Fish & Shellfish Immunology 31 (2011) 993–1004

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Intracellular localisation and innate immune responses following *Francisella noatunensis* infection of Atlantic cod (*Gadus morhua*) macrophages

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ARTICLE INFO

Article history:
Received 15 April 2011
Received in revised form
19 August 2011
Accepted 20 August 2011
Available online 28 August 2011

Keywords:
Innate immune response
Francisella noatunensis
Macrophages
Intracellular infection
LPS

ABSTRACT

The facultative intracellular bacterium *Francisella noatunensis* causes francisellosis in Atlantic cod (*Gadus morhua*), but little is known about its survival strategies or how these bacteria evade the host immune response. In this study we show intracellular localisation of *F. noatunensis* in cod macrophages using indirect immunofluorescence techniques and green fluorescent labelled bacteria. Transmission electron microscopy revealed that *F. noatunensis* was enclosed by a phagosomal membrane during the initial phase of infection. Bacteria were at a later stage of the infection found in large electron-lucent zones, apparently surrounded by a partially intact or disintegrated membrane. Immune electron microscopy demonstrated the release of bacterial derived vesicles from intracellular *F. noatunensis*, an event suspected of promoting phagosomal membrane degradation and allowing escape of the bacteria to cytoplasm.

Studies of macrophages infected with *F. noatunensis* demonstrated a weak activation of the inflammatory response genes as measured by increased expression of the Interleukin (IL)-1β and IL-8. In comparison, a stronger induction of gene expression was found for the anti-inflammatory IL-10 indicating that the bacterium exhibits a role in down-regulating the inflammatory response. Expression of the p40 subunit of IL-12/IL-17 genes was highly induced during infection suggesting that *F. noatunensis* promotes T cell polarisation. The host macrophage responses studied here showed low ability to distinguish between live and inactivated bacteria, although other types of responses could be of importance for such discriminations. The immunoreactivity of *F. noatunensis* lipopolysaccharide (LPS) was very modest, in contrast to the strong capacity of *Escherichia coli* LPS to induce inflammatory responsive genes. These results suggest that *F. noatunensis* virulence mechanisms cover many strategies for intracellular survival in cod macrophages.

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1. Introduction

Francisellosis caused by *Francisella noatunensis* (previously termed *Francisella piscicida* and *Francisella philomiragia* subsp. *noatunensis*) is a severe systemic granulomatous inflammatory disease in Atlantic cod [1,2]. The severity and prevalence of the disease has increased in Norway since its first occurrence in 2004 and today represents one of the biggest threats to the Norwegian cod farming industry [3,4]. Infected fish have high levels of granulomas in visceral organs especially in the haematopoietic organs (head kidney and spleen), but they are also frequently found in heart and liver [1]. The bacterium has been characterised as a Gram-negative facultative intracellular bacteria mainly based on its taxonomy and growth characteristics [1,2,5]. Histopathological studies and confocal microscopy have located *F. noatunensis* in or around phagocytic cells associated with epithelial cells in cod [1,2,6].

The bacterium has cocco-bacillus morphology with a heterogeneous size ranging from 300 to 700 nm and belongs to the genus *Francisella* [1,2]. Many studies have described the intracellular lifestyle of the highly contagious human pathogen *Francisella*

Abbreviations: anti-FN, rabbit anti-*F. noatunensis* serum.

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doi:10.1016/j.fsi.2011.08.020

tularensis and how it efficiently evades the host immune response (reviewed in [7,8]). This bacterium infects various mammalian cell types, among which macrophages constitute a survival and replication niche essential to its virulence. Initially, *F. tularensis* enters the host cell through a process termed "looping" phagocytosis and resides in a tight membrane enclosed vacuole [9]. Shortly after infection (2–4 h), the bacterium avoids the usual degradation in the phagolysomal pathway by escaping to the cytoplasm where replication takes place (4–20 h) [10,11]. Following replication in cytoplasm, *F. tularensis* has a post-replication stage which involves re-entering the endocytic compartment through an autophagy mediated process and known to reside in large fusogenic vacuoles [12].

Intracellular bacteria have created a favourable niche for replication inside macrophages, an apparent paradox since the primary role of macrophages is to provide the first line of host defence by recognising bacteria and performing phagocytosis [13]. This recognition normally facilitates internalisation of the bacteria and fusion to lysosomes where bacteria are degraded by proteolytic enzymes and antimicrobial peptides. The innate immune system senses invasion of bacteria through recognition of characteristic pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) and the more recently defined intracellular nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) (reviewed in [14,15]). These PAMPs, include amongst others, formylated bacterial peptides, LPS, lipoteichoic acid, peptidoglycans, flagellin and CpG motifs. In mammals are LPS the major component of the outer membrane of Gram-negative bacteria recognised by TLR4, which usually provides a strong innate immune response [14,15]. However, the role of TLR4 in fish has been questioned since the gene encoding the receptor is absent in the genomes of spotted green pufferfish (*Tetraodon nigroviridis*), pufferfish (*Fugu rubripes*) [16] and cod [17]. In contrast, TLR4 has been identified in zebrafish (*Danio rerio*) [18], but was not found to be a receptor for LPS [19,20].

The innate immune response is a fine-tuned release of cytokines to detect the presence of infection and provide the first line of host defence [14,15]. The inflammatory response generated by IL-1β, IL-6 and IL-8 is tightly regulated, whereas IL-10 has a key function in suppressing this response in mammals [21–23]. We have previously characterised IL-1β, IL-8 and IL-10 and indicated an anti-inflammatory role for IL-10 in cod [24]. During the past decades it has become evident that innate immunity has an essential role, not only in repelling an infection, but also in directing the adaptive immune response. Cell-mediated immunity has been assigned a crucial function in the eradication of intracellular infections and the different T cells play distinct and complementary roles in protective immunity. Interferon (IFN) γ and IL-12 have an important function in the differentiation of CD4 positive cells into T_H1 cells, while the pleiotrophic IL-12 also primes CD8 T cell proliferation and development of cytotoxic function [25,26]. Both the IFNγ gene and the IL-12p40 subunit have been identified in cod [27,28]. In mammals, the IL-12p40 subunit is shared by the heterodimeric IL-12 and IL-23, but has quite different roles by priming CD4 positive cells into T_H1 and T_H17, respectively [29]. The latter cell type is usually associated with autoimmune diseases, but recent results show that the IL-23-T_H17 pathway is critical for optimal induction of T_H1 cell responses and protection against *F. tularensis* live vaccine strain [30].

Intracellular bacteria have developed several strategies to escape the immune defence and by different mechanisms replicate inside host cells. The survival strategies of intracellular bacteria infecting fish are relatively unknown and few have reported the cellular host response in infected cells. The aim of this study was to confirm *F. noatunensis* as an intracellular bacterium and determine its localisation within macrophages. Gene expression studies were performed to examine cod macrophages response to *F. noatunensis* infection and whether live and inactivated bacteria were discriminated. Further, it was of interest to study the immunoreactivity of LPS isolated from *F. noatunensis* in comparison to *Escherichia coli* LPS.

2. Material and methods

2.1. Isolation of head kidney derived macrophages

Atlantic cod (approx. 400–800 g) were obtained from the Aquaculture Research Station (Tromsø, Norway). The fish were kept in 900 L circular, centrally drained, fibreglass tanks in seawater (3.4%) at natural seawater temperature and fed *ad libitum* with amber neptun 100 or 300 (Skretting, Cambridge, Tasmania, Australia). Head kidney derived macrophages were isolated based on previously described protocols [31,32] with some modifications. Fish were rapidly killed by cranial concussion and blood was removed by bleeding the fish from the *Vena caudalis*. Head kidneys were aseptically removed and transferred to L-15++ (L-15 (Gibco, Invitrogen, Carlsbad, CA, USA or PAA Laboratories, GmbH, Parsching, Germany) supplemented with 25 mM HEPES, 2 mM L-glutamine, 20.5 mM NaCl, 1.8 mM glucose, 4.2 mM $NaHCO_3$, 20 U/ml penicillin and 20 μg/ml streptomycin) with 10 U/ml heparin (LEO Pharma AS, Oslo, Norway). Head kidneys were minced through a 100 μm nylon Falcon cell strainer (BD Bioscience, Bedford, MA, USA) and diluted in 30 ml L-15++ (with heparin). The cell suspensions were loaded on discontinuous 34%/49% Percol (Amersham Pharmacia Biotec, Uppsala, Sweden) gradients and separated by centrifugation at 350 × *g* for 40 min at 4 °C. The interphase containing purified macrophages was washed twice in 50 ml L-15++ followed by centrifugation at 300 × *g* for 10 min at 4 °C. In the last washing step, cells were diluted in L-15++ with 0.1% Gold foetal calf serum (FCS; PAA). Cells were seeded at an equal density for all studies; 5–8 × 10^6 cells per well in 24-well culture plates (Nunc, Roskilde, Denmark), 2.1 × 10^4 cells per well for μ-slide IV (Ibidi, Munich, Germany) and 2.5 × 10^6 cells per SlideFlasks (Nunc).

