Fish and Shellfish Immunology 94 (2019) 190–198

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

In vitro immune response of chinook salmon (*Oncorhynchus tshawytscha*) peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide

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ARTICLE INFO

Keywords:
Fish peripheral blood
Mononuclear cells
Fish immunology
E. coli
LPS
PMA

ABSTRACT

We investigated cellular functional and targeted immune cytokine responses of farmed Chinook salmon (*Oncorhynchus tshawytscha*) peripheral blood mononuclear cells (PBMCs) *in vitro* to LPS from *Escherichia coli* (*E. coli*) serotypes O111: B4 and O55: B5, and a phorbol ester phorbol 12-myristate 13-acetate (PMA). Bacterial LPS and PMA significantly ($p < 0.05$) induced reactive oxygen species (ROS) production in *O. tshawytscha* PBMCs, and enhanced by interferon (IFN)-inducible cytokine production. Cellular phagocytosis was significantly enhanced with PMA and *E. coli* serotype O111: B4 LPS after 1 and 2 h respectively. At the molecular level, LPS and PMA significantly ($p < 0.05$) upregulated pro-inflammatory cytokine gene transcripts for IFNγ, TNF-α, and anti-inflammatory IL-10, 24 h post-stimulation. This response is postulated to be mediated via the MyD88 and TRIF pathways in TLR4, or synergistic TLR1 and TLR2 receptors. This is the first report of LPS induced immune related *in vitro* responses in farmed *O. tshawytscha* PBMCs.

1. Introduction

Salmonids are the third most farmed finfish species after cyprinids and tilapines, and they accounted for 6% of global finfish production in 2016, mostly from Atlantic salmon (*Salmo salar*), and rainbow trout (*Oncorhynchus mykiss*) [1]. Norway, Chile, Scotland, Canada, and the Faroe Islands are the major *S. salar* producers [2,3]. Norway and Scotland farm *S. salar* and *O. mykiss* [4,5], and the Faroe Islands only farm *S. salar* [6]. In Chile, production comes from S. *salar*, *O. mykiss*, Coho salmon (*Oncorhynchus kisutch*) [reviewed in Ref. [7]] and *O. tshawytscha* [8] in order of production volume. Canada has the most diverse salmon industry composed of *S. salar, O. kisutch, O. tshawytscha*, brown trout (*Salmo trutta*), Lake trout (*Salvelinus namaycush*), brook trout (*Salvelinus fontinalis*) and *O. mykiss* [9]. New Zealand leads in global *O. tshawytscha* farming, and produced up to 13,000 ton in 2016 worth over US$ 176 million [10]. Fifty percent of this production is marketed internationally under the trade name King salmon, and constitutes about half of the global supply chain.

New Zealand's current *O. tshawytscha* stock was successfully established in 1904 as Chinook salmon, after initial introductions in 1870s failed [11]. The species is currently New Zealand's main farmed finfish after the Greenshell™ mussels (*Perna canaliculus*) [10]. The New Zealand aquaculture industry is also known for its sustainable practices, making salmon farming relatively disease free. However, we understand that farmed fish co-exist with bacteria, viruses, fungi, and parasites, which rapidly proliferate following deviations from optimal culture conditions and compromise the innate immune system. Consequently, disease outbreaks routinely lead to heavy industry losses in aquaculture [reviewed in Ref. [12]]. Indeed, there have been recent reports of *Tenacibaculum maritimum* associated with summer mortality [13,14] and New Zealand rickettsia-like organism (NZ-RLO) [15]. The situation may be complicated with challenges posed by climate change

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https://doi.org/10.1016/j.fsi.2019.09.003
Received 10 July 2019; Received in revised form 24 August 2019; Accepted 2 September 2019
Available online 03 September 2019
1050-4648/

(e.g., rising seawater temperatures and ocean acidification). The above scenarios, thus make fish immunology understanding an indispensable asset to allow routine detection and monitoring of health problems [reviewed in Ref. [16]].

The fish immune system consists of the innate or nonspecific (physical, cellular and humoral) components, and acts as the first line of defence [reviewed in Ref. [17-18]]. The adaptive or specific immune components rely on humoral and cellular responses, and is characterized by specific antigen recognition, which invokes a quick secondary pathogen specific response [18]. Consequently, fish mostly rely on the innate immune system for defence [19]. Evolutionally, pathogenic Gram-negative bacteria and viruses have common conserved pathogen associated molecular patterns (PAMPs) [20]. They include viral double stranded ribonucleic acid (dsRNA) and bacterial deoxyribonucleic acid (DNA), fungal β1, 3-glucans (β-glucan), bacterial cell wall peptidoglycans, polysaccharides and Gram-negative bacterial endotoxin or lipopolysaccharides (LPS) [21].

These PAMPs can be recognised by the vertebrate immune system through pattern recognition receptors (PRRs) on cell surfaces, following breach of physical barriers (e.g. cellular epithelia and the skin). The binding of the host cellular PRRs and the PAMPs initiates cellular responses specifically designed to kill and eliminate the microbial pathogen [22]. Endotoxic bacterial LPS, a cell wall component of Gram-negative bacteria, is widely used in aquaculture immunological investigations [23–27]. Structurally, LPS consists of lipid A, a polysaccharide core, and an O-polysaccharide of variable length. Lipid A confers the endotoxic effects that lead to innate immune responses [reviewed in Ref. [28]].

Bacterial LPS induces reactive oxygen species (ROS) production via activation and induction of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and the suppression of anti-oxidative enzymes involved in ROS clearance [29]. The enzyme NADPH oxidase facilitates transfer of electrons to molecular oxygen resulting in superoxide anion production [30]. In teleosts, the process of ROS production has been reviewed thoroughly by several authors [31,32], and ROS progression explained in a review by Grayfer et al. [31]. Also, genes for the enzyme NADPH oxidase have been linked with ROS production in *O. mykiss*, *S. salar*; have been cloned and sequenced in *O. mykiss* [31,33]. Earlier work showed that *O. mykiss* leucocytes isolated from head kidney (HK), thymus (TH), peripheral blood (PB) and spleen (S), responded to LPS and concanavalin A (con A) *in vitro* [34].

