

Molecular Innate Immunity in Teleost Fish: Review and Future Perspectives

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Innate immune response is a primitive form of defense mechanism existent in plants and animals. It is a complex system which, in vertebrates, is composed of cellular and humoral responses. The vertebrate teleost fish, which diverged from the tetrapod lineage about 450 million years ago, has an innate immune component that shows considerable conservation with higher vertebrates particularly in mammals highlighted by the presence of orthologous pattern recognition receptors (PRRs) and stimulated cytokines. However, there is also increasing evidence of teleost fish components and functions that are not observed in mammals suggesting complexity and diversity in teleost fish innate immune function. Knowledge about the network and function of these innate immune-related molecules in model and/or economically important fish is therefore important and new molecular biological tools such as large scale gene expression profiling including DNA microarrays and protein-based techniques will help elucidate such information. This review focuses on the molecular innate immune mechanisms in teleost fish in reference to the known mammalian system. It also includes future perspectives on how this basic knowledge coupled with existing molecular biological techniques can contribute to the control of fish disease and thereby improve aquaculture.

KEYWORDS molecular immunity; teleost fish; pathogen recognition receptors; inflammatory cytokines

1. Introduction

Innate immunity is the first line of defense against infection and is regarded as the pri-

meval and hence the universal form of host defense (Janeway and Medzhitov 2002). It exists in animals (vertebrates and invertebrates) and plants, and recent data suggests that it is a product of convergent rather than

divergent evolution (Ausubel 2005). Common features of the system across species include conserved receptors for microbe-recognition, antimicrobial peptides and mitogen-associated protein kinase signaling cascades.

Innate immunity is generally subdivided into two parts, the cellular and humoral defense responses. Cellular responses include the physical barrier such as mucus and epithelial tissues lining the skin, gills and stomach, which keeps infectious microorganisms from entering the body, and specialized cells (like monocytes/macrophages, granulocytes and nonspecific cytotoxic cells) capable of killing and digesting pathogens if the latter breaches the physical barriers. These cells are recruited in the infection site primarily by inflammatory cytokines. Humoral responses, on the other hand, employ a variety of proteins and glycoproteins capable of destroying or inhibiting growth of infectious microorganisms, which include among others anti-bacterial peptides, proteases, complement, transferrins and the antiviral myxovirus resistance-1 protein (Mx1).

In mammals, innate immunity is triggered by the recognition of conserved microbial products by receptors generally called pathogen recognition receptors or pattern recognition receptors (PRRs) (Fig. 1). These molecules can distinguish 'infectious nonself' from the 'noninfectious' self. PRRs, which include both extracellular and cytosolic recognition, mediate several pathways that give rise to the production of inflammatory cytokines and interferons (Lee and Kim 2007). In teleost fish, such mechanisms are believed to be conserved mainly because of the presence of PRR and cytokine orthologs (Plouffe *et al.* 2005; Stein *et al.* 2007). Furthermore, microarray analysis has revealed transcriptional modulation of various cloned fish PRRs and cytokines following bacterial agents (Kurobe *et al.* 2005; Gerwick *et al.* 2007; Peatman *et al.* 2007;

Peatman *et al.* 2008). On the other hand, increasing evidence also shows that there are molecules and mechanisms that are specific to teleost fish (Plouffe *et al.* 2005; Stein *et al.* 2007).

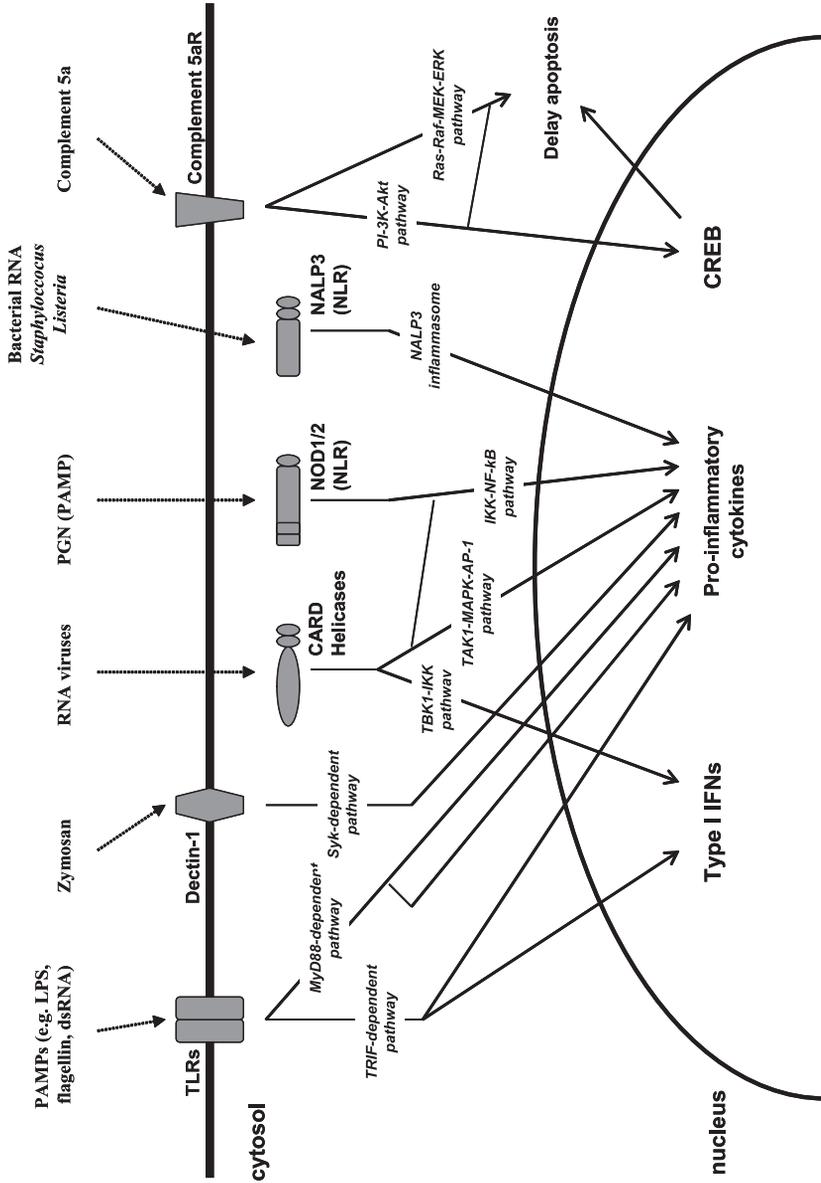
In this paper, we review the innate immune system network in fish gathered from available information and studies including pathogen recognition and the ensuing cytokine cascade output as well as innate immune-related cellular functions. We draw comparisons of these with the known molecules and mechanisms in mammals, in the absence/limited functional information in teleost fish, as well as highlight recent and growing innate immune functions that are teleost fish-specific. We also present some future perspectives on the basic study of fish immunology and the techniques that would help produce this knowledge, as well as the possible applications that could be generated for the improvement of aquacultural productivity.

