Studies on the effects of LPS, ß-glucan and metabolic inhibitors on the respiratory burst and gene expression in Atlantic salmon macrophages

Running title: Modulation of respiratory burst in salmon macrophages

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Conflict of interest statement
The authors declare no competing financial interests.

Author contribution statement
R.A.D., T.S. and J.S.U conceived and designed the study. J.S.U., J.K., H.C. executed the experiments. R.A.D., J.S.U., J.K., T.S. and H.C. analyzed the results. R.A.D., J.S.U., H.C. and T.S wrote and reviewed the manuscript.

Abstract

Reactive oxygen species (ROS) production in macrophage-like cells is induced as an antimicrobial defence against invading pathogens. In the present study, we have explored how different stimuli and metabolic inhibitors affects the level of respiratory burst in Atlantic salmon (*Salmo salar* L.) head kidney macrophage-like cells. Cells stimulated *in vitro* by bacterial lipopolysaccharide (LPS) and ß-glucan showed increased production of ROS compared to unstimulated cells. Both stimulation and co-stimulation by curdlan (ß-glucan) induced a higher production of ROS compared to stimulation and co-stimulation by LPS. Metabolic inhibitors co-incubated with the

stimulants did not, in most cases, perturb the level of ROS generation in the salmon macrophage-like cells. The NAD^+ content as well as the NAD^+/NADH ratio increased in curdlan, and LPS + curdlan stimulated cells compared to control cells, which indicated increased metabolic activity in the stimulated cells. Supporting these findings, gene analysis using real-time quantitative PCR showed that arginase-1 and IL-1ß genes were highly expressed in the stimulated cells.

Key words: Respiratory burst, salmon, macrophages, metabolism, gene expression

1 Introduction

Bacterial lipopolysaccharide (LPS) (often called bacterial endotoxin) is composed of lipid and carbohydrate moieties, the latter extruding from the outer cell membrane. LPS may induce immune responses in fish, even though the lipopolysaccharide receptor, toll-like receptor 4 (TLR4) appears to be absent in most fish species (Palti, 2011). Since the classical TLR4 is absent, other LPS recognising receptors that confers signalling events must exists, as suggested by Sepulcre et al. (2009). It may likely be other pattern recognition receptors or members of the large superfamily of scavenger receptors (Canton, Neculai, & Grinstein, 2013; Li, Li, Cao, Jin, & Jin, 2017; Seternes et al., 2001).

ß-glucans are a heterogeneous group of homo-polysaccharides consisting of glucose monomers with ß1-3 or/and ß1-6 linkages in the backbone. They may be termed immunostimulants since they may modulate the host immune response (Bricknell & Dalmo, 2005). In nature, ß-glucans are widespread and are found in plants, algae, bacteria, yeast and mushrooms. ß-glucans from various sources possess differences in molecular weights and degree of branching, rendering them to be fully or moderate aqueous soluble or even insoluble (Dalmo & Bogwald, 2008). It is acknowledged that the specific receptors for ß-glucans is dectin-1 that belongs to the scavenger receptor family (PrabhuDas et al., 2017). As for TLR4, the gene for dectin-1 has not been found in any fish species yet, thus it is speculated that receptors such a TLR-2, complement receptor 3 or not yet identified lectins (sugar binding proteins) may be responsible for binding and intracellular signaling (Petit & Wiegertjes, 2016). It has been shown that fish macrophages respond to LPS and ß-glucans by increased respiratory burst activity followed by production of reactive oxygen species (ROS) and pro-inflammatory molecules (Castro, Couso, Obach, & Lamas, 1999; Cook, Hayball, Hutchinson, Nowak, & Hayball, 2001; Dalmo & Seljelid, 1995; Neumann, Stafford, Barreda, Ainsworth, & Belosevic, 2001; Novoa, Figueras, Ashton, & Secombes, 1996; Solem, Jorgensen, & Robertsen, 1995; Tahir & Secombes, 1996). Activation of cells by certain "danger" signals induces production of molecules that may enable researchers to distinguish different mammalian macrophage subsets; e.g. pro-inflammatory M1 polarised macrophages and immune suppressive M2 macrophages (Murray et al., 2014). The M1 and M2 dichotomy (also termed classical and alternatively activated

macrophages) has also been applied to fish macrophages (Buchmann, 2014; Edholm, Rhoo, & Robert, 2017; Forlenza, Fink, Raes, & Wiegertjes, 2011; Hodgkinson, Grayfer, & Belosevic, 2015; M. Joerink et al., 2006; Nguyen-Chi et al., 2015; Wiegertjes, Wentzel, Spaink, Elks, & Fink, 2016), even though considerable macrophage heterogeneity may exist (Murray et al., 2014).

It has been documented, in some mammalian species, that both LPS and ß-glucans activate, via initial receptor binding, the Akt kinases (protein kinases B/serine-threonine-specific protein kinases) downstream of phosphoinositide 3-kinase (PI3K) in monocytes / macrophages / dendritic cells. In case of LPS, it may bind to TLR4 in the cell membrane that interacts with the signalling entity MyD88; which activate several pathways, one of them being the RIP-PI3K-Akt pathway (Bauerfeld et al., 2012; McGuire et al., 2013; Saponaro et al., 2012). RIP is "death domain kinase receptor interacting protein 1". The RIP-PI3K-Akt pathway is of vital importance during cell metabolism (Covarrubias, Aksoylar, & Horng, 2015). Curdlan, a linear ß-glucan, may bind to different receptors such as dectin-1, TLR4, complement receptor 3 and scavenger receptors where activation of Akt is one of the signalling mechanisms involved following curdlan stimulation of cells (Kim et al., 2016).

