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Studies on the stimulation of Atlantic salmon macrophage-like cells with emphasis on respiratory burst

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Master's Thesis in Aqua Medicine (60 credits)
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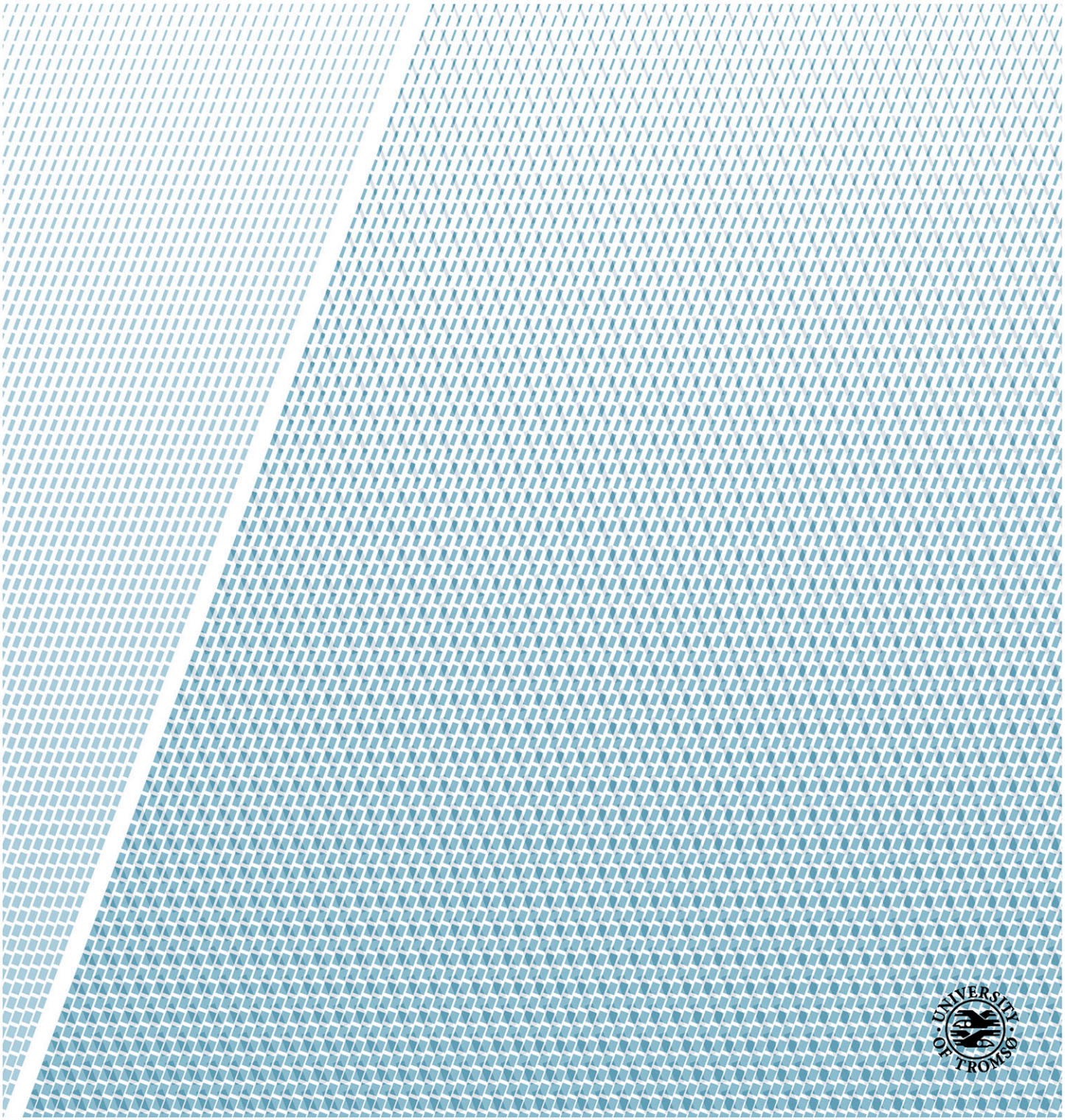

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Johanne Skår Ulvestad

Abstract

Reactive oxygen species (ROS) production in macrophage-like cells is induced as an antimicrobial defence against invading pathogens. In this 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 (developed for mammals) 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 SYBR green real-time quantitative PCR showed that the genes *Arignase-1* and *IL-1ß* were highly expressed in the stimulated cells.

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1 Introduction

Bacterial lipopolysaccharide (LPS) (often called bacterial endotoxin) is composed of lipid and carbohydrate moieties, the latter extruding from the outer cell membrane. The carbohydrate moiety (O-antigen) serves important applications with respect to serotyping of bacteria within a species allowing discrimination between different subspecies. LPS may induce immune responses in fish, even though the lipopolysaccharide receptor, toil-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 (Seternes et al., 2001, Canton et al., 2013, Li et al., 2017).

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 and 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 and 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 and Wiegertjes, 2016). It has been shown that fish macrophages respond to perturbation using “danger” signals such as LPS and ß-glucans with increased production of reactive oxygen species (ROS) and pro-inflammatory molecules (Dalmo and Seljelid, 1995, Novoa et al., 1996, Solem et al., 1995, Castro et al., 1999, Neumann et al., 2001, Cook et al., 2001, Tahir and 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 (Joerink et al., 2006a, Buchmann, 2014, Wiegertjes et al., 2016a, Edholm et al., 2017, Nguyen-Chi et al., 2015, Forlenza et al., 2011, Hodgkinson et al., 2015), even though considerable macrophage heterogeneity exists. Indeed, many more subsets than the original

