*, *tlr3*, *irf3*, *myd88* and *i-muc*) while for others it was lower (*villin*, *tril* and *ifn1*) or higher (*il6* and *il8*). *Tnfα* expression was slightly lower in control RTgutGC cells compared to the tissue but higher after immune stimulation.

Time dependency of gene expression *Tril* expression was downregulated following LPS and Poly(I:C) exposure, but this downregulation was not statistically significantly changing over time (Fig. 2, Panel A, SI Fig. 2, Panel B). In contrast, *tlr3* expression was not statistically significantly regulated by LPS but was significantly upregulated by Poly(I:C). However, the change over time was not significant for both exposures (Fig. 2, Panel A, SI Fig. 2 D). Even if not significant, for both genes, the strongest response was detected after 24 h.

Adaptor molecules (*myd88*, *ticam*) and *nfκb* expression levels were mainly within a two-fold change. Thus, these genes were considered as not regulated in biological terms even though significant changes were detected based on the statistical analyses, such as an upregulation of *myd88* over time by Poly(I:C) (SI Fig. 2, Panel F) and a shift in expression of *ticam* from down-to upregulation by LPS (SI Fig. 2, Panel H). Similiarly, *nfκb* expression changed from an initial upregulation followed by a significant downregulation by LPS (SI Fig. 2, Panel J). Poly (I:C) did not affect the regulation of *nfκb* over time (SI Fig. 2, Panel J). *Irf3* was not regulated by LPS (< 2-fold) but upregulated by Poly(I:C) with no significant time dependent effect (Fig. 2, Panel A, SI Fig. 2, Panel L).

Recorded fold-changes for cytokines were the strongest among all genes and all showed an upregulation in response to either LPS or Poly (I:C), which was significantly changing over time. The highest expression was recorded after 6 h of exposure. *Ifn1* was more strongly upregulated by Poly(I:C) than by LPS (Fig. 2, Panel A, SI Fig. 2, Panel N, P, R, T, V). Since additional time points were not assessed between 1 and 6 h of exposure, it cannot be fully excluded that certain molecules, e.g. *myd88* and *ticam*, show a stronger regulated at earlier time points.

Concentration dependency Generally, expression for all genes in the LPS pathway was more strongly regulated by LPS, whereas expression of genes in the Poly(I:C) pathway were more strongly regulated by Poly (I:C) (Fig. 2, Panel B, SI Fig. 2). When exposed to Poly(I:C), expression of all genes, besides *ticam*, was upregulated and none of the tested genes showed a statistically significant concentration dependent expression. When exposed to LPS, expression for all genes, besides *tlr3* and *ticam*, where upregulated and some showed a significant concentration dependency.

Tril expression was significantly upregulated with the highest expression level detected upon exposure to the highest LPS concentration of 100 mg/L (SI Fig. 2, Panel A). *tlr3* expression was upregulated by Poly(I:C) and only 50 mg/L showed a significant effect compared to the control (SI Fig. 2, Panel C). The expression of *myd88* was not regulated (< 2-fold) (SI Fig. 2, Panel E). *Ticam* expression was not regulated by LPS exposure and Poly(I:C) induced expression change was slightly higher than 2-fold however not significant (SI Fig. 2, Panel G). The expression of *nfκb* was upregulated by LPS and Poly(I:C) to similar extents but did not dependent on the added stimulus concentration (SI Fig. 2, Panel I). In contrast, *irf3* expression was stronger upregulated by Poly(I:C) but as well without significant concentration dependency (SI Fig. 2, Panel K).

Expression of all cytokines was more strongly upregulated by LPS, except *ifn1* expression, which showed a stronger reaction to Poly(I:C) treatment (SI Fig. 2, Panel M, O, Q, S, U). Expression of *ifn1* was upregulated by both stimuli but no statistically significant difference between the concentrations was detected. Expression of *il8, il6*, *tnfα* and *il1β* was significantly concentration-dependently regulated after exposure to LPS but not to Poly(I:C).

