Effects of *Moritella viscosa* antigens on pro-inflammatory gene expression in an Atlantic salmon (*Salmo salar* Linnaeus) cell line (SHK-1)

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**A B S T R A C T**

*Moritella viscosa* is the causative agent of winter ulcer disease in salmonids reared in North-Atlantic countries. In this study the effects of selected *M. viscosa* antigens on cytotoxicity and pro-inflammatory gene expression in an Atlantic salmon (*Salmo salar* Linnaeus) macrophage-like cell line (SHK-1) were examined. SHK-1 cells were stimulated with live and heat-killed bacterial cells, extracellular products (ECP) and an extracellular vibriolysin, termed MvP1. Following incubation, cytotoxicity and expression levels of interleukin-1β (IL-1β) and interleukin-8 (IL-8) were examined at different time points. Both live *M. viscosa* cells and ECP were cytotoxic, but neither heat-killed cells, nor the MvP1 peptidase caused cell death. Expression levels of both IL-1β and IL-8 increased significantly after stimulation with live cells, but heat-killed cells only caused increased IL-8 expression. ECP did not affect IL-1β expression, but did stimulate IL-8 expression. The isolated MvP1 peptidase stimulated both IL-1β and IL-8 expression at the highest concentration tested. This study reveals a difference in the induction of pro-inflammatory gene expression in salmon SHK-1 cells between live and heat-killed *M. viscosa* cells, and also that an unknown secreted factor is the main stimulant of IL-1β and IL-8 expression.

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**1. Introduction**

*Moritella viscosa* causes winter ulcer disease in farmed salmonids in North-Atlantic countries, at temperatures below 10 °C. Infections in Atlantic cod (*Gadus morhua* Linnaeus) have also been reported [1]. Infected fish often develop ulcers, which can extend deep into the underlying musculature, and the disease poses a serious fish welfare problem. Internally, hemorrhages and tissue necrosis are observed in various tissues and organs [2,3].

The virulence mechanisms of *M. viscosa* are still poorly understood. Lipooligosaccharides and a 17–19 kDa outer membrane antigen seem to be potential protective antigens in salmon [4]. *M. viscosa* extracellular products (ECP) cause mortalities in Atlantic salmon (*Salmo salar* Linnaeus), and internal disease signs similar to those seen in infected fish. Additionally, ECP have been shown to have both cytotoxic and hemolytic activities [5].

A metallopeptidase, termed MvP1, was isolated from the ECP of *M. viscosa*, and partially characterised. The peptidase, which is a vibriolysin (EC 3.4.24.25), is non-lethal to salmon at concentrations below 0.22 μg protein/g fish, but has virulence-related activities. It causes extensive hemorrhaging and tissue necrosis in salmon, degrades host tissue components and IgM, and affects cell–cell adhesion. It has been proposed that MvP1 aids in the invasion and dispersion of *M. viscosa* in its host [5].

A continuous cell line derived from Atlantic salmon head kidney leukocytes, SHK-1, has been used as a model system to study salmon immune responses at both the translational and transcriptional levels, under various conditions [6–9]. The cell line displays macrophage-like properties and is able to phagocytose the fish pathogen *Aeromonas salmonicida*, but does not exhibit bactericidal activity or have a macrophage-like appearance [10]. The cell line expresses both major histocompatibility complex class I and II [11], and C-type lectin receptors [12].

Most infectious agents induce an inflammatory response in their host; this response initiates activation of the innate immunological response. Cytokines such as interleukin-1β (IL-1β) and...
interleukin-8 (IL-8), which promote the inflammation process, are called pro-inflammatory cytokines; they are good markers of the inflammatory response. Both genetic and biological evidence imply that the fundamental properties of the pro-inflammatory cytokines in mammals and teleost fish are similar [13–17]. IL-1β is an important and central mediator of immune and inflammatory responses and initiates a wide variety of functions, such as the expression of other cytokines and macrophage activation. It is primarily produced by monocytes and macrophages [14,15]. IL-8 (also known as CXCL8) is a chemokine, which attracts neutrophils to the site of infection or injury. It is produced by many different cell types and can be induced by different factors, including IL-1β [17].