classical M1 and M2 paradigm has been described in certain mammalian species (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 kinase (protein kinase B/serine-threonine-specific protein kinase) 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 a death domain kinase receptor interacting protein 1. This pathway is of vital importance during cell metabolism (Covarrubias et al., 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). The Akt kinase 1, 2 and 3 are activated by phosphoinositide 3-kinase (PI3K); which previously have been activated by tyrosine kinase receptors (e.g. cell membrane receptors containing tyrosine kinase motifs) by different ligands (Vivanco and Sawyers, 2002) such as LPS (Okutani et al., 2006, Saponaro et al., 2012). Akt are recruited to the plasma membrane in cells stimulated with e.g. growth factors, cytokines, and some danger signals (Fayard et al., 2010, Manning and Cantley, 2007). Akt kinases associates with PIP3 (phosphatidylinositol (3,4,5)-trisphosphate; product of PI3K), this event phosphorylates Akt. PI3K generates metabolites that may activate Akt and protein kinase C-ζ (PKC-ζ), this transcription factor may in turn, under certain inflammatory circumstances, induce respiratory burst and modulate Akt activity (Cosentino-Gomes et al., 2012). PMA (phorbol 12- myristate 13-acetate) may directly target PKC isoforms (Chang and Beezhold, 1993) both in mammalian immune cells, and also in fish leucocytes and macrophage-like cells (Burnett and Schwarz, 1994, Olavarria et al., 2010). Mechanistic target of rapamycin complex (mTORC) is a serine/threonine kinase of the PI3K family. In addition to its function in the regulation of cellular metabolism, mTORC has been shown to be involved in the regulation of both innate and adaptive immune responses. Following stimulation of cells with e.g. growth factors and cytokines, signalling events lead to generation of mTORC2 that activates (phosphorylates) Akt that subsequently activates mTORC1; which regulates the glucose transport, thus glycolysis (Soliman, 2013) and ROS production - as also activation of mTORC2 may do (Chen et al., 2008).

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 TCA cycle activity (Kelly and O'Neill, 2015, Langston et al., 2017). These features are reminiscent of the known Warburg effect (Kelly and O'Neill, 2015). Aerobic metabolism produces ATP, which is the key energy molecule from glycolysis and from TCA cycle and oxidative phosphorylation – through intermediates such as NADH and FADH. These two reducing agents provide electrons, in the form of hydride anions, to the electron transport chain of oxidative phosphorylation. The third energy generating process is metabolism of fatty acid oxidation that yields acetyl-CoA, NADH and $FADH_2$ that goes in the TCA cycle and also into the oxidative phosphorylation. Resting immune cells are relatively metabolically inactive, with minimal biosynthetic demands beyond housekeeping processes (Gaber et al., 2017). During the response, this activated macrophage phenotype produces pro-inflammatory molecules, reactive oxygen metabolites (ROS) and nitric oxide (Iles and Forman, 2002, Beyer et al., 2012, Mosser and Edwards, 2008). Even though not as studied as in mammalian species, the principle of macrophage activation has been suggested to be similar in fish (Hodgkinson et al., 2015, Boltana et al., 2017, MacKenzie et al., 2006). But macrophage metabolism during activation of fish macrophages has not been studied before, nor has the dependence of the metabolism on the formation of ROS from the respiratory burst activation been explored. The objective of the current study was to examine the formation of ROS and NAD^+/NADH 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 marker genes potentially discriminating M1 and M2 macrophages – be means of quantitative real time PCR.

2 Materials & Methods

2.1 Reagents

Roy A. Dalmo (University of Tromsø) kindly provided ultrapure bacterial lipopolysaccharide from *Aeromonas salmonicida* spp. *salmonicida* and curdlan (*Alcaligenes faecalis)*. Chemicals were from SigmaAldrich if not specified.

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

2.2 Fish

The Aquaculture Research Station in Kårvika provided the non-vaccinated Atlantic salmon used in this study. Being fed commercial dry feed at frequencies recommended by the feed producer (Skretting, Norway), the size of the fish varied from 300 g – 1100 g during the two years this study lasted. The fish were kept in tanks at ambient temperature (3-12 °C) with a constant flow of seawater. The fish for cell isolation had priory been analysed clinically, and by RT-qPCR analysis for any presence of pathogens. 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 characterisation of macrophage-like cells

Macrophage-like cells from the head kidney were isolated using a Percoll-gradient, as described by Braun-Nesje et al. (1981), with some modifications.

In short, the head kidney was aseptically removed from the fish into a tube (Falcon) of cell medium (L-15) supplemented with, penicillin (60 μg ml^{-1}), streptomycin (100 μg ml^{-1}), 2 % fetal bovine serum (FBS) and heparin (20 U/ml) (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 400x 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.

Freshly isolated cells were analysed by flow cytometry using a BD FACS Aria III (BD Biosciences, USA) flow cytometer. Dot blots of forward against side scatter were generated by BD FACSDiva™ (BD Biosciences) and three regions were overlaid (R2, R3, and R4). R2 represented the small cells with little granularity, R3 the small-to-medium sized and medium granular cells, whereas the R4 region contained dot blots from detection of medium sized and granulated cells.

2.4 Stimulation of macrophage-like cells

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.

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. LPS and curdlan were 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. The cells were stimulated approximately 24 hours after isolation – where the cells were stimulated with either LPS or curdlan or a combination of both. The re-stimulation of the cells did occur 24 h after the first stimulation. The control cells were cultivated in cell medium only.

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 (Tocris Bioscience, UK), Oligomycin A (Merckmillipore, Germany), Rapamycin (Selleckchem, Germany), 2-deoxyglucose (Carbosynth MD, UK), Dactolisib (Selleckchem) and Wortmannin (LC Laboratories, USA). 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 (Supplementary figure 1). The cells were incubated for approximately 48 hours after the addition of the metabolic inhibitors. Then, the cells were assayed for their respiratory burst activity (cf. 2.5).

2.5 Quantification of respiratory burst activity

To quantify the generation of ROS in macrophages, the respiratory burst assay described by Solem et al. (1995) was followed. In general, the cells were stimulated with LPS or/and curdlan for 24 h or 48 h before the assay was started. 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 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 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 one and two 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.

2.7 NAD^+/NADH measurements

The kit "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 completed 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, SYBR green real-time quantitative PCR (qPCR) were used 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 for 48 hours before harvested. The cells were lysed in a RLT-buffer containing 2-mercaptoethanol and kept at -80 ℃. 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 RT- 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 (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.