2.2. Phagocytosis study

The phagocytic capacity of macrophages was studied *in vitro* using *E. coli* conjugated fluorescent beads (pHrodo™ *E. coli* BioParticles® Conjugate; Molecular Probes, Inc., Eugene, OR, USA). Macrophages were seeded in μ-slide IV (Ibidi), incubated for 16 h and washed two times with L-15+ to remove non-adherent cells. Then, macrophages were incubated (0.5, 1, 2 and 4 h) with *E. coli* conjugated beads diluted in L-15+ with 2% FCS. To remove non-ingested beads, cells were washed 6 times with L-15+ and subsequently fixed for 10 min in 4% paraformaldehyde (PFA) followed by washing 2 times with Phosphate buffered saline (PBS). The slides were mounted in ProLong Gold antifade reagent with 4′, 6-diamidino-2-phenylindole (DAPI; Molecular probes). A Zeiss Axio Observer Z1 (Zeiss, Munich, Germany) was used for capturing fluorescence images of the preparations.

2.3. Transformation of F. noatunensis with a plasmid encoding green fluorescent protein

In order to study the intracellular localisation of *F. noatunensis* in cod macrophages, *F. noatunensis* strain NCIMB 14265 isolated from farmed cod [5] were transformed using the pKK289 plasmid encoding green fluorescent protein (GFP) [33,34]. The *F. noatunensis* strain was maintained on CHAB plates [35] at 20 °C, while the *E. coli*

HB101 strain [36] used for plasmid maintenance and propagation was grown at 37 °C in Luria Bertani medium [37]. Liquid culture medium for *F. noatunensis* consisted of Bacto Eugon broth (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 2 mM $FeCl_3$ (EB–Fe).

The antibiotics used for selection of transformants were 50 μg/ml kanamycin for *E. coli*, 25 μg/ml kanamycin and 75 μg/ml polymyxin for *F. noatunensis*. Plasmids were introduced into *F. noatunensis* by electroporation as previously described [38] with some modifications. Briefly, a pre-culture of *F. noatunensis* was grown for 48 h in EB–Fe at 21 °C with slow shaking, then 5 ml was subcultured in 50 ml EB–Fe and grown to $OD_{540nm} = 2.0$. Bacterial cells were harvested by centrifugation at 12,000 × *g* for 20 min at 4 °C. The cell pellet was washed twice with ice cold 0.5 M sucrose and resuspended in 1 ml 0.5 M sucrose. For electroporation 1 μg of plasmid DNA was mixed with 200 μl of electro competent cells, incubated at room temperature for 10 min and electroporated in a 0.2 cm cuvette (2.5 kV, 25 μF, and 600 Ω). Immediately after electroporation, the cells were resuspended in 1 ml of EB–Fe and incubated for 4 h at 21 °C with slow shaking before transformants were selected on CHAB plates containing the appropriate antibiotics.

The GFP-expressing strain of *F. noatunensis* was constructed by electro transformation with plasmid pKK289:*gfp* [34] resulting in strain HWL108. Verification of the presence of GFP plasmids in the HWL108 strain was confirmed by PCR using *gfp* specific primers FGFP289 (5′-TTGAAGGTGATGCCCTTGTT-3′) and RGFP289 (5′-AAAGGGCAGATTGTGTGGAC-3′). The expression of GFP was verified microscopically using an Olympus IX81 inverted microscope (Olympus, Hamburg, Germany). Analysis of plasmid stability was tested by growing strain HWL108 exponentially under antibiotic selection in EB–Fe at 21 °C. Cultures were then sequentially diluted and grown overnight in non-selective media. After each dilution the presence of the plasmid and expression of GFP was verified by PCR and microscopic analysis. Plasmid stability of GFP-expressing *F. noatunensis* was studied over several generations without any significant plasmid loss.

2.4. Infection of macrophages with GFP-expressing F. noatunensis

Macrophages were seeded in μ-slide IV (Ibidi), incubated for 40 h and washed twice with L-15+ (L-15++ without antibiotics) to remove non-adherent cells and antibiotics. The GFP-expressing strain stored at −80 °C in freeze medium (PHARMAQ AS, Oslo, Norway) was grown at 20 °C in nutrition broth (PHARMAQ AS) to $OD_{600nm} = 1$. The number of colony forming units (cfu) was determined by spreading tenfold dilutions on CHAB agar plates [35]. Growth of pure colonies was counted after 7 days. Colonies were easily grown and clearly visible colonies were easy to count macroscopically. Macrophages were infected for 3 h with 50 moi (multiplicity of infection) GFP-expressing *F. noatunensis* diluted in L-15+ with 2% FCS. Negative controls were macrophages with only L-15+ added. Subsequently cells were washed 3 times with L-15+ and treated for 30 min with 50 μg/ml gentamicin (Sigma–Aldrich, Saint Louis, MO, USA) followed by three additional washing steps. Cell membranes were stained for 10 min with 1 μg/ml Alexa wheat germ agglutinin 555 (Molecular probes), followed by 3 washes in Hank's Buffered Salt Solution (HBBS) and fixed for 10 min in 4% PFA. The slides were washed 3 times in HBBS and mounted in ProLong Gold antifade reagent with DAPI (Molecular probes). Microscopy was carried out on a Zeiss Axiovision Z1 equipped for structured illumination (Apotome). Cellular localisation in macrophages of *F. noatunensis* expressing GPF was addressed by studying a 3D model created from a z-stack (Zeiss Axiovision).

2.5. Indirect immunofluorescence studies of macrophages infected with F. noatunensis

Intracellular infection was studied by infecting macrophages with *F. noatunensis* strain AL-1102 (PHARMAQ AS) originally isolated from cod showing clinical signs of francisellosis in Norway. The strain was characterised by the Norwegian Veterinary Institute (Oslo, Norway) using established methods such as growth characteristics [1] and PCR [3]. The bacterial isolate was propagated as described above. Macrophages were seeded on glass cover slips in a 24-well cell culture dish (Nunc), incubated for 16 h and washed twice with L-15+. Cells were incubated for 24 h with 1 ml of *F. noatunensis* ($OD_{600nm} = 1$) diluted 1:10 in L-15+ supplemented with 2% FCS. Negative controls were macrophages with only L-15+ added. Following infection, cells were washed 3 times with L-15+, treated with 50 μg/ml gentamicin (Sigma–Aldrich) for 1 h and washed 3 times. Cells were fixed in methanol: acetone (1:1) for 5–10 min and washed once in PBS. Fixed cells were incubated for 20 min with diluted (1:500) polyclonal rabbit anti-*F. noatunensis* serum (anti-FN; PHARMAQ AS) and washed in PBS (3× 3 min). The cover slips were stained with a 1:50 dilution of TRITC 550 $F(ab')_2$ fragment, swine anti-rabbit IgG (DakoCytomation, Glostrup, Denmark) for 20 min. The cover slips were washed once in PBS, stained with 0.2 mg/ml DAPI (Sigma–Aldrich) and mounted in Fluorescent mounting medium (DakoCytomation). Negative controls were stained using the same procedure. Preparations were studied in a fluorescent microscope (Zeiss Axiovision).

2.6. Electron microscopy of macrophages infected with F. noatunensis

Macrophages were seeded in SlideFlask (Nunc), incubated for 16 h and washed 2 times with L-15+. Cells were infected with 50 moi *F. noatunensis* diluted in L-15+ with 2% FCS for 3 h, while control macrophages received only L-15+. Following infection, the cells were washed once in L-15+ and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight. Cells were washed in 0.1% cacodylate buffer (pH 7.2), post fixed in mixture of 2% osmium tetroxide/1.5% potassium ferrocyanide [39] in 0.1% cacodylate for 30 min. Staining was performed with 1% tannic acid and 1% uranyl acetate, followed by dehydration in a graded series of ethanol (70%, 90%, 96%, 2 × 100%). The cells were treated with acetonitrile as an intermediate step before infiltration with an Epon substitute (AGAR 100 resin, Agar Scientific, Stansted, England) and polymerised at 60 °C overnight. Ultrathin sections (70 nm) were made using a Leica Ultracut S Ultramicrotome (Vienna, Austria) with a Diatome diamond knife (Biel, Switzerland). The sections were mounted on carbon coated formvar films on copper grids and contrasted with 5% uranyl acetate for 8 min and Reynolds lead citrate [40] for 5 min. Micrographs were taken on a Jeol 1010 JSM (Tokyo, Japan) with a Morada 11 Mpixels digital camera (Olympus).