During e.g. infection, changed local microenvironments by virtue of oxygen tension, accessibility of metabolites and nutrients may be challenging for the immune cells, especially innate immune cells such as macrophages. During activation of macrophages by e.g. "danger signals", the cells may undergo substantial changes with respect to metabolism to support cell growth, proliferation, functional transition and synthesis and release of molecules. This requires metabolic adaptation to new microenvironments. Activated macrophages may have increased glycolytic activity (utilizing glucose, glutamine and fatty acids to support the increased energy demand), reduced oxidative phosphorylation activity (hence reducing the formation of ATP) and modified tricarboxylic acid cycle (TCA) activity (Kelly & O'Neill, 2015; Langston, Shibata, & Horng, 2017). These features is reminiscent of the known Warburg effect (Kelly & O'Neill, 2015). Resting immune cells are relatively metabolically inactive, with minimal biosynthetic demands beyond housekeeping processes (Gaber, Strehl, & Buttgereit, 2017). High contents of NADH from e.g. fatty acid oxidation, unlike low NAD^+ levels, favours generation of ROS (Kussmaul & Hirst, 2006).

Activated macrophages, from stimulation with e.g. LPS and certain ß-glucans, may differentiate into a distinct phenotype that produce pro-inflammatory molecules, certain ROS levels and nitric oxide (NO) (Beyer et al., 2012; Iles & Forman, 2002; Mosser & Edwards, 2008) – a feature of M1-type macrophages (pro-inflammatory) (Tan et al., 2016). Highly elevated ROS levels may, on the other hand, lead to a M2 phenotype (pro-resolving) (Tan et al., 2016). Even though not that well studied as in mammalian species, the principle of macrophage activation has been suggested to be similar in fish (Boltana et al., 2017; Hodgkinson et al., 2015; MacKenzie et al., 2006). Macrophage metabolism in fish macrophages has not been studied before, nor has the dependence of the metabolic inhibitors on the respiratory burst activity been explored. The objective of the current study was to examine the formation of ROS, analysis of NAD^+ and NADH contents upon stimulation with LPS and curdlan (ß-glucan), and to study how different metabolic inhibitors affect the generation of ROS. In addition, we assessed the expressions of arginase-1, hypoxia inducible factor-1α, glut-1, TNF-α, IL-10 and IL-1ß genes potentially discriminating M1 and M2 macrophages – by means of quantitative real time PCR.

2 Materials & Methods

2.1 Reagents

Ultrapure bacterial lipopolysaccharide from *Aeromonas salmonicida* spp *salmonicida* was obtained in our lab (Dalmo & Bogwald, 1996). Curdlan (*Alcaligenes faecalis*) was a gift from Takeda Chemical Industries (Osaka, Japan). Other chemicals were from SigmaAldrich if otherwise not specified.

All the steps from cell isolation, centrifugation, cell maintenance and stimulation were carried out using Leibovitz's L-15 Medium (L-15) (SigmaAldrich) supplemented with 60 µg penicillin ml^{-1} and 100 µg streptomycin ml^{-1}. However, Dulbecco's Modified Eagle Medium (DMEM) (Gibco) (pyruvate-free) was used in the experiment involving metabolic inhibition. Approximately 5% CO_2 was supplied to cells incubated in DMEM.

2.2 Fish

The Aquaculture Research Station in Kårvika (NOFIMA and University of Tromsø – the arctic university of Norway) provided non-vaccinated Atlantic salmon used in this study. Fish were fed commercial pelleted feed without ß-glucan supplements at frequencies recommended by the feed producer (Skretting, Norway). The fish weighted approx. 600 g. All the fish were kept in one tank at ambient temperature (6 -12°C) with a constant flow of fresh seawater. The fish for cell isolation had priory been analysed clinically and by RT-qPCR analysis for any presence of pathogens. The fish were free from infection and disease. The Norwegian Animal Health Authority approved the use of fish for experimental purpose – to be compliance with the Animal Welfare Act.

2.3 Isolation and characterization of macrophages

Macrophage-like cells from the head kidney were isolated using a Percoll gradient, as described by Braun-Nesje, Bertheussen, Kaplan, and Seljelid (1981), with some modifications. In short, the head kidney was aseptically removed from fish into a tube (Falcon) of cell medium (L-15) supplemented with, penicillin (60 µg ml^{-1}), streptomycin (100 µg ml^{-1}), 2 % inactivated fetal bovine serum (FBS) and heparin (20

U ml^{-1}) (LEO Pharma, Denmark). The head kidney and the contents of the tube were then meshed through a cell strainer (100 µm) (Falcon), layered onto a 25%/54% percoll (GE healthcare, Sweden) density gradient and centrifuged at 400 x g at 4 °C for 40 minutes. The cloudy macrophage-enriched layer that appeared in the interface between the two gradients were collected and washed twice in L-15 by centrifugation (15 min and 10 min at 450 x g respectively). The cell suspension was diluted in L-15 containing 1% FBS to achieve a cell number of 5 x 10^6 cells per ml. Thereafter, 100 µl of the cell suspension was transferred to wells in 96-well plates (Nunc, Denmark). To allow the macrophages to adhere the plates were incubated overnight at 16 °C. The number of cells prior to seeding and after treatment (cell number and viability) were analysed using NucleoCounter® NC-200™ (Chemometec, Denmark). The results are given as optical density (OD) which were normalised to 1 x 10^5 cells.