Table 1: Sequences of primers used for RT-PCR 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

2.8.3 Statistics

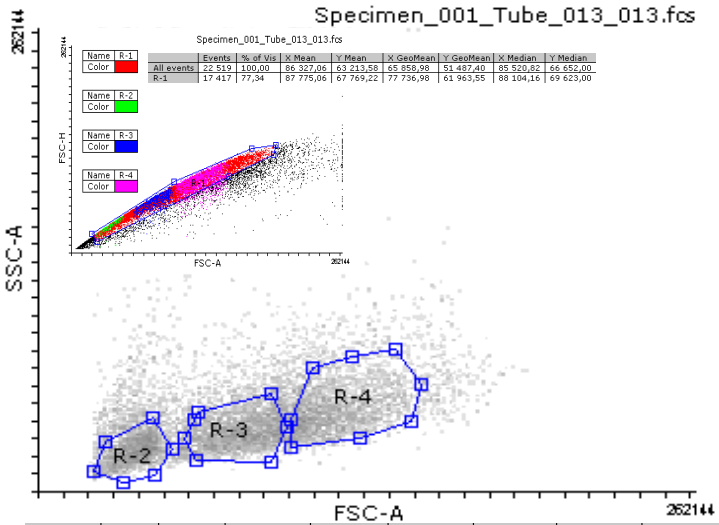
All the experiments were carried out in a duplicate or triplicate fashion (except cell stimulation followed by qPCR), with at least three technical replicates. Statistical analysis was performed using one-tailed, paired Student's T-test. QPCR: The data was log-transformed and analysed by one-way analysis of variance (ANOVA). Thereafter, Tukey's test was completed using

SPSS 25.0 software. In all experiments, statistically significance between the datasets (treatments) were considered if $p<0.05$.

3 Results

3.1 Flow cytometry of isolated cells

The ratio of salmon head kidney cell subpopulations used for *in vitro* studies was evaluated by flow cytometry right before seeding. Approximately 77 % of the cells were small to medium granulated and sized (Fig. 1) (R: 2-4; small to medium forward- and side scatter (FSC and SSC)), resembling lymphocytes and macrophage-like cells at different developmental stages while the rest was evaluated to be other cells and debris (Fig. 1). The proportion of macrophage-like cells (medium FSC and SSC) from R: 3-4 was found to be approximately 56 % (Fig. 1).



	Events	% of Vis	X Mean	Y Mean	X GeoMean	Y GeoMean	X Median	Y Median
All events	17 417	100,00	87 775,06	50 117,62	77 736,98	42 355,13	88 104,16	42 156,60
R-2	2 505	14,38	38 628,36	22 472,09	38 001,31	21 292,21	39 309,27	22 236,00
R-3	3 524	20,23	80 730,47	33 557,39	80 074,13	32 561,13	81 517,59	32 868,65
R-4	4 052	23,26	121 445,44	47 998,48	120 749,56	46 497,96	119 558,66	46 483,95

Figure 1: Flow cytometry of salmon head kidney cells obtained right after the gradient centrifugation and subsequent washing. The flow cytometry was performed on cells from one fish.

3.2 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 intracellular respiratory burst activity of PMA-elicited salmon macrophages – measured as the formation of superoxide anion oxidising NBT to formazan (Fig. 2).

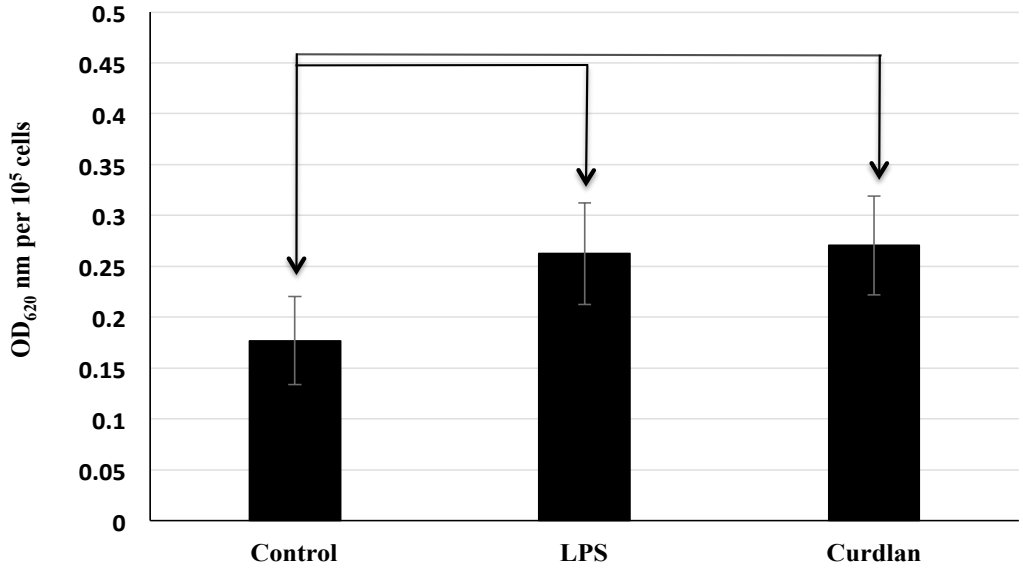


Figure 2: The formation of superoxide anion assayed by the NBT method. Cells were stimulated with A. *salmonicida* LPS (1µg ml-1) and curdlan (10 µg ml-1) for one day, and the respiratory burst activity of PMA elicited macrophages was analyzed. The result is a representative of triplicate analysis, whereas the values are means of at least three replicate analyses (±SD). The horizontal lines connected with arrows indicate statistically significant differences.

3.3 Repeated stimulation and dose relationship

To establish whether repeated stimulation with these two immunostimulants would further increase 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 superoxide generation in cells stimulated with LPS and re-stimulated with curdlan ($p=0.05$), and in cells that underwent twice curdlan stimulation ($p=0.02$) was significantly higher than in control cells (Fig 3A and 3B respectively). No significant differences were found between the other stimulation regimes.

