Fish and Shellfish Immunology 91 (2019) 172–179

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Leucocyte integrins, but neither caspases nor NLR inflammasome are associated with lipopolysaccharide recognition and response in barramundi (*Lates calcarifer*)

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ARTICLE INFO

Keywords:
Caspase
Integrin
Leucocyte
Lipopolysaccharide
NLR
Inflammasome

ABSTRACT

The inflammatory response of fish to LPS is subdued, attributed to absence of TLR4, a key pro-inflammatory receptor for LPS in mammals. Nevertheless, LPS is processed in fish in a T-independent manner and is a protective antigen in fish vaccines, yet pathways for processing LPS in fish remain to be elucidated. Here, we report that caspases and NOD-like receptor inflammasomes typically responsible for LPS recognition and processing in mammals lack critical domains or are absent in barramundi (*Lates calcarifer*). On the other hand, leucocyte integrins MAC-1 and LFA-1 were detected on the surface of neutrophil- and lymphocyte-like cells respectively in the barramundi spleen by immunocytochemistry, and leucocytes displaying MAC-1 or LFA-1 bound to Factor X and ESM-1 respectively. Exposure to MAC-1 and LFA-1 induced significant IL-1β expression post-stimulation with LPS compared to unstimulated and isotype controls, but the differences observed in TNF-α expression were inconclusive. Our findings implicate MAC-1 and LFA-1 involvement in immune processing of LPS in barramundi and in antigen processing in fish.

1. Introduction

Teleost fish live in permanent contact with relatively high concentrations of bacteria compared with their terrestrial counterparts. A large-scale metagenomics survey by the Tara Oceans project identified that more than 90% of bacteria found in global oceans are gram-negative [1]. With the exception of the Chloroflexi, all gram-negative bacteria are didermic, comprising an internal cytoplasmic membrane, a thin peptidoglycan layer and an outer membrane comprising lipopolysaccharides (LPS) [2]. Consequently, fish epithelial and barrier surfaces are continuously exposed to LPS. LPS is typically divided into three structural sections: lipid A, core polysaccharide and repeating O-antigen units [3]. The LPS lipid A, or endotoxin, is the region of LPS that is recognised by the innate immune system and is highly stimulatory, even at low doses [4]. LPS recognition in mammals occurs primarily through the toll-like receptor (TLR) 4, which is present on an array of phagocytic immune cells including antigen-presenting cells (APCs) [5]. Briefly, the lipopolysaccharide binding protein (LBP) mediates the interaction between LPS on the bacterial cell surface and the glycoprotein CD14 on phagocytic cells [6]. CD14 then concentrates LPS to facilitate its binding to the TLR4/myeloid differentiation protein 2 (MD-2), which in turn triggers the inflammatory cascade [7]. Lipid A is an essential component of gram-negative bacteria, but it is also highly variable, which can affect its detectability by the immune system [4]. In fact, there seems to be a correlation between TLR4 recognition of bacterial lipid A and the severity of a disease, with a lipid A poorly recognised by TLR4 more likely to cause severe disease (reviewed in Ref. [4]). In mammals, including humans, lipid A encountered during infections of the bloodstream often causes endotoxic shock, a general inflammatory response which is characterised by fever, hypotension and eventually organ failures that can lead to death [8]. Fish, on the other hand, seem to be resistant to endotoxic shock caused by LPS [9].

Fish show an attenuated regulation of inflammatory cytokines using standard LPS dosages employed in mammalian models, or require approximately 1000 fold higher LPS concentrations to induce a response similar to that observed in mammals (reviewed in Ref. [9]). Recently, advances in bioinformatics have established that TLR4 is absent from most of the published genomes and immune transcriptomes from

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https://doi.org/10.1016/j.fsi.2019.05.015
Received 15 March 2019; Received in revised form 8 May 2019; Accepted 8 May 2019
Available online 16 May 2019

teleost species [9–11] and, that when present (in *Danio rerio* and *Gobiocypris rarus* for example), the other molecules necessary for recognition of LPS through TLR signalling (LBP, CD14, and MD-2) were absent and/or truncated, non-functional [9]. However, there is evidence of LPS-induced cytokine production in fish [12,13], which suggest that other molecules are likely involved in LPS recognition and processing in teleosts. In previous work, LPS stimulation induced TNFα transcription through the C-type lectin Mincle in barramundi, but seemed to induce IL-6 transcription through other molecular pathways [11]. Thus, there is potential for alternative LPS receptor families, including inflammatory caspases and leukocyte integrins, to be involved in barramundi leucocyte activation.

Caspases are cysteine proteases that mediate cell death and inflammation in mammals [14,15]. Caspases are composed of a CASc domain (comprised of a large p20 and a small p10 subunit), as well as a variable pro-domain. Typically, in humans, caspases can be grouped into three sub-categories: cell death initiators, which possess a double death effector domain (DED) motif pro-domain or caspase activation and recruitment domain (CARD) motif pro-domain (caspase-2, -8, -9 and -10); cell death effectors, which only have a short pro-domain (caspase-3, -6 and -7); and inflammatory, which normally possess a CARD motif pro-domain (caspase-1, -4, -5, and -12). Caspase-4/5, in conjunction with caspase-1/NOD-like receptor (NLR) inflammasomes, are potent contributors to pyroptosis (a type of cell death), mediating the activation of the inflammatory cytokine IL-1β [16,17]. Inflammatory caspases have also been shown to stimulate the transcription of nuclear factor-κB (NF-κB), which in turn promotes the transcription of other inflammatory cytokines such as interferons, tumour necrosis factors and interleukins (IL)-6 and −8 [18].