Cytokine expression in two different exposure media Gene expression of three cytokines (*il8*, *il6* and *tnfα*) was additionally explored in MuS medium (SI Fig. 3). In MuS, LPS exposure resulted in a similar pattern of upregulation of the cytokine expression compared to the exposures conducted in L-15/ex even though the absolute fold-changes significantly differed for *il6* and *tnfα*. For Poly(I:C) in MuS, only the highest concentration upregulated *il8* expression but did not affect *il6* and *tnfα* expression compared to the control (< 2-fold).

3.2. Expression of barrier genes following immune stimulation

The expression levels for the three barrier genes were mainly within a two-fold regulation. *Zo-1* and *i-muc* expression was downregulated and the expression level was not significantly changing over exposure time or concentration (Fig. 3, Panel A–D). With exposure time, villin expression transitioned from down-to up-regulation after 24 h exposure (Fig. 3, Panel E). Increasing concentration of LPS upregulated villin (Fig. 3, Panel F). However, the basal expression for villin was always very low (SI Table 1) and expression changes over time and concentration were not significant.

3.3. Effect of LPS and Poly(I:C) in mixture

In order to evaluate the effect of LPS and Poly(I:C) in mixture, the mean fold changes were plotted according to their factorial design and connected with straight lines (Fig. 4, SI Fig. 4, Table 1). Graphically, parallel lines indicate effect addition (= no interaction) whereas diverging lines indicate interaction between stimuli [33]. Visual inspection was confirmed via two-way ANOVA with interactions (Table 1). Three genes, for which different effects can be demonstrated, are exemplary shown in Fig. 4 and a panel summarizing all genes can be found in the supplement (SI Fig. 4).

For the expression of *tlr3*, *ticam*, *ifn1*, *zo-1*, only the Poly(I:C) treatment significantly contributed to the observed regulation in