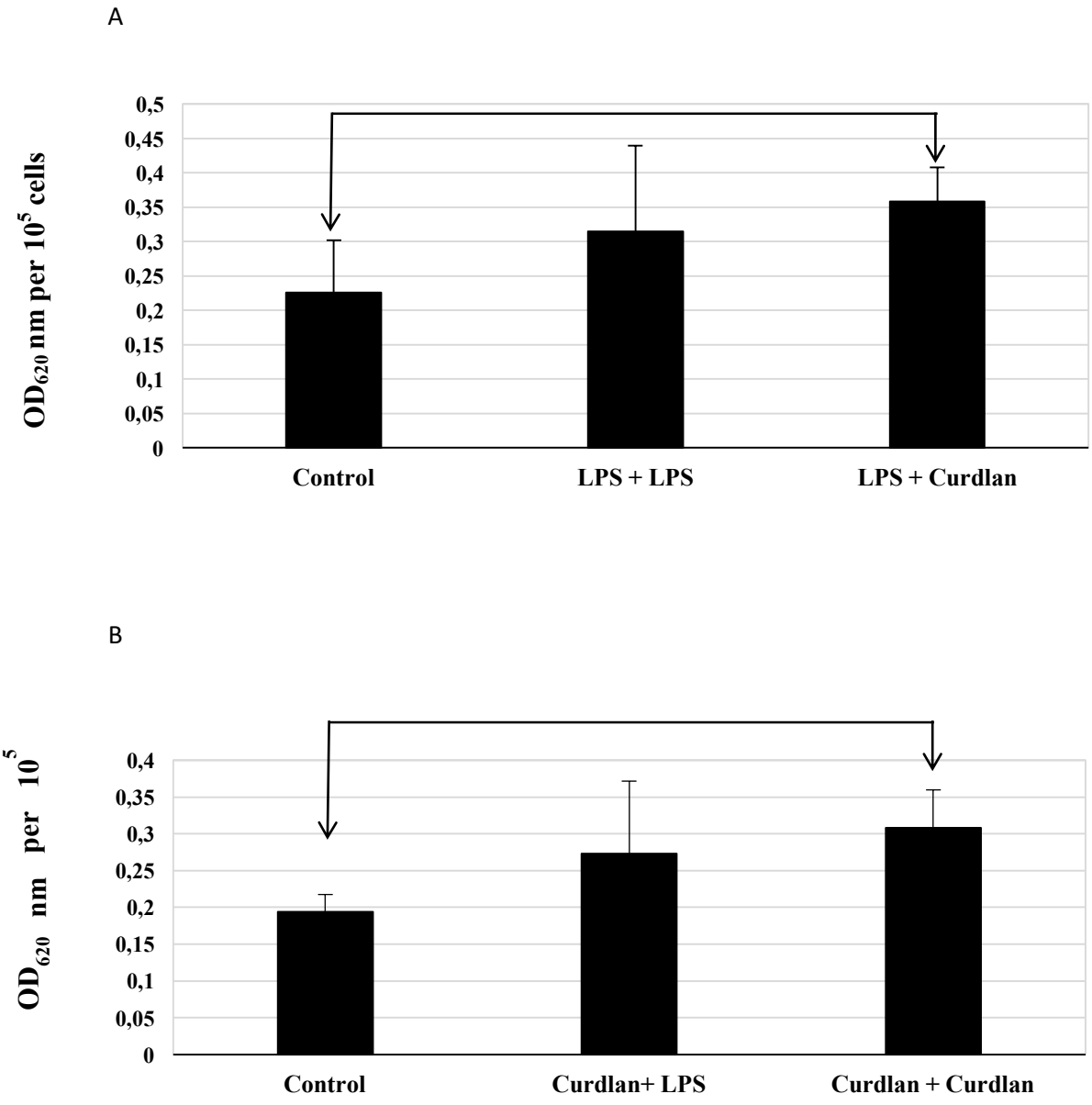


Figure 3 A and B: The formation of superoxide anion assayed by the NBT method. Cells were stimulated with A. *salmonicida* LPS (1 µg ml^{-1}) (A) or curdlan (10 µg ml^{-1}) (B) for one day, then repeatedly with LPS or curdlan stimulants the next day. The respiratory burst activity of PMA-elicited macrophages was analysed. The result is a representative of triplicate analysis, whereas the values are means of at least three technical replicates (±SD). The horizontal lines connected with arrows indicate statically significant differences.

Since the re-stimulation experiments using fixed doses of LPS (1 µg ml^{-1}) and curdlan (10 µg ml^{-1}) appeared not to induce significant increased formation of superoxide anion compared to what obtained from single stimulations, we were interested to see whether this was due to a dose-relationship phenomenon. Thus, 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) the day after (Fig. 4).

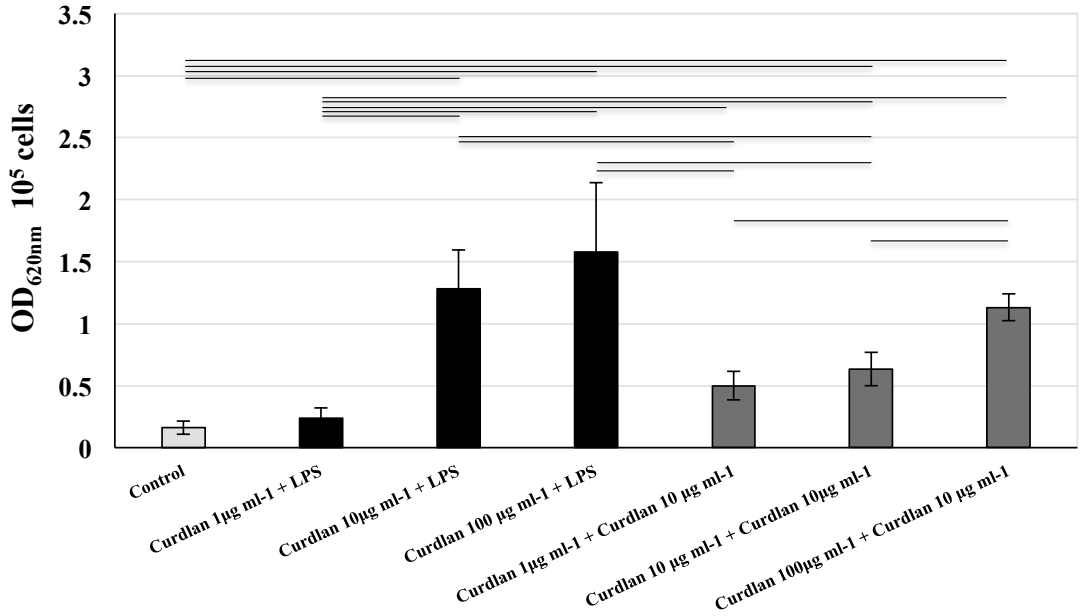


Figure 4: Dose-relationship by repeated stimulation. The cells were incubated with different doses of curdlan (1-100 µg ml^{-1}) for 24h; subsequently the cells were washed and subjected to a second dose of fixed amount of either curdlan (10 µg ml^{-1}) or LPS (1 µg ml^{-1}). The result is a representative of duplicate analysis, whereas the values are means of at least three technical replicates (±SD). The horizontal lines connected indicate statically significant differences.