2.4 Stimulation of macrophages

Approximately 24 hours after seeding, the cells were washed with cell medium, causing removal of non-adherent cells. The remaining adherent cells were cultivated in cell medium with antibiotics and FBS.

2.4.1 Stimulation by LPS and curdlan

If otherwise not specified the concentrations of LPS and curdlan remained at 1 µg ml^{-1} and 10 µg ml^{-1} respectively – based on prior work (Dalmo & Seljelid, 1995; Pietretti, Vera-Jimenez, Hoole, & Wiegertjes, 2013). LPS and curdlan was solubilized by microwave treatment (probe sonication) in required cell medium containing penicillin (60 µg ml^{-1}) and streptomycin (100 µg ml^{-1}) to achieve the desired concentrations. 24 h after cell isolation, the cells were stimulated with either LPS or curdlan or a combination of both for 24 (single stimulation) or 48 h (metabolic inhibition experiment and for repeated stimulation). The control cells were cultivated in only cell medium. To wash away LPS or curdlan from the wells, the cells were washed twice with medium.

2.4.2 Co-incubation with metabolic inhibitors

In two sets of experiments, stimulated cells were co-incubated with metabolic inhibitors. The inhibitors were Akt 1/2 inhibitor (Akt 1/2 kinase inhibitor) (Tocris Bioscience, UK), oligomycin A (Merckmillipore, Germany), rapamycin (Selleckchem, Germany), and 2-deoxyglucose (Carbosynth MD, UK). The inhibitors were given at a concentration of 100 nM, except 2-deoxyglucose, which were given in a concentration of 100 µM. These concentrations were selected based on literature studies and from dose-response studies where their effects on the respiratory burst were assayed (not shown). The cells were incubated with LPS and curdlan for approximately 48 hours in presence of the metabolic inhibitors. Control cells were incubated in the cell medium only. The cells were assayed for their respiratory burst activity (cf. 2.5) using PMA as the respiratory burst facilitator.

2.5 Quantification of respiratory burst activity

To quantify the generation of ROS in macrophages, the respiratory burst assay described by (Secombes, Chung, & Jeffries, 1988) and Solem et al. (1995) was followed. In general, the cells were stimulated with LPS or/and curdlan for 24 or 48 h before the respiratory burst assay was started. Control cells were not stimulated by LPS or curdlan. The cells were washed in PBS before addition of a solution containing 20 mg nitro blue tetrazolium (NBT) (Sigma Aldrich) and 20 µl phorbol 12-myristate 13-acetate (PMA) (1 mg ml^{-1}) in PBS. The stimulated and control cells were incubated for 40-50 min at 16 °C, and thereafter fixed with 70% methanol. To solve the formazan crystals, a solution of 120 µl KOH (2 M) and 140 µl dimethylsulfoxid (DMSO) was added and mixed well together. The optical density (OD) was measured at 620 nm in an ELISA reader (VersaMax ELISA microplate reader, USA).

2.6 Measurements of nitric oxide production

The amount of produced nitric oxide (NO) was measured according to the Griess assay described by Wu and Yotnda (2011). The formation of nitric oxide was assayed 24 and 48 h days after stimulation. In short, a standard curve was made by a 1:2 serial dilution of "blank" L-15 (with no phenol red added) and 100 µM nitrite (Alfa Aesar, Germany), followed by addition of a solution made of 1% sulphanilamide (Alfa Aesar) in 5%

phosphoric acid (Alfa Aesar). After stimulation, 50 µl of the cell medium from each well were transferred to wells in a new 96-well plate, where 50 µl of the sulphanilamide solution was added to each well. The plates were incubated for 10 minutes at room temperature before 50 µl of N-1-naptylethylenediamine dichloride (Alfa Aesar) was added to each well. The plates were incubated as described earlier. Thereafter OD was measured at 520 nm. This experiment was repeated trice.

2.7 NAD^+/NADH measurements

"Amplite Flurometric NAD/NADH Ratio Assay Kit" from AAT Bioquest (USA) was used to calculate the NAD^+/NADH ratio in the control and stimulated cells. The procedure was as described in the protocol from the manufacturer.

In short, a standard curve was made with a dilution ratio of 1:2. The test samples were tested for total NAD^+ and NADH, and NAD^+ alone; the two groups of cell samples were therefore given different extraction solutions. To detect total NAD^+/NADH the cells were given a NAD^+/NADH control solution, while both NADH and NAD+ extraction solution were given to the cells to be assayed for NAD^+. In the end, a NADH reaction mixture was added to yield the standard curve, the total NADH plus NAD^+ and NAD^+ in control cells and the test samples. The OD was measured at 576 nm in an ELISA reader.

2.8 RT-qPCR of gene expression

To determinate the levels of gene expression, quantitative real-time quantitative PCR (qPCR) using SYBR green were performed for gene expression analysis.

2.8.1 Isolation of RNA and cDNA synthesis

Macrophage-like cells from the head kidney were isolated and treated with LPS and curdlan 48 hours before harvested. The cells were lysed in a RT-buffer containing 2-mercaptoethanol and kept at -80°C. RNA was isolated using *RNeasy Mini Kit* by Qiagen (Germany) - according to the manufacturer's guidelines. The yield and purity of the RNA was determined using a NanoDrop (Nano-Drop Technologies, Wilmington, DE, USA). The samples having $OD_{260/280}$ values between 1.9 and 2.1 was processed further. To avoid the risk of having contaminating DNA, interfering with the assay, in the

samples - the samples were pre-treated with DNase I (1 U μg^{-1} RNA; Invitrogen, USA). To synthesize first-strand cDNA, a SuperScript III RNase reverse transcriptase (Invitrogen) was used, as described by Kumari et al. (2015).