The aim of this study was to examine the effects of selected M. viscosa antigens on gene expression of the pro-inflammatory cytokines IL-1β and IL-8 in an Atlantic salmon cell line, SHK-1, and to determine their potential role in triggering the host immune response following infection with M. viscosa.

2. Materials and methods

2.1. Moritella viscosa culturing and isolation of antigens

M. viscosa strain K58 was used in the current study. The strain originates from the head kidney of Atlantic salmon suffering from winter ulcer disease in Iceland [18,19]. This strain was passed in salmon prior to the study, by re-isolation from kidney of intraperitoneally (i.p.) infected fish. The bacterium was grown in batch cultures, following cultivation on 5% horse blood agar supplemented with 2% NaCl (BA-NaCl) at 4 °C. For each batch culture, Brain-Heart infusion broth (BHI, BD, Franklin Lakes, NJ, USA) containing 1.5% NaCl (BHI-NaCl) was inoculated with strain K58 (1% inoculation from a starter culture), and cultivated at 4 °C with agitation (200 rpm). M. viscosa cells and ECP were harvested from a logarithmic phase culture, grown for 48 h. Cells were washed twice in Dulbecco’s phosphate buffered saline (DPBS, with Ca2+ and Mg2+, Sigma, Mississauga, ON, Canada), re-suspended in the same buffer and kept on ice. A part of the washed cell suspension was heat-killed in a water bath at 60 °C for 1 h. Colony forming units (cfu) were estimated by plate counting on BHI-NaCl agar at 4 °C. ECP were isolated from cells through centrifugation (3200 × g, 10 min, 4 °C) and filtration (22 μm, Whatman, Dassel, Germany). The MvP1 peptidase was isolated from a stationary phase culture as previously described [5]. Protein content of M. viscosa ECP and isolated MvP1 was measured in duplicates using a Bradford protein assay kit (Coomassie Plus, Pierce, Rockford, IL, USA) and bovine serum albumin (Sigma) used for plotting a standard curve. Absorbance was measured at 590 nm and each sample measured in duplicate.

2.2. M. viscosa growth in cell cultures, MvP1 stability and cytotoxicity assay

The ability of M. viscosa to grow at 20 °C in BHI-NaCl, SHK-1 cell cultures and cell culture medium alone was tested at 4 and 24 h. Bacterial numbers were estimated by plating out ten-fold dilutions of the medium onto BA-NaCl plates.

The stability of MvP1 activity in DPBS and culture medium, both fresh and conditioned, was verified for up to 72 h at 20 °C. Medium collected from SHK-1 culture flasks after 3 days of culturing was used as conditioned medium. MvP1 was diluted 1:1 in the respective buffer or medium. The buffer used for MvP1 isolation (50 mM Na-phosphate buffer, pH 7, containing 15 mM NaCl) was used as a control. Casenolytic activity in each sample was measured in duplicate using the azocasein assay, as previously described [5], and one unit of azocasein activity defined as an increase in absorbance of 0.01 under the assay conditions at 430 nm.

Concentration of antigens used for SHK-1 stimulation was determined via a cytotoxicity detection kit (LDH, Roche, Roche, Basel, Switzerland), as stimuliants should not affect cell viability. The assay was performed in triplicate in 96 well plates, according to the manufacturer's instructions, with 5 × 103 SHK-1 cells/well. After culturing for 2 days, under the conditions described below, cells were washed twice with DPBS and incubated with stimulants diluted in DPBS at 20 °C for 4, 24 or 72 h. Controls, lactate dehydrogenase measurements and calculation of percentage cytotoxicity were performed as described by Fast et al. [20].