The results revealed that 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 and cells stimulated with the other combinations ($p<0.05$), except cells stimulated with curdlan (10 µg ml^{-1}) and LPS (Fig. 4). Cells pre-stimulated with curdlan (10 µg ml^{-1}) followed by LPS produced significant more ROS than control cells ($p<0.05$), 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. 4). 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.

3.4 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), PI3K (wortmannin, dactolisib), Akt 1/2 (Akt 1/2 inhibitor), complex V in the electron transport chain (mitochondria) (oligomycin A) and mTORC (rapamycin). (Unfortunately, we were not able to include wortmannin and dactolisib in the main experiment).

In the dose response study where LPS and curdlan-stimulated cells were co-incubated with each inhibitor, only 2-deoxyglucose, rapamycin and wortmannin resulted in a dose dependent decrease in superoxide anion formation by increased inhibitor concentration (1nM - 1μM) (Supplementary figure 1). The Akt 1/2 inhibitor resulted in an increase of respiratory burst at 100 nM compared to a lower (1-10nM) and a higher (1μM) concentration of Akt 1/2 inhibitor (Supplementary figure 1). 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. 5), 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 + curdlan + 2-deoxyglucose ($p=0.002$). The glucose transport inhibitor, 2-deoxyglucose (100 μM), also increased the formation of superoxide anion when co-incubated with LPS ($p=0.03$), but decreased the respiratory burst activation when the cells where incubated with curdlan ($p=0.0001$) (Fig. 5).

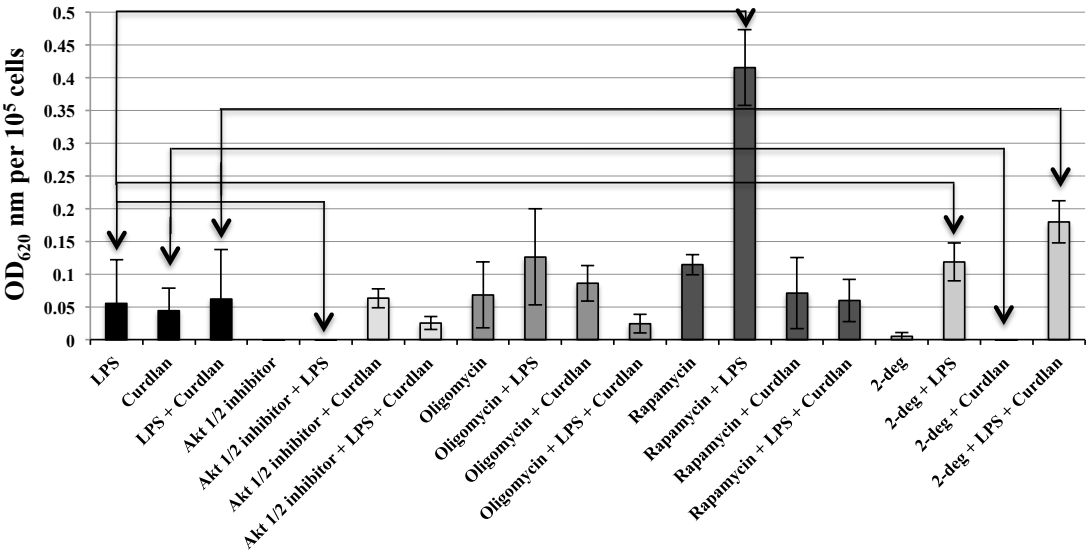


Figure 5: The effect of metabolic inhibitors on the formation of superoxide anion. Macrophages were co-incubated with Akt 1/2 inhibitor, oligomycin A, rapamycin and 2-deoxyglucose for two days, subsequently the cells were assayed for their content of superoxide anion by the NBT method. A representative experiment is presented from duplicate experiment; the number of technical replicates was four or more. Horizontal lines connected with arrows denote statistical significance.

3.5 Measurements of nitric oxide production

The quantification of produced nitric oxide in macrophage-like cells did not give any results. After the Griess assay and incubation time was completed, there were no colour change in the cell samples. Due to the lack of colour change, there was no difference in the OD values between the blank controls and test samples.

3.6 NAD^+/NADH measurements

The NAD^+/NADH ratio may differ between resting and activated cells; activated cells may have increased ratio. After stimulation of macrophages for two days with curdlan (10 µg ml-1), curdlan + LPS (1 µg ml^{-1}) the NAD^+/NADH ratio increased 2.3 -and 1.5-fold, respectively. The "opposite" was found when we calculated the ratio for LPS stimulated cells (Fig. 6). 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.