2. Mammalian Innate Immune Responses

2.1. Pathogen recognition

In mammals, innate immune response is initiated through the recognition of pathogens by the PRRs. PRR families that have been identified thus far include the extracellular Toll-like Receptors (TLRs), C-type lectins and complement receptors CRs, and the cytosolic nucleotide-binding domain and LRR proteins (NLRs) (Table 1). It is known that these PRRs are able to trigger various innate immune responses including complement pathway, apoptosis, leukocyte activation and migration, and cytokine production (Lee and Kim 2007).

TLRs, now one of the most studied biological molecules discovered because of the Toll receptor in *Drosophila*, are considered to be vital to microbial pathogen recognition. Its extracellular leucine-rich repeat (LRR)



Source: modified from Lee and Kim, 2007

Fig. 1. Molecular mechanism of innate immunity in mammals: from recognition to signaling to inflammatory cascade. Orthologous molecules/mechanisms known in teleost fish are shown (discontinuous ring).

Table 1. Selected mammalian and teleostean pattern-recognition receptors

Family	Function/Structure	Taxon	Member	Source
TLRs	Pathogen associated molecular patterns (PAMPs) recognition on the cellular membrane. Extracellular leucine-rich repeats (LRRs) and intracellular Toll/IL-1 receptor (TIR) domain.	Mammals	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12, TLR13	Lee and Kim 2007; Roach <i>et al.</i> 2005 Jault C, <i>et al.</i> , 2004
		Teleost Fish	TLR1*, TLR2, TLR3, TLR4*(Z), TLR5, soluble TLR5, TLR7*, TLR8*, TLR9, TLR14 (P), TLR21*, TLR22, TLR23 (P)	
Lectins	Involved in the carbohydrates recognition. Secretion form and membrane form are exist, and generally function as multimeric structure.	Mammals	Mannose binding lectin (MBL), Dectin-1#, Intelectin, Pentraxins	Lee and Kim 2007; Russell and Lumsden
		Teleost Fish	MBL, Intelectin, Pentraxins	
Complement receptors (CRs)	Involved in the leukocytes activation and migration.	Mammals	CR3 and CR4 (integrins), C5aR	Lee and Kim 2007; Boshra H, <i>et al.</i> 2004 Qian Y <i>et al.</i> 1999
		Teleost Fish	CD18 (β chain for the CR3 and CR4), C5aR	
NLRs	Involved in the PAMPs recognition in the cytoplasm. The receptors posses the central nucleotide oligomerization domain (NOD), caspase activation and recruitment domain (CARD) and LRRs.	Mammals	NOD1, NOD2, NALPs, IPAF, NAIP5	Lee and Kim 2007; Stein <i>et al.</i> 2007
		Teleost Fish	NOD1, NOD2	
CARD helicases	Implicated in the cytoplasmic detection of viruses. The molecules possess the RNA helicase activity in its C-terminal portion and CARD.	Mammals	Retinoic acid-inducible gene 1 (RIG-I), melanoma-differentiation-associated gene 5 (MDA5)	Lee and Kim 2007
		Teleost Fish	Unknown	

*Isotypes were reported.

(Z) Reported from only the zebrafish.

(P) Reported from only the pufferfish.

Only dectin-1 is a lectin that be able to mediate the intracellular signaling directly after the ligand recognition.

domains are responsible for specifically recognizing molecular signatures of various pathogens while the cytoplasmic Toll/IL-1R (TIR) domain directs signals that induces pro-inflammatory cytokine, Type I interferon and chemokines production (Kawai and Akira 2005). To date, 10 members of TLRs have been identified in human, and 13 in mouse (Krishnan *et al.* 2007).

On the other hand, C-type lectins, of which the mannose-binding lectin (MBL) the best characterized, recognize enveloped virus, bacteria and fungi structure via cell to cell interactions following Ca^{2+} -dependent manner (Epstein *et al.* 1996; Vasta *et al.* 1999). Through the binding of extracellular MBL to the pathogen then to MBP-associated serine proteases (MASPs), the lectin complement pathway is induced and produces pro-inflammatory cytokines (Gupta and Suroliya 2007). Recently, another C-type lectin, dectin1, was found to recognize fungus and β -glucan (Robinson *et al.* 2006). Upon binding of dectin1 to pathogen, the immunoreceptor tyrosine-based activation motif (ITAM) also induces immune responses (Ariizumi *et al.* 2000; Brown 2006). Another lectin, the mammalian intelectin (IntL), is a Ca^{2+} -dependent enteric molecule that has specific affinities to D-pentoses and D-galactofuranosyl residues as well as recognizes *Nocardia rubra* arabinogalactan (Tsuji *et al.* 2001). Pentraxins recognize a wide range of pathogenic substances and altered self molecules and acute phase proteins (Mantovani *et al.* 2007). Examples of which are the C-reactive protein (CRP), serum amyloid P component (SAP) and Pentraxin 3 (PTX3) (Mantovani *et al.* 2007).

