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A transcriptomic and functional approach to investigation of antigen processing and adaptive immunity in Barramundi (*Lates calcarifer*)

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Abstract

Due to a constantly growing world population, food security has become an increasingly important issue. Controlling diseases in aquaculture can significantly increase food production while minimising the need for new infrastructures. Vaccination has been a major success story in the global aquaculture industry, revolutionising disease control and almost eliminating antibiotic use in modern fish farms. However, there are some infections that remain difficult to control by vaccination including diseases caused by highly variable bacterial pathogens such as *Streptococcus* species. In Australia, for example, barramundi (*Lates calcarifer*, also known as Asian sea bass) are a significant aquaculture fish but the industry is hindered by the hypervariable bacterium *Streptococcus iniae*. Antigen presentation by the cellular immune system is essential for development of a protective adaptive immune response, therefore, understanding it is critical to improve vaccine efficacy. In mammals, antigen presentation is coordinated by dendritic cells (DCs), and the recent identification of dendritic-like cells in rainbow trout and zebrafish strongly suggests the existence of those cells in teleosts. However, some antigens such as lipopolysaccharides (LPS), a bacterial sugar often used to stimulate cells in mammalian systems, do not seem to trigger a strong primary inflammatory response in teleosts. Nevertheless, it has also been shown that vaccination with LPS in fish induced the production of specific antibodies, warranting further investigation into the kinetics of antibody production in teleosts. The main objective of my project was thus to gain a better understanding of the early adaptive immunity, such as pathogen recognition, and how it links to antibody production as well as the role of dendritic cells in teleosts, using both functional *in vitro* assays and new technologies such as Illumina sequencing and bioinformatics analysis.

To do so, I used barramundi as an industry relevant study organism. Following the generation of an immune transcriptome for barramundi, pathogen recognition receptors (PRRs) were identified for a number of pathogen associated molecular patterns (PAMPs), including peptidoglycans and nucleic acids. However, the PRR mainly associated with LPS recognition, the Toll-Like Receptor (TLR)-4 was not found in the immune transcriptome, and neither were the molecules classically associated with LPS recognition through TLR-4. Potential LPS receptors were thus investigated further in barramundi. The C-type lectin receptor Mincle was identified

as a partial LPS receptor in barramundi, as LPS stimulation did induce some cytokine transcription through the Mincle pathway (Chapter 2).
Caspases and NOD-like receptor inflammasomes were also investigated but they did not seem to be conserved in barramundi, or were missing molecular domains crucial to pathogen binding, suggesting that they were not participating in LPS recognition in barramundi. Beta2-integrin, on the other hand, were well conserved in barramundi compared with mammalian and other teleosts. Moreover, barramundi cells adhered to substrates specific to those β2-integrins and were bound by specific anti-integrin antibodies, suggesting that the αMβ2 and αLβ2 integrin molecules identified in the transcriptome were folding correctly and displayed binding sites similar to those of their mammalian counterpart (Chapter 3).
Cells that were morphologically and functionally similar to DCs were identified from barramundi primary cell cultures. Those cells were shown to migrate out of the spleen and head kidney of barramundi after injection of bacterial components in the peritoneum, before returning to the spleen, where they probably induce the adaptive immune response. Interestingly though, those cells did not seem to react strongly to LPS alone (Chapter 4).
Finally, immunoglobulin was investigated further due to its role in pathogen clearance, and the genomic region coding for variable, junction and constant immunoglobulin genes was identified and annotated in barramundi. A vaccine trial with subsequent challenge was also performed to assess the kinetics and amplitude immunoglobulin production in barramundi, and different adjuvants were used to assess their potential role on the quality and quantity of immunoglobulin produced. Overall, all adjuvants increased the number of antibodies produced, and one adjuvant was identified to significantly improve the secondary production of antibodies (Chapter 5).
Globally, the present work investigated pathogen recognition, in particular LPS recognition, in barramundi as well as the onset of immune memory with a focus on the role of DCs and the mechanisms underlying immunoglobulin production. The results highlighted in this thesis suggest that barramundi, and likely other teleost species, use a combination of non-classical molecular pathways to process LPS. Moreover, it was demonstrated that the quantity and quality of antibodies could be improved with the use of adjuvants in barramundi, providing invaluable information to improve vaccines for warm water species aquaculture in the future.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed papers

Zoccola E, Delamare-Deboutteville J, Barnes AC (2015) Identification of Barramundi (*Lates calcarifer*) DC-SCRIPT, a Specific Molecular Marker for Dendritic Cells in Fish. PLoS ONE 10(7): e0132687. doi:10.1371/journal.pone.0132687

Zoccola E, Kellie S, Barnes AC (2017) Immune transcriptome reveals the mincle C-type lectin receptor acts as a partial replacement for TLR4 in lipopolysaccharide-mediated inflammatory response in barramundi (*Lates calcarifer*). Molecular Immunology, 83, 33-45

Conference abstracts

Zoccola E, Delamare-Deboutteville J, Barnes AC. Identification of Barramundi (*Lates calcarifer*) DC-SCRIPT, a Specific Molecular Marker for Dendritic Cells in Fish. Presented at the third FRDC Australasian Aquatic Animal Health Scientific Conference, Cairns, 6-10 July 2015.

Zoccola E, Kellie S, Barnes AC. *Lates calcarifer* transcriptome reveals the Mincle CLR as an alternative to the classical LPS recognition pathway. Presented at the ASFB-OCS joint conference, Hobart, 4-7 September 2016.

Zoccola E, Li A, Barnes AC. Linking antibody response and efficacy of the adaptive immunity in vaccine development: How adjuvants modulate the immune response in barramundi. Presented at the fourth FRDC Australasian Aquatic Animal Health Scientific Conference, Cairns, 10-14 July 2017.

Zoccola E, Kellie S, Barnes AC. Mechanisms of lipopolysaccharide recognition and processing in barramundi (*Lates calcarifer*). Presented at the 18th International Conference on Diseases of Fish and Shellfish, Belfast, 4-7 September 2017.

Conference abstracts for the above references can be found in Appendix 1.

Publications included in this thesis

Chapter 2: **Zoccola E**, Kellie S, Barnes AC (2017) Immune transcriptome reveals the mincle C-type lectin receptor acts as a partial replacement for TLR4 in lipopolysaccharide-mediated inflammatory response in barramundi (*Lates calcarifer*). Molecular Immunology, 83, 33-45

Contributor	Statement of contribution
Emmanuelle Zoccola (Candidate)	Conceived experiments (40%) Designed experiments (70%) Conducted the work and analysed the data (70%) Wrote the paper (70%)
Stuart Kellie	Conceived experiments (20%) Designed experiments (10%) Helped with analysis of the data (10%) Wrote and edited paper (10%)
Andrew C. Barnes	Conceived experiments (40%) Designed experiments (20%) Helped with analysis of the data (20%) Wrote and edited the paper (20%)

Chapter 4: **Zoccola E**, Delamare-Deboutteville J, Barnes AC (2015) Identification of Barramundi (*Lates calcarifer*) DC-SCRIPT, a Specific Molecular Marker for Dendritic Cells in Fish. PLoS ONE 10(7): e0132687. doi:10.1371/journal.pone.0132687

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Emmanuelle Zoccola (Candidate)	Conceived experiments (20%) Designed experiments (40%) Conducted the work and analysed the data (60%) Wrote the paper (60%)
Jérôme Delamare-Deboutteville	Designed experiments (40%) Conducted the work and analysed the data (30%) Wrote and edited paper (15%)

Andrew C. Barnes	Conceived experiments (80%) Designed experiments (20%) Conducted the work (10%) Wrote and edited the paper (25%)

Contributions by others to the thesis

Associate Professor Andrew Barnes (primary supervisor) helped to conceive and design the experiments, helped with some of the technical work, and helped to write and review the papers and this thesis. He provided guidance throughout this project. Associate Professor Stuart Kellie (secondary supervisor) helped conceive and design some of the experiments, and helped to write and review the papers and this thesis. Dr. Jérôme Delamare-Debouteville assisted with the conception and design of some of the experiments, helped with some of the technical work, and helped write and review Chapter 4. Angus Li (Honours student) conducted some of the technical work and data analysis for Chapter 5 under my supervision and helped write and review Chapter 5.

Statement of parts of the thesis submitted to qualify for the award of another degree

For chapter four, the functional and morphological assays were submitted for BMarSt Honours, The University of Queensland, 2013, degree awarded with Class I December 2013. The resulting publication was included in this thesis as most of the work which was needed for publication was conducted during the early stages of my PhD.

Zoccola E, Delamare-Debouteville J, Barnes AC (2015) Identification of Barramundi (*Lates calcarifer*) DC-SCRIPT, a Specific Molecular Marker for Dendritic Cells in Fish. PLoS ONE 10(7): e0132687. doi:10.1371/journal.pone.0132687

For chapter five, the ELISAs, avidity and immunoglobulin composition assays were performed under my co-supervision by Angus Li and submitted for BSc Honours, The University of Queensland, 2017, degree awarded with Class I December 2017. The work was included in the present PhD thesis as I designed most of the experiments and performed the main vaccination trial, as well as all the sampling on which those assays were performed.

Research Involving Human or Animal Subjects

NEWMA 078/15 Understanding the early onset of adaptive immunity in fish.

SBS/037/16/ARC/ELANCO Effective vaccination of barramundi against Streptococcosis.

SBS/056/13/ARC Cellular immunity in fish: Robust defence against infection or Achilles heel?

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Immunology, pathogen recognition, aquaculture, fish health, lipopolysaccharide processing, immune pathway, immune cells, molecular immunology

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FoR code: 0704, Fisheries Sciences, 30%

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List of Included Abbreviations

18S – 18S ribosomal RNA

α-tub – alpha tubulin

aa – amino acid

Ab – antibody

Ag – antigen

AGRF – Australian Genome Research Facility

AID – activation-induced cytidine deaminase

ANOVA – analysis of variance

APC – antigen presenting cell

BCPI/NBT – 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium

BCR – B-cell receptor

BIR – baculovirus inhibitor of apoptosis protein repeat

BLAST – Basic Local Alignment Search Tool

bp – base pair

BSA – bovine serum albumin

CARD – caspase activation and recruitment domain

CD – conserved domain

cDNA – complementary DNA

CFSE – carboxyfluorescein succinimidyl ester

CIITA – class II major histocompatibility complex transactivator

CLEC – C-type lectin domain

CLR – C-type lectin receptor

CpG-ODN – cystidine-phosphate guanosine oligodeoxynucleotides

CPS – capsular polysaccharide

CRD – carbohydrate-recognition domain

DAPI – 4',6-diamidino-2-phenylindole

DC – dendritic cell

DC-SCRIPT – dendritic cell-specific transcript

DED – death effector domain

DNA – deoxyribonucleic acid

DNP – 2,4-dinitrophenol

EAFP – European Association of Fish Pathologists

ef1α – elongation factor 1 alpha

ELISA – enzyme-linked immunosorbent assay

ELISPOT – enzyme -linked immunospot

ESM1 – endothelial cell-specific molecule 1

EST – expressed sequence tag

FAO – Food and Agriculture Organisation

FBS – foetal bovine serum

Fc – fragment crystallisable

FIIND – function to find domain

FISH – fluorescent *in situ* hybridisation

FISNA – Fish-specific NACHT associated domain

FPKM – fragment per kilo-base of transcript per million fragments mapped

GC – germinal centre

gDNA – genomic DNA

GO – gene ontology

HI – heat inactivated

HK – head kidney

ICC – immunocytochemistry

IHC – immunohistochemistry

ICE – interleukin-1beta converting enzyme

IFN – interferon

Ig – immunoglobulin

IL – interleukin

IMGT – ImMunoGeneTics

IPAF – ICE protease-activating factor

ITAM – immunoreceptor tyrosine-based activation motif

ITGAL – integrin alpha L

ITGAM – integrin alpha M

KASS – KEGG automatic annotation server

KEGG – Kyoto Encyclopedia of Genes and Genomes

KLH – keyhole limpet hemocyanin

LBP – lipopolysaccharide binding protein

LPS - lipopolysaccharide

LRR – leucine rich repeat

m.y.a – million years ago

MAPK – mitogen-activated protein kinase

MD-2 – myeloid differentiation protein 2

MHC – major histocompatibility complex

MLR – mixed leucocyte reaction

MLT – maximum-likelihood tree

MMC – melano-macrophage centre

MOI – multiplicity of infection

N – Nystatin

NAIP – NLR family apoptosis inhibitor protein

NCBI – National Center for Biotechnology Information

NF-κB – nuclear factor kappa B

NK – natural killer

NLR – NOD-like receptor

NLRC – NOD-like receptor CARD

NLRP – NOD-like receptor PYD

NOD – nucleotide-binding oligomerization domain

nt – nucleotide

OD – optic density

PAMP – pathogen associated molecular pattern

PBS – phosphate buffered saline

PCR – Polymerase chain reaction

PFA – paraformaldehyde

pH – potential of hydrogen

PI – propidium iodine

pNPP – p-Nitrophenyl Phosphate

ppt – part per thousand

PRR – pattern recognition receptor

PS – penicillin streptomycin

PTG – peptidoglycan

PYD – pyrin domain

qRT-PCR – quantitative reverse transcription polymerase chain reaction

RACE – Rapid amplification of cDNA ends

RBC – red blood cell

REST – relative expression software tool

RIG-I – retinoic acid-inducible gene I

RIN – RNA integrity number

RLR – RIG-I-like receptor

RNA – Ribonucleic acid

rpm – round per minute

RPMI – Roswell Park Memorial Institute

RT – room temperature

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SHM – somatic hypermutation

SMART – simple modular architecture research tool

SOP – standard operating procedure

spp. – species

SRBC – sheep red blood cells

TBS – tris-buffered saline

TBST – tris-buffered saline tween

TD – T-cell dependent

TDB – trehalose-6,6-dibehenate

Tfh – follicular helper T-cell

TI – T-cell independent

TigGER – Tool for Immunoglobulin Genotype Elucidation via Rep-Seq

TIR – toll/interleukin-1 receptor

TLR – toll-like receptor

TNF – tumour necrosis factor

UK – United Kingdom

UQ – the University of Queensland

USA – United States of America

V(D)J – Variable (Diverse) Junction

VNN – viral nervous necrosis

VWA – Von Willebrand factor type A

ZNF – zinc finger protein

Chapter One – General Introduction

1.1. Innate vs. adaptive immunity

1.1.1. Basic concepts

Both the innate and the adaptive immune systems, which are respectively responsible for the clearance of pathogens and the development of immune memory, have been extensively studied in mammals. The key effector cells of the innate immune system engulf non-self substances, non-specifically, and work towards breaking them down in order to clear potential infections. However, the innate immune system can be by-passed by pathogens and, as no memory of the pathogens is developed, re-infections can occur easily. The adaptive immune system, on the other hand, facilitates pathogen clearance through specific labelling of “non-self” particles and the formation of immune memory [1]. Antigen recognition and antigen-presentation are the processes that form the junction between innate and adaptive immunity, and are largely coordinated by dendritic cells (DCs). DCs are known as “professional” antigen-presenting cells (APCs), due to their ability to prime naïve lymphocytes, leading to the establishment of the immune memory [1, 2]. In brief, by presentation of specific antigen, DCs initiate maturation of naïve T-cells into either cytotoxic or helper T-cells which, respectively, lyse infected cells or activate other immune cells through cytokines release. Subsequently, B-lymphocytes, activated via interaction with the same antigen, mature into plasma cells under direction of mature helper T-lymphocytes and produce specific antibodies that label foreign and infected cells [2]. A small subset of differentiated, mature B- and T-cells with receptors specific to the antigen remain active after the infection is cleared, and thereby form the basis of immune memory [1].

1.1.2. Pathogen recognition

For any immune response to be initiated, specific structures present on foreign micro-organisms (pathogen-associated molecular patterns (PAMPs)) have to be recognised by the host’s immune system, through pattern recognition receptors (PRRs). Of the four main categories of PRRs able to successfully induce a cellular response (Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding, oligomerization domain (NOD)-like receptors (NLRs) and RIG-I-like receptors (RLRs) [3]), three have been described in fish: TLRs, CLRs and NLRs [4].

TLRs are the most commonly studied PRRs in vertebrates due to their key ability to activate inflammation. TLR-mediated pathogen recognition occurs notably through the activation of the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) and mitogen-activated protein kinase (MAPK) signalling pathways, which in turn initiate the production of pro-inflammatory molecules responsible for the activation of adaptive immunity, including tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and IL-6 [5-7]. Functional TLRs are transmembrane proteins comprising leucine-rich repeat (LRR) domains, a trans-membrane domain as well as a Toll/interleukin-1 receptor (TIR) domain [5]. TLRs can be grouped into two sub-families depending on their ability to recognise and bind either cell surface elements (microbial sugars, lipids and proteomes) or nucleic acid [8]. TLRs have been identified in a number of cartilaginous and bony fish species with high levels of homology to mammalians TLRs, providing a foundation for further investigation in fish.

Many of the lectin or lectin-like receptors found on APCs have been identified as members of the calcium dependant CLRs family [9], implicating CLRs as critical components in the onset of adaptive immunity. Indeed, pathogen recognition by CLRs is closely associated with antigen capture and presentation and several of the membrane-bound CLRs are specific to APCs, particularly DCs [10-12]. CLRs can be transmembrane or secreted proteins and use conserved carbohydrate recognition domains (CRDs) to bind sugars in a calcium dependant manner [9, 13]. Currently, two types of CLRs have been described, based on the orientation of their amino (N)-terminus and on the amount of CRDs they possess [9]. Type I CLRs (mannose receptors) have their N-termini pointing outwards of the cell and contain several CRDs at the N-terminus, whereas type II CLRs (collectins) currently identified have their N-termini pointing inside of the cell's cytoplasm and only one CRD at their carboxyl (C)-terminus [9]. CLRs have a central role in the initiation of fungal immunity in mammals (reviewed in [3]), can recognise viruses through mannose specificity [14], and also seem to play a critical part in bacterial clearance and recognition of non-self particles in marine invertebrates and vertebrates [8, 15-20]. Moreover, CLR activation seems to result in cytokine signalling either through direct nuclear factor kappa B (NF-kB) activation or through coinciding TLR activation [21]. It has also

become increasingly clear that CLRs play a part in DCs migration and in subsequent communication with lymphocytes [9], suggesting CLRs can regulate adaptive immune responses.

NLRs constitute a large family of receptors involved in bacterial and viral recognition, as well as apoptosis and immune regulation. Proteins from this family share a domain organisation, with a nucleotide-binding domain at the N-terminus (such as a caspase activation and recruitment domain (CARDs) or a pyrin domain (PYD)) and leucine rich repeats (LRRs) at the C-terminus [22]. NLRs have been classified into three distinct subfamilies, the NODs, the NLRPs (also known as NALPs) and the IPAF subfamilies, based on the phylogeny of their NACHT domain [23], but other nomenclatures have also been proposed [24]. Many NLRs have been extensively studied, in particular NOD1 and NOD2, which are found abundantly on APCs and have been shown to recognise bacterial peptidoglycan (PTG) [22]. NOD1 and NOD2 stimulated with PTG have also been shown to activate the production of inflammatory cytokines through NF-kB activation, although lipopolysaccharide (LPS), another bacterial component, seemed to greatly increase the production of inflammatory cytokines via NOD in mammals [25]. Although the role of many NLRs is yet to be elucidated, it is known that some of them can activate inflammatory caspases (see below) through the formation of inflammasomes [23, 26, 27]. Many NLRs belonging to all three mammalian NLR subfamilies have been identified in teleosts, and a NLR subfamily unique to teleosts was also identified [28], suggesting that those receptors are well conserved and have most likely a core function in the recognition of some pathogens.

Other molecules such as enzymes also play an important part in the development of inflammation and the onset of adaptive immunity. Caspases are protease enzymes and play a significant role in mediating cell death and inflammation in mammals [29, 30]. 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, based on their function and phylogeny, and linked to the nature of their pro-domain: caspases responsible for cell death initiation (double death effector domain (DED) motif pro-domain or CARD motif pro-domain, caspase-2, -8, -9 and -10), execution (short pro-domain, caspase-3, -6 and -

7) and inflammation (CARD motif pro-domain, caspase-1, -4, -5, and -12). Inflammatory caspases are key activators of pro-inflammatory cytokines (IL-1β, IL-6, IL-18 and TNF among others) [27]. Moreover, caspase-4 and -5 have recently been shown to recognise and process intracellular lipopolysaccharide (LPS), a PAMP found in abundance on gram-negative bacteria [26, 31]. Caspases have been identified in a range of organisms, from sponges to ascidians to mammals, and it is speculated that the function of caspases has been conserved through evolution, especially in regards to IL-1β processing by caspase-1 [29, 32].

Finally, heterodimeric transmembrane glycoproteins termed leucocyte integrins have also been identified as PRRs in mammals. Members of the leucocyte integrins are composed of a unique α-subunit (αD / CD11d, αL / CD11a, αM / CD11b or αX / CD11c) and a shared β-subunit (β2 / CD18) (Table 1.1). Leucocyte integrins play a crucial role in leucocyte adhesion and each α-subunit has its own set of ligands, but it seems that their association with the β2-subunit is necessary for any adhesion to occur (reviewed in [33]). Moreover, although the αM/β2 and αX/β2 were identified as LPS receptors in the mid-1990s [34, 35], the binding sites for LPS were later actually identified on the β2-subunit [36]. Literature on β2-integrins in fish is limited, with β2 molecules identified in zebrafish, carp and channel catfish [37-39], but as they have a pivotal role in LPS recognition and in inflammation in mammals [33, 40], it can be hypothesised that leucocyte integrins will be conserved in fish and should thus be investigated further.

Table 1.1. Different nomenclatures for β2-integrins.

Protein name	**α-subunit names**		**β-subunit names**	
LFA-1	αL	CD11a	β2	CD18
MAC-1 / CR3	αM	CD11b	β2	CD18
p150/95	αX	CD11c	β2	CD18
-	αD	CD11d	β2	CD18

1.1.3. Development of adaptive immunity

PRRs' primary purposes are to assist with the capture of foreign particles and to trigger inflammatory responses, but they also help with the activation and maturation of APCs, thereby linking innate and adaptive immunity [9]. Adaptive immunity is characterized by the development of an immune memory. This memory of previously encountered pathogens allows a fast and specific response when reinfection by the same pathogen occurs. Adaptive immunity involves antigen recognition by antigen presenting cells (APCs) and an associated antibody response and pathogen elimination, generally performed by previously primed specialist B and T-lymphocytes [1]. B-lymphocytes are specialized in producing antibodies that label pathogens and make them easier to identify by phagocytic cells for elimination from the host [1]. T-lymphocytes, on the other hand, either help other white blood cell (leucocyte) activation or directly destroy tumorous and virus infected cells [1]. APCs present antigens via the major histocompatibility complex (MHC) molecules, which bind internalised foreign and local peptides produced by protein degradation and transport them for display on their surface via the endoplasmic reticulum [41]. After presentation of foreign antigens bound on MHC to naïve T-cells, those cells become activated [41]. Non-professional APCs, which can be any cell in the organism, can only activate cytotoxic T-cells via the display of antigenic structures on their internal MHC class I receptors or via stimulation by cytokines (cell-signalling molecule) [42]. Professional APCs, on the other hand, can activate both helper and cytotoxic T-cells by displaying antigenic structures on MHC class II receptors at their surface and by producing co-stimulatory molecules [43]. B-cell proliferation can also be indirectly activated by professional APCs. Indeed, some B-lymphocytes need a co-stimulation from helper T-lymphocytes ($CD4^+$) to mature and proliferate [1]. B-cells in turn produce specific antibodies, which label the foreign particles and infected cells and direct other immune cells towards clearing the infection (Figure 1.1).

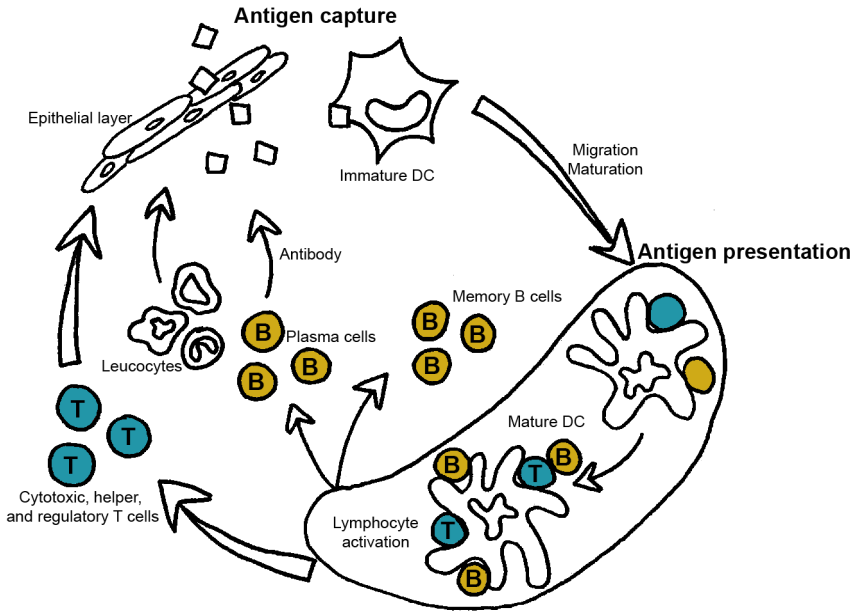

Figure 1.1. Schematic representation of innate and adaptive immunities adapted from Banchereau *et al.* [1].

In mammals, the principal professional APCs are DCs, due to their extended ability to engulf pathogens, present antigens and activate potent helper lymphocyte T-cells [1, 44, 45]. Indeed, DCs interact directly with immature T-cells and induce them to produce interleukin-2 (IL-2), and to multiply and mature [46]. IL-2 is a type of cytokine that stimulates immature cell development and differentiation by binding to IL-2 receptors on T-cell surfaces, in both autocrine and paracrine fashions. This cytokine is also required for the maturation of T-cell immunological memory [1, 47].

Understanding of innate and acquired immunity, and especially of the mechanisms leading to immune memory (antigen recognition and presentation), has enabled vaccination to revolutionise disease management, eradicating smallpox and rinderpest globally as well as preventing hundreds of millions of cases of illnesses such as measles, varicella and pneumococcus-related diseases [48, 49]. Moreover, since the implementation of the Vaccines for Children program in 1994, eligible children in the USA have been granted free routine immunisation, predicted to prevent over 732,000 deaths [50]. However, there are still gaps in our understanding of immunity, in particular in non-mammalian species, which, combined with fast

evolving pathogens, lead to some vaccination failures, for example in the aquaculture industry.

1.2. Vaccines against bacterial diseases in aquaculture

Because of the high economic losses associated with disease emergence in aquaculture, due to fish mortality, lower growth rates and decrease of product quality, it is crucial to efficiently protect farmed fish stocks [51, 52]. Antibiotics were commonly used as a disease control method before the introduction of vaccines in the aquaculture industry. However, due to their detrimental commercial impact and the inevitable development of bacterial resistance, leading to a reduced antibiotic efficacy, antibiotic use has significantly decreased in fish farming [53, 54]. Vaccination, on the other hand, has been a major success story in the global aquaculture industry since its introduction in the 1970s, revolutionising disease control and almost eliminating antibiotic use in modern fish farms [53, 54]. In the Norwegian salmon industry, for example, vaccine use has allowed for a significant increase in production while reducing the use of antibiotics [55, 56]. However, there are some infections that remain difficult to control by vaccination including diseases caused by highly variable bacterial pathogens such as *Streptococcus* or *Vibrio* species.

Streptococci are gram-positive cocci typically growing in chains or in pairs. *Streptococcus iniae* is a β-haemolytic aquatic pathogen identified in over 27 fish species and it affects economically important farmed species such as tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss)*, yellowtail (*Seriola lalandi*) and barramundi (*Lates calcarifer*) [57]. *S. iniae* is also a zoonotic pathogen that causes, amongst other symptoms, meningitis and septicaemia in farmed animals and can be transmitted to humans, especially to immuno-deficient individuals, through handling of infected animals [58, 59]. Vaccine failures against *S. iniae* have been reported in Israeli trout farms and in Australian farms, with re-emergence of infections post vaccination [60]. Vaccination failure in Israel resulted in a massive disease outbreak in 1997 and was due to the emergence of a new *S. iniae* serotype, caused by capsular variations [60]. To date, no generic vaccine against *S. iniae* is registered in Australia, so the barramundi farming industry relies on an autogenous vaccine program (vaccines developed from bacteria strain(s)

isolated from an outbreak and used exclusively on the farm from which the pathogens were isolated) [57]. However, vaccinated fish are occasionally re-infected by different *S. iniae* serotypes with re-emergence of the pathogen resulting from mutation on the genes responsible for synthesis of the bacterial polysaccharide capsule [61]. It seems that *S. iniae*, through mutations that change the capsular polysaccharide, is able to effectively avoid detection and/or evade its hosts' adaptive immune system, leading to diseases outbreaks in already vaccinated fish. Those disease outbreaks are highly problematic, as septicaemia caused by *S. iniae* often leads to over 50% mortality in 3 weeks or less in cultured animals [57].

Vibrios are motile gram-negative bacteria possessing polar flagella and a curved rod shape. *Vibrio* species are typically marine bacteria and are hard to differentiate at the species level, due to their highly plastic overlapping genomes [62, 63]. Several species of *Vibrio* are pathogenic and are often associated with marine organisms such are crabs, prawns and fish but they can also be zoonotic, causing gastroenteritis and potentially septicaemia in marine mammals or even in humans, mostly after ingestion of raw seafood [64-66]. Many aquaculture species are affected by *Vibrio*, including but not limited to white shrimp (*Litopenaeus vananmei*) [67], Atlantic cod (*Gadus morhua*) [68], European seabass (*Dicentrarchus labrax*) [65] and Australian barramundi (*Lates calcarifer*) [69], and those bacteria currently are the leading disease causing agent in the industry. Losses due to vibriosis in prawns were estimated at approximately US $1 billions in 2011 in Viet Nam only, and those numbers have likely increased with the development of the industry [70]. Vibriosis caused by *V. harveyi* is also a recurring issue in fingerling barramundi culture in Australia, causing heavy mortalities over a short time frame [69].

In order to improve the efficacy of vaccines, adjuvants are typically added to the injection mix. This practice is valuable both economically and immunologically, as adjuvants are usually cheap to produce, they allow for lower antigen concentrations in the vaccine, and they greatly enhance the antibody response [71]. For example, in chickens, the addition of cystidine-phosphate guanosine oligodeoxynucleotides (CpG-ODN) as an adjuvant increased the specific antibody response to *Escherichia coli* fimbrial adhesin F4 up to 480%, compared to Freund's incomplete adjuvant [72]. There are currently two main types of adjuvants: depots or irritants. Depot adjuvants,

mostly composed of oil emulsions, attract effector cells to the injection site, increasing the delivery of antigen to APCs [73]. Oil emulsions also maximize the time during which the antigens are presented, thus maximizing the immune response. Irritant adjuvants cause the body to trigger and amplify the immune response. Many of the components found in adjuvants act on PRRs, which enhance and quicken APC maturation for a boosted immune protection. For example, a combination of CpG-ODN and polyI:C with an inactivated salmon alphavirus significantly increased the titres of neutralising antibodies against this antigen, which were maintained post cohabitation challenge [73]. Adjuvants have played a big part in the success of vaccines aquaculture, with vaccines in salmon only becoming commercially viable with the introduction of oil emulsions in the early 1990s [53]. However, although current adjuvants in aquaculture have been optimised for slow delivery of antigens in cold water species, new adjuvant formulations need to be tested in vaccine trials in order to optimize dose and antigen delivery for better vaccine release in warm water species. Moreover, additional work is necessary to understand not only the pathogens' camouflage/evasion techniques, but also their hosts' immune adaptive mechanisms.

1.3. Focus on the adaptive immunity of teleosts

Antigen-presentation by the cellular immune system is essential for the development of a protective adaptive immune response, therefore, understanding it is critical to improve vaccine efficacy. However, until recently, no equivalent of DCs, which are the main APCs in mammals, had been discovered in fish. For years, the existence of DCs in teleosts was debated, due to the differences between teleostean and mammalian immune systems and due to the lack of suitable identification tools, such as specific markers, in fish. One of the major differences between fish and mammals is the location of the main hematopoietic centres, where immune cells and their precursors are generated. Unlike mammalian bones, fish bones are not hollow and, consequently, they lack bone marrow, which is one of the main hematopoietic centres in mammals [74, 75]. Instead, immune cells in fish arise mainly in the pronephros or head-kidney (HK), which seems to effectively supplant bone marrow as the primary hematopoietic organ [74]. Moreover, as lymph nodes are also absent in fish, the spleen is considered to be the primary site for T-cells/ APCs interactions, including antigen processing and presentation [76]. Another significant difference

between the adaptive immunities of mammals and teleosts is the variety of immunoglobulin (Ig) classes they each possess. Igs are also known as antibodies or B-cell receptors which are secreted or membrane bound respectively [77]. In mammals, antibodies are classified into five main isotypes, namely IgA, IgD, IgE, IgM and IgG. IgG and IgM constitute the bulk of circulating antibodies, with pentameric IgM being the principal antibody during the primary innate response, then undergoing class switch to a monomeric IgG during the secondary adaptive response [78]. In teleost fish however, only three main isotypes have been described, IgD, IgM and IgT/Z, with the later thought to play a similar role to mammalian IgA [79, 80]. IgM, which is conserved in both fish and mammals, functionally defines B-lymphocyte lineages, and is thus an important component of the immune response [77]. Moreover, in teleosts, IgM is found from monomers to tetramers, and polymerisation seems to correlate with antibody affinity [81], unlike what is observed in mammals. Finally, there are important discrepancies in PAMPs recognition between mammals and fish. Indeed, some TLRs, such as TLR14, have only been identified in fish and still need functional characterisation [82]. On the other hand, an equivalent to mammalian TLR4 is yet to be found in teleosts [4]. Due to the differences between teleost and mammalian immunity and the challenging identification of immune cells in fish, complex processes such as antigen sampling and presentation are still poorly understood in teleosts. However, because putative DCs were recently discovered and characterised in rainbow trout (*Oncorhynchus mykiss*) and in zebrafish (*Danio rerio*) among other fish species [76, 83], further work is now warranted to investigate the role of those putative DCs in fish adaptive immunity. This will provide an excellent platform for understanding antigen presentation in fish and will subsequently enable a faster development of protective vaccines against complex fish pathogens such as *S. iniae.* To investigate the early onset of adaptive immunity and in particular putative DCs in teleosts, the Australian barramundi (*Lates calcarifer*) will be used as an industry-relevant model species.

1.4. Barramundi aquaculture overview

Barramundi (*Lates calcarifer*) is a tropical Australian native fish [84]. Because they are euryhaline organisms (they can migrate between fresh and saltwater) and have a fast growth rate, barramundi are relatively easy to farm and are therefore economically important to the aquaculture industry in Australia and throughout

South-East Asia [85]. In 2010, the barramundi industry had a gross value of $AU20.7 million in Queensland alone, and the industry is still expanding [86]. Due to this expansion, the occurrence of diseases, including vibriosis, lymphocystis, viral nervous necrosis (VNN), Picorna-like virus, streptococcosis and gastrointestinal cryptosporidiosis, has considerably increased on barramundi farms and hatcheries [57, 87-90]. Several simple measures can be taken to reduce disease outbreaks, such as managing the water quality, keeping stressful conditions to a minimum and reducing fish density, but the most effective method of protection against bacterial pathogens in aquaculture remains vaccination [53, 91]. However, vaccine failures can occur, especially when targeting highly variable pathogens such as *S. iniae* and *V. harveyi*.

1.5. Barramundi as an industry model species

Barramundi belong to the Perciformes order, which include over 40% of all bony fish species identified to date. Perciformes also encompass other commercially important species such as tilapia, mackerel, grouper and European seabass, however, most research on teleosts focusses on the model species *Danio rerio* or on high value species such as salmonids. Moreover, Perciformes diverged much later that cyprinids (zebrafish, carps, koi) or than salmonids (trouts, salmons), which makes them a valuable link between the highly-studied zebrafish and terrestrial vertebrates in the evolutionary story of the immune system [92]. More importantly, barramundi are warm water fish, with an optimal growth temperature ranging between 25 and 32°C [93]. With the development of aquaculture in tropical and subtropical areas and with global warming coming into play, it is increasingly important to understand how the immune system of warm water fish species functions. Investigating pathogen recognition and the onset of immune memory in barramundi would provide meaningful insight into the immune system of warm water fish and such research would also be applicable to many currently farmed species. Further, considering that most teleost species are classified as Perciformes, with over 10,000 species, it is likely that when considering new species for aquaculture, many of those will belong to this order. It is thus time to fill the knowledge gaps regarding warm water fish immunology, and in particular Perciformes' immunology. By using barramundi as an industry relevant model species, the outcomes of this thesis are ensured to be

applicable to many aquaculture species, especially ones farmed in developing countries where the need for high yield and low loss protein sources is crucial.

1.6. Techniques overview and methods development

Although fish immunology has steadily developed and improved over the last few decades, specific tools and techniques are still scarce. With the development of aquaculture, there is an increased need for the study of fish immune system, following the emergence of new diseases.

