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Characterization of PAMP/PRR interactions in European eel (*Anguilla anguilla*) macrophage-like primary cell cultures

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ABSTRACT

The eel (*Anguilla anguilla*) has been identified as a vulnerable species with stocks dramatically declining over the past decade. In an effort to support the species from overfishing of wild stocks increased interest in eel aquaculture has been notable. In order to expand the scarce knowledge concerning the biology of this species significant research efforts are required in several fields of biology. The development of cell culture systems to study the immune response is a key step towards an increased understanding of the immune response and to develop resources to support further study in this threatened species. Macrophages are one of the most important effector cells of the innate immune system. The capacity to engulf pathogens and orchestrate the immune response relies on the existence of different surface receptors, such as scavenger receptors and toll-like receptors. We have developed and described an eel macrophage-like *in vitro* model and studied its functional and transcriptomic responses. Macrophage-like cells from both head kidney and purified peripheral blood leukocytes were obtained and phagocytic activity measured for different whole bacteria and yeast. Moreover, based on PAMP-PRR association the innate immune response of both head kidney and PBL derived macrophage-like cells was evaluated against different pathogen-associated molecular patterns (PAMPs). Results highlight that peptidoglycan stimulation strongly induces inflammatory mRNA expression reflected in the up-regulation of pro-inflammatory genes IL1β and IL18 in PBL derived cells whereas IL8 is upregulated in head kidney derived cells. Furthermore TLR2 mRNA abundance is regulated by all stimuli supporting a multifunctional role for this pathogen recognition receptor (PRR) in eel macrophage-like cells.

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1. Introduction

The teleost immune system presents both innate and adaptive responses [1], however the innate immune response is of central importance in mounting a successful defence in fish whereas the adaptive response is commonly delayed [2]. The innate response is an evolutionary ancient system present in both invertebrates and vertebrates that acts as the first line of defence against invading pathogens, detects the presence and the nature of infection and initiates the defence system. The innate system is basically composed of two components, humoral including cytokines, antimicrobial peptides, lysozyme, lectins, complement and cellular components such as phagocytes and non-specific cytotoxic cells, both with efferent and afferent systems [3,4].

The innate immune system perceives infection by detecting well conserved pathogen structures known as pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs). Toll-like receptors (TLRs) were the first PRRs to be identified and their key role in pathogen recognition, induction of antimicrobial genes and the control of adaptive immune response has been well described [5]. Different TLRs recognize specific PAMPs from different microbes, such as bacteria, viruses, fungi and protozoan parasites. After recognition, these receptors orchestrate the activation of signalling pathways that generate specific immunological responses tailored to the suite of PAMPs expressed by the pathogen in question [6]. All TLRs trigger signalling pathways using different adaptor molecules of which MyD88 is a common element to all of them (with the exception of TLR3). These pathways culminate in NF-κB and MAP kinase activation and the induction of inflammatory cytokines and chemokines [7].

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The mononuclear phagocytic system comprises of monocytes, tissue macrophages and dendritic cells. The primary function of mature macrophages is the clearance of pathogens by phagocytosis and orchestration of the response by humoral signalling. Therefore, macrophages are one of the most important effector cells of the innate immune system and also may play an important role in the priming and activation of the adaptive immune response [8]. The capacity to engulf a wide array of particles relies on the existence of different surface receptors, such as scavenger receptors and the already mentioned TLRs [9].

The European eel (*Anguilla anguilla*) is an emblematic species in Europe that currently occupies a position in the IUCN red list as a critically endangered species due to multiple anthropogenic factors including habitat destruction, pollution and overfishing [10,11]. One of the measures to fight against population decline would be to culture eels in captivity and restore natural stocks with those eels. Currently, this is not possible due to the complexity of the eel life cycle [12], which is only partially understood and is non-reproducible in captivity. Furthermore, disease caused by bacteria, viruses and parasites affects eels at any stage of development thus limiting survival in both the wild and in farmed conditions. Due to the afore-mentioned difficulties eels are a difficult species to research and as a non-model species few molecular and cellular tools are available. The anguillids are phylogenetically distant to the most of the more common teleost species used making progress even more challenging. Concerning to eel immune system little information are available so new advances in the description of the eel immune system are necessary to improve eel health under rearing conditions [13], and increase the efforts to protect this species.

The aim of the present work was to study the role of macrophages in the innate immune response of eels against different PAMPs. To this end, we developed a primary macrophage-like cell culture from the eel and characterized a series of functional and transcriptomic studies.

2. Material and methods

2.1. Fish and maintenance conditions

Adult European eels (*A. anguilla*) weighing 200–250 g were obtained from Valenciana de Acuicultura (Puçol) and reared in Autonomous University of Barcelona facilities under controlled conditions. Eels were maintained in a 12:12 h dark:light cycle in a recirculation freshwater system at 18 ± 2 °C before experimentation and fed daily with a commercial diet. All experimental animals were housed, handled and used in accordance with internationally recognized guidelines.