2.7. Immune electron microscopy of macrophages infected with F. noatunensis

Macrophages grown on 0.2 mm cell culture cover slips (Nunc) were infected and washed as described above. Negative controls were macrophages with only L-15+ added. Infected and control macrophages were fixed in 4% PFA. Cultured *F. noatunensis* ($OD_{600nm} = 1$) grown in broth medium (PHARMAQ AS) were centrifuged, washed once in PBS and fixed in 4% PFA. Further processing: the specimens were moved to a Leica EM AFS2 Freeze Substitution Unit (Vienna, Austria) were a Progressive Lowering of Temperature (PLT) procedure [41] was performed. Briefly, 30% ethanol at 0 to −15 °C for 1 h, 50% ethanol at −15 to −30 °C for 1 h,

75% ethanol at −30 °C for 1 h, three changes of 100% ethanol at −50 °C, followed by infiltration at −50 °C with increasing concentrations of Lowicryl HM20 (Electron Microscopy Sciences, Hatfield, PA, USA). Polymerisation was performed in UV light starting at −50 °C and ending up at room temperature for 48 h. Ultrathin sections (70 nm) were made using a Leica EM UC6 Ultramicrotome (Vienna, Austria) with a Diatome diamond knife (Biel, Switzerland) and mounted on formvar coated copper grids. Immunolabelling was performed as previously described [42]. In short, blocking of unspecific labelling with 1% fish skin gelatine (FSG; Sigma–Aldrich), incubation with 1:3000 anti-FN (diluted in 1% FSG), washed in PBS and incubated in a dilution 1:60 of Protein A-bound gold particles (10 nm in diameter; University of Utrecht, the Netherlands). As a negative control for the anti-FN specificity, parallel sections were incubated with 1% FSG without antibody, followed by Protein A-gold. These sections showed no labelling on corresponding structures labelled with anti-FN. After washing in PBS and distilled water, the sections were contrasted in 5% uranyl acetate for 3 min and Reynolds lead citrate for 1 min. Micrographs were taken with TEM electron microscope as described above.

2.8. Isolation of *F. noatunensis* LPS

A suspension of formalin inactivated *F. noatunensis* (AL-1102) in PBS (180 ml, $OD_{600nm} = 6.5$) was dialysed for 4 days against deionised water and lyophilised. The lyophilised cells (2 g) were suspended in 200 ml Tris–HCl buffer (50 mM, pH 7.5) and treated simultaneously for 16 h at 37 °C with RNase and DNase I, both from bovine pancreas (Boehringer Ingelheim, Nové Mésto, Czech Republic). The cells were then treated with trypsin (Serva, Prague, Czech Republic) at 37 °C for 90 min followed by proteinase K (*Tritirachium album*, Sigma–Aldrich). After enzyme treatment, the cell suspension was centrifuged (14,000 × *g*, 50 min, 10 °C) and the sediment was washed with acetone. To remove phospholipids, the dried cells were extracted with chloroform–methanol (2:1, v/v) overnight at 20 °C. The extraction was repeated with fresh solvent mixture for 2 h. The cell suspension was centrifuged at 3000 × *g* for 20 min and the sediment was suspended in 200 ml preheated (68 °C) deionised water and extracted with an equal volume of aqueous 90% phenol as previously described [43]. The yield of crude LPS was 19.2 mg (0.96%) calculated on the basis of weight of lyophilised *F. noatunensis* cells. The polymer was again treated sequentially with RNase, DNase I and proteinase K as described above and the enzyme-treated material was subjected to repeated (3×) ultracentrifugations (120,000 × *g*, 4 h, 4 °C). The precipitated gels were dissolved in water and lyophilised to yield 11 mg of LPS. The protein content in the purified LPS was estimated colorimetrically to 1% as previously described [44]. The LPS was slowly dissolved in nuclease free water (Ambion, Austin, TX, USA), homogeneously dispersed using magnetic stirrer and sonication. The LPS were further examined by SDS–PAGE electrophoresis using the X-cell SureLock electrophoresis system (Invitrogen). The samples (40 μg) were boiled for 5 min prior to separation in a pre-cast 12% Bis-Tris gel premixed running buffer and LDS sample buffer with reducing agent according to the manufactures protocol (ClearPage; CBS Scientific, Del Mar, CA, USA). The pre-stained molecular marker (Two color SDS marker, CBS Scientific) was used and the gel was silver stained using Focus Fast Silver staining (G-Bioscience, St Louis, MO, USA). The *F. noatunensis* LPS were pure and only a single band was visible with molecular weight of approx. 22 kDa.

2.9. Gene expression studies following infection and stimulation

Macrophages were seeded in 24-well cell culture plates (Nunc) and treated with LPS or infected with *F. noatunensis* AL-1102. Cells were treated with 20 μg/ml *F. noatunensis* LPS or *E. coli* LPS (026:B6; Sigma–Aldrich) diluted in L-15+ with 2% FCS. Cells were exposed to 100 moi live or formalin inactivated bacteria diluted in L-15+ with 2% FCS. The cell culture plates were centrifuged at 500 × *g* for 5 min to enhance the initial contact between bacteria and cells. After 2 h of infection were cells washed 3 times with L-15+, treated with 50 μg/ml gentamicin, followed by 3 washes in L-15+. As control, culture medium alone was added to the cells. After incubation for sequential time points (3, 6, 12, 24, 48 and 72 h) cells were harvested by removing medium and adding 1× lysis buffer (Applied Biosystems, Foster City, CA, USA) to the cells.

A dose response study of macrophages subjected to different concentrations (20, 50, 100 and 200 μg/ml) of *F. noatunensis* LPS was performed similarly as described above. After incubation for 6 h cells were harvested by adding 1× lysis buffer (Applied Biosystems).

Total RNA was isolated using the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems) with the recommended on-column DNase treatment. Reverse transcription was performed in a total reaction volume of 20 μl with 150 ng RNA using High Capacity RNA-to-cDNA master mix (Applied Biosystems) and 2.5 μM poly dT primer (Promega, Madison, WI, USA). The parameters were as follows; 25 °C for 5 min, 42 °C for 60 min and 85 °C for 5 min. The cDNA was diluted three times in nuclease free water (Ambion) for further use in quantitative real time PCR. The absence of genomic DNA in the RNA (non-template control) was verified by a selection of samples being subjected to real time PCR without prior cDNA synthesis.

Real time PCR was performed in duplicates in 384 well plates using the 7900HT Fast Real-Time PCR System and Power SYBR Green PCR Master Mix as recommended by the manufacturer (Applied Biosystems). Real time primers for target genes (IL-1β, IL-6, IL-8, IL-10 and IL-12p40) and endogenous control (Elongation factor 1α, eF1a) are listed in Table 1. Primers for the genes IL-1β,

Table 1
Real time PCR primers used in this study.

Name	Primer name	Sequence (5′–3′)	PCR efficiency	r^2
Interleukin-1β [24]	IL1β-658F	GGAGAACACGGACGACCTGA	98.8%	0.998
	IL1β-708R	CGCACCATGTCACTGTCCTT		
Interleukin-6 [JF309111][a]	IL6-80F	TGAAGAAGGAGTACCCCGACAAT	97.1%	0.994
	IL6-172R	GGTGCCTCATCTTTTCCTCAATG		
Interleukin-8 [24]	IL8-446F	GGTTTGTTCAATGATGGGCTGTT	96.8%	0.995
	IL8-516R	GACCTTGCCTCCTCATGGTAATACT		
Interleukin-10 [24]	IL10-618F	CCTATAAAGCCATCGGCGAGTTA	100%	1.000
	IL10-693R	TGAAGTCGTCGTTTTGAACCAAG		
Interleukin-12 [28]	IL12p40-1F	AGCGAGACTTCATTCTGGAGGA	91.0%	0.995
	IL12p40-103R	GCCATGGTTGCATTCACCTT		
Elongation factor 1α [24]	eF1α-148F	ATGTGAGCCGGTGTGGCAATC	96.4%	1.000
	eF1α-220R	TCATCATCCTGAACCACCCTG		

[a] GenBank accession no.

IL-8, IL-10, IL-12p40 and eF1a has been reported previously [24,28,31]. IL-6 was identified in a normalised cDNA library [24] and was submitted to GenBank with accession no JF309111.

All gene expression data were analysed with SDS 2.3 software (Applied Biosystems) and exported to Microsoft Excel for further analysis. The efficiency of the PCR reactions was close to 100%, determined by analysis of 2-fold dilutions of cDNA (Table 1) allowing the use of the $2^{-\Delta\Delta CT}$ method for calculation of relative gene expression levels [45]. The non-stimulated control (medium only) for each time point served as calibrator. The mean quantity $\pm$ standard error of mean (SEM) was calculated from relative quantification values obtained from 3 individual fish. Statistical analyses between groups were made with a paired two-tailed Student *t*-test and $P < 0.05$ was considered significant. The stimulation and infection experiments were repeated three times with similar results.