2.8.2 qPCR

The qPCR was performed in triplicates from samples obtained from three fish using ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) using Fast SYBR® Green (Applied Biosystems). The procedure was the same as described by Kumari et al. (2015). In short, the reaction mixtures were incubated at 95℃ (10 min), thereafter, 40 cycles of 95 ℃ (15 s), 60 ℃ (1 min) and 95 ℃ for 15 seconds. Amplifications were specific in all cases, and amplification was not observed in any of the negative controls (non-template control). The relative quantification method by (Pfaffl, 2001) was used to convert the Ct values for each sample into fold differences. The most stable reference gene was *EF-1α*, hence, gene expression was normalized by this gene in each sample. The primers used in this study are listed in table 1.

2.8.3 Statistics

All the experiments were carried out in a duplicate or triplicate fashion (except cell stimulation followed by qPCR), with at least three technical replicates. Statistical analysis was performed using one-tailed, paired Student's T-test. For QPCR, the log-transformed data were analyzed by ANOVA and a Tukey's multiple range test to determine the differences between groups using SPSS 25.0 software. In all experiments, statistically significance between the datasets (treatments) were considered if $p<0.05$.

3 Results

3.1 Stimulation of macrophage-like cells by curdlan and LPS

Both LPS (1 µg ml^{-1}) ($p=0.01$) and curdlan (10 µg ml^{-1}) ($p=0.07$) increased the intracellular respiratory burst activity of PMA-elicited salmon macrophages – measured as the formation of superoxide anion reducing NBT to formazan (Fig. 1).

3.2 Repeated stimulation and dose relationship

To establish whether repeated stimulation with these two immunostimulants would further increase or decrease the respiratory burst activity compared to control cells, the cells were stimulated with one of these two stimulants for one day, and subsequently re-stimulated with the same or another stimulant for one more day. The cells were firstly stimulated with curdlan (1, 10 and 100 µg ml^{-1}) for one day and then re-stimulated with fixed doses of curdlan and LPS (10 and 1 µg ml^{-1}, respectively) during the day after (Fig. 2). Stimulation with 100 µg ml^{-1} curdlan for 24h and subsequent 1 µg ml^{-1} LPS (24h) induced significantly higher ROS generation compared to control cells, curdlan and LPS-stimulated cells and cells stimulated with the other combinations ($p<0.05$), except cells stimulated with curdlan (10 µg ml^{-1}) and LPS (Fig. 2). Cells pre-stimulated with curdlan (10 µg ml^{-1}) followed by LPS produced significant more ROS than control cells ($p<0.05$), LPS and curdlan-stimulated cells, cells stimulated with 1 µg ml^{-1} curdlan + LPS, and cells stimulated twice with curdlan (1 +10 µg ml^{-1} and 10 + 10 µg ml^{-1}) (Fig. 2). Cells pre-stimulated with 100 µg ml^{-1} curdlan followed by curdlan stimulation (10 µg ml^{-1}) showed significantly more ROS generation compared to cells stimulated with 1 + 10 µg ml^{-1} curdlan and 10 + 10 µg ml^{-1} curdlan (Fig. 2). The results after pre-stimulation with different doses of LPS followed by curdlan stimulation showed no consistent pattern (not shown).

3.3 Effect of metabolic inhibitors on respiratory burst

Metabolic inhibitors may alter the cells ability to produce superoxide anion via respiratory burst. Hence, we co-incubated different metabolic inhibitors together with stimulants and evaluated their effects on the respiratory burst activity. We included inhibitors against glucose uptake (2-deoxyglucose), Akt 1/2 (Akt 1/2 kinase inhibitor), complex V in the electron transport chain (mitochondria) (oligomycin A) and mTORC (rapamycin). In the dose response study where LPS and curdlan-stimulated cells were

co-incubated with each inhibitor, only 2-deoxyglucose, and rapamycin resulted in a dose dependent decrease in superoxide anion formation by increased inhibitor concentration (1nM - 1μM) (not shown). The Akt 1/2 inhibitor resulted in an increase of respiratory burst at 100 nM compared to a lower (1-10 nM) and a higher (1 μM) concentration of Akt 1/2 inhibitor (not shown). We were interested to see how the inhibitors affected cells when co-incubated with LPS and curdlan. The Akt 1/2 inhibitor (100 nM) significantly decreased the formation of superoxide anion in cells stimulated with LPS compared to cells without the inhibitor ($p=0.03$) (Fig. 3), whereas no significant inhibition of superoxide formation in cells stimulated with curdlan alone or LPS + curdlan. Oligomycin A (100 nM) and rapamycin (100 nM), in general, increased respiratory burst activation – even in non-stimulated cells ($p=0.04$ and $p<0.0001$, respectively). The highest increase in superoxide anion formation was found in cells stimulated with LPS + rapamycin ($p<0.0001$) and LPS + oligomycin A (not significant). The glucose transport inhibitor, 2-deoxyglucose (100 μM), also increased the formation of superoxide anion when co-incubated with LPS, curdlan + LPS ($p=0.03$ and $p=0.002$, respectively), but decreased the respiratory burst activation when the cells where incubated with curdlan ($p=0.0001$) (Fig. 3).

3.4 Nitric oxide

There was no production of NO assessed by the Griess assay on cells stimulated with LPS or curdlan – evaluated from the standard curve using the Griess reagents (N-(1-naphtyl) ethylenediamine dihydrochloride, sulphanilamide, phosphoric acid and nitrite standard solution.