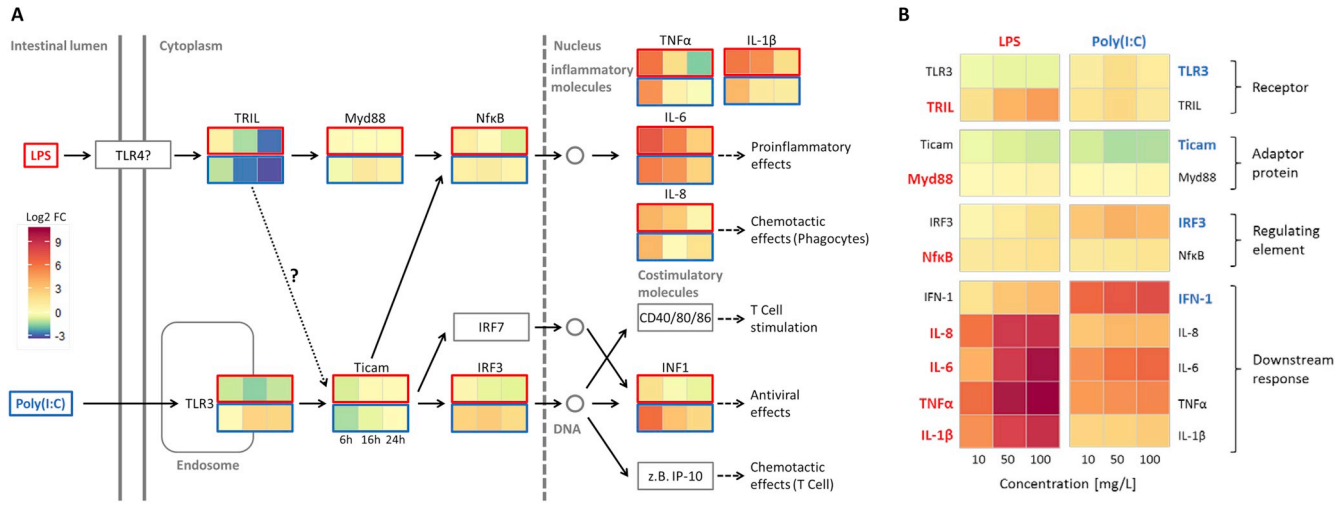

Fig. 2. Time- and concentration-dependency of gene expression following LPS and Poly(I:C) exposure
Heatmap analysis of time- (Panel A) and concentration-dependency (Panel B) of the RTgutGC cell layer exposed to LPS or Poly(I:C). Time dependency data are shown as integration in a simplified toll-like receptor signalling pathway (Panel A). Each box is divided into two rows thereby the upper row (red framed) represents the results for LPS exposure whereas the lower row (blue framed) shows the results for Poly(I:C) exposure. Each row is further divided into three boxes which represent the results for three exposure time points (Panel A, 50 mg/L of LPS and Poly(I:C) for 6, 16, 24 h) or concentrations (Panel B, 10, 50, 100 mg/L). Concentration-dependency is shown as full heatmap (Panel B) with genes ordered according to their topology from the receptor to the downstream response to LPS (left column) and Poly(I:C) (right column). Genes selected for the LPS signalling pathway are highlighted in bold and red and genes selected on the Poly(I:C) pathway are highlighted in bold and blue. For both heatmaps, log2 fold changes are color coded from downregulation (blue) to upregulation (red). An alternative representation of the data presented as mean ± SD can be found in SI Fig. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mixture. For *tril* and *il1β* expression, only LPS significantly influenced expression change. For expression of *myd88*, *nfκb*, *irf3*, *il8*, *il6* and *tnfα*, both stimuli significantly contributed to the regulation. The expression of *i-muc* and *villin* was not significantly regulated at all. A significant interaction between both stimuli was detected for *irf3*, *il8*, *il6* and *tnfα*, indicating synergistic effects.

To compare the effect of the mixture exposure to the exposure with single stimuli, the fold differences were calculated. For this calculation, the expression induced by the mixture was divided by the sum of expressions induced by the single stimuli (Table 2). A fold difference > 1 would indicate synergistic effects, whereas a fold difference < 1 indicates antagonistic effects. The fold difference for receptor (*tlr3*), adaptor protein (*ticam*) and signalling molecule (*irf3*) on the viral pathway was higher than 1, as was the difference for two of the barrier genes (*zo-1*, *i-muc*). On the bacterial pathway (*tril*, *myd88*, *nfκb*), all expression ratios were below 1, as was the difference for the barrier gene *villin*. The ratio for cytokines was either around 1 or clearly higher than 1, such as for *il6*.

4. Discussion

4.1. Immune regulation in the in vitro fish intestinal barrier model

This study focused on evaluating the immune responses of the RTgutGC fish intestinal cell line, grown on permeable supports, to individual PAMPs and their mixture, evaluated by assessing the mRNA abundance of purposefully selected genes. The mRNA abundance was chosen because the regulation of gene expression represents the first level of biological reaction to perturbation. Since changes in mRNA expression level do not necessarily correlate with protein abundance and functional activity, future studies should built on the presented expression results to further evaluate the immune regulation on different levels of biological organisation. The presented results indicate that Poly(I:C) and LPS stimulated corresponding pattern-recognition-pathways and, as expected from knowledge in fish *in vivo*, have interactive effects in the cell line.

Reaction to a viral stimulus DsRNA, such as Poly(I:C), is suspected to be recognized internally by the pattern recognition receptor, TLR3, located within the endosomes, sensing viral infection or cellular damage [7–9,34]. Since TLR3 is located intracellularly, it has been shown that some cells only react to Poly(I:C) after successful transfection of Poly(I:C) into the cell [35]. In contrast, the RTgutGC cells appeared to directly recognize and react to Poly(I:C) as indicated by upregulation of the antiviral response, leading to enhanced expression of *ifn1*. Here, class A scavenger receptors (SR-As), which bind dsRNA in the extracellular space and transport nucleic acids into endosomes, could be an important part of viral detection. Indeed, the RTgutGC cell line has been characterized to functionally express SR-As and to exhibit an SR-As dependent dsRNA uptake mechanism opposed to, for example, another rainbow trout cell line, the RTgill-W1 [36].