3. Results

3.1. The phagocytic capacity of macrophages

We have studied whether macrophages isolated from cod head kidney could be a host to infection with the intracellular bacterium *F. noatunensis*. Initially, the ability of macrophages to maintain their phagocytic capacity *in vitro* was tested using inactivated *E. coli* cells conjugated to fluorescent beads. These commercially available *E. coli* beads stain red after ingestion into the acidic environment inside phagosomes, while no or low fluorescence should be detected outside cells. Fluorescence microscopy revealed a clear red colour surrounding the blue DAPI stained macrophage nuclei thereby demonstrating that many cells had ingested *E. coli* bead conjugates (Fig. 1). The level of ingestion increased during prolonged co-incubation (0.5, 1, 2 and 4 h), which strongly indicates time dependent phagocytosis in the isolated macrophages. Thus, cod macrophages have maintained the ability to perform phagocytosis of bacteria in culture conditions after isolation from head kidney.

3.2. Invasion of macrophages with GFP-expressing F. noatunensis

After establishing that cod macrophages maintained their phagocytic capacity *in vitro*, macrophages were co-incubated with *F. noatunensis* to elucidate whether live bacteria could be found intracellularly. To determine intracellular localisation of *F. noatunensis* we constructed a bacterial strain constitutively expressing a plasmid-contained GFP. Prior to this work, no biological tools for genetic manipulations or transformation of *F. noatunensis* strains have been reported. This strain was constructed by electro transformation of the plasmid pKK289:*gfp* [33] into *F. noatunensis* NCIMB 14265. Several other transformation procedures previously described as functional for *F. tularensis* were also tried. Conjugation [46] resulted in transformants of *F. noatunensis* using the same plasmid derivate, while cryotransformation [47] was not successful. The use of broad host range plasmids such as pJT19 [48] or pCNB1 [49] did not result in electotransformants or transconjugants, which indicates that *F. noatunensis* is akin to *F. tularensis* in the ability to discriminate foreign DNA.

Macrophages were exposed to the *F. noatunensis* GFP-expressing strain for 3 h, followed by removal of extracellular bacteria. Cell membranes were visualised with wheat germ agglutinin (WGA) to obtain a clear outline of the cell membranes, while the nuclei were stained with DAPI. Macrophage preparations with GFP-expressing *F. noatunensis* were visualised using fluorescent microscopy and by creating a transparency 3D model of a cell containing

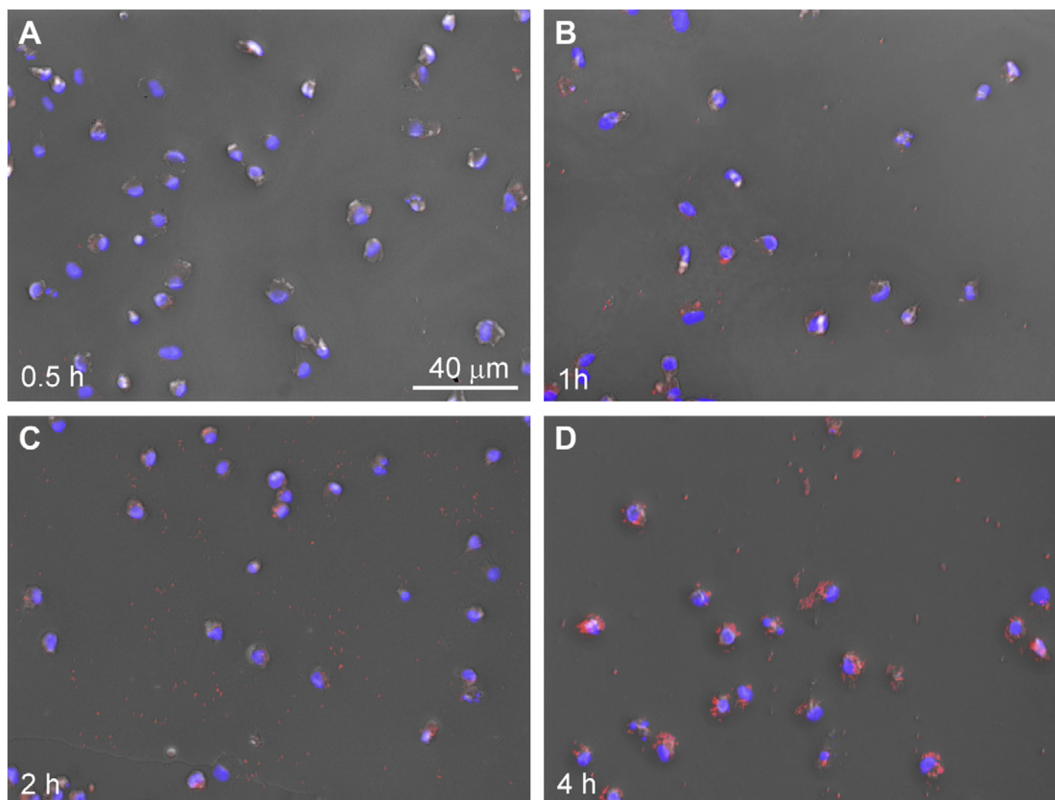

Fig. 1. Time course study of phagocytosis of inactivated *E. coli* by Atlantic cod macrophages. Macrophages were co-incubated with *E. coli* conjugated fluorescent beads (red) for A) 0.5 h, B) 1 h, C) 2 h and D) 4 h. Preparations were stained with DAPI (blue) and visualised by fluorescence microscopy. Fluorescent images are superimposed on a DIC image.

a fluorescent bacteria. Image analysis of the macrophages visualised intracellular localisation of *F. noatunensis*, as shown by the inset in Fig. 2.

3.3. Studies of macrophages infected with F. noatunensis

Immunofluorescence studies with anti-*F. noatunensis* serum (anti-FN) was used to visualise intracellular bacteria in macrophages 24 h after infection. The majority of bacteria were found associated with macrophages and few bacteria were located separated from cells (Fig. 3). The bacteria were distributed differently in adjacent cells indicating different stages of the intracellular infection. In some cells, bacteria were tightly clustered, whereas in others they had accumulated in apparent large vacuolar compartments (Fig. 3). These observations show that bacteria were able to persist intracellularly for at least 24 h indicating that cod macrophages were not able to eradicate bacteria during the studied time frame.

3.4. Intracellular localisation of F. noatunensis in macrophages using electron microscopy

Transmission electron microscopy was performed following 3 h of infection to study the intracellular localisation of *F. noatunensis* in cod macrophages. After internalisation, bacteria could be found inside intact membrane enclosed vacuoles (Fig. 4). The bacteria were separated from the phagosomal membrane only by a narrow electron-lucent space. Internalised bacteria exhibited an intact morphology compared to extracellular bacteria (results not shown). The same macrophage also contained a larger area of electron-lucent space apparently containing two bacteria without a clear surrounding membrane or possibly a partially disintegrated membrane. In close proximity to the intracellular bacteria small amorphous vesicular bodies could be observed (Fig. 4). These results show that *F. noatunensis* could be found in macrophages enclosed in vacuoles, but might also reside surrounded by apparently incomplete vacuole membranes.

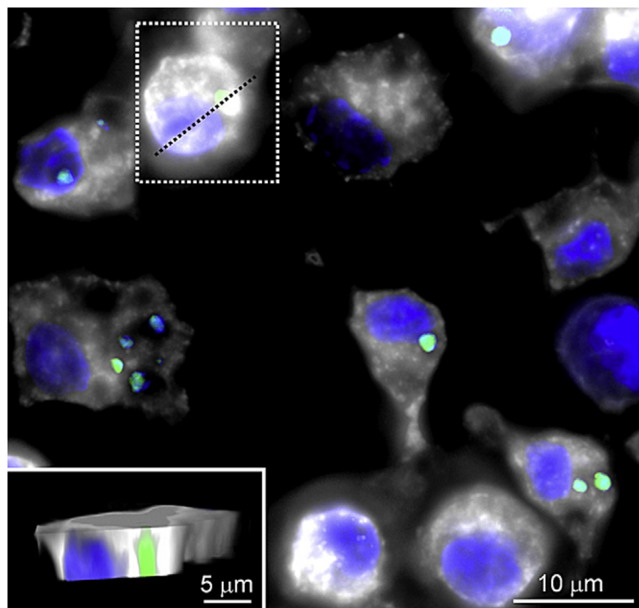

Fig. 2. Atlantic cod macrophages infected with GFP-expressing *F. noatunensis* for 3 h. Macrophages were co-incubated with GFP-expressing *F. noatunensis* (pseudocoloured green) and subjected to fluorescence microscopy studies. Macrophage membranes were stained with WGA (pseudocoloured white) and nuclei by DAPI (blue). The cell in the stippled box is shown in a transparency 3D model in the lower left corner with GFP-expressing *F. noatunensis* (green) inside the macrophage.