Transcriptomes are sets of RNA transcripts produced by the genome at a given time, and thus are used to study gene expression under specific circumstances [94]. Transcriptomes have been generated, sequenced and analysed for a large variety of fish species, including, but not limited to, grey bamboo shark (*Chiloscyllium griseum*), Miiuy croaker (*Miichthys miiuy*), pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*), turbot (*Scophthalmus maximus*), African coelacanth (*Latimeria chalumnae*) and half-smooth tongue sole (*Cynoglossus semilaevis*) [95-101]. Transcriptomes from these fish species have mainly been used in order to identify their phylogenetic relationships with other vertebrates, in particular mammals, as well as to get a better understanding of chondrichthyan and teleostean immune processes. Moreover, they have increased the amount of expressed sequence tags (EST) available for fish species on databases such as the National Center for Biotechnology Information (NCBI), providing valuable data for further genetic and genomic research on ichthyes. However, the genomic data available for perciform fish, which represent about 40% of all bony fish and include several farmed species, is still limited. Hence, the generation of a spleen and head-kidney immune transcriptome (transcriptome generated during an immune response) for barramundi using *de novo* assembly would prove a valuable tool for future research on farmed fish immunology. Specifically, the processes underlying early stages of adaptive immunity are of particular importance as they set the base for the immune memory of pathogens, on which the development of vaccines relies.

1.7. Objective and aims

The main objective of this project was to gain a better understanding of the early onset of adaptive immunity and the role of dendritic cells in teleosts, using

barramundi (*L. calcarifer*) as an industry relevant study organism. To achieve this objective, the project was divided into the following aims:

1- Generation and assembly of a transcriptome from the major immune organs in barramundi (*Lates calcarifer*), the head-kidney and spleen

A spleen and head-kidney barramundi immune transcriptome was generated using next generation sequencing from RNA derived after immunisation with a mixture of lipopolysaccharide (LPS) and peptidoglycan, from gram-negative and gram-positive bacteria respectively. The immune transcriptome was assembled using *de novo* assembly and was annotated using gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG), in order to identify pathways of interest for further study into the recognition and presentation of key antigens in fish. LPS is of particular interest because teleosts have an attenuated innate response to LPS, but strong and type-specific adaptive response to LPS, which is critical to the efficacy of several vaccines. Particular attention was given to TLRs and CLRs identified in the immune transcriptome, as they are the main receptors involved in initiating presentation of major PAMPs in fish. The Mincle CLR was identified as a partial alternative to TLR4 recognition of LPS in barramundi.

2- Identification of LPS receptors in barramundi (*Lates calcarifer*) through transcriptomic analysis and functional assessment of those receptors

Interleukin-1β-converting enzymes (ICE)-caspases have recently been identified as intracellular LPS receptors in humans (caspase-4, -5) and in mice (caspase-11). Using the previously generated barramundi transcriptome, a range of caspases were identified in barramundi. Moreover, inflammasome complexes (combining caspase-1 and diverse NLRs) were also investigated in barramundi transcriptome, due to their ability to recognise PAMPs and induce inflammation. Finally, two leucocyte integrins were identified in barramundi, with similar binding sites and functions to mammalian leucocyte integrins, suggesting a role in LPS recognition in barramundi.

3- Identification and characterisation of dendritic cells in barramundi (*Lates calcarifer*) and first description of a specific molecular marker for dendritic cells in fish

As the key antigen presenting cells in higher animals, dendritic cells are at the primary link between innate and adaptive immunity. DC-SCRIPT, a protein specifically associated with dendritic-cells in humans and mice, was identified in barramundi (*L. calcarifer*) and its expression pattern were characterised in head-kidney and spleen post injection with TLR-ligands. Using this protein as a molecular marker, DC-like cells were also morphologically and functionally identified in barramundi for the first time.

4- Assessment of antibody response kinetics and affinity maturation potential to various adjuvanted vaccines in barramundi (*Lates calcarifer*)

Different adjuvant formulations were tested in a large scale vaccine trial using a model antigen in the tropical farm species *L. calcarifer* (barramundi) to determine the feasibility of increasing speed of antigen release early in the farm cycle, whilst retaining longevity of protection. Primary and secondary response kinetics were measured, in terms of antibody-response to vaccination using several antigen/adjuvant formulations. The avidity of those antibodies was also quantified and the genomic region coding for heavy chain V(D)J region of immunoglobulin was identified in barramundi.

Chapter Two – Immune transcriptome reveals the Mincle C-Type Lectin Receptor acts as a partial replacement for TLR4 in lipopolysaccharide-mediated inflammatory response in barramundi *(Lates calcarifer)*

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2.1. Introduction

Teleost fishes are the most abundant extant vertebrates on the planet and are critical in the global food supply. Diverging from “bony vertebrates”, including birds, amphibians and mammals around 420 m.y.a., teleosts underwent an additional genome duplication event around 300 m.y.a. [102] that contributed to their high diversity through both accelerated gene loss [102, 103], and through sub- and neo-functionalisation of genes under diversifying selection [92, 104]. They are also one of the earliest diverging animal infraclass with an adaptive immune system based on antigen recognition by MHC I and II molecules [105] and by immunoglobulins [106]. This trait has been exploited to great effect in the aquaculture industry through the development and implementation of mass vaccination programs for farmed fish [53]. The aquaculture industry currently supplies more than half of the fish for global human consumption, yet has only latterly been recognised as a key foundation for ensuring future food security for a growing population [70]. Increasing production to meet future demand in a sustainable manner is a challenging problem that requires a multifactorial approach, with reduction of stock losses to bacterial and viral diseases critical to increasing efficiency from existing aquaculture infrastructure. There has been some substantial success with the introduction of oil-adjuvanted injectable vaccines against major bacterial diseases in the Norwegian salmon industry since the early 1990s [53]. However, high loss to disease still occurs, especially where aquaculture is growing fastest, in the tropical and subtropical regions [54, 107], with many of the pathogens associated with disease in warm water species difficult to control by vaccination. For example, the gram-positive streptococcal pathogens *Streptococcus iniae* and *Streptococcus agalactiae* undergo rapid serotype switching under stress in the immune host [61, 108, 109], and the gram-negative pathogen *Aeromonas hydrophila* and other motile Aeromonads are also highly diverse, making

effective targeting of vaccines difficult [110]. *Edwardsiella ictaluri*, which causes devastating losses in the burgeoning *Pangasius* catfish industry in Vietnam, is facultatively intracellular, thus difficult to control with conventional killed vaccines whilst live attenuated vaccines are fraught with difficulties in the milieu of fish farm ponds where reversion to virulence has occurred [111-113]. In order to solve these complex problems through vaccination, a much more thorough understanding of how adaptive immunity develops in warm water fish species is required. This presents a challenge as teleosts represent the most diverse extant vertebrate infraclass.

Vaccination relies upon the formation of immune memory, which is coordinated by specialist antigen presenting cells (APCs), particularly dendritic cells (DCs) [1, 2]. Putative DCs have recently been identified in several teleost species, including rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*) and barramundi (*Lates calcarifer*) [76, 83, 114], representing respectively Salmonid, Cyprinid and Perciform orders, and providing a basis for improved understanding of acquired immunity in teleost fishes. In vertebrates, an immune response is initiated following the recognition of specific structures from invading microorganisms (pathogen-associated molecular patterns (PAMPs)) by the host's pattern recognition receptors (PRRs). PRRs not only play a major role in the initial capture of foreign particles and onset of inflammation, but also in the activation and maturation of DCs and other APCs, thereby providing a link between innate and adaptive immune responses [9]. There are four major types of PRRs capable of inducing cellular responses: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) such as MDA5 [3]. All of these PRRs have been described in teleost fish [4, 115].

TLRs have a central role in the vertebrate immune response by activating inflammatory pathways such as the NF-kB and MAPK signalling cascades, responsible for the production of pro-inflammatory molecules and for the initiation of adaptive immunity [5-7]. Functional TLRs are transmembrane proteins presenting leucine-rich repeat (LRR) domains, a trans-membrane domain as well as a Toll/interleukin-1 receptor (TIR) domain [5]. TLRs can be grouped in two sub-families depending on their ability to recognise and bind either cell surface elements (microbial sugars, lipids and proteomes) or nucleic acid [8]. TLRs have been

identified in a number of cartilaginous and bony fish species, with close phylogenetic relationship to mammalian TLRs based on amino acid alignment [116, 117]. One exception is TLR4, responsible for lipopolysaccharide (LPS) recognition in mammals. Although *tlr4* genes have been identified in cyprinids (including *Danio rerio* and *Cyprinus carpio*), a functional fish ortholog to human TLR4 protein has not yet been identified [4, 118]

Bacterial polysaccharides and lipopolysaccharides can also be recognised by lectin receptors such as C-type lectin receptors (CLRs). CLRs can be transmembrane or secreted proteins and use conserved carbohydrate recognition domains to bind sugars in a calcium-dependant manner [9, 13]. Pathogen recognition by CLRs is closely associated with antigen capture and presentation and several of the membrane-bound CLRs are specific to APCs, particularly DCs [10-12]. CLRs have a central role in the initiation of anti-fungal immunity in mammals (reviewed in [3]), can recognise viruses through mannose specificity [14], and also seem to play a critical role in bacterial clearance and recognition of non-self particles in marine invertebrates and vertebrates [8, 15-20]. Moreover, CLR activation can result in cytokine signalling either through direct NF-kB activation or through coinciding TLR activation, suggesting CLRs can partially regulate adaptive immune responses and inflammation [21]. Some CLRs have been described in fish, though not in Perciforms [119-121], and the functional roles of CLRs in innate and adaptive immunity in teleosts remain unclear.

In the present study, the recognition and processing of the carbohydrate-rich bacterial cell wall components (LPS and peptidoglycan (PTG)) are investigated in a perciform fish, the barramundi or Asian sea bass, *Lates calcarifer*, to elucidate potential pathways linking innate and adaptive immune responses to these essential pathogen-associated molecules. To achieve this, a broad immune transcriptome was initially generated from the major haematopoietic organ, the head-kidney (HK) or pronephros, and the major antigen processing organ, the spleen, of barramundi challenged with LPS and PTG. Subsequently, the role of the Mincle C-type lectin receptor pathway in processing of LPS by spleen and head-kidney leucocytes was further analysed *in vitro*.

2.2. Materials and Methods

2.2.1. Experimental animals and husbandry

Barramundi (*Lates calcarifer*) juveniles of approximately 30–100 g were obtained from a commercial aquaculture facility in Cairns, Queensland, Australia. Fish from this farm are specific pathogen free fish as the direct supply of filtered bore water to the hatchery and farm coupled with strict biosecurity prevent exposure to common fish pathogens. Fish were transported to The University of Queensland by air freight and 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 and the salinity were maintained at 28 ± 2°C and 15 part per thousand (ppt) respectively. Water quality was checked regularly for ammonia, nitrite, nitrate and pH, and water exchanges were applied as required. Fish were fed to satiation twice daily with a commercial diet for barramundi (Ridley Aqua Feed).

2.2.2. Fish immunisation, RNA isolation and preparation for Illumina sequencing

Juvenile barramundi (all males) were injected in the peritoneal cavity with either phosphate buffer saline (PBS 1X) or with a cocktail of lipopolysaccharide (LPS from *Escherichia coli* 0111:B4, Sigma; 10 µg/mL) and peptidoglycan (PTG from *Staphylococcus aureus*, Sigma; 10 µg/mL) diluted in 100 µL PBS. LPS and PTG were chosen as they are bacterial polysaccharides often used to induce innate immune responses both *in vivo* and *in vitro* [122-124]. Moreover, LPS is both a requirement and type specific antigenic molecule used for full protection in some bacterial fish vaccines [125], and is therefore of significant interest in both adaptive and innate immunity in fish. At 6 h, 24 h, 72 h and 7 d, four fish were sampled from both control and treatment groups. From each fish, the spleen and head-kidney were dissected aseptically and kept in RNAlater until further processing.

RNA was extracted using the RNeasy extraction kit (QIAGEN), according to the manufacturer's instructions. Tissues kept in RNAlater were disrupted by reflux passage through a 25 *x g* needle mounted on a sterile 1 mL syringe before processing. RNA was eluted in 40 µL of nuclease free water. Prior to library preparation, samples were pooled to reach 3 µg of RNA into four different categories: control spleen (SC), control head-kidney (HKC), treatment spleen (ST), and treatment head-kidney (HKT). Pooled samples were then concentrated to 100

ng/μL using a sodium acetate and ethanol precipitation. Briefly, nuclease free water was added to the samples to bring the volume to 450 μL, and 50 μL of 3M sodium acetate and 1 mL of nuclease free ethanol were then added to the samples. The samples were then incubated at -20°C overnight. The next day, the samples were spun at 4°C for 30 min at max speed in a microcentrifuge (Eppendorf). The supernatant was carefully removed and the samples were washed in 500 μL ice-cold 70% ethanol by centrifugation at 4°C for 10 min at max speed. The samples were then allowed to dry at room temperature by inversion of the tubes on tissue paper for 1 h and re-diluted in nuclease free water. Quality was checked using a bioanalyser (Agilent, courtesy of the Australian Genome Research Facility (AGRF)) and RNA quantity was assessed by Qubit 2.0 fluorimetry (Invitrogen). RIN values from the bioanalyser analysis ranged from 6.1-9.4. Whilst the single sample with a RIN value of 6.1 was lower than normal (a cut-off of 7 is usually applied), the gel and trace output did not show evidence of significant degradation, merely lower peak heights reflecting the lower concentration of RNA in this sample. Samples were kept frozen at -80°C until TruSeq paired-end library preparation and sequencing using Illumina Hi-seq 2000 by the AGRF (Melbourne, Australia). Subsequent to sequencing, no 3' bias was detected in the resulting Illumina reads in the low concentration (RIN 6.1) sample.

2.2.3. De novo assembly and annotation

Raw Illumina reads were uploaded to the NCBI database under the BioProject PRJNA307522 with the accession numbers SRR3170685, SRR3170702, SRR3170703 and SRR3170704. Illumina read quality was checked using the FastQC software on the Queensland Galaxy virtual interface (http://galaxy-qld.genome.edu.au/). Sequences were quality trimmed with Trimmomatic before being processed through the Trinity assembly package (available at http://galaxy-qld.genome.edu.au/workflow/list_published) using paired reads assembly and default parameters. Assembly was conducted for the four treatment groups independently as well as pooled in order to generate an immune transcriptome. The generated transcripts were then annotated using BLASTX against non-redundant NCBI database and mapped to gene ontology (GO) in the Blast2Go program, and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were generated using the KEGG automatic annotation server (KAAS, available at

http://www.genome.jp/kaas-bin/kaas_main) and the KEGG pathway reconstruction tool (http://www.kegg.jp/kegg/tool/map_pathway.html) [126-129].

2.2.4. Differential expression and comparison of immune organ transcripts

Using Bowtie2 in Galaxy, the trimmed high-quality reads from the four different categories (SC, HKC, ST and HKT) were mapped back to their corresponding assembled transcriptome in order to obtain a differential read count. The read count for each category was then normalised to FPKM (fragments per kilo-base of transcript per million fragments mapped) values and further analysed to identify differentially expressed genes between control and treatment groups as well as between organs using the Tuxedo pipeline (Cufflinks, Cuffmerge and Cuffdiff) and Edge R analysis on Galaxy.

2.2.5. Phylogenetic analysis

From the KEGG annotation, transcripts associated with the development of immunity were chosen. The TLRs and CLRs agonists were chosen for further phylogenetic analysis due to their role in the early onset of an immune response. Barramundi transcripts were compared to several mammalian and teleost sequences obtained on the NCBI database (GeneBank; accession numbers listed in Table A2.2.1 and A2.2.2). MEGA6.0.6 software was used to align sequences (ClustalW, codon) to infer phylogenetic relationships by maximum likelihood with node probability supported by 2000 boostrap replicates.

2.2.6. LPS stimulation and bDCs maturation

Spleen and HK were dissected aseptically from euthanised (anaesthetic overdose, Aqui-S, Upper Hutt, New Zealand) healthy juvenile barramundi and placed in supplemented L-15 medium (1 % penicillin-streptomycin (P/S, 10,000 U), 10 % foetal bovine serum (FBS, heat inactivated)). The organs were then gently pushed through a 100 µm cell strainer using the plunger from a sterile disposable syringe in order to obtain a cell suspension. Cells were then either incubated with LPS (*E. coli* 0111:B4, Sigma) at 0.01, 0.05, 0.1, 0.5 and 1 µg/mL, with PTG at 1 µg/mL or with nothing (control) for 5 days. Cells were sampled at day 5 and stained using a fluorescent *in*

situ hybridization (FISH) probe for DC-SCRIPT as previously described [114]. The sampled cells were then analysed by flow cytometry for CAL Fluor 590 (red fluorescence) and DAPI (blue fluorescence) for each treatment.

2.2.7. Western blotting

Barramundi Mincle/CLEC4E cDNA sequence was identified in the transcriptome by homology to human, murine and teleost Mincle using BLASTX. A primary mixed spleen and HK cell culture was obtained by sterile sampling of the spleen and HK post anaesthesia by overdose of Aqui-S. A single cell suspension was obtained by gently pushing the organs through a 100 µm cell strainer as described in paragraph 2.6. Any erythrocytes still present in the suspension were removed by layering the cell suspension on a 34% / 51% Percoll density gradient (Sigma) and separating by centrifugation at 800 x g, 30 min, RT, acc. 6, no brake in an Eppendorf 5810R centrifuge. Cells at the interphase between the medium and the 34% Percoll layer (macrophages, neutrophils and granulocytes) and at the interphase between the two Percoll layers (lymphocytes and monocytes) were collected and washed twice in 1X PBS (400 x g, 10 min, RT, acc. 9, brake 9). The obtained leucocytes were either stimulated with the Mincle pathway inducer Trehalose-6,6-dibehenate (TDB, 1 µg/mL) for 21 h (Integrated Sciences Pty Ltd) or were left unstimulated. Both stimulated and unstimulated cells were then lysed and the proteins were separated on a 15% SDS-PAGE gel. Subsequently, the proteins were transferred onto a PVDF membrane (Immobilon P, Millipore) using a semidry blotting apparatus (Transblot TE44, GE Healthcare Biosciences) in Towbin buffer at 80 mA for 1 h. The blots were probed for Mincle using monoclonal mouse IgG vs human Mincle antibody (clone 15H5, InVivoGen via Integrated Sciences Pty Ltd), diluted to 1 µg/mL from 100 µg/mL stock in tris-buffered saline-Tween (TBST),) for 3 h at room temperature, before using a conjugated alkaline phosphatase goat anti-mouse IgG secondary antibody (1:15000 dilution in TBST - Sigma) for 1 h at room temperature. A negative control (to test for non-specific secondary antibody binding) was performed by omission of the primary antibody (incubation in TBST alone). An immunoglobulin isotype control was performed by substituting the primary antibody with mouse anti-*S. agalactiae* monoclonal IgG (Abcam AB21114), diluted in TBST to 1 µg/mL final concentration. Development was performed at room temperature for 15 min using BCPI/NBT Liquid Substrate (Sigma).

2.2.8. Cytokine regulation by the Mincle pathway assessed by qRT-PCR

Spleen and HK were sampled aseptically from healthy juvenile barramundi and a cell suspension was obtained as described in paragraph 2.2.6. After 5 days of incubation at 28°C, cells were incubated with LPS (*E. coli* 0111:B4) at 0.05 and 0.5 µg/mL for 1 h at 28°C. The blank controls consisted in cells alone, the negative controls consisted of cells incubated with 1 µg/mL of mouse anti-human Mincle blocking antibody for 1 h at 28°C followed by a 1 h incubation with 1 µg/mL of TDB or with 0.05 or 0.5 µg/mL of LPS at 28°C, and the positive controls consisted in cells incubated with 1 µg/mL of TDB 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 10 ng of starting RNA per sample using the QuantiTect Reverse Transcription kit (Qiagen). Subsequently, the cDNA was used to assess the relative expression of *IL6* and *TNFA* by qRT-PCR, using *18S*, *elongation-factor-1α*, *CLEC4E*, *FcRgamma* and *Syk* as normalisers, on a ABI-ViiA7 cycler (Applied Biosystems). Subsequently, only *CLEC4E*, *FcRgamma* and *Syk* were used as normalisers as they presented the most stable expression levels across treatments and presented similar efficiencies (within 10% of target genes and of each other). The primers used are listed in Table A2.2.3 and were designed to span across exons when possible to eliminate gDNA amplification.

2.2.9. Statistical analysis

Data analysis was performed with RStudio v0.99.486 (RStudio, Inc) and with Prism 6 (GraphPad). 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) [130-132]. Data from flow cytometry analysis and the qRT-PCR were analysed using analysis of variance (ANOVA) tests, using RStudio and GraphPad Prism respectively. Where ANOVA indicated significant differences, it was followed by pairwise comparison t-tests for the flow cytometry data (RStudio) and by Tukey *post-hoc* tests for the qRT-PCR data (GraphPad Prism) with α 0.05 in each case.

2.3. Results

2.3.1. De novo assembly

The spleen and HK cDNA sequenced by Illumina HiSeq 2000 generated 40,801,246 (spleen) and 37,724,291 (HK) raw reads for control and 40,072,015 (spleen) and 38,363,780 (HK) raw reads for treatment. The read length was 100 x 2 (paired end reads) and the total raw data generated for spleen and HK were 19.8 Gb and 18.6 Gb respectively, for a total of 38.4 Gb. The low quality base pairs were trimmed and the reads from both tissues and conditions were assembled together to generate a reference transcriptome for *Lates calcarifer*. The *de novo* transcript assembly produced a total of 163,661 transcripts, with the mean transcript length being approximately 1,502 bp and the longest transcript being 17,480 bp (Table 2.1). The transcripts differentially expressed between treatments and between organs were filtered and resulted in 90 transcripts and 19,713 transcripts respectively.

Table 2.1. Summary of *Lates calcarifer* transcriptome transcripts, assembly and annotation.

Total number of reads (spleen)	80,873,261 total (40,801,246 control and 40,072,015 treatment)
Total number of reads (head-kidney)	76,088,071 total (37,724,291 control and 38,363,780 treatment)
GC percentage (%)	44.56
Total number of transcripts	163,661
Mean length of transcripts (bp)	1,502
Longest length of transcripts (bp)	17,480
Differentially expressed (DiffExpressed) transcripts between organs	19,713
Total DiffExpressed transcripts between organs with BLASTX hit	9,213
Total DiffExpressed transcripts between organs with annotation	7,450

2.3.2. Transcriptome GO annotation and functional annotation

The 163,661 transcripts were grouped in 5 KEGG categories, further classified into 31 KEGG sub-categories (Figure 2.1), 71,718 transcripts were matched through BLASTX hit and 53,282 transcripts generated an annotation for Gene Ontology (GO). Of the GO annotated transcripts, 31,451 were categorised in biological process, 22,056 in cellular component and 44,122 in molecular function (Figure 2.2). Transcripts annotated through the KEGG pathways were most numerous in the 'Signal Transduction', 'Endocrine System' and 'Immune System' sub-categories. The detailed transcript groupings for those sub-categories are presented in Figure 2.1B, 2.1C and 2.1D. Moreover, out of the 90 transcripts differentially expressed between control and treatment, 63 were matched through BLASTX hit and 42 generated an annotation for GO. Of the 19,713 transcripts differentially expressed between organs, 9,213 were matched through BLASTX hit and 7,450 generated an annotation for GO. Edge R analysis of the top 100 differentially expressed transcripts also revealed that some transcripts were differentially expressed between control and treatment groups, both in spleen and HK (Figure 2.3). Moreover, when spleen

and HK were compared for GO in regards to ‘Immune system process’ (GO:0002376), more transcripts were identified in spleen overall. Interestingly, transcripts related to ‘Leukocyte migration’ (GO:0050900) and ‘Leukocyte activation’ (GO:0045321) were only identified in HK whereas transcripts related to ‘Antigen processing and presentation’ (GO:0019882) and ‘Immune effector process’ (GO:0002252) were only identified in spleen (Figure 2.3C and Figure A2.2.1).

2.3.3. TLR and CLR phylogeny

The transcriptome was screened for TLRs and CLRs, due to the pivotal role TLRs and CLRs play in the onset of an immune response. The presence of TLR, NLR and RIG transcripts was established by the KEGG analysis and TLR and CLR transcripts were confirmed using TBLASTX through the barramundi immune transcriptome using human, murine or piscine (*D. rerio*, *T. rubripes* or *S. partitus*) mRNA sequences obtained from the NCBI database (Table A2.2.1 and A2.2.2). Barramundi TLRs and CLRs sequences were then used to construct Maximum Likelihood Trees (MLTs) to infer their phylogenetic relationships with TLRs and CLRs from other vertebrate species (Figures 2.4 and 2.5). Barramundi TLRs all grouped with their respective piscine and mammalian counterparts. TLR1, TLR6 and TLR10 grouped closely together, being more similar within a given species than within the TLR group. It was noted that TLR4, TLR4ba, TLR4bb and CD180 (also involved in LPS recognition) were not found in the barramundi immune transcriptome, although the fish were injected with LPS (Figure 2.4). A total of six C-type lectin receptors were identified in *L. calcarifer*, grouping to their mammalian and/or piscine counterparts (Figure 2.5). Two isoforms of CD209 (DC-SIGN) were observed, one closely related to human CD209 and the other related to piscine CD209. Furthermore, as human CLEC4E is inducible by LPS and participates in bacterial recognition, identifying a CLEC4E-like transcript in the barramundi immune transcriptome was of particular interest.

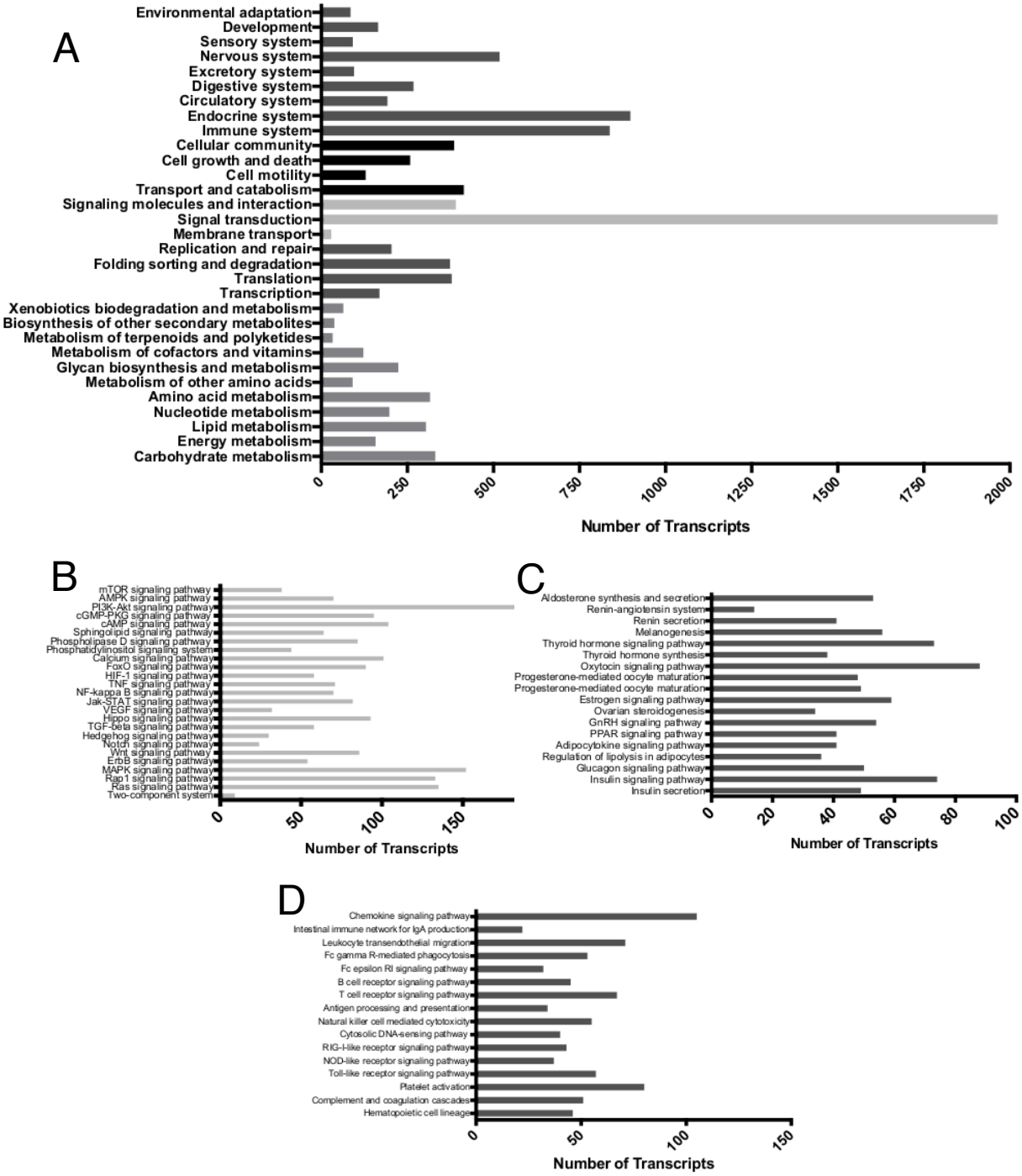

Figure 2.1. KEGG pathway transcriptome annotation. Transcript grouping into the different KEGG sub-categories (A). Detailed transcript grouping for the 'Signal Transduction' KEGG sub-category (B). Detailed transcript grouping for the 'Endocrine system' KEGG sub-category (C). Detailed transcript grouping for the 'Immune System' KEGG sub- category (D).

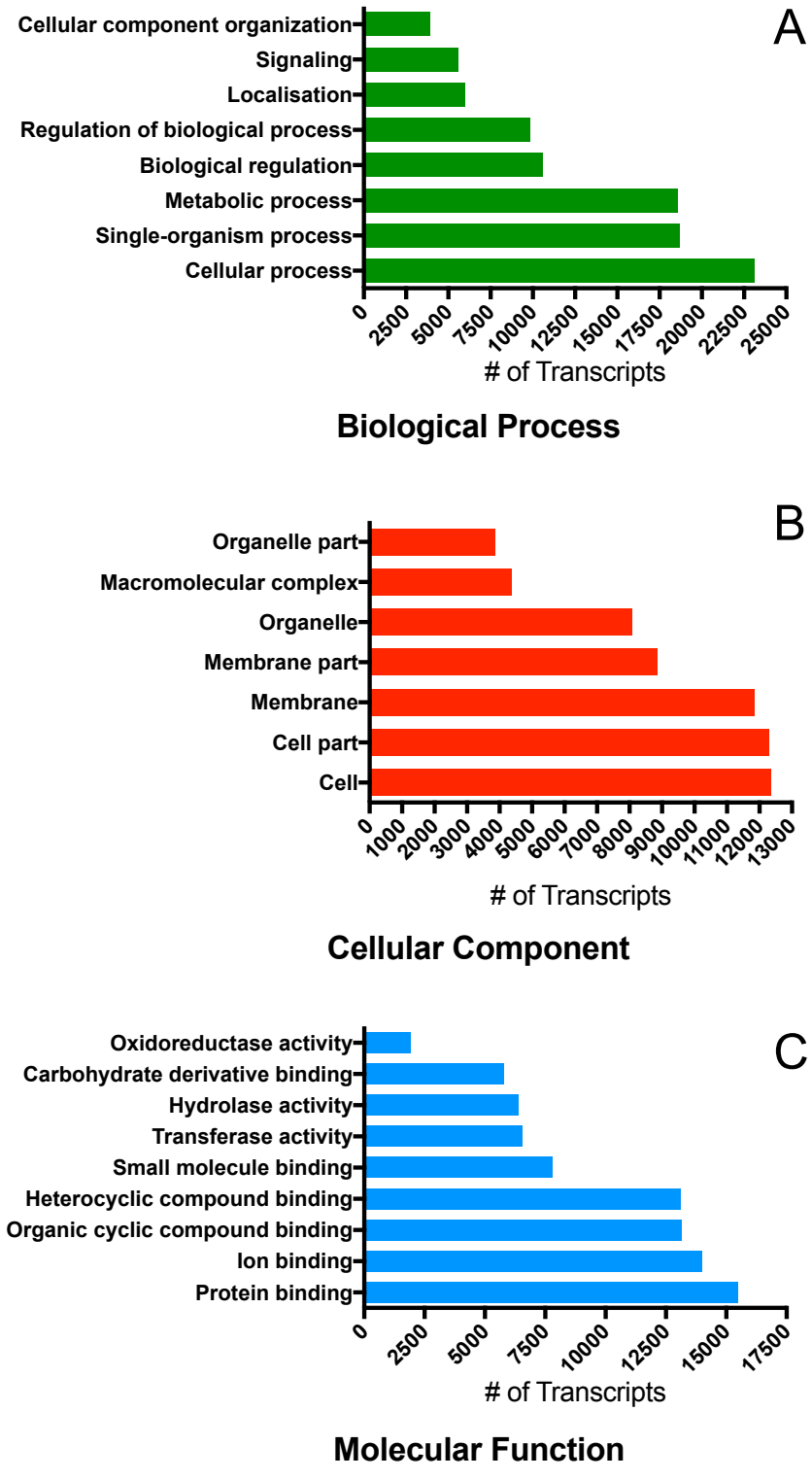

Figure 2.2. Functional annotation of *Lates calcarifer* transcripts using BLAST2GO. The transcripts were sorted into different categories including (A) Biological process, (B) Cellular components and (C) Molecular functions.

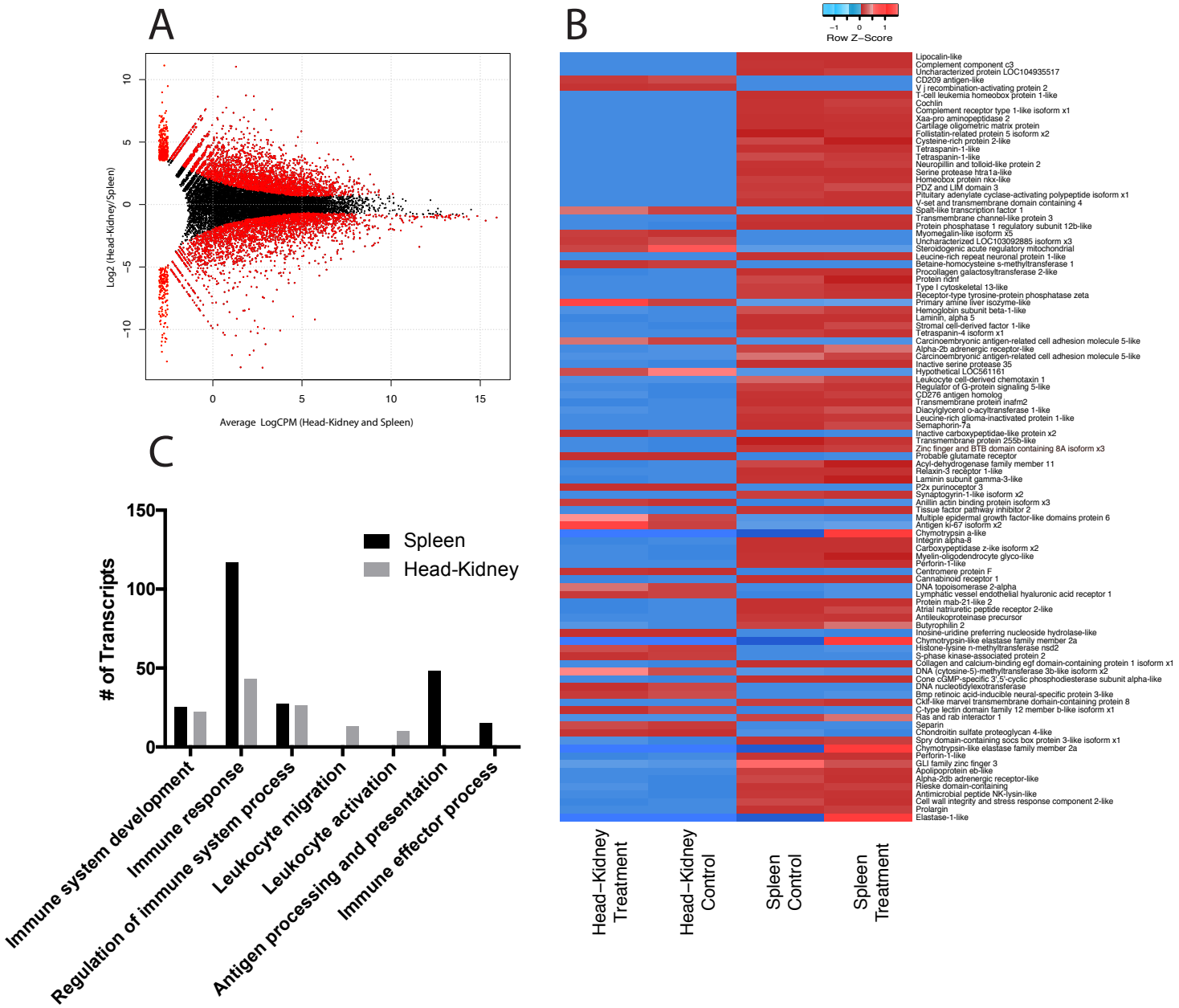

Figure 2.3. Differential gene expression analysis. (A) Edge-R scatter plot showing the differentially expressed transcripts across HK and spleen. (B) Top 100 differentially expressed transcripts based on fold change across HK and spleen for both control and treatment groups. Note that most transcripts are differentially expressed between organs but some transcripts are differentially expressed between treatment groups, suggesting inducible genes. (C) Gene ontology of the differentially expressed transcripts in the 'Immune Process' category for spleen and HK.