At the molecular level, LPS induced expression of interleukin-1 (IL-1) like compounds in channel catfish (*Ictalurus punctatus*) [35], and upregulated interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) in HK leucocytes [36]. LPS also induced expression of macrophage stimulating factor 1 receptor (M-CSFR), major histocompatibility complexity II (MHC-II), and interleukin-6 (IL-6) in half smooth tongue sole (*Cynoglossus semilaevis*) PB leucocytes [37]. In established *S. salar* TO cell line, LPS induced significant upregulation of cluster of differentiation 88 (CD83) gene transcript, indicative of dendritic cell (DC) origin [38]. Symbiotically, β-glucan and bacterial LPS administration enhanced accumulation of lysozyme gene transcripts in *S. salar* HK macrophages [39]. In addition, *S. salar* HK macrophage *in vitro* stimulation with bacterial LPS and β-glucan highly induced gene expression for arginase-1 and IL-1β [40].

In previous immunological studies on *O. tshawytscha,* researchers mostly used LPS *in vitro* to assess HK and SP immunocompetence following fish exposure to environmental chemical stressors [41–44]. Done *in vitro*, Milston et al. [45] investigated the effect of cortisol on *O. tshawytscha* HK and SP leucocytes immunocompetence via blastogenesis and surface IgM positive cells activation using LPS. In addition, Slater and Schreck [46] used HK leucocytes to study effects of steroid hormones on fish immunocompetence via plaque forming cells, following LPS activation. In this study, we isolated peripheral blood mononuclear cells (PBMCs) from health animals to demonstrate immunocompetence against Gram-negative bacterial LPS products *in vitro*. The study characterised the cellular functional and molecular immunomodulatory responses of farmed *O. tshawytscha* PBMCs *in vitro* using LPS from *Escherichia coli* (*E. coli*) serotypes O111: B4 and O55: B5 plus PMA. These findings contribute towards the understanding of immunocompetence in farmed and wild fish stocks under different conditions.

2. Materials and methods

2.1. O Fish husbandry and blood sampling

Captive two year old *O. tshawytscha* postsmolt (weight = 480.00 ± 119.11 g; total length = 36.67 ± 2.02 cm) were obtained from the Nelson Marlborough Institute of Technology (NMIT) aquaculture facility (Glenduan, Nelson, New Zealand). Fish had been maintained in a saltwater recirculating system (temp = 16.02 ± 0.94 °C; DO = 7.34 ± 0.32 mgL^{-1}; pH = 8.18 ± 0.17; NH_4^+ = 0.17 ± 0.12 mgL^{-1}; NO_2^- = < 0.01 mgL^{-1}) at NMIT aquaculture facility at 16 °C and fed to satiation with Orient 3.0 mm commercial diet (Skretting, Australia) containing 44% crude protein and 28% lipid. Fish were randomly taken by scoop net from the culture tank and, individually euthanized by Ikijime for isolation of PBMCs. All fish included in the study were free of physical abnormality, had intact skin, without lesions and tail rub. Approximately 2.5 mL of blood were collected from each fish via caudal vein puncture using a chilled non-heparinised 3.0 mL syringe attached to a 20 gauge Terumo needle. Blood was quickly distributed into six 400 μL BD Microtainer® Lithium Heparin tubes (Becton Dickinson, USA) and used for PBMC processing as illustrated below. Ethical approval was obtained from the NMIT Animal Ethics Committee (NMIT-AEC-2018-NMIT-03).

2.2. O PBMC isolation

Fish PBMCs were isolated from blood following a protocol from our previous work [47] using Ficoll-Paque Premium density gradient medium (1.077 gmL^{-1}) (Sigma-Aldrich, New Zealand). Briefly, 284 μL of heparinised blood were quickly diluted 1:1 with sterile filtered (40 μm) phosphate-buffered saline (PBS) (pH 7.4) in triplicates. PBMCs were obtained by centrifuging the diluted blood samples at 971 g for 20 min over a layer of 682 μL of Ficoll-Paque. Cells at the interface were aspirated with a precision pipette, pooled together and gently washed twice with 1 mL of sterile PBS at 674 g for 7 min. The cell pellet was resuspended to a final concentration of 1×10^5 cells mL^{-1} in 2 mL of PBS supplemented with 2% fetal calf serum (FCS). This working cell suspension was kept chilled at 4 °C for further analysis. Cell viability was determined using the Muse® Count and Viability Assay Kit as previously described [47].

2.3. O Preparation of bacterial LPS and PMA

Bacterial LPS products from *E. coli* 0111: B4, *E. coli* serotype O 55:B5 previously used on *O. tshawytscha* [41,44,45] and PMA were obtained from Sigma Aldrich, New Zealand. These LPS products were prepared by suspending the solute in sterile filtered PBS (pH 7.4) to a concentration of 1 mg mL^{-1} and stored in small aliquots at −20 °C. Similarly, a PMA solution was prepared to a final concentration of 200 ng mL^{-1} in sterile PBS (pH 7.4) and kept at −20 °C until further use.

2.4. O In vitro LPS and PMA challenge

Fish PBMCs isolated from 3 fish were divided into four 450 μL aliquots, centrifuged at (16,250 g; 4 min), and resuspended in an equivalent volume of PBS containing 10 μg mL^{-1} of LPS *E. coli* serotype 0111: B4, *E. coli* serotype O 55:B5 and 200 ng mL^{-1} of PMA according to Solem, Jørgensen, and Robertsen [48]. Cells were incubated at room

Table 1
Primer sequences used for PBMC cytokine salmon gene expression.