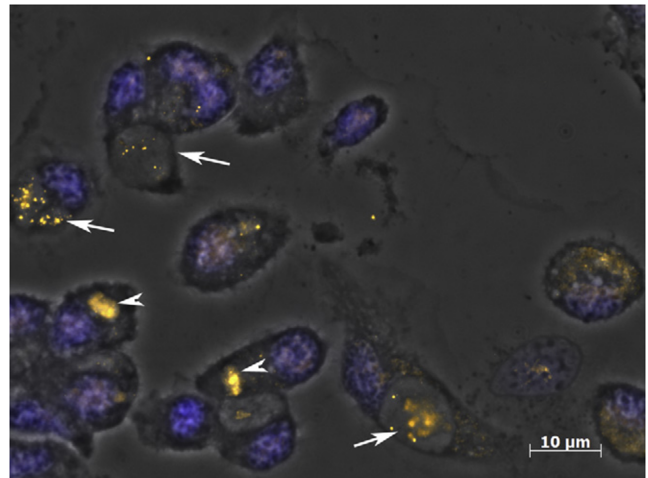

Fig. 3. Atlantic cod macrophages infected with *F. noatunensis* for 24 h. Macrophages were infected with *F. noatunensis* and subjected to indirect immunofluorescence studies using anti-*F. noatunensis* serum (anti-FN; yellow by TRITC 550). Nuclei were stained with DAPI (blue). Preparations were visualised microscopically by merging fluorescence and phase contrast images. Bacteria were apparently either evenly distributed in large vacuoles (arrows) or clustered tightly (arrowheads) in macrophages.

3.5. Intracellular localisation of F. noatunensis in macrophages using immune electron microscopy

Further verification of the intracellular localisation of *F. noatunensis* was performed using immune electron microscopy with the same antiserum (anti-FN) as used in the immunofluorescence studies. Immune electron microscopy was initially carried out on *F. noatunensis* grown in broth to identify the localisation of the antigens recognised by the antiserum. A highly specific electron dense gold labelling of extracellular bacteria was observed with an apparently immunoreactive material along the outer perimeter of the bacteria (approx. 100 nm; Fig. 5A).

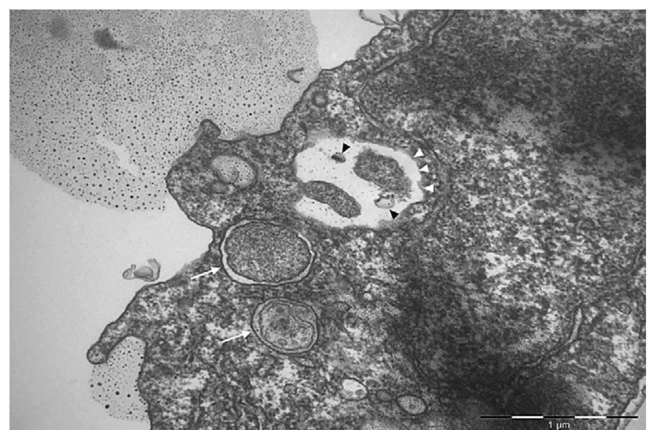

Fig. 4. Intracellular localisation of *F. noatunensis* in Atlantic cod macrophages. Macrophages were infected with *F. noatunensis* for 3 h and subjected to transmission electron microscopy. The macrophage contains two tight membrane enclosed vacuoles each containing one bacterium (arrows). There is also a large electron-lucent area containing two bacterial structures without an apparent intact membrane (white arrowheads). In close proximity to the bacteria small vesicular bodies could be observed (black arrowheads).

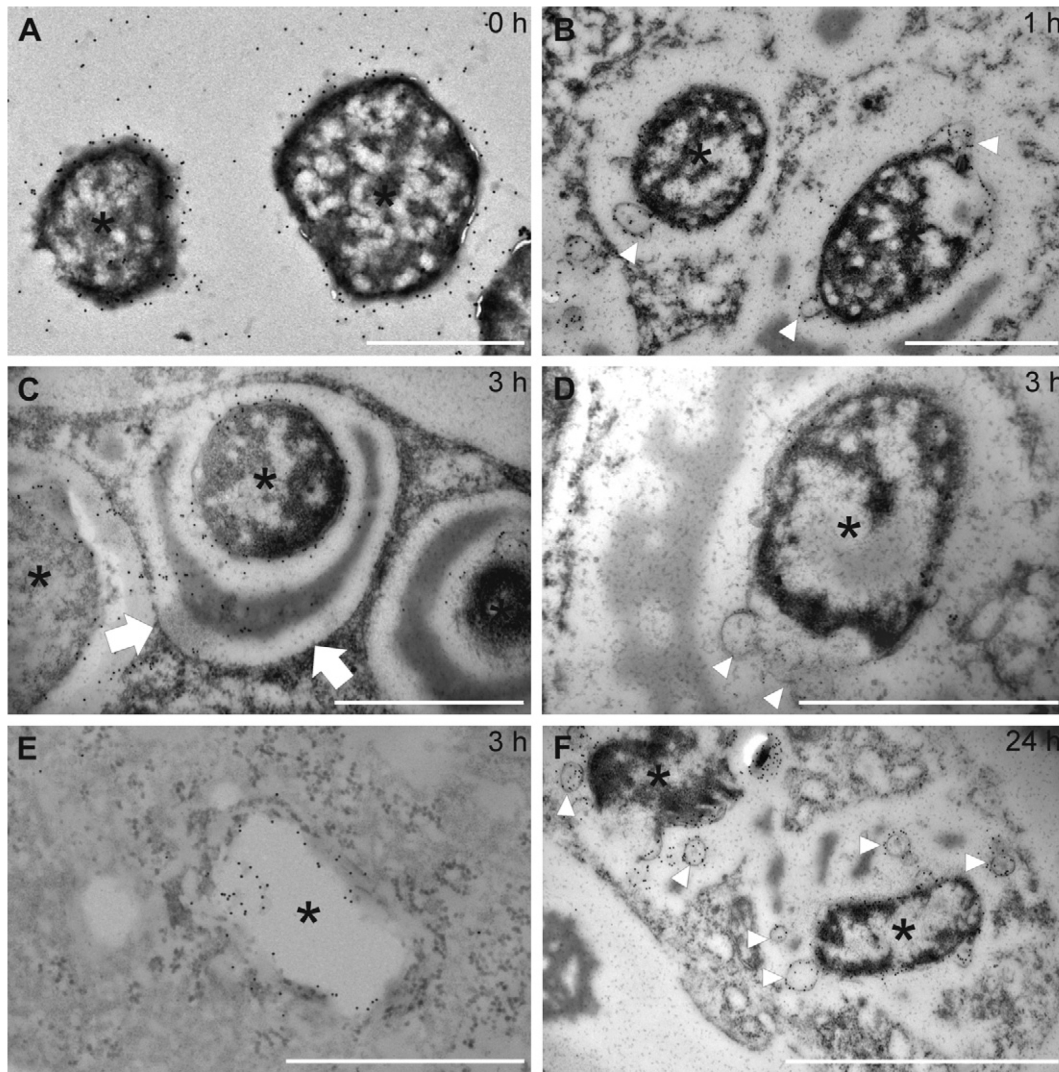

Fig. 5. Immune electron microscopy of intracellular localised *F. noatunensis* in Atlantic cod macrophages. Macrophages were infected with *F. noatunensis* and subjected to immune electron microscopy 1, 3 and 24 h after infection using anti-*F. noatunensis* serum (anti-FN; gold labelled). A) Localisation of anti-FN reactive material surrounding extracellular bacteria (asterisks). B) Intracellular bacteria (asterisks) without a clearly visible membrane and surrounded by a few bacterial derived vesicles (arrowheads). C) Intracellular bacteria (asterisks) are apparently surrounded by intact vacuole membranes (arrows). D) Intracellular bacteria (asterisks) weakly stained by anti-FN with many embedding vesicles. E) Empty electron-lucent areas in infected macrophages that stained clearly for bacterial antigens. F) Intracellular bacteria (asterisks) without a vacuole membrane and many bacterial derived vesicles (arrowheads). The scale bar represents 1 μm.

To determine whether intracellular *F. noatunensis* were stained with the anti-FN reactive material similarly to the extracellular bacteria, immune electron microscopy of macrophages infected with *F. noatunensis* for different time points (1, 3, 24 h) was performed. Infected macrophages showed clear intracellular staining with anti-FN demonstrating the presence of bacterial antigens inside macrophages (Fig. 5). Most intracellular bacteria displayed similar morphology and contained an evenly distributed staining with anti-FN similar to extracellular bacteria (Fig. 5A–D, F), while some bacteria were marginally stained or almost devoid of staining (Fig. 5D). The immunoreactive material was commonly found diffusely localised in the electron-lucent area surrounding the bacteria or associated with the transition to the more electron dense cytoplasm. In the early stage of the infection (1–3 h) some bacteria could be found surrounded by an electron-lucent space that showed a clear separation from the surroundings indicating the presence of a phagosomal membrane (Fig. 5C). In the same time span bacteria could also be found in electron-lucent spaces, where seemingly only partially intact membranes were present (Fig. 5B, D). Later in the infection (24 h) bacteria could be found more loosely associated within the macrophage surrounded by a larger electron-lucent space (Fig. 5F). Intracellular bacteria were surrounded by different sized vesicles, which were of bacterial origin since they clearly stained positive for bacterial antigens (Fig. 5B, D, F) resembling the ones observed earlier (Fig. 4). Some bacteria appeared to be in the process of forming buds from their outer membrane. In addition, the number of vesicles increased from 1–3 h to 24 h (Fig. 5B–D, F). Interestingly, infected cells contained empty electron-lucent areas that stained clearly for bacterial antigens and this labelling was mainly centred on the edges of the empty space (Fig. 5E).