2.3. SHK-1 cell culture and stimulation

SHK-1 cells were cultured at 18 °C in 75 cm² flasks (Costar, Fisher Scientific, Ottawa, ON, Canada), as described by Fast et al. [6], without antibiotics. Cells used in this study were passaged between 61 and 63 times. SHK-1 cells were seeded in 25 cm² flasks (Costar, Fisher Scientific) at approximately 1 × 10⁶ cells/flask, 72 h before stimulation, and cultured at 20 °C. Then, medium was removed from each flask and 5 ml fresh medium, containing the stimulants, was added. The stimulations were performed in two separate experiments. In experiment 1, SHK-1 cells were stimulated with M. viscosa cells, either live or heat-killed, with estimated multiplicity of infection (MOI) of 2, or ECP in final concentrations of 0.01 or 0.05 μg protein/ml. In experiment 2, isolated MvP1 was added in final concentrations of 0.1, 0.4, 0.7 and 1.0 μg protein/ml. Cultures were incubated with stimulants at 20 °C for 4, 24 or 72 h, and cells incubated with medium and dilution buffer only were used as control. Each treatment was carried out in triplicate.

2.4. RNA isolation, cDNA synthesis, and real-time Q-PCR

Following stimulation, total RNA was isolated from SHK-1 cells using 3 ml Trizol Reagent (Invitrogen, Burlington, ON, Canada), according to the manufacturer’s instructions, and as described by Fast et al. [20]. RNA concentrations were determined using a NanoDrop-1000 (v3.2.1, Thermo Scientific, Delaware, CO, USA) spectrophotometer, and samples were stored at −80 °C until reverse transcription. For reverse transcription, 2.0 μg total RNA from each sample was used, dissolved in molecular biological grade water. Two-step reverse transcription real-time Q-PCR was carried out using the SuperScript III Platinum Two-Step qRT-PCR kit with SYBR green (Invitrogen) on an iCycler iQ Real-Time detection system (BioRad, Mississauga, ON, Canada). The manufacturer’s instructions were followed, with the exceptions described by Fast et al. [20]. Following first strand synthesis, samples were stored at −20 °C until use in real-time Q-PCR. Primers for real-time Q-PCR of elongation factor-1α (EF-1α), which was used as a reference gene, IL-1β, and IL-8 were as described in Table 1, and based on previously published Atlantic salmon sequences. The PCR products of each primer pair were cloned and sequenced, and isolated plasmid DNA was sequenced as described below.
vectors used as standards for real-time Q-PCR as previously described [6]. The EF-1A primers were designed to span an intron/exon splice site and single-product amplification was confirmed through melt curve analysis, to ensure that genomic DNA was not quantified in real-time Q-PCR. Cycle conditions were as described by Fast et al. [20]. Ten-fold dilutions of the standards (between 1 × 10^{-5} ng and a blank without cDNA were run in each real-time Q-PCR, along with the duplicate samples. The relationship between the threshold cycle (C_T) and log(RNA) was linear (-3.18 < slope < -3.02) for all reactions.

2.5. Data analysis

All data are presented as means ± SEM. Statistical analyses on cytotoxicity assay data and gene expression data were performed using two-way ANOVA (variables: antigen, sampling time, and interaction). The Tukey HSD test was then used to discern differences among means for those effects that had significant differences (p < 0.05) (SPSS 15.0 for Windows) and the data were segregated by variable and compared to the relative control.

Expression of IL-1β and IL-8 was calculated as relative to the expression of the EF-1A reference gene. Due to significant differences in the expression of EF-1A between sampling times, the relative expression of IL-1β and IL-8 was calculated from the mean expression of EF-1A at each time point, for each experiment, with values from control and stimulated cells combined. Gene expression of IL-1β and IL-8 in stimulated cells is presented as fold expression compared to expression in control cells.

3. Results

3.1. M. viscosa antigens

M. viscosa cfu were counted on agar plates and the MOI calculated. No colonies grew on plates streaked out with heat-killed cells. The protein content of freshly collected ECP was estimated at 120 μg/ml, and casinase activity of 15 U. The protein content of isolated MvP1 was estimated at 25 μg/ml and casinase activity of 50 U.