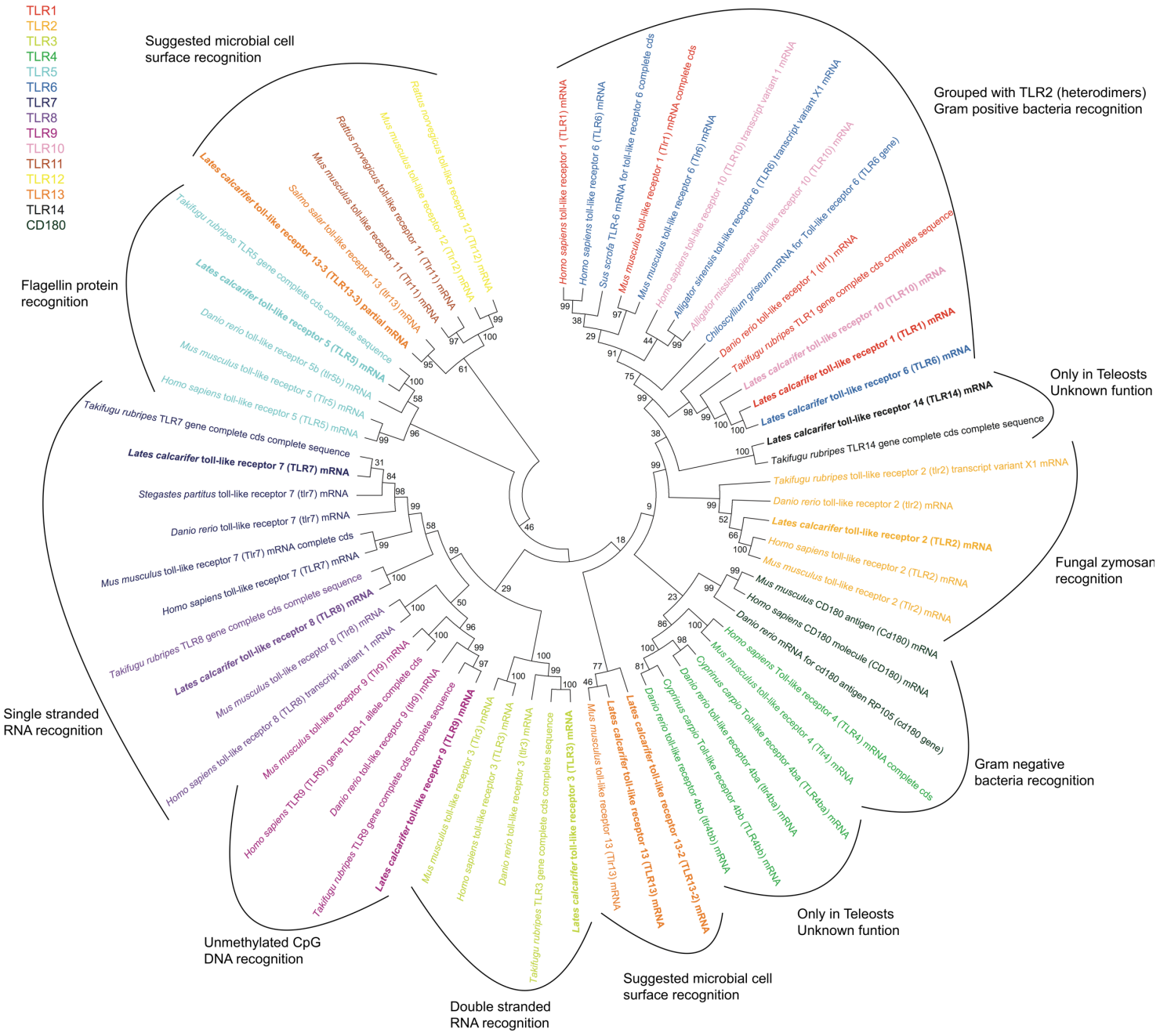

Figure 2.4. Maximum likelihood phylogenetic relationships of the TLR transcripts identified in the *Lates calcarifer* immune transcriptome. The TLR sequences of *L. calcarifer* were aligned with the sequences listed in Supplementary Table A2.2.1 and are presented on the figure in bold. The TLR clusters were further categorised by known function (outer groupings).

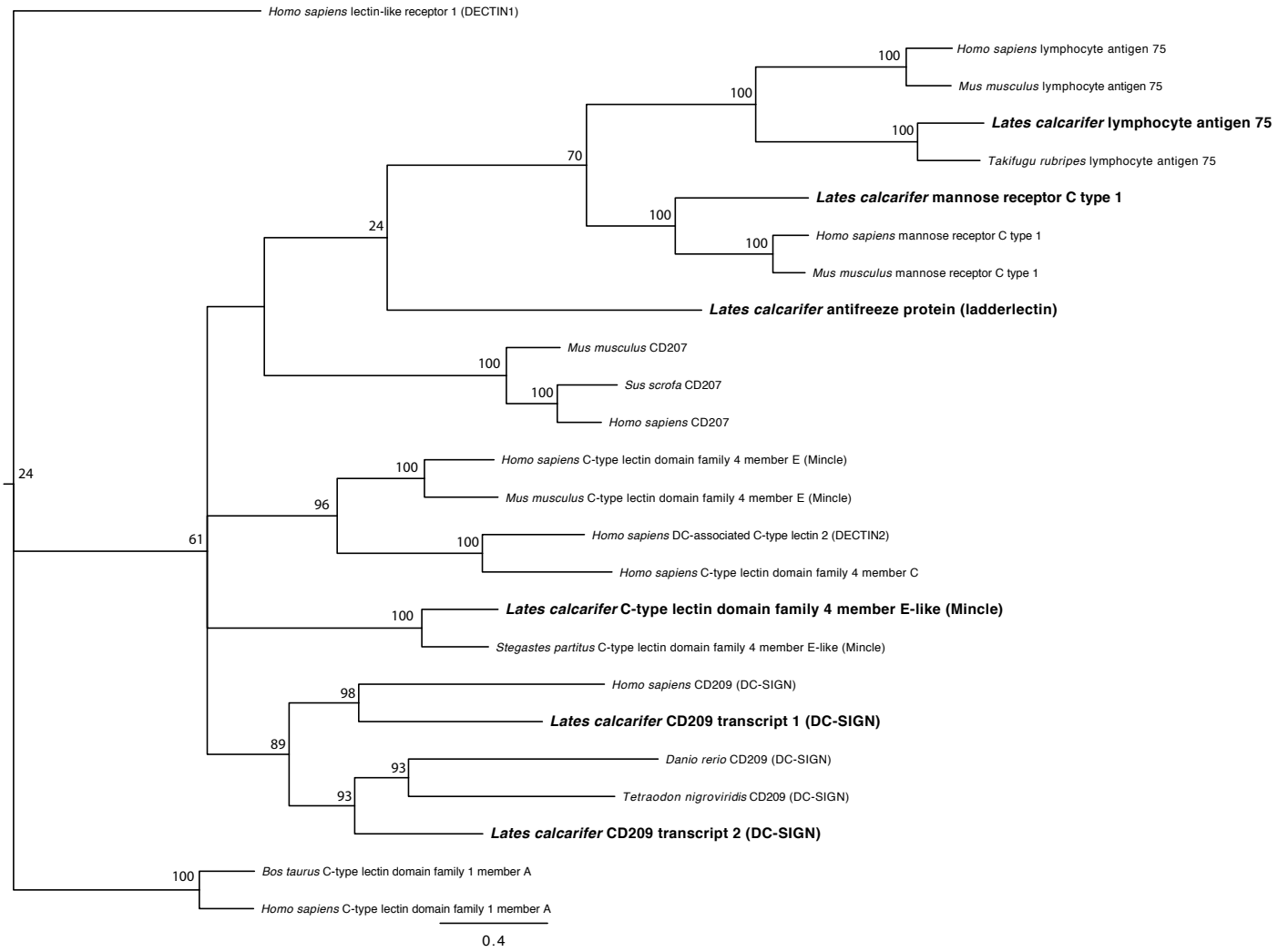

Figure 2.5. Maximum likelihood phylogenetic relationships of the CLR transcripts identified in the *Lates calcarifer* immune transcriptome. The CLR sequences of *L. calcarifer* were aligned with the sequences listed in Supplementary Table A2.2.2 and are presented on the figure in bold.

2.3.4. Barramundi DCs do not mature in response to LPS activation

When barramundi leucocytes were stimulated with LPS over a period of five days, a lower percentage of bDCs were observed as a result of the stimulation when compared to non-stimulated control cells (Figure 2.6). The different concentrations of LPS seemed to hinder the maturation of bDCs, with higher concentrations leading to a significantly lower percentage of DC-SCRIPT-positive cells after five days. In contrast, PTG stimulation lead to a significantly higher number of bDCs than high concentrations of LPS stimulation after the same time, but the number was not significantly different from untreated cells (Figure 2.6).

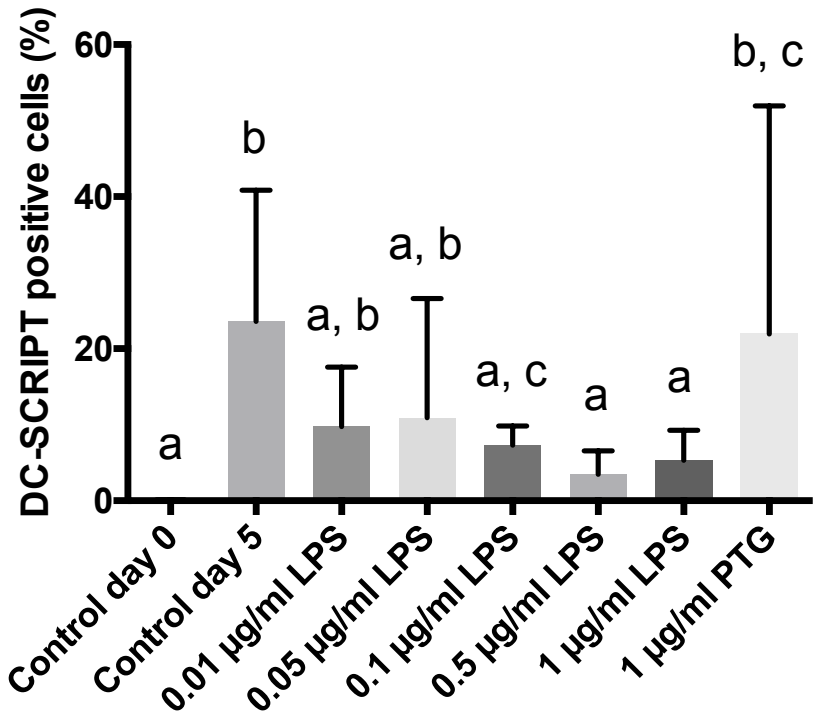

Figure 2.6. Effect of LPS on DCs maturation assessed by flow cytometry reading of DC-SCRIPT FISH probe. Percentages of DC-SCRIPT positive cells (mean ± SEM) are compared when stimulated with different concentrations of LPS or PTG. Same letters represent non-statistically different treatments ($p > 0.05$). PTG was used as a control drug ($n = 6$).

2.3.5. Mouse anti-Human Mincle antibody cross-reacts with barramundi Mincle

In order to determine if the *CLEC4E* transcript identified was translated to a protein in barramundi cells, a SDS-PAGE was performed, followed by a Western Blot. The molecular weight of the putative *Lates calcarifer* Mincle protein was calculated using the Compute pI/Mw tool on the ExPASy server and was estimated at approximately 24.2 kDa [133-135] (http://web.expasy.org/compute_pi/). When the SDS-PAGE-separated proteins extracted from enriched barramundi leukocytes were probed by Western Blot with human anti-Mincle antibody, a single protein between 22 and 25 kDa was detected (Figure 2.7). An isotype control comprising mouse monoclonal IgG against *S. agalactiae* (Abcam AB21114) and used at the same protein concentration as the anti-Mincle mAb did not cross-react with Mincle (Figure 2.7). These data, coupled with amino acid sequence (36% identity) and protein domain comparisons (similar structures) between human and barramundi Mincle suggest that human and

barramundi Mincle present homologous binding epitopes and protein structures. Moreover, the lack of additional bands detected in the extracts from enriched cells with the monoclonal antibody indicates that it is specific for Mincle in barramundi and suitable as a blocking agent in subsequent experiments (Figure 2.7).

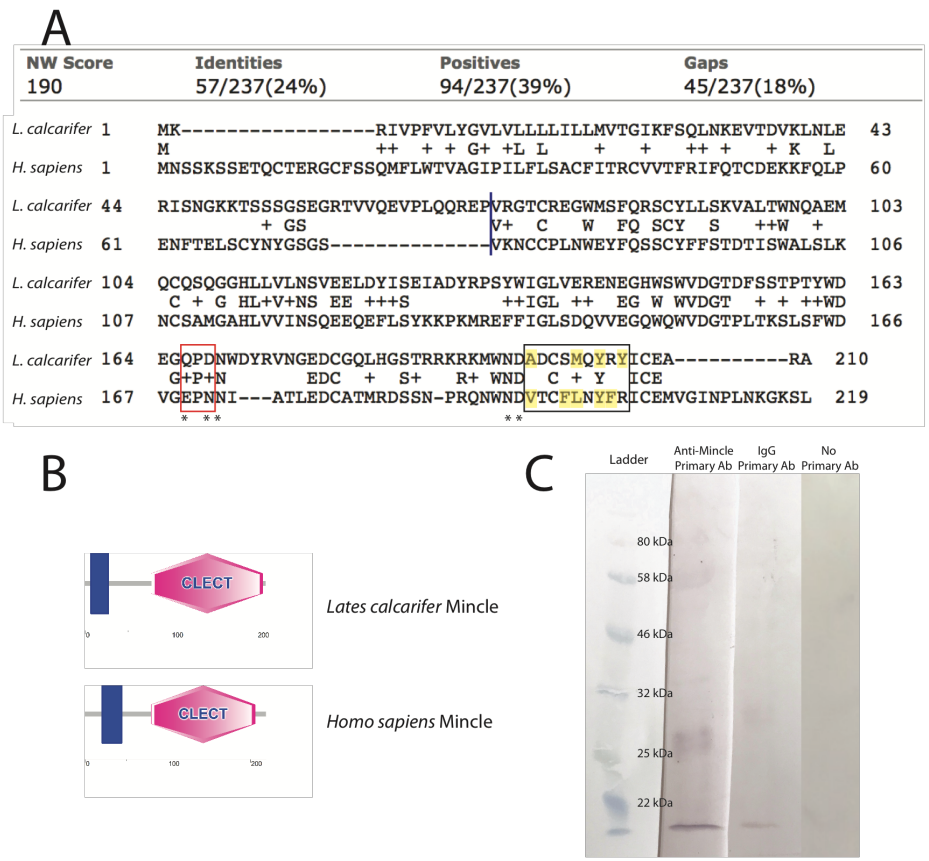

Figure 2.7. Comparison between putative barramundi Mincle and human Mincle. (A) Amino acid alignment between barramundi and human Mincle. Blue line represents the start of the ectodomain. Red box indicates motif normally involved in carbohydrate recognition by C-type lectins and black box represents hydrophobic amino acid loops, with residues highlighted in yellow being hydrophobic residues. The asterisks below the sequences represent the residues implicated in calcium binding. (B) Protein domain structure of barramundi and human Mincle predicted by SMART (http://smart.embl-heidelberg.de). (C) Western blotting of barramundi leukocytes probed with anti-human Mincle primary antibody, polyclonal non-specific IgG primary antibody or control with omission of primary antibody. The development of colouration indicates a positive binding around 25 kDa when probed with anti-human Mincle primary antibody, which coincides with the size of barramundi Mincle.

2.3.6. The barramundi Mincle pathway is activated by LPS

In barramundi leucocytes, LPS induced the transcription of *TNFA* and inhibited the transcription of *IL6* in a dose dependant manner, with transcription levels induced by 0.5 µg/mL of LPS almost identical to the transcription levels induced by the TDB positive control (no significant statistical difference - Figure 2.8). TDB is highly specific for Mincle. The cells incubated with the anti-Mincle antibody prior to exposure to TDB, on the other hand, had increased *IL6* transcription and reduced *TNFA* transcription compared to TDB-stimulated control cells, confirming that anti-Mincle antibody blocks the Mincle receptor in cell culture and is consequently a suitable negative control for Mincle activation (Figure 2.8). When Mincle was blocked by incubating cells with the anti-Mincle antibody and then stimulated with LPS, *TNFA* transcription levels were also lower than when the cells were incubated with LPS alone, suggesting that LPS can regulate *TNFA* through the Mincle pathway. However, the transcription of *IL6* was inhibited by LPS by a similar amount in barramundi cells whether blocked with anti-Mincle antibody or not, suggesting that the effect of LPS on *IL6* transcription levels does not depend exclusively on the Mincle pathway.

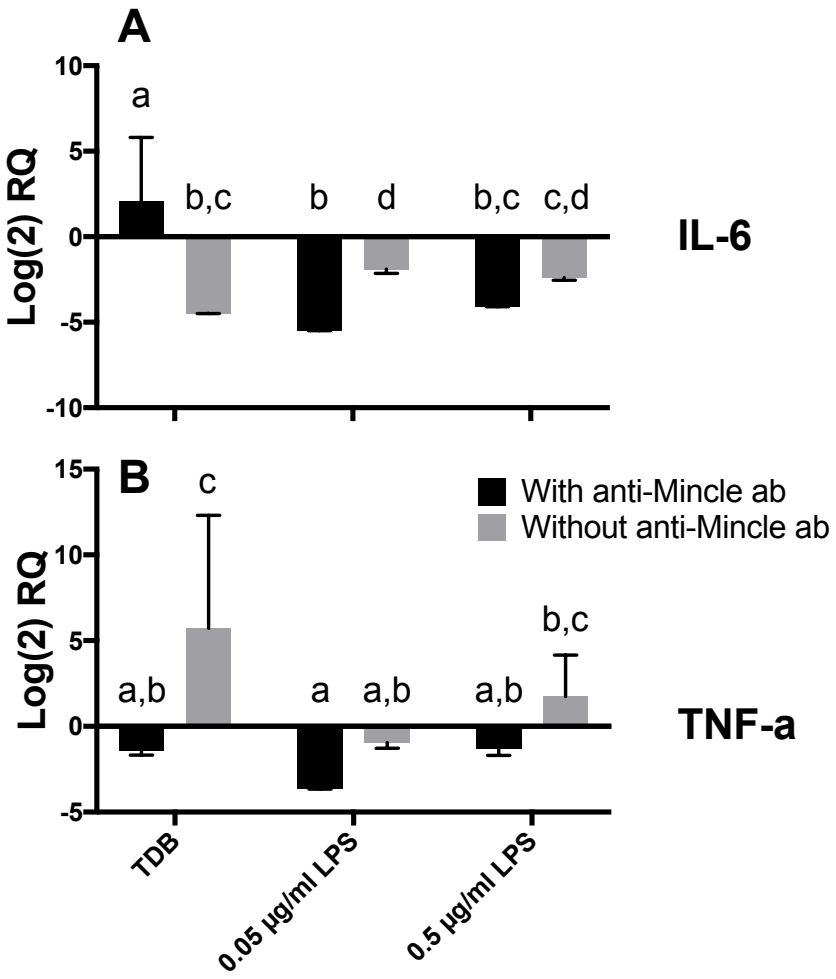

Figure 2.8. Differential expression of cytokines in response to LPS by qRT-PCR. In each graph (IL-6 (A) and TNF-α (B)) different letters indicate statistically significant difference in expression level (mean ± SEM) under different conditions ($p < 0.05$, three biological replicates, i.e. cells from three different fish ($n = 3$)).

2.4. Discussion

Understanding how the bacterial cell wall is recognised and processed by fish hosts is critical to the development of more effective protective methods against bacterial pathogens. In this study, a transcriptomic approach was used to investigate the recognition and processing of two bacterial cell wall components, LPS and PTG, by *L. calcarifer* immune cells.

Functional analysis of transcriptome data using Gene Ontology and KEGG classifications revealed that the ‘Signal Transduction’ and ‘Immune System’ sub-categories presented a high number of transcripts after a model immunisation with a

mixture of PTG and LPS. It was also noted that the 'Endocrine System' sub-category also presented a large number of transcripts. For many decades, interactions between the endocrine and immune systems have been reported in humans, with hormones influencing both the function and proliferation of leucocytes [136]. *In vitro*, several hormones, such as glucocorticoids, androgens, progesterone and adrenocorticotropic hormones, can depress the immune response whereas growth hormones, prolactin and insulin have been shown to increase the immune response [137]. Beta-endorphin, in particular, has been shown to impact on the activity of natural killer cells as well as to increase the production of IFN-γ in rat spleen cells [138]. The high number of transcripts present in the 'Immune System' and 'Endocrine system' KEGG categories in this analysis suggests that endocrine-immune interactions occur in barramundi as well. In particular, transcripts involved in oxytocin, insulin and thyroid hormone signalling pathways were identified in the highest numbers in the 'Endocrine System' category (88, 74 and 73 transcripts respectively), probably driven by cytokine production, with 105 transcripts present in the chemokine signalling pathway being the highest number of transcripts in the 'Immune System' category (Figure 2.1). Immune derived cytokines (mostly interleukins) have indeed been shown to increase the production of adrenocorticotropic hormone, luteinizing hormone, prolactin, vasopressin and oxytocin in the pituitary and of corticosterone, vasoactive intestinal peptide and mRNA insulin-like growth factor II in the adrenal gland *in vitro* [137], supporting the information highlighted by the functional analysis.

Genes differentially expressed between immune organs were also identified in the barramundi transcriptome, with several genes potentially inducible during an immune response (genes with high expression variability between control and treatment). Notably, most of those inducible genes in spleen were identified as coding for proteolytic enzymes, which are involved in regulation and modulation of the inflammatory response. Both chymotrypsin A and elastase 1 and 2a are endo-peptidases belonging to the clan PA peptidases (Proteases of mixed nucleophile, superfamily A) and their main function is to break down polypeptides into shorter chains, hydrolysing peptide bonds downstream of large hydrophobic residues (chymotrypsin A) or downstream of smaller amino acids (elastase) [139]. PA peptidases play a role in several critical functions such as blood coagulation,

digestion and the immune response [139]. In particular, key functions of DCs, including maturation and cell-surface expression of several DC-specific antigens, heavily rely on a functional chymotrypsin-like peptidase [140]. Moreover, the elastase 2 gene is known to be preferentially expressed in early myeloid progenitor cells and to be regulated during early myeloid differentiation by a hematopoietic specific transcription factor [141-143]. Lastly, it is noteworthy that both chymotrypsin and elastase transcripts were expressed over 10-fold more in spleens that underwent treatment with PTG and LPS than in control spleens in the current study, further suggesting the involvement of chymotrypsin and elastase in immune related processes.

Most of the known TLRs were transcribed in the barramundi spleen and HK transcriptome, with the exception of TLR4, which is primarily responsible for LPS recognition in mammals [144]. Moreover, the MD-2 protein, which typically couples with the extracellular domain of TLR4 and is critical to LPS binding through TLR4 [145], was also absent from the transcriptome, strongly suggesting that barramundi immune cells do not recognise LPS through a TLR4 activated pathway. Another accessory molecule associated with LPS recognition in mammals, CD14, was also absent from the transcriptome. Furthermore, although zebrafish have been shown to recognise LPS through the RP105 protein (also known as CD180 antigen), which is very closely related to mammalian TLR4, with MD-1 playing the role of MD-2 [146], both RP105 and MD-1 were also absent from the barramundi immune transcriptome. Fish are particularly insensitive to LPS compared to their mammalian counterparts [16, 147], perhaps due to the absence of TLR4 orthologs in most fish species [116, 147], or by TLR4-like mediated down-regulation of pro-inflammatory response in cyprinids such as zebrafish and common carp [147]. From the transcriptomic data gathered in this study, barramundi insensitivity to LPS is likely coupled to their lack of TLR4 and TLR4-associated molecular orthologs. It is noteworthy that DC maturation was inhibited by LPS in barramundi head kidney and spleen extracts, which contrasts with the induction of DCs maturation in mammals through the TLR4 pathway [148], corroborating the absence of TLR4 from the barramundi transcriptome. As the mucosal surfaces of teleosts are continuously challenged by a high density of environmental bacteria (relative to their terrestrial counterparts), this lack of pro-inflammatory sensitivity to LPS may confer an evolutionary advantage.

Environmental bacteria are represented predominantly by the α- and γ-proteobacteria in the surface waters [149]. Consequently, continuous activation of a pro-inflammatory innate immune response due to this abundance of LPS at the mucosal surfaces would represent a substantial draw on energy reserves, and subsequent reduction in fitness of fish competing for energy in an oligotrophic environment. Nevertheless, there is good evidence of a specific adaptive response to LPS O-antigen in several teleost species [150-152], so LPS O-antigen is clearly processed through antigen-presentation during the adaptive immune phase whilst only weakly pro-inflammatory during the early onset of innate immunity. A gene coding for a molecule similar to the lipopolysaccharide binding protein (LBP) was also present in the transcriptome, suggesting that although LPS only triggers a weak inflammatory response in fish, there are still molecules that are able to recognise LPS in fish. Moreover, a C-type lectin inducible by LPS in rainbow trout (*O. mykiss*) was recently identified, further suggesting that fish can respond to LPS to some extend [153]. In order to further uncover how barramundi cells recognise bacterial LPS O-antigen, CLRs were investigated in the transcriptome.

Several transcripts coding for putative CLRs in barramundi were uncovered (Figure 2.5). Particular interest was given to the putative barramundi Mincle. The Mincle CLR, coupled with FcR-γ, is inducible by LPS in mammals, leading to the phosphorylation of ITAMs, the recruitment of Syk and the transcriptional regulation of *TNFA* and *IL6* through the Card9-Bcl10-Malt1 complex [154]. The Mincle pathway is heavily dependent on protein phosphorylation and recruitment, and hence the transcription levels of many of the proteins necessary to this pathway were not expected to vary. The qRT-PCR analysis was thus targeted towards investigating the transcription levels of *IL6* and *TNFA*. IL-6 has previously been shown to suppress *TNFA* transcription levels when induced by LPS, as well as to stop TNF-α from inducing high cellular death *in vitro* [155, 156]. Although IL-6 and TNF-α are both classified as pro-inflammatory molecules, it seems that the balance between those two molecules in particular plays an essential role in the conservation of homeostasis within the immune system, especially in preventing auto-immunity. As the anti-Mincle antibody bound specifically to barramundi Mincle in Western blot, and pre-treating barramundi cells with the anti-Mincle antibody prior to exposure to the Mincle-specific agonist TDB blocked induction of *TNFA* and repression of *IL6*, the

blocking by the anti-Mincle of *TNFA* induction by LPS is strongly supportive that LPS induce *TNFA* via the Mincle pathway. The results presented in this paper therefore support that *TNFA* transcription can be induced by LPS through the Mincle pathway in *L. calcarifer* (Figure 2.8B). However, the anti-Mincle antibody did not block the inhibition of *IL6*, suggesting that *IL6* transcription is probably controlled through another molecular pathway, independent of Mincle (Figure 2.8A). Therefore, Mincle seems to partially supplant TLR4 in barramundi in its ability to recognise and process LPS, and to further induce an immune response through the regulation of inflammatory cytokines. Nevertheless, barramundi Mincle seems to be less sensitive to LPS than TLR4 in mammals, as higher concentrations of LPS were needed to induce a response (minimum of 500 ng/mL in *L. calcarifer* compared to 100 ng/mL in *H. sapiens*). Moreover, although the transcription of *TNFA* was upregulated by LPS stimulation, this upregulation was low compared to observations in mammals, corroborating the muted response to LPS in teleosts. Previous studies suggest that the involvement of the TLR4 complex in LPS recognition occurred after the divergence between fish and tetrapods [147], implying that other molecules might be involved in LPS recognition in earlier vertebrates. Beta-2 integrins such as the CD11/CD18 heterodimer, for example, have been shown to interact with LPS and have been hypothesised to play an important role in teleost LPS signalling [34, 118]. In humans and mice, the inflammatory caspase-4/5/11 have been shown to bind intracellular LPS through their CARD domain, and to induce immunity [31]. Putative β-2 integrins and caspases have been identified in the present barramundi transcriptome (data not shown) but further work is warranted in order to determine if those molecules can act as receptors for LPS in barramundi and other fish species and orders.

Chapter Three – Leucocyte integrins, but not caspases or NLR inflammasome are associated with lipopolysaccharide recognition and response in barramundi (*Lates calcarifer*)

3.1. 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 [149]. 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) [157]. 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 [158]. 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 [159]. 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) [160]. Briefly, the lipopolysaccharide binding protein (LBP) mediates the interaction between LPS on the bacterial cell surface and the glycoprotein CD14 on phagocytic cells [161]. CD14 then concentrates LPS to facilitate its binding to the TLR4/myeloid differentiation protein 2 (MD-2), which in turn triggers the inflammatory cascade [162]. 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 [159]. 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 [159]). 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 [163]. Fish, on the other hand, seem to be resistant to endotoxic shock caused by LPS [118].

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 [118]. Recently, advances in bioinformatics have established that TLR4 is absent from most of the published genomes and immune transcriptomes from teleost species [4, 118, 164] 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 [118]. However, there is evidence of LPS-induced cytokine production in fish [119, 165], which suggest that other molecules are likely involved in LPS recognition and processing in teleosts. In previous work, LPS stimulation induced TNF-α transcription through the C-type lectin Mincle in barramundi, but seemed to induce IL-6 transcription through other molecular pathways [164]. 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 [29, 30]. 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 [23, 26]. 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 [27].

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) [33, 34]. Both CD11b/CD18

(MAC-1) and CD11c/CD18 (p150/95) have been identified as LPS receptors in mammals [34, 166]. 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 [33]. 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 [34, 36, 166]. More specifically, MAC-1 has been shown to enable LPS uptake and subsequent inflammatory pathway activation independently of TLR4 signalling [145] and p150/95 has been shown to activate a cellular response after binding to LPS in a CD14-independant manner [34].

In the present study, putative barramundi caspases, NLRs and leukocyte integrins were investigated in the barramundi immune transcriptome [164]. Barramundi inflammatory caspases, inflammasomes and leukocyte integrins were identified and characterised, providing further insight into the processes underlying LPS recognition in *Lates calcarifer*.

3.2. Materials and Methods

3.2.1. 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 and the salinity were maintained at 28 ± 2°C and 15 parts per thousand (ppt) respectively. Water quality was checked regularly for ammonia, nitrite, nitrate and pH, and water exchanges were 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.

3.2.2. Bioinformatics analysis

Using the previously generated and annotated barramundi immune transcriptome [164], 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 A2.3.1, A2.3.2 and A2.3.3 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 A2.3.1 for caspases and with the cDNA sequences listed in Table A2.3.3 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 A2.3.2), using the Minimum Evolution method with bootstrap replication (2000) in MEGA6.06, after ClustalW amino-acid alignment [167]. 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 [29, 30]. Moreover, each mRNA sequence was aligned using the published barramundi genome [168] to ensure that all the caspase, NLR and integrin genes were correctly identified from the transcriptome (Figure A2.3.1, A2.3.2 and A2.3.3 for caspases, NLRs and integrins respectively).

3.2.3. 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 cells. 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 [169, 170]. Collagen was used as a positive control as it has been identified as a ligand for the

αL, αM and αX subunits [33]. Briefly, wells of a high-binding 96 well plate was coated overnight at 4°C with either 100 µL of 20 µg/mL fibronectin, ESM-1, factor X or collagen, or with 100 µL undiluted Poly-L-Lysine solution or 1% BSA as negative controls (all from Sigma). The wells were then washed three times in 1M phosphate buffer saline (PBS) 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 1M 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 1M 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.

3.2.4. 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 [164] and leucocytes were isolated on a Percoll gradient as adapted and modified from Tumbol, Baiano and Barnes [171]. Briefly, the cell suspension was layered over a 34% / 51% discontinuous Percoll gradient and centrifuged for 30 min at RT (800 *x g*, acc. 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*, acc. 9, brake 9, RT, Eppendorf 5810R), before being resuspended in L-15 with 5% heat inactivated barramundi serum 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 $2x10^6$ 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 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).

3.2.5. Inflammatory cytokine regulation assessed by qRT-PCR

TNF-α, 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 [34, 36, 166]. 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) 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 *TNFA, IFNA, NF-κappaB*, *IL1B* and *IL6* 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 3.1). After primary optimisation, only *TNFA*, *NF-κappaB* and *IL1B* expression levels were assessed, as the primers for *IFNA* and *IL6* could not be optimised to the template.

Table 3.1. List of primers for qRT-PCR.

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

3.2.6. 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) [130-132]. 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 through REST.

3.2.7. Ethics statement

All animal work was conducted in accordance with Animal Care and Protection Act 2001 (QLD) and the NHMRC Code of Practice 2013. 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."

3.3. Results

Putative barramundi caspases were identified by homology and in most cases grouped clearly with their human counterpart (Figure 3.1), and their gene organisation was determined (Figure A2.3.1). 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. Moreover, although identified by homology in barramundi, caspase 5 and caspase 9 were lacking a CARD pro-domain, thus differing from their human counterparts (Figure 3.1).

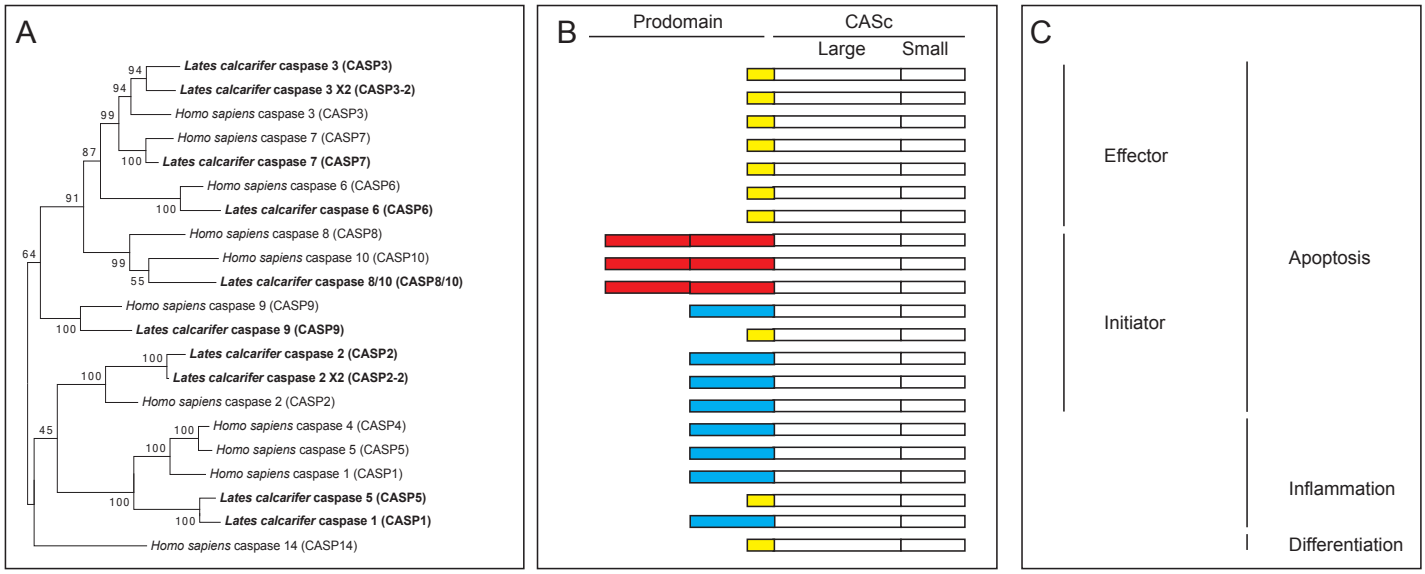

Figure 3.1. Characterisation of human and barramundi caspases. (A) Phylogenetic relationship between human and barramundi (in bold) caspases are linked with (B) domain organisation of each corresponding protein and (C) functional categorisation based on literature. Yellow boxes correspond to a short 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.

Putative barramundi NLRs were identified by homology and grouped with their human and murine counterparts (Figure 3.2), and their gene organisation was determined (Figure A2.3.2). 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)) [23], only sequences coding for proteins 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 (Figure 3.2).