Genes	Sequence (5′–3′)	Product size (bp)	Gene function	Species & GenBank Accession no.	Reference
β-Actin	(F)- GTCACCAACTGGGACGACAT (R)- GTACATGGCAGGGGGTGTTGA	175	ATP binding, determines cell shape and controls motility	*O. tshawytscha* FJ546418	[49]
IFNγ	(F)- CAACATAGACAAACTGAAAGTCCA (R)- ACATCCAGAACCACACTCATCA	129	Triggers cellular response to viral and microbial infections	*O. tshawytscha* GT897806	[50]
IL-1β	(F)- ACCGAGTTCAAGGACAAGGA (R)- CATTCATCAGGACCCAGCAC	181	Potent pro-inflammatory cytokine	*O. tshawytscha* DQ778946	[50]
IL-6	(F)- CAGTTTGTGGAGGAGTTTCAGA (R)- TGTTGTAGTTTGAGGTGGAGCA	118	Potent pleiotropic cytokine	*O. mykiss* NM_001124657	[50]
TNF-α	(F)- ACCAAGAGCCAAGAGTTTGAAC (R)- CCACACAGCCTCCATAGCCA	154	Multifunctional pro-inflammatory cytokine	*O. tshawytscha* DQ778945	[50]
IL-10	(F)- CTACGAGGCTAATGACGAGC (R)- GATGCTGTCCATAGCGTGAC	97	Anti-inflammatory roles; limits excessive tissue damage caused by inflammation	*O. mykiss* AB118099	[50]

F: forward primer, and R: reverse primer.

temperature (18 °C) and monitored for ROS production, and phagocytosis activity at 0, 0.5, 1, 2, 3, 6, 12 and 24 h. After 24 h, the remaining cells were centrifuged in cryo-vials at (16,250 g; 4 min), the pellet snap frozen in liquid nitrogen and stored at −80 °C for gene expression analysis.

2.5. O Reactive oxygen species (ROS) assay

Intracellular ROS production in PBMCs was measured using the Muse® Oxidative Stress Kit protocol (Merck KGaA, Darmstadt, Germany), with the Muse® Cell Analyzer at 0, 0.5, 1, 2, 3, 6, 12 and 24 h. At each time point, 20 μL of PBMC suspension were incubated with 180 μL of Muse® Oxidative Stress working solution following the manufacturer's procedures. Subsequently, the samples were mixed thoroughly and run on the Muse® Cell Analyzer and monitored for ROS production using the Muse® Oxidative Stress Kit (Merck KGaA, Darmstadt, Germany) at the specified time points.

2.6. O Phagocytic activity and phagocytic index determination

Isolated PBMCs were also monitored for phagocytosis activity at 0, 0.5, 1, 2, 3, 6, 12 and 24 h. Ten microliters of PBMC suspension (1×10^5 cells mL^{-1}) were pipetted onto sterile glass microscope slides and incubated at room temperature for 1 h. Non-adherent cells were washed off using sterile filtered PBS with 2% FCS. Then, 10 μL of fluorescent latex beads (1×10^5 mL^{-1}) amine-modified polystyrene suspension (1.0–1.3 μm in diameter, Sigma-Aldrich, New Zealand) were added and incubated for another 2 h at room temperature. Non engulfed latex beads were washed off using sterile filtered PBS with 2% FCS. The slides were subsequently fixed with absolute methanol and later Giemsa stained. Exactly 100 cells were counted microscopically at 400x magnification and the percent phagocytic activity (PA) and phogocytic index determined according to the following equations:

$$Phagocytosis = (Number\ of\ phagocytic\ leucocytes) /(Number\ of\ total\ leucocytes)\ x\ 100$$

While, the phagocytic index was determined by the following equation:

$$Phagocytic\ index = (Number\ of\ latex\ beads\ per\ cell) /(Number\ of\ phagocytic\ cells)$$

2.7. O Molecular assessment of immune genes

2.7.1. Analysis of mRNA expression in PBMCs

Total RNA was extracted from each PBMC sample using the Roche MagNA Pure LC 2 instrument with the MagNA Pure LC RNA Isolation Kit – High Performance (Roche Life Sciences, New Zealand). Subsequently, 100 μL of eluted mRNA were collected and stored at −80 °C prior to gene expression analysis. Relative quantification of gene expression by one step reverse transcriptase polymerase chain reaction (RT-PCR) was performed (LightCycler 480 instrument II [Roche Diagnostics, Auckland, New Zealand]).

Primers (Integrated DNA Technologies, Inc., Singapore) at a working concentration of 0.9 μmol L^{-1} and RNA in a final volume of 20 μL were used. The reactions were performed using the following thermocycling conditions: 60 °C for 15 min (reverse transcription/cDNA synthesis), 95 °C for 10 min (transcriptase inactivation and initial denaturation step) and 40 cycles of amplification (95 °C for 15 s for denaturation and 60 °C for 1 min for annealing and extension). Upon completion, dissociation/melting curve analyses were performed to reveal and exclude nonspecific amplification or primer-dimer issues (all melting analyses in this study presented single reproducible peaks for each target gene suggesting amplification of a single product). The housekeeping gene used for mRNA expression analysis was β-actin due to its stability within mitogen-stimulated PBMCs. Primer sequences used in this study are provided in Table 1. Relative gene expression levels were calculated using the comparative Ct (△△Ct) equation [51]. Gene expression was calculated as $2^{-\triangle\triangle Ct}$ and expressed as a fold-change. The mRNA transcripts for all target genes were normalised to the reference gene (β-actin) within the same sample, condition and time point, and to a calibrator (control with PBS).

2.8. O Data processing and statistical analysis

Data from fish PBMC cellular functional characterisation for ROS production, phagocytosis and phagocytic index were processed to assess the effect of *in vitro* PMA and LPS from *E. coli* serotype 0111: B4 and *E. coli* serotype 055: B5 on fish immune system by one-way time series ANOVA using Minitab 17 statistical software at 95% confidence interval. Data for immune genes were expressed as relative gene expression using the “delta-delta method” [51], and graphical presentation in Microsoft Office Excel 2016. One-way ANOVA was used to detect differences induced by PAMPs and PMA using Minitab 17 statistical software at 95% confidence interval.