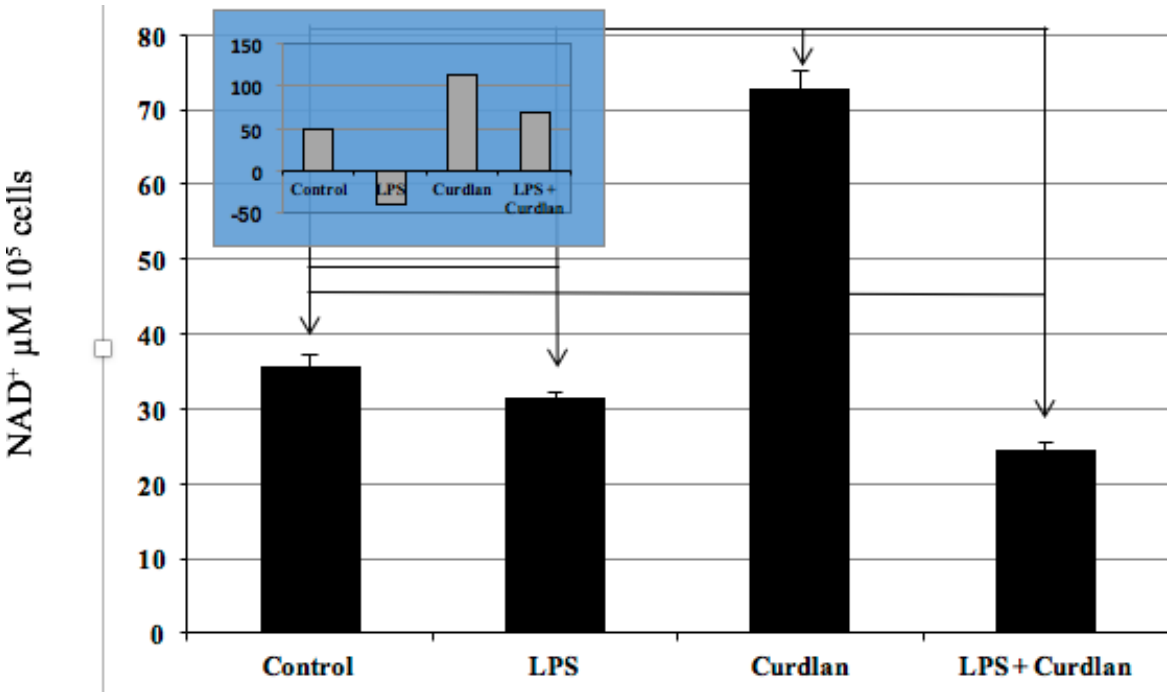


Figure 6: The contents of NAD^+ (µM per 10^5 cells) in macrophage-like cells after stimulation with LPS and curdlan. The cells were stimulated for 24 h. A representative experiment is presented from duplicate experiment; the number of technical replicates was eight. Horizontal lines connected with arrows denote statistical significance ($p<0.05$). Insert shows the calculated NAD^+/NADH ratio – based on initial analyses of total NAD^+ plus NADH, and NAD^+

3.7 Gene expression after LPS and curdlan stimulation

During fungus-elicited activation of macrophages, the cells may undergo a metabolic switch featured by increased arginase 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 and 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. 7).

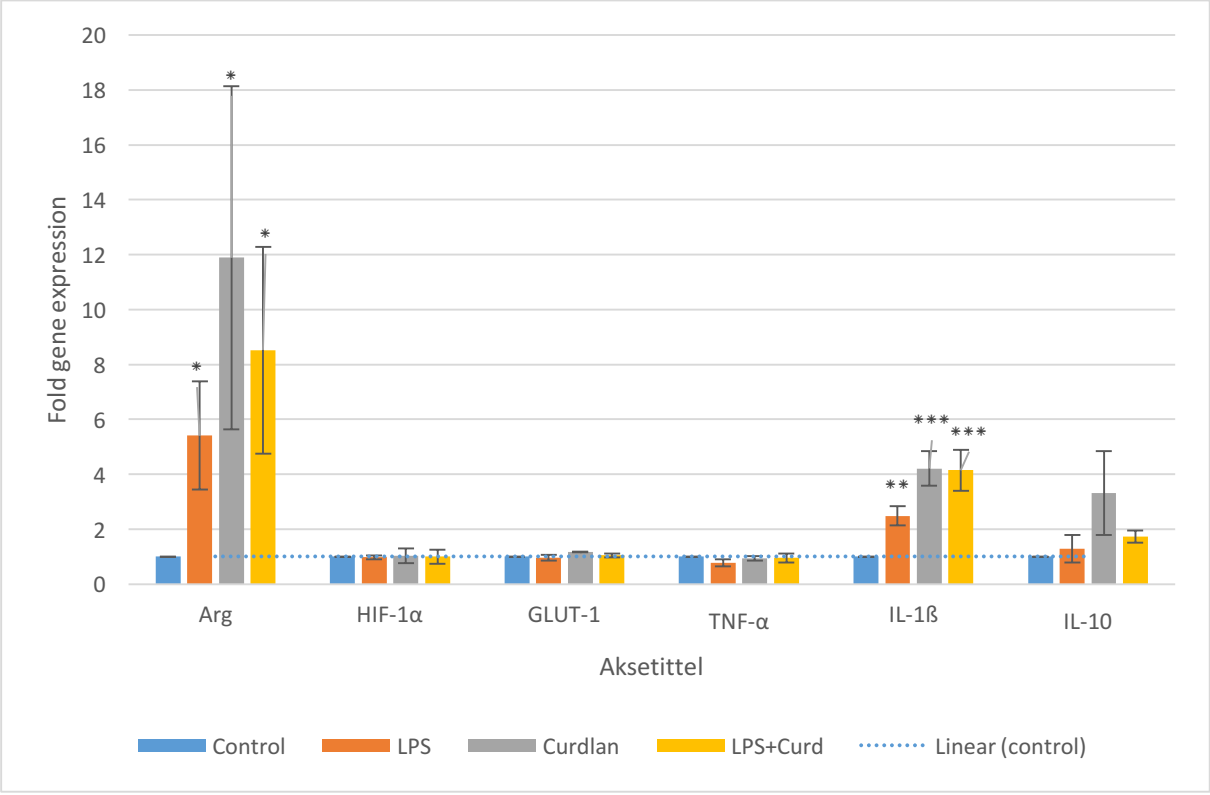


Figure 7: Expression of different genes in the macrophage-like cells (5 x 10^6 cells) after 48 h stimulation by qPCR. Gene expression data was normalized to EF-1 α expressions, set to a numerical value 1. Bars represent mean values ±SD. Asterisk (*) above the bar shows significant difference. One asterisk 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.

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, NAD^+ and NADH, and gene expression in the cells after stimulation with LPS and β-glucan (curdlan) was analysed.

4.1 Isolation and characterisation of head kidney cells

According to the results from the flow cytometry, the proportion of macrophages in the cell population was found to be approximately 56%. The yield of macrophage-like cells after density centrifugation was quite similar to what reported for rainbow trout (Lovoll et al., 2007). The distribution of cells observed from the flow cytometry was also similar to what have been reported from other teleost fish species (Haugland et al., 2012, Overland et al., 2010, Ronneseth et al., 2005, Morgan et al., 1993).

4.2 Stimulation of macrophage-like cells

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 (Jorgensen and Robertsen, 1995, Paulsen et al., 2001, Dalmo and Seljelid, 1995). In the current study, macrophages stimulated with curdlan showed, in general, the highest increase in ROS production. Re-stimulation with curdlan induced higher increase of superoxide anion production compared to co-stimulation with LPS. As far as we know, combined and repeated stimulation of salmon macrophages by virtue of respiratory burst has not been done before; thus, any direct comparison with findings by others is not possible.

4.3 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. The activation of the PI3K/Akt/mTOR pathway is described in Figure 8. Inhibitor targets are also included in this figure.