Leukocyte integrins are transmembrane heterodimeric glycoproteins found on the surface of leukocytes and play a role in several cellular interactions associated with immune functions. The main integrins expressed on leucocytes, β_2-integrins, are composed of a unique α subunit (CD11a, CD11b, CD11c or CD11d), which is non-covalently attached to a common β_2 subunit (CD18) [19,20]. Both CD11b/CD18 (MAC-1) and CD11c/CD18 (p150/95) have been identified as LPS receptors in mammals [19,21]. MAC-1 is the most abundant integrin on neutrophils and is also found on natural killer (NK) cells, fibrocytes, B- and T-cells, whereas p150/95 is primarily found on myeloid dendritic cells and macrophages, although it is also found on NK, B- and T-cells [20]. Moreover, the identification of LPS binding sites on CD18 suggests that β_2-integrins are able to directly bind and process LPS, translocating NF-κB to the nucleus and inducing inflammatory cytokine release [19,21,22]. More specifically, MAC-1 has been shown to enable LPS uptake and subsequent inflammatory pathway activation independently of TLR-4 signalling [23] and p150/95 has been shown to activate a cellular response after binding to LPS in a CD14-independant manner [19].

In the present study, putative barramundi caspases, NLRs and leukocyte integrins were investigated in the barramundi immune transcriptome [11]. Barramundi inflammatory caspases, inflammasomes and leukocyte integrins were identified and characterised, providing further insight into the processes underlying LPS recognition in *Lates calcarifer*.

2. Materials and methods

2.1. Experimental animals and husbandry

Barramundi (*L. calcarifer*) juveniles of approximately 30–50 g were obtained from Australian Native Finfish, Burpengary, Queensland and transported by road to the University of Queensland. Fish were acclimatised for 2 weeks in a recirculating system of eight 84 L cylindrical food-grade plastic tanks with individual aeration, all connected to a 260 L sump equipped with a protein skimmer and a bio-filter. The water temperature was maintained at 28 ± 2 °C by means of 2 × 2 kw submerged thermostatically controlled heaters and salinity at 15 parts per thousand (ppt), checked by conductivity meter (Thermo, Australia) and corrected for evaporation by adding dechlorinated (in-line activated carbon column) tap water. Water quality was checked regularly for ammonia, nitrite, nitrate using aquarium test kits (API, Australia) and pH with a pH meter (Thermo, Australia), with water exchanges applied as required. Fish were fed to satiation twice daily with a commercial diet for barramundi (Ridley Aqua Feed). Fish were graded (segregated into different tanks by size) weekly to prevent cannibalism until they reached around 80–90 mm in size, after which they were distributed into their experimental groups.

Resource	Source	Identifier
Antibodies		
goat-anti-rabbit IgG conjugated with Alexa Fluor 488	ThermoFisher	A-11034
polyclonal rabbit IgG anti-ITGAL	Aviva Systems Biology	OAAN00447
polyclonal rabbit IgG anti-ITGAM	Aviva Systems Biology	OAAN00404
Chemical		
Lipopolysaccharide from E.coli O111:B4	Sigma	L3034
ProteinPeptide		
Bovine Factor X, native protein	ThermoFisher	RP-43073
Collagen from rats tail	Sigma	C7661
ESM-1, recombinant human	ABCAM	ab182825
Fibronectin from Bovine Plasma	Life technologies (Gibco)	33,010–018

2.2. Bioinformatics analysis

All sequences used in the paper were derived from the Transcriptome Shotgun Assembly project PRJNA307522, deposited at DDBJ/ENA/GenBank under the accession GHJR00000000. The version described in this paper is the first version, GHJR01000000. Original reads are also available for additional curation of the assembly under Accession numbers: SRR3170685, SRR3170702, SRR3170703 and SRR3170704 [11]). Several caspases, β_2-integrins (both α and β subunits) and NLRs were identified by homology in barramundi through local Basic Local Alignment Search Tool (BLAST), using human, murine, crustacean and teleostean caspases sequences (Table S1, S2 and S3 for caspases, NLRs and integrins respectively). Phylogenetic relationships were inferred by maximum likelihood from alignments of barramundi cDNA sequences from the transcriptome, with human sequences listed in Table S1 for caspases and with the cDNA sequences listed in Table S3 for β_2-integrins. Trees were inferred in MEGA6.06 using ClustalW nucleotide alignment (codon) and supported by 2000 bootstrap replicates. NLR phylogeny was performed using only the proteins' NACHT domain, identified using the National Center for Biotechnology Information (NCBI) CD-search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Only proteins with a nucleotide-binding and oligomerization domain (NACHT domain) containing the conserved motif G-(4X)-GK-(10X)-W at the N-terminus were used for phylogenetic analysis (Table S2), using the Minimum Evolution method with bootstrap replication (2000) in MEGA6.06, after ClustalW amino-acid alignment [24]. The protein domains were predicted using a combination of SMART (http://smart.embl-heidelberg.de) and CD-search tool on the NCBI website, using each corresponding protein sequence. Functional categorisation for caspases was based on current literature on human and teleostean caspases [14,15].