3. Results

3.1. O Fish PBMC cellular functional characterisation

In this study, we assessed the immunological response of primary *O. tshawytscha* PBMCs to LPS from *E. coli* serotype 0111: B4 and *E. coli* serotype 055: B5 and PMA *in vitro* 24 h post challenge at 18 °C. Cellular functional parameters of ROS production, phagocytosis and phagocytic index, and targeted immune gene expression were determined.

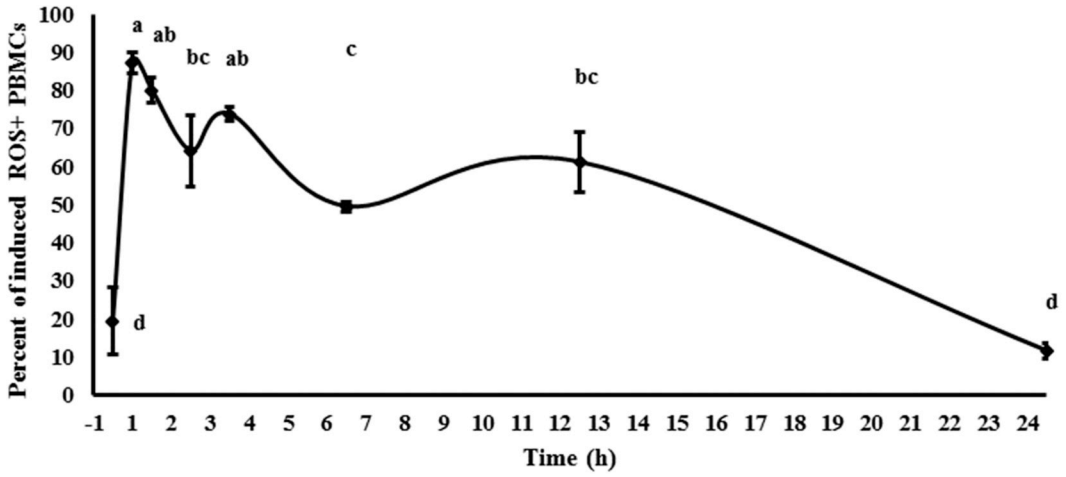

Fig. 1. *In vitro* induction of ROS production in *O. tshawytscha* PBMCs by 200 ng mL^{-1} of PMA over 24 h incubation at 18 °C. Data points are mean ± SEM from duplicate technical readings of three biological triplicates at each time point. Data points with different superscripts are significantly different, One-way time series ANOVA at p = 0.05.

3.1.1. Reactive oxygen species (ROS) production

Following *in vitro* stimulation with PMA, *O. tshawytscha* primary PBMCs exhibited significantly ($p < 0.05$) high peak ROS production 30 min post induction. Twenty-four hours post stimulation, ROS production reduced below initial levels, with a general biphasic trend (Fig. 1).

Similarly, bacterial LPS from *E. coli* serotype 0111:B4, induced a biphasic ROS response that significantly ($p < 0.05$) peaked after 2 h, followed by a second major but nonsignificant peak ($p > 0.05$) after 12 h and remained elevated above initial levels 24 h later (Fig. 2).

Likewise, bacterial LPS from *E. coli* serotype 055:B5, induced low bimodal ROS production in *O. tshawytscha* primary PBMCs that significantly ($p < 0.05$) peaked after 2 h following induction, followed by a second peak ($p > 0.05$) at 12 h, and remained above initial levels 24 h later (Fig. 3).

3.1.2. Phagocytosis activity

Also, bacterial LPS and PMA significantly ($p < 0.05$) enhanced *O. tshawytscha* primary PBMCs latex bead phagocytosis; particularly at 1 h post PMA incubation and at 2 h following incubation with LPS from *E. coli* serotype 0111:B4. Conversely, LPS from *E. coli* serotype 055:B5 did not significantly ($p > 0.05$) induce phagocytosis in the fish PBMCs for the entire experimental period (Table 2).

3.2. O Phagocytic index

Although PMA and LPS significantly ($p < 0.05$) enhanced *O. tshawytscha* primary PBMCs latex bead phagocytosis, the phagocytic index was not significantly ($p > 0.05$) affected by the LPS and PMA in fish PBMCs for the entire experimental period (Table 3).

3.3. O Fish PBMC immune gene expression

When challenged with bacterial LPS from *E. coli* serotype 055:B5 and *E. coli* serotype 0111:B4, the expression levels for pro-inflammatory cytokines IFNγ and TNF-α, plus anti-inflammatory IL-10 were significantly upregulated in *O. tshawytscha* primary PBMCs 24 h post challenge (Fig. 4).

Comparatively, PBMCs challenged with PMA significantly ($p < 0.05$) induced over 90, 30 and 70 fold increase in pro-inflammatory IFNγ, TNF-α and anti-inflammatory IL-10 expression. Cytokine levels for IL-1β were significantly ($P < 0.05$) downregulated in treated fish PBMCs while IL-6 was not affected in *O. tshawytscha* primary PBMCs 24 h post challenge (Fig. 5).