3.2. M. viscosa growth in cell cultures, MvP1 stability and cytotoxicity assay

M. viscosa cells were unable to grow in SHK-1 cell culture medium at 20 °C. After 4 and 24 h of incubation only 2 and 0.5% of the initial bacterial numbers were culturable, respectively. Attempts to culture M. viscosa from SHK-1 cell cultures were unsuccessful. However, the bacterium grew normally in BHI-NaCl at 20 °C (results not shown).

The MvP1 peptidase was stable and retained its caseinolytic activity at the experimental temperature in the buffers and medium used, as shown in Fig. 1.

The effects of M. viscosa antigens on SHK-1 cell viability are shown in Fig. 2. P-values from two-way ANOVA were significant (p = 0.000) for all variables. Live M. viscosa cells caused significant cytotoxicity in SHK-1 cells after 72 h incubation in all densities tested, MOI 6, 3 and 0.3, and after 24 h with the highest density. However, heat-killed M. viscosa cells did not affect SHK-1 cell death at any time point when tested in the same densities. Only the results from the highest density of heat-killed cells (MOI 6) are shown in Fig. 2. M. viscosa ECP caused significant cell death after 24 and 72 h of incubation at a concentration of 0.5 μg protein/ml, but a concentration of 0.05 μg protein/ml ECP did not affect cell viability. The MvP1 vibriolysin did not cause significant cell death at a 1 μg protein/ml concentration. The results of the cytotoxicity assay were subsequently used to determine the upper limits of M. viscosa antigen doses used in cell stimulation experiments, so that they would not affect the viability of the SHK-1 cells.

3.3. EF-1A as a reference gene

EF-1A was chosen as a reference gene for this study, and the mean expression of EF-1A at each time point was calculated from values of both stimulated and unstimulated cells. In experiment 1 the average expression was 5.4 × 10^{6} ± 0.91 × 10^{6}, 4.7 × 10^{6} ± 0.29 × 10^{6} and 4.8 × 10^{6} ± 3.6 × 10^{6} copy numbers per μg RNA at 4, 24 and 72 h, respectively. P-values from two-way ANOVA were non-significant for all variables: 0.052, 0.181, and 0.490 for antigen, sampling time, and interaction, respectively. However, in experiment 2, mean EF-1A copy numbers per μg RNA were 1.2 × 10^{5} ± 0.07 × 10^{5}, 6.7 × 10^{5} ± 0.72 × 10^{5} and 6.0 × 10^{5} ± 0.89 × 10^{5} at 4, 24 and 72 h, respectively. Sampling time was found to have a significant effect on expression (p = 0.000), where the expression at 4 h was higher than at 24 and 72 h. Antigen type or interaction between antigen and sampling time were not found to have significant effect on EF-1A expression (p = 0.107 and 0.062, respectively). Due to the differences in the expression of EF-1A, the average copy number at each time point was used to calculate the relative expression of both IL-1β and IL-8.

3.4. Stimulation experiment 1; M. viscosa cells and ECP

The mean relative expression of IL-1β in control cells in experiment 1 was 5.2 × 10^{-4} ± 0.84 × 10^{-4}, 4.9 × 10^{-4} ± 0.95 × 10^{-4}, 5.4 × 10^{-4} ± 0.5 × 10^{-4} at 4, 24 and 72 h, respectively. The values did not differ significantly. The type and dose of antigen was found to have a significant effect on IL-1β expression (p = 0.000), but sampling time (p = 0.486) or interaction (p = 0.892) did not have a significant effect. Following stimulation with live M. viscosa cells for 24 h, expression of IL-1β increased significantly, and was 7.1 times higher than in control cells, despite a high degree of variability in the treatment group. Heat-killed cells did not cause a significant increase in IL-1β expression compared to control cells, with only a two-fold increase. ECP did not cause a significant increase in the expression of IL-1β, causing only a 2.3-fold or lower increase (Fig. 3A).