The complement receptors such as CR3 and CR4 are able to mediate phagocytosis of opsonized pathogens and/or apoptotic cells. Further, C5aR is known as immunoregulatory signal generator. C5a is a potent inflammatory agent that has diverse functions including chemotaxis, phagocytosis enhancement, reduction of neutrophil

apoptosis, and IL-12 production through C5aR. The PI-3K-Akt and ERK signaling pathways are known C5aR cascade that lead to the expression of the anti-apoptotic protein Bcl2 so far (Lee and Kim 2007).

The NLR family, are recently discovered PRRs involved in the cytoplasmic recognition of whole bacteria and their products such as flagellin and peptidoglycan (Kaparakis *et al.* 2007). It is composed of 2 major sub-families, the NODs and the NACHT-LRR-PYD-containing proteins, or NALPs. NODs and NALPs induce the production of pro-inflammatory cytokines and even antimicrobial peptides upon pathogen recognition via the NLR signaling pathway and the "inflammasome" pathway, respectively (Kaparakis *et al.* 2007). The other cytoplasmic PRRs of retinoic acid-inducible gene I (*RIG-I*) and melanoma-differentiation-associated gene 5 (*MDA5*) possesses RNA helicase activity to mediate the antiviral responses (Kaparakis *et al.* 2007).

2.2. Cytokine cascade

Cytokines, specifically pro-inflammatory cytokines and Type I interferons, are known to be induced following the activation by signal pathways as result of the stimulation of PRRs (Table 2). Cytokines have pleiotropic (multiple), overlapping and sometimes contradictory functions such that their classification is oftentimes difficult. These molecules have been grouped based on general names (e.g. lymphokines, monokines, chemokines, interleukins, interferons, tumor necrosis factors, colony stimulating factors etc); on functions (e.g. pro- and anti-inflammatory or innate and adaptive immunity-related); on structure (e.g. short and long chain cytokines); and on receptors used (immunoglobulin superfamily, hematopoietic growth factor (type 1-family), interferon family (type 2-family), tumor necrosis factor (type 3-family) and chemokine receptors (7 transmembrane helix

family). Cytokines here were grouped based on available recent reviews for each of the stated group; Cytokine Class 1, Cytokine Class 2, Chemokines, TNF Super-family and IL1 Family (Table 1).

Class 1 cytokines are often involved in the proliferation, recruitment to inflammation/infection sites, survival and maturation of cells (Huisin *et al.* 2006). Because of their pleiotropic nature, they can also be involved in other physiological processes like reproduction, food intake and metabolism. They share similar 4α -helical- 2β sheet 3D structure and use the class 1 cytokine receptors. Although they share little primary sequence identity, these cytokines are considered to have expanded from a single ancestor (Bazan 1990).

Class II cytokines, in contrast to Class I, generally acts on minimizing damage to host after noninfectious and infectious insult, and include the interferons which mediates antiviral responses and IL10 known to be anti-inflammatory in nature (Krause and Pestka 2005). Their structure is likewise different with Class 1 cytokines in that their β sheets have been replaced by α -helices (Langer *et al.* 2004).

Chemotactic cytokines or chemokines, are mostly involved in leukocyte migration and are known as "second-order" cytokines, being induced by pro-inflammatory "first-order" cytokines like IL-1s, TNFs, IFNs. These are a family of small proteins that regulate cell migration under both inflammatory conditions or homeostasis (Peatman and Liu 2007). Chemokines are subdivided based on the arrangement of the 1st 2 of the 4 cysteine residues; CC, CXC, C and CX3C (Bacon *et al.* 2003).

TNF ligand superfamily (TNSF) cytokines, which includes the tumor necrosis factor- α (TNF- α), lymphotoxin- α and lymphotoxin- β , are structurally related proteins, either soluble or membrane-bound, that are produced during lymphoid organ development and inflammation. (Gruss 1996)

These molecules are involved mainly in cellular regulation such as tumor cell killing during immune responses and inflammatory reactions.

Interleukin 1 family is one of the critical early pro-inflammatory cytokines that down- or up-regulate other cytokines during infection or inflammation (Huisin *et al.* 2004). These molecules are produced by monocytes, activated macrophages, neutrophils, endothelial cells, fibroblasts, Langerhans cells of the skin and other types of cells. IL18, which is a recently discovered member, was observed to stimulate (IFN)- γ production, T-helper1 (Th1) cell differentiation and enhances cell cytotoxicity of natural killer cells (Akira 2000).

3. Teleost Innate Immune Responses

3.1. Pathogen recognition

Many components of the innate immune signaling are conserved between teleost fish and mammals, with very clear orthologous relationships. Examples of the latter are the presence of teleost fish PRRs such as TLRs, Lectins, complement receptors, NLRs (Table 1).

Teleost fish have been shown to functionally react to a number of TLR agonists and that this recognition induces cytokine expression similar to those observed in mammals (Purcell *et al.* 2006). In particular, TLR signaling molecules have been established to be evolutionary conserved in teleost fish including myeloid differentiation factor 88 (MYD88), TIR domain-containing adapter protein (TIRAP), TIR-domain containing adaptor including INF- β (TRIF), TRIF-related adaptor protein (TAM), sterile α and HEAT-Armadillo motifs (SARM), interferon regulatory factor 3 (IRF3) and interferon regulatory factor 7 (IRF7) (Purcell *et al.* 2006). As such, it is now believed that the basic program of gene regulation by TLR-signaling pathway is conserved across

vertebrates. A total of 11 types of teleost fish TLRs (TLR 1, 2, 3, 5, 7, 8, 9, 14, 21, 22 and 23) have been including isotypes for TLR 1, 4, 8 and 21 (Jault *et al.* 2004; Roach *et al.* 2005). Their orthology suggests that they also possess the same type of pathogen recognition mechanism as with their mammalian counterparts.