2.2. Isolation of phagocytes and cell culture

Eels were anaesthetized with 250 mg/l of benzocaine (Sigma–Aldrich) and 2.5 ml of blood from the caudal vein were extracted with heparinized syringe and needle to obtain circulating monocytes/macrophages-like cells from peripheral blood leukocytes (PBLs). Blood were centrifuged at 600× *g* during 2 min, the plasma were collected, and inactivated at 57 °C for 30 min, centrifuged at 720× *g* for 10 min to remove the debris, and stored at −80 °C until use. 5 ml of saline buffer were added to the pellet of cells and centrifuged at 600× *g*, 5 min, cells were recovered and resuspended in 3 ml of PBS1x, the suspension were added to 6 ml of Lymphoprep™ (Axis-Shield) and PBL separation were carried out by centrifugation at 720× *g* for 30 min, these protocol were performed twice per sample. White blood cells were collected, washed twice in PBS1x and resuspended in fresh supplemented L-15 medium to be plated in 6 wells of 12-well poly-L-Lysine treated plates (Nunc) and incubated at 20 °C.

Head kidney derived macrophage-like cells were obtained from killed individuals by an overdose of benzocaine, The head kidney was dissected out [14], placed in a complete L-15 medium, and minced using a 100 μm nylon mesh cell-strainer (BD). Cells were recovered by centrifugation at 720× *g* for 5 min and resuspended in L-15 complete medium. 1 ml of homogenate was placed into each well, using 6 wells of a 12-well poly-L-lysine treated plate. Then, plates were incubated at 20 °C until cells were completely differentiated, at 72 h.

In both cell cultures, non-adherent cells were removed 24 h after culture and fresh medium was added until use. Complete medium were composed of Leibobitz-15 with Glutamax (Gibco®) supplemented with 10% of inactivated FBS (v/v) (Sigma–Aldrich) and 100 μg/ml Primocin (Invitrogen).

Cell morphology was monitored everyday by inverted microscopy (Olympus CKX-31) and images were taken (Leica DC200 camera) as well as, cells were visualized by confocal laser microscopy Zeiss LSM 700 (Carl Zeiss Microscopy) where the nuclei was labelled with Hoechst 33342 (20 μg/ml) and plasma membrane was stained with CellMask™ Deep red (2.5 μg/ml) for 5 min in darkness followed by further washing of the cell culture and fresh L-15 medium addition. Furthermore, cultures were analysed by Flow cytometry (BD FACScalibur™) and data were treated with FlowJo 8.7 software, to determine different cell populations and homogeneity of the primary cell cultures. Homogenates from minced head-kidney and purified peripheral blood leukocytes were used as samples for day 0 of cell culture. Once cells were morphologically differentiated *in vitro*, they were washed three times with PBS1x to remove non-adherent cells. Adherent cells, potentially macrophage-like cells, were trypsinized with TrypLE™ Select (Gibco®) for 10 min at room temperature and washed with PBS1x to be analysed by cytometry.

2.3. Phagocytosis assays

Phagocytosis assays were performed by incubating the differentiated eel cell cultures ($n = 3$ per assay) with fluorescent-labelled dead microorganisms; fluorescein conjugated *Escherichia coli*, *Staphylococcus aureus* and *Saccharomyces cerevisiae* (zymosan) (Molecular probes®) or 2 μm red-labelled polystyrene beads (Molecular probes®) for 1 h at room temperature at a ratio (dead microorganism or bead/eel cell) of 40:1 for *E. coli*, *S. aureus* and beads, and of 4:1 for *S. cerevisiae*. Before incubation, dead microorganisms were opsonized using 10^6 particles/ml in 10% plasma solution for 30 min, plasma used on each incubation were provided by the same individual that the culture come from. After incubation, eel cells were repeatedly washed with PBS1× to remove non-phagocytosed particles and other cellular debris, monitored by fluorescence microscopy (Leica DMRB microscopy) and pictures were taken (Leica DC200 camera). Immediately, cells were trypsinized for 10–15 min at RT (TrypLE™ Select, Gibco®) to be analysed by Fluorescence activated cell sorting (FACS) by using FACSCalibur cytometer (BD). Counterstaining with propidium iodide (1 mg/ml) was done to test cell viability.

Discrimination of free fluorescent particles, as well as, selection of the target cells was carried out using the combined measurement of complexity (SSC) and size (FSC) in dot plots by selecting the cell population of interest. Furthermore, the exclusivity of the phagocytic process by our cells of study was checked by the combined measurement of their green fluorescence (FITC) and size (FSC) in histograms.

Moreover, phagocytosis assay using zymosan-FITC particles were also evaluated by confocal microscopy to discriminate

between intra- and extracellular particles. Assay was done with control cells and zymosan treated cells. The nuclei and plasma membrane were labelled as mentioned before with Hoechst 33342 and CellMask™. Cells were examined using Zeiss LSM 700 (Carl Zeiss Microscopy) and Z-series were collected at 0.6 μm intervals for 3D representation. Images were taken using a 40× W Plan-apochromatic objective. Afterwards, images were analysed and processed by Imaris software (Bitplane AG).