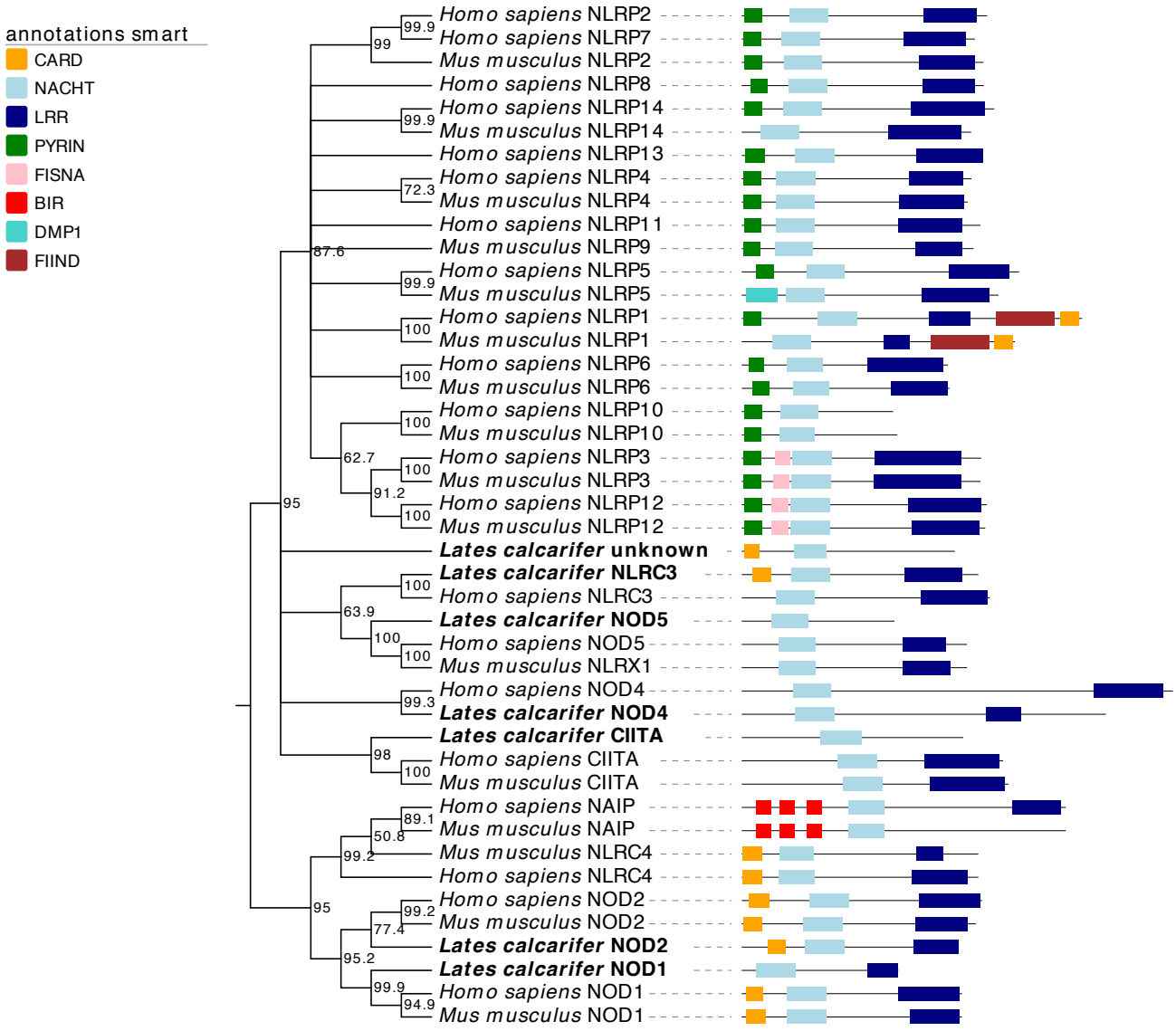

Figure 3.2. Phylogenetic relationship between human, murine and barramundi (in bold) NLRs based on their NACHT domain. Protein domain organisation is depicted on the right of the phylogenetic tree. CARD: caspase activation and recruitment domain; NACHT: nucleotide-binding and oligomerization domain; 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.

Putative barramundi α- and β2-integrin subunits were identified by homology with their human, murine and teleost counterparts (Figure 3.3), and their gene organisation was determined (Figure A2.3.3). 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.

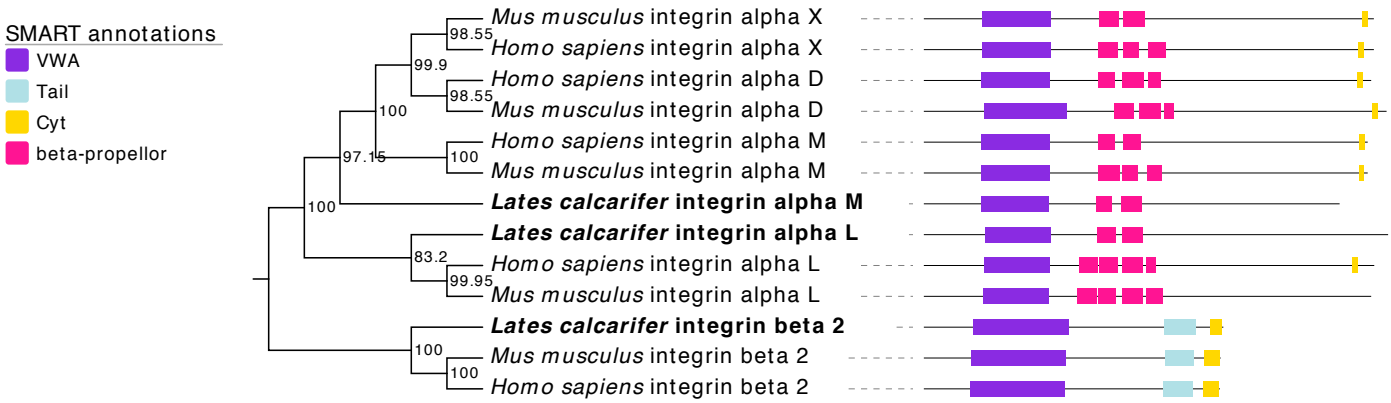

Figure 3.3. Phylogenetic relationship between human, murine, teleost and barramundi (bold) α- and *β*2-integrin subunits. Protein domain organisation is depicted on the right of the phylogenetic tree. VWA: Von Willebrand factor type A; Tail: tail domain; Cyt: cytoplasmic domain.

When incubated with substrates specific to the D, M and L α-sub-units, 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) (Figure 3.4). Interestingly, when incubated with anti-ITGAM antibody, cells from barramundi spleen were more adherent to the integrin αM substrate factor X (Figure 3.4). Similarly, cells incubated with anti-ITGAL antibody were more adherent to integrin αL substrate ESM-1 (Figure 3.4).

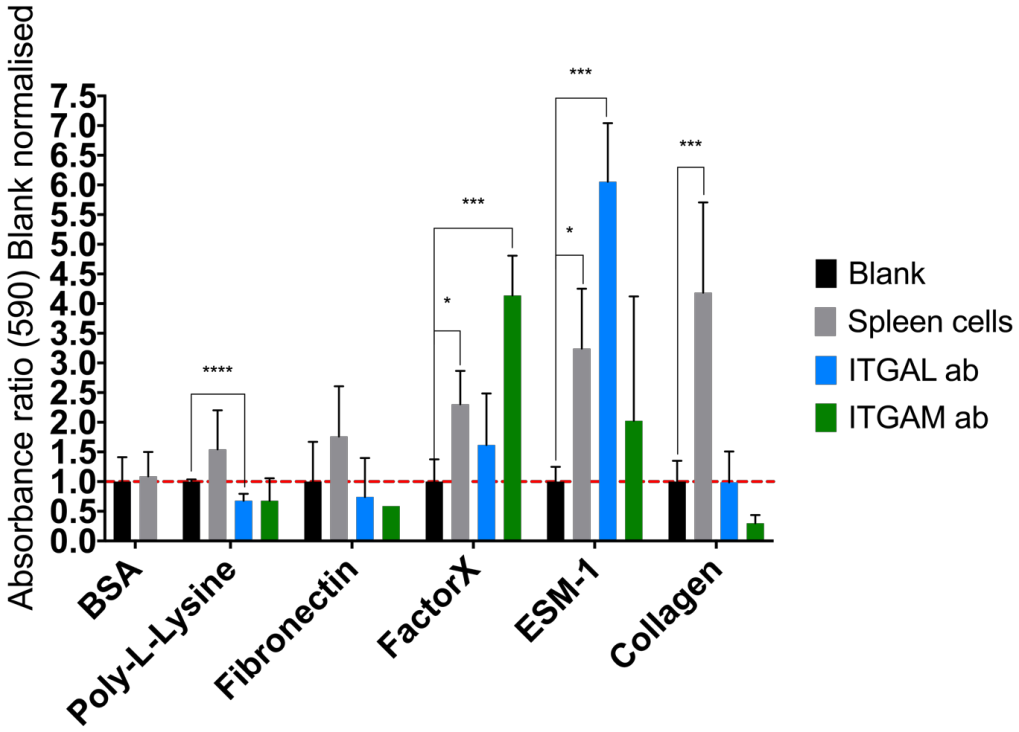

Figure 3.4. Adherence of barramundi spleen cells to different substrates. Significant differences between blank incubated and cells incubated wells are represented with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ and **** $p < 0.001$.

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 (Figure 3.5).

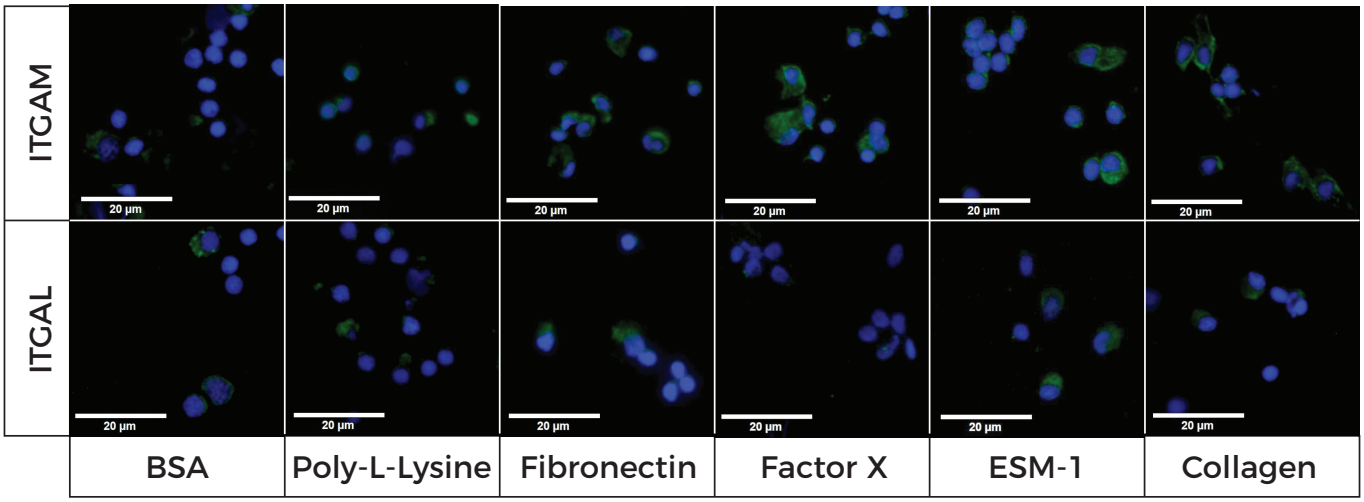

Figure 3.5. Micrographs of leukocytes adhering to each substrate. DAPI stain (nucleic acid) shown in blue and anti-ITGAM or anti-ITGAL specific antibodies stain shown in green.

To determine whether integrins might be involved in LPS recognition and response, a stimulation assay was conducted and the expression of a cohort of cytokines determined by qPCR. The specificity of anti-ITGAM and anti-ITGAL antibodies was confirmed by the use of a polyclonal rabbit IgG isotype control, showing that pre-incubation with either anti-ITGAM or anti-ITGAL specifically impacted the expression of *IL1B* through the activation of integrin αM and L respectively (Figure A.2.3.4). LPS stimulation upregulated the expression of *IL1B* and, to a lower level of *TNFA*, but did not impact the expression of *NF-kappaB* (Figure 3.6). Pre-incubation with anti-ITGAM and anti-ITGAL significantly upregulated the expression of *IL1B* compared to unstimulated and isotype controls when further exposed with LPS. However, *TNFA* 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 *TNFA* expression after a short incubation with LPS do not depend on integrins αM and L activation.

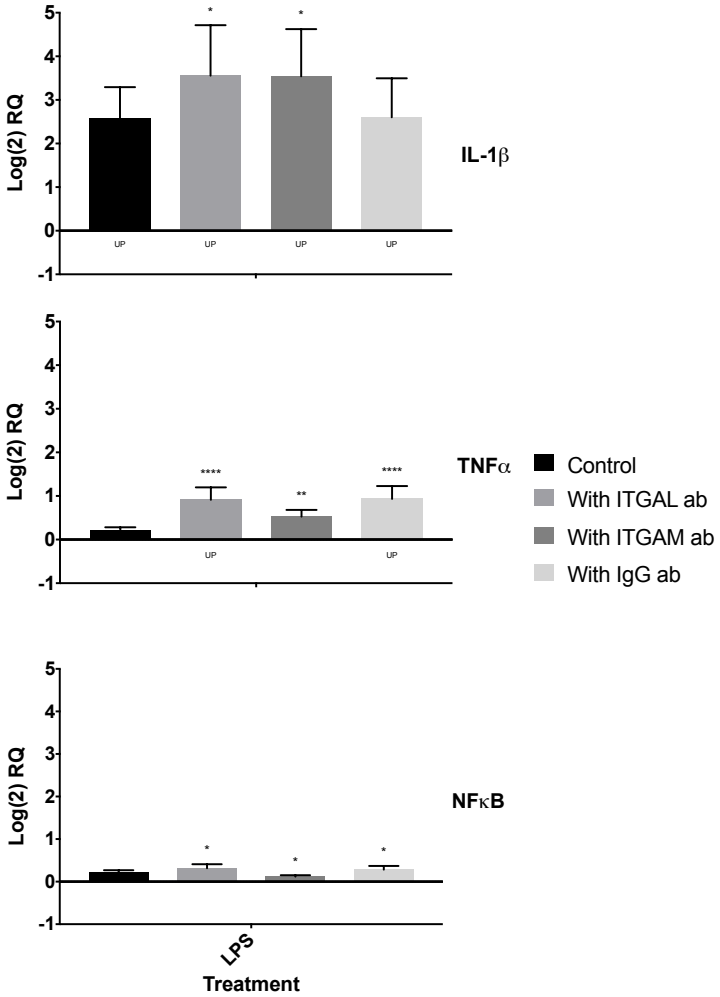

Figure 3.6. Differential expression of cytokines and transcription factor in response to LPS stimulation. Expression of *IL1B* (A), *TNFA* (B) and *NF-κappaB* (C) in barramundi splenocytes is shown with or without pre-incubation with anti-ITGAL, anti-ITGAM or rabbit IgG isotype antibody. Significant 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 *** $p < 0.005$.

3.4. 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 [26, 31]. 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 [32]. 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 [27, 172]. 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 [23, 27, 173]. 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 [173]. 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 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 [114].

Leukocyte integrins are found at the surface of white blood cells and are involved in pathogen recognition through conformational changes after activation by ligands [174]. 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 [169], 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 [169], 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 [175]. Indeed, integrin αMβ2 (MAC-1) and αLβ2 (LFA-1) can assume two conformations, open (active) and closed (inactive), which differentially recognise ligands [174, 176]. Changes in configuration are crucial to integrin function

and can also influence avidity and affinity of the leucocyte integrin [174, 177], impacting adhesion to ligands, as detected in the adhesion assay reported here (Figure 3.4). However, in view of the data obtained, it seems that collagen is in fact not a specific ligand for either integrin αMβ2 or αLβ2. Instead, collagen seemed to bind spleen cells without discrimination, regardless of αMβ2 or αLβ2 being present at the surface of those cells (Figure 3.4).

Currently, two binding sites for LPS have been identified on the β2 chain of leucocyte integrins [36]. Moreover, integrin αMβ2 has been shown to enable LPS recognition and activation independently of TLR4 [145], and processes depending on MAC-1 activation have been shown to induce the inflammatory cytokine IL-1β's expression [145, 178]. 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 [179, 180]. Almost all cells that bound to ESM-1 expressed integrin αL (ITGAL) and were more rounded, resembling B- or T-lymphocytes [33]. Considering that MAC-1 has recently been identified as a LPS receptor in mammals [145], 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. The expression of *IL1B* was statistically higher in cells stimulated by LPS, however LPS did not affect *TNFA* expression or *NF-kappaB* activation in control cells (Figure 3.6). Moreover, prior incubation with anti-ITGAL and anti-ITGAM antibodies activated integrins on barramundi splenocytes evidenced by significantly increased expression of *IL1B* post-stimulation with LPS when compared to either unstimulated cells or isotype control cells pre-incubated with rabbit IgG (Figure 3.6A). Pre-incubation with antibodies (both treatments as well as the isotype control) resulted in a significant upregulation in the expression of *TNFA* when compared to the unstimulated control, suggesting that *TNFA* 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 *IL1B* upregulation but not impacting the expression of *TNFA* [181]. However, before integrins can act as receptors, they need to be activated, which was likely effected by incubation with anti-ITGAL and anti-ITGAM antibodies

prior stimulation, supporting the difference in *IL1B* regulation by LPS between pre-stimulated and control cells (Figure 3.6A).

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 [164]. In the current study, we show that 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.

Chapter Four – Identification of barramundi (*Lates calcarifer*) DC-SCRIPT, a specific molecular marker for dendritic cells in fish

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4.1. Introduction

Jawed fish are the earliest vertebrate order possessing both innate and adaptive immunity [74, 77]. Adaptive immunity is characterised by the development of an immune memory of previously encountered pathogens that allows a fast and specific response when reinfection by the same pathogen occurs. Adaptive immunity involves antigen recognition by antigen presenting cells (APCs) and an associated antibody response and pathogen elimination, generally performed by primed B and T-lymphocytes [2]. B-lymphocytes are specialised in producing antibodies that label pathogens and make them easier to identify by phagocytic cells [2]. T-lymphocytes assist in leucocyte activation or they destroy tumorous and virus infected cells, depending on the cell type [2]. T-cell activation is initiated by and is dependent upon the major histocompatibility complex (MHC) receptors which bind foreign and local peptides produced by protein degradation [41]. Naïve T-cells are activated after they are presented with foreign antigens that are bound on the MHC [41]. “Non-professional” APCs can be any cell in the organism and can only activate cytotoxic T-cells by displaying antigenic structures on MHC class I receptors or via stimulation by cytokines [42]. In contrast, “professional” APCs are specialised in priming naïve T-cells and can activate both cytotoxic and helper T-cells by displaying antigenic structures on MHC class I and II receptors respectively and by producing co-stimulatory molecules [43]. B-cell proliferation can also be indirectly activated by professional APCs [2]. The principal professional APCs in mammals are dendritic cells (DC), and are defined by their extended ability to engulf pathogens, present antigens and activate potent helper T-cells [1, 44, 45].

The existence of DC-like cells in teleosts has been controversial for many years, in part due to the absence of specific identification tools such as cell surface receptor markers, and due to significant differences between the immune systems of teleosts

and mammals. For example, the lymph nodes and the bone marrow, which are essential to mammalian immunity by seeding the lymphoid organs, are absent in fish [74, 75]. Moreover, the lymph nodes are also the major site for antigen presentation, thus the main site where DCs interact with T-cells in mammals [182], and their absence in fish is an important consideration in the study of fish acquired immunity. Consequently, processes such as antigen presentation are still poorly understood in fish, and the lack of specific cell markers makes the study of these immune processes even more challenging. In spite of these challenges, dendritic-like cells have been identified recently in head kidneys (HK) and/or spleens of zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) [76, 83, 183, 184]. Organs such as the HK and the spleen in fish seem to effectively replace the missing leucocyte-producing mammalian organs. The HK has a major role in immune-endocrine interactions and, in fish, supplants the bone marrow as the main hematopoietic organ [74]. Fish spleen, which is analogous to mammalian spleen and is a likely site for T-cell-APC interactions [76]. However, the lack of DC-specific markers in fish means that the cells require laborious functional and morphological characterisation making the study of antigen presentation in fish extremely difficult. Recently, a zinc-finger protein, DC-SCRIPT, which is preferentially expressed in all subsets of dendritic cells in humans [185] and in mice [186], was discovered. Importantly, DC-SCRIPT was found to be identical to *Homo sapiens* ZNF366, which is homologous to the teleost fish *Fugu rubripes* fZnf1 gene [185]. This, in conjunction with the lack of confinement of DC-SCRIPT to a particular subset of DCs, makes it a logical target as a first molecular marker for fish DCs.

In the present study we identify DC-SCRIPT for the first time in a Perciform fish, the barramundi, *Lates calcarifer* and show specific expression in barramundi dendritic-like cells (bDCs). The order Perciformes represents the largest extant group of vertebrates (comprising 40% of known fish species) and comprise an increasing proportion of the globally expanding aquaculture industry, particularly high value fish such as tilapias, snappers, sea bass, breams, jacks and barramundi, yet little work has been conducted to date on immunity in this order. Barramundi represent a good candidate for further research on perciform immunity as hatchery technology is well established, they are easy to keep under controlled conditions in recirculating systems and are of significant value to aquaculture throughout south east Asia [85].

Improved tools for in vitro models of the cellular processes leading to specific adaptive immunity are therefore significant in the refinement of vaccines for the aquaculture industry and our understanding of antigen presentation in early diverging vertebrate orders.

4.2. Materials and Methods

4.2.1. Experimental animals and husbandry

Barramundi (*Lates calcarifer*) juveniles of approximately 30–100 g were obtained from a commercial aquaculture facility near Cairns, Queensland, Australia. Fish from this farm are specific pathogen free fish as the direct supply of filtered bore water to the hatchery and farm coupled with strict biosecurity prevent exposure to common fish pathogens. Fish were transported to The University of Queensland by air freight and acclimatised for 2 weeks in a recirculating system in 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 and the salinity were maintained at 28 ± 2°C and 15 part per thousand (ppt) respectively. Water quality was checked daily for ammonia, nitrite, nitrate and pH, and water exchanges were applied as required. Fish were fed to satiation once daily with a commercial diet for barramundi (Ridley Aqua Feed).

4.2.2. Serum collection

Barramundi were bled aseptically from the caudal vein and the blood was allowed to clot for 1 h at room temperature. Samples were centrifuged at 12,100 *x g* for 10 min and the supernatant (serum) was stored at -20°C until further use. Prior to use, serum was heat inactivated (HI) for 30 min at 56°C.

4.2.3. Identification of Barramundi DC-SCRIPT

DNA and RNA extraction

DNA was extracted from cultured barramundi HK and spleen cells (in 75 cm^3 flasks, 10^7 cells/mL) using Nucleospin Tissue DNA kit (Machery Nagel) according to the manufacturer's instructions. Extracted DNA was subsequently used for the construction of four DNA-libraries as described in the Universal Genome Walker 2.0 kit (Clontech Laboratories, Inc). RNA was extracted from HK and spleen cultured cells (in 75 cm^3 flasks, 10^7 cells/mL) using the RNeasy Mini Kit (QIAGEN) according

to the manufacturer's instructions. Extracted RNA was subsequently synthesized into complementary DNA (cDNA) RACE libraries using the SMARTer RACE cDNA Amplification kit (Clontech Laboratories, Inc) section V.

Primer design, genome walking and 5' 3' RACE PCR

Initially, degenerate primers for teleost DC-SIGN were designed from conserved sequences derived from multiple alignments (CLUSTALW) of hypothetical DC-SCRIPT identified by nucleotide Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/) of completed teleost genomes using human DC-SCRIPT (NM_152625.1) as the query sequence. Sequences included in the alignment were derived from the genomes of *Pundamilia nyererei* (XM_005741743), *Maylandia zebra* (XM_004547137), *Haplochromis burtoni* (XM_5919046), *Oreochromis niloticus* (XM_005452162), *Takifugu rubripes* (XM003974872) and *Danio rerio* (XM_693094.5). Amplicons derived from genomic DNA by degenerate PCR were sequenced and those with homology to DC-SCRIPT by BLAST were employed to design gene specific primers for genome walking and RACE in accordance with the manufacturer's instructions (Clontech). New primers were designed following each round of walking or RACE to obtain sufficient sequence for further experimentation.

mRNA Fluorescence In Situ Hybridization (FISH) and immunocytochemistry

To identify bDCS in mixed and enriched cell cultures, mRNA FISH was employed. Around 500 base pairs of DC-SCRIPT cDNA sequence, downstream of the zinc finger region, was used to design mRNA custom FISH probes using the Stellaris® FISH Probe Designer tool available online at www.biosearchtech.com/stellarisdesigner (Biosearch Technologies). In all 32 FISH probes were designed and conjugated to CAL Fluor 590 (Biosearch Technologies). Mixed-cell populations were then allowed to adhere on round glass coverslips at 28°C, were subsequently stimulated for 2 h with peptidoglycan (PTG; 10 µg/mL), and underwent immunocytochemistry (ICC) fluorescent staining procedures. Different treatments included anti-IgM (1:1000 primary antibody sheep IgG anti-barramundi IgM; 1 µg/mL secondary antibody donkey anti-sheep IgG conjugated with AlexaFluor 405 for combined staining or with AlexaFluor 594 for separate staining), anti-MHCIIa (1:1000 primary antibody rabbit IgG anti-zebrafish MHCIIa (Sapphire Bioscience,

Product LS-C210021); 1 µg/mL secondary antibody goat anti-rabbit IgG conjugated with AlexaFluor 488) and *in-situ* hybridisation with the DC-SCRIPT probes (50 ng), either combined or stained separately. Antibodies and Stellaris® FISH probe titrations were previously performed to determine optimal concentrations. Controls consisted of the same procedure while omitting the primary antibodies or the Stellaris® FISH probe. The adherent cells were hybridised and stained, with some modifications, following the Stellaris® FISH probes manufacturers instructions (For simultaneous Stellaris FISH and immunofluorescence using adherent cells) available online at www.biosearchtech.com/stellarisprotocols. Briefly, the cells were fixed in 4% paraformaldehyde (Electron Microscopy Science) for 10 min at room temperature (RT), washed in 1X PBS (5 min; RT) and permeabilized with Triton-X for 3 min (Sigma; RT). The cells were then washed in 1X PBS twice (5 min; RT) and blocked in 5% donkey serum, 5% goat serum, 0.1% bovine serum albumin for 30 min at RT, followed by two more washes in 1X PBS (5 min; RT) and an incubation in the dark at 37°C for 4h in hybridisation buffer containing 50 ng of FISH probe and primary antibodies at the dilutions specified above. Subsequently, the cells were washed three times in 1X PBS (5 min; RT) and incubated in wash buffer as recommended by the manufacturer (Stellaris® FISH Probes protocol) containing the appropriate secondary antibodies (see above) for 30 min at 37°C in the dark. When stains were combined, the coverslips were mounted at this point using VECTASHIELD® HardSet Mounting Medium (Vector Laboratories). When stains were separated, cell nuclei were stained with DAPI (Invitrogen; 2.5 µg/mL) for 15 min at RT in the dark before the coverslips were mounted using VECTASHIELD® HardSet Mounting Medium (Vector Laboratories).

Fish injection and RNA extraction for qRT-PCR

Barramundi were injected in the peritoneal cavity with 100 µL of either sterile phosphate buffered saline (PBS control) or a mixture of lipopolysaccharide (LPS; 10 µg/mL) and peptidoglycan (PTG; 10 µg/mL) diluted in PBS. LPS and PTG were chosen based on both *in vivo* and *in vitro* induction of immune response in fish/fish cells previously reported [123, 124]. At 6 h, 24 h, 72 h and 7 d post-injection, four fish were sampled from both control and treatment groups. For each fish, the spleen and head-kidney were dissected aseptically and kept in RNAlater until further processing.

RNA was extracted using the RNeasy extraction kit (QIAGEN), according to the manufacturer's instructions. Tissues kept in RNAlater were disrupted by serial passage through a 25-gauge needle mounted on a 1 mL syringe before processing. RNA was eluted in 40 µL of nuclease free water.

Quantitative real-time PCR for relative quantification of DC-SCRIPT expression
RNA samples were treated with DNAse (RNAse-free DNAse set, Qiagen) to remove any trace genomic DNA then converted to cDNA using the QuantiTect RT kit (QIAGEN) according to the manufacturer's instructions. cDNA was quantified by Qubit Fluorimetry and 0.5 ng per sample was optimised as template to yield approx. 100% efficiency in 10 µL real-time PCR reactions with 0.2 picomoles of each primer pair specific for DC-SCRIPT and three endogenous reference genes (Table 4.1) [187]. Primers were designed to span the intron/exon boundaries to eliminate amplification of any traces of genomic DNA. Amplification of 3 technical replicates from each of the 4 biological replicate samples per treatment and control was performed in 384 well plates on a ViiA 7 Real-Time PCR system (Applied Biosystems) using SYBRgreen MasterMIx (Invitrogen) and cycling parameters as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 54 °C for 30 sec and 62 °C for 30 sec, 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. The PCR exponential amplifications of each gene were calculated based on the slope estimated by the Cq value of a serial dilution in a preliminary validation experiment against Log_{10} of the cDNA amount per reaction, described by $E = 10^{-1/slope}$ [188]. Any variation in amplification efficiency was then accounted for during normalisation of relative gene expression differences between the treatment samples and the control samples. The normalisation factor was calculated based on the geometric mean of the relative quantities of three reference genes using REST [189, 190]. Cq data were analysed with REST based on a pairwise fixed reallocation randomisation test [190], in which the normalised relative quantities were calculated based on the ratio of the group means for the target gene against the normalised reference genes. The result represented the up- or down-regulation of the treatment group (i.e. LPS/PTG-injected) compared to the control group (i.e. PBS injected).

4.2.4. Cell culture & functional assays

Cell culture from hematopoietic organs

Barramundi were killed by overdose of anaesthetic (Aqui-S). Fish were bled from the caudal vein before the spleen and HK were removed aseptically. The organs were placed on ice in complete L-15 medium: L-15 Leibovitz medium with phenol red (Invitrogen) supplemented with HI 10% foetal bovine serum (FBS), 2% Nystatin (N; 10,000 U/mL stock) and 1% penicillin-streptomycin (PS; 10,000 U/mL stock). The organs were then gently pushed through a 100 µm cell strainer with the plunger of a 1 mL tuberculin syringe to obtain a single cell suspension in supplemented medium. Cells were counted and adjusted to a concentration of 1 x 10^7 cells/mL in supplemented L-15 and plated in 25 cm^2 or 75 cm^2 culture flasks. Cells were incubated at 28°C for 5–14 days. During this period, non-adherent cells were harvested and the removed medium was replaced by fresh supplemented medium.

Barramundi DCs harvesting and enrichment

Prior to harvest, culture flasks were gently agitated and medium containing the suspended non-adherent cells was collected. The suspension was layered over a discontinuous Percoll gradient (Sigma) at d = 1.058 g/mL and d = 1.048 g/mL and centrifuged for 30 min at 800 *x g* with no brake at 23°C, as adapted and modified from Bassity and Clark [76]. The cells at the interface between the Percoll layers were collected and washed at 23 °C in fresh medium (10 min, 400 *x g*) before further use.

Transwell migration assay

For this study, two isolates of *Streptococcus iniae* (strains QMA0076 and QMA0248) from disease outbreaks in Australian barramundi farms (in Queensland and New South Wales respectively) were chosen. These strains had previously been employed in a vaccination trial in which experimental fish responded poorly to vaccines prepared from QMA0076, but responded strongly with consistently high antibody response to vaccines prepared with QMA0248 [191]. The migratory ability of bDCs was assessed using a 12-well Transwell culture plate (Corning) with membranes presenting 3 µm pores which allowed active migration between upper and lower compartments, but were substantially smaller than the cells (10-20 µm) to minimise passive transfer between the chambers. For each treatment, 1.5 mL of

0.5% FBS/L-15 was placed into the lower part of the Transwell and either lipopolysaccharide (LPS; 5 µg/mL), peptidoglycan (PTG; 10 µg/mL) or live *S. iniae* (strains QMA0076 or QMA0248 at a multiplicity of infection (MOI) of 0.1 and 1) were added to the medium. The control was composed of medium alone in the lower chamber. Barramundi DCs (500 µL of 4 x 10^5 cells/mL), resuspended in 0.5% FBS/L-15, were added to the upper chamber. The Transwell plate was then incubated for 4 h at 28°C to allow migration. After 4 h, the cells remaining in the upper chamber were removed by gently swabbing a cotton bud against the membrane. The cells attached on the lower part of the membrane were detached with trypsin (Sigma; 10 µg/mL) for 1.5 min. Cells were then stained with DAPI (Invitrogen; 2.5 µg/mL for 15 min) and counted on a haemocytometer using fluorescent microscopy.

Phagocytosis assay

The preliminary assessment of bDCs phagocytic capacity was performed in 24-wells plates using fluorescent latex beads. The bead stock suspension (Fluoresbrite Yellow Green microspheres 0.2 µm, ~5.68 x 10^{12} particles/mL, 2.65% latex, Polysciences, Inc.) was diluted 50-fold in phosphate buffered saline (PBS) pH 7.4 and opsonised by addition of HI barramundi serum to 10% (v/v). Barramundi DCs concentration was adjusted to 5 x 10^6 cells per well in 500 µL L-15 in a 24-well tissue culture plate. Then opsonised beads were added to each well, at a 1:10 dilution in 10% FBS/L-15 and plates centrifuged at 500 *x g*, 23°C for 5 min to bring the beads and cells into contact, as adapted and modified from Bassity and Clark [76]. After 2 h incubation at 28°C, the plates were put on ice to stop the cell activity and were observed by microscopy directly in culture or as cytospins (see below).

Further analysis of bDCs phagocytic capacities was performed using flow cytometry. When fluorescent beads were used, the protocol remained the same as described above, with the following exceptions. After incubation (2 h at 28°C), the cells were washed from the plates in medium and transferred to 5 mL cytometry tubes before being put on ice to stop the cell activity until flow cytometry analysis.

For phagocytosis of bacterial strains, *S. iniae* QMA0076 and QMA0248 were adjusted to an optical density at 600 nm (OD_{600}) of 1 (~10^8 bacteria/mL). They were then stained with 1 µM BacLight Green (BLG)(Life technologies) for 15 min at RT before being washed extensively in PBS and diluted 100-fold in 10% FBS/L-15.

Barramundi DCs concentration was adjusted to 5 x 10^6 cells per well in 500 µL and the medium containing stained bacteria was added resulting in a final multiplicity of infection (MOI) of 1.0. After incubation for 2 h at 28°C, the cells were washed from the plates in medium, transferred to 5 mL flow cytometry tubes and put on ice to stop the cell activity until flow cytometry analysis.

T-lymphocyte isolation by E-rosette

After sterile removal of the spleen from euthanized barramundi, a single cell suspension was obtained by forcing the spleen through a 100 µm cell strainer with the plunger from a 1 mL tuberculin syringe. The suspension was then centrifuged on a d = 1.072 g/mL and d = 1.050 g/mL discontinuous Percoll gradient at 800 *x g*, 23°C for 30 min, as adapted and modified from Tumbol, Baiano and Barnes [171]. The leucocytes, located at the interface between the two Percoll layers, were collected and diluted 2-fold in L-15 medium. Cells were washed by centrifugation at 400 *x g*, 23°C for 10 min. After centrifugation, the supernatant was discarded and the cell pellet resuspended in L-15 medium supplemented with 10% FBS/1% PS.

To obtain a pure culture of live T-cells to test in the proliferation assay, the E-rosette method was used, as adapted and modified from Madsen, Johnsen, Hansen and Christiansen [192]. Sheep red blood cells (SRBC) in Alsever's solution were diluted 1:1 in complete L-15 medium. Equal volumes of spleen leucocytes and SRBC in L-15 were then mixed in a 1.5 mL Eppendorf tube and centrifuged for 5 min at 5870 *x g* to bring the leucocytes and SRBC in contact. The tubes were then incubated at 28°C overnight. On the next day, the cells were resuspended and layered and centrifuged over a d = 1.072 g/mL and d = 1.050 g/mL discontinuous Percoll gradient at 800 *x g*, 23°C for 30 min. The supernatant was discarded and the pellet containing the SRBC and the T-cells was washed twice in PBS. RBC lysis buffer (1 mL) was then added to the pellet and, when the suspension became translucent, complete L-15 medium was added to stop the reaction. The enriched T-cells, released from the rosettes, were then washed twice and resuspended in L-15/10% FBS medium (Figure A2.4.1).

T-cell proliferation assay

Responder cells (T-cells) and stimulator cells (bDCs) from different fish were counted and resuspended in L-15 supplemented with 5% barramundi serum, 1% PS

at a concentration of 5 x 10^5 cells/mL. Responder cells were stained using 5 μM/mL carboxyfluorescein succinimidyl ester (CFSE; Sigma) for 30 min at 28°C. Cells were then washed twice in PBS by centrifugation. T-cells and bDCs were mixed together at a 1:1, 1:2, 1:4 and 1:8 stimulator to responder ratio and incubated for three days (experimentally determined to be optimal) at 28°C in the dark. Negative controls were composed of responder cells alone. On day 3, cells were analysed by flow cytometry using decrease in CFSE fluorescence as an indicator of proliferation.