The complement system of teleost fish, which is composed of soluble plasma proteins involved in immune responses, has been reviewed recently (Boshra *et al.* 2006). It has been shown to be involved in opsonization, phagocytosis and inflammation. Fish complement components include the C1q/MBL family, C1r/C1s/MASP family, C2/Bf and factor D molecules, C3/C4/C5 family, membrane attack complex (MAC) assembly, complement regulatory proteins and complement receptors.

Lectin is well studied in various teleost fish, although the detail of correlation to the fish immunity is unclear (Russell and Lumsden 2005). Teleost fish MBL is able to recognize mannose, N-acetyl-D-glucosamine (GlcNAc) and glucose (Nakao *et al.* 2006). This has also been demonstrated with lamprey MBL suggesting that lectin complement pathway exist as early as in agnathans (Takahashi *et al.* 2006). IntL homologs have likewise been identified in teleost fish through gene expression profiling (Gerwick *et al.* 2007; Peatman *et al.* 2007; Peatman *et al.* 2008).

3.2. Cytokine cascade

Similar to PRRs, there are also clear orthologous cytokines in teleost fish, particularly of the Class I, Class II, chemokines, TNF superfamily and IL1 families (Table 2). Coupled with the PRR orthologues, this information suggests that the innate immune system in teleost fish also exhibits the same general mechanism of recognition and cascade seen in mammals. Recent reviews also discussed the similarities of innate immune

mechanisms between teleost fish and mammals where the presence of fish orthologues for antimicrobial peptides, pro-inflammatory cytokines, e.g. TNF- α , IL-1 β , IL-18, interferons, chemo-kines and IL-8, and complement have been established (Plouffe *et al.* 2005).

Gene or protein orthology and conservation allows speculation for similarity of function but functional genomics and protein assays are desirable to ascertain function. With the whole genome sequences in fish, recent publications have started to report functionalities of immune-related genes and their proteins. An example of gene structure and function conservation between fish and humans is the action of erythropoietin (EPO) and erythropoietin receptor (EPOR) in red blood cell formation and hematopoiesis and under hypoxic conditions (Paffett-Lugassy *et al.* 2007). Another is the macrophage colony-stimulating factor (CSF-1), which like in mammals, was observed to induce proliferation of monocytes to macrophages (Hanington *et al.* 2007).

4. Only in Teleost Fish

The Whole Genome Duplication (WGD) that happened early in the life of ray-finned fishes is now increasingly believed to have happened about 350 to 450 million year ago and is the main reason for the explosion of the fish species diversity at >23,500 spp. (Volf 2005). The gene duplication that happened resulted in the creation of numerous novel or semi-novel genes and functions in fish, known as “more genes in fish than mammals” concept (Ohno 1970). It is therefore not surprising that there are already 5 genomic databases sequenced thus far for this taxon; the zebrafish, medaka, stickleback, tiger pufferfish and the green spotted pufferfish (www.ensembl.org). Large scale teleost fish genome analysis has revealed numerous gene duplications that are thought to originate from the WGD (Stein 2007). This

Table 2. Selected mammalian and teleostean cytokines

Family	Function/Structure	Taxon	Selected members	Source
Cytokine Class 1	Involved in expansion and differentiation of cells. Have a 4- α helix bundle structure	Mammals	IL6, GCSF, IL11, IL12p35, IL23p19, IL27p28, ILF, OSM, CNTF, CLC, CT-1, CT-2 NP, Leptin, Epo, PRL, GH	Bazan 1990; Huising <i>et al.</i> 2006; Santos <i>et al.</i> 2006; Hwang <i>et al.</i> 2007
		Teleost Fish	IL6-a and -b, IL11-a and -b*, GCSFa and -b*, Leptin, Epo, PRL, GH, M17* and M17 Homologue (MSH)*	
Cytokine Class 2	Involved in minimizing damage to host after insult. Contain more than 4- α helices.	Mammals	Type I IFN (IFN α 2, IFN α 4, IFN β , IFN ω 1, IFN γ 1), Type II IFN, IFN- γ , IL10, IL28-like, IL19, IL20, IL22, IL24, IL26	Krause and Pestka 2005; Robertson 2006
		Teleost Fish	Type I IFN (IFN α -1, IFN α -2), IFN- γ , IL10, IL20, IL24	
Chemokines	Regulate cell migration under both inflammatory and homeostasis. Small proteins with 4 conserved Cys residues	Mammals	CXC (IL8, PF4, PBP, NAP-2), CC (MIP-1 α , MIP-1 β , MCP-1, MCP-2, MCP-3, RANTES); C (Lymphotactin)	Bacon <i>et al.</i> 2003; Peatman and Liu 2007; Kim <i>et al.</i> 2007
		Teleost Fish	CXC (CXC8-like, CXC-10, -12, -13, -14) CC (CCL19/21/25, CCL20, CCL27/28, CCL17/22, MIP, MCP)	
TNF Super Family	Involved in inflammation and lymphoid organ development. Compact trimers as membrane bound or soluble proteins	Mammals	TNF- α , lymphotactin- α (TNF- β) and lymphotactin- β	Gross 1996; Kono <i>et al.</i> 2006
		Teleost Fish	TNF- α 1, TNF- α 2, TNF-N, lymphotactin- β 1 and - β 1	
Interleukin 1 Family	Involved in pro-inflammatory responses. Fold rich in β -strands	Mammals	IL1- α , IL1- β , IL18	Huising <i>et al.</i> 2004
		Teleost Fish	IL1- α , IL1- β , IL18	

* Only found in fish.

was clear with genes involved in signal transduction downstream of the transmembrane receptors but not with molecules involved in interactions with pathogen components. Genetic diversity translates to protein diversity and as such it is therefore very possible that in teleost fish, there will be a lot of unique and differing functionalities amidst the background of conserved functions. In fact, many of these fish-specific features are now starting to be unraveled.