2.4. PAMP challenges

Differentiated macrophage-like cell cultures, either, from PBLs and from head kidney, were stimulated with several PAMPs to determine the ability of these primary cell cultures to recognize and respond against pathogen molecules. Before stimulation, cells were kept in serum-free L-15 medium for 3 h to maintain a basal state. Then, cultures were stimulated for 12 h with 50 μg/ml of lipopolysaccharide from *E. coli* 0111:B4 (LPS; Sigma–Aldrich)), or 10 μg/ml of peptidoglycan from *E. coli* 0111:B4 (PGN-EC; Invivogen), *S. aureus* (PGN-SA; Invivogen) or zymosan from *S. cerevesiae* (Zym; Sigma–Aldrich).

2.5. Gene expression analysis

Total RNA was isolated from control and PAMP stimulated cell cultures using 1 ml of Tri-reagent (Sigma–Aldrich) for each 2 wells of treatment, following the manufacturer's protocol. RNA quality was determined by using a BioAnalyzer RNA chip (Agilent technologies) and RNA was quantified by Nanodrop1000 (Thermo scientific). RNA was reverse transcribed by Superscript™ III RT (Invitrogen) and oligo-dT primer (Promega) obtaining a final volume of 20 μl of cDNA from 500 ng of RNA.

Genes selected for the analysis were; TLR2, as a recognition molecule, MyD88, a transduction molecule, and three response molecules, putative IL-1β, IL-18 and the chemokine, IL-8. Specific primers were designed *in silico*, using Prime3 (v. 0.4.0) software and were analysed by IDT OligoAnalyzer 3.1. All primers (Table 1) amplified the selected targets with optimal PCR efficiencies (94%–103%).

Absolute quantification was carried out to evaluate gene expression levels. Real-time PCR reactions were performed in triplicate using 1:100 of diluted cDNA as a template, the specific primers (Table 1) and SYBR green dye (Bio-Rad) in a volume of 10 μl. Reactions were run on 384-well plates in CFX384 Touch™ detection system (Bio-Rad) and data were analysed with CFX Manager™. The number of transcript molecules was calculated from the linear regression of the standard curve (Ct-Threshold cycle *versus* log copy number), using a 10^9 to 10^2 copies/ml dilutions of the DNA plasmid and normalized to control samples. Previously, PCR products were cloned into pGEM vector to obtain the DNA plasmids (pGEM) and to confirm their identity.

Table 1
List of primers used for gene expression analysis by RT-qPCR.

Gene	5′–3′	Primer sequence
IL1β	Fw	CGTGCCACGTGCTCTCACAA
	Rv	CAGCACCACCTAGTGGCTGAACC
IL8	Fw	TAGGGGTGGATCTGCGGTGT
	Rv	GCTGCTTGTGTGTCTAACTTGTGC
IL18	Fw	CCAGCCACAATGCCGTGTTC
	Rv	CCCAGCCTCTCTCAGCACCA
MyD88	Fw	CCAGGAGCGCAAAAGAAACG
	Rv	AGGCTGTCGGGCCTTGAACT
TLR2	Fw	ATGACCTGGGCTTGGCTTCA
	Rv	GGGCTCCAGCAGAATCAGGA

2.6. Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) followed by the post hoc multiple comparison by Bonferroni's method was run for each mRNA to determine differences between groups. And significant differences relative to control are denoted * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

3. Results

3.1. Macrophage-like cell culture description

Two different methodologies were used to obtain macrophage-like cells from the European eel using a modification of the methodology described by Mackenzie et al. [14] for both head kidney-derived cells (HK) and peripheral blood leukocytes (PBLs). Adherent cells from head kidney or PBLs samples spontaneously differentiated exhibiting a macrophage-like cell phenotype after 72 h or 48 h in culture, respectively (Figs. 1A–B and 3A). The morphology of the macrophage-like cells from both tissues was assessed by optical microscopy showing a very similar morphology being heterogeneous and oval with branched extensions (Fig. 1A–B). Moreover, PBL-derived cells were also evaluated by confocal microscopy. Nuclei were labelled by Hoechst and allow us to determine its bilobulated shape and the staining of the membrane by CellMask™ corroborate the heterogeneous morphology of the cell with extensions and irregular surface (Fig. 3A).

The macrophage-like cell recovery rate from PBLs was almost 3 times higher than the head kidney derived cells, with $3.8 \times 10^4 \pm 1.2 \times 10^4$ cells/cm^2 against $1.3 \times 10^4 \pm 3.4 \times 10^3$ cells/cm^2 obtained from head kidney cultures (Fig. 1C). Due to this reason, only PBL derived cell cultures were used in the functional assays.