The mean relative expression of IL-8 in control cells was 2.1 × 10^{-2} ± 0.21 × 10^{-2}, 2.0 × 10^{-2} ± 0.34 × 10^{-2}, 1.0 × 10^{-2} ± 0.07 × 10^{-2} at 4, 24 and 72 h, respectively, with no significant differences. Significant differences on IL-8 expression were found for all variables: antigen and interaction (p = 0.000),
and sampling time \((p = 0.008)\). Both live and heat-killed cells caused increased expression of IL-8, showing 9.3- and 6.6-fold increased expression, respectively. The difference between stimulation with live and heat-killed cells was not significant.

**M. viscosa** ECP \((0.01 \text{ mg protein/ml})\) caused a significant 34.1-fold increase in IL-8 expression at 72 h, but not at 4 or 24 h. At a higher concentration \((0.05 \text{ mg protein/ml})\), ECP caused a significant increase in IL-8 expression from control cells at all sampling times \((5.8–19.1\text{-fold})\) (Fig. 3B), but there was not a significant difference between the three sampling times at that concentration.

### 3.5. Stimulation experiment 2; **M. viscosa** MvP1 vibriolysin

In experiment 2, the mean relative IL-1\(\beta\) expression in control cells was \(6.5 \times 10^{-4} \pm 1.9 \times 10^{-4} \), \(1.2 \times 10^{-3} \pm 0.29 \times 10^{-3} \), \(8.9 \times 10^{-4} \pm 0.88 \times 10^{-4} \) at 4, 24 and 72 h, respectively. There was no significant difference between the control values. Significant differences on IL-1\(\beta\) expression were found for all variables: antigen dose \((p = 0.007)\), sampling time \((p = 0.036)\) and interaction \((p = 0.004)\). The change in IL-1\(\beta\) expression of MvP1 stimulated cells ranged from 0.5 to 2.6-fold, and only cells stimulated with the highest concentration showed a significant increase in expression at 4 h (Fig. 4A).

The mean relative expression of IL-8 in control cells was \(8.0 \times 10^{-3} \pm 0.47 \times 10^{-3} \), \(7.4 \times 10^{-3} \pm 4.1 \times 10^{-3} \), \(1.2 \times 10^{-2} \pm 0.25 \times 10^{-2} \) at 4, 24 and 72 h, respectively, and did not show any significant differences. All variables were found to have a significant effect on IL-8 expression: antigen dose \((p = 0.000)\), sampling time \((p = 0.016)\) and interaction \((p = 0.001)\). The expression of IL-8 exhibited dose-dependence on the concentration of MvP1 at both 4 and 24 h, but was only significantly higher from control cells at the highest concentration \((1.0 \text{ mg protein/ml MvP1})\), or 4.9- and 9.4-fold, respectively. After stimulation for 24 h with 0.7 \text{ mg protein/ml} MvP1, two of the samples showed a 6.3- and 7.3-fold increase in IL-8 expression, while the third sample showed only a 2.3-fold increase in expression. Therefore, the data were not significantly different from control cells. No significant differences were detected at 72 h (Fig. 4B).

### 4. Discussion

In this study, the effects of **M. viscosa** antigens on pro-inflammatory gene expression in an Atlantic salmon macrophage-like cell line (SHK-1) were evaluated. The results show that live **M. viscosa** cells and MvP1 peptidase caused increased expression of IL-1\(\beta\), but heat-killed cells or ECP did not. However, all the antigens stimulated expression of IL-8. Furthermore, both live **M. viscosa** cells and ECP affected the SHK-1 cell viability.

EF-1A, which is an important part of the translational machinery in eukaryotes, was chosen as a reference gene in this study. EF-1A has previously been used successfully as reference gene in expression experiments in Atlantic salmon [20–22]. In our study, however, some significant differences in expression levels were observed. EF-1A expression was significantly higher at 4 h than at
24 or 72 h in cell stimulation experiment 2, but no significant differences were detected in experiment 1. In order to correct for the differences, the average EF-1A expression of both unstimulated and stimulated cells at each time point in each experiment was used to calculate the relative expression of IL-1β and IL-8. The increased expression of EF-1A in experiment 2 at 4 h could possibly be due to some short-term effects of handling of the cells or addition of medium. Also, EF-1A may have variable expression throughout the life cycle of SHK-1 cells, and that in experiment 2 sampling was performed at a time of increased expression. Further research into the temporal variation of EF-1A in SHK-1 cells would be valuable.