3.6. Gene expression studies in macrophages after exposure to F. noatunensis

The innate immune response is “first-responders” to infectious agents like bacteria and consequently expression of inflammatory as well as anti-inflammatory responsive genes were monitored

after incubation with live or inactivated *F. noatunensis*. Macrophages were co-incubated with *F. noatunensis* for 3 h followed by removal of extracellular bacteria. Next, macrophages were lysed at sequential time points (3 h, 6 h, 12 h, 24 h, 48 h and 72 h) and subjected to real time PCR to study gene expression of interleukins. Exposure of macrophages to *F. noatunensis* leads to a weak but significant elevation of inflammatory responsive genes showed by elevated expression of IL-1β and IL-8 (Fig. 6A, C), while IL-6 gene expression showed only a modest but not significant induction (Fig. 6B). The highest levels of up-regulation of IL-1β (8–12 fold) and IL-8 (7–8 fold) were at 6 h and 12 h, respectively and had almost diminished by 48 h post-infection. Compared to the inflammatory responsive genes, the anti-inflammatory gene IL-10 (Fig. 6D) showed a delayed, but more elevated expression level with a peak at 24 h (17–21 fold). Expression of IL-10 was still elevated at a low level at the last sampling point at 72 h (8–9 fold). Gene expression of IL-12p40, one of the factors associated with T cell polarisation, was significantly elevated at all studied time points with a peak in expression level at 24 h (34–42 fold, $p < 0.05$) after infection with *F. noatunensis* (Fig. 6E). The expression of IL-12p40 displayed a delayed stimulation peak similar to the IL-10 expression profile.

In general, the studied expression levels in macrophages exposed to live compared to inactivated bacteria were not significantly different although live bacteria tended to induce a more marked immune response, i.e. for IL-1β (Fig. 6A).

3.7. Gene expression studies in macrophages following LPS stimulation

The immunoreactivity of *F. noatunensis* LPS (20, 50, 100 and 200 μg/ml) was compared to *E. coli* LPS (20 μg/ml) to evaluate its potency to activate the immune response in cod macrophages. Real time PCR analyses of expressed interleukin genes demonstrated that *F. noatunensis* LPS was a poor inducer of all studies genes (Fig. 7). This was verified by performing a dose response study of macrophages subjected to different concentrations of *F. noatunensis* LPS showing that very high concentrations were necessary to induce up-regulation (Fig. 8). A very modest induction (2–8 fold increase on average) was shown for IL-1β, IL-6, IL-8 and IL-10 genes at the lowest LPS concentration, while for the highest LPS concentration this was elevated slightly (7–16 fold increase at average) (Fig. 8A–D). This shows that the induction level did not increase tenfold even though the concentration of LPS was increased from 20 to 200 μg/ml. High concentrations of *F. noatunensis* were also observed to be toxic to the cells (results not shown). The expression levels of IL-12p40 were not changed due to *F. noatunensis* LPS stimulation (Figs. 7E and 8E).

However, high immune response was obtained when stimulating macrophages with *E. coli* LPS (Fig. 7). These responses were even higher than the induction level observed for *F. noatunensis* bacterial infection (Fig. 6). The induction by *E. coli* LPS of IL-1β (50-fold) and IL-8 (40-fold) showed a pronounced peak in expression 6 h post incubation (Fig. 7A, C), but otherwise followed the same pattern as in the previous *F. noatunensis* infection study (Fig. 6A, C). Similarly, IL-6 was weakly up-regulated (3-fold, $p = 0.01$) (Fig. 7B). However, the IL-10 gene showed a somewhat different expression profile in response to *E. coli* LPS compared to the *F. noatunensis* infection. This was manifested by a faster induction and lower expression level compared to infection (10-fold increase at 3 and 6 h), followed by a slight decrease throughout the studied time points (Fig. 7D). Gene expression of IL-12p40 also showed a different expression pattern compared to *F. noatunensis* infection (Fig. 6E). The expression peak of IL-12p40 after exposing macrophages to *E. coli* LPS was at 6–12 h post incubation (8–9 fold). It remained elevated throughout the studied time points, albeit at a lower level than for infection. The genes IL-10 and IL-12p40 were the only ones that were up-regulated with higher expression levels due to *F. noatunensis* infection than to *E. coli* LPS stimulation.

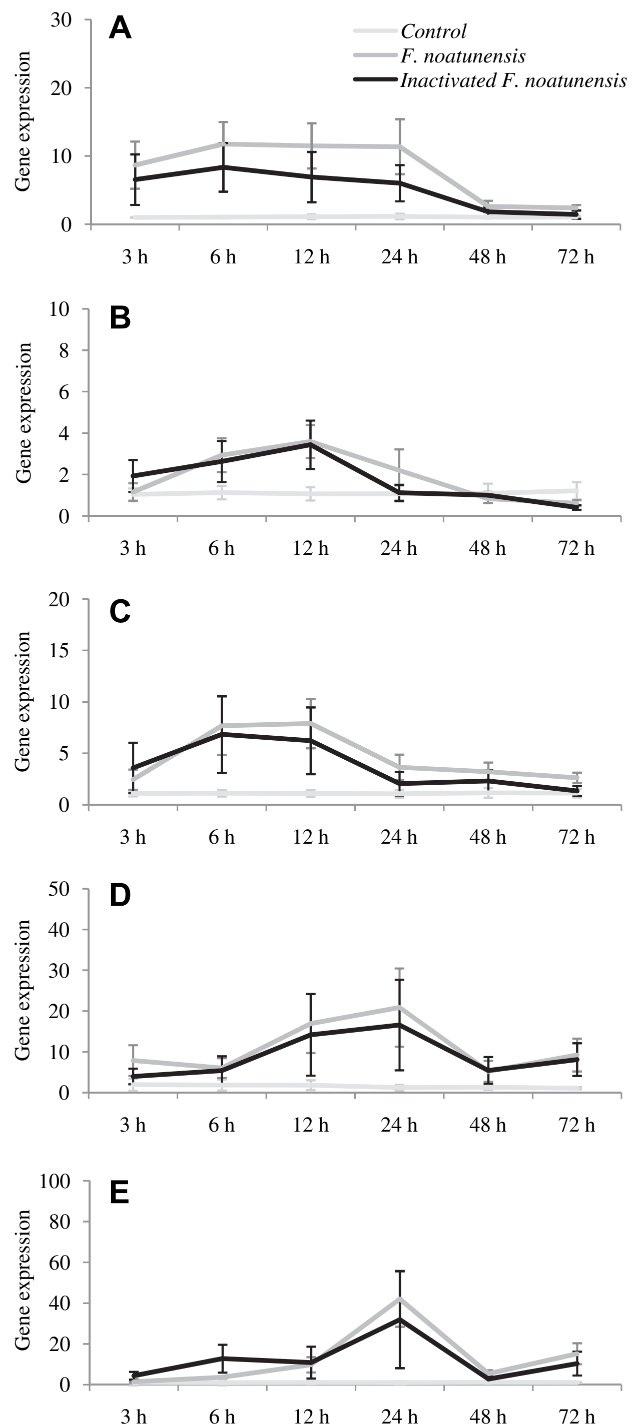

Fig. 6. Time course study of gene expression in Atlantic cod macrophages following exposure to live and inactivated *F. noatunensis*. Macrophages were co-incubated with live or killed *F. noatunensis*, lysed at sequential time points and subjected to real time PCR analysis. Gene expression of A) IL-1β, B) IL-6, C) IL-8, D) IL-10 and E) IL-12p40. All target genes were normalised to eF1α and calibrated to the control at each time point. Relative quantification values of three individual fish were obtained and the mean quantities ± SEM are shown. The experiment was repeated three times with similar results.