Cell culture populations from PBL-derived cultures were analysed at day 0 and at 48 h by flow cytometry. As expected for primary cell cultures from homogenates, at day 0, three main cell populations were observed they may correspond to monocytes, neutrophils and the main mixed population of lymphocytes + thrombocytes (data not shown) according to the previously described by Inoue et al. [15]. However, after 48 h and the required cell culture washes, a decrease in the non-adherent cell number and cellular debris led to a main population of 71.5 ± 17.8% of cells corresponding to macrophage-like cells (Fig. 1D).

3.2. Phagocytosis assays

The ability to engulf microorganisms is one of the main roles of macrophages. In this study, we demonstrate that the eel macrophage-like cells are phagocytic. Based on that, flow cytometry and confocal laser microscopy are powerful tools for analysing pathogen-host cell interaction [16]. Here, we show the FACS-analysis results where more than 20,000 events were counted per sample and confocal laser microscopy were about 20 cells per sample were evaluated. The macrophage-like population may significantly interiorized the bacteria and yeast as well as polystyrene beads; a 15.44 ± 5.88% were able to phagocytose *E. coli* (Fig. 2A and C), a 31.73 ± 3.00% *S. aureus* (Fig. 2A and D), a 75.17 ± 13.90% *S. cerevisiae* (Fig. 2A and E) and, finally, 16.5 ± 1.27% the non-opsonized polystyrene-beads. Regarding the number of potential internalized cells or beads per phagocytic cell, around 50% of the cell population engulf one particle while more than 20% of the population engulf two, 13% three and 12% more than three

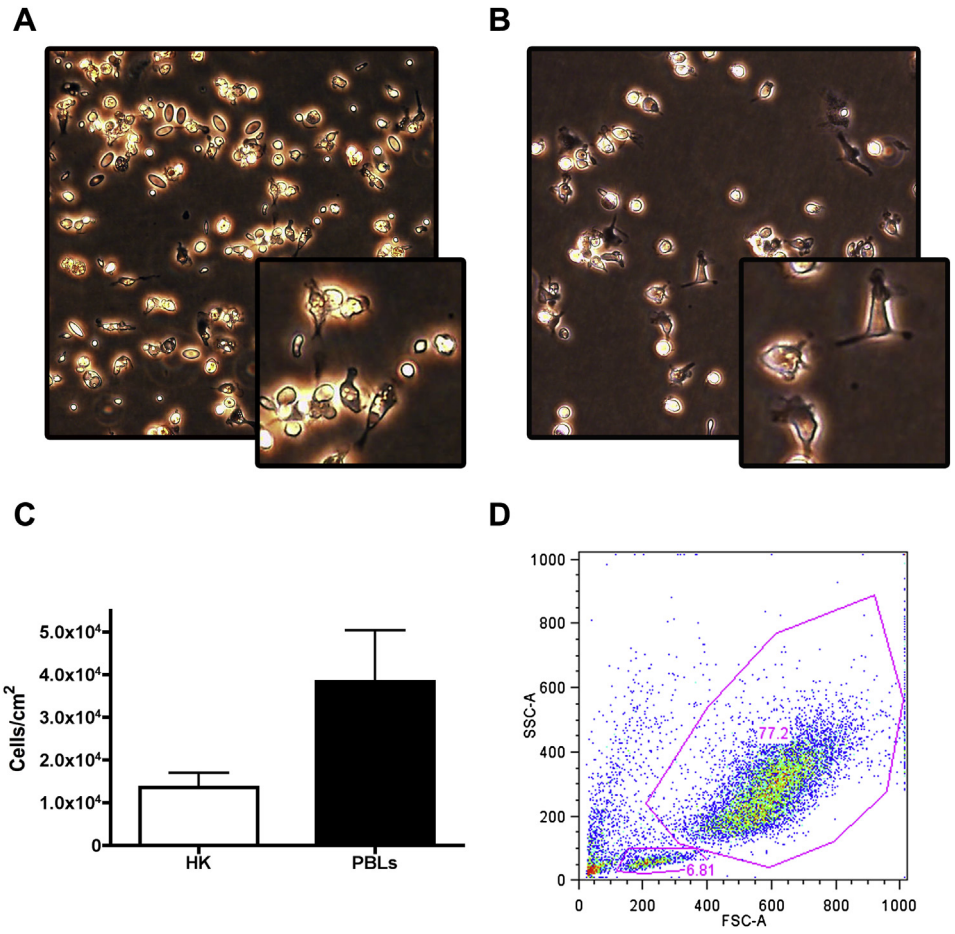

Fig. 1. Cell phenotype and homogeneity of macrophage-like cell cultures. Morphology of the cells after spontaneous differentiation under *in vitro* conditions was evaluated by inverted microscopy. (A) Differentiated macrophage-like cells obtained from head kidney homogenates after 3 days in culture. (B) PBLs derived cell phenotype after 48 h *in vitro*. (C) Relative cell yield obtained from head kidney homogenates (open bars) with a mean value of $1.3 \times 10^4 \pm 6.8 \times 10^3$ cells/cm^2 *versus* PBL derived cells (closed bars) with a mean value of $3.8 \times 10^4 \pm 2.4 \times 10^4$ cells/cm^2. (D) FACS analysis of cell populations derived from PBLs after 48 h; 71.5 ± 17.7% of differentiated macrophage-like cells.