3.5 NAD^+/NADH contents

The NAD^+/NADH ratio may differ between resting and activated cells; activated cells may possess increased ratio. After stimulation of macrophages for 48 h with curdlan (10 μg ml^{-1}), curdlan + LPS (1 μg ml^{-1}) the NAD^+/NADH ratios were 2.3 -and 1.5-fold, respectively. The "opposite" was found when we calculated the ratio for LPS stimulated cells (Fig. 4). This ratio turned out to be negative since the OD value in the reaction mixture was lower than for zero-controls calculated from the standard curve. The intracellular content of total NAD^+ increased in cells stimulated with curdlan, compared with control cells ($p<0.05$), LPS and LPS + curdlan stimulated cells ($p<0.05$). There

was even a significant decrease of NAD^+ levels in LPS stimulated cells compared to control cells ($p<0.05$).

3.6 Gene expression after LPS and curdlan stimulation

During fungus-elicited activation of macrophages, the cells may undergo a metabolic switch featured by increased arginase-1 and IL-10 expression in so-called alternatively activated macrophages (M1) (Roszer, 2015). In the current study, the expression of arginase-1 was significantly higher in cells stimulated with LPS, curdlan and LPS + curdlan compared to control cells ($p<0.05$). The expression of IL-10 by stimulated cells was not significantly higher than control cells. Marker for classical activated macrophages, e.g. elicited by LPS or IFN-γ, has been suggested to be hypoxia-inducible factor 1α (HIF-1α), glucose transporter protein-1 (GLUT-1), TNF−α and IL-1β (Kelly & O'Neill, 2015). The expression of IL-1β was significantly higher in cells stimulated by LPS, curdlan and LPS + curdlan ($p<0.01$) compared to control cells. TNF-α, GLUT-1 and HIF-1α mRNA expressions were not regulated by the stimulants (Fig. 5).

4 Discussion

The aim for this study was to evaluate the effects from LPS and β-glucan stimulation on respiratory burst, and how different stimuli plus metabolic inhibitors affected ROS production by macrophages extracted from Atlantic salmon head kidney. In addition, we analysed total NAD^+ contents in cells, the NAD^+/NADH ratio, and gene expression in the cells after stimulation with LPS and β-glucan (curdlan).

4.1 Stimulation of macrophages

The present work demonstrated that stimulation by both LPS and curdlan resulted in an increased ROS production by the stimulated macrophages. Previous studies on the respiratory burst activity in salmon macrophages stimulated with LPS and β-glucan showed similar tendencies (Dalmo & Seljelid, 1995; Jorgensen & Robertsen, 1995; Paulsen, Engstad, & Robertsen, 2001). In the current study, macrophages stimulated with both curdlan and LPS showed increased ROS production compared to non-stimulated cells. When curdlan-primed cells (10 and 100 µg ml^{-1}) were re-stimulated with LPS, the respiratory burst activity was, in most cases, even higher than after re-

stimulation with curdlan alone. Such synergistic effect has been observed before in pink snapper macrophages (Cook et al., 2001). We also tried to prime the salmon macrophages with LPS followed by stimulation with curdlan. This set-up gave no consistent results. The reason for this inconsistency is not known. It is not clear why the salmon head kidney macrophages did not produce nitric oxide upon LPS and curdlan stimulation - assayed by the Griess method. The close relative rainbow trout macrophages/mononuclear cells have previously been shown to produce NO in response to LPS stimulation (Fierro-Castro et al., 2012; Zvizdic, Licek, & Lam, 2012). Macrophages from other teleost fish species have also been reported to produce NO following stimulation (Buentello & Gatlin, 1999; Pietretti et al., 2013; Stafford, Galvez, Goss, & Belosevic, 2002; Yang et al., 2013). Probably, optimisation with respect to the mode of salmon macrophage stimulation together with the optimal read out choice (e.g. colorimetric versus fluorescent detection of NO). Whether the washing steps between stimulation completely removed any remains of LPS or curdlan is not known. We assume, however, that any unwanted (sub-optimal) amounts of remains would not infer significantly to the assayed ROS production.

4.2 Effect of metabolic inhibitors on respiratory burst

As previously mentioned (cf. introduction), the PI3K/Akt/mTOR pathway is important in cell metabolism. How metabolism affects ROS production in fish macrophages is yet to be discovered. Thus, it was desirable to look into the PI3K/Akt/mTOR pathway in relation to respiratory burst activity in macrophages. The reason for including the metabolic inhibitors was to see how they affected the ROS production by inhibiting different parts of the metabolism that may be connected to respiratory burst. Receptor engagement (e.g. on the cell surface) activates PI3K; which through cascades of activation leads to activation of Akt, the two mTORC, and activating the multicomponent enzyme NADPH oxidase - which initiate and perform ROS production.

It is known that activated macrophages may have an increased glycolytic activity; hence, it was of interest to use inhibitors targeting parts of the energy metabolism. The oxidative phosphorylation, a highly energy-yielding part of cell metabolism in the mitochondria, goes through a metabolic switch towards production of reactive oxygen species rather than ATP generation in activated macrophages (the Warburg effect)

(Kelly & O'Neill, 2015). Production of ROS occurs when the rate of electrons transferred in the electron transport chain are mismatched (Nelson, Lehninger, & Cox, 2008). During the switch, most of the NADPH produced in the pentose phosphate pathway (PPP) is utilized by the NADPH-oxidase to generate ROS, rather than going to the ATP yielding oxidative phosphorylation.