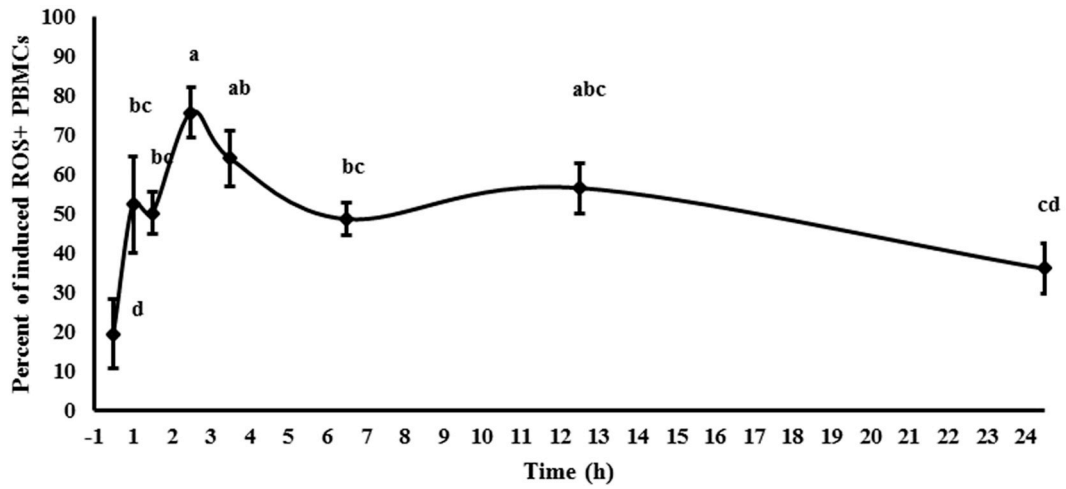

Fig. 2. *In vitro* induction of ROS production in *O. tshawytscha* PBMCs by 10 μg mL^{-1} of LPS from *E. coli* serotype 0111: B4 over 24 h incubation at 18 °C. Data points are mean ± SEM from duplicate technical readings of three biological triplicates at each time point. Data points with different superscripts are significantly different, One-way time series ANOVA at p = 0.05.

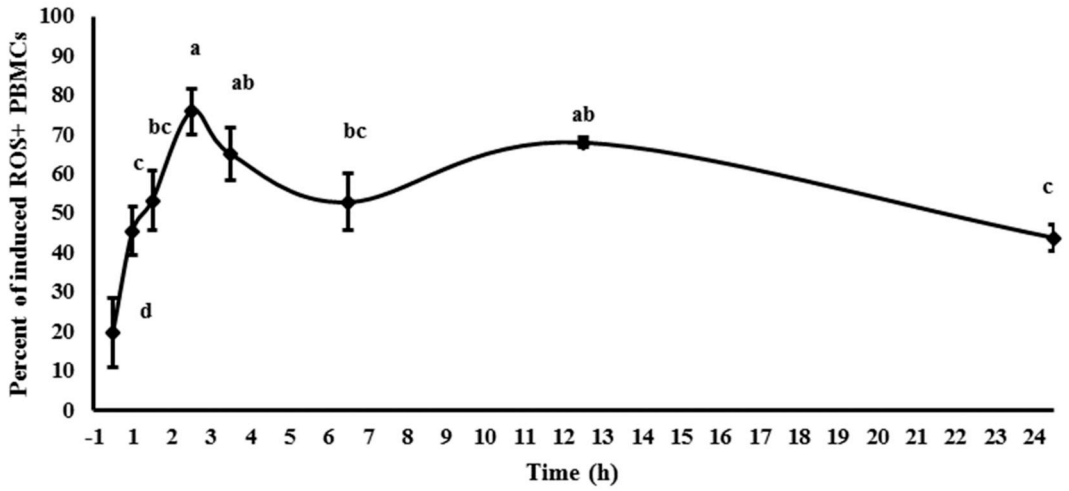

Fig. 3. *In vitro* induction of ROS production in *O. tshawytscha* PBMCs by 10 μg mL^{-1} of LPS from *E. coli* serotype 055: B5 over 24 h incubation at 18 °C. Data points are mean ± SEM from duplicate technical readings of three biological triplicates at each time point. Data points with different superscripts are significantly different, One-way time series ANOVA at p = 0.05.

Table 2
In vitro phagocytosis (%) of latex beds in *O. tshawytscha* PBMCs using PMA and two bacterial PAMPs over a 24 h incubation period at 18 °C. Data are mean ± StDev from duplicate readings of three fish at each time point. Values in a column with different superscripts are significantly different, One-way time series ANOVA at p = 0.05.

Time (h)	PMA	LPS serotype 0111:B4	LPS serotype 055:B5
0	9.00 ± 6.00^{b}	9.00 ± 6.00^{c}	9.00 ± 6.00^{a}
0.5	18.33 ± 6.35^{ab}	18.33 ± 2.08^{abc}	13.00 ± 3.00^{a}
1.0	22.67 ± 2.52^{a}	22.00 ± 2.65^{ab}	13.00 ± 5.00^{a}
2.0	15.67 ± 2.08^{ab}	25.33 ± 9.07^{a}	12.67 ± 0.57^{a}
3.0	16.67 ± 0.58^{ab}	17.00 ± 1.73^{abc}	17.67 ± 2.08^{a}
6.0	–	16.67 ± 0.58^{abc}	16.67 ± 2.52^{a}
12.0	–	12.67 ± 1.53^{bc}	16.00 ± 3.46^{a}
24.0	–	13.33 ± 3.06^{abc}	16.00 ± 6.56^{a}
F-value	4.22	4.56	1.43
P-value	0.029	0.006	0.260

Table 3
In vitro phagocytic index in *O. tshawytscha* PBMCs following challenge with a macrophage activating factor PMA and two bacterial PAMPs over a 24 h incubation period at 18 °C. Data are mean ± StDev from duplicate readings of three fish at each time point. Values in a column with different superscripts are significantly different, One-way time series ANOVA at p = 0.05.

Time (h)	PMA	LPS serotype 0111:B4	LPS serotype 055:B5
0.0	1.80 ± 1.06^{a}	1.80 ± 1.06^{a}	1.80 ± 1.06^{a}
0.5	2.59 ± 0.43^{a}	2.13 ± 0.17^{a}	2.25 ± 0.80^{a}
1.0	2.93 ± 0.06^{a}	2.11 ± 0.40^{a}	2.21 ± 0.30^{a}
2.0	2.60 ± 0.33^{a}	1.92 ± 0.68^{a}	2.14 ± 0.34^{a}
3.0	2.73 ± 0.46^{a}	1.96 ± 0.34^{a}	2.16 ± 0.21^{a}
6.0	–	2.07 ± 0.45^{a}	2.19 ± 0.22^{a}
12.0	–	2.05 ± 0.39^{a}	2.07 ± 0.16^{a}
24.0	–	2.49 ± 0.05^{a}	1.80 ± 0.17^{a}
F-value	1.71	0.44	0.37
P-value	0.225	0.865	0.908

4. Discussion

In the current study, we investigated *in vitro* immunomodulatory effects of two bacterial LPS products and PMA on captive *O. tshawytscha* primary PBMCs via cellular functional parameters of ROS production, phagocytosis and phagocytic index, plus targeted mRNA immune gene expression.