2.3. Immunohistochemistry and fluorescent microscopy

Glass coverslips were sterilised using ethanol and a flame, and were placed in each well of a 24-well plate. The coverslips were then coated overnight at 4 °C with either 20 μg/mL fibronectin, ESM-1, Factor X or

collagen, or with undiluted Poly-L-Lysine or 1% BSA as negative controls. Spleens from three fish were homogenised into cell suspensions as previously described [11] and leucocytes were isolated on a Percoll gradient as adapted and modified from Tumbol, Baiano and Barnes [25]. Briefly, the cell suspension was layered over a 34%–51% discontinuous Percoll gradient and centrifuged for 30 min at RT (800 *x g*, acceleration 6, brake 0, Eppendorf 5810R). The buffy layer between the two Percoll densities was collected and washed twice in PBS by centrifugation (400 *x g*, acceleration 9, brake 9, RT, Eppendorf 5810R), before being resuspended in L-15 with 5% heat inactivated (56 °C, 30 min) barramundi serum (obtained from barramundi blood, allowed to clot overnight at 4 °C and recovered by centrifugation 10,000 x *g*, 5 min [26]) and incubated in a 6-well plate overnight at 28 °C. The next day, the 24-well plate containing the cover slips was washed thrice in PBS and blocked with 1% BSA at 37 °C for 1 h. Cells incubated overnight were washed in PBS to remove serum and concentration was adjusted to 2×10^6 cells/mL and the cells were plated into the 24-well plate. The plate was incubated for 4 h at 28 °C before the coverslips were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C.

The coverslips were then washed three times in PBS and permeabilised in Triton X for 3 min at RT, before being washed again thrice in PBS. Coverslips were then blocked in 1% BSA for 1 h, and following a further wash in PBS, they were incubated with either rabbit-anti-ITGAM or rabbit-anti-ITGAL primary antibodies at 1:100 or with PBS as a negative control for 4 h at RT. After a further three PBS washes, coverslips were incubated with goat-anti-rabbit IgG conjugated with Alexa Fluor 488 (1:500) in the dark at RT. After 1 h, DAPI was added at 5 μg/mL and the coverslips were incubated for a further 30 min for a total incubation time of 1 h 30 min.

Coverslips were viewed with an Olympus BX41 epifluorescent microscope. Images were captured with an Olympus DP26/U-CMAD3 camera and optimised with the imaging software CellSens (Olympus Optical Co. Ltd, Japan).

2.4. Adhesion assay

Following the identification of leukocyte integrins, a cell adhesion assay was performed to support the presence of MAC-1 and LFA-1 on barramundi splenocytes. Leukocyte integrins share common ligands but fibronectin, ESM-1 and Factor X were identified as specific ligands for the αD, αL and αM subunits respectively [27,28]. Collagen was used as a positive control as it has been identified as a ligand for the αL, αM and αX subunits [20]. Briefly, wells of a high-binding 96 well plate was coated overnight at 4 °C with either 100 μL of 20 μg/mL fibronectin (from bovine plasma, Sigma), ESM-1 (recombinant human (ABCAM ab182825)), Factor X (from bovine plasma, Sigma) or collagen (type I, from rats tail, Sigma), or with 100 μL undiluted Poly-L-Lysine solution or 1% BSA (globulin-free) as negative controls (both from Sigma). The wells were then washed three times in 1 M phosphate buffer saline (PBS), pH 7.4 before blocking for 1 h at 37 °C with 1% bovine serum albumin (BSA) to prevent non-specific binding to the plastic. Half the wells containing ESM-1 and Factor X were simultaneously incubated with antibodies specific for Integrin αM (polyclonal rabbit IgG anti-ITGAM antibody, OAAN00404, Aviva Systems biology) and integrin αL (polyclonal rabbit IgG anti-ITGAL antibody, OAAN00447, Aviva Systems biology) subunits respectively (diluted 1:100), as supplementary controls. After the wells were washed thrice in PBS, cells were isolated from spleen by passing the organ through a 100 μm mesh and subsequently washed in RPMI by centrifugation (300 *x g*, room temperature (RT), 5 min). The cells were then resuspended in RPMI at 10^6 cells/mL and seeded at 100 μL/well. After 1 h incubation at 28 °C, the wells were inverted to remove the media and non-adherent cells before being washed twice using 300 μL of ice-cold 1 M PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The cells were then fixed and permeabilised using ice-cold methanol for 10 min at RT. After three washes in 1 M PBS, the wells were stained at RT for 10 min using a crystal violet solution (0.5% w/v crystal violet in 20% ethanol). After three washes by immersion in deionised water, the crystal violet retained was dissolved using 150 μL of 100% methanol for 15 min at RT and quantified by absorbance at 590 nm with a Fluostar Optima plate reader (BMG Labtech, Melbourne, Australia). This plate assay was repeated on three separate occasions using a different fish each time and data collated for statistical analysis.