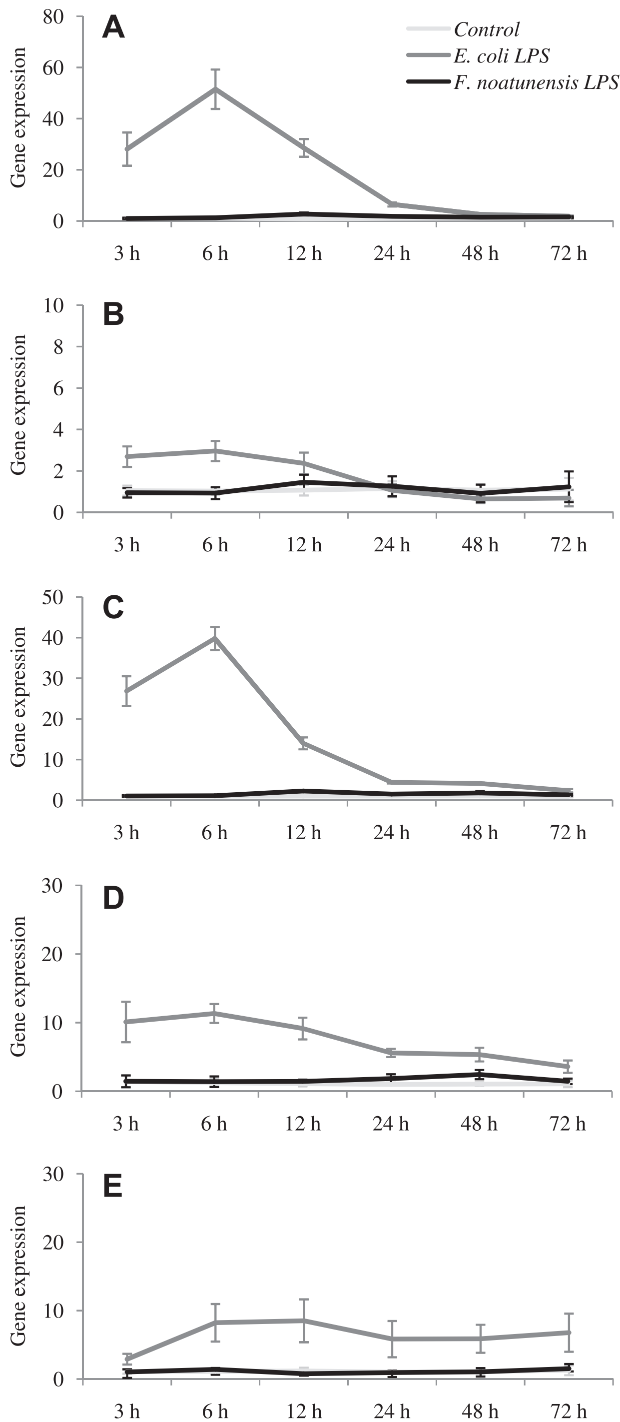

Fig. 7. Time course study of gene expression in Atlantic cod macrophages after stimulation with *E. coli* LPS and *F. noatunensis* LPS. Macrophages were stimulated with 20 μg/ml LPS from *E. coli* and *F. noatunensis*, lysed at sequential time points and subjected to real time PCR analysis. Gene expression of A) IL-1β, B) IL-6, C) IL-8, D) IL-10 and E) IL-12p40. All target genes were normalised to eF1α and calibrated to the control at each time point. Relative quantification values of three individual fish were obtained and the mean quantities ± SEM are shown. The experiment was repeated three times with similar results.

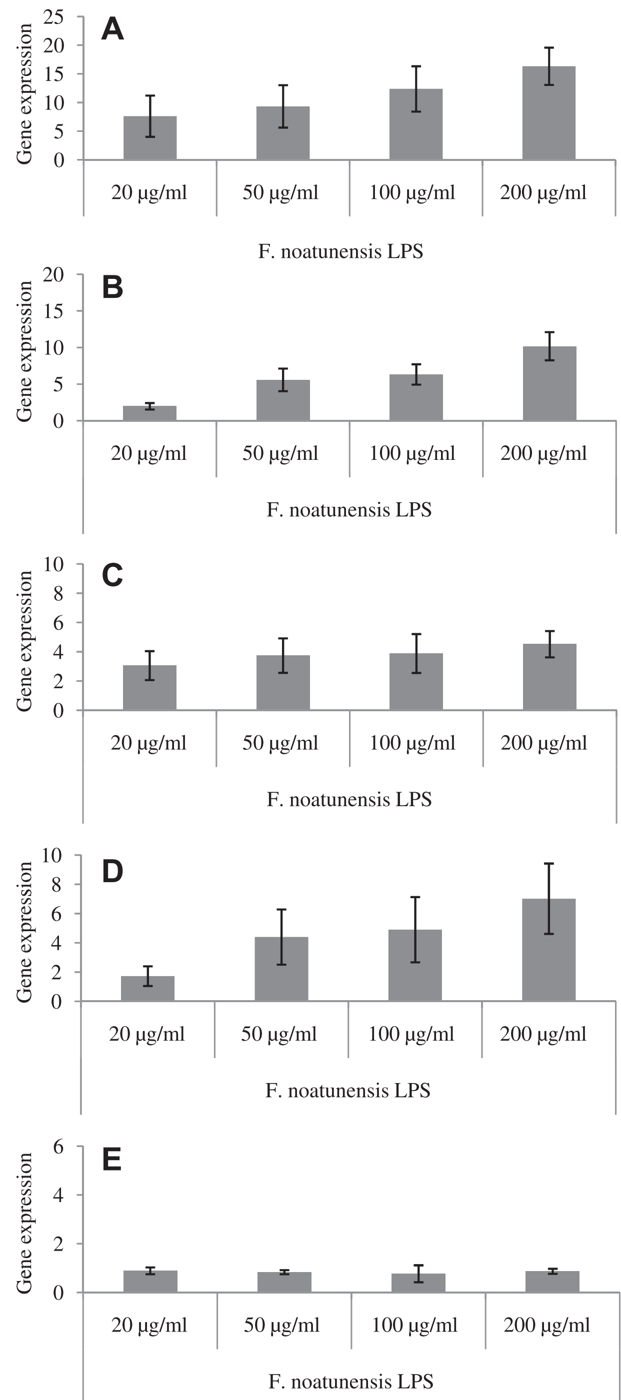

Fig. 8. Gene expression in Atlantic cod macrophages stimulated with different concentrations of *F. noatunensis* LPS. Macrophages were stimulated for 6 h with *F. noatunensis* LPS (20, 50, 100 and 200 μg/ml), lysed and subjected to real time PCR analysis. Gene expression of A) IL-1β, B) IL-6, C) IL-8, D) IL-10 and E) IL-12p40. All target genes were normalised to eF1α and calibrated to the control for the same time point. Relative quantification values of four individual fish were obtained and the mean quantities ± SEM are shown.

4. Discussion

Phagocytic cells such as macrophages are usually highly efficient killers of bacteria through their ability to carry out phagocytosis leading to bacterial degradation in phagolysosomes, simultaneously as they secrete cytokines to initiate immune responses that further facilitate the eradication of the infection. However, pathogenic intracellular bacteria have evolved multiple strategies to infect, avoid destruction and commonly, to suppress the immune response in macrophages. Knowledge of intracellular infections in fish and the subsequent immune response is scarce compared to that

available for such infections in humans. In this study, cod macrophages were exposed to *F. noatunensis* and the bacteria were localised intracellularly. In addition, the innate immune response was studied following bacterial infection and stimulation with *F. noatunensis* LPS.

Initial studies of macrophages co-incubated with inactivated *E. coli* conjugated beads were performed to study whether the cells maintained their functionality in culture conditions. The phagocytic capacity was highly intact and increased in a time dependent manner (Fig. 1) indicating that the isolated macrophages were functional. Next, macrophages were exposed to live *F. noatunensis* transformed with a GFP-expressing plasmid. Shortly following co-incubation, the bacteria were clearly localised inside the macrophage (Fig. 2). Intracellular *F. noatunensis* was also visualised by indirect immunofluorescence applying anti-*F. noatunensis* serum (anti-FN). The localisation of the anti-FN marked bacteria inside macrophages appeared to be either tightly clustered or dispersed in large vacuole enclosed inclusions (Fig. 3). Macrophages exposed to *F. noatunensis* for 24 h might contain cells that are in different stages of infection such as invasion, replication and preparation for escape from the macrophage. On the other hand, the vacuole inclusions observed might also resemble bacteria in phagolysosomes on their way to degradation.

A notable finding of the present study was that only a subset of the macrophages contained *F. noatunensis*, even when using a high infectious dose. Apparently, even fewer intracellular bacteria were present using the GFP-expressing strain compared to the strain used in the immunofluorescence study. Among other factors this could reflect differences in virulence properties between the two isolates used. However, compared to the rapid accumulation of inactivated *E. coli* in macrophages it could be suggested that *F. noatunensis* do not effectively gain access to macrophages, as previously described for *F. tularensis* [10,50,51]. Furthermore, it should be emphasised that only a few bacteria are required during early stages to cause fatal disease and that is why *F. tularensis* is characterised as a potential biological weapon [7].