Phorbol ester PMA mimics diacyl glycerol (DAG) to activate the enzyme protein kinase C (PKC) [52] in cells initiating, protein biosynthesis, DNA, polyamine, cell differentiation, and gene expression activities [53]. Also, PMA induces cellular differentiation via enhanced cellular surface adherence, phagocytosis and superoxide production [52]. Data in the current study revealed that *in vitro* administration of PMA, significantly ($p < 0.05$) induced a rapid and substantial increase in ROS production within 30 min of stimulation in primary PBMCs (Fig. 1). Similarly, previous studies have demonstrated in *O. mykiss* HK macrophages that PMA induces ROS [54] and a greater respiratory burst activity compared to stimulation with PAMPs [55]. PMA targets cellular membrane phospholipid receptors for attachment and interaction to augment uptake of 2-deoxyglucose, induces arachidonic acid (ARA) release and prostaglandin production, alters cellular receptor activities, obstructs cellular surface receptor binding of epidermal growth factor (EGF), and affects lipid metabolism [reviewed in Ref. [53]]. The rapid induction of ROS production by PMA as observed in this study could be attributed to surface binding and damage that PMA induces during interaction with cellular membranes [56,57]. This process is facilitated by enhanced release of proteases, cytokines, and activation of NADPH oxidase [53], which damages cells. This theory is supported by the elevated expression of IFNγ and TNF-α cytokines in PMA treated PBMCs. In particular, TNF-α has been linked with cellular necrosis and/or apoptosis [58].

LPS from *E. coli* serotype 0111:B4, and *E. coli* serotype 055:B5 also induced high ROS production 2 h post stimulation. Similar findings have been reported previously among salmonids as LPS *in vitro* enhanced cellular functions and ability to kill an avirulent A-layer lacking strain of *Aeromonas salmonicida* in *S. salar* HK macrophages [48] and activated respiratory burst in *O. mykiss* HK macrophages [59]. In addition, supplementary LPS *in vivo* enhanced cellular functions, and bactericidal effects against pathogenic *Aeromonas hydrophila* in *O. mykiss* [26]. Overall, *O. tshawytscha* PBMCs exhibited the ability to recognise bacterial LPS as in other vertebrates. These results provide invaluable information as to the mechanisms by which the host interacts with bacterial pathogens [22], via strong respiratory burst activity facilitated by the NADPH oxidase complex [60].

In addition, a biphasic ROS production trend was observed following stimulation with PMA and bacterial LPS with a second, but nonsignificant ($p > 0.05$) ROS peak after 12 h. The initial ROS peak is due to direct cellular LPS and PMA response facilitated by the NADPH oxidase complex, followed by a temporal ROS clearance after 6 h, probably due to cellular anti-inflammatory cytokine production to abate damage. However, the second strong ROS peak is due to PMA and