The effects of the metabolic inhibitors on the respiratory burst were in most cases moderate, as there were not as much modulation of ROS production as expected. In some instances, inhibitors rather increased the rate of respiratory burst rather than the opposite. The Akt 1/2 inhibitor induced a decrease of ROS production in cells stimulated with LPS – compared to cells stimulated with a combination of LPS and curdlan. As mentioned, the Akt 1/2 inhibitor inhibits transcription factors Akt 1 and Akt 2 in the cell cytoplasm. Akt plays an important role in many processes of the cell, such as glucose metabolism and downstream gene expression. Previous inhibition studies using Akt inhibitors suggested that activation of Akt is necessary, but by itself insufficient for respiratory burst activity (Chen et al., 2003).

Oligomycin A possessed no inhibitory effect on the rate of respiratory burst on the PMA-elicited salmon macrophages. The ROS production appeared to be even higher in cells co-incubated with oligomycin A than in cells without the inhibitor. A study by Fossati et al. (2003) presented results that showed enhancement of ROS production, for up to 120 min, in cells incubated with oligomycin A (at low concentrations; 1 $\mu g\ ml^{-1}$) rather than inhibition in PMA-elicited human neutrophils. As such there may be similarities between the current results and the results from neutrophil stimulation. However, neutrophils are different from macrophages, therefore comparison between these two cell types is difficult.

The mTOR1 has, as previously mentioned (cf. introduction), a vital role in cell metabolism, in particular cell growth, protein synthesis and translation. It has therefore become an important therapeutic target in cancer treatments; hence, there is a high amount of research reports on possible inhibitors of mTORC1 in humans. Rapamycin is a well-known allosteric inhibitor of the mTOR1 complex in mammals. Despite the well-studied effects of mTOR and the impact of rapamycin in mammals, there has not been much research on how it affects fish cells (Dai, Panserat, Terrier, Seiliez, & Skiba-

Cassy, 2014). In the current study, the rapamycin did not give any inhibitory effect on the ROS production; it rather induced an increase in the respiratory burst activity - in particular in LPS stimulated cells. The LPS stimulated cells co-incubated with rapamycin resulted in approximately an 8-fold increase of ROS production compared to LPS stimulated cells (without the inhibitor). The reason for this increase is unknown.

The inhibitor 2-deoxyglucose is known to inhibit glucose uptake, hence limiting glucose metabolism. 2-deoxyglucose had a significant inhibitory effect on ROS production from curdlan-stimulated cells, and led to nearly no ROS production. A study presented by Rist et al. (Rist, Jones, & Naftalin, 1991) found that the rate of superoxide production in PMA-elicited rat peritoneal macrophages increased as the concentration of added 2-deoxyglucose increased. 2-deoxyglucose possessed no activity on LPS-stimulated cells with respect to ROS production.

4.3 NAD^+ and NADH

The intracellular contents of NADH and NAD^+ is important during the respiratory burst as a high NADH content favor ROS generation (Mills & O'Neill, 2016).

Unfortunately, there are no previous correlative reports on the contents of NAD^+ and NADH on cells from fish. A very few reports exist on muscle tissue and egg contents of NAD^+ and NADH. The salmon muscle tissue content of NAD^+ has been estimated to be in the order of 0.38 mM (Bailey & Lim, 1977), whereas in goldfish the NAD^+ content is reported to be up to 394 nmoles per gram fresh tissue (lateral red muscle). In the same report, the NAD^+/NADH ratio was 0.98 (Vandenthillart, Vanwaarde, Dobbe, & Kesbeke, 1982). Following on, the NAD^+/NADH ratio has been found to be 1.8 and 0.7 in oocytes and eggs of the loach (Yermolaeva & Milman, 1974). The calculated NAD^+ contents in macrophage-like cells, in the current study, were 35.5 µM (controls), 31.5 nM (LPS), 24.5 µM (curdlan + LPS) and 72.6 µM (curdlan) – quite lower than the previous reported figures in fish muscle, oocytes and eggs. In the present study, the NAD^+/NADH ratio was found to be close to 55 in control cells, 64 in LPS + curdlan stimulated cells and 117 in curdlan stimulated cells. The LPS stimulated cells showed even a negative ratio because the readings were lower than the lowest standard curve point. It is not known why the NAD^+/NADH ratio, in our study, was considerable higher than previously reported ratios in fish. When compared to mammalian

macrophages (THP-1 cell line), this ratio increased during LPS stimulation, reaching 2.5 at 24 h relative to time-matched non-stimulated control cells (Liu, Vachharajani, Yoza, & McCall, 2012). A similar finding has been revealed where the mice macrophage NAD^+/NADH increased during LPS stimulation (Haschemi et al., 2012). These reports normalized the NAD^+/NADH ratio to controls. If we normalize the results from LPS and curdlan stimulation from control values, we find a 1.4-fold increase after LPS + curdlan stimulation, and 2.3-fold increase after curdlan stimulation. These results are, however, comparable with those where LPS induced an increased cell NAD^+/NADH ratio (Haschemi et al., 2012).