4.1. Pathogen recognition

Teleost fish TLR14, 21, 22 and 23 have not been found from mammals so far. TLR 14 is reported from lamprey, teleosts and amphibians, TLR 21 in teleosts, amphibians and birds, TLR 22 in teleosts and amphibians, TLR 23 in Fugu (Roach *et al.* 2005). The soluble form TLR5 (sTLR5) is also unique in teleost fish (Tsujita *et al.* 2004). Its production is facilitated by the NF- κ B activated by membrane TLR5 (mTLR5) upon recognition of Gram-negative bacteria flagellin (Tsujita *et al.* 2004). Conversely, there are no reports yet of mammalian TLR6 orthologues in fish.

Fish complement/anaphylatoxin PUEF-8, which is composed of C3a, C4a and C5a, greatly enhance particle uptake in teleost fish leukocytes, increased the number of phagocytic cells by 3- to 4-fold and acted as strong chemoattractant to peripheral blood and kidney leukocytes. These observations were not observed in mammalian anaphylatoxins and are therefore novel functions only found in fish (Boshra *et al.* 2006).

NLRs have been identified in teleost fish including a conserved NACHT-domain protein which is unique in fish and species-specific subgroup of NLRs with a novel N-terminal domain were found from zebrafish and pufferfish (Stein *et al.* 2007). Taken together, the divergence of the fish PRRs may indicate the species-specific pathogen recognition pathways and immune responses.

4.2. Cytokine cascade

The differing functional attributes between teleost fish and mammals is now also being observed not only at the genetic but at the protein level as well. Th2 cytokines (IL4, IL5, IL13) as well as the neighbor IL3 and granulocyte-macrophage colony stimulating factor (GM-CSF) are apparently missing in teleost fish (Huising *et al.* 2006). Recently, there have been reports of members of the IL6-cytokine subfamily that appear to only be found in teleosts. A novel class-1 helical cytokine M17 (named after the original clone number of the carp leukocyte cDNA library), cloned in 2003, was shown to be induced by alginate by (Fujiki *et al.* 2003) and later shown to be induced by nitric oxide and can stimulate proliferation of monocytes to macrophages (Hanington and Belosevic 2007). Its apparent paralogue named M17 homologue (MSH) have been cloned recently and was shown to be induced by bacterial agents, lipopolysaccharide, peptidoglycan and the dsDNA, viral mimic polyI:C (Hwang *et al.* 2007). Another recently published fish-specific report showed that goldfish possesses a soluble macrophage colony stimulating factor receptor (CSF1R), In terms of cytokine which can inhibit CSF1-induced proliferation of monocytes into macrophages and monocyte-like cells (Hanington *et al.* 2007).

In terms of cytokine receptors, there have also been reports of such genes that do not possess clear orthology to known receptors and appears to be more of ancestral in structure. The Leukemia Inhibitory Factor Receptor (LIFR)-like molecule identified in goldfish was suggested to be the ancestral molecule for mammalian LIFR and Oncostatin M Receptor (OSMR) (Hanington and Belosevic 2005) although it still not clear whether the LIFR-like genes predicted in the chromosome 12 of the green spotted pufferfish is orthologous to the goldfish

LIFR-like genes or to teleost OSMR as well (Jaillon *et al.* 2004). Another class 1-cytokine receptor in fish which appears to be ancestral in structure is the Japanese flounder glycoprotein 130 homologue or JfGPH discovered very recently (Santos *et al.* 2007). This receptor, which contains the classic WSXWS motif, the cytokine binding domains, and the Jak/STAT signaling boxes 1 to 3 in its cytoplasmic region, does not possess a detectable Ig-like N-terminal domain. It appears to be an ancestral gene for class 1 cytokine receptors that is ubiquitously expressed in tissues and down-regulated during viral-induced pathways.

Novel immune-type receptor (NITRs) is another group of molecules belonging to the immunoglobulin superfamily that have no known homolog in mammals. These are expressed mainly in spleen, kidney and intestine and were suggested to play important roles in the regulation of cellular immune responses (Hawke *et al.* 2001). Recently, we have identified an NITR in Japanese flounder that is localized in both the T and B cells pointing to its specific importance in adaptive immunity (Piyaviriyakul *et al.* 2007).

4.3. Cellular mechanisms

Fish rely heavily on innate immune responses to deal with pathogens. With this is the use of cells to combat pathogens. Novel types of cells that have been found in teleost fish but not mammals include non-specific cytotoxic cells (NCCs) (Plouffe *et al.* 2005).

Because of cellular and morphological differences, blood cells between mammals and teleost fish, has always been suggested to be functionally different (Rowley *et al.* 1988). Recently, direct studies have identified immune-related cellular functions in fish that defies present paradigms in mammals. For example, unlike mammalian B cells, rainbow trout B cell populations were shown to have potent phagocytic capability i.e. following uptake of particles, these cells could

induce “downstream” degradation pathways leading to formation of phagolysosome and intracellular killing of microbes (Li *et al.* 2006). Zebrafish primitive macrophage lineage was shown to give rise to a number of neutrophilic granulocytes in contrast to mammalian primitive macrophage which is considered to give rise to no other cell types. Only a small fraction of these zebrafish larval neutrophils phagocytose microbes as compared to the macrophages and are more attracted to stressed and malformed tissues suggesting that these cells are involved in a wider role than biodefense (Guyader *et al.* 2007). The same functional adaptation has been seen in seabream professional phagocytic granulocytes, where these cells, other than immune surveillance, have a role in the reorganization of the testis during post-spawning (Chaves-Pozo *et al.* 2005).

5. Future Perspective

5.1. Basic knowledge in fish innate immunity

There are undoubtedly numerous innate immune mechanisms in teleosts that resemble or are conserved with mammalian processes. This is not unexpected since it is regarded that innate immunity is the universal and ancient form of host defense against infection (Janeway and Medzhitov 2002). Present studies on orthologous teleost fish genes, albeit many are only up to expression studies, points to general similarity in function to higher vertebrates. Going forward, however, it is the fish-specific molecules and processes that would usher a new era in basic teleost fish immunological studies. The apparent duplication of many of the teleost fish genes adds to the complexity of fish immunology and could potentially reveal new mechanisms unheard of in the study of immunology. The availability of 5 fish genomic databases (for zebrafish, fugu, green spotted pufferfish, stickleback and medaka)

will certainly fast track the study of these fish-specific phenomenon. This will allow for a better understanding of fish immunity as well as how this relate to the evolution of innate immunity in vertebrates.