TLR3 has been shown to use Ticam as an adaptor; thus one would expect an activation and upregulation of *ticam* expression as a protein required downstream of TLR3 [37]. Interestingly, *ticam* expression was downregulated after exposure to Poly(I:C) and not regulated by LPS in the RTgutGC cells. Yet, research in human lung tissue supports our finding with the fish cells: it was suggested that dsRNA-induced downregulation of *ticam* expression presents an essential step in TLR3 desensitization in order to limit IFN production because overproduction of IFN results in cell growth inhibition and cell death [4]. In the RTgutGC cells, the downregulation of *ticam* expression was followed by a time-dependent downregulation of *ifn1* expression, which further supports this suggestion. Remarkably, the strength of *ticam* downregulation decreased over time. Simultaneously, *tlr3* receptor expression increased over time, which could be part of the regulation feedback. Together, these results indicate that longer exposure to dsRNA could lead to a reactivation of the antiviral signalling. Indeed, in the setup used here, cells are continuously exposed, which mimics a non-defeated viral infection *in vivo*. However, it is also possible that an alternative Poly(I:C) signalling pathway, not covered by the selected genes, led to an induction of *ifn1*. A Ticam-independent pathway is one such candidate. Such a pathway could be activated by a different PRR, such as the cytosolic antiviral RIG-I-like receptor family [7] or the fish specific surface receptor TLR22 [37]. TLR22 has been identified at the transcript level in RTgutGC cells but has thus far not been further