4.4 Gene expression after LPS and curdlan stimulation

During fungus-induced activation of macrophages, the cells may undergo a metabolic switch featured by increased arginase-1 and IL-10 expression in so-called alternatively activated macrophages (M2) (Roszer, 2015). This polarization may also arise during stimulation with immune complexes through Fc receptors, IL-4 and IL-13 (Th2 cytokines) (Martinez & Gordon, 2014). There is a plasticity where M2 macrophages may be classified into at least four subset phenotypes dependent on cytokine profile, cell membrane markers and activity (Roszer, 2015). Markers for classically activated macrophages, e.g. elicited by LPS or IFN-γ, has been suggested to be hypoxia-inducible factor-1α (HIF-1α), glucose transporter protein-1 (GLUT-1), TNF-α and IL-1ß (Kelly & O'Neill, 2015). Classical activated macrophages (M1) express high levels of e.g. iNOS, IL-6, TLR-2 and TLR-4. HIF-1α is also central in its role for nitric oxide generation (Wang, Liang, & Zen, 2014). There are reports showing that carp and zebra fish macrophages may undergo plasticity resembling classical (innate) and alternative activation (Maaike Joerink et al., 2006; Wiegertjes, Wentzel, Spaink, Elks, & Fink, 2016). In the current study, there was significant increase of arginase-1 mRNA expression after LPS and curdlan stimulation – suggesting that the cells may be skewed to a M2-like phenotype, rather than M1-like phenotype. In our study, IL-1ß was also increased compared to non-stimulated cells, while HIF-1α, GLUT-1 and TNF-α remained non-regulated during LPS or curdlan stimulation. In the report by Maaike Joerink et al. (2006), there was an increased TNF-α and IL-1ß expression in carp macrophages after LPS stimulation, whereas the arginase-1 and IL-10 expression remained relatively non-regulated. As such, there are both similarities and

dissimilarities between our results and the results obtained from carp macrophages. Obviously, a more extended analysis on the expression levels of marker molecules and genes must be done to ascertain the existence of M1 and macrophages M2 during stimulation in fish including salmonids.

5 Conclusion

In conclusion, both LPS and curdlan stimulated the macrophage-like cells to induce the formation of ROS; but the effect of the metabolic inhibitors on stimulated salmon macrophages, with respect to ROS formation, proved to be moderate in most instances. However, the Akt 1/2 inhibitor seemed to down regulate the ROS formation, while 2-deoxyglucose up regulated the formation of ROS in some cases. The inhibitors used in this study are produced and optimised for use in mammalian systems and hence their bioactivities in fish are much unexplored. Due to the lack of studies on how metabolic inhibitors affects respiratory burst in salmon macrophages, the underlying mechanisms in fish are yet fairly unknown. The content of NAD^+ increased during stimulation (curdlan, and curdlan + LPS); which may suggest an increased metabolic activity, and also supported by the gene expression studies where the stimulated cells expressed more arginase-1 and IL-1ß.

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778 Table 1: Sequences of primers used for qPCR analysis.

Gene	Primer	Oligonucleotide sequence (5'-3')	Amplicon (bp)	GenBank acc. no.	R^2	% Efficiency (E)
EF-1α	For Rev	TCGTTTTGCTGTGCGTGAC CAGACTTTGTGACCTTGCCG	98	AF308735	0.996	99,86
IL-10	For Rev	CTGTTGGACGAAGGCATTCTAC GTGGTTGTTCTGCGTTCTGTTG	129	EF165028	0.996	106
TNF-α	For Rev	TGTCCATCAAGCCACTACACTC GCACTCACACACCCTGTCATT	129	BT049358	0,994	87,7
IL-1β	For Rev	GCTGGAGAGTGCTGTGGAAGA TGCTTCCCTCCTGCTCGTAG	73	AY617117	0,996	103,7
Arginase-1	For Rev	AGCCATGCGTATCAGCCAA AAGGCGATCCACCTCAGTCA	122	EG929369	0,994	99,99
HIF-1 α	For Rev	GCTCAGAAAGTCGGTTGTCC GCCAGCTCGTAGAACACCTC	132	NM_001140022.1	0,987	92,47
GLUT-1	For Rev	CGCCAGCCCATCTTCATC GAAAACAGCGTTGATGCCAGA	69	AF247728	0,998	107,6

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Figure legends

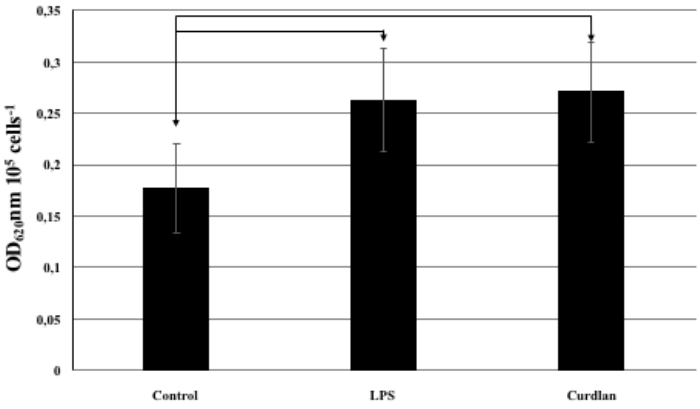
Figure 1: The formation of superoxide anion assayed by the NBT method. Cells were stimulated with A. *salmonicida* LPS (1µg ml^{-1}) and curdlan (10 µg ml^{-1}) for one day, and the respiratory burst activity of PMA elicited macrophages was analyzed. This experiment was repeated trice. The figure presents results from a representative experiment and the values are means (±SD) of 12 technical replicates (N=12). The horizontal lines connected with arrows indicate statistically significant differences.