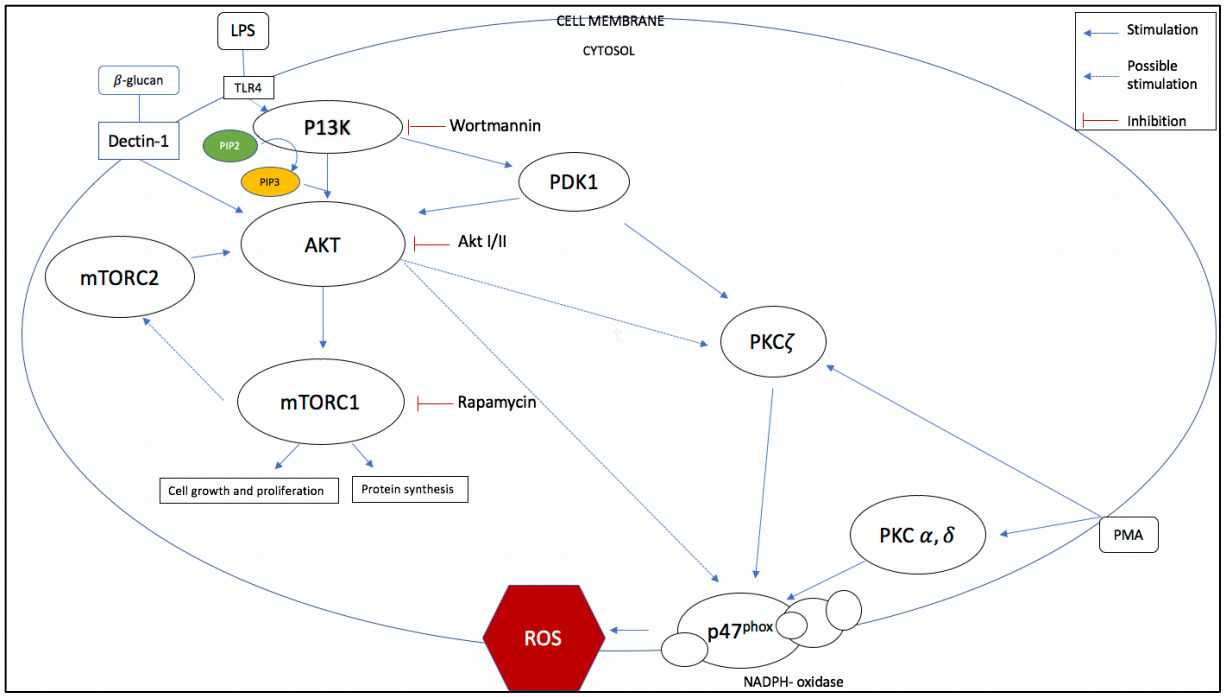


Figure 8: A simplified view on the PI3K/Akt/mTOR pathway in mammals. Stimuli by e.g. LPS activate the TLR4 pathway – inducing PI3K-AKT-mTORC1 cascade, partly resulting in ROS production. In contact with the cell, PMA activates NADPH oxidase through direct activation of protein kinase C (PKC) isoforms. The components of the NADPH oxidase migrate to the cell membrane when activated; the active multicomponent enzyme is then formed and able to generate ROS. The inhibitors wortmannin inhibits PI3K, Akt I/II inhibits Akt and rapamycin inhibits mTORC1. Adapted from Zhao et al. (Zhao et al., 2010) and Cheng et al. (Cheng et al., 2014).

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 and O'Neill, 2015). Production of ROS occurs when the rate of electrons transferred in the electron transport chain are mismatched (Nelson et al., 2008). During the switch, most of the NADPH produced in the pentose phosphate pathway (PPP) (Fig. 9) is utilized by the NADPH-oxidase to generate ROS (Fig. 8), rather than going to the ATP yielding oxidative phosphorylation.

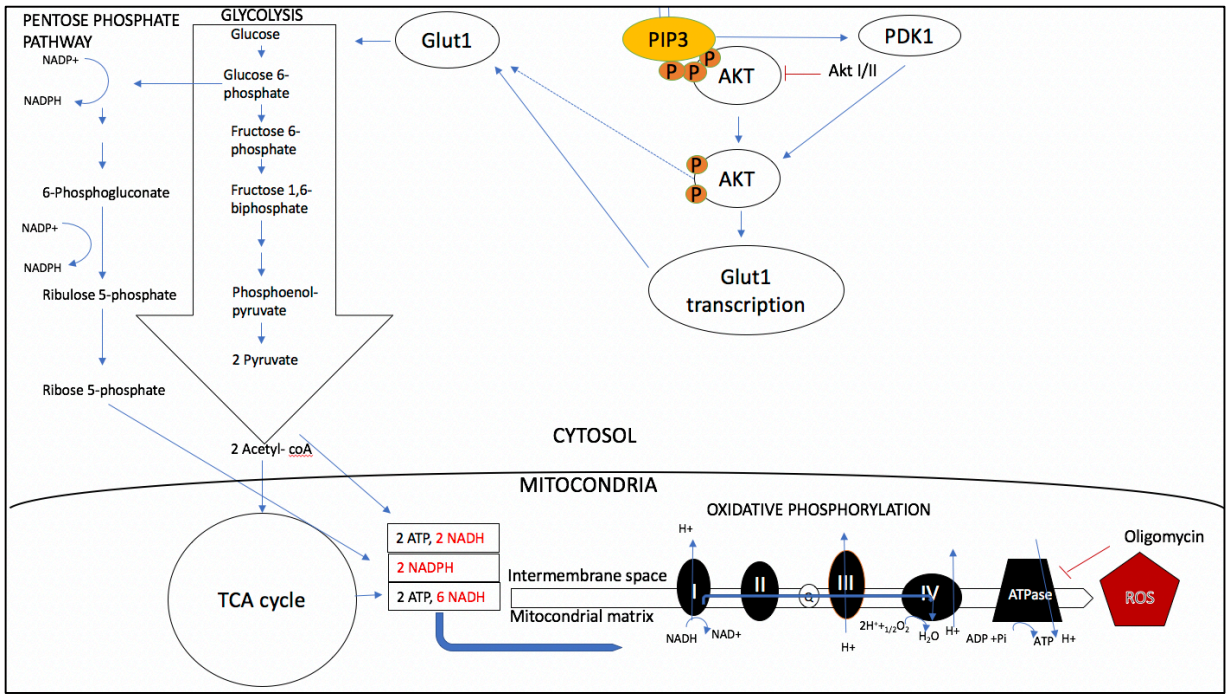


Figure 9: Parts of the glycolytic metabolism and the oxidative phosphorylation. Akt regulates transcription of Glut1, which is a glucose transporter central to feed glucose in the glycolysis. Both the glycolysis, PPP and the tricarboxylic acid (TCA) cycle generates NADH or NADPH, which can either go to ATP generation or ROS generation via the electron transport chain in the oxidative phosphorylation. NADPH from the PPP can also be utilized by the NADPH-oxidase (Fig. 8) to generate ROS. The inhibitor Akt 1/2 inhibits transcription factors Akt1 and Akt2, thus, the glucose transport to the glycolysis can be affected. Oligomycin A inhibits ATPase (complex V) in the electron transport chain. Adapted from (Chan et al., 2012, Marie and Shinjo, 2011, Hecker et al., 2013, Nelson et al., 2008)

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 µg 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 et al., 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-deoxyglucse 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 et al., 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.