(Fig. 2B–E). Therefore, besides FACS analysis, we assessed the internalization of the zymosan particles by confocal laser microscopy to differentiate between intra- and extracellular bacteria (Fig. 3). Results indicate that zymosan particles were completely interiorized (Fig. 3B) and no particles attached to the membrane were observed.

3.3. Gene expression studies

To determine if there were differences in the gene expression pattern of PAMP recognition and cytokine response, phenotypically differentiated PBLs and head kidney derived cell cultures were challenged with different PAMPs (LPS, PGN and zymosan).

Expression profiling results revealed that the relative expression of IL-1β mRNA was significantly upregulated ($P < 0.05$) after peptidoglycan stimulation regardless of the origin of the cell culture, however, PBL derived macrophage-like cells show a stronger expression of this cytokine (Fig. 4A). The relative mRNA abundance of IL-8 was significantly upregulated ($P < 0.05$) in response to PGN-EC in head kidney derived cultures but not in PBL derived cells (Fig. 4B). Expression levels of IL-18 were significantly increased ($P < 0.05$) after PGN-SA challenge in PBL derived cell cultures (Fig. 4C), while the rest of samples exhibited no variation relative to control. MyD88 and TLR2 were expressed in similar levels irrespective of the stimulus and the origin of the culture. Furthermore, MyD88 mRNA abundance did not show differences relative to the control (Fig. 4D) while TLR2 mRNA abundance was significantly downregulated ($P < 0.05$) after all challenges with the exception of PGN-SA in PBL derived macrophages-like (Fig. 4E).

4. Discussion

In the present study, we have developed a macrophage-like primary cell culture from *Anguilla anguilla*, and have characterized phagocytic and PAMP inducible gene expression activity. Firstly, we obtained undifferentiated myeloid cells from HK and purified PBLs *in vitro* left to differentiate until a typical macrophage-like phenotype was observed. Cells obtained in the culture over the specific culture period reached a maximum homogeneity of >70% after 72 h and 48 h for HK and PBLs respectively. Secondly, functionality of the cells were analysed focussing our studies in its phagocytic hability and assessing the gene expression pattern of several immune related genes, involving recognition (TLR2), signal transduction (MyD88) and response molecules (IL-1β like, IL-18 and IL-8).

During the last few decades the interest in fish immunology has steadily increased and one of the principal reasons and the most practical is that the aquaculture industry has expanded and therefore infection problems have became a major problem. From a theoretical perspective, increased knowledge of the fish immune system can help to understand both the evolution of immunity and provide new insights to solve practical problems [1,17]. Therefore,