Flow cytometry

Flow cytometry data were obtained using the BD FACSAria II (BD Bioscience). Data were analysed using the DIVA software. Hoechst and propidium iodine (PI) fluorescent dyes (both from Life Technologies) were used according to the manufacturer's instructions to identify and exclude debris and dead cells respectively. The FITC voltages for the phagocytosis and proliferation functional assays were set at 419 V and 520 V respectively and the 605/12 violet voltage was set at 485 V for the Stellaris® FISH probe labelling.

4.2.5. Microscopy and non-fluorescent staining

Cultures were viewed with an Olympus CKX41 inverted microscope and cytospin slides were viewed using an Olympus BX41 epifluorescent microscope. On both microscopes, images were captured with an Olympus DP26/U-CMAD3 camera and optimised with the imaging software CellSens (Olympus Optical Co. Ltd, Japan). Cytospins were performed with the Cellspin I (Tharmac GmbH), stained with the Hemacolor staining kit (Merck Millipore), according to manufacturer instructions, and subsequently mounted with Permount medium (Fisher Scientific). Cytospins of cells previously stained with fluorochromes were mounted using DABCO anti-fading medium (Sigma).

4.2.6. Statistical analysis

Data analysis was performed with R v2.15.0 (R Core Team, 2012). Before analysis, homogeneity of variance was checked using Cochran's test. Data from the qRT-PCR and the migration assay were analysed using analysis of variance (ANOVA) tests. Where ANOVA indicated significant differences, it was followed by pairwise

comparison t-tests. As variances were not homogeneous for the phagocytosis assay, the non-parametric Mann-Whitney U test was used.

4.2.7. Ethics statement

All animal work was conducted in accordance with Animal Care and Protection Act 2001 (QLD) and the NHMRC Code of Practice 2013. Work was conducted under the University of Queensland Animal Ethics Committee Approval No. SBS/056/13/ARC "Cellular immunity in fish: Robust defence against infection or Achilles heel?"

4.3. Results

4.3.1. DC-SCRIPT is a potential marker for DCs in barramundi

A putative gene for DC-SCRIPT was identified in barramundi genomic DNA and in cDNA derived from RNA extracted from stimulated enriched dendritic cells, by genome walking and RACE PCR respectively. A complete cDNA was obtained comprising 2385 nucleotides (nt) translating to a 795 amino acid (aa) putative protein with 11 conserved C_2H_2-like zinc fingers, a proline rich region at the N-terminal and an acidic region at the carboxy terminal end (Figure 4.1A). Nucleic acid binding and metal ion binding functions were identified with InterProScan [193]. Domain organisation was predicted using SMART online software (http://smart.embl-heidelberg.de) and was compared to a panel of DC-SCRIPT/Zinc-finger protein 366 from mammalian, aviary and fish species (Figure 4.1B). Comparison of RACE-derived cDNA sequence with gDNA obtained by genome walking revealed that Barramundi DC-SCRIPT comprises 6 exons (Figure 4.1C). Complete cDNA and gDNA sequences have been deposited in Genbank with accession number KM386621. The cDNA sequence and the translated protein sequence were highly homologous to predicted zinc-finger protein 366 from multiple teleost and bird hosts (including damselfish (*Stegastes partitus*), puffer fish (*Taifugu rubripes*), rock dove (*Columba livia*) and American crow (*Corvus brachyrhynchos*)) and to DC-SCRIPT from human (*Homo sapiens*) and mouse (*Mus musculus*) using BLAST (http://blast.ncbi.nlm.nih.gov/). The close homology was supported by phylogenetic analysis using maximum likelihood (MEGA 5.2.2 for Macintosh, Figure 4.1D).

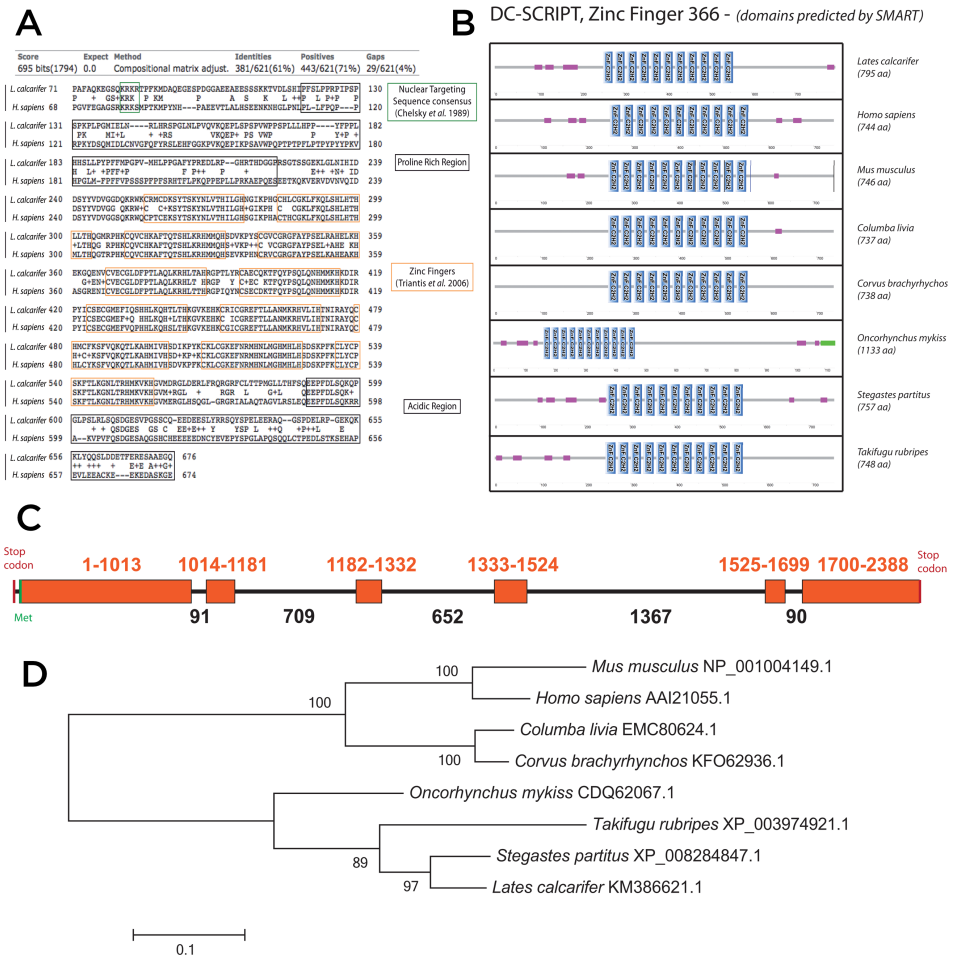


Figure 4.1. Amino-acid sequence alignment, domain organisation, gene organisation and phylogeny of DC-SCRIPT from *L. calcarifer*. (A) Amino acid sequences comparison between barramundi (*L. calcarifer*) and human (*H. sapiens*) DC-SCRIPT, aligned on the NCBI website (http://www.ncbi.nlm.nih.gov) by protein BLAST. The green box represents the nuclear targeting sequence consensus (KRK*) as per Chelsky *et al.* [194]. The black boxes represent a proline rich region and an acidic region at the N- and C-ends respectively. The 11 orange boxes represent the 11 zinc finger sequences as identified by Triantis *et al.* [185]. (B) Domain organisation of DC-SCRIPT from different species predicted using SMART online software. Blue rectangles represent C2H2 zinc fingers, pink rectangles represent low complexity regions and the green rectangle represents a coiled coil region. (C) Gene organisation of barramundi DC-SCRIPT. The orange boxes represent the exons. Numbers are representative of the number of base pairs, totalling 5337 bp between stop codons. (D) Phylogenetic tree is representative of a Maximum Likelihood analysis performed on CLUSTALW aligned sequences (MEGA 5.2.2).

To determine specificity of DC-SCRIPT to barramundi DCs, mRNA FISH was used, employing 32 CAL Fluor 590 labelled probes downstream of the zinc finger motif to eliminate possible non-specific hybridisation to other zinc-finger containing transcription factors. Cells exhibiting dendrite-like protrusions were positively labelled with Stellaris® DC-SCRIPT FISH probes as well as with anti-MHCII antibody but were not labelled with anti-IgM antibody (Figure 4.2D). Smaller, rounded cells were stained with both anti-MHCII and anti-IgM antibodies, but not with Stellaris® DC-SCRIPT FISH probes, indicative of probable B-cells (Figure A2.4.2). Macrophages appeared as irregularly shaped cells with regular nuclei and stained positively with anti-MHCII antibodies, but were negative for DC-SCRIPT and IgM (data not shown). To investigate DC-SCRIPT expression in haematopoietic tissues in fish, head kidney and spleen samples were analysed at 4 times between 6 h and 7 days post-injection with PTG and LPS. DC-SCRIPT was up regulated in both spleen and HK at 6 h post injection compared to PBS injected controls. However, after 24 h, DC-SCRIPT expression decreased in spleen but remained elevated in HK relative to controls. After 72 h, relative expression of DC-SCRIPT was lower in both spleen and HK and finally, after 7 d, DC-SCRIPT was once again up regulated in spleen but remained down regulated in HK relative to PBS injected controls (Figure 4.3).

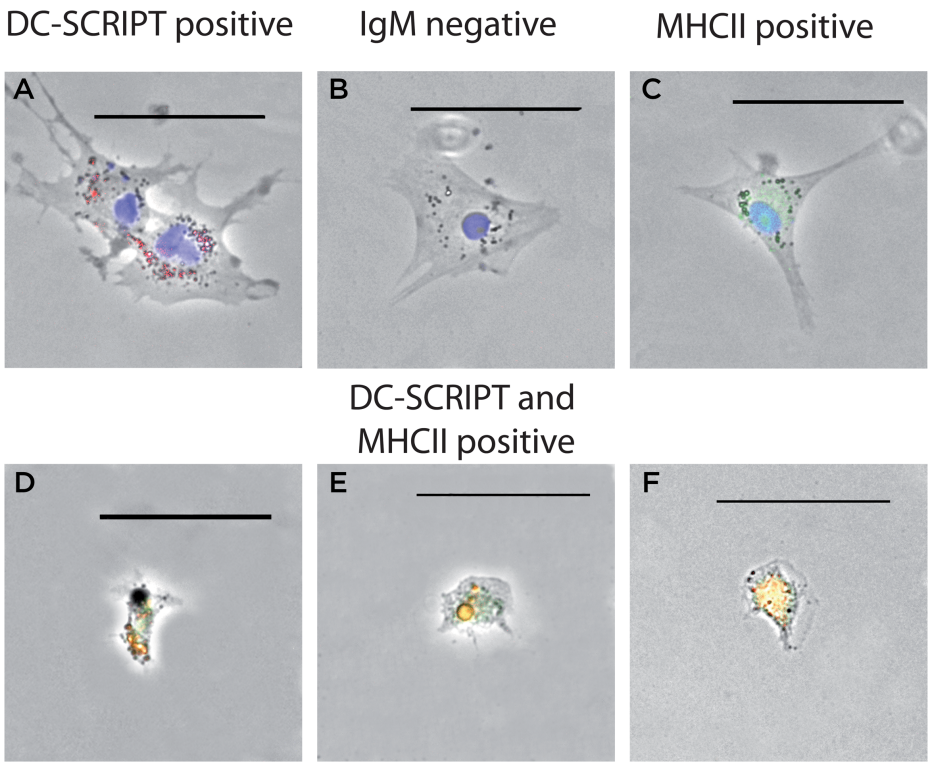


Figure 4.2. Antibody labelling of putative DCs. Antibody and Stellaris DC-SCRIPT FISH probe labelling of putative DCs: (A) DC-SCRIPT FISH probe in red and DAPI (nuclear stain) in blue, (B) DAPI (nuclear stain) in blue, (C) MHCII antibody in green and DAPI (nuclear stain) in blue, (D-F) DC-SCRIPT FISH probe in red, MHCII antibody in green. Scale bars represent 50 µm.

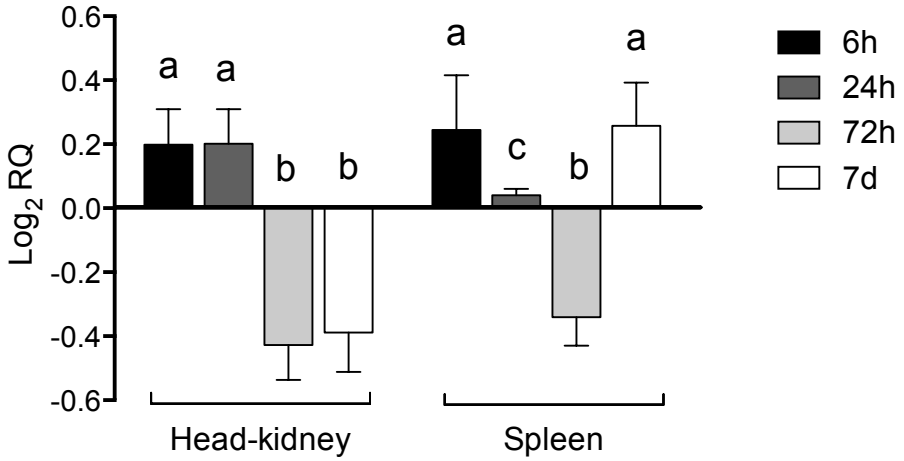


Figure 4.3. DC-SCRIPT expression in barramundi spleen and HK following a time course post-injection with LPS and PTG. Different letters represent statistically significant differences ($p < 0.05$) in gene expression (mean ± SEM). Data are derived from 4 biological and 3 technical replicates per treatment/control.

4.3.2. Morphological identification

Identification of dendritic-like cells in hematopoietic cell cultures

The morphological traits used to identify bDCs were the presence of dendrites on the cells' surface and their irregular nuclei, based on Steinman *et al.* [195], Lu *et al*. [196], Ganassin and Bols [197] and Bassity and Clark [76] (Figure 4.4A-B). After overnight incubation of HK and spleen-derived cultures, many cells, comprising large poly-nucleated melano-macrophages and macrophages, had adhered and formed clumps. Non-adherent cells were composed of small, round cells suggestive of lymphocytes/thrombocytes and larger, round, monocyte-like cells. On further sub-culture, these monocyte-like cells acquired an irregular shape with a branched morphology. These cells could also be enriched by isopycnic separation on a 1.058 g/mL Percoll gradient resulting in a population of larger more granular cells, which suggested a low buoyant density, similarly to mammalian DCs (mDCs) [195] (Figure 4.4C-D).

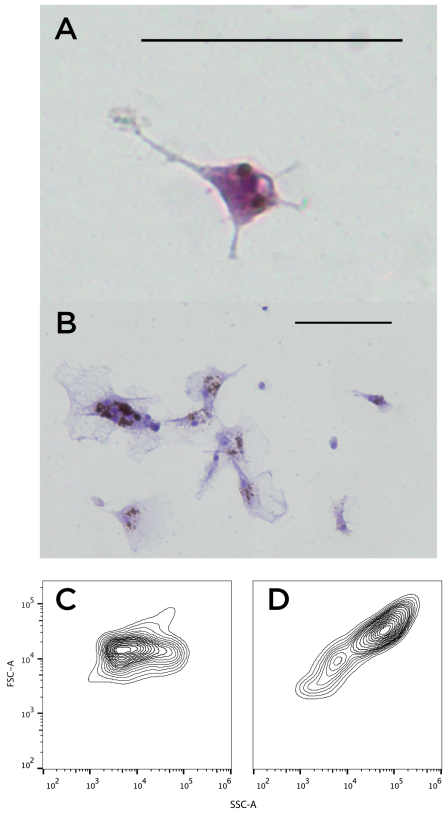


Figure 4.4. Barramundi DC morphology and isopycnic enrichment. (A-B) Dendritic-like cells isolated from *L. calcarifer* and stained with Hemacolour. (C) Forward and side scatter of barramundi spleen/HK cell populations before isopycnic enrichment and (D) after isopycnic enrichment.

4.3.3. Functional identification

Barramundi DCs are migratory and phagocytic cells

Trans-membrane migration and flow cytometry were used to assess chemotactic migration and phagocytosis capacity of bDCs. When exposed to Toll-like receptor (TLR) ligands and bacteria in the migration assay, bDCs actively migrated through the pores in the membrane towards PTG (TLR2) and both *S. iniae* strains ($p < 0.01$) but not significantly towards LPS (TLR4) ($p = 0.076$) (Figure 4.5). The migration was defined “active” because the cells, measuring approximately 10 µm, had to pass through 3 µm pores in order to reach the stimulants. Notably, bDCs migration towards either of *S. iniae* strains was not dose dependent, but at MOI 0.1 there was a significant difference in migration between the two isolates ($p < 0.05$, Figure 4.5). When incubated with micro beads for 2 h, bDCs were able to ingest them, demonstrating their ability to phagocytose foreign particles (Figure 4.6). Dendritic cells were also able to ingest two strains of the barramundi primary bacterial pathogen *S. iniae* (Figure 4.6).

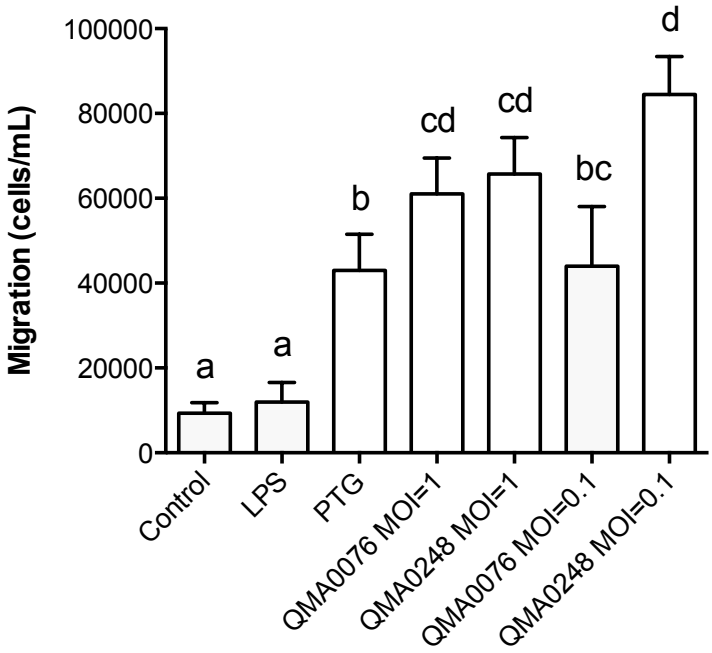


Figure 4.5. Barramundi DCs migration towards different chemical cues. Different letters represent statistically significant differences. Barramundi DCs migrated significantly towards all cues ($p < 0.001$ for PTG, QMA0076 and QMA028 at MOI 1 and QMA0248 at MOI 0.1; $p < 0.01$ for QMA0076 at MOI 0.1) with the exception of LPS, which bDCs did not migrate towards significantly ($p = 0.076$).

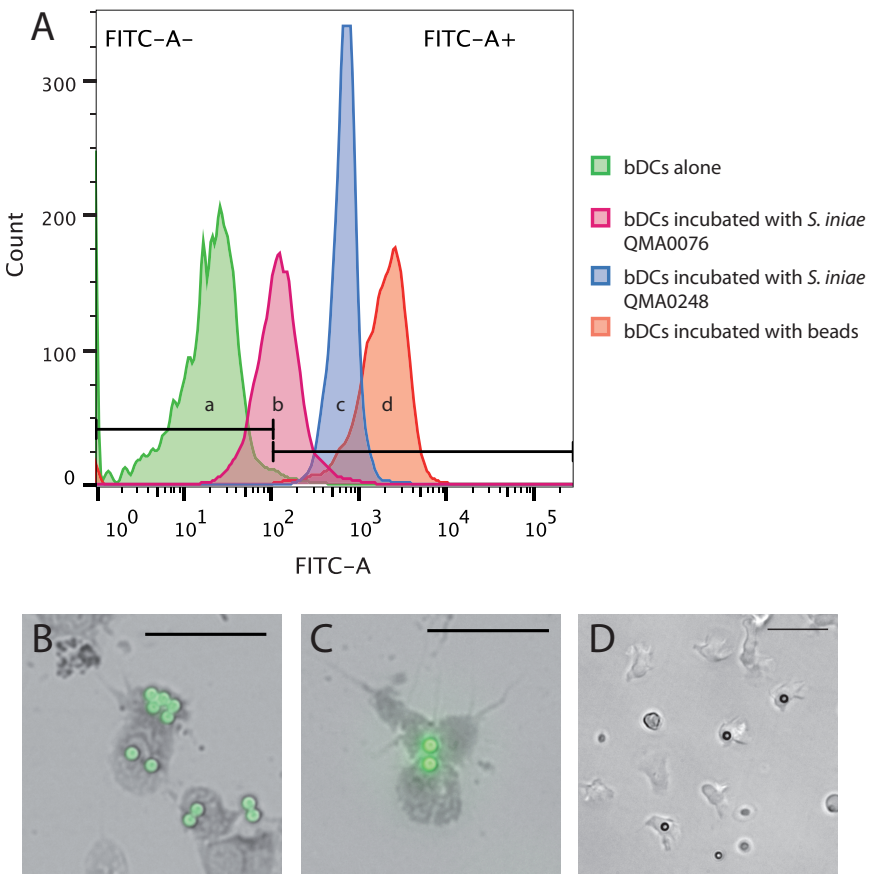


Figure 4.6. Phagocytic capability of bDCs. (A) Histograms showing barramundi dendritic cells alone (a), in presence of stained bacteria (b) QMA0076 and (c) QMA0248 and (d) in presence of fluorescent beads. Note that ingestion of beads and bacterial cells shifts fluorescence of DCs fluorescence significantly the right along the x-axis ($z > 1.96$) in b, c and d when compared to a. (B-C) Fluorescent microscopy and (D) inverted light microscopy of dendritic-like cells incubated with fluorescent beads. Scale bars represent 50 µm.

Barramundi DCs trigger the proliferation of responder T-cells

The defining role of antigen presenting cells, and especially of dendritic cells, is to stimulate naïve lymphocytes and trigger their proliferation. To determine if enriched cells were able to trigger T-lymphocyte proliferation, a proliferation assay, was performed. CFSE fluorescence of cells is typically reduced each generation by 50%, making the distinction of daughter cells possible. To determine background cell division, responder cells were incubated alone. After three days, the CFSE fluorescence of responder cells alone decreased slightly, but not significantly ($p > 0.05$) when compared to fluorescence at day 0 (data not shown). However, when

incubated for three days with bDCs (Figure 4.7), the CFSE fluorescence of T-cells decreased significantly in comparison to the fluorescence of responders alone after the same time. Decrease in fluorescence occurred in a dose-dependent manner, with maximum decrease in CFSE fluorescence achieved with a bDC:responder ratio of 1:1 (Figure 4.7). The CFSE fluorescence of responders and stimulators combined at day 3 was also significantly lower than the CFSE fluorescence of responders and stimulators at day 0, whereas the CFSE fluorescence of responders alone after three days was not significantly different from the CFSE fluorescence at day 0 (data not shown).

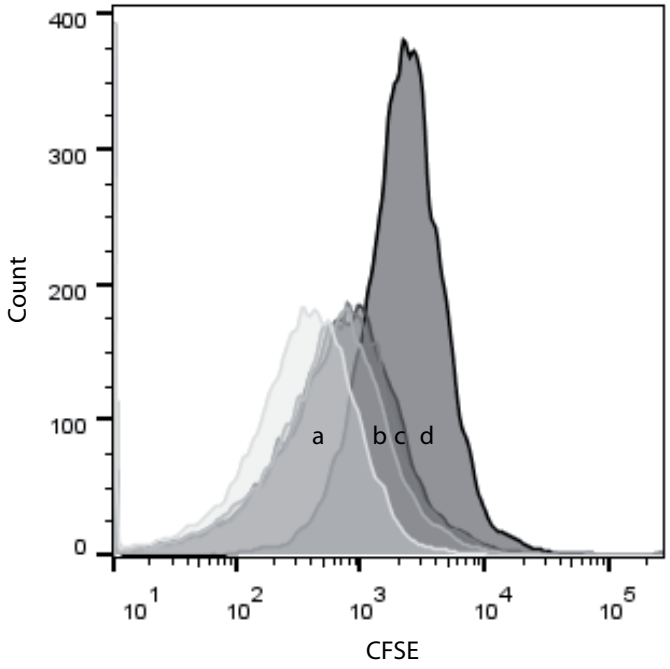


Figure 4.7. Histograms showing the fluorescence of CFSE stained T-cells. (a) At day 3, the fluorescence of responders/stimulators at 1:1 ratio significantly shifted to the left when compared to the (d) fluorescence of responders alone ($p = 0.02564$). Effect was ratio dependent with 1:2 (b), 1:4 (c) bDCs:responders showing less decrease in fluorescence over three days than 1:1 (a). Data presented are representative of three biological replicate data sets.

4.4. Discussion

A subset of barramundi cells derived from head kidney and spleen monocytes expressed DC-SCRIPT and exhibited morphological and functional characteristics of

mammalian dendritic cells (mDCs). DC-SCRIPT is a transcriptional regulator of the Zinc finger family that is preferentially expressed in all subsets of human DCs [185]. The putative DC-SCRIPT protein identified in this study was highly similar to human and murine DC-SCRIPT, with a similar structure and highly conserved zinc fingers. A homologue has been previously identified in the fugu genome *in silico*, but this study represents the first identification of DC-SCRIPT in putative teleost DCs. DC-SCRIPT did not appear to be expressed in other blood cells in mixed cell cultures from barramundi, similar to findings in human blood cells [185, 198], making teleost DC-SCRIPT a potentially useful molecular marker for DCs in fish. In fluorescence microscopy, bDCs were labelled with fluorescent mRNA probes directed against DC-SCRIPT and were also positive for MHCII using anti-zebrafish MHCIIa. The anti-MHCII antibody employed is a commercial polyclonal antibody raised in rabbits against a peptide comprising the transmembrane MHCIIa and MHCIIa-Ig superfamily domains from zebrafish (NP_571565.1) and was chosen as likely to be cross reactive with barramundi MHCII based on protein homology by BLAST with the barramundi MHCIIa Ig superfamily region (derived from partial gDNA sequence), and that of fish from several orders including other cyprinids (*C. carpio*), salmonids (*S. salar*) and perciforms (*O. niloticus, M. zebra*). Epitope mapping revealed highly conserved B-cell epitopes in both the MHCIIa domain and the Ig superfamily domain of the zebrafish peptide (Figure A2.4.2 A-B). Moreover co-labelling with specific anti-barramundi IgM and the anti-zebrafish MHCII in barramundi B-cells was evident (Figure A2.4.2 C).

DC-SCRIPT expression determined by qRT-PCR indicated that bDCs are present in spleen and HK at the beginning of an infection and that DC-SCRIPT is induced, possibly via cytokine signalling from primary responder cells at the site of injection, within 6 h post injection. As the innate and adaptive immune response develops, DC-SCRIPT expression decreases in spleen, possibly indicative that bDCs have migrated from the spleen towards the site of infection. At 72 h post infection, DC-SCRIPT expression is also down regulated in HK, also suggesting a migration from the HK. However, after 7 days, DC-SCRIPT is again up regulated in spleen but not in HK, which suggests that bDCs have migrated back to the spleen, possibly for maturation and antigen presentation. These kinetics appear to be consistent with previous studies on appearance of antibody secreting cells in tissues in European

sea bass [199]. This is important against a background of fish vaccination for sustainable disease control in aquaculture. Previously we have demonstrated that a number of antigens elicit poor antibody responses in vaccinated barramundi [200]. In human DCs, DC-SCRIPT appears to control glucocorticoid (GC) suppression of antigen presentation by binding to the GC receptors [201]. It will be interesting to determine how DC-SCRIPT is expressed in fish DCs post-vaccination with vaccines that elicit both strong and poor antibody responses. Novel adjuvants that enhance DC-SCRIPT expression may be a way of promoting presentation of critical but poorly immunogenic antigens.

Barramundi cells, cultured from spleen and HK, were able to differentiate into highly motile, irregular shaped cells with low buoyancy, similar to mDCs and rainbow trout dendritic cells (tDCs) [76, 195-197]. These cells could also arise in HK and spleen cultures individually as well as in blood leucocyte cultures, although no functional studies have been performed yet on these culture and blood-derived cells. As observed in cultures from rainbow trout head kidney, anterior trunk kidney, spleen, and mouse spleen, barramundi DCs seemed to be generated from hematopoietic tissues without addition of an exogenous growth factor source [76, 202]. This could indicate the presence of endogenous growth factors in primary barramundi spleen and HK cultures. This theory is supported by the quick de-differentiation of these cells when cultured in fresh supplemented medium (L-15/10%FBS/1%PS) after enrichment and by the slow/non differentiation of spleen and HK cultures into bDCs when the medium was changed every day instead of every 4–5 days (data not shown).

In addition to identifying DCs by their morphological properties, the current study characterised their function as putative APCs, using a proliferation assay. Classically, in mixed leucocyte reactions, CFSE histograms present several peaks, each representing a unique cell division. In this study, however, CFSE histograms only presented one peak, which shifted to the left when T-cells were exposed to bDCs for 3 days. This dissimilarity with other studies is likely explained by the method used to isolate the T-cells subsequently used in the proliferation assays. As no T-cell markers are available in barramundi to enable enrichment of T-cells from mixed leucocyte populations we used the E-rosette method to obtain a

homogeneous population of viable T-cells. E-rosetting is a method that relies on the binding of T-cells CD2 by a LFA-3 homologue present at the surface of sheep red blood cells, forming “rosettes” [203]. This method has been extensively used in humans and mice until the commercialisation of magnetised beads, and results in high yields of viable active T-cells [192, 204]. More recently, the E-rosette method has also been used to isolate T-cells from mud catfish peripheral blood, indicating that this technique is efficient not only in mammals but also in fish [205]. In contrast, many previous studies obtain responder cell populations by removing B-cells from mixed leucocyte cultures by FACS or magnetic beads sorting, resulting in a mixed leucocyte population. The presence of cells other than T-cells in the MLR can account for the presence of several well-defined CFSE peaks through differing cell division rates. In contrast in this study, bDCs were able to stimulate proliferation of the whole T-cell population, resulting in the CFSE fluorescence decreasing by half after 3 days compared to T-cells alone, or bDCs plus T-cells at day 0. This suggests that isolated cells are functionally equivalent to mDCs.

Another key characteristic of APCs that is critical to antigen presentation is their ability to sense non-self substances and migrate towards them by chemotaxis [44]. Barramundi DCs were attracted to TLR-ligands and whole bacteria and were able to phagocytose particulates including bacteria and opsonised beads. Barramundi DCs did not migrate towards LPS, which suggests that bDCs were unable to recognise and bind TLR4 ligands in contrast to mammalian DCs. TLRs are proteins capable of recognising a range of exogenous and endogenous ligands, including pathogen associated molecular patterns (PAMPs). In mammals, the lipopolysaccharide binding protein (LBP), located in serum, mediates the interaction between LPS on bacteria surface and the glycoprotein CD14 on phagocytic cells [161]. Additionally, TLR4 must be coupled with the myeloid differentiation protein 2 (MD-2) to functionally interact with LPS [162]. The lack of significant attraction of bDCs to LPS in the present study is likely due to a lack of LBP in the culture system or to a lack of CD14/MD-2 on bDCs surface: to date, CD14 and MD-2 are absent from all fish genomes sequenced [147]. It may also be that higher concentrations of LPS than used in the present study are required to activate fish immune cells [206]. The lack of reaction to LPS by bDCs could present an issue both for future vaccine design and for *in vitro* studies on *L. calcarifer*, as TLR4 ligands are frequently used to stimulate

DCs for *in vitro* studies in mammals [122, 207]. Further work is warranted to determine whether barramundi do indeed have intermediate LBP that could be included in the assays system to mediate interaction with TLR4.

Finally, the characterisation of DCs in *L. calcarifer* provides a baseline for future vaccine design against *S. iniae* and other pathogens. Vaccination in fish has experienced some failures due to rapid evolution of key immunogens and consequent vaccine escape [60, 61]. For example, killed whole-cell vaccines against *S. iniae,* which is a major pathogen of warm water fish, are often compromised by rapid-evolution of novel serotypes [61, 208]. Vaccines against *S. iniae* are effective against the specific serotype in the vaccine, with the fish adaptive immune system responding to the capsular polysaccharide (CPS), which is immunodominant [61, 108, 209]. However, the CPS is highly variable and new CPS variants of *S. iniae* rapidly emerge, leading to reinfection and disease outbreaks in previously vaccinated fish [61, 108]. Moreover, although there are conserved critical virulence factors in *S. iniae*, such as the surface-expressed M-protein, the immune system does not seem to develop an effective response against them [191]. Enhancing antigen presentation of non-immunodominant conserved virulence factors is therefore a critical step in the development of broadly cross-protective vaccines against these highly variable and rapidly mutating strains. The main cells that need to be targeted during vaccination are APCs, and in particular dendritic cells that will activate pathways leading to immune memory [1, 2]. The capacity to evaluate multiple antigens across a single population of APCs *in vitro*, rather than vaccinating cohorts of fish with each strain or antigen has immense value in terms of rapid high throughput screening of putative antigens. *In vitro* experiments also have great merit in terms of animal welfare as these assays greatly reduce animal numbers required and refine (reduce the stress of) the procedures conducted on animals, in line with the ethical requirements of the three R's (Replacement, Reduction and Refinement). The identification of a molecular marker for dendritic cells in barramundi provides a potentially useful tool for new flow-cytometric investigations of antigen presentation in fish. Work is ongoing to determine the function of DC-SCRIPT in bDCS and elucidate its role in antigen presentation.

Chapter Five – Evidence for affinity selection of IgM in fish: A high avidity secondary antibody response to T-dependent antigen with antibody-mediated suppression

5.1. Introduction

The introduction of mass vaccination in the salmonid industry based on water-in-oil emulsions is one of the major success stories in the growth of the global salmon farming industry [53], from a few hundred thousand tonnes during the early 1990s to more than 1.3 million metric tonnes in 2012 [70]. The practice of vaccination by intraperitoneal injection has been slowly transferring to non-salmonid species and this is an important transition with the major growth in finfish aquaculture now occurring in warm-water species [53, 210, 211]. The role of the oil-adjuvant in the success of vaccination of salmonids is often underplayed, but it was the stability and slow release of the antigen allowing single injection protection throughout the 2-4 year grow-out period that made vaccination an economical option for preventing disease in fish, and lead to almost universal adoption by salmon farmers within a few years. In contrast to salmon farming, warm water finfish have a much shorter grow-out period. Many fish species are farmed only for plate size and the farming cycle, from hatchery to market, is less than one year, often between 6 and 9 months [93, 212, 213]. This can lead to issues in vaccine efficacy: the rapid onset of protection and low cost of aqueous vaccination makes them attractive, but protection is short-lived with disease occurring in vaccinated fish after only 3-6 months [208]. In contrast, water-in-oil emulsion vaccines are perceived as expensive. Moreover, the slow antigen release afforded by water-in-oil emulsions, coupled with the difficulties in achieving sufficiently high antigen dose for target pathogens such as *Streptococcus agalactiae* and *S. iniae*, reduces vaccine efficacy early in the farming cycle, and can lead to lower protection than desired. It is timely, therefore, to investigate the kinetics of immune response in warm water finfish to determine whether vaccines can be better formulated to address disease prevention in this rapidly developing industry.

Successful vaccination relies on eliciting long-term immune memory and is dependent upon correct activation and interplay of both innate and adaptive immunity. Innate immunity is typically characterised by the development of a non-

antigen-specific inflammatory response, following exposure to foreign particles, through production of cell-signalling molecules (cytokines) and recruitment of phagocytes and lymphocytes [214]. This initial response is also crucial in eliciting the antigen-specific adaptive response, as it provides the basis for foreign particle recognition on which the adaptive response relies. Some pro-inflammatory cytokines, such as interleukin (IL)-1β, have been shown to stimulate T-lymphocytes, both in teleosts and mammals [215, 216], thus linking innate and acquired immunities. Moreover, the binding of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), which constitute the base of innate immunity, triggers an array of molecular cascades, some of which initiate the expression of genes involved in maturation of dendritic cells (DCs), that are critical to presentation of some antigen types [1, 217]. In humans, DC maturation is activated by cytokines (e.g. CD40 L and IFN-γ) and pathogen products such as capsular polysaccharide or nucleic acids [1]. DCs are the primary antigen presenting cells (APCs) responsible for the priming and proliferation of antigen-specific T-lymphocytes, allowing the formation of immune memory, and are thus at the core of the adaptive response on which vaccination is based (reviewed in [1]). Indeed, the adaptive response occurs when T-cell receptors, located at the surface of T-lymphocytes, recognise PAMPs bound on major histocompatibility complex (MHC), located at the surface of APCs. MHC class II (MHCII) molecules found at the surface of DCs, in particular, prime CD4+ helper T-lymphocytes, which results in localised inflammation and B-lymphocyte activation [105]. B-lymphocytes are crucial in the elimination of foreign particles, as they produce antibodies, which non-specifically bind to foreign particles during their first encounter, but can quickly become antigen-specific after B-cell maturation [218]. Through a process termed affinity maturation, the binding site of the antibodies is mutated to create a stronger bond with the specific antigen [219]. Briefly, variable (V), diversity (D) and junction (J) immunoglobulin (Ig) genes already have many isotypes present in the genome, which allows B-cells to produce around 10^{10} different antibodies through selective gene rearrangement, without all those antibodies having to be individually encoded in the genome [220]. This represents the primary antibody repertoire. Moreover, V(D)J genes also possess hyper-mutable regions within their coding sequence, which allows for further affinity maturation through a process called somatic hypermutation [218, 221]. When a foreign particle is detected, only the B-cells that have higher affinity due to initial V(D)J

rearrangement and accumulated mutations are selected to become memory B-cells within the germinal centres [218, 222]. This selection shifts the B-cell population from a very diverse antibody pool to a clonal population producing antibodies targeted against a specific antigen [219]. Specific memory B-lymphocytes stay in the organism and diversify into plasma B-cells upon re-encounter with the same antigen, improving and accelerating the immune response due to the faster production of higher affinity antibodies [219].