4.4 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 and O'Neill, 2016).
Unfortunately, there are no previous correlative reports on the contents of NAD^+ and NADH on cells from fish. A very few report 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 and 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 ration was 0.98 (Vandenthillart et al., 1982). Following on, the NAD^+/NADH ration has been found to be 1.8 and 0.7 in oocytes and eggs of the loach (Yermolaeva, Lp. and 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 standard curve points. 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 et al., 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).

4.5 Gene expression after LPS and curdlan stimulation

During fungus-induced activation of macrophages, the cells may undergo a metabolic switch featured by increased arginase 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 and Gordon, 2014). To complicate the concept further, 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). 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 phenotype, rather than M1 phenotype. But more examination on the expression levels of marker molecules and genes must be done to ascertain the existence of M2 during stimulation in fish including salmonids. However, there are reports showing that carp and zebra fish macrophages may undergo plasticity resembling classical (innate) and alternative activation (Joerink et al., 2006b, Wiegertjes et al., 2016b). 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 and O'Neill, 2015). Classical activated macrophages (M1) express also high levels of

e.g. iNOS, IL-6, TLR2 and TLR4. HIF-1α is also central in its role for nitric oxide generation (Wang et al., 2014). In the report by Joerink et al. (2006b), 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. We were not able to detect any NO production from salmon macrophage-like cells – which was a bit strange since NO production has been found in macrophages from other fish species (Wiegertjes et al., 2016b). Anyway, increased loads of data from work on fish macrophages on the plasticity during stimulation is underway – that together may define the existence of M1 and M2 subsets in fish, and may also suggest whether there are differences with respect to different fish species.

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.* Genes encoding for LPS receptor (e.g. TLR4) and curdlan (e.g. dectin-1) has not been found in the salmon genome, suggesting the presence of other and unknown LPS and ß-glucan receptors inducing macrophage activation. This adds, however, more questions whether the traditionally classical and alternative macrophage phenotype dichotomy exists in salmon.

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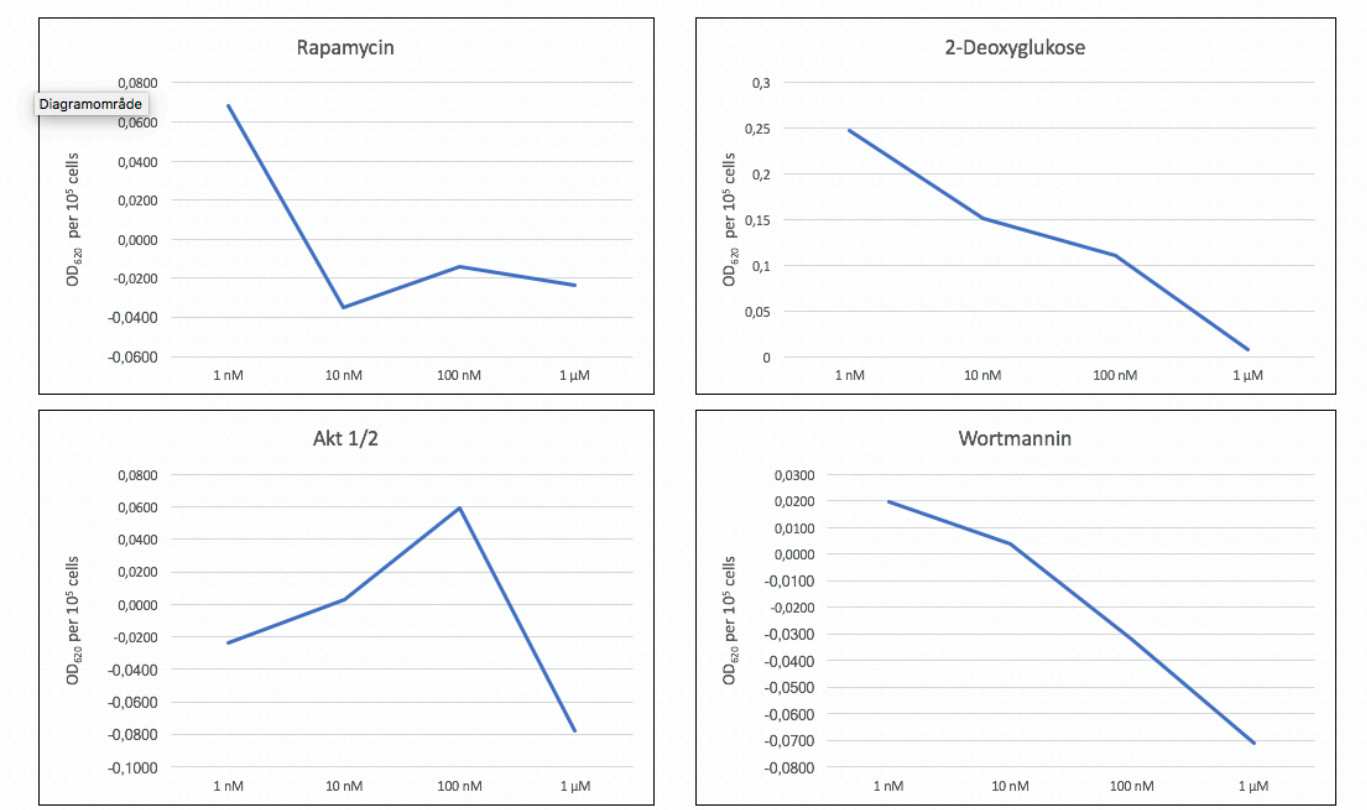
Supplementary materials



Supplementary figure 1: The dose-relationship of different metabolic inhibitors on the formation of ROS by macrophage-like cells. The experiment was repeated once.