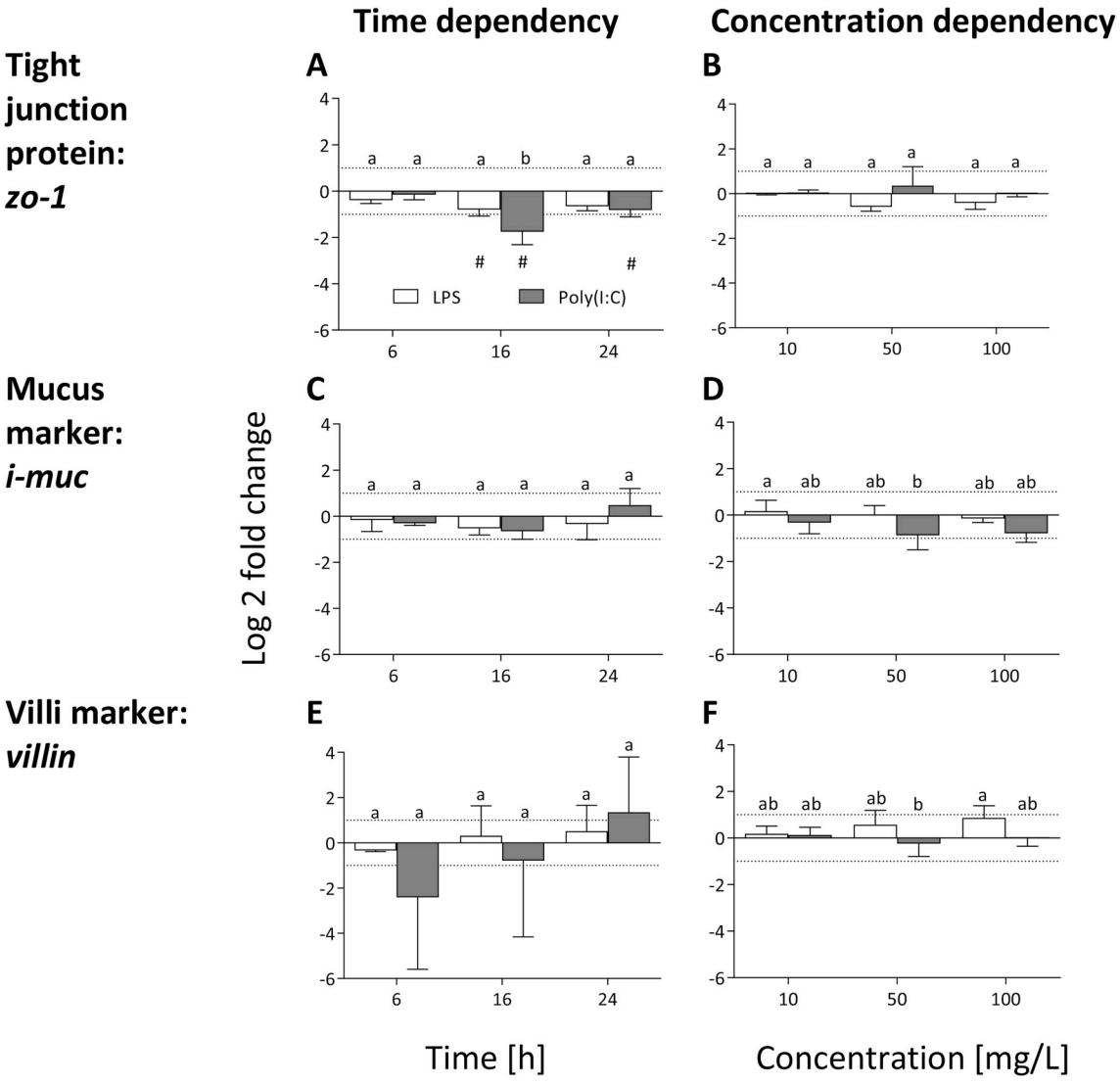

Fig. 3. Gene expression of three barrier property markers following LPS and Poly(I:C) exposure
Time dependent (Panel A,C,E) and concentration dependent (Panel B,D,F) expression of barrier related genes were evaluated in RTgutGC monolayer in 6-well sized *trans*-well inserts, exposed to LPS (white bars) or Poly(I:C) (grey bars). Mean normalized expression (MNE) was normalized to 1h of exposure (time dependency) or L-15/ex control (concentration dependency). Bars represent mean ± SD of three biological replicates. Dashed horizontal lines indicate a 2-fold up- or downregulation. Means with different letters were determined to be statistically different via two - way ANOVA followed by a Tukey's post test ($p < 0.05$). Hashtags # indicate significant difference compared to the control.

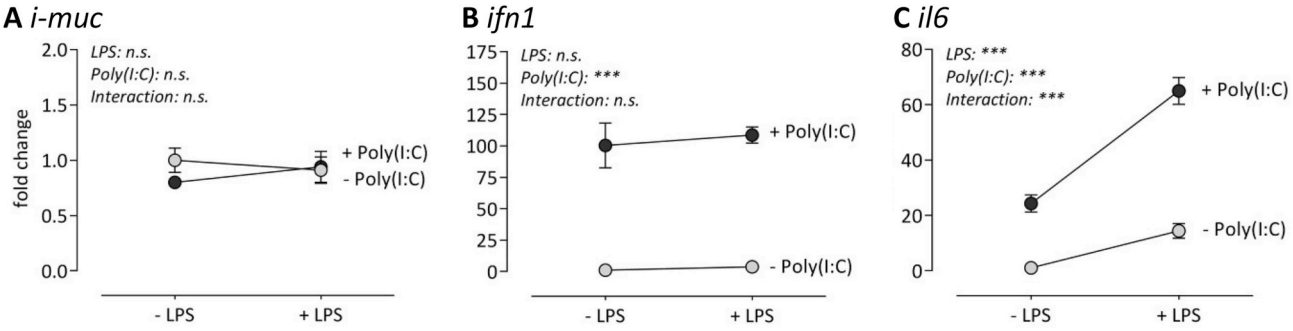

Fig. 4. Effect of a Poly(I:C) and LPS mixture based on three examples
RTgutGC monolayers in 6-well sized *trans*-well inserts were exposed to single immune stimuli (10 mg/L LPS or 10 mg/L Poly(I:C)) or a mixture of both for 6 h and the fold change was calculated as described in the materials and methods. Data for three genes are shown as examples for no significant effect and no interaction (Panel A, *i-muc*), significant effect of a single stimuli but no interaction (Panel B, *ifn1*) and significant effect and interaction (Panel C, *il6*). Data are presented as mean ± SD of three technical replicates (= three *trans*-well inserts per exposure condition). Data representation for all genes can be found in SI Fig. 4. Difference were further confirmed by two - way ANOVA. N.s. indicates no statistical difference. Asterisks denote statistically significant differences with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. P-values for all genes can be found in Table 2.

characterized [36].