This process, however, only occurs with T-lymphocyte dependent antigens. From an immunological point of view, there are two kinds of antigens, T-lymphocyte dependent (TD) and T-lymphocyte independent (TI). Most proteins and peptides are TD, requiring T-cell activation by presentation through MHCII to induce B-cell proliferation and differentiation into plasma and memory cells [223, 224]. Antibodies produced against TD antigens typically have high affinity and undergo isotype switching from IgM to IgG in mammals [224]. On the other hand, TI antigens include most carbohydrates, and do not require any T-cells to induce an immune response. There are two types of TI antigens, type 1 and type 2, based on their ability to interact with B-cells. TI type 1 can induce B-cell proliferation and differentiation into plasma cells, in both naïve and mature cells, whereas TI type 2 can only activate mature B-cells, most likely through Ig crosslinking [223]. In either case, the antibodies produced by TI induced B-cells are highly antigen-specific and do not generally show a strong avidity. Moreover, TI antigens fail to induce immune memory, as B-cells do not differentiate into memory cells, which is characterised by a lack of response to challenges with the same antigen [224, 225]. Fortunately, it has previously been shown that coupling TI antigens with a protein carrier could lead to a T-dependent response, combined with the development of memory B-cells producing antibodies against that TI antigen [226]. This model is based on hapten-carrier antigens, which include an often small non-immunogenic hapten and a bigger immunogenic carrier protein [227]. Vaccines based on the hapten-carrier model have already proven to be successful, for example in the prevention of meningococcal diseases caused by *Neisseria meningitidis* [226]. Indeed, polysaccharide vaccine usually do not confer immune memory, but conjugate vaccines against *N. meningitidis* serotype C have proven to induce antibody production, confer immune memory and even to help with herd immunity [228]. As rapid advances and

decreases in cost of whole genome sequencing enable the identification of new protein antigens (likely processed via T-dependent pathways) for improved, cross-protective vaccination, it is likely that hapten-carrier vaccine will before increasingly used in aquaculture, especially against TI capsular polysaccharides. Thus, a better understanding of T-dependent primary and secondary responses is required in fish systems.

In the current study, primary and secondary antibody responses against a model T-dependent antigen were determined in barramundi (*Lates calcarifer*) over the course of a three-month prime-boost trial in the presence and absence of commercial adjuvants. Speed, magnitude and quality (avidity) of antibody elicited during primary and secondary responses was assessed. Moreover, the affinity of those antibodies was also determined, as well as the proportion of their isomers under the differing conditions. Finally, the barramundi transcriptome and genome were examined for immunoglobulin coding genes [164, 168], identifying the heavy chain V(D)J genomic region in barramundi for the first time.

5.2. Materials and Methods

5.2.1. 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 and the salinity were maintained at 28 ± 2°C and 15 parts per thousand (ppt) respectively. Water quality was checked regularly for ammonia, nitrite, nitrate and pH, and water exchanges were 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.

5.2.2. Vaccine formulation

A hapten-carrier complex was employed as a model T-dependent antigen in all vaccine groups, as previously employed in sea bass [229] and rainbow trout [230]. Dinitrophenol covalently linked to keyhole limpet haemocyanin (DNP-KLH) was dissolved in phosphate buffered saline, pH 7.4 such that the final antigen concentration when mixed with adjuvant at the correct ratio was 100 µg/mL (equivalent to 10 µg/fish post-injection). Adjuvants requiring water in oil (w/o) emulsions (Montanide ISA 763A VG, Essai 1616101 and Essai 1616102) were formulated with antigen by weight (Table 5.1) and mixed for 3 min at 15,600 rpm using an IKA Ultra Turrax T25 mixer fitted with an S25 N-18G head. To prepare w/o/w emulsion with adjuvant Montanide Essai 1632102, antigen and adjuvant were first warmed to 31°C in a water bath. The adjuvant was then stirred under low-shear at 350 rpm with the 4 blade impeller head. The aqueous antigen phase was then added quickly and stirring continued at 350 rpm for 5 min. The emulsion was then transferred to a cooling water bath at 20°C for 1 h prior to storage at 4°C. For the gel adjuvant Montanide Gel 1632101, the adjuvant was added to the antigen solution and stirred at room temperature for 10 min at 200 rpm using the 4 blade impeller head. Antigen only control was mixed 1:1 with sterile PBS for injection. All formulated vaccine mixtures were stored at 4°C and inspected over 5 days for emulsion stability before further use.

Table 5.1. Vaccine treatment groups in the prime-boost experiment.

Vaccine	Adj:Ant Ratio (w/w)	Experimental purpose
PBS	N/A	Background nonspecific response
Antigen (DNP-KLH)	50:50 (PBS)	Response without adjuvant
Antigen + ISA 763A VG	70:30	Water in oil emulsion
Antigen + essai w/o/w 1632102	50:50	Water in oil in water emulsion
Antigen + essai gel 1632101	50:50	Gel adjuvant
Antigen + essai w/o 1616101	60:40	Water in oil experimental 1
Antigen + essai w/o 1616102	60:40	Water in oil experimental 2

5.2.3. Vaccination

Fish missed their routine feed the evening prior to vaccination to ensure an empty gut and thereby minimise the chance of accidental vaccination into the gut. For vaccination, barramundi fish were anaesthetised with Aqui-S according to the University of Queensland SOP AHP44. Fish were vaccinated by intraperitoneal injection with 100 µL of antigen vaccine in aqueous form, antigen vaccine formulated with either adjuvant (ISA 763A VG, Essai w/o 1616101, Essai w/o 1616102, Essai w/o/w 1632102 or Gel 1632101), or PBS control. This volume has been shown to be safe for IP injection and cause minimal stress to the barramundi of this size (evidenced by no stress-induced darkening and by rapid return to feeding post vaccination, within a few hours). Periodically, 6 fish from each treatment group were removed and re-vaccinated ("challenged" or boosted) with DNP-KLH to elicit a secondary specific immune response. Revaccination occurred 1 week, 2 weeks, 9 weeks and 13 weeks post initial vaccination.

5.2.4. Sampling

Seven days after primary vaccination, 6 vaccinated fish per treatment were euthanised and sampled for blood. Moreover, seven days after challenges, 6 re-injected and 6 non-re-injected fish per treatment were also euthanised and blood collected. Fish were euthanised by overdose of Aqui-S and bled from the caudal vein. Blood was allowed to clot overnight at 4°C and sera collected by centrifugation at 7,500 *x g* for 10 min and frozen at -20°C until subsequent antibody analysis by ELISA.

5.2.5. Enzyme linked immunosorbent assay

Antibody response was detected via indirect ELISA. A series of optimisation experiments was first conducted to optimise resolution of positive and negative control sera. Binding surface (Greiner high and medium binding plates), primary antibody dilution (8 x to 256 x), blocking reagent (1% bovine serum albumin, 2% normal donkey serum, 5% skim milk) and development time for the p-nitrophenyphosphate (pNPP) chromogen (10-50 min) were optimised, whilst dilution of secondary antibody (sheep vs. barramundi IgM, 1:2,000) and tertiary antibody conjugate (donkey anti-sheep IgG alkaline phosphatase conjugate, Sigma A5187, 1:15,000) were fixed. Following optimisation experiments, a standard protocol was

devised for experimental samples as follows: Greiner high binding 96 well ELISA plates were coated overnight at 4°C with DNP-BSA dissolved at 5 µg/mL in carbonate bicarbonate buffer, pH 9.0 (100 µL/well). Plates were washed thrice in tris-buffered saline containing 0.01% Tween 20 (TBST) and then blocked with 1% BSA (100 µL/well) for 1 h. Plates were rinsed with TBST, then primary antibody at 1:128 dilution in TBST was added to each well and incubated overnight at 4°C. Plates were then thrice washed in TBST before adding secondary antibody sheep anti-barramundi IgM, diluted 1:6,400 in TBST and incubating for 1 h at RT. After three further washes, antibody-conjugate reagent (donkey anti-sheep IgG) diluted 1:15,000 in TBST was added and the plates incubated for 1 h. Plates were then washed five times in TBST and once in TBS. Then 100 µL chromogen (pNPP liquid substrate, Sigma) was added to each well using a multi-channel pipette and the plate immediately placed in the incubator tray of a temperature controlled 96-well plate reader (BMG Fluostar Optima) and incubated at 28°C and the absorbance of each well read at 405 nm every 10 min for 30 min. Addition of chromogen was staged to enable sequential reading of plates. A standard positive control and negative control were included in triplicate on each plate to enable inter-plate calibration and normalisation of optical density *post-hoc*.

5.2.6. Avidity assay by chaotrope titration ELISA

Antibody avidity was quantified via indirect ELISA by titration of ammonium thiocyanate (NH4SCN) as follows: Greiner high binding 96 well ELISA plates were coated overnight at 4°C with DNP-BSA dissolved at 5 µg/mL in carbonate bicarbonate buffer, pH 9.0 (100 µL/well). Plates were washed three times in tris-buffered saline containing 0.01% Tween 20 (TBST) and then blocked with 1% BSA (100 µL/well) for one hour. Plates were rinsed with TBST, then primary antibody at 1:128 dilution in TBST was added to each well and incubated overnight at 4°C. Plates were then washed three times in TBST before adding four dilutions of ammonium thiocyanate (0.5 g/mL, 0.3 g/mL, 0.1 g/mL and 0.05 g/mL diluted in TBS pH 6) in order to obtain an avidity curve for each serum. The plates were then incubated for 15 min at RT and washed thrice in TBST before addition of secondary antibody sheep anti-barramundi IgM, diluted 1:6,400 in TBST and incubating for 1 h at room temperature. After three further washes, antibody-conjugate reagent (donkey anti-sheep IgG) diluted 1:15,000 in TBST was added and the plates

incubated for 1 h. Plates were then washed five times in TBST and once in TBS. Then 100 µL chromogen (pNPP liquid substrate, Sigma) was added to each well using a multi-channel pipette and the plate immediately placed in the incubator tray of a temperature controlled 96-well plate reader (BMG Fluostar Optima) and incubated at 28°C and the absorbance of each well read at 405 nm every 10 min for 40 min. Addition of chromogen was staged to enable sequential reading of plates. A standard positive control and negative control were included in triplicate on each plate to enable inter-plate calibration and normalisation of optical density *post-hoc*. Titration curves were fitted for antiserum and control from the normalised data by non-linear regression in Prism 7 (Graphpad) and an avidity index calculated as the concentration of ammonium thiocyanate that resulted in 50% reduction in ELISA optical density.

5.2.7. Immunoglobulin polymer composition by SDS-PAGE

To detect the proportion of monomer, dimer, trimer and tetramer among Ig molecules in barramundi sera, three serum samples of fish vaccinated with ISA 763A VG, Essai 1632101 gel and Essai 1616102 w/o adjuvanted vaccines, sampled on day 28 and 98, were chosen, in both challenged and non-challenged groups. Optimisation experiments were conducted to optimise the resolution of the barramundi IgM protein bands, by varying the gel concentrations (12%, 10%, 6% and 4%) and the concentrations of fish sera (1:64 – 1:256). Diluted sera (1:64) were mixed with non-reducing sample buffer and were loaded on 6% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE). An electrophoretic separation was conducted at 140 V and 40 mA for approximately 3 h, until the samples had been appropriately separated through the gel. Resulting gels were stained in Coomassie Brilliant Blue 250R (Bio-Rad, UK). Immunoglobulin band intensities were semi-quantitatively analysed densitometrically by ImageJ (National Institutes of Health, USA). The intensity of each band was calculated as a proportion of total IgM for a given sample (proportion of total Ig = intensity of the polymer investigated in the sample/total intensity of all Ig polymers in the sample).

5.2.8. LPS immunisation and ELISA

Lipopolysaccharide (LPS) from *E. coli* 0111:B4 (Sigma) was dissolved in PBS, such that the final antigen concentration was 100 µg/mL (equivalent to 10 µg/fish).

Similarly, DNP-KLH was dissolved in PBS, to a final concentration of 100 µg/mL. Fish missed their routine feed the evening prior to vaccination to ensure an empty gut, thereby minimising the likelihood of accidental vaccination into the gut. For vaccination, barramundi fish were anaesthetised with Aqui-S according to the University of Queensland SOP AHP44 and were vaccinated by intraperitoneal injection with 100 µL of either LPS of DNP-KLH vaccine. Negative controls consisted of fish mock-vaccinated with PBS. Fourteen and 21 days after vaccination, 6 vaccinated fish per treatment (including PBS control) were euthanised and blood drawn from the caudal vein. Blood was allowed to clot overnight at 4°C and sera collected by centrifugation at 7,500 *x g* for 10 min and frozen at -20°C until subsequent antibody analysis by ELISA.

Antibody response was detected via indirect ELISA. Equal volumes of Polymyxin B (1 mg/mL) and LPS 0111:B4 (Ultra-pure – 10 µg/mL) were mixed and incubated at RT, on a magnetic stirring plate for 30 min. A Greiner high binding 96 well ELISA plate was then coated overnight at 37°C with either LPS dissolved at 1 µg/mL in carbonate bicarbonate buffer, or with DNP-BSA dissolved at 5 µg/mL in carbonate bicarbonate buffer (100 µL/well). The plate was washed thrice in tris-buffered saline containing 0.01% Tween 20 (TBST) and then blocked with 1% BSA (100 µL/well) for one hour. The plate was rinsed with TBST, then primary antibody at 1:128 dilution in TBST was added to each well and incubated overnight at 4°C. The same PBS control sera were added to both LPS and DNP coated wells, to allow for comparison between both anti-LPS and anti-DNP antibodies. The plate was then thrice washed in TBST before adding secondary antibody sheep vs barramundi IgM, diluted 1:6,400 in TBST and incubating for 1 h at room temperature. After three further washes, antibody-conjugate reagent (donkey anti-sheep IgG) diluted 1:15,000 in TBST was added and the plates incubated for 1 h. Plates were then washed five times in TBST and once in TBS. Then 100 µL chromogen (pNPP liquid substrate, Sigma) was added to each well using a multi-channel pipette and the plate immediately placed in the incubator tray of a temperature controlled 96-well plate reader (BMG Fluostar Optima) and the absorbance of each well read at 405 nm. This procedure was repeated for sera samples from 14 and 21 days post vaccination.

5.2.9. Immunoglobulin genomic assembly and mapping

The immunoglobulin coding region was identified in the barramundi genome though Basic Local Alignment Search Tool (BLAST) using Ig coding sequences identified from the annotated transcriptome. Within this genomic sequence, the variable (V) and junction (J) regions were identified using the TigGER package in R (http://tigger.readthedocs.io/en/0.2.10/) [231]. Briefly, the barramundi transcriptome sequences were first IMGT-gapped formatted, using *Danio rerio* as a reference on the IMGT/HighV-QUEST online software (http://www.imgt.org) [232-234]. IMGT-gapped V(D)J regions from *Danio rerio* were also imported as references, before performing the *inferGenotype* command on TigGER. The resulting V and J sequences were subsequently mapped to the barramundi genome in BAM format using the *MAP with BWA-MEM* on the Galaxy web interface (https://galaxy-qld.genome.edu.au/galaxy) [235], before being visualised in Artemis. Constant immunoglobulin regions were identified by homology using BLASTx, open reading frames containing those constant regions were identified and subsequently mapped to the genome as described above before being visualised in Artemis.

5.2.10. Statistical analysis

ELISA and avidity assay data were analysed via two-way analysis of variance (ANOVA). A Sidak's *post-hoc* was then performed to examine the differences between boosted and non-boosted fish sampled on the same day from the same treatment group.

Densitometry data derived from electrophoretic separation of barramundi IgM were analysed via one-way type I ANOVA to examine whether the polymerisation forms of IgM were different in fish sera samples (challenged and non-challenged) within each adjuvant group. Tukey's *post-hoc* tests were also conducted to compare specific Ig forms between samples within the same adjuvant group.

5.3 Results

To assess vaccine effectiveness, antibody levels in the serum of immunised organisms are often evaluated by routine ELISAs. In the current study, barramundi serum antibody titres were measured post-immunisation, comparing different vaccine formulations using the same model antigen. Control fish that received only PBS via intraperitoneal injection showed that background serum antibody was

detectable and significant compared to primary serum-free reagent controls. Whilst there was some variation in ELISA OD through the trial ranging between 0.35 and 0.7, the variation was not significant over time. Vaccinated fish, either with the antigen in aqueous phase or with the addition of adjuvants, developed serum antibody titres significantly higher than the PBS handling controls, in spite of the background, indicative of a specific response to the antigen (Figure 5.1). In the absence of adjuvant, the primary serum antibody response to DNP peaked at or before the first sample point of 21 days and then decreased throughout the 100 days of the experimental period (Figure 5.1B). However, with addition of adjuvants, the primary response peaked around 28 days, and was maintained throughout the experiment with all the adjuvants except for the Essai 1632102 w/o/w, for which antibody levels decreased after the 28 day peak (Figure 5.1F). The addition of the Essai 1632101 gel adjuvant resulted in a higher primary response than for any other treatment, with those high levels maintained for the duration of the experiment (Figure 5.1G). When fish were vaccinated, a secondary injection with aqueous DNP-KLH resulted in significantly higher antibody titres at 98 days in all cases (Figure 5.1B-G). Overall, secondary injection in PBS control fish did not significantly increase antibody titres, except in one individual fish at day 98, which resulted in a significant statistical test (Figure 5.1A). Surprisingly, the addition of adjuvants resulted in significantly lower levels of secondary antibodies at 28 days, coinciding with the peak of the primary antibodies in all adjuvanted treatments except for the Essai 1616102 w/o (Figure 5.1E).

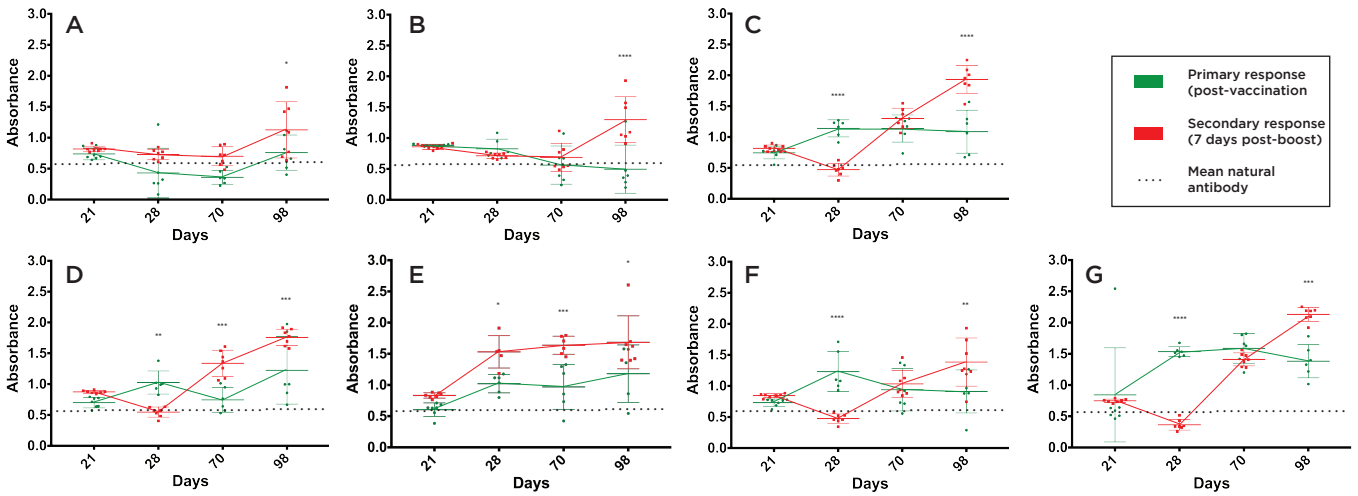


Figure 5.1. Kinetics of primary (green) and secondary (red) antibodies following vaccination. Vaccine treatments used were PBS (A), DNP-KLH in aqueous solution (B), or DNP-KLH with ISA 763A VG (C), with Essai 1616101 w/o (D), with Essai 1616102 w/o (E), with Essai 1632102 w/o/w (F) or with Essai 1632101 gel (G). Each secondary antibody measure corresponds to a sampling 7 days post-challenge with DNP-KLH in aqueous solution. Stars represent statistically significant differences between primary and secondary antibody titres (mean ± SEM) at a given time point with *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$ and ****: $p < 0.001$.

Because of the particularities observed by ELISA for the vaccines adjuvanted with Essai 1616102 w/o (no inhibition of the secondary response at 28 days post-vaccination) and Essai 1632101 gel (increased primary response maintained over 98 days), avidity was also determined for those treatments, along with the three control treatments (PBS, aqueous DNP-KLH vaccine and ISA 763A VG adjuvanted vaccine). Overall, the avidity of natural antibodies (produced by PBS control) was very low and consistent throughout the experiment. A similar pattern was observed with the aqueous vaccine (Figure 5.2A and 5.2B), with consistently low avidity which was not significantly different from the PBS control. However, with the addition of adjuvants, the avidity was significantly improved, with significant differences observed between PBS controls and primary antibody avidity overall, as well as between primary and secondary antibody avidity for all adjuvanted treatments at day 98 (Figure 5.2C-E). Moreover, for the Essai 1616102 w/o adjuvant, the avidity of secondary antibodies was also significantly higher than the one of primary antibodies at day 70, displaying an overall avidity superior to all other treatments (Figure 5.2D).

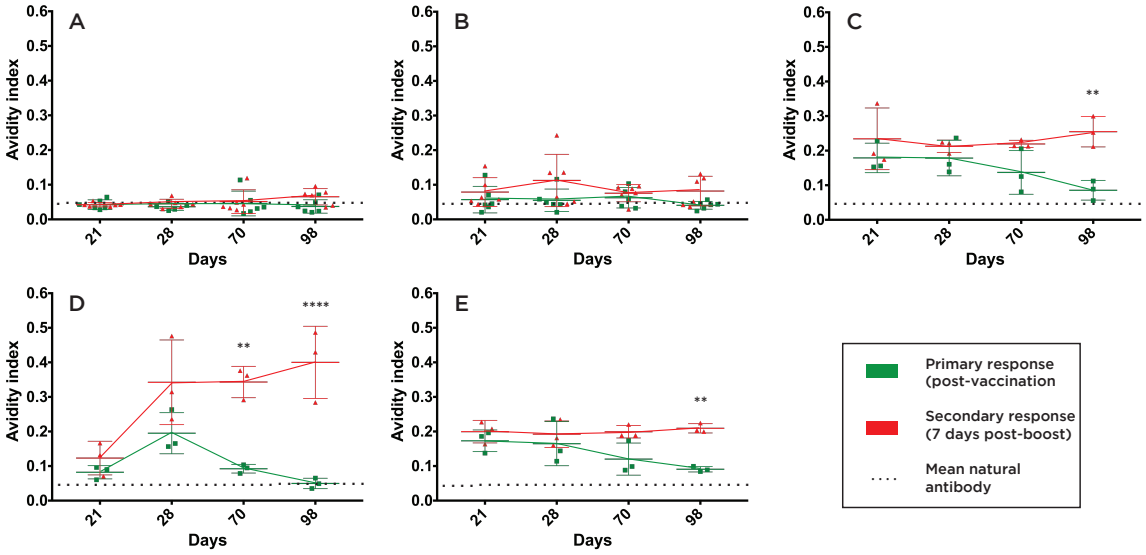
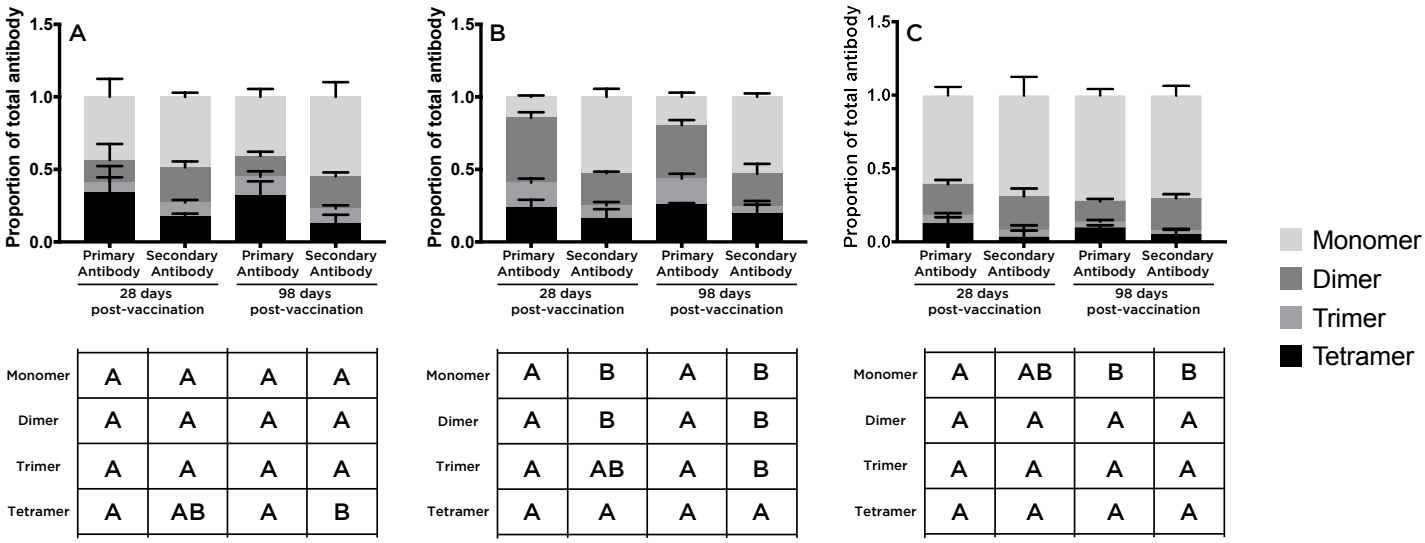


Figure 5.2. Avidity of primary (green) and secondary (red) antibodies following vaccination. Vaccine treatments used were PBS (A), DNP-KLH in aqueous solution (B), or DNP-KLH with ISA 763A VG (C), with Essai 1616102 w/o (D), or with Essai 1632101 gel (G). Each secondary antibody measure corresponds to a sampling 7 days post-challenge with DNP-KLH in aqueous solution. Stars represent statistically significant differences between primary and secondary antibody titres (mean ± SEM) at a given time point with **: $p < 0.01$ and ****: $p < 0.001$.

IgM protein bands separated by SDS-PAGE were identified in Western blot by specific anti-barramundi IgM antibody. The barramundi serum Ig was composed of monomers, dimers, trimers and tetramers (Figure A2.5.1). IgM monomers comprised most of the total antibody (48%), while trimers were the lowest proportion (12%) in barramundi serum. On day 98, non-challenged fish vaccinated with ISA 763A VG adjuvant had significantly higher proportion of tetramers in sera than those in challenged fish ($p < 0.05$) (Figure 5.3A). On days 28 and 98, in fish vaccinated with Essai 1616102 w/o adjuvant, non-challenged fish had significantly higher proportion of dimers and trimers, and fewer monomers than re-injected fish ($p < 0.05$) (Figure 5.3B). In fish vaccinated with Essai 1632101 gel adjuvant, there were fewer monomers in fish at day 98 compared to non-challenged fish from day 28 ($p < 0.05$) (Figure 5.3C).



	A			
Monomer	A	A	A	A
Dimer	A	A	A	A
Trimer	A	A	A	A
Tetramer	A	AB	A	B

	B			
Monomer	A	B	A	B
Dimer	A	B	A	B
Trimer	A	AB	A	B
Tetramer	A	A	A	A

	C			
Monomer	A	AB	B	B
Dimer	A	A	A	A
Trimer	A	A	A	A
Tetramer	A	A	A	A

Figure 5.3. Isomer composition of barramundi immunoglobulin. Samples were taken at 28 and 98 days, from not-challenged (primary) or challenged (secondary) fish vaccinated with DNP-KLH and ISA 763A VG (A), Essai 1616102 w/o (B) or Essai 1632101 gel (C). For each sample, the proportion of monomer, dimer, trimer and tetramer is represented (mean ± SEM). Tables represent statistical analysis, with each isomer analysed separately across the different conditions for each treatment. Different letters represent statistically significant differences in the proportion of each isomer ($p < 0.05$).

When fish were vaccinated with LPS, there was a significantly higher titre of anti-LPS antibodies after 14 days than when fish were mock-vaccinated with PBS (Figure 5.4A). Additionally, it was worth noting that the level of anti-LPS antibodies decreased back significantly after 21 days, reaching levels similar to the mock-vaccinated fish. Interestingly, the titre levels of anti-DNP antibodies were much higher during the same time course, with no difference in antibody levels between day 14 and day 21 (Figure 5.4B).

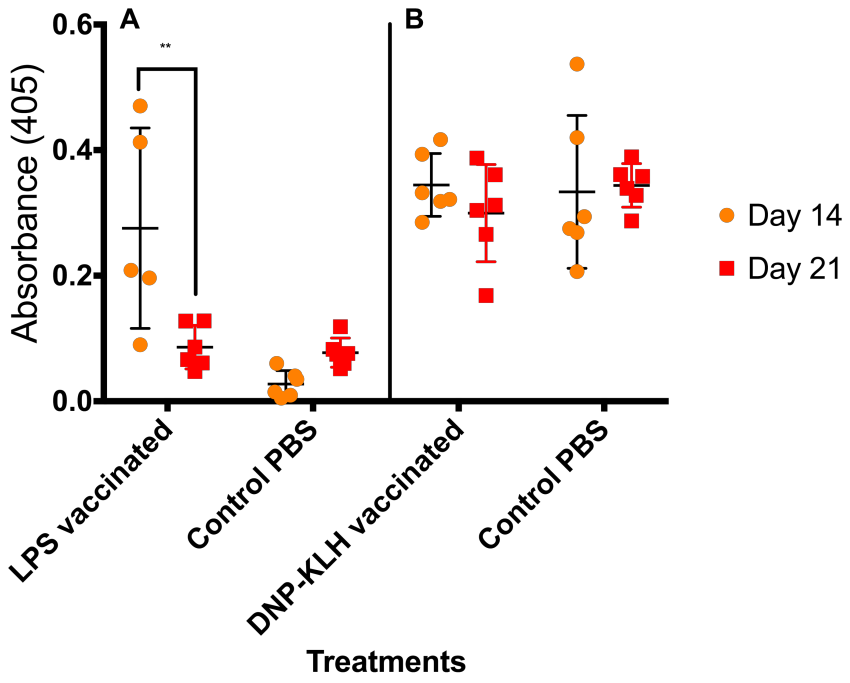


Figure 5.4. Antibody titres post vaccination with either LPS or DNP-KLH. PBS used as a mock-vaccination control. Titres against LPS (A) and DNP-KLH (B) are represented. Each fish (n = 6) is plotted with mean ± SEM represented in black for each treatment. Significant differences between time points are represented with ** $p < 0.01$.

V, J and C gene segments were all identified from a 270 kilo-base (kb) region of Scaffold 42 in the barramundi genome available from http://seabass.sanbi.ac.za [168], subsequently referred to as barramundi *igh* locus. A region of 200 kb at the 5' end of the *igh* locus contained 43 V_H gene segments identified using the TigGER package on R (Figure 5.5) [231]. Most of those V genes were duplicates (23 out of 43) and they ranged mostly between V1 and V5, with one V10, one V11 and one V13 genes identified through homology with *Danio rerio* V_H genes. At the 3' end of the *igh* locus, C genes were identified by sequence homology, along with J regions identified by the TigGER package on R [231]. The four constant region gene segments identified included Cα, Cτ, Cμ and Cδ. Several Cμ and Cδ regions were identified in barramundi *igh* locus, suggesting a gene duplication in barramundi, as previously seen in other fish species (reviewed in [236] and [237]). Associated with each of the constant region genes, upstream J regions were also identified, reminiscing of the arrangement of Ig gene segments in zebrafish [238]. Interestingly, the gene presently identified as Cτ in barramundi shares a relative position in the *igh*

locus with the previously identified Cζ region from zebrafish [238]. However, when performing a phylogenetic analysis using sequence homology with 2,000 bootstrap replicates, this gene identified in barramundi closely clustered with *Salmo trutta* Cτ rather than with *Danio rerio* Cζ, corroborating its identity as barramundi Cτ (Figure 5.6).

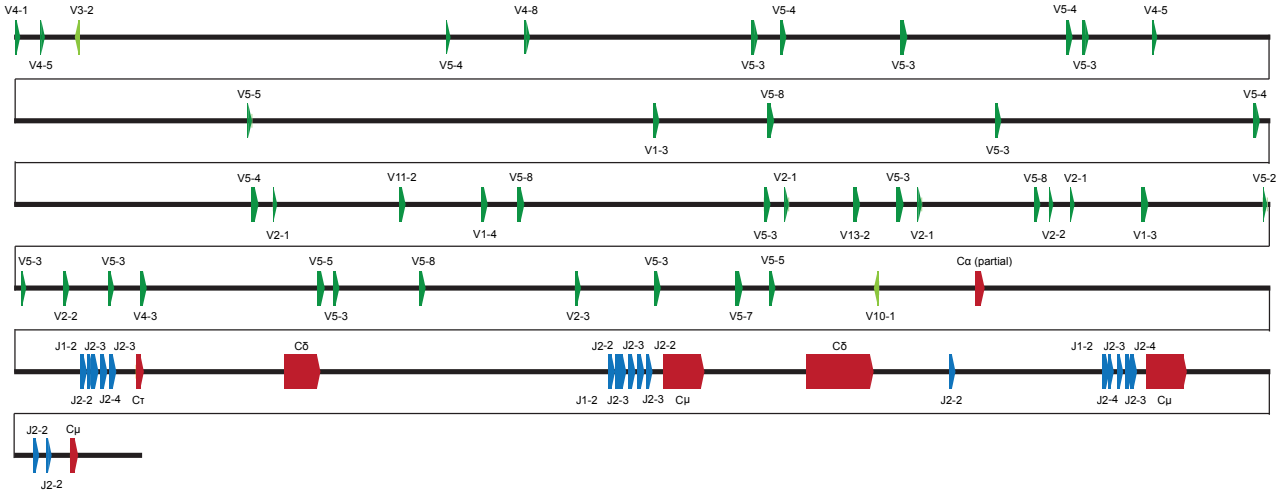


Figure 5.5. Gene arrangement of the barramundi *igh* locus. Gaps and genes are to scales and arrows represent the direction of the genes. Variable genes are represented in green, junction genes in blue and constant genes in red.

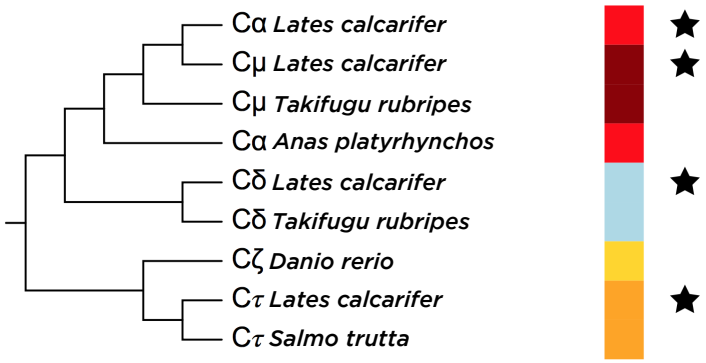


Figure 5.6. Phylogenetic arrangement of constant immunoglobulin genes from different teleost and avian species. Colours represent the different constant genes, with Cα in light red, Cμ in dark red, Cδ in blue, Cζ in yellow and Cτ in orange. Stars represent genes from barramundi (*Lates calcarifer*).

5.4 Discussion

Little is known about prime-boost responses in teleost fish and this is the first sequential analysis of prime-boost response to our knowledge. Previous studies have been conducted in European sea bass [199] and in rainbow trout [230], but both of these studies employed a single homologous boost applied many weeks after primary exposure. Both experiments demonstrated rapidly increasing antibody response either by ELISA [230] or by ELISPOT quantification of antibody positive cells [239]. In the current study, fish were boosted at 14, 21, 63 and 91 days post primary immunisation and secondary response examined 7 days post-boost. Significant increase in secondary antibody compared to primary antibody was only detected in fish boosted 91 days post-primary immunisation.