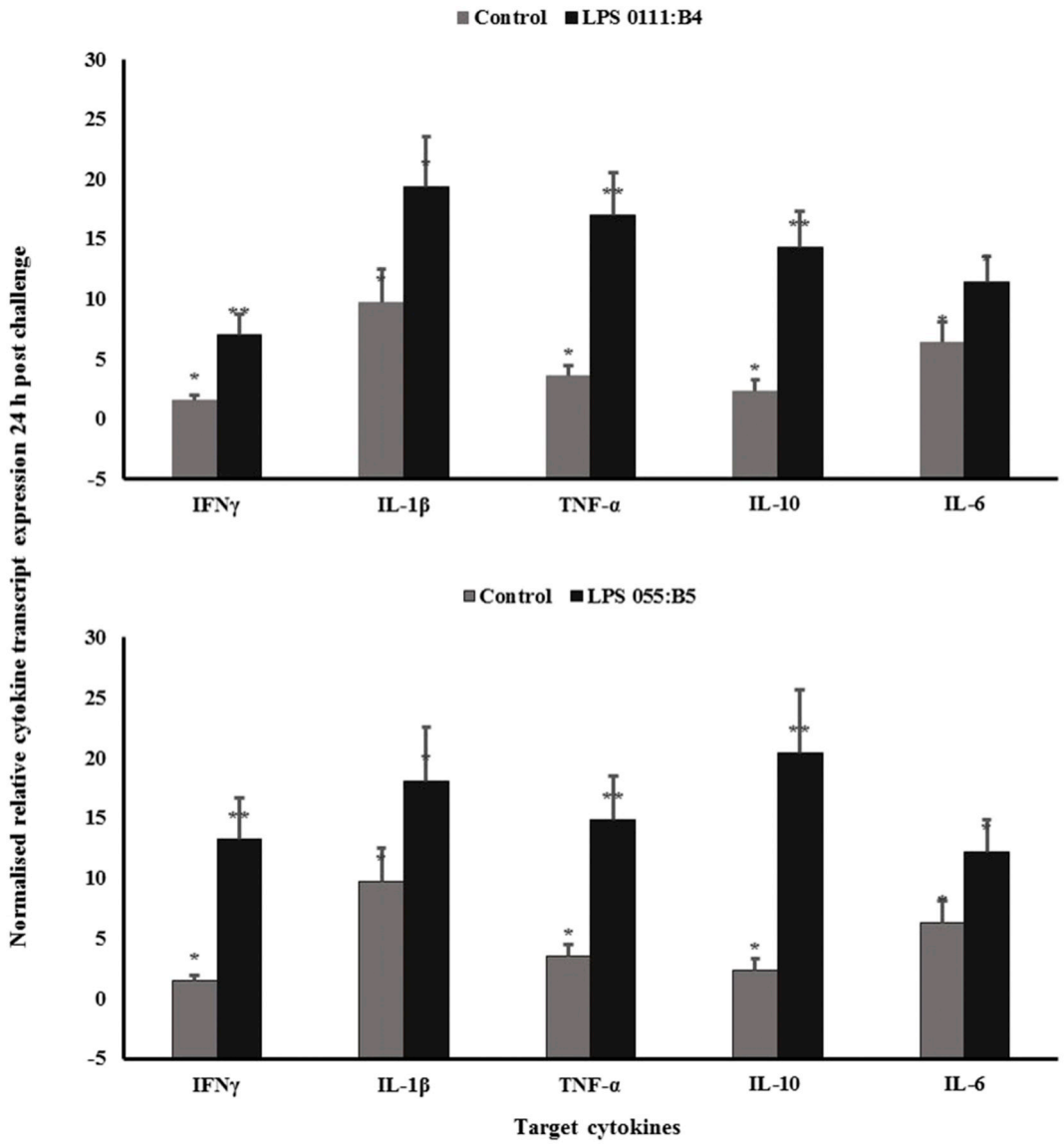

Fig. 4. Relative expression of mRNA PBMC cytokines by RT-qPCR following *in vitro* challenge of *O. tshawytscha* PBMCs by 10 μg mL^{-1} of LPS from *E. coli* serotype 055: B5, LPS from *E. coli* serotype 0111: B4 over 24 h incubation at 18 °C. Data are mean ± SEM of three technical replicates of three fish 24 h post challenge, One-way time series ANOVA at $p = 0.05$.

LPS induced pro-inflammatory TNF-α and IFN-inducible IFNγ cytokine production [61]. While ROS levels remained elevated above basal levels 24 h later for LPS (Figs. 2 and 3), lower than initial levels were observed with PMA after 24 h (Fig. 1), probably due to cellular damage [57,62]. This is evidenced by the over expression of pro-inflammatory IFNγ and TNF-α in PMA challenged PBCs, which probably resulted into cellular necrosis and/or apoptosis [58]. In addition, PMA as a DAG analogue reportedly hyper activates PKC, and unlike DAG, PMA is hardly metabolised by cells, which induces cellular proliferation, and carcinogenic effects. Similarly, longer PMA incubation results into PKC down regulation [53], corresponding with the observed results.

Phagocytosis is the most important innate immune defence mechanisms in fish due to its limited disturbance by temperature [reviewed in Ref. [16]]. The phagocytic process involves uptake of pathogens by engulfment into phagosomes [63]. In this study, PAMPs and PMA significantly ($p < 0.05$) enhanced *O. tshawytscha* primary PBMCs latex bead phagocytosis; 1 h post PMA incubation and at 2 h after LPS from *E. coli* serotype 0111:B4 stimulation (Table 2). Similarly *in vitro* LPS exposure enhanced phagocytic activity in *S. salar* HK macrophages [48]. *In vivo*, supplementary LPS enhanced HK leucocyte phagocytosis, and bactericidal effects against pathogenic *A. hydrophila* in *O. mykiss* [26]. In PMA challenged PBMCs, cellular damage [57,62], observed as aggregations resulted into phagocytosis and phagocytic index determination up to only 3 h post stimulation. This is further suggestive that PMA may have induced *O. tshawytscha* primary PBMC apoptosis and or necrosis [58].

The above findings are supported by the significant ($p < 0.05$) enhancement of pro-inflammatory cytokines IFNγ and TNF-α by PAMPs and PMA (Fig. 4), which are quick responders to infection [50]. Comparably, the magnitude of pro-inflammatory cytokine expression was considerably higher with PMA than in both PAMPs probably due to PMA's strong inflammatory action in PBMCs. This is supported by the rapid PMA induced ROS production, making this agent extremely potent. Previous findings show that PMA binds and damages cellular surfaces during membrane interaction [56,57], while LPS binds cell surfaces via specific Toll-like receptors (TLRs) including TLR4, TLR1 and TLR2, which are not universal to all fish [reviewed in Ref. [64]]. Several studies have demonstrated that teleost fish TLRs can immunologically detect LPS [65–67], and bacterial PAMPs; J.; X.-T [64,67-70]. As strong upregulation of pro-inflammatory cytokines were