5.2. Technologies for fish innate immunity

The advent of various molecular biological techniques have paved the way in increasing our knowledge of fish innate immunity and these same technologies will still be very useful in uncovering this new information. For example, we have successfully used the Expressed Sequence Tag (EST) platform to identify orthologous, paralogous and novel genes in Japanese flounder (Aoki and Hirono, 2005). We likewise have successfully employed cDNA microarray platform to evaluate efficacy of vaccination using different vaccine agents like formalin-killed cells, DNA and recombinant protein vaccines (Byon *et al.* 2006; Byon *et al.* 2005) as well as comparing between vaccine agents like viral G and N protein DNA vaccines (Yasuike *et al.* 2007). Technologies that are important for the future include PCR-based techniques including Quantitive Real-Time PCR, immunological-bases assays, recombinant protein analysis and microarray studies. In mammals, these assays have been very effective in elucidating innate immunity and is expected that it will do the same for fish.

5.3. Application to aquaculture

The detailed analysis of the innate immune-related molecules fish including their function and network will certainly generate new technologies that can be applied to improve aquaculture.

5.3.1. Biomarkers

The production of healthy quality fish is important to consumers in terms of food safety and nutrient content, and to aquacul-

ture in terms of profitability. Biomarkers could be utilized to monitor such quality. In innate immune responses, several important molecules are involved and the up- or down-regulation of these molecules provides some measure of the biological state of the fish, whether it is in normal condition or stressed due to infection. For instance, C-reactive protein is an indicator for the internal inflammatory responses in mammals. From our studies of the innate immune responses of Japanese flounder at the molecular level, we have discovered a number of potential biomarker genes such as antimicrobial agents, acute stress response factors, and various cytokines during the infectious pathogen invasion (Aoki and Hirono 2005). The antiviral Mx1 gene is an interesting example of a biomarker to measure vaccine efficiency. We observed that it was only expressed by rhabdovirus glycoprotein gene vaccinated but not in rhabdovirus nucleocapsid gene vaccinated fish (Yasuike *et al.* 2007).

5.3.2. Vaccine/adjuvant

Our immunostimulation studies with the aid of extensive gene expression profiling like ESTs, cDNA microarray and Serial Analysis of Gene Expression (SAGE) generated information on various pro-inflammatory cytokines that are potential for vaccine/adjuvant development. For example, we have previously shown that Japanese flounder IL-1 β can induce various cytokines, cell surface antigens, signal transduction genes, suggesting that IL-1 β is a potential vaccine adjuvant (Emmadi *et al.* 2005).

5.3.3. Transgenic

Fish transgenesis to produce transgenic fish with commercially important traits such as growth enhancement and disease resistance is still being pursued and many molecules involved in innate immunity are target molecules for such. We have, for example, successfully shown that the TNF promoter can

be a tissue-specific, inducible promoter that can be utilized to express foreign genes in Japanese flounder (Yazawa *et al.* 2005a–c). The transgenic technique itself could be used to augment or complement the fish innate immune response. For instance, the recombinant Japanese flounder lysozyme has been shown to be infective against Japanese flounder pathogens like *Edwardsiella tarda* (Hikima *et al.* 2001). However, when chicken

lysozyme was expressed in transgenic zebrafish, the fish acquired resistance against the bacteria (Yazawa *et al.* 2006).

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References

- Akira S. The role of IL-18 in innate immunity. *Curr. Opin. Immunol.* 2000; **12**: 59–63.
- Aoki T, Hirono I. Molecular immunity in fish-pathogen interactions. In: Leung, KY (ed). *Current Trends in the Study of Bacterial and Viral Fish and Shrimp Diseases*. World Scientific Publishing, Singapore. 2004; 256–291.
- Ariizumi K, Shen GL, Shikano S, Xu S, Ritter R III, Kumamoto T, Edelbaum D, Morita A, Bergstresser PR, Takashima A. Identification of a novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. *J. Biol. Chem.* 2000; **275**: 20157–20167.
- Ausubel FM. Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol.* 2005; **10**: 973–979.
- Bacon K, Baggolini M, Broxmeyer H, Horuk R, Lindley I, Mantovani A, Matsushima K, Murphy P, Nomiyama H, Oppenheim J. Chemokine/chemokine receptor nomenclature. *Cytokine* 2003; **21**: 48–49.
- Bazan JF. Haemopoietic receptors and helical cytokines. *Immunol. Today* 1990; **11**: 350–354.
- Boshra H, Li J, Peters R, Hansen J, Matlapudi A, Sunyer JO. Cloning, expression, cellular distribution, and role in chemotaxis of a C5a receptor in rainbow trout: the first identification of a C5a receptor in a nonmammalian species. *J. Immunol.* 2004; **172**: 4381–4390.
- Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* 2006; **6**: 33–43.
- Byon JY, Ohira T, Hirono I, Aoki T. Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. *Fish Shellfish Immunol.* 2005; 135–147.
- Byon JY, Ohira T, Hirono I, Aoki T. Comparative immune responses in Japanese flounder, *Paralichthys olivaceus* after vaccination with viral hemorrhagic septicemia virus (VHSV) recombinant glycoprotein and DNA vaccine using a microarray analysis. *Vaccine* 2006; **24**: 921–930.
- Chaves-Pozo E, Mulero V, Meseguer J, Ayala AG. Professional phagocytic granulocytes of the bony fish gilthead seabream display functional adaptation to testicular microenvironment. *J. Leukoc. Biol.* 2005; **75**: 345–351.
- Emmadi D, Iwahori A, Hirono I, Aoki T. cDNA microarray analysis of interleukin-1 β induced Japanese flounder *Paralichthys olivaceus* kidney cells. *Fish. Sci.* 2005; **71**: 519–530.
- Epstein J, Eichbaum Q, Sheriff S, Ezekowitz RA. The collectins in innate immunity. *Curr. Opin. Immunol.* 1996; **8**: 29–35.
- Fujiki K, Nakao M, Dixon B. Molecular cloning and characterisation of a carp (*Cyprinus carpio*) cytokine-like cDNA that shares sequence similarity with IL-6 subfamily cytokines CNTF, OSM and LIF. *Dev. Comp. Immunol.* 2003; **27**: 127–136.
- Gerwick L, Corley-Smith G, Bayne CJ. Gene transcript changes in individual rainbow trout livers following an inflammatory stimulus. *Fish Shellfish Immunol.* 2007; **22**: 157–171.
- Gruss HJ. Molecular, structural and biological characteristics of the tumor necrosis factor ligand superfamily. *Int. J. Clin. Lab. Res.* 1996; **26**: 143–159.
- Gupta G, Surolia A. Collectins: sentinels of innate immunity. *Bioessays* 2007; **29**: 452–464.
- Guyader DL, Redd MJ, Colucci-Guyon E, Murayama E, Kissa K, Briolat V, Mordelet E, Zapata A, Shinomiya H, Herbomel P. Origins and unconventional behavior of neutrophils in developing zebrafish. *Blood* 2008; **111**: 134–141.