Downstream of Ticam, signalling molecules, such as IRF3 and IRF7, are activated. They translocate to the nucleus and start the anti-viral response by initiating IFN-1 expression. In contrast to mammalian IRF3 regulation [38], in the present study, *irf3* expression has been shown to be upregulated following Poly(I:C) exposure in RTgutGC cells. Similarly, an upregulation of *irf3* expression after Poly(I:C) or IFN stimulation has been reported for crucian carp (*Carassius auratus* L.) [39], for the rainbow trout macrophage cell line RTS11 and the gonadal cell line RTG-2 [40]. Thus, there are *in vivo* and *in vitro* indications that IRF3 regulation in fish differs from that in mammals.

In addition to IRF3, Ticam indirectly activates NfκB and the expression of pro-inflammatory cytokines in a Myd88-independent manner. Also in the RTgutGC cell layer, Poly(I:C) upregulated *nfκb* expression and the expression of pro-inflammatory cytokines downstream, as presented here by *il6*, *tnfα*, *il1β* and the chemokine *il8*. In general, the Poly(I:C) induced change in expression was lower compared to LPS. However, also *myd88* expression was upregulated over time, which suggests the involvement of the adaptor molecule to some extent. In Japanese flounder [7], it has been found that the antiviral response required TLR3 and one additional dsRNA receptor of the RIG-I receptor family and was negatively regulated by overexpressed Myd88. However, in the RTgutGC cells, both *tlr3* and *myd88* expression were upregulated following Poly(I:C) exposure. Clearly, more in depth studies *in vitro* and *in vivo* are needed to shed light on the precise regulation of TLR3 in fish.

Reaction to a bacterial stimulus In contrast to mammals, fish have been shown to tolerate high concentrations of LPS [11], which has been debated as a general lack of the LPS-specific receptor TLR4 and the complete genomic absence of essential co-stimulatory molecules [16–18]. Also the RTgutGC cells showed little sensitivity with regard to impact of LPS exposure on cell viability but clearly responded to the exposure with regulation of LPS-related genes. LPS-related gene expression indicates that LPS can potentially be sensed via receptors other than TLR4 [37], such as β2-integrins (CD11/CD18 heterodimers) [13], TLR5 and TLR9 as shown in muscles tissue of rainbow trout [41], TLR9 as shown in the rainbow trout fibroblast cell line RTHDF [42], or NOD1 as shown in zebrafish [18].

In the present work, the TLR4 interactor with leucine rich repeats (TRIL) was detected at the transcript level in RTgutGC cells and was upregulated by LPS and Poly(I:C). In human studies, TRIL has been shown to be a component of the TLR4 complex responding to LPS exposure in a similar manner [10] as well as being important as adaptor molecule for TLR3 mediated responses at the endosomes [34]. In fish, TRIL has been identified in zebrafish and common carp and suggested to inherit similar functionality compared with humans based on the highly conserved sequence [43]. However, exactly these species were also shown to possess TLR4 orthologs in comparison to rainbow trout, where TLR4 has been confirmed to be absent [16–18,44]. Nevertheless, the abundance and regulation of *tril* expression in the RTgutGC cells suggests that TRIL is functioning as receptor interactor for LPS and Poly (I:C) signalling, interacting with TLR3, with a potentially stronger involvement in the recognition of LPS. To further elucidate the LPS recognition in the RTgutGC cells, different types of receptors, putatively involved in the LPS signalling, should be the focus of further research.

Myd88 expression was not regulated by LPS treatment in RTgutGC cells, suggesting that LPS might act via a Myd88-independent pathway as it has been shown for zebrafish [11]. Expression of *nfκb* changed from initial up- to down-regulation. In contrast, *ticam* was initially downregulated by LPS in our study but upregulated after 16 h. Similar to the Poly(I:C) treatment, we suspect desensitization to limit final IFN-1 production to be the reason for this observation [4]. However, regulation for both was close to the considered threshold of 2-fold and thus was considered as not biologically significant. *Irf3* and *ifn1* expression was also increased following LPS exposure, indicating that LPS interacts with the viral signalling pathway in RTgutGC cells.