The cytotoxic effects of M. viscosa antigens on SHK-1 cells were evaluated, to determine their effect on cell viability during stimulation experiments. Live cells were highly cytotoxic, but heat-killed cells had no effect on cell viability. Since ECP isolated from M. viscosa were also highly toxic, it may suggest that at least some of the bacterial virulence is related to ECP secretion. However, our results show that only a small percentage of the live cells survived in the cell culture medium. Another possibility is that the heat treatment, during killing of M. viscosa, affected the ability of some bacterial antigens to cause cytotoxicity. Isolated MvP1 peptidase had no significant effect on cell viability. The peptidase was shown to be active at the experimental conditions, therefore, one or more unidentified components of the ECP are responsible for cell death. Microscopic examination of cells revealed that cell morphology was slightly affected by live cells, ECP, and MvP1 at concentrations that did not affect viability. Only heat-killed cells had no detectable effect on cell morphology. It has been shown previously that M. viscosa ECP are cytotoxic to EPC and BF-2 fish cell lines, and that MvP1 affects cell–cell adhesion in the same cells [5].

Based on cytotoxicity assay results, SHK-1 cells were stimulated with M. viscosa cells at MOI 2. Cells were stimulated for 24 h, but not for longer, as incubation with live cells at MOI of only 0.3 resulted in significant cell death at 72 h. At 24 h, live cells caused a significant increase in IL-1β expression, but heat-killed cells did not. Due to how highly cytotoxic the ECP were to SHK-1 cells at a concentration of 0.5 μg protein/ml, cells were subsequently only stimulated with a concentration of 0.05 or 0.01 μg protein/ml. At those concentrations the ECP did not affect IL-1β expression. Some cytotoxicity, although not significant, may have been associated with live cells at MOI 2. Subsequently these dead or dying SHK-1 cells may have acted as danger signals inducing IL-1β expression in the remaining culture. Interaction of M. viscosa with cellular membranes and/or continuous ECP production of live cells may also be reasons for the differences in IL-1β expression between SHK-1 cells stimulated with heat-killed and live cells. Yet another possibility is that gene expression was only stimulated through the synergistic effects of M. viscosa cells and ECP. The continuous production and release of ECP by live cells in culture with SHK-1 cells may have allowed a higher, or more stimulatory, concentration to be achieved with minimal cytotoxicity, whereas ECP at the concentrations tested on their own did not stimulate IL-1β expression. It should also be noted that the ECP used in the stimulation were from an exponential phase broth culture cultivated at 4 °C, whereas during the stimulation, M. viscosa cells were in contact with salmon cells at 20 °C. The possible factors that the cells may have produced during stimulation may thus have been different from the factors present in broth culture ECP. However, as previously discussed, the heat killing of bacterial cells may have destroyed a stimulatory antigen(s).

In this study, SHK-1 cells were stimulated at 20 °C, whereas M. viscosa usually causes infections at temperatures below 10 °C. Therefore, it is important to note that the cells can respond differently to the antigen stimulation at lower temperatures. However, the antigens were produced from 4 °C cultures, so that they would represent factors produced at those temperatures. Temperature has been shown to have a pronounced effect on expression levels of IL-1β in rainbow trout head kidney leucocytes, where expression at 22 °C was significantly higher than at both 4 and 14 °C, following LPS stimulation for 4 h [23].

Both live and heat-killed cells caused significant increases in IL-8 expression at 24 h. M. viscosa ECP caused a significant increase in IL-8 expression at 72 h in both concentrations, but also at 4 and 24 h in the higher concentration. Therefore, a lower concentration of ECP did not affect IL-8 expression as quickly as the higher concentration, which stimulated expression throughout the experiment.