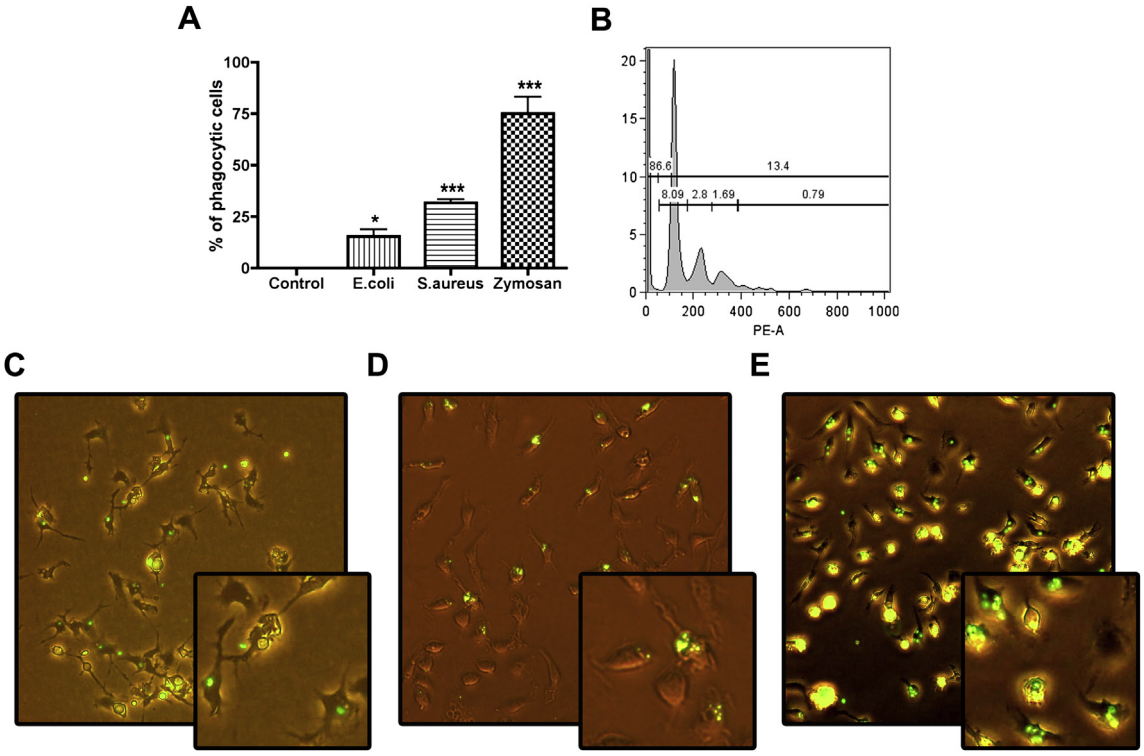

Fig. 2. Evaluation of the phagocytic activity in differentiated PBL-derived cell cultures. (A) FACS analysis of phagocytic cells after 1 h of incubation with different Bioparticles®; One-way ANOVA analysis ($P < 0.05$) were run to determine differences between particle interiorization, differences against control are denoted as * ($P < 0.05$) and *** ($P < 0.001$). (B) Relative number of phagocytosing cells in population after 1 h of incubation with different PAMPs. (C–E) Fluorescence microscopy for phagocytosis of labelled *E. coli* (C), *S. aureus* (D) and Zymosan (E) in differentiated macrophages-like cells.

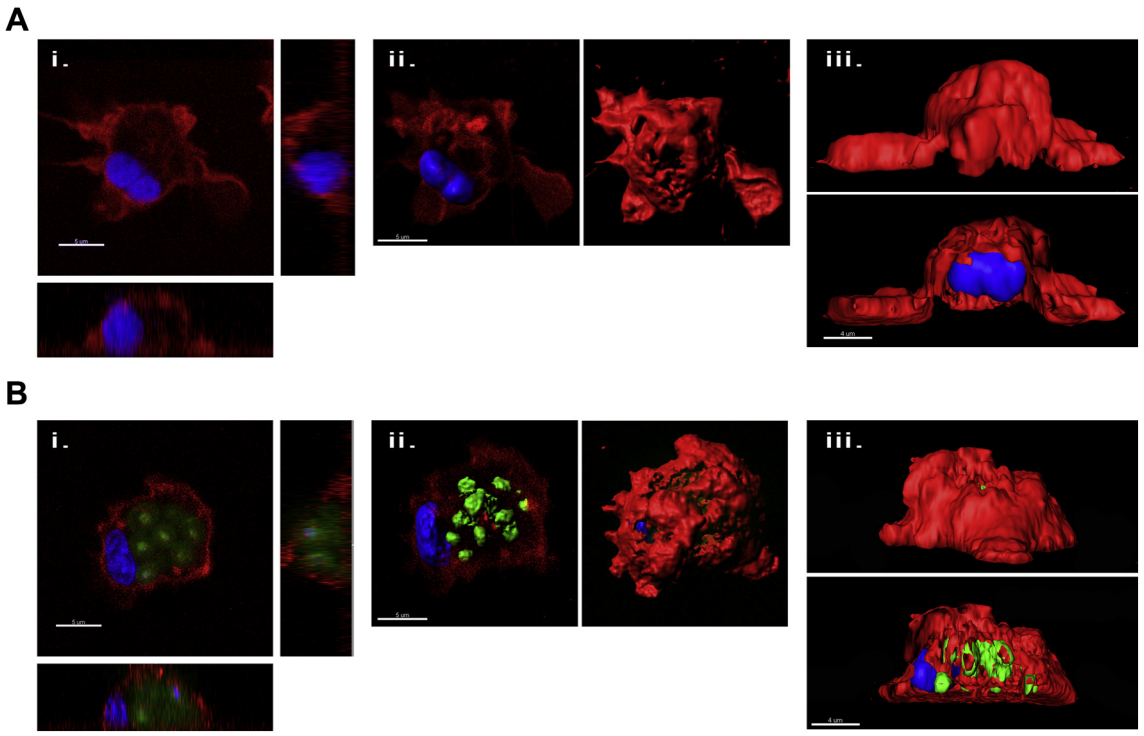

Fig. 3. Representative confocal images of eel macrophage-like cells. (A) Control cells and (B) eel macrophage-like cell after zymosan uptake. CellMask™ (red) was used for plasma membrane staining and Hoechst 33342 (blue) for nuclei labelling. (i) orthogonal view of a single cell. (ii) On the left, a combination of Z′ stack with nuclei and zymosan particles masking, on the right, a 3D reconstruction of the whole cell. (iii) On the top, a 3D longitudinal reconstruction of the cell, on the bottom a longitudinal plane of the digitalized image. i–ii: 5 μm, iii: 4 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