Adjuvants had a significant effect on the kinetics and magnitude of both the primary and secondary immune response. Primary immune response was elevated by all adjuvants tested compared to aqueous antigen alone, in line with previous research [73, 240-242]. The gel adjuvant resulted in a significantly higher and more sustained primary antibody response than any of the oil adjuvants tested. A decrease in secondary antibody production was detected at the 21-day post-vaccination boost with all adjuvants except Essai 1616102. Interestingly, in our vaccine trials against *Streptococcus iniae* in barramundi, challenges are routinely carried out at this time and protection is usually around 100% against a homologous bacterin [200]. However, it is likely that at least some of the antigen present in complex vaccines, notably peptidoglycan, LPS or other capsular polysaccharide, are presented through TLR-mediated T-independent routes. In contrast, the current study investigates only the T-dependent pathways of B-cell response. It may be that protection at that time (± 600 degree-days) post-primary immunisation is compromised, for vaccines in which protection is entirely founded on response to T-dependent antigens (e.g. recombinant proteins and most viral antigens). In this case, timing of vaccination well in advance of the disease season should be a priority to allow the primary response to pass, thereby reducing risk of feedback inhibition. Moreover, investigation of other adjuvants, such as Essai 1616102 w/o and the gel adjuvant (due to the very high and extended primary response) may well be worthwhile in full challenge models against *S. iniae* in barramundi and *S. agalactiae* in tilapia. It was also interesting that the secondary response appeared to only be significantly higher than the primary response when the secondary exposure occurred at least 12 weeks post primary

immunisation. This suggests that there may be a pre-requisite for memory formation that takes substantial time in fish, at least in Perciform fish.

Secondary immunisation at 14 days resulted in no significant change in circulating antibody. In contrast, secondary injection at 21 days resulted in significant decrease in circulating antibody in all treatment groups with exception of those groups vaccinated with antigen alone or with Essai 1616102 w/o emulsion. This decrease in circulating antibody may result from feedback mechanisms caused by antibody complexing at the peak of the primary response [243]. In mammals, only high affinity IgG antibody results in complexes and negative feedback, and this is likely due to antibody masking antigenic epitopes, preventing further B-cell responses when circulating antibody levels are already high [244]. This phenomenon has not previously been reported in fish, due to the absence of Ig Gamma heavy chains in all fish classes studied to date [245]. However, while fish do not have IgG and do not undergo classical isotype switching from IgM to IgG, there is evidence for affinity maturation of antibody over time in trout [246]. Consequently, it seemed likely that, at 21 days post vaccination in barramundi at 28°C, the combination of high levels of circulating primary antibodies coupled with some affinity maturation was sufficient to result in complexing and negative feedback. Complexing would likely occur until circulating antibody levels subsided sufficiently to permit a normal secondary immune response post boost. In humans and mice, antibody-mediated suppression of secondary response is generally associated with particulate antigens, such as erythrocytes [247], but has been noted also for soluble or subunit antigens when delivered with adjuvant [248]. The level of suppression of immune response is correlated directly with IgG affinity for the antigen [249] and both primary and secondary antibody responses are supressed but T-cell priming appears to occur normally [244]. Consequently, suppression may result from one or more of three scenarios.

(1) By masking antigenic epitopes, high or same affinity antibody may prevent specific B-cells from recognizing and binding to the antigen. (2) Opsonisation of the antigen in IgG/antigen complexes may facilitate capture and elimination via Fc receptor mediated phagocytosis, consequently removing antigen from immune-sensitised areas prior to recognition by B-cells. (3) IgG/antigen complexes may co-crosslink the B-cell receptor (BCR) with the inhibitory FccRIIB, also expressed on the B-cell surface resulting in negative regulation of the B-cell [250].

Epitope masking would function without the IgG(Fc) (or in fish, IgM(Fc))-portion of the antibody whereas the two latter mechanisms would require intact IgG/IgM(Fc)-portions. Whilst studies examining suppressive function of Fc-deleted monoclonal antibodies have provided conflicting results, IgG suppresses antibody response equally well in both wild-type mice and mice lacking all known Fc-receptors, including FccRIIB [251]. It appears, then, that most of the adjuvants used in the present study resulted in high affinity antibodies that suppressed secondary response during peak response by epitope masking. Although largely investigated in mammals in the context of high-affinity IgG, under certain conditions, IgM may also suppress antibody response provided it is of sufficiently high affinity [249, 252]. To test this, an avidity assay was performed by ELISA. Interestingly, we found that the avidity of the antibodies was not significantly different at day 21 between the primary and the secondary response, except for the samples adjuvanted with Essai 16161062 w/o. Therefore, when the avidity of primary and secondary antibodies was of similar strength, meaning that B-cell receptors (BCRs) from affinity selected primed B-cells would not bind preferentially to antigens, amounts of circulating antibodies were decreased, supporting that the immune suppression observed was due to epitope masking.

Whilst epitope masking via high affinity IgM explains the immune suppression noted with most of the adjuvants, it does not account for the lack of suppression detected when water-in-oil emulsion formulated with Essai 1616102 w/o and antigen was used as the primary immunogen. This lack of suppression did not result from a low avidity IgM response, as the primary antibody response at that time was similar in terms of avidity and higher in terms of magnitude than obtained with ISA 763A VG. However, the avidity of the secondary antibodies was higher for Essai 1616102 w/o at that time, suggesting that this adjuvant allowed for a faster antibody affinity maturation. Antibodies, particularly IgM, can also promote immune response to complex antigens such as KLH when antigen dose is suboptimal [253]. This appears to be mediated through complement, as functional C1 and C2 receptors of the classical pathway are required for IgM-dependent immune-enhancement [253]. It is thus possible that Essai 1616102 w/o adjuvant slowed the release of the antigen to maintain the dose at sub-optimal levels, thereby invoking a faster IgM somatic hypermutation and increasing IgM avidity more quickly. In mice, secreted IgM and complement collaborate to form immune complexes that facilitate interaction with

follicular dendritic cells and accelerate formation of germinal centres [254]. Fish do not have classical secondary lymphoid germinal centres where class switching and affinity maturation occur as they do not have lymph nodes or peripheral lymphatic system. However, melanomacrophage centres (MMC) in spleen, head-kidney and occasionally liver [255-257] have been implicated in antigen processing and it is postulated that these may be primordial germinal centres in early vertebrates [243]. Because Essai 1616102 w/o resulted in significantly higher avidity antibody, it is likely that the adjuvant promoted the accelerated development of germinal centres. As affinity maturation occurs in germinal centres under the mediation of follicular T-helper cells (Tfh) [258, 259], a faster development of germinal centres would result in faster affinity maturation. Histological examination of spleen and HK over time post-vaccination, using antibodies to identify cell types, may clarify a role for MMCs in antibody response. Moreover, analysis of tissue samples from the present study by deep-sequencing for a broad array of cytokine signals associated with follicular DC and T-lymphocytes would be particularly relevant. In mice, IgM-immune complexes first locate to the spleen where they interact with complement receptors on B-cells in a complement dependent manner [254] and it is possible that Essai 1616102 w/o promoted complement activation for improved collaboration with IgM. This may also be a critical step in successfully initiating adaptive immune responses.

The results highlighted in the current study suggest affinity maturation and selection for high affinity IgM is both possible in fish and can be promoted by some adjuvants, both novel findings. The mechanisms that underlie this affinity selection process in fish need to be unravelled as this process presents interesting questions on the evolution of adaptive immunity in higher animals. Moreover, only by understanding how affinity maturation is initiated can we actively develop better adjuvants to target these pathways for more effective immunisation, and even modulate them if immune suppression proves to be problematic during vaccine deployment in aquaculture.

Chapter Six – General Discussion

Amongst all vertebrate taxa, the fishes are the earliest extant class that have both innate immunity and adaptive immunity based on major histocompatibility complex (MHC), T-cell receptors and antibody. Whilst there are many such similarities with the immune systems of higher vertebrates, there are also fundamental differences, not least amongst the diversity of immunoglobulin light and heavy chains. Moreover, the bony fishes themselves are the most diverse of the vertebrate taxa and there are substantial differences in immune process between divergent orders of the Teleostei. Most studies of teleostean immunity to date focus on model species such as zebrafish, or high value commercial species like Salmonids. For example, locally induced immunoglobulins at the mucosal surface of a poikilothermic animal was first described in salmonids [260]. Salmonids include many commercial aquaculture species such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), which are high value but are limited to temperate climates. Moreover, zebrafish have been widely employed as a model research animal due to their small size, ease of husbandry and breeding, leading to early sequencing of the genome [261] and a diverse array of tools for research. Zebrafish are part of the Cypriniformes, which include some commercial aquaculture species such as carps that are critical to food supply in some developing countries and popular for recreational fishing throughout Europe. Cypriniformes also mostly include fish popular with the international aquarium fish trade (such as loach, goldfish and koi) and, though representing a substantial global trade, they are not fundamental to global food security. From an immunological perspective, functional dendritic cells were first characterised in fish in the zebrafish [83], followed more recently by rainbow trout [76]. However, of more than 20,000 extant species of teleost fish, Salmonids are only comprised of around 220 species and Cypriniformes of around 4,250 species. In contrast, Perciformes include over 10,000 species, including many commercially important fish such as the European sea bass (*Dicentrarchus labrax*), tilapia (*Oreochromis niloticus* and *O. mozambicus*), sea bream (*Sparus aurata*), many grouper species (*Epinephelus spp.)*, various snappers (*Pagrus spp*.), jacks, trevally and pompanos (*Caringidae*), along with the Australian barramundi or Asian sea bass (*Lates calcarifer*). As one of the latest diverging bony fish orders (second

only to Beloniformes) [92] this incredibly diverse order represents highly adapted and successful animals that are foundational to food supply throughout the world.

Global population growth is concentrated in tropical and subtropical regions of the world, and with increased per capita consumption of sea food, production of fish for human consumption needs to double by 2030 to meet goals for food security [70]. It makes sense that aquaculture of warm and tropical species should be a focus for sustainable development due to proximity to growing populations and the higher productivity that can be achieved in warmer climates. In these regions, farming of Perciform species dominates high value aquaculture production. Here, it is critical to replicate the success of vaccination for disease prevention experienced in salmon aquaculture [53] to improve farm animal welfare, reduce economic losses and eliminate antibiotics from food production in line with the One Health ethos adopted in 2009 [262]. Furthering our nascent understanding of Perciform adaptive immunity will therefore be critical to optimising vaccine performance in these animals in a warm water environment.

For this thesis, the interlink between innate and adaptive immunity in barramundi, an iconic Australian fish, but popular throughout tropical and subtropical South-East Asia, was explored. As a foundation on which to build the understanding of the immune response in barramundi, an immune transcriptome was generated from pronephros and spleen after injection with PTG and LPS, or with saline, in order to capture the full scope of the transcripts activated over time during an antibacterial response (Chapter 2, [164]). Many genes linked to the endocrine, immune and nervous systems were regulated in the transcriptomes, underlining the importance of a holistic approach to maintaining healthy aquaculture animals. The neuro-endocrine-immune axis has become a fertile area of research in humans and mice over the last few decades, with interactions between systems evidenced by shared signalling molecules and receptors [137, 263]. For example, cytokines released by immune cells following a secondary immune response can induce a set of behaviours associated with disease by crossing the blood-brain barrier and binding to corresponding receptors in the brain, leading to reduced appetite and social interactions, and increased sleeping patterns among other symptoms [263]. Hormones can also play an important role in regulating an immune response.

Indeed, leptin, an adipose hormone, has been shown to critically control the balance between immunity and other physiological functions [264]. As such, leptin has been postulated as a key factor in the development of seasonal responses to disease, with loss of body fat associated with loss of immune functions, such as the production of antibody [265, 266]. This is further evidenced by the finding of leptin receptors on specific leukocytes, such as monocytes, neutrophils, DCs, B- and T-cells and natural killer cells [267-272]. With the bulk of neuro-endocrine-immune studies focussing on humans or model rodents, however, it is hard to draw conclusions about those interactions in other animal species, especially those used as agriculture or aquaculture products. Nonetheless, the nervous, endocrine and immune systems in barramundi were all regulated in response to bacterial components, providing a stepping stone for further investigations into those interactions (Chapter 2, Figure 2.1, [164]).

6.1 LPS recognition in teleosts

The transcriptome analyses, supported by corresponding analyses of the barramundi genome (published elsewhere during my doctoral studies [168]), revealed that the molecules classically involved in lipopolysaccharide (LPS) recognition (as part of the TLR-4 recognition system) in mammals were absent from barramundi. However, teleosts, including barramundi, are able to mount an inflammatory response to LPS, albeit when relatively high concentrations are employed. Lipopolysaccharides are a major component of the outer membrane in gram-negative bacteria. Typically, LPS molecules are composed of a core polysaccharide linked to an inward facing phospholipid termed lipid A, as well as an outer carbohydrate O-antigen consisting of up to 25 repeating sugar units [163, 273]. In most cases, the core polysaccharide and lipid A show conserved structures across species, whereas the O-antigen is more variable [273]. However, lipid A structure is also known to be somewhat dependent on the bacteria's external environment, with adaptability to the host potentially leading to intra-specific variability [274, 275]. The lipid A portion of LPS has been shown to be the main toxic moiety in mammals (reviewed in [273]), inducing the release of many inflammatory cytokines such as TNF-α, IL-1β and IL-6, triggering the production of reactive oxygen species such as nitric oxide and activating the C3 and C5 complement cascades [163]. In fact, the inflammatory response produced by LPS in mammals is so potent that it often results in endotoxic

shock, a generalised inflammation of the bloodstream which leads to severe hypotension, organ failure and potentially death [163].

In mammals, LPS recognition and processing by the immune system have been extensively studied since the early 1990s, with LPS-binding protein (LBP) and CD14 identified as key components of the LPS receptor complex in mammals [161, 276]. In the late 1990s, TLR4 was identified as the key signalling receptor for LPS and the identification of MD-2 as a molecule required for LPS signalling completed our current knowledge of the TLR4 associated LPS receptor complex [160, 277]. Briefly, LPB binds several LPS molecules together which then aggregate on CD14 before binding in turn to the MD-2/TLR4 complex and triggering signal transduction leading to immune activation (reviewed in [278]).

LPS recognition in fish, however, has proven more problematic to elucidate. Several studies using LPS to stimulate fish cells *in vitro* have shown that doses of LPS necessary to induce a cellular and molecular response in fish were 100-1000-fold higher than doses needed in mammals [114, 119, 165, 279, 280]. Moreover, several molecules associated with the TLR4 associated LPS recognition complex in mammals could not be identified in fish. For example, LBP is lacking in trout and cod (reviewed in [118]) and, more importantly, TLR4 is lacking from most fish species (reviewed in [4, 116, 147]) including from barramundi, on which this thesis is based [164]. However, several studies have also demonstrated cytokine activation in fish post-stimulation with LPS [18, 119, 281-283], indicating other receptors are likely involved in LPS recognition in teleosts [164, 284]. Moreover, recent evidence suggests that LPS is an essential component in some fish vaccines, for example against enteric redmouth disease (ERM) caused by *Yersinia ruckeri* [125]. Indeed, exposure to highly purified LPS O-polysaccharide from *Y. ruckeri* was enough to confer complete protection in rainbow trout despite the apparent lack of TLR4 in those fish, implicating alternative co-stimulatory pathways [8, 116].

Because LPS remains the main immunogen in many effective fish vaccines in spite of lacking classical pro-inflammatory TLR4 LPS recognition molecules, we investigated how fish might alternatively recognise and process LPS. With our transcriptomic work and the timely publication of *Lates calcarifer* genome, we were

able to show that barramundi lack all the classical LPS recognition molecules, including TLR4, MD-2 and CD14 [164]. Moreover, we identified several receptors that, complementarily, could account for the recognition of LPS in barramundi, namely Mincle and leucocyte integrins (Chapter 2, [164]). Putative DCs were also identified in barramundi and, although LPS did not seem to greatly activate those cells *in vitro*, injection with combined LPS and PTG *in vivo* did induce migration of DCs towards spleen (secondary immune organ), potentially for T-dependent processing of those bacterial components (Chapter 4, [114]). Additionally, barramundi were able to mount an antibody response against LPS when injected intraperitoneally, even without the addition of oil adjuvant (Chapter 5), confirming that LPS is processed by the adaptive immune system in *Lates calcarifer*.

LPS comprises a major specific protective antigen during adaptive immune response to gram-negative bacterial fish pathogens. However, a plethora of alternate PRRs have been found to bind to LPS in mammals, which provided some direction for further investigation of the barramundi transcriptome. The C-type lectin receptor Mincle was identified as a LPS receptor in barramundi that is present on dendritic cells and consequently may underlie early adaptive immune processing of LPS as an antigen via T-dependent pathways (Chapter 2, [164]). However, whilst LPS affects expression of both TNF-α cytokine and IL-6, it was only able to block the effect on TNF-α, but not IL-6 expression through Mincle with a specific anti-Mincle antibody, which implicates other receptors in LPS recognition and response (Chapter 2, [164]) thus stimulating further investigation.

Among the caspases identified in barramundi, two were inflammatory caspases, caspase-5 and caspase-1. Caspase-5 had previously been identified as a LPS receptor through its CARD domain, however, the caspase-5 identified in barramundi lacked a CARD domain, thus is unlikely a functional pro-inflammatory LPS receptor in barramundi. Caspase-1 typically combine with NLRs to form inflammasomes. Six NLRs, all belonging to the NOD family, were identified in the barramundi transcriptome. However, none of these NLRs are known to form inflammasomes, suggesting there are no inflammasomes in barramundi. There are four leucocyte integrins in mammals, composed of a different α-subunit and a common β-subunit. The β-subunit, as well as two of the α-subunits were identified in barramundi.

Barramundi leucocytes adhered to ligands specific to integrin-αM and -αL, and were also successfully labelled with specific fluorescent antibodies against the α-subunits. Moreover, when spleen cells were activated using integrin antibodies before stimulation with LPS, the pro-inflammatory cytokine IL-1β was up-regulated, strongly suggesting that leukocyte integrins play a role in LPS processing in barramundi, and potentially other teleost fish (Chapter 3).

Putative DCs were identified for the first time in barramundi and their migratory pattern post-injection was determined, thanks to the localisation of DC-SCRIPT, a specific DC marker (Chapter 4, [114]). Evidence strongly suggested that spleen in barramundi has a role very similar to lymph nodes, where antigen presentation occurs, as barramundi DCs migrated back to the spleen within seven days post-injection. Interestingly, it was shown that barramundi DCs responded to some bacterial peptides such as PTG whereas they did not respond strongly to LPS, further confirming the likely lack of strong inflammatory LPS receptors in barramundi (Chapter 4, [114]).

6.2 Antibody production and implications for immune memory in fish

After investigating PRRs and APCs in barramundi, subsequent work focused on the development of adaptive immunity post vaccination (Chapter 5). The kinetics and magnitude of the secondary immune response were investigated through a long-term immunisation experiment in barramundi. The production of antibodies is a crucial part of foreign antigen clearance and secondary antibodies are an indication that memory B-cells have been formed during the first encounter with this antigen. The genomic region coding for the variable (V), junction (J) and constant genes of barramundi immunoglobulin heavy chain was identified through genome analysis, identifying over 40 V regions and four different constant regions (Chapter 5, Figure 5.5). Some of those constant region genes were present several times, suggesting a genome duplication in barramundi, similarly to findings in several other fish species. Moreover, through a partnership with industry, different experimental adjuvants were also tested to determine if commercially available adjuvants could be improved for warm water fish species production. It was shown that barramundi process TD antigens, likely through antigen presentation from DCs to T-cells, and that memory B- and T-cells were subsequently present, as demonstrated by the detection of

secondary antibodies post challenge. It was also shown that barramundi can produce primary antibodies against TI antigens such as LPS, with a significantly higher titre of LPS antibodies just 14 days post vaccination. However, it was also shown that some adjuvants were more suited to barramundi than others, with higher, more sustained production of primary antibodies in Essai 1632101 gel, and a faster, more potent secondary antibody response is Essai 1616102 w/o. Interestingly, the strength of the bond between antigen and antibody (avidity) also differed based on the adjuvant used, suggesting that the formulation of those adjuvants, rather than their preparatory mode, impacts the onset of adaptive immunity (Chapter 5).

The production of antibodies has been closely linked to the secondary immune response in mammals and other vertebrates. In fact, antibodies production is a key component of adaptive immunity, with jawed fish (including Chondrichthyes and Teleosts) being the earliest diverging phyla with an immunity based on antigen recognition by major histocompatibility complex and antibodies [285]. Antibodies, also known as immunoglobulins are large Y-shaped quadripartite molecules that include two small light chains covalently bound to two larger heavy chains (Figure 6.1). The tip of the junction between light and heavy chains form what is known as an antigen binding site or paratope, and are highly variable to accommodate for the incredibly large diversity of antigens (Figure 6.1).

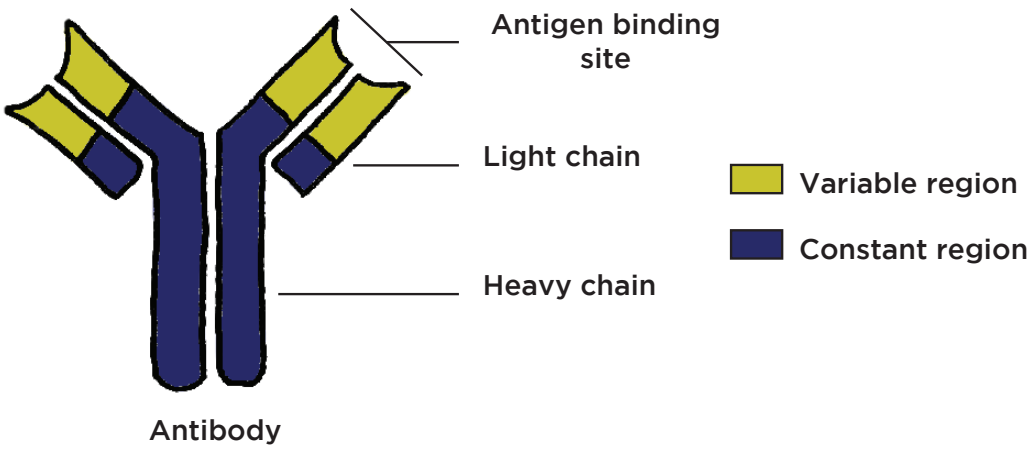


Figure 6.1. Graphical representation of an antibody. Antibodies can be either membrane bound (on B-cells) or secreted in blood plasma.

Antibodies are present in two different forms, membrane-bound at the surface of B-cells (also known as B-cell receptors (BCRs)) or secreted by plasma cells and circulating freely in blood plasma. Natural antibodies, which are present in the animal without prior external stimulation [286], are part of the first line of defence in vertebrates' innate humoral system, including teleosts [287]. In mammals, natural antibodies are mostly comprised of immunoglobulin M (IgM), can bind to an array of unspecific foreign particles, have low binding strength, and are produced in a small subset of B lymphocytes, B-1 cells [288-291]. Because they are constantly exposed to foreign microorganisms, fish are thought to have high level of circulating natural antibody [292-294]. To induce the production of specific antibodies on the other hand, B-cells need to be activated by antigen presenting cells and often require co-stimulation by T-cells (discussed in more details towards the end of this section). In mammals, there are three major known mechanisms that increase the diversity of the antigen binding site, namely V(D)J recombination, somatic hypermutation and class switching [285]. Those mechanisms allow for the optimal binding of foreign antigens and fast response of the immune system upon re-encounter of the same antigen, and form the base of adaptive immunity in mammals. There is preliminary indication that antibodies in rainbow trout (*Oncorhynchus mykiss*) have increased affinity over time after an immunisation [295-297], similarly to what was observed in barramundi (Chapter 5, Figures 5.1 and 5.2). Therefore, it is suggested that there is one or several mechanisms in fish, potentially the same as in mammals, that contribute to the improvement of antibody affinity post-immunisation, despite critical differences between teleost and mammals.

Variable, diversity and junction (V(D)J) recombination is the mechanism that forms the base of the antibody repertoire diversity. Because of the diversity of antigens in existence, the diversity of antibodies needs to be equally large. Consequently, each light and heavy chain immunoglobulin gene includes many variable and junction gene segments (44 and 6 in humans respectively). Moreover, heavy chain genes also include diversity gene segments (27 recorded in humans), bringing the number of possible combinations to around $3x10^{11}$ different immunoglobulin molecules.

Somatic hypermutation (SHM) is a mechanism that increases the affinity of antibodies to antigens by allowing high mutation rates at the complementary-

determining regions (CDRs) of the immunoglobulin, thus expanding the antibody repertoire even further [298]. Those mutations occur in germinal centres (GCs) in proliferating B-cells at a rate that is 10^5-10^6 times higher than the mutation rate in the rest of the genome, and require the enzyme activation-induced cytidine deaminase (AID) [298-302]. Within the thousands of slightly different B-cells produced as a result to V(D)J recombination and somatic hypermutation, only the B-cells with the greatest affinity (binding strength) to the antigen are selected to mature into plasma cells and memory B-cells [298, 301].

Class switching, also known as isotype switching, occurs post-activation of mature B-cells in mammals, once the V(D)J recombination and SHM have already occurred. Unlike during V(D)J recombination and SHM, only the constant region of the heavy chain in the immunoglobulin molecule is affected, changing from one class of Ig to another (e.g. from IgM to IgG). Because the variable region of the Ig molecule is not affected, the antigen specificity stays the same but the molecule can now interact with different effector cells, allowing for a stronger and faster response of the immune system. Immunoglobulin diversity in fish has been extensively reviewed in the last decade [106, 236], with the sequencing and publication of teleost genomes helping to establish Ig diversity and structure in many fish species. Three classes of Ig have currently been identified in teleosts, IgM [77], IgD [303] and IgT/Z [238, 304]. IgM and IgD have both been identified as primordial classes of Ig, as they are well conserved across the vertebrates with homologs found even in Chondrichthyan [236]. On the other hand, IgT/Z is restricted to fish species, and is thought to be associated with mucosal immunity, playing a role similar to IgA in mammals [236]. Surprisingly, IgG and IgE have not been identified in teleosts to date, despite their ability to mount potent immune responses against secondary infections and fish being affected by parasites [305]. Classical GCs are also absent in fish [292, 301], however, melanomacrophage centres (MMC) present in abundance in fish head-kidney, spleen and liver, are structurally similar to mammalian GCs and have often been termed “primitive GCs” in the literature [243, 306-309]. Moreover, AID-positive cells normally found in mammalian germinal centres have been found in or close to melanomacrophage centres in fish spleen, suggesting that MMCs perform a GC-like function in fish [300, 310-313]. Nonetheless, to date, MMCs have not been

documented to host SHM [313], but a systematic investigation of MMCs in fish could lead to a definite characterisation of their function [314].

The typical structure of Ig heavy chain loci comprises of a region containing variable genes at the 5' end, followed by diversity, junction and constant clusters for each different constant region (Cμ/δ and Cτ/ζ) at the 3' end [236, 238]. The same typical Ig locus organisation was observed in barramundi (Chapter 5, Figure 5.5). There seems to be an incredible heterogeneity of loci number, organisation and splicing patterns for immunoglobulin in teleost fish, likely contributing to the natural antibody diversity found in fish [236]. Moreover, several IgM molecules can be produced in zebrafish, due to different transcription patterns, which increases the number of immunoglobulin isotypes expressed [315]. Although different functions for those isotypes have not been determined yet, it is possible that heterogeneous IgM isotypes may account for the lack of IgG in fish.

The production of antibodies against a given antigen and the magnitude of this response have been used extensively to determine the ability of an organism to respond and fight against said antigen. Indeed, as antibodies can immobilise antigens and act as beacons for antigen clearance by immune cells, the quantity and quality (i.e. binding strength) of antibodies produced over time can be directly correlated with the effectiveness of an immune response. Antibody quantification, over the course of primary and secondary immunisation (kinetics), has been used in mammals and fish for many decades to this effect [68, 316-318]. Because fish are poikilothermic, the water temperature in which the animal lives directly dictates the rate of metabolic responses, including during an immune response [310]. This parameter makes the study of immune kinetics even more crucial in fish, as big variations are expected between species living in different climates. More importantly, the formulation of vaccines as oil emulsion with an adjuvant can also impact the speed at which antibodies are produced, both after primary and secondary immunisations. Work on immune kinetics and adjuvants in fish has mostly focused on salmonids, which are raised in cold water ranging from 2^{o}C to 18^{o}C [53, 319]. On the other hand, sub-tropical and tropical aquaculture fish such as barramundi (*Lates calcarifer*), catfish and tilapia are farmed at much higher temperatures ranging between 28°C and 32°C [320]. Because increased

temperature correlates with increased metabolic rate, the response of warm water fish to pathogen is likely to be proportionately faster. However, oil adjuvants, known to slow the onset of the immune response but to prolong its duration, are still used in vaccines for warm water fish species [321]. Because of the discrepancies in farming cycle (6 to 9 months for warm water fish versus 2 to 3 years for cold water fish), and because the kinetics of immune responses are affected by adjuvant use and water temperature, it is likely that warm water fish vaccinated with vaccines optimised for cold water fish will not develop a full immune response during their farming cycle, putting them at risk of disease even if vaccinated.

Consequently, investigation of antibody kinetics using different adjuvant formulations and compositions in warm water fish species was warranted, to maximise vaccine efficiency (i.e. antibody production post vaccination) for the industry. Interestingly, this thesis highlighted that adjuvants not only affected the magnitude of antibody production, but also affected the quality of binding (affinity) of the resulting antibodies (Chapter 5). Furthermore, there also seemed to be a difference in antibody kinetics depending on the type of antigen used in the vaccine formulation. Antigens can be either T-cell dependent (TD), meaning that B-cell proliferation against those antigens is dependent on prior T-cell activation, or T-cell independent (TI), which means that the antigens can activate B-cells directly without needing T-cells stimulation. Most protein and peptides are TD antigens, and the antibodies produced against those antigens typically have higher binding strength and they undergo isotype switching in mammals [223, 224]. TI antigens, on the other hand, include mostly carbohydrates, and are divided into two categories, type 1 and type 2, based on their interactions with B-cells. TI type 1 antigens can induce naïve and mature B-lymphocyte proliferation and differentiation into plasma cells, whereas TI type 2 antigens can only activate mature B-cells [223]. In both cases, the antibodies produced against TI antigens do not have a strong avidity and are highly antigen-specific (they do not cross-react with other antigens). Moreover, there is generally a lack or low response to secondary exposure to TI antigens, suggesting they fail to induce immune memory and to drive B-cells differentiation into memory cells [224, 225]. As most microbial vaccines are formulated with inactivated bacteria, and the primary protective antigens are primarily carbohydrates such as capsular polysaccharide or LPS O-antigen, vaccine design ought to consider how to induce a TD response using such TI antigens.

Previous research has shown that the development of memory B-cells against a TI antigen is possible, by coupling the TI antigen with a protein carrier [226]. Vaccines based on the hapten-carrier model, with a generally smaller non-immunogenic hapten and a bigger immunogenic protein carrier, have already proven to be successful in mammals, for example in the prevention of meningococcal diseases caused by the bacterium *Neisseria meningitidis* [226, 227]. Polysaccharide vaccines (which include most Streptococcal vaccines) are generally poorly immunogenic and the resulting serum antibody response is short lived [322, 323], but a conjugate vaccine combining the protein carrier CRM197 and *N. meningitidis* serotype C oligosaccharide successfully induced antibody production, conferred long term immune memory and even helped with herd immunity [228]. In aquaculture, there are many bacterial pathogens hindering the development of the industry, such as *Yersinia ruckeri*, *Aeromonas spp*., *Photobacterium spp*. and *Streptococcus spp*. to only name a few [110, 208, 324, 325]. However, the immuno-dominant antigens in these bacteria are composed primarily of carbohydrates (namely capsular polysaccharide and lipopolysaccharide) [158], which highly strain-variable and likely TI, resulting in highly type-specific, low quality antibody response. Consequently, one issue with designing highly effective vaccines against such pathogens is that their carbohydrate layer will be detected preferentially by the host's immune system (immuno-dominant) but they will not induce a strong secondary immune response based on memory cells (T-independent). It is thus possible that a hapten-carrier approach may be developed for the aquaculture industry against bacterial pathogens, as the need for more efficient polysaccharide vaccines arise and the costs associated with producing conjugate vaccines decrease. Moreover, with recent advances in whole genome sequencing for fish-pathogenic bacteria [326, 327], conserved protein antigens in these pathogens are being identified. The challenge facing vaccine design and formulation will lie in how to encourage the fish to process these conserved TD-antigens in place of the immuno-dominant type-specific TI-polysaccharides.

6.3 The future of vaccines for warm water fish aquaculture

Loss due to disease is one of the major hindrances to the aquaculture industry [54], with loss of stock sometimes reaching over 75% in a single outbreak (e.g. infectious hematopoietic necrosis virus in salmonids or viral nervous necrosis in barramundi) [51, 90]. Overall, vaccination has been shown to be the most effective way to control for disease in aquaculture. Indeed, since the introduction of vaccination in the 1990s, some pathogens such as *Vibrio* and *Aeromonas spp.* in the salmon industry have been largely controlled, which has allowed for a steady increase in production [53]. Moreover, vaccination against bacterial diseases, which account for the majority of disease outbreaks in aquaculture, has also benefited the environment by limiting the use of antibiotics and their possible spread to the adjacent marine life [53]. Vaccine formulation and administration mode are also of utmost importance, with oil-based vaccines injected intraperitoneally being the current optimal combination for long-term protection in cold water species. However, oil-based adjuvants need to be adjusted to warm water species, as discussed above (Section 6.2). Additionally, individual injection into the peritoneal cavity can be labour intensive and single dose vaccines too expensive, especially in warm water species, where the fish are only farmed for a few months and the value of the final fish product is relatively low. Such economical parameters mean that intraperitoneal vaccination, although very effective at stopping disease occurrence, is less cost-effective for the industry, which highlights the need for easier administration routes and cheaper vaccine formulations.

In the last few decades, research has thus focussed on assessing and improving immersion and oral vaccinations, both routes allowing for faster immunisation of high number of animals, decreasing costs and intensity of labour. Unfortunately, most studies have shown that fish do not respond as well to immersion and gut (oral or anal) vaccination compared to intraperitoneal or intramuscular injections [69, 328, 329], as evidenced by lower levels of antibodies detected by ELISA and lower relative percent survival (RPS) post challenge. However, in some of those trials, the RPS post oral vaccination and boost was still around 60%, demonstrating that immunisation through this route, although not optimal, is possible [329, 330]. The main challenge with oral vaccination is that antigens administered this way are often degraded in the foregut due to very low pH levels before they can arrive to the

hindgut, where they may be absorbed [331-333]. Moreover, oral vaccination stimulates mucosal immunity first, which could be an issue if the pathogen directly enters the host's bloodstream through cutaneous lesions, a potentially frequent occurrence under poor husbandry, rapidly changing water conditions and high stocking densities [334]. On the other hand, stimulating the host's mucosal immunity could be beneficial, as healthy gut, skin and gills, which are potential first points of entry into the host, are protected by a mucus layer. Furthermore, it has been shown that oral vaccination, in some cases, elicited an increase in antibody levels not only in gut mucus, but also in skin mucus and even serum [335], suggesting that oral vaccination has the potential to induce a generalised immune response in the host. Recent research has also identified vectors that seem suitable to the delivery of oral vaccines. For example, alginate microspheres and chitosan nanoparticles appear like effective vectors for DNA vaccine delivery, both when administered manually into the mouth of the fish and when incorporated to food pellets [242, 336, 337]. The use of bacterial spores, obtained from species such as *Bacillus subtilis*, genetically modified to express antigens of interest have also been successful in eliciting protection when administered as oral vaccine, with the added benefit of bacterial spores also acting as adjuvants [335, 338, 339]. Despite those promising steps towards oral vaccination, major concerns are still associated with the use of oral vaccines, such are the cost-effective production of large quantities of antigen needed or the possible development of immune tolerance in the gut. Further work is thus still necessary to optimise oral vaccines production and delivery, but due to the attractiveness of this administration route, research will undoubtedly focus on oral vaccines in the next decades.

Vaccine formulation (i.e. antigen(s) and adjuvant(s) included in a vaccine) is crucial to vaccine effectiveness. Indeed, even if an antigen is delivered appropriately to the immune system, the vaccine will not be effective if the antigen is not recognised on the pathogen once an infection occurs. With the development of bioinformatics and the easier access to sequencing technologies, it is becoming increasingly easy to identify potential antigens, through a process termed reverse vaccinology [340]. As fish pathogen genomes are becoming increasingly available, novel antigens that are not necessarily immuno-dominant but still immunogenic can be detected and purified for use in trials. Using the same principle, antigens that are shared among different

pathogen strains can also be identified, allowing for the simpler development of cross-protective vaccines, for example against Group B *Streptococcus* [341]. Moreover, with new knowledge on fish immunity available, it is also easier to quickly screen for effective antigens, by conducting small injection trials and assessing expression levels of immune genes in plate based assay, before committing to larger and more expensive vaccine/challenge trials. Indeed, the regulation of cytokines and chemokines during an immune response have now been well characterised, and often even liked with RPS post-challenge in fish [242, 335, 342, 343]. Therefore, based on immune molecule profiling, it is now possible to infer immunogenicity of a large quantity of antigens using qRT-PCR, before conducting full scale vaccine trials on the most promising of those antigens.