In experiment 2, SHK-1 cells were stimulated with MvP1, an extracellular M. viscosa vibriolysin. MvP1 has virulence-related activities and may have a role in bacterial invasion and dispersion [5]. MvP1 did not affect SHK-1 cell viability, and could thus be used in much higher concentrations for stimulation than total ECP, which were highly cytotoxic. Therefore, cells were stimulated with up to 200-fold higher concentrations of isolated MvP1 than would be expected from the ECP in experiment 1. However, the concentrations are believed to be within biologically relevant limits, as MvP1 is a major component of in vitro produced ECP [5]. The relative production of MvP1 in vivo is, however, not known.

At the highest concentration, MvP1 stimulated IL-1β expression at 4 h, but not at any other concentrations or time points. Similarly, IL-8 expression increased significantly only after stimulation with the highest MvP1 concentration, both at 4 and 24 h. Thus, the increased expression of IL-8 seen after stimulation with ECP has not been caused by MvP1, or unknown factor or factors in the ECP have enhanced the stimulatory effects of MvP1. Also, the long-term
increase in IL-8 expression seen after ECP stimulation is not detected after MvP1 stimulation, where the expression increase is short-term. A previous study has shown that another vibriolysin, Vibrio cholerae, has not induce IL-8 expression or affect cell viability in human intestinal epithelial cells [24]. High variability was seen in IL-8 expression after 24 h stimulation with 0.7 µg protein/ml MvP1, where two samples showed a clear increase in expression, but one sample showed only a minor increase. This may indicate that during that time, cells were going through a shift in expression. This may also apply to other time points where gene expression was highly variable.

Several reports have been published on the effects of bacterial products on IL-1β expression in Atlantic salmon. IL-1β expression in SHK-1 cells has been examined in two previous studies by Fast et al. [6,7]. Both studies found a rapid increase in IL-1β expression following LPS stimulation for 4 h. In another study, Fast et al. [20] showed that LPS stimulation of head kidney macrophages isolated from Atlantic salmon increased IL-1β expression after 24 h, but not after 1 or 3 h. M. viscosa produces LOS [4], which presumably were present on both live and heat-killed cells. Therefore, since heat-killed cells did not stimulate IL-1β expression, the LOS do not seem to have affected IL-1β expression under the assay conditions. A multivalent salmon vaccine, containing an oil adjuvant and selected bacterial antigens, including M. viscosa, has also been shown to stimulate increased IL-1β expression in head kidney of i.p. and intramuscularly (i.m.) vaccinated Atlantic salmon at 24 h post injection, but not at 4 or 6 h [25]. Our results of increased IL-1β expression at 24 h following stimulation with live cells, and at 4 h following stimulation with MvP1 are in agreement with the previous studies, where the changes in expression seem to be rapid and short-term.

To our knowledge, the expression of IL-8 in Atlantic salmon in response to bacterial antigens has not been reported previously. IL-8 expression has been shown to be up-regulated in rainbow trout following bacterial infections [26], and also in pink salmon (Onco- rhynchus gorbuscha (Walbaum)) and chum salmon (Oncorhyncus keta (Walbaum)) following vaccination against A. salmonicida [27]. Increased expression of pro-inflammatory genes thus seems to be a common response in salmonids to bacteria, as in higher vertebrates. Cytokines usually have short half-lives in vivo and are highly regulated, both at the transcriptional and the post-transcriptional levels. Therefore, their increase following infection or tissue damage is usually only detectable over a limited time. High and prolonged expression of pro-inflammatory cytokines can lead to local tissue damage. Following stimulation with 0.05 µg protein/ml ECP, IL-8 expression was up-regulated for up to 72 h. Therefore, the pathology observed in fish suffering from winter ulcer disease may possibly be associated with the host’s prolonged inflammatory reaction to ECP, and not only with direct effects of the bacterium.

The results of the study show that M. viscosa antigens have different stimulatory effects on pro-inflammatory gene expression in SHK-1 cells. It would be of interest to examine further the differences between the ability of live and heat-killed cells in inducing IL-1β expression, and the possible importance for vaccine production. Further studies on finding the main inflammatory-stimulating factors in ECP would also be of interest.

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