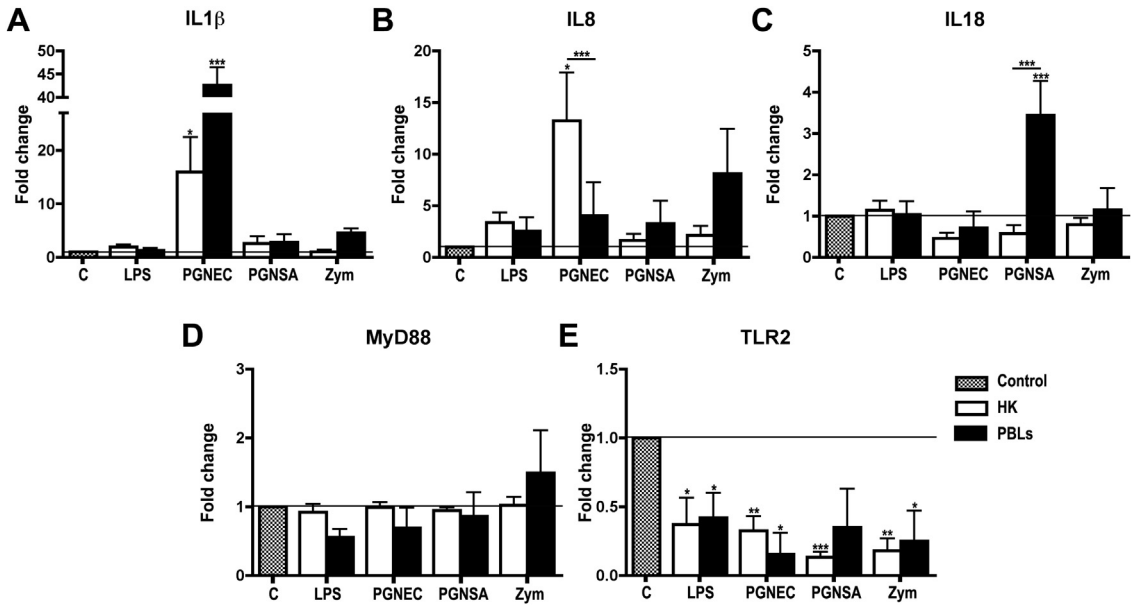

Fig. 4. Gene expression analyses of immune-related mRNAs after 12 h of stimulation with different PAMPs; 10 μg/ml of peptidoglycan from *E. coli*, *S. aureus* and of zymosan from *S. cereveisae*, and 50 μg/ml of lipopolysaccharide from *E. coli*. (A) IL-1β, (B) IL-8, (C) IL-18, (D) MyD88 and (E) TLR2. Statistical analyses ($P < 0.05$) were carried out using a one-way analysis of variance (ANOVA) followed by the post hoc multiple comparison by Bonferroni's, significant differences relative to control are denoted * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$); significant differences between PBL and head kidney derived cells are marked with a horizontal line (y axis = 1) denotes normalized control values.

from the early 90's onwards several macrophage-like cell cultures from different fish species have been developed, reviewed recently by Forlenza et al. [18] including goldfish [19,20], rainbow trout [14], seabream [21,22] and cyprinid fishes [23].

For the development of a macrophage-like primary cell culture from European eel, first of all, we tried to obtain cells from head kidney homogenates as the kidney is the primary haematopoietic tissue in fish [24,25]. However, due to the morphology of the eel head kidney, composed of a pair of thin filaments that run parallel to the body [26], the obtention of tissue in adequate quantities by dissection is difficult and the cell yield is very low. Differentiated macrophages-like from head kidney homogenates were obtained after 3 days *in vitro* however due to low yields analytical studies are both laborious and limited. As an alternative source of macrophages [27], we have developed a macrophage-like cell culture from circulating monocytes obtained from two consecutive rounds of density gradient purification from blood leucocytes (PBLs) that produces differentiated macrophages-like after 48 h of culture. These cells present a characteristic macrophage heterogeneous phenotype where cells are amoeboid with elongated spindle-like lamellipodial extensions, furthermore, nuclei presents a kidney-shaped morphology, commonly described for macrophage cells. Monocytes originate in the head kidney from a common myeloid progenitor and are released into the peripheral blood, where they circulate for several days. These cells are in a specific differentiated functional state and therefore require shorter incubation time in culture in comparison to HK derived cells to display the characteristic macrophage morphology and phenotype. Heterogeneity of macrophage cell cultures across all vertebrates has been extensively reported and our cell culture conforms to reported values [18,28].

A characteristic function of macrophages, is their phagocytic capacity that is shared with granulocytes [29]. Many reports have described the ability of mononuclear phagocytes to uptake different pathogens. Phagocytosis of polystyrene beads was demonstrated in leukocytes purified from both head kidney and blood [30,31] and yeast and bacteria uptake was enhanced by cytokine stimulation in different teleost species [32–36]. Our results show that PBLs derived cells are able to successfully phagocyte both, gram negative (*E. coli*) and gram positive (*S. aureus*) bacteria, yeast (*S. cerevisiae*) and polystyrene beads. HK-derived cells also phagocyte (A Callol, personal observation) however due to the small cell numbers these cultures are not adequate for flow cytometry studies.