To further support this assumption, the gene regulation following a mixture exposure was compared to the single exposures. Biologically, the co-exposure was thought to mimic a co-infection with heterologous pathogens as, in their natural environment, fish are likely confronted by a mixture of bacterial and viral pathogens rather than by one of the two alone [45]. Even though being more likely, hardly any mechanistic knowledge on effects of co-stimulation on immune signalling is available.

Reaction to a LPS/Poly(I:C) mixture Three main outcomes can theoretically be proposed following a mixture exposure: effect addition, antagonism or synergism. Assuming synergism, one would expect an enhanced gene regulation upon mixture exposure compared to the calculated sum of individual component exposures. Indeed, expression of three downstream cytokines, *il8*, *il6* and *tnfα*, was significantly increased upon exposure to the mixture of LPS/Poly(I:C). A synergistic response has been previously observed in experiments with specialised immune cells, i.e. mammalian and chicken monocytes [46–50] and in an *in vivo* experiment using fish [45]. Such responses have been suggested to result from complementary and/or cooperative recruitment of components of TLR signalling pathways.

No significant interactions on other components of the signalling cascade were detected with the exception of *irf3* expression. Assuming a strong amplification between the different levels of signalling, very small changes in upstream expression, such as for *irf3*, could lead to strong effects in the downstream response. Such small changes might, however, be difficult to detect and might therefore have gone unnoticed in our study for upstream signalling components, i.e. receptors adaptor and signalling molecules, apart from *irf3*. Yet, it is also possible that signalling pathways other than those explored here are responsible for the synergistic signalling when exposed to a mixture [48] or regulation occurs at other levels than gene expression, i.e. at the protein level. It should also be noted that the change in expression for some genes was enhanced nonlinearly with added concentrations of one stimulus (Fig. 2 B, SI Fig. 2). Thus, if signalling occurs via similar pathways, the apparent synergism could simply result from the increased concentration in the mixture compared to the single exposures. Nevertheless, biologically it seems reasonable that not all cytokines are enhanced simultaneously in order to limit collateral damage caused by exaggerated inflammation [51], which requires that downstream responses result from a fine-tuned cascade.

Interestingly, even though not significant, genes related to the bacterial pathway were rather lower expressed as expected from effect addition (fold-difference < 1), while genes on the viral pathway were regulated to similar or stronger levels (fold-difference ≥ 1). Expression of barrier genes showed indications for effect addition (e.g. *zo-1*), synergism (e.g. *i-muc*) and antagonism (e.g. *villin*) as well. Thus, albeit no conclusion on the mechanism of synergism can be provided based on this study, the RTgutGC cells as epithelial cell line likely possess interacting immune signalling pathways with the potential for synergistic effects. This will enable future studies on the interaction between immune stimulants in a systematic manner.

Gene expression in fresh- and saltwater In addition to a freshwater model, the RTgutGC cell line based barrier system has great potential to serve as a model for saltwater adaptation [1]. At the current state it is difficult to compare the presented results with other studies, since no similar studies, evaluating short-term exposure (< 6 h) to buffers with different salt composition, are available yet. Nevertheless, it has been shown that the transitioning of fish from fresh to sea water *in vivo*, referred to as smoltification, affects the gene expression in different organs [52–54], which might be due to the change in salinity. A downregulation of expression of immune genes has been reported in the intestine and other tissues, potentially connected to increased susceptibility to pathogens in seawater compared to freshwater [52]. Also the RTgutGC cells react to both immune-stimuli in both buffer solutions, with significant lower cytokine expression detected in the seawater buffer. Thus, differences in regulation and expression levels might

provide clues as to the role of saline composition in the mechanisms of salinity adaptation in future studies.