2.5. Inflammatory cytokine regulation assessed by qRT-PCR

TNFα, IFN-α, NF-κB, IL-1β and IL-6 were chosen for assessment by qRT-PCR due to their inducible nature by LPS through processing by β-integrins [19,21,22]. Complete cDNA sequences for cytokine and reference genes were derived from the assembled barramundi transcriptome (Assembly version GHJR01000000). Spleens were sampled aseptically from three healthy juvenile barramundi and a cell suspension was obtained as described above. Cells were incubated with ultrapure LPS (*E. coli* 0111:B4, Sigma L3034) at 0.05 μg/mL for 1 h at 28 °C. Blank controls consisted of cells alone, antibody controls consisted of cells incubated with 1 μg/mL of polyclonal rabbit IgG anti ITGAM or polyclonal rabbit IgG anti ITGAL blocking antibody for 1 h at 28 °C followed by a 1 h incubation with 0.05 μg/mL of LPS at 28 °C. Isotype controls consisted of cells incubated with 1 μg/mL of polyclonal rabbit IgG for 1h at 28 °C followed by a 1 h incubation with 0.05 μg/mL of LPS at 28 °C. Cells from all treatment groups were then harvested in RNAlater. For each sample, RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Contaminating genomic DNA was removed by on-column digestion with the RNAse-Free DNAse set (Qiagen) and the resulting RNA was converted to cDNA from a total of 12 ng of starting RNA per sample using the QuantiTect Reverse Transcription kit (Qiagen). Subsequently, the cDNA was used to assess the relative expression of TNFα, IFN-α, NF-κB, IL-1β and IL-6 by qRT-PCR, using elongation-factor-1-α and Syk as normalisers, as they present the most stable expression levels across treatments and presented similar efficiencies (within 10% of target genes and of each other), on a ABI-ViiA7 cycler (Applied Biosystems). The primers were designed to span across exons when possible to eliminate gDNA amplification (Table 1) with amplicon sizes of approximately 200 bp. After primary optimisation, only TNFα, NF-κB and IL-1β expression levels were assessed, as the primers for IFN-α and IL-6 could not be optimised to the template. Gene expression was quantified in 10 μL reactions using SYBR chemistry on the ViiA7 qPCR systems. Reaction mixtures were prepared with the epMotion 5075 Robot to avoid pipetting errors in 384-well PCR plates, and thermal cycling was performed by the ABI ViiA7. Primer and input cDNA per reaction were optimised at 5 μM and 2 ng respectively. In each reaction, there was 0.5 μL of forward primer, 0.5 μL of reverse primer, 5 μL of SYBR Green, 2 μL of cDNA (1 ng/μL), and 2 μL of nuclease free water. Cycling parameters were: 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 57 °C for 20 s and 60 °C for 20 s, then a final melt curve at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. All temperature cycling was performed with acceleration at 1.6 °C/s.

Table 1
List of primers for qRT-PCR. Sequences are derived from Shotgun Transcriptome Assembly version GHJR01000000.

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
NF-kappaB	TGAAAGAGGCCAAGGAGCTG	CGCACGTCTTATCCATCCGA
IL1B	ACAACGTCATGGAGCTCTGG	TCTTTGTCCTTCACCGCCTC
INFA	TCAAGAGACTGTCAGGCCAC	GTGAGCAGAGATGAAACCAGC
IL6	CAGCTGACTGCCGTGATAAA	TCCAGGGTTCCTCATCTTTG
TNFA	GCCATCTATCTGGGTGCAGT	AAAGTGCAAACACCCCAAAG
18S	CGCCTGAATACCGCAGCTAG	AGAACGGCCATGCACCACCAC
EF-1α	AAATTGGCGGTATTGGAAC	GGGAGCAAAGGTGACGAC

2.6. Statistical analysis

Prior to qRT-PCR analysis, the stability of the internal control genes was assessed and the relative expression for each gene was computed using the Relative Expression Software Tool (REST) [29–31]. Data from the adhesion assay were analysed using multiple T-tests in GraphPad Prism, with statistical significance determined using the Holm-Sidak method, with alpha = 0.05. Each row was analysed individually, without assuming a consistent SD. Data from qRT-PCR were analysed with REST [30].

2.7. Ethics statement

All animal work was conducted in accordance with Animal Care and Protection Act and the NHMRC Code of Practice. Work was conducted under the University of Queensland Animal Ethics Committee Approval No. NEWMA/078/15 "Understanding the early onset of adaptive immunity in fish."

3. Results

Putative barramundi caspases were identified by homology, and their gene organisation was determined (Figure S1). In most cases, barramundi caspases grouped clearly with their human counterpart (Fig. 1), with apoptosis initiator and effector caspases 8/10 and 3, 6 and 7 respectively present and structurally complete in barramundi (Fig. 1C). However, while some barramundi caspases had several isoforms (caspase 3 and caspase 2) the inflammatory caspase 4 and the differentiation caspase 14 were not identified in barramundi (Fig. 1A, C). Moreover, although identified by homology in barramundi, caspase 5 and caspase 9 were lacking a CARD pro-domain, thus differing from their human counterparts (Fig. 1A and B).

Putative barramundi NLRs were identified by homology and grouped with their human and murine counterparts (Fig. 2), and their gene organisation was determined (Figure S2). Out of the three distinct NLR sub-families (nucleotide-binding oligomerization domain (NOD), NLR family, pyrin domain containing (NLRP) and Ice protease-activating factor (IPAF)) [17], only sequences coding for protein from the NOD sub-family were identified in barramundi, with members from the NLRP and IPAF families lacking. Although most protein domains were conserved between human, mouse and barramundi NLRs, some mouse and barramundi proteins were lacking either the pyrin domain (PYD) or caspase activation and recruitment domain (CARD) at the N-terminus or were lacking leucine-rich repeat (LRR) domains at the C-terminus (Fig. 2).