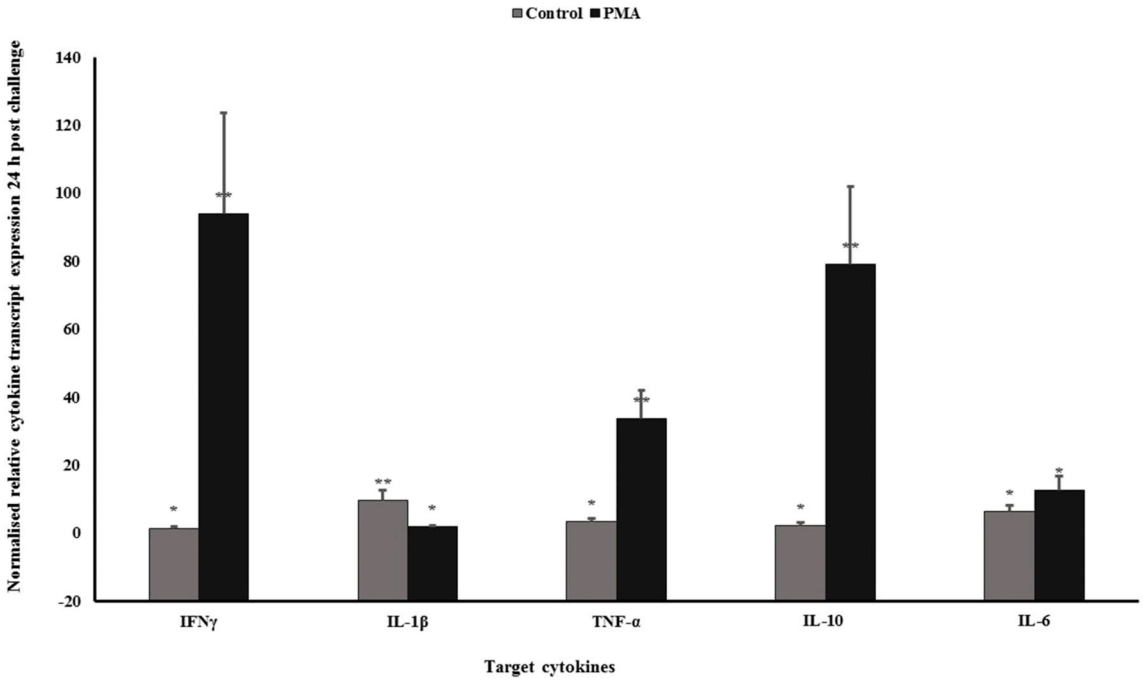

Fig. 5. Relative expression of mRNA PBMC cytokines by RT-qPCR following *in vitro* challenge of *O. tshawytscha* PBMCs by 200 ng mL^{-1} of PMA over 24 h incubation at 18 °C. Data are mean ± SEM of three technical replications from three fish 24 h post challenge, One-way time series ANOVA at $p = 0.05$.

observed with the PAMPs and PMA in this study, the associated TLRs could probably be involved in the myeloid differentiation primary response gene 88 (MyD88) and the Toll-interleukin-1 receptor (TIR) domain containing adaptor inducing interferon-β (TRIF) signalling pathways [71]. The MyD88 pathway activates nuclear factor kappa (NF-κB) to produce pro-inflammatory genes observed in this study. The TIR domain pathway activates IFN-regulatory factor 3 (IRF3) and the expression of IFN-β and IFN-inducible genes [reviewed in Ref. [71], also detected in the current study. Since TLR4 orthologues were reported for salmonids during an in-depth blast survey using catfish and zebrafish TLR4 as baits [72], the discovery helps to elucidate the salmonid immune system recognition mechanism of LPS, which has been previously unclear [66].

Also, IFNγ and TNF-α expression were concurrently upregulated following stimulation with bacterial LPS and PMA, and remained relatively high 24 h post stimulation. This is suggestive of a synergistic action of the two cytokines, as demonstrated by their concurrent expression with IL-10. Concurrently, the high expression of anti-inflammatory IL-10 observed in challenged O. *tshawytscha* primary PBMCs with PAMPs and PMA 24 h post incubation confirms the suppressive effect of this cytokine against cellular damage by the highly expressed anti-inflammatory cytokines previously reported in *S. salar* and *O. mykiss* HK and SP leucocytes [73,74]. The extremely low levels of pro-inflammatory IL-6 and IL-1β, 24 h post LPS and PMA stimulation confirms the anti-inflammatory effect of IL-10 and suggestive of possible earlier upregulation of IL-6 and IL-1β, as previously reported in S. *salar* HK macrophages [40]. This is in line with previous studies with mrigal (*Cirrhinus mrigala*) and Indian major carp (*Catla catla*), where LPS induced IL-10 expression to suppress IL-1β through TLR4 signalling and activation of NF-κB via the MyD88 pathway [71,75].

Administration of LPS *in vitro* has been reported to induce expression of immune related genes in *O. mykiss* HK leucocytes; Jun [76-77]. Also, LPS *in vitro* induced increased trout (tTNF-α) expression in *S. trutta* HK leucocytes [78], TNF-α in *O. mykiss* HK leucocytes; J [79-80], and IL-1β and TNF-α in *S. salar* HK leucocytes [36]. Thus, we postulate that LPS induced immune stimulation in O. *tshawytscha* could be regulated via TLR4, or synergistic TLR1 and TLR2 most probably via the MyD88 and TRIF pathways. However, further studies will have to be conducted to confirm the exact bacterial LPS TLRs in this species. These findings improve our understanding of LPS signalling in O. *tshawytscha* immune cells and provide the foundation for further investigations on immunomodulatory effects that maybe induced due to environmental influences, farm-related stresses, pathogenic exposures and dietary manipulations. The research also contribute towards development of sustainable strategies for improving farmed fish health.

4.1. O Conclusions and recommendations

The response of O. *tshawytscha* primary PBMCs to two *E. coli* LPS products were investigated via cellular functional ROS production and phagocytosis, plus targeted immune gene expression. Findings demonstrate significant induction of cellular ROS production and phagocytic ability, in O. *tshawytscha* primary PBMCs *in vitro*. The results also suggest that LPS significantly ($p < 0.05$) induced upregulation of pro-inflammatory IFNγ, TNF-α and anti-inflammatory IL-10 *in vitro*. We suggest that immune related cytokine release could be regulated via candidate TLR4, or synergistic TLR1 and TLR2 ligands most probably via the MyD88 and TRIF pathways. This is the first report to illustrate *in vitro* LPS effects in farmed O. *tshawytscha* PBMCs. The findings provide valuable information for studying physiological and immune related effects in farmed fish stocks. Further long term *in vitro* and *in vivo* studies are recommended to model bacterial pathogenic effects.

Acknowledgements

The authors are grateful to the aquaculture technicians at the Nelson Marlborough Institute of Technology (NMIT) Glenduan Aquaculture Park in Nelson, New Zealand, Kathryn Hattersley at the NMIT medical laboratory, and the technical and logistical support team at the Auckland University of Technology (AUT) during the course of this study. The authors also extend kind thanks to Dr. Seumas Walker at Cawthron Institute for technical support offered. This project was supported by a New Zealand AID doctoral scholarship to Ronald Lulijwa under the supervision of Andrea C. Alfaro and Fabrice Merien and co-funded by AUT's Aquaculture Biotechnology Research Group (ABRG). All authors, confirm that there is no financial or nonfinancial

conflict of interest in the topic covered and the manuscript as a whole.

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