4.2. Barrier regulation in the fish intestinal barrier model following interaction with immune-modulating stimuli

The selected barrier genes represent major characteristics of the *in vivo* intestinal barrier, namely the apical surface mucus (*i-muc*), the apical surface enlarging microvilli (*villin*) and the cell-cell connection by tight-junctions (*zo-1*). Thus far, only the tight junction marker ZO-1 had been confirmed in RTgutGC cells on the protein level by immunofluorescence [1]; in that aspect the current study adds information on the ZO-1 regulation on the level of the mRNA. For *villin* and *i-muc*, basal mRNA expression levels are presented for the first time.

I-muc expression was downregulated following immune stimulation albeit non-significantly and to a small extent. A similar observation has been reported from viral infections in carp, where expression of the mucin gene *muc2* was consistently but not significantly downregulated in the intestine and gill, while expression of *muc5* was upregulated in the skin [55]. The same study showed that different types of stress, i.e. shear stress or immune stimulation, such as by viral infection, tend to increase mucus production. Both, *muc2* and *muc5* were suspected to belong to the group of gel-forming mucins produced by specialised goblet cells. Nonetheless, the thin transmembrane mucin layer has been considered important for immediate cell layer protection [56].

Even though the overall mRNA abundance was very low, *villin* expression was regulated by LPS and Poly(I:C) exposure in RTgutGC cells. In mammalian intestinal cells, villin has been shown to be essential for pathogen-host interactions by facilitating bacterial infection through severing actin filaments [57]. No such information exists as of yet for fish. However, as for humans, villin has been suggested as an enterocyte differentiation marker of the fish intestine [58]. Despite one suggestion about microvilli formation in RTgutGC cells [59], a conclusive proof does not exist yet [1]. However, the basal expression and the regulation of villin mRNA abundance upon immune stimulation indicate potential for microvilli differentiation in RTgutGC cells.

LPS and Poly(I:C) exposure overall led to downregulation of *zo-1* expression in the RTgutGC barrier system, however, no effect on epithelial permeability was detected. Since the tight junctions regulate the cell-cell connections, the downregulation or rearrangement of tight junction proteins have been suspected to manifest in changed paracellular permeability, which *in vivo* could result in disease patterns [21–23]. To evaluate a change in permeability in the RTgutGC barrier system, in addition, up to 21 day exposures using LPS were conducted. This long-term exposure slightly affect barrier integrity (SI Fig. 5), which provided a first indication that LPS is indeed affecting the space between the cells. *In vivo*, similar effects have been shown using rainbow trout: IL-1β and IL-6 decreased physical barrier tightness of isolated rainbow trout tissues while IFN strengthened barrier functionality [24]. In a study using carp, the upregulation of claudin gene expression following virus infection was connected to the upregulated expression of cytokines, which likely indicated a rearrangement of tight junction complexes [60].

4.3. The RTgutGC cells as immunological and physical barrier model

Adding to the growing knowledge about the features of RTgutGC cells, we here demonstrated the responsiveness of the intestinal barrier setup to a model viral and bacterial immune-modulating stimulus with the downstream activation of pro-inflammatory cytokines and interferon. Two signalling pathways, roughly dividable into Myd88-and Ticam-dependent, were shown to be involved, which is well comparable to the *in vivo* situation. Even though a receptor for LPS recognition has not yet been identified in rainbow trout, the RTgutGC cells reacted towards stimulation with upregulation of pro-inflammatory cytokines and chemokines. This finding provides support that the *in vitro* fish intestinal barrier model can be used to further elucidate LPS recognition in fish in more detail. With regard to the viral challenge, the RTgutGC cells activated the anti-viral response comparable to *in vivo* and conveniently, no Poly(I:C) transfection is needed. Finally, proof of the presence and susceptibility to regulation of the transcripts of three major intestinal markers provide impetus for linking explorations on immune regulation with the physical intestinal barrier function. In addition, these barrier genes provide a tool for studies on further RTgutGC cell differentiation to increase the physiological relevance of this unique *in vitro* intestinal barrier model for fish.

Conflicts of interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.036.

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