Putative barramundi α- and β2-integrin subunits were identified by homology with their human, murine and teleost counterparts (Fig. 3), and their gene organisation was determined (Figure S3). Out of the four possible α subunits forming leukocyte integrins (D, M, L and X), only two were identified in barramundi: M (forming MAC-1) and L (forming LFA-1). Both include a Von Willebrand factor type A, which is required for metal ion ligand binding, and several β-propellor repeats. Moreover, the putative barramundi integrin αM identified was missing a transmembrane domain, which suggests that integrin αM may be secreted rather than membrane bound in barramundi.

When incubated with substrates specific to the D, M and L α-subunits, barramundi spleen leucocytes did not bind significantly to any of the negative controls or to fibronectin (specific substrate for integrin αD subunit) but did bind significantly to Factor X and ESM-1 (specific substrates for integrin αM and L respectively) (Fig. 4). Interestingly, when incubated with anti-ITGAM antibody, cells from barramundi spleen were more adherent to the integrin αM substrate Factor X (Fig. 4). Similarly, cells incubated with anti-ITGAL antibody were more adherent to integrin αL substrate ESM-1 (Fig. 4). The numbers of barramundi spleen leucocytes bound to the positive control, collagen (specific substrate for integrin αM, L and X subunits), was also significantly higher than BSA-coated control. When the adherent cells were observed by microscopy, the cells binding to Factor X were larger and more granular, resembling granulocytes whereas cells binding to ESM-1 were rounded and slightly smaller, resembling lymphocytes (Fig. 5).

To determine whether integrins might be involved in LPS recognition and response in barramundi, a stimulation assay was conducted and the expression of a cohort of cytokines determined by qPCR in the presence and absence of anti-ITGAM, anti-ITGAL or isotype control (rabbit IgG) antibodies. Pre-incubation of cells with either anti-ITGAM or anti-ITGAL increased expression of IL-1β through the activation of integrin αM and L respectively, while the isotype control had no effect (Fig. 6A). Stimulation of leucocytes with LPS upregulated the expression of IL-1β and, to a lesser extent, TNFα, but did not impact the expression of NF-κB (Fig. 6B–D). Pre-incubation with anti-ITGAM and anti-ITGAL significantly upregulated the expression of IL-1β compared to unstimulated and isotype controls (Fig. 6A). However, TNFα expression was significantly upregulated by both anti-ITGAM and anti-ITGAL antibodies, as well as by the rabbit IgG isotype control compared to the unstimulated control, suggesting that changes in TNFα

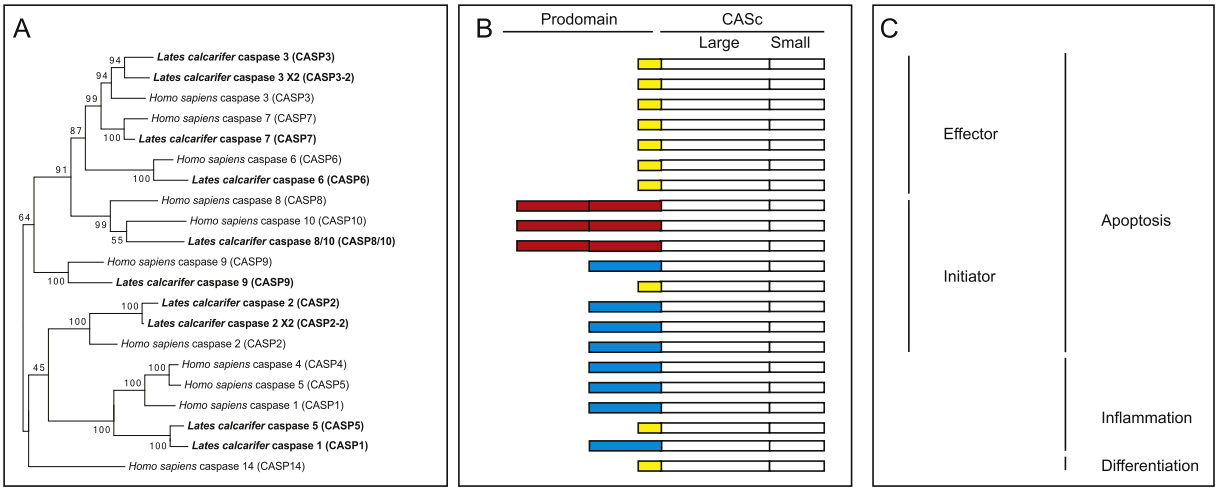

Fig. 1. Characterisation of human and barramundi caspases. (A) Phylogenetic relationship between human and barramundi (in bold) caspases are linked with (B) domain organisation of each corresponding protein and (C) functional categorisation based on literature. Yellow boxes correspond to a short pro-domain; Red boxes correspond to a DED (death-effector domain) protein domain; Blue boxes correspond to a CARD (caspase activation and recruitment domain) protein domain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

