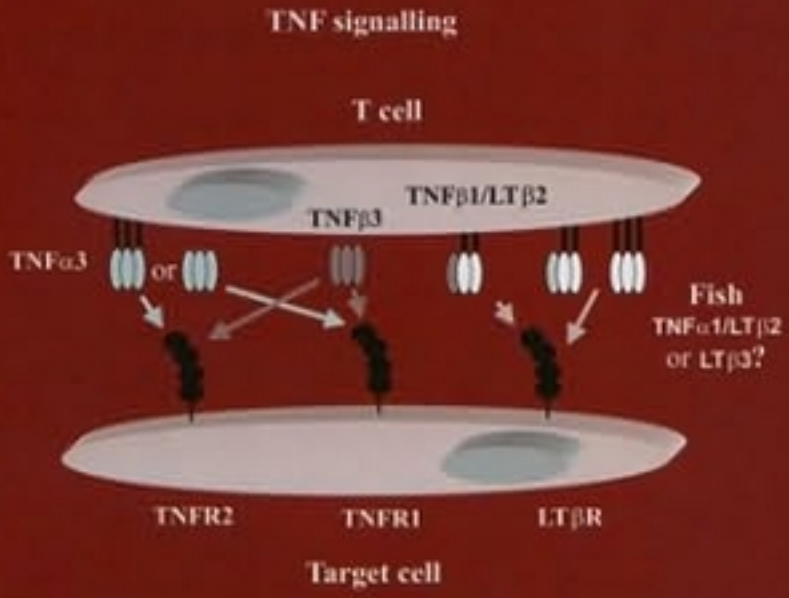
FISH DEFENSES

Volume 1: Immunology



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Volume 1: Immunology

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"…in ***Mozart*** the Nature has produced exceptional, an unrepeatable one, in any case never more repeated, masterpiece."

Wolfgang Hildesheimer

Preface

In its widest sense the term defense refers to all the mechanisms used by living organisms as protection against foreign environmental agents such as microorganisms and their products, chemicals, drugs, animal actions, etc. Among these, immunity is the main endogenous mechanism of defense which helps to distinguish between self and non self. This recognition mechanism originated with the formation of cell markers probably involving cell surface molecules that were able to specifically bind and adhere to other molecules present on opposing cell surfaces. This simple method seems to have evolved into the full complexity of what we call an immune response.

The greatest complexity of the immune response is shown by vertebrates which are endowed with innate and acquired immunity, although, increasingly, evidence for types of acquired immunity is coming to light within the invertebrates. Immunological studies performed mostly in mammals have been the reference for studies in other vertebrates. However, the efforts of research scientists around the world have now produced findings that allow us to identify significant differences among the immune system of the major vertebrate groups. Fish immunity, particularly, shows striking differences from that observed in homoeothermic animals.

The study of immunological fish defenses has advanced considerably in recent decades. This has been due to the key position of fish in terms of the evolution of acquired immunity and to the rapid expansion of aquaculture over this period, where disease control is of prime concern. In

addition, some fish species are seen as powerful scientific models for different field of study.

The objective of this book is to present a compilation of some of the main findings that reflect current thinking on fish immune defenses.

Two chapters are devoted to fish innate immunity: the antimicrobial peptides and the cellular processes involved in macrophage-mediated host defense. Also, two chapters look at adaptive immunity in fish and review current knowledge on the molecular organization of antibody genes, the structural and functional features of the antibody molecule, the development of antibody-producing cells and the organization and function of the system which leads to an antibody response. The discovery of a new immunoglobulin class and the characterization of teleost IGH loci are discussed. Another chapter is dedicated to the fish immune response to eukaryotic parasites (immune responses to surface and internal parasites) as well as the evasion and suppression of the immune response by such pathogens (elusive parasite).

The fish cytokine network, immune regulatory peptides coordinating innate and adaptive responses, is analysed in a further chapter, outlining their discovery, activities and potential application. Two chapters are devoted to immune-endocrine interactions in fish, namely, the effects of estrogens as fish immunoregulators. Linked to this, the morpho-functional features of leukocytes and cytokines present in the fish testis are also described.

Finally, two chapters give up-to-date reviews on applied aspects of manipulating fish immune defenses in aquaculture. Current knowledge of the immune system of sea bass (*Dicentrarchus labrax* L.), a teleost species of immense commercial interest for Mediterranean aquaculture, is reported, and the potential use of CpG ODNs as immunostimulants in aquaculture is presented, together with their possible use to improve future vaccine formulations.

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We especially thank Professor Chris Secombes (University of Aberdeen, Scotland, UK) for his encouragement, suggestions and collaboration during the preparation of this book.

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CHAPTER

1

Fish Cytokines: Discovery, Activities and Potential Applications

C.J. Secombes*, J. Zou and S. Bird

INTRODUCTION

Over the last few years, there has been a tremendous increase in our knowledge of the fish cytokine network, largely due to the increasing number of EST sequences in the databases and the availability of sequenced fish genomes. This chapter will highlight some of these advances in terms of genes discovered and, where elucidated, the role of the proteins in the immune system of fish. The potential applications of these molecules in studies to improve fish health in aquaculture will also be discussed.

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CYTOKINES INVOLVED IN INNATE IMMUNITY

Cytokines are the key regulators of the immune system. Their role in initiating inflammatory events in response to bacterial exposure is well known in mammals, where a cytokine cascade leads to the attraction of particular leucocyte types and activation of their antimicrobial pathways. Tumor necrosis factor-α (TNF-α) is the first cytokine released in this cascade and leads to the downstream expression of interleukin-1β (IL-1β) and chemokines such as IL-8. Following infection with viruses, cytokines can again activate various cellular pathways but also have direct effects on cells that lead to an antiviral state, as seen with the interferons (IFN). Such molecules represent a crucial component of the innate defences, although cross talk and activation of adaptive (specific) immunity may also be triggered in the medium term.

Pro-inflammatory Cytokines

Discovery

One of the first cytokine genes discovered in