- Hanington PC, Belosevic M. Characterization of the leukemia inhibitory factor receptor in the goldfish (*Carassius auratus*). *Fish Shellfish Immunol.* 2005; **18**: 359–369.
- Hanington PC, Wang T, Secombes CJ, Belosevic M. Growth factors of lower vertebrates: characterization of goldfish (*Carassius auratus*) macrophage colony-stimulating factor–1. *J. Biol. Chem.* 2007; **282**: 31865–31872.
- Hawke NA, Yoder JA, Haire RN, Mueller MG, Litman RT, Miracle AL, Stuge T, Shen L, Miller N, Litman GW. Extraordinary variation in a diversified family of immune-type receptor genes. *Proc. Natl. Acad. Sci. USA* 2001; **98**: 13832–13837.
- Hikima J, Minagawa S, Hirono I, Aoki T. Molecular cloning, expression and evolution of the Japanese flounder goose-type lysozyme gene, and the lytic activity of its recombinant protein. *Biochim. Biophys. Acta* 2001; **1520**: 35–44.
- Huising MO, Stet RJM, Savelkoul HFJ, Lidy Verberg-van Kemenede BM. The molecular evolution of the interleukin-family of cytokines; IL 18 in teleost fish. *Dev. Comp. Immunol.* 2004; **28**: 395–413.
- Huising MO, Kruiswijk CP, Flik G. Phylogeny and evolution of class-I helical cytokines. *J. Endocrinol.* 2006; **189**: 1–25.
- Hwang JY, Santos MD, Kondo H, Hirono I, Aoki T. Identification, characterization and expression of novel cytokine M17 homologue (MSH) in fish. *Fish Shellfish Immunol.* 2007; **23**: 1256–1265.
- Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A *et al.* Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* 2004; **431**: 946–957.
- Janeway CA, Medzhitov R. Innate immune recognition. *Ann. Rev. Immunol.* 2002; **20**: 197–216.
- Jault C, Pichon L, Chluba J. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. *Mol. Immunol.* 2004; **40**: 759–771.
- Kaparakis M, Philpott DJ, Ferrero RL. Mammalian NLR proteins; discriminating foe from friend. *Immunol. Cell. Biol.* 2007; **85**: 495–502.
- Kawai T, Akira S. Toll-like receptor downstream signaling. *Arthritis. Res. Ther.* 2005; **7**: 12–19.
- Kim HJ, Yasuike M, Kondo H, Hirono I, Aoki T. Molecular characterization and gene expression of a CXC chemokine gene from Japanese flounder, *Paralichthys olivaceus*. *Fish Shellfish Immunol.* 2007; **23**: 1275–1284.
- Kono T, Zou J, Bird S, Savan R, Sakai M, Secombes CJ. Identification and expression analysis of lymphotoxin-beta like homologues in rainbow trout *Oncorhynchus mykiss*. *Mol. Immunol.* 2006; **43**: 1390–1401.
- Krause CD, Pestka S. Evolution of the class 2 cytokines and receptors, and discovery of new friends and relatives. *Pharmacol. Ther.* 2005; **106**: 299–346.
- Krishnan J, Selvarajo K, Tsuchiya M, Lee G, Choi S. Toll-like receptor signal transduction. *Exp. Mol. Med.* 2007; **39**: 421–438.
- Kurobe T, Yasuike M, Kimura T, Hirono I, Aoki T. Expression profiling of immune-related genes from Japanese flounder *Paralichthys olivaceus* kidney cells using cDNA microarrays. *Dev. Comp. Immunol.* 2005; **29**: 515–523.
- Langer JA, Cutrone EC, Kotenko S. The class II cytokine receptor (CRF2) family: overview and patterns of receptor-ligand interactions. *Cytokine Growth. Factor. Rev.* 2004; **15**: 33–48.
- Lee MS, Kim YJ. Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Ann. Rev. Biochem.* 2007; **76**: 447–480.
- Li J, Barreda DR, Zhang YA, Boshra H, Gelman AE, LaPatra S, Tort L, and Sunyer JO. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nature Immunol.* 2006; **7**: 1116–1124.
- Mantovani A, Garlanda C, Doni A, Bottazzi B. Pentraxins in Innate Immunity: From C-reactive protein to the long Pentraxin PTX3. *J. Clin. Immunol.* 2008; **23**: 1–13.
- Nakao M, Kajiya T, Sato Y, Somamoto T, Kato-Unoki Y, Matsushita M, Nakata M, Fujita T, Yano T. Lectin pathway of bony fish complement: identification of two homologs of the mannose-binding lectin associated with MASP2 in the common carp (*Cyprinus carpio*). *J. Immunol.* 2006; **177**: 5471–5479.
- Ohno S. *Evolution by Gene Duplication*. Springer, New York. 1970.
- Paffet-Lufassy N, Hsia N, Fraenkel PG, Paw B, Leshinsky I, Barut B, Bahary N, Caro J, Handin R, Zon LI. Functional conservation of erythropoietin signaling in zebrafish. *Blood* 2007; **110**: 2718–2726.
- Peatman E, Liu Z. Evolution of CC chemokines in teleost fish: a case study in gene duplication and implications for immune diversity. *Immunogenetics* 2007; **59**: 613–623.