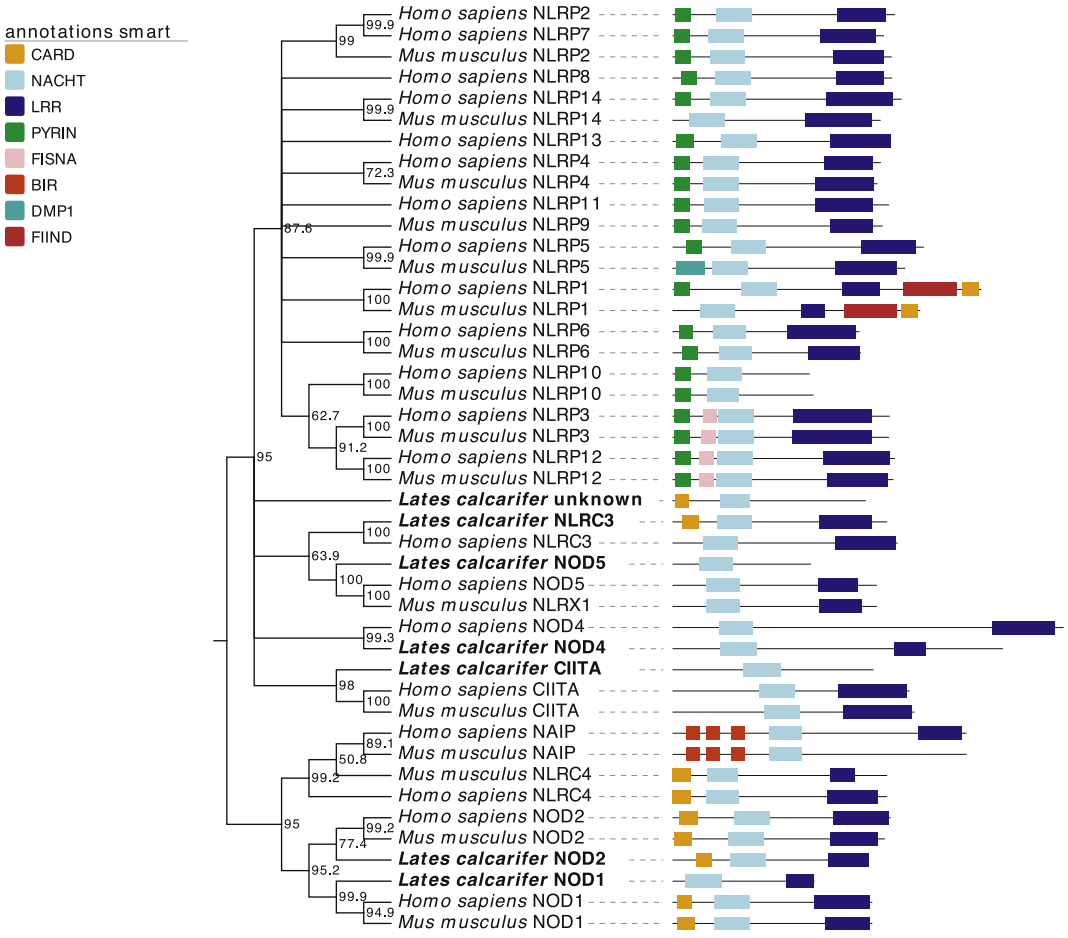

Fig. 2. Phylogenetic relationship between human, murine and barramundi (in bold) NLRs based on their NACHT domain. Protein domain organisation is depicted on the right of the phylogenetic tree. CARD: caspase activation and recruitment domain; NACHT: nucleotide-binding and oligomerization domain; LRR: leucine-rich repeat; PYR: pyrin domain; FISNA: Fish-specific NACHT associated domain; BIR: baculoviral inhibition of apoptosis protein repeat domain; DMP1: Dentin matrix protein 1; FIIND: domain with function to find.

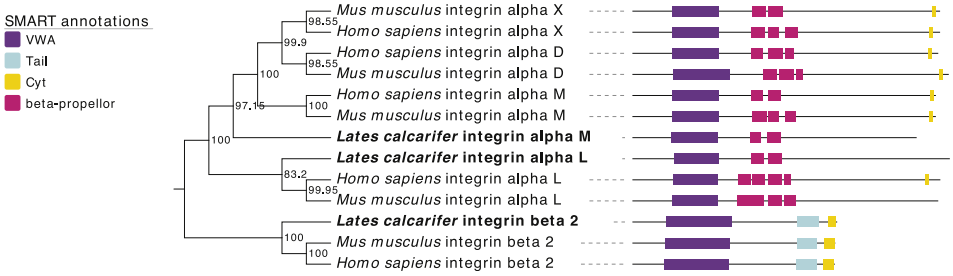

Fig. 3. Phylogenetic relationship between human, murine, teleost and barramundi (bold) α- and β2-integrin subunits. Protein domain organisation is depicted on the right of the phylogenetic tree. VWA: Von Willebrand factor type A; Tail: tail domain; Cyt: cytoplasmic domain.

expression after a short incubation with LPS do not depend on integrins αM and L activation (Fig. 6C and D).