Figure 2: Dose-relationship by repeated stimulation. The cells were incubated with different doses of curdlan (1-100 µg ml^{-1}) for 24h; subsequently the cells were washed twice with medium and subjected to a second dose of fixed amount of either curdlan (10 µg ml^{-1}) or LPS (1 µg ml^{-1}). This experiment was repeated twice. The figure presents results from a representative experiment and the values are means (±SD) of 12 technical replicates (N=12). The horizontal lines connected indicate statically significant differences.

Figure 3: The effect of metabolic inhibitors on the formation of superoxide anion. Macrophages were co-incubated with Akt 1/2 inhibitor, oligomycin, rapamycin and 2-deoxyglucose for two days, subsequently the cells were assayed for their content of superoxide anion by the PMA-NBT method. A representative experiment is presented from duplicate experiment; the number of technical replicates was four (N=4). Horizontal lines connected with arrows denote statistical significance.

Figure 4: The contents of NAD^+ (µM per 10^5 cells) in macrophage-like cells after stimulation with LPS and curdlan. The cells were stimulated for 24 h before they were lysed and assayed for their contents of total NAD^+. A representative experiment is presented from duplicate experiment; the number of technical replicates was eight (N=8). Horizontal lines connected with arrows denote statistical significance ($p<0.05$). Insert shows the calculated NAD^+/NADH ratio – based on initial analyses of total NAD^+ plus NADH, and NAD^+.

Figure 5: Expression of different genes in the macrophage-like cells (5 x 10^6 cells) after 48 h stimulation by qPCR. Gene expression data was normalized to EF-1 α expressions, set to a numerical value 1. Bars represent mean values ±SD. Asterisk (*) above the bar shows significant difference. One asterisk equals $P<0.05$, two equals $P<0.01$, and three equals $P<0.001$. Three fish were used in this study and qPCR analysis included three technical replicates (N=9).



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814 Figure 1

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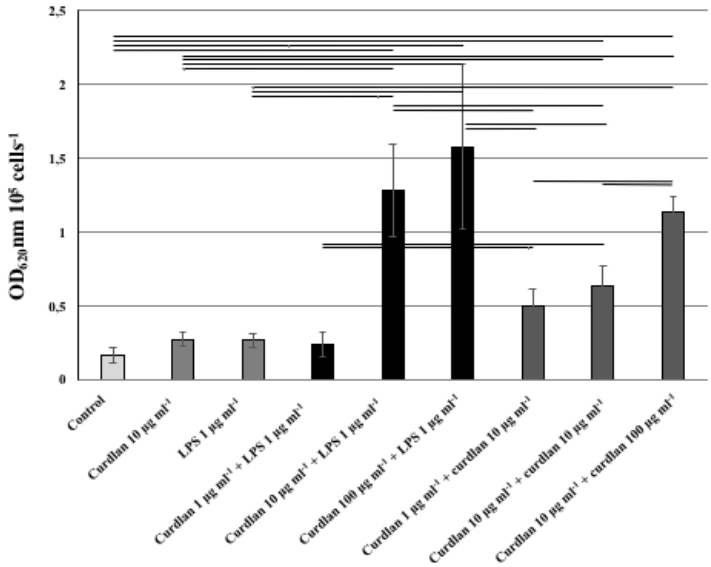

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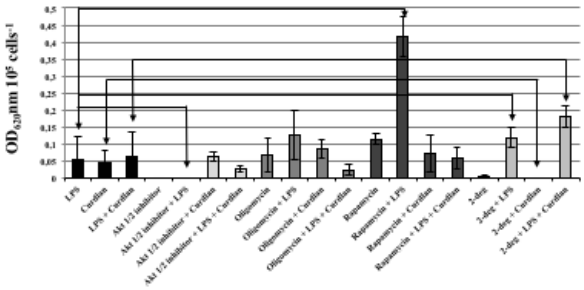
818 Figure 2



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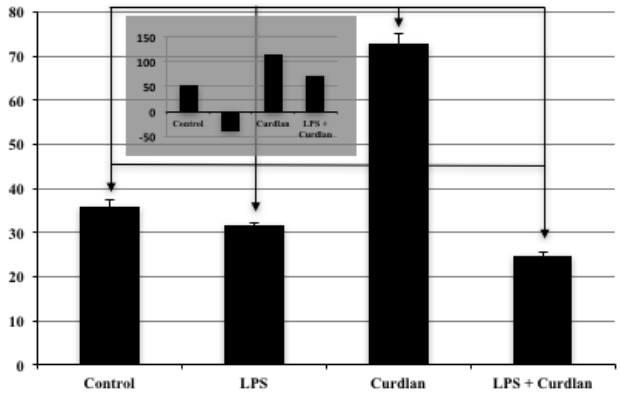
820

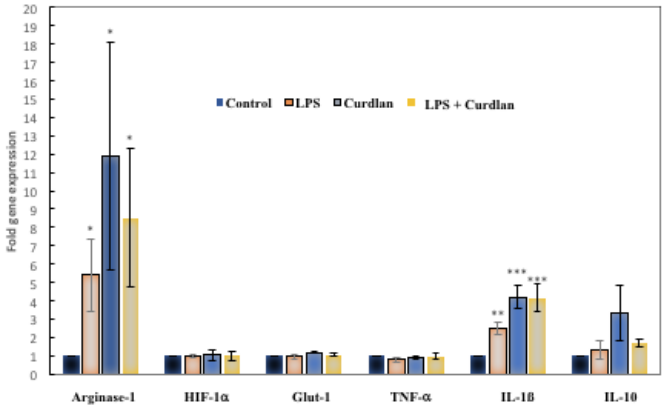
821 Figure 3



822

823 Figure 4

824



825

826 Figure 5