- Peatman E, Baoprasertkul P, Terhune J, Xu P, Nandi S, Kucuktas H, Li P, Wang S, Somridhivej B, Dunham R, Liu Z. Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. *Dev. Comp. Immunol.* 2007; **31**: 1183–1196.
- Peatman E, Terhune J, Baoprasertkul P, Xu P, Nandi S, Wang S, Somridhivej B, Kucuktas H, Li P, Dunham R, Liu Z. Microarray analysis of gene expression in the blue catfish liver reveals early activation of the MHC class I pathway after infection with *Edwardsiella ictaluri*. *Mol. Immunol.* 2008; **45**: 553–566.
- Piraviriyakul P, Kondo H, Hirono I, Aoki T. A novel immune type receptor of Japanese flounder (*Paralichthys olivaceus*) is expressed in both T and B lymphocytes. *Fish Shellfish Immunol.* 2007; **22**: 467–476.
- Plouffe DA, Hanington PC, Walsh JG, Wilson EC, Belosevic M. Comparison of select innate immune mechanisms of fish and mammals. *Xenotransplantation* 2005; **12**: 266–77.
- Purcell MK, Smith KD, Hood L, Winton JR, Roach JC. Conservation of Toll-Like Receptor Signaling Pathways in Teleost Fish. *Comp. Biochem. Physiol. Part D. Genomics. Proteomics.* 2006; **1**: 77–88.
- Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE, Aderem A. The evolution of vertebrate Toll-like receptors. *Proc. Natl. Acad. Sci. USA* 2005; **102**: 9577–9582.
- Robertsen B. The interferon system of teleost fish. *Fish Shellfish Immunol.* 2006; **20**: 172–191.
- Robinson MJ, Sancho D, Slack EC, LeibundGut-Landmann S, Reis e Sousa C. Myeloid C-type lectins in innate immunity. *Nat. Immunol.* 2006; **7**: 1258–1265.
- Rowley AF, Hunt T, Page M, Mainwaring G. Fish. In: Rowley AF, Ratcliffe NA (eds). *Vertebrate Blood Cells*. Cambridge University Press, Cambridge, UK. 1988; 19–128.
- Russell S, Lumsden JS. Function and heterogeneity of fish lectins. *Vet. Immunol. Immunopathol.* 2005; **108**: 111–120.
- Santos MD, Yasuike M, Kondo H, Hirono I, Aoki T. A novel type-1 cytokine receptor from fish involved in the Janus kinase/signal transducers and activators of transcription (Jak/STAT) signal pathway. *Mol. Immunol.* 2007; **44**: 3355–3363.
- Stein C, Caccamo M, Laird G, Leptin M. Conservation and divergence of gene families encoding components of innate immune response systems in the zebrafish. *Genome Biol.* 2007; **8**: R251.
- Takahashi M, Iwaki D, Matsushita A, Nakata M, Matsushita M, Endo Y, Fujita T. Cloning and characterization of mannose-binding lectin from lamprey (Agnathans). *J. Immunol.* 2006; **176**: 4861–4868.
- Tsuji S, Uehori J, Matsumoto M, Suzuki Y, Matsuhisa A, Toyoshima K, Seya T. Human intelectin is a novel soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cell wall. *J. Biol. Chem.* 2001; **276**: 23456–23463.
- Tsujita T, Tsukada H, Nakao M, Oshiumi H, Matsumoto M, Seya T. Sensing bacterial flagellin by membrane and soluble orthologs of Toll-like receptor 5 in rainbow trout (*Onchorhynchus mykiss*). *J. Biol. Chem.* 2004; **279**: 48588–48597.
- Vasta GR, Quesenberry M, Ahmed H, O'Leary N. C-type lectins and galectins mediate innate and adaptive immune functions: their roles in the complement activation pathway. *Dev. Comp. Immunol.* 1999; **23**: 401–420.
- Volff JN. Genome evolution and biodiversity in teleost fish. *Heredity* 2005; **94**: 280–294.
- Yasuike M, Kondo H, Hirono I, Aoki T. Difference in Japanese flounder, *Paralichthys olivaceus* gene expression profile following hirame rhabdovirus (HIRRV) G and N protein DNA vaccination. *Fish Shellfish Immunol.* 2007; **23**: 531–541.
- Yazawa R, Hiono I, Ohira T, Aoki T. Functional analysis of tumor necrosis factor gene promoter from Japanese flounder, *Paralichthys olivaceus*, using fish cell lines. *Dev. Comp. Immunol.* 2005a; **29**: 73–81.
- Yazawa R, Hiono I, Ohira T, Aoki T. Induction of Japanese flounder TNF promoter activity by lipopolysaccharide in zebrafish embryo. *Mar. Biotechnol.* 2005b; **7**: 625–633.
- Yazawa R, Hiono I, Aoki T. Characterization of promoter activities of four different Japanese flounder promoters in transgenic zebrafish. *Mar. Biotechnol.* 2005c; **7**: 231–235.
- Yazawa R, Hiono I, Aoki T. Transgenic zebrafish expressing chicken lysozyme show resistance against bacterial disease. *Transgenic Res.* 2006; **15**: 385–391.