4. Discussion

Inflammatory caspases have recently been shown to recognise and process intracellular LPS in humans (caspase-4 and -5) and in mice (caspase-11), binding to LPS through their CARD domain [16,32]. In barramundi, the inflammatory caspases −5 and −1 were identified, and grouped tightly with their human counterparts. However, the CARD domain from barramundi caspase-5 was lacking, suggesting a potential loss of function as CARD is necessary for LPS recognition and CARD oligomerisation is also necessary for caspase activation [33]. Another way for caspases to recognise PAMPs is through the coupling of caspase-1 with other specific pattern recognition molecules (mostly NLRs) through their CARD domain, forming functional inflammasomes [18,34]. As barramundi caspase-1 displayed a CARD pro-domain, inflammasomes were plausible actors in LPS recognition so NLRs and other inflammasome sensor proteins were subsequently investigated in barramundi. To date, there are six characterised NLR-inflammasomes, formed using six different NLRs: NLRP1, NLRP3, NLRP6, NLRP7, NLRP12 and NLRC4/IPAF [17,18,35]. Other molecules such as RIG-I, AIM2 and IFI16 have also been described as inflammasome sensor proteins but recognise nucleic acid, rather than carbohydrate-based ligands such as LPS [35]. None of the NLRs involved in inflammasomes were identified in *L. calcarifer* in the current study, suggesting that inflammasomes in barramundi are not involved in detecting LPS thereby implicating other pathways in LPS recognition in these fish. As

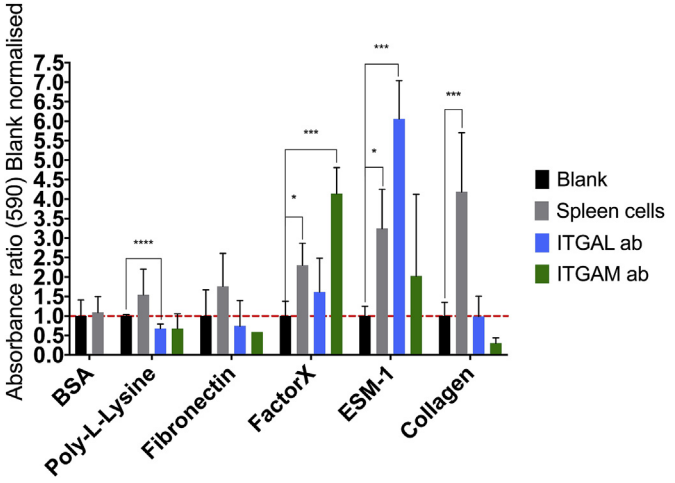

Fig. 4. Adherence of barramundi spleen cells to different substrates. The x-axis indicates the treatment of the cell culture surface of the plate. Significant differences between blank incubated and cells incubated wells are represented with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ and **** $p < 0.001$, $n = 6$ wells per treatment.

inflammasomes typically trigger an inflammatory response, the lack of NLR inflammasomes in barramundi correlates with the weak inflammatory response observed in barramundi and other fish post-stimulation with LPS [26].

Leukocyte integrins are found at the surface of white blood cells and are involved in pathogen recognition through conformational changes after activation by ligands [36]. Out of the four possible genes coding for leukocyte integrin α-subunits, two were identified in barramundi: the subunit αM (CD11b) which forms MAC-1 and the subunit αL (CD11a) which forms LFA-1. Cells incubated with Factor X, a specific ligand for the integrin αM subunit [27], bound significantly more to Factor X than to negative control substrates. This suggests that barramundi leucocytes express a protein that is conformationally similar to the αM subunit of MAC-1, hence able to bind to Factor X. The same was true for cells incubated with ESM-1, a specific ligand for the integrin αL subunit [27], suggesting that barramundi leucocytes also produce a protein with binding sites similar to the αL subunit of LFA-1. Incubation of spleen cells with anti-ITGAL or anti-ITGAM antibody increased adhesion of barramundi spleen cells to the corresponding substrates. The antibodies may activate β2-integrins by configurational change, increasing the molecules' binding strength as previously reviewed [37]. Indeed, integrin αMβ2 (MAC-1) and αLβ2 (LFA-1) can assume two conformations, open (active) and closed (inactive), which differentially recognise ligands [36,38]. Changes in configuration are crucial to integrin function and can also influence avidity and affinity of the leucocyte integrin [36,39], impacting adhesion to ligands, as detected in the adhesion assay reported here (Fig. 4). Currently, two binding sites for LPS have been identified on the β2 chain of leucocyte integrins [22]. Moreover, integrin αMβ2 has been shown to enable LPS recognition and activation independently of TLR4 [23], and processes depending on MAC-1 activation have been shown to induce the inflammatory cytokine IL-1β's expression [23,40]. When observed by fluorescence microscopy, most spleen cells that bound to Factor X expressed integrin αM (ITGAM) and resembled granulocytes, being larger and more granular than cells not expressing ITGAM, similarly to previous observations in peritoneal cells [41,42]. Almost all cells that bound to ESM-1 expressed integrin αL (ITGAL) and were more rounded, resembling B- or T-lymphocytes [20]. Considering that MAC-1 has recently been identified as a LPS receptor in mammals [23], and that barramundi leucocytes express MAC-1, it is possible that LPS in barramundi is processed through MAC-1. The expression of inflammatory cytokines following stimulation with LPS was thus investigated by qRT-PCR in barramundi spleen cells. Although the effect size (fold change in expression) was small, expression of IL-1β was statistically higher in cells stimulated by LPS, however LPS did not affect TNFα or NF-κB expression in control cells (Fig. 6). Moreover, prior incubation with anti-ITGAL and anti-ITGAM antibodies activated integrins on barramundi splenocytes evidenced by significantly increased expression of IL-1β post-stimulation with LPS when compared to either unstimulated cells or isotype control cells pre-incubated with rabbit IgG (Fig. 6B), which is consistent with conformational activation of integrins being a requirement for interaction with LPS as in mammals [23]. Pre-incubation with antibodies (both treatments as well as the isotype control) resulted in a significant upregulation in the expression of TNFα when compared to the unstimulated control, suggesting that TNFα expression might be dependent on exposure to antibody rather than on incubation with LPS. Indeed, LPS activates outside-in signalling, binding directly to the integrin receptor and activating caspase-1, resulting in IL-1β upregulation but not impacting the expression of TNFα [43]. However, before integrins can act as receptors, they need to be activated [23], which was likely effected in this case by pre-incubation with anti-ITGAL and anti-ITGAM antibodies, supporting the difference in IL-1β regulation by LPS between pre-stimulated and control cells (Fig. 6B).