In mammals different physiological roles have been described for macrophages that are dependent upon the cell developmental stage and environment [28]. Our results indicate that in macrophage-like cells derived from different compartments, HK *versus* PBL, cellular functions may also be distinct. In order to explore this we analysed the gene expression of key molecules within the inflammatory pathways in the two cell populations. The TLR pathways provide excellent routes to study the activation of the innate immune system and its linkage to the adaptive response [37,38]. TLR2 could be considered as one of the most versatile PRRs as it may function alone as a homodimer or be linked to TLR1 or TLR6 as a heterodimer [39]. This promiscuity confers the capacity to recognize more than one PAMP for example peptidoglycans, lipoteichoic acids and lipopeptides [5,40]. After binding, a series of mechanisms are triggered via activation of the MyD88 cytoplasmatic adaptor protein to induce the expression of several cytokines and chemokines [41–43]. European eel TLR2 mRNA appears to respond to PAMP stimulation by a down-regulation, similar to results obtained *in vivo* in immune-related tissues of catfish [44] and indian major carp [43]. It has been described in other fish species an up-regulation of TLR2 prior to a down regulation in response to a bacterial infection [43,45]. At 12 h post stimulation TLR2 could be down regulated right after an initial up regulation, moreover, our data support the hypothesis that TLR2 in eels would recognize PAMPs from different pathogens. However, further studies are necessary. Hence, our results suggest the involvement of eel TLR2 in bacterial and fungal PAMP-mediated recognition. The functional activity of TLR2 via MyD88 has been widely described, and its essential role to trigger the synthesis of cytokines and chemokines has been well reported. However, our results demonstrate that MyD88 mRNA abundance remains unchanged at least at 12 h post stimulation with all PAMPs tested.

Activation of monocytes/macrophages is dependent upon PAMP-PRR interaction leading to the production of pro-inflammatory cytokines such as IL-1β and IL-18 amongst others and these cytokines are released as inactive precursors. Consequently these cytokines are regulated at two distinct levels, transcriptional rate as pro-IL-1β and pro-IL-18 mRNAs and post-translational processing mainly via the caspase-1 dependent pathway [46], although it is not always required [47]. Furthermore, secretion of specialized cytokines with chemoattractant functions are essential to maintain and regulate the immune response, IL-8 (also known as CXCL8) was the first described in bony fish and therefore, the most studied [48–50]. In our studies the reported differences in response to PAMPs at the level of cytokines in macrophage-like cells from distinct origins likely reflect diverse differentiation stages prior to culture. The functional status of a cell related to the microenvironment of the tissue of origin could influence the response of the differentiated cultures to challenge as well as, the required time to phenotypic differentiation [28]. This could be reflected in our results where PBL derived macrophages-like cells show a significantly higher up-regulation of effector cytokines, IL-1β and IL-18, suggesting a more mature phenotype whereas head kidney derived cultures exhibit a higher up regulation of the chemokine IL-8 suggesting an earlier recruitment/monocyte type response. Disparity of cytokine mRNA abundance have been previously reported during rainbow trout macrophage differentiation after PAMP stimulation [51] as well as in human monocyte/macrophage for TNF-α and IL-1β [52,53].

In general terms, both cell cultures have a stronger response to peptidoglycans (PGN), whether from gram negative or gram positive when compared to LPS response. The unresponsiveness of fish macrophages to LPS has been previously described and also have a considerably weaker systemic response when compared to mammals [54]. However, secretion of TNF-α was demonstrated after LPS stimulation indicating that mRNA transcription is not essential for an effective immune response over short time periods [55]. Recently, the role of peptidoglycans as key mediators of inducible cytokine expression forming the major stimulatory component of LPS preparations has been shown in trout macrophages [56]. Interestingly, in eel macrophages-like cells the most significant PAMP-induced changes were noted using a PGN challenge. The structure and architecture of peptidoglycan have been widely described and a high structural diversity that is dependent on the bacterial species reported [57]. Furthermore, in parallel to inter-species differences in PGN structure differences in inflammatory outcomes have been shown in trout macrophages treated with PGNs from different bacterial serotypes of *E. coli* [58]. Our results show that PGN from both gram positive and negative bacteria acts strongly to induce cytokine expression in eel macrophages-like and support the hypothesis that the origin of the PGN provokes differential activation patterns and this could be related to the tissue origin and differentiation status of the culture.

In conclusion, we have developed an *in vitro* model of eel macrophages-like cells as a valuable tool for further work on the immune system and its response in eels. The establishment of a valid macrophage-cell like model in which functional and transcriptomic studies can be carried out will greatly contribute to our knowledge of immune responses in eels. Furthermore this cell culture system will provide a critical resource to identify molecular tools and provide a useful model to further study specific eel–pathogen interactions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2013.07.037.

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