Based on this thesis, barramundi have the potential to mount an immune response against protein-based antigens (i.e. TD antigens), with immune memory and affinity maturation of antibodies occurring (Chapter 5). Recombinant protein antigens have also been shown to induce immune protection in other fish species such as flounder, carp and channel catfish [344-346], suggesting that TD antigens are good vaccine candidates in teleosts. On the other hand, carbohydrate-based vaccines (including most whole-cell killed bacterial vaccines), especially those including LPS as their main antigen (i.e. TI antigens), should be treated with caution. Although our research suggests that barramundi can recognise and process LPS (Chapter 2 and 3), it is doubtful that a fish vaccine containing only carbohydrate-based antigens would be efficient in the long term and is likely to be highly strain specific [61], most likely because they do not activate TD immune memory. Further research could thus focus on the development of glycoconjugate vaccines [224], as combining highly detectable TI antigens with TD protein carriers could significantly improve the efficacy of vaccines used in aquaculture. Moreover, using the right adjuvant for the vaccination target species will be crucial in ensuring disease protection, especially with the development of warm water species aquaculture requiring faster protection.

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Appendices

Appendix 1.1. Conference abstract for the research presented at the third FRDC Australasian Aquatic Animal Health Scientific Conference, Cairns, 6-10 July 2015

Conference abstract for the citation:
Zoccola E, Delamare-Debouteville J, Barnes AC. Identification of barramundi (*Lates calcarifer*) DC-SCRIPT, a specific molecular marker for dendritic cells in fish. Presented at the third FRDC Australasian Aquatic Animal Health Scientific Conference, Cairns, 6-10 July 2015.

Identification of barramundi DC-SCRIPT, a specific molecular marker for dendritic cells in fish

Antigen presentation is a critical step bridging innate immune recognition and specific immune memory. In mammals, the process is orchestrated by dendritic cells (DCs) in the lymphatic system, which initiate clonal proliferation of antigen-specific lymphocytes. However, fish lack a classical lymphatic system and there are currently no cellular markers for DCs in fish, thus antigen-presentation in fish is poorly understood. Recently, antigen-presenting cells similar in structure and function to mammalian DCs were identified in various fish, including rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*).
The present study aimed to identify a potential molecular marker for DCs in fish and therefore targeted DC-SCRIPT, a well-conserved zinc finger protein that is preferentially expressed in all sub-types of human DCs. Putative dendritic cells were obtained in culture by maturation of spleen and pronephros-derived monocytes in barramundi (*Lates calcarifer*). DC-SCRIPT was identified in barramundi by homology using RACE PCR and genome walking. Specific expression of DC-SCRIPT was detected in barramundi cells by Stellaris mRNA FISH, in combination with MHCII expression when exposed to bacterial derived peptidoglycan, suggesting the presence of DCs in *L. calcarifer*. Moreover, morphological identification was achieved by light microscopy of cytospins prepared from these cultures. The cultured cells were morphologically similar to mammalian and trout DCs. Migration assays

determined that these cells have the ability to move towards pathogens and pathogen associated molecular patterns, with a preference for peptidoglycans over lipopolysaccharides. The cells were also strongly phagocytic, engulfing bacteria and rapidly breaking them down. Barramundi DCs induced significant proliferation of responder populations of T-lymphocytes, supporting their role as antigen presenting cells. DC-SCRIPT expression in head kidney was up-regulated 6 and 24 h following intraperitoneal challenge with peptidoglycan and lipopolysaccharide and declined after 3 days. Expression was also down-regulated in the spleen at 3 days post challenge but increased back at 7 days. This may indicate initial migration of immature DCs from head kidney and spleen to the injection site, followed by return to the spleen for maturation and antigen presentation.
DC-SCRIPT may be a valuable tool in the investigation of antigen presentation in fish and facilitate optimisation of vaccines and adjuvants for aquaculture.

Appendix 1.2. Conference abstract for the research presented at the ASFB-OCS joint conference, Hobart, 4-7 September 2016

Conference abstract for the citation:
Zoccola E, Kellie S, Barnes AC. *Lates calcarifer* transcriptome reveals the Mincle CLR as an alternative to the classical LPS recognition pathway. Presented at the ASFB-OCS joint conference, Hobart, 4-7 September 2016.

Lates calcarifer transcriptome reveals the Mincle CLR as an alternative to the classical LPS recognition pathway

Aquaculture, currently supplying over half of the world's fish supply, is acknowledged as one of the major foundations of future food security for a growing population. However, successfully developing this industry requires the reduction of stock loss due to diseases, in particular in the fast developing sector of warm water fish aquaculture. Vaccination has previously shown some potential but it requires a deeper understanding of the development of immunity in warm water fish. An immune transcriptome was thus generated against lipopolysaccharide (LPS) and peptidoglycan (PTG) to elucidate potential pathways involved in the onset of adaptive immunity, on which vaccination relies. The immune transcriptome,

generated from barramundi primary immune organs, was assembled and annotated, generating 163,661 transcripts. Putative proteins from the TLR and CLR families were identified in barramundi. Interestingly, TLR4, responsible for LPS recognition, was absent from the transcriptome. Using qRT-PCR, barramundi Mincle (a CLR) was identified as an alternative to the classical TLR4 mediated LPS recognition pathway as LPS induced the transcription of IL-6 and TNF-α through Mincle.

Appendix 1.3. Conference abstract for the research presented at the fourth FRDC Australasian Aquatic Animal Health Scientific Conference, Cairns, 10-14 July 2017

Conference abstract for the citation:
Zoccola E, Li A, Barnes AC. Linking antibody response and efficacy of the adaptive immunity in vaccine development: How adjuvants modulate the immune response in barramundi. Presented at the fourth FRDC Australasian Aquatic Animal Health Scientific Conference, Cairns, 10-14 July 2017.

Linking antibody response and efficacy of the adaptive immunity in vaccine development: how adjuvants modulate the immune response in barramundi

Vaccination of finfish has been a successful practice for reducing both disease outbreaks and antibiotic use in aquaculture. Vaccination relies on activation of adaptive immunity, usually by injection, which results in immunological memory that can provide the animal with long-term protection against the pathogen in the vaccine. However, full efficacy of vaccines in aquaculture, similarly to humans and other terrestrial animals, is dependent upon the use of adjuvants added to the injection mix. Indeed, oil emulsion adjuvants were a core enabling technology that underpinned the adoption of single injection multivalent vaccines that offered lifetime protection in salmonid aquaculture. In warm water aquaculture, where expansion is most rapid, vaccination faces a new set of challenges, not least the shorter farming cycle and the increased metabolic rate at high temperature. Consequently, there is a need to explore alternatives to emulsions that were developed for cold-water salmonids.

In order to evaluate effects of adjuvants on development of immunity in a warm water species, a vaccination prime-boost experiment was conducted in barramundi at 28 °C using a model antigen formulated with five different adjuvant treatments. The different treatments included four new experimental adjuvants (one gel, one water-in-oil-in-water (w/o/w), and two water-in-oil (w/o1 and w/o2)) along with a current commercial adjuvant (w/o; ISA763AVG). These were formulated with DNP-KLH antigen in aqueous solution. Barramundi juveniles (96 each) were vaccinated with the vaccine treatments, an antigen control (non-adjuvanted aqueous vaccine) or a PBS injection control. After a holding period, replicate cohorts of fish were boosted using the DNP-KLH aqueous control vaccine and blood was collected subsequently from both non-boosted and boosted fish to assess the primary and secondary immune responses respectively over time by measuring antibody via indirect ELISA. Generally, barramundi had very high background antibody binding by ELISA. Adjuvants significantly slowed the development of primary immunity with antigen-only peaking before the 21 days initial sample point. Inclusion of any adjuvant in the vaccination delayed the antibody response by around 7 days with antibody peaking at or later than 28 days post immunisation. The gel adjuvant resulted in a significantly elevated and prolonged immune primary immune response compared to all other treatments, with significantly higher circulating antibody remaining in serum until the end of the trial (3 months). In terms of secondary response, boosts resulted in depleted circulating antibody at 28 days, coincident with peak primary antibody levels in all adjuvant treatment groups except w/o2. This is likely explained by complexing of antigen with high affinity antibody resulting in inability to mount a secondary B-cell response. There are consequently implications for timing of vaccination when using oil adjuvants with T-dependent antigens (eg. virus, recombinant proteins, capsular polysaccharides). All adjuvants resulted in strong secondary response when animals were boosted 3 months post-primary immunisation. Further work to determine the affinity of the resulting antibody responses is currently ongoing. Further vaccine and challenge trials should investigate the efficacy of the gel and w/o2 compared to ISA 763A at differing times post immunisation. These results imply that vaccination strategy for T-dependent and independent vaccines can be optimised for warm water fish.

Appendix 1.4. Conference abstract for the research presented at the 18th International Conference on Diseases of Fish and Shellfish, Belfast, 4-7 September 2017

Conference abstract for the citation:
Zoccola E, Kellie S, Barnes AC. Mechanisms of lipopolysaccharide recognition and processing in barramundi (*Lates calcarifer*). Presented at the 18th International Conference on Diseases of Fish and Shellfish, Belfast, 4-7 September 2017.

Mechanisms of lipopolysaccharide recognition and processing in barramundi (*Lates calcarifer*)

Aquaculture, currently supplying over half of the world's fish supply, is acknowledged as one of the major foundations of future food security for a growing population. However, successfully developing this industry requires the reduction of stock loss due to diseases, particularly in the fast-developing sector of warm water fish aquaculture. Vaccination has previously shown some potential but it requires a deeper understanding of the development of immunity in warm water fish, in particular when exposed to complex and diverse antigens such at lipopolysaccharides (LPS). Moreover, fish have a subdued innate response to LPS, but adaptive response is generally high and type-specific, warranting further investigation in the way fish recognise and process LPS.
An immune transcriptome was generated against bacterial cell wall polysaccharides to elucidate potential pathways involved in the onset of adaptive immunity, on which vaccination relies. Putative sequences coding for proteins from the TLR, CLR, NLR and integrin families were identified in barramundi by homology with other teleosts and mammals. Those proteins were further investigated as potential receptor/initiators of the immune response in barramundi by qRT-PCR and western blotting.
TLR4, responsible for LPS recognition, was absent from the barramundi transcriptome. Proteins normally involved in the formation of inflammasomes (potent inflammatory inducers), NLRPs, were also absent from the transcriptome. Using qRT-PCR, barramundi Mincle (a CLR) was identified as a partial alternative to the classical TLR4 mediated LPS recognition pathway as a high dose of LPS induced

TNF-α expression via Mincle. However, IL-6 regulation, whilst still regulated in response to LPS, did not depend upon the Mincle pathway, suggesting other routes of activation.

LPS induces TNF expression in barramundi via the Mincle CLR, but induces IL6 via a different route that has yet to be elucidated. As the adaptive response to LPS is type-specific and critical to protection in many fish vaccines, determining pathways for LPS processing in fish may aid with modulating response. This might enable better immune targeting of more conserved protein antigens in new cross-serotype vaccines.

Appendix 2.2. Supplementary information for chapter 2

Table A.2.2.1. TLRs accession numbers from NCBI database.

Organism	Name	NCBI accession number
Homo sapiens	TLR1	NM_003263.3
	TLR2	NM_003264.3
	TLR3	NM_003265.2
	TLR4	U88880.1
	TLR5	NM_003268.5
	TLR6	NM_006068.4
	TLR7	NM_016562.3
	TLR8	NM_016610.3
	TLR9	DQ019992.1
	TLR10	NM_030956.3
	CD180	NM_005582.2
Mus musculus	TLR1	AY009154.1
	TLR2	NM_011905.3
	TLR3	NM_126166.4
	TLR4	NM_021297.3
	TLR5	NM_016928.3
	TLR6	NM_011604.3
	TLR7	AY035889.1
	TLR8	AY035890.1
	TLR9	NM_031178.2
	TLR11	NM_205819.3
	TLR12	NM_205823.2
	TLR13	NM_205820.1
	CD180	NM_008533.2
Danio rerio	TLR1	NM_001130593.1
	TLR2	NM_212812.1
	TLR3	NM_001013269.3
	TLR4ba	NM_001131051.1
	TLR4bb	NM_212813.2

Table A2.2.1. continued

	TLR5	NM_001130595.1
	TLR7	XM_003199261.3
	TLR9	NM_001130594.1
	CD180	LN624114.1
Takifugu rubripes	TLR1	AC156430.1
	TLR2	XM_011617903.1
	TLR3	AC156436.1
	TLR5	AC156437.1
	TLR7	AC156438.1
	TLR8	AC156438.1
	TLR9	AC156439.1
	TLR14	AC156431.1
Rattus norvegicus	TLR11	NM_001144779.2
	TLR12	NM_001108682.1
Cyprinus carpio	TLR4ba	KF582561.1
	TLR4bb	KF582562.1
Alligator sinensis	TLR6	XM_006029236.2
Alligator mississippiensis	TLR10	XM_006029243.1
Sus scrofa	TLR6	AB208698.2
Chiloscyllium griseum	TLR6	HG964646.1
Stegastes partitus	TLR7	XM_008275650.1
Salmo salar	TLR13	NM_001140388.1

Table A.2.2.2. CLRs accession numbers from NCBI database.

Organism	Name	NCBI accession number
Homo sapiens	CD209	NP_066978.1
	Lymphocyte antigen 75	NP_002340.2
	Mannose receptor, C-type 1	NP_002429.1
	CD207	NP_056532.4
	Lectin-like receptor 1 (DECTIN1)	NP_922938.1

Table A2.2.2. continued

	DC-associated C-type lectin 2 (DECTIN2)	NP_001007034.1
	C-type lectin domain family 4, member C	NP_987099.1
	C-type lectin domain family 1, member A	NP_057595.2
	C-type lectin domain family 4, member E	NP_055173.1
Mus musculus	Lymphocyte antigen 75	NP_038853.2
	Mannose receptor, C-type 1	NP_032651.2
	CD207	NP_659192.2
	C-type lectin domain family 4, member E	NP_064332.1
Danio rerio	CD209	ADB55613.1
Tetraodon nigroviridis	CD209	ADB55615.1
Takifugu rubripes	Lymphocyte antigen 75	NP_001177292.1
Sus scrofa	CD207	NP_001123429.1
Bos taurus	C-type lectin domain family 1, member A	NP_001069549.1
Stegastes partitus	C-type lectin domain family 4, member E	XP_008274887.1

Table A2.2.3. Primers used for qRT-PCR.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
***CLEC4E* (Mincle)**	GACCCAGCTACTGGATTGGA	CTTGCGTTTGCGTCTTGTAG
FcRgamma	AAGACTGTCCCACATCCTGG	CCTGCTTCAGACTCACACCA
Syk	AACTGGGCTCTGGGAATTTT	GTTGAGAGGTCCCAGCTCAG
IL6	CAGCTGACTGCCGTGATAAA	TCCAGGGTTCCTCATCTTTG
TNFA	GCCATCTATCTGGGTGCAGT	AAAGTGCAAACACCCCAAAG
18S	CGCCTGAATACCGCAGCTAG	AGAACGGCCATGCACCACCAC
EF-1α	AAATTGGCGGTATTGGAAC	GGGAGCAAAGGTGACGAC

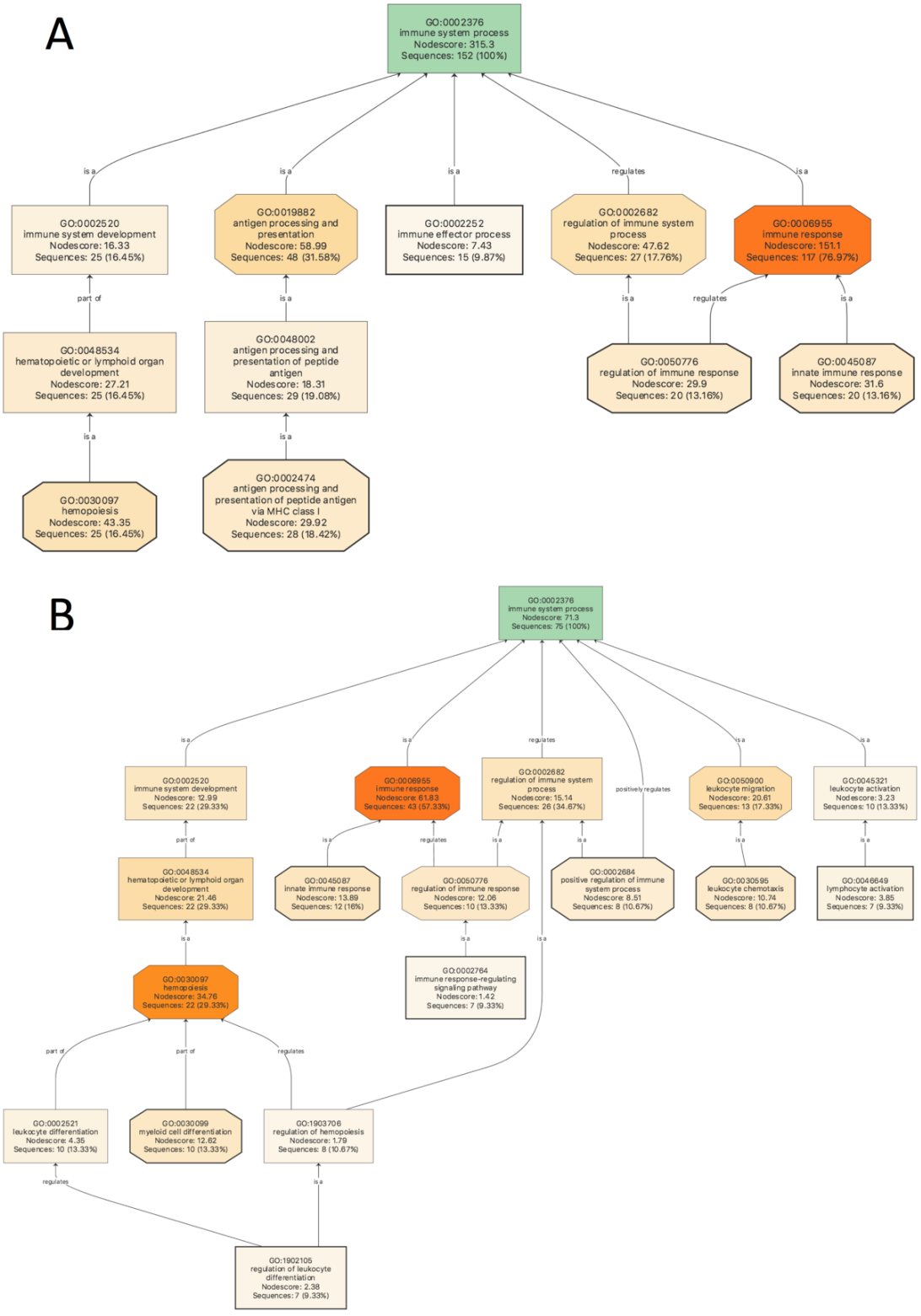

Figure A2.2.1. Gene ontology trees for 'Immune system process' (green boxes) from BLAST2GO. Organs represented are (A) spleen and (B) head-kidney. Darker shades of orange represent higher nodescore values.

Appendix 2.3. Supplementary information for chapter 3

Table A2.3.1. NCBI accession numbers used to identify barramundi caspases and to infer phylogeny.

Organism	Name	NCBI accession number
Homo sapiens	Caspase 1	NM_033292.3
	Caspase 2	NM_032982.3
	Caspase 3	NM_004346.3
	Caspase 4	NM_001225.3
	Caspase 5	NM_004347.3
	Caspase 6	NM_001226.3
	Caspase 7	NM_001320911.1
	Caspase 8	XM_005246885.1
	Caspase 9	NM_001229.4
	Caspase 10	NM_032974.4
	Caspase 14	NM_012114.2
Litopenaeus vannamei	Caspase 4	KC660105.1
	Caspase 5	KC660104.1
Mus musculus	Caspase 11	Y13089.1
Danio rerio	Caspase a	AF233434.1
	Caspase b	NM_152884.2

Table A2.3.2. NCBI accession numbers used to identify barramundi NLRs and to infer phylogeny.

Organism	Name	NCBI accession number
Homo sapiens	NOD1	AF113925.1
	NOD2	NM_022162.2
	NOD3	XM_017023027.1
	NOD4	EF452236.1
	NOD5/NLRX1	EF452237.1
	CIITA	NM_001286402.1
	IPAF/NLRC4	NM_021209.4
	NAIP	NM_004536.2

Table A2.3.2. continued

	NLRP1	NM_033004.3
	NLRP2	NM_017852.3
	NLRP3	NM_004895.4
	NLRP4	NM_134444.4
	NLRP5	NM_153447.4
	NLRP6	NM_138329.2
	NLRP7	NM_139176.3
	NLRP8	NM_176811.2
	NLRP10	NM_176821.3
	NLRP11	NM_145007.3
	NLRP12	XM_017027460.1
	NLRP13	NM_176810.2
	NLRP14	NM_176822.3
Mus musculus	NOD1	NM_172729.3
	NOD2	NM_145857.2
	NOD5/NLRX1	NM_001163743.1
	CIITA	NM_001243760.2
	IPAF/NLRC4	NM_001033367.3
	NAIP	NM_008670.2
	NLRP1	NM_001004142.2
	NLRP2	NM_177690.3
	NLRP3	NM_145827.3
	NLRP4	NM_001004194.2
	NLRP5	NM_011860.3
	NLRP6	NM_001002894.2
	NLRP9	XM_006539938.2
	NLRP10	NM_175532.3
	NLRP12	NM_001033431.1
	NLRP14	NM_001002894.2

Table A2.3.3. NCBI accession numbers used to identify barramundi leukocyte integrins and to infer phylogeny.

Organism	Name	NCBI accession number
Homo sapiens	Integrin alpha L	NM_002209.2
Table A2.3.3. continued		
	Integrin alpha M	J03925.1
	Integrin alpha X	NM_001286375.1
	Integrin alpha D	NM_001318185.1
	Integrin beta 2	NM_000211.4
Mus musculus	Integrin alpha L	NM_001253872.1
	Integrin alpha M	X07640.1
	Integrin alpha X	NM_021334.2
	Integrin alpha D	NM_001029872.3
	Integrin beta 2	M31039.1

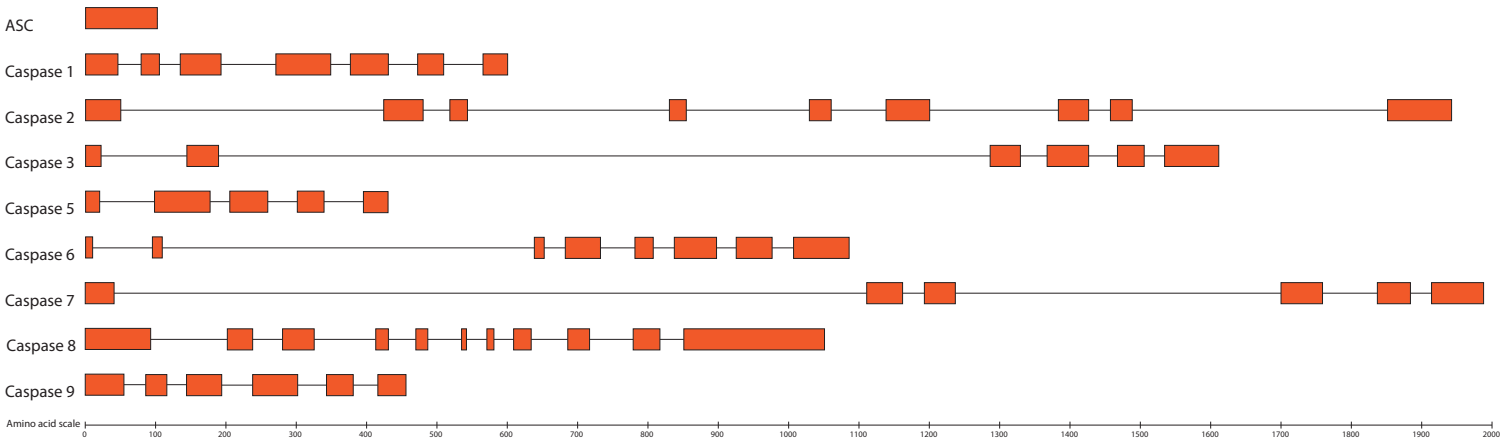

Figure A2.3.1. Gene organisation of barramundi caspases. The orange boxes represent the exons and the black lines the introns.

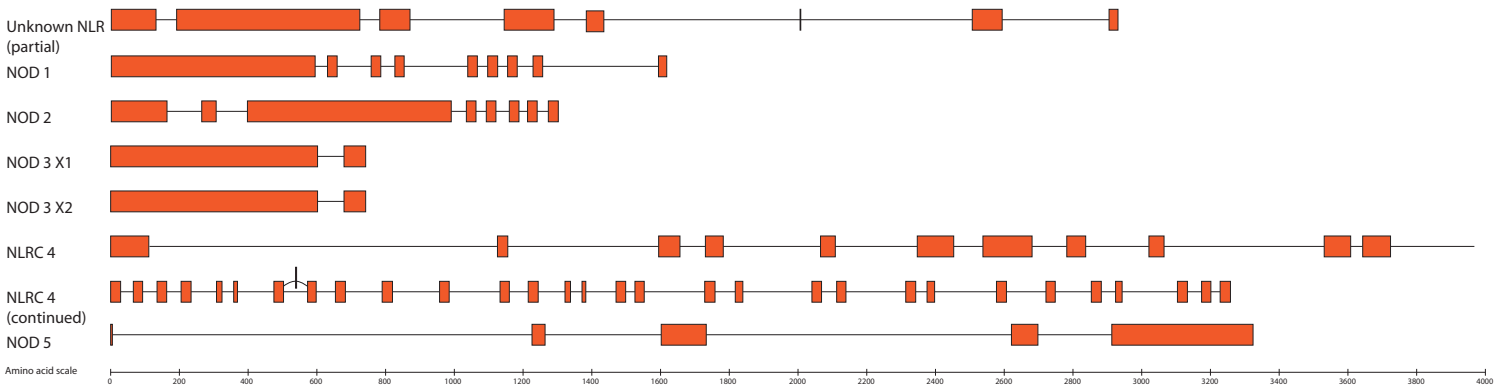

Figure A2.3.2. Gene organisation of barramundi NLRs. The orange boxes represent the exons and the black lines the introns. Red boxes represent exons present in the transcriptome but not in the genome.

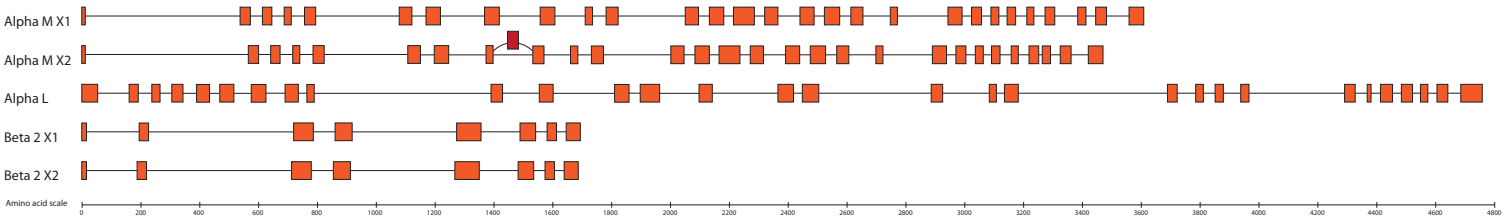

Figure A2.3.3. Gene organisation of barramundi integrins. The orange boxes represent the exons and the black lines the introns. Red boxes represent exons present in the transcriptome but not in the genome.

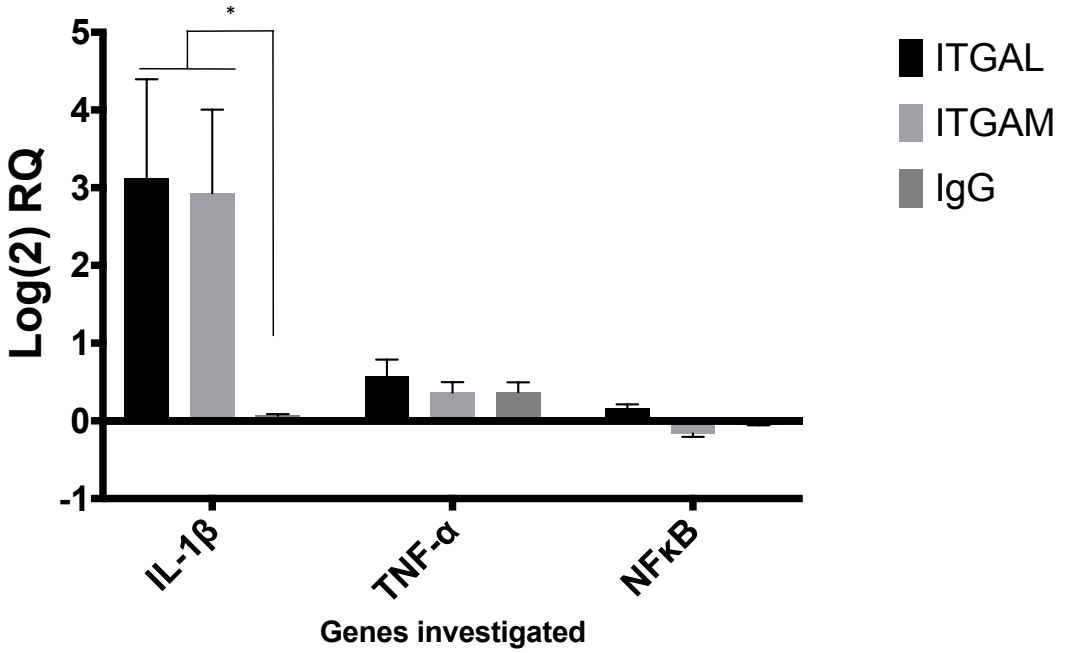

Figure A2.3.4. Differential expression of immune genes in cells stimulated by different antibodies. Rabbit IgG anti-ITGAL and rabbit IgG anti-ITGAM are used as treatments and polyclonal rabbit IgG is used as an isotype control. Significant differences between treatments and control are represented with * $p < 0.05$.

Appendix 2.4. Supplementary information for chapter 4

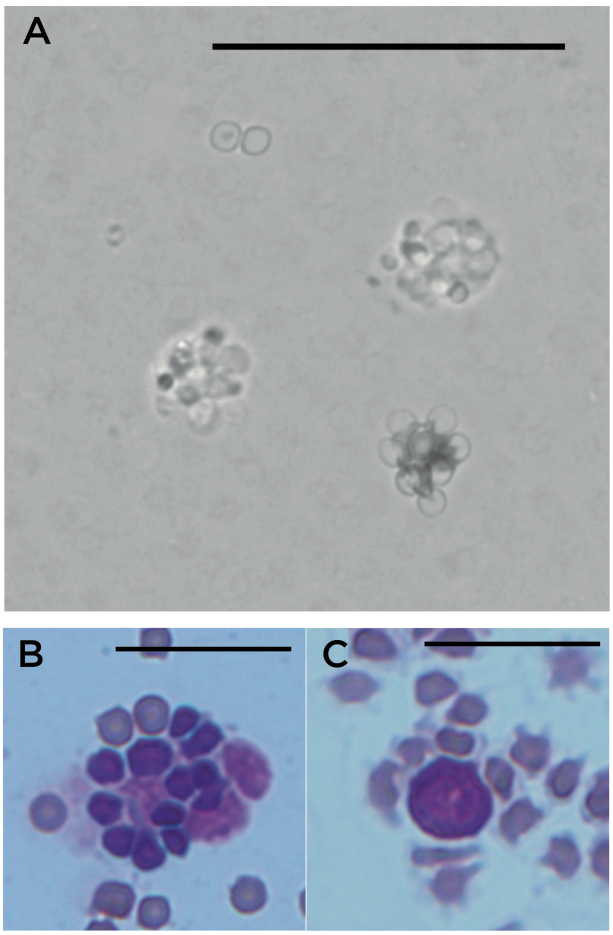

Figure A2.4.1 E-rosettes. (A) Inverted light microscopy of rosette formations. (B-C) Rosettes stained with Hemacolour. Scale bars represent 50 μm in (A) and 20 μm in (B) and (C). In each case, putative T-cells are surrounded by sheep erythrocytes.

A

NP_571565.1| major histocompatibility complex class II integral membrane alpha chain precursor [Danio rerio]

MFFLALRKMEVYVFILTLSVFVSSEVNVVHEDIVMDGCSDTEKEYISVLDGEEMYHT
DFSGKRGEMTLPDFADPFTYPGTYEQSLADYETCKHNLDVAAKAYKSPLEKLDPPQT
SIYSRDDVQPDIENKLICHVTGFFPPPVRVSWTKNNEIVTEGMSVSQYRPNNDGTYN
IFSTLRFTPVEGDIYSCSVNHKTLEQPQTKAWEVEVAMPSVGPAVFCGVGLFLGLLG
VAAGTFFLIKGNNCN

B

D. rerio	LICHVTGF**FPPPVRVSWT**KNNEIVTEGMSVSQYRPNNDG
L. calcarifer	LICHVSGF**YPAPVNVSWT**KNEQKVTEGTSINVPFPLKDG
M. zebra	LICHVTGF**YPAPVNVSWT**KNGQKVT-GSSINVPYPNKDG
O. niloticus	LICHVTGF**YPAPVNVSWT**KNQQKVT-GSTINVPYPNKDG
	******:**:*.**.****** : **** ::. * :**

C

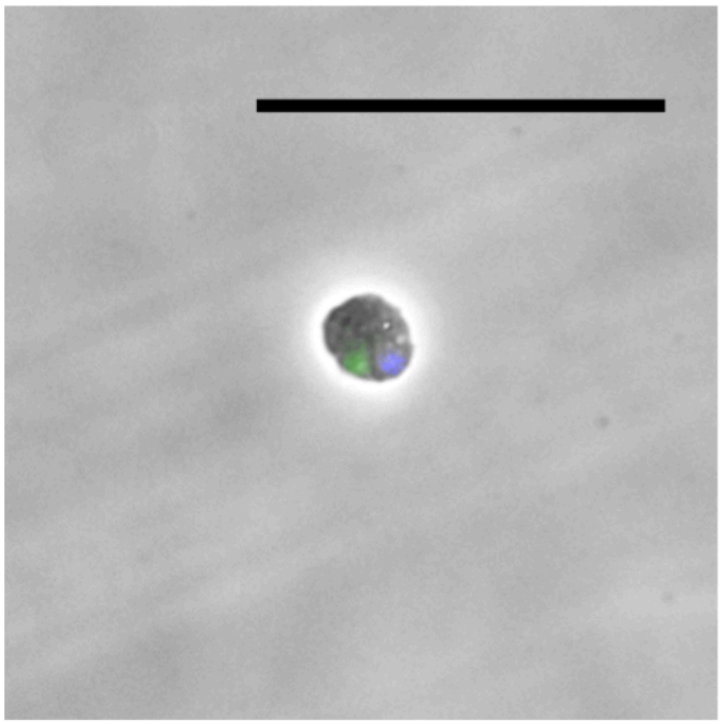

Figure A2.4.2 Rabbit Anti-zebrafish MHCIIa (Sapphire Bioscience). A) Synthesised peptide amino acid sequence, showing MHCIIa antigen (green) and MHCII-Ig superfamily domains (purple). Alignment: B) subsection of exon from *L. calcarifer* partial gDNA sequence is highly conserved across *D. rerio, L. calcarifer, M. zebra* and *O. niloticus*. B-cell epitope region is indicated (shaded, bold) showing very high conservation across several fish orders (Standard ClustalW2 notation to indicate similarity/identity). C) Co-staining of B-lymphocyte from *L. calcarifer* with rabbit anti-zebrafish MHCIIa antibody (green), sheep anti-barramundi IgM (blue).

Appendix 2.5. Supplementary information for chapter 5

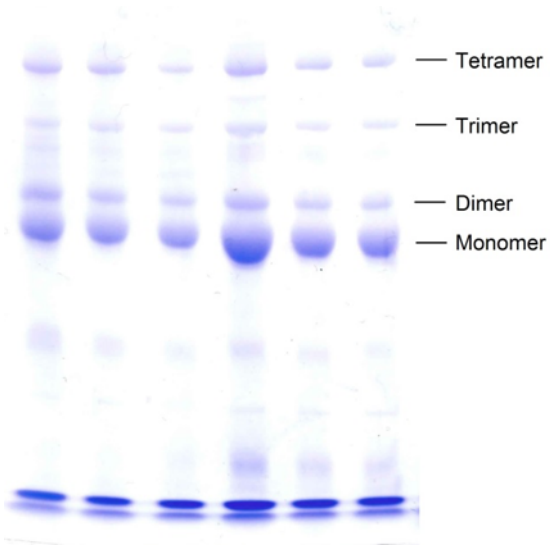

Figure A2.5.1 SDS-PAGE gel of barramundi IgM showing separation into tetramers, trimers, dimers and monomers.