Intracellular infection is commonly mediated by phagocytosis of bacteria, but instead of bacterial degradation, virulent bacteria escape destruction and survive within macrophages either in membrane enclosed vacuoles or free in cytoplasm. In this study, morphologically intact *F. noatunensis* were present inside cod macrophages 3 h post-infection in tightly enclosed vacuoles separated by only a narrow electron-lucent space (Fig. 4). In the same macrophage, bacterial structures could also be found in a larger electron-lucent space surrounded by an apparently incomplete vacuole membrane. Immune electron microscopy supported these results where shortly after infection (1–3 h) bacteria were located inside macrophages either surrounded by an intact membrane or an apparently partially degraded membrane (Fig. 5). After 24 h morphologically intact bacteria were still present inside macrophages within an enlarged electron-lucent space, while the phagosomal membrane appeared disintegrated. This might be interpreted as that *F. noatunensis* quickly infect cod macrophages, prior to apparent phagosomal membrane disruption allowing bacterial release to the macrophage cytoplasm. In head kidney derived tilapia (*Oreochromis niloticus*) macrophages it was shown that *F. asiatica* reside in tight vacuoles early in the infection, while at later stages in spacious vacuoles or free in cytosol. The bacteria were also able to propagate in tilapia cells [52]. A recent study suggested that *F. noatunensis* were able to replicate in cod cells since the amount of microscopically observed bacteria increased from 4 h to 24 h. They also suggested that *F. noatunensis* follows the same escape pathway as *F. noatunensis* and could be found freely in cytosol [6]. At the present time we do not know if *F. noatunensis* is able to replicate intracellularly or if they escape into cytoplasm in cod macrophages, but live bacteria could be recovered from infected cells (3 h–24 h) and grown on CHAB plates (manuscript in prep). Further verification of the localisation is difficult since co-localisation of bacteria with endosomal markers is commonly used for such studies and there are few markers available for fish. A further limitation for localisation studies is that misinterpretations might occur (e.g. [50,51]) given the difficulties in clarifying whether bacteria localise to intact or partially degraded vacuoles as demonstrated by our results.

Despite much research effort the actual mechanism of phagosome membrane disruption remains elusive in the pathogenesis of *F. tularensis* and the effector proteins of phagosomal disruption have yet to be characterised at a functional level [8]. In this work, intracellular *F. noatunensis* were frequently surrounded by differently sized bacterial derived vesicles and several bacteria were in the process of forming vesicular buds (Fig. 5). The formation and secretion of vesicles could not be observed in extracellularly grown bacteria indicating that this is an intracellular event occurring only during infection. Additionally, these bacterial vesicles were only present when the membrane appeared partially degraded. The presence of similar vesicles in intracellular bacterial infections has been shown previously [10,11,53], but the molecular events leading to this are still not elucidated. Golovliov et al. [10] hypothesised that *F. tularensis* derived vesicles could be involved in permeabilising the phagosomal membrane allowing bacteria to remain partially enclosed in vacuoles or to reside freely in cytoplasm. It was also suggested that this event could influence the composition of the bacterial membrane rendering the intracellular bacteria only marginally stained. The observation of some intracellular *F. noatunensis* that were almost devoid of anti-FN staining could support this notion. We frequently observed anti-FN staining in the electron-lucent space and at the outer perimeter of apparently empty translucent areas. This could represent vesicular remains of bacteria after being released to cytoplasm, but it might also be bacterial remains after degradation in phagolysosomes. Further experiments are thus necessary to verify how *F. noatunensis* deviate from the classic phagolysosomal pathway and replicate inside cod cells, which are likely to be of critical importance to the virulence of the bacterium.

Other virulence factors known for intracellular bacteria are their capacity to subvert the host immune response and to create a favourable environment for intracellular replication. Gene expression studies were used to measure activation of the innate immune response after exposure to *F. noatunensis* and stimulation with LPS. Initially, a comparison of the immune response due to live or inactivated *F. noatunensis* revealed small differences (Fig. 6). Live bacteria resulted in higher induction of interleukin genes, but not significant different from inactivated bacteria. Similar gene expression results were obtained in human macrophages after exposure to live or killed *Francisella* sp., but only live bacteria were able to promote secretion of IL-1β from infected cells [54].

Infection of macrophages with *F. noatunensis* generated a low induction of inflammatory responsive genes measured by small fold increases in IL-1β and IL-8 expression (Fig. 6), while in comparison a higher fold induction of the anti-inflammatory responsive gene IL-10 was observed. However, in the case of stimulation with *E. coli* LPS this pattern was reversed. The bacterium *F. tularensis* has a virulence mechanism delaying the inflammatory response by down-regulating the transcriptional nuclear factor κB (NF-κB) signalling pathway, thus inhibiting the production of inflammatory cytokines [55]. This delay probably allows sufficient time to facilitate bacterial replication before the host can mount an immune response [56]. Interestingly, studies from mammals have shown that virulent *Francisella* sp. cause a low inflammatory response probably facilitating bacterial replication,

whereas avirulent strains promote a high inflammatory response that could assist bacterial clearance [54,57]. Challenge of cod has shown that this *F. noatunensis* isolate is obviously virulent (unpublished results), but whether it posses similar virulence mechanisms as in mammalian *Francisella* species are currently not known.

The major adaptive immune response against intracellular bacteria is cell-mediated immunity, but recently T_H17 cells have been given a role in defence against *F. tularensis* infection [30]. The high activation of the p40 subunit and its dual role in activating mammalian cell-mediated immunity and T_H17 cells through IL-12 and IL-23, respectively, could imply that *F. noatunensis* activates T cell polarisation in cod. However, cod lack a functional major histocompatibility complex (MHC) class II pathway where genes encoding MHC class II, CD4 and invariant chain are all absent in the cod genome [17]. This, most likely renders the CD4 pathway including T_H1 and T_H17 cells absent in cod. Nevertheless, the p40 subunit might still have a role in IL-12 promoting the proliferation and cytotoxicity of CD8 cells [25,26]. The cod genome has a functional major class I pathway and this could suggest a greater importance for cytotoxic T cells in pathogen clearance in cod. However, further studies of how cod present antigens to its immune system and activate cell-mediated immune response are important to reveal the function of the unique immune system in cod.

The LPS in Gram-negative bacteria is normally highly exposed to potential host cells and recognised by TLR4 in mammals, which initiates the inflammatory response through NF-κB activation [58]. In fish the role of TLR4 has been questioned and the receptor is most likely not functionally equivalent to mammalian TLR4 [16,18–20]. Another difference is that fish are almost resistant to the endotoxic effect of LPS [59] and 1000-fold higher concentrations are necessary to induce inflammation [60], which was also confirmed in this study. The low sensitivity to LPS might be explained by the presence of other receptors for LPS with low binding affinity [60], which requires higher concentrations in order to activate the immune response. Macrophages stimulated with *F. noatunensis* LPS showed a poor inflammatory response, in contrast to the high response obtained when stimulating with *E. coli* LPS (Figs. 7 and 8). These results show that immunoreactivity of *F. noatunensis* LPS was very modest akin to *F. tularensis* LPS [54,58,61], which could represent a virulence mechanism used to avoid recognition by the immune defence.

This report shows that *F. noatunensis* are located intracellularly in cod macrophages. The intracellular localisation of *F. noatunensis* was during the initial phase of infection in membrane enclosed vacuoles, while at later stages, apparently surrounded by partially intact membranes. The release of bacterial derived membrane vesicles from intracellular *F. noatunensis* was demonstrated, an event suggested of promoting phagosomal membrane degradation allowing escape of the bacteria to cytoplasm. Infected macrophages displayed a low fold induction of inflammatory responsive genes and a higher fold induction of the anti-inflammatory response gene, which could represent a virulence mechanism of *F. noatunensis* used to suppress the immune response. Another potent virulence mechanism could be the poor ability of *F. noatunensis* LPS to activate the inflammatory response. These results suggest that the bacterium has many virulence mechanisms to subvert the immune response in cod macrophages.

Acknowledgements

The authors wish to thank Prof. Rudolf Toman for isolation of *F. noatunensis* LPS. Anne Grethe Hestnes is highly appreciated for her indispensable help and enthusiasm with the light microscopy studies. Kari Steiro, Audny Johansen and Elin Sandaker are appreciated for excellent technical assistance with the experiments. Dr Marie Cooper is thanked for her proofreading of the manuscript, while Dr. Eirin F. Pettersen and Prof. Heidrun Wergeland are acknowledged for their advice on isolation of cod macrophages. This project was funded by the Research Council of Norway (183204 and 199672/S40) and MABIT (AF0042). Partial support for C.V. and H. C. W-L were by the triple alliance funds from the Norwegian School of Veterinary Sciences, Norwegian Veterinary Institute and Norwegian University of Life Sciences. H.C.W.-L. was supported in part by the Research Council of Norway (158882/I10).

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