The C-type lectin Mincle was previously identified as a receptor for LPS in barramundi, but other unidentified receptors were also implicated in recognition of bacterial polysaccharides in perciform fish [11]. In the current study, we show that leucocyte integrins likely have a critical role in LPS recognition and processing in *Lates calcarifer* and are worthy of further investigation throughout the Teleostei.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.015.

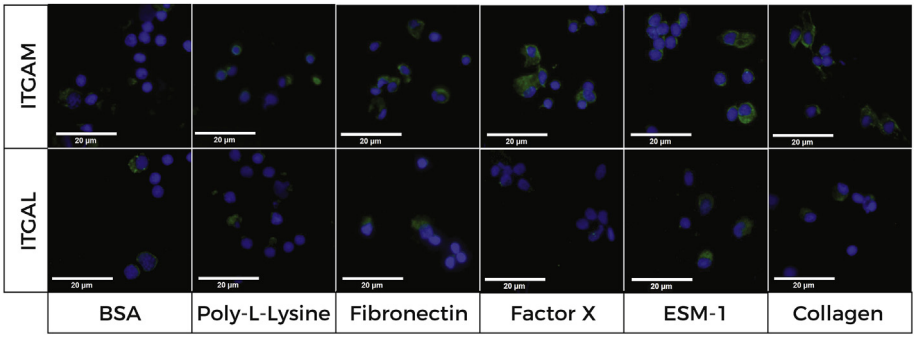

Fig. 5. Micrographs of leukocytes adhering to each substrate. DAPI stain (nucleic acid) shown in blue and anti-ITGAM or anti-ITGAL specific antibodies stain shown in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

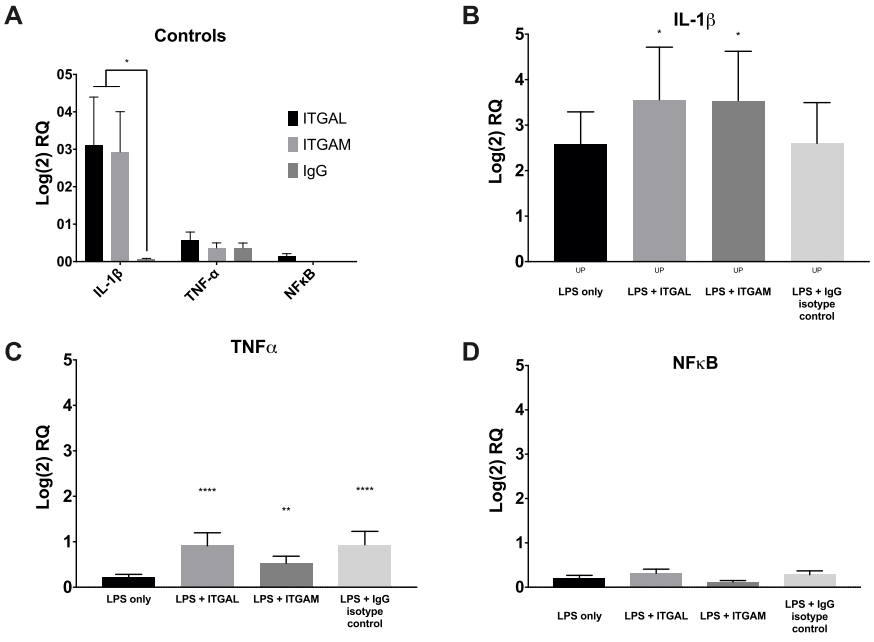

Fig. 6. Differential expression of immune genes in cells stimulated by LPS in the presence and absence of anti-integrin antibodies or isotype control. A) Direct stimulation of cells by antibody where rabbit IgG anti-ITGAL and rabbit IgG anti-ITGAM are considered treatments and polyclonal rabbit IgG is used as an isotype control. X-axis indicates the cDNA amplicon under measurement. Significant differences between treatments and control are represented with * $p < 0.05$. B) Expression of *IL1B*, (C) *TNFalpha* and (D) *NF-κB* in barramundi splenocytes is shown with or without pre-incubation with anti-ITGAL, anti-ITGAM or rabbit IgG isotype control antibody (X-axis "Treatment"). Significant upregulation (as determined by REST analysis) of each gene is indicated underneath the relevant bar by 'UP'. Significant differences between control unstimulated cells gene expression and stimulated cells are represented with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$, $n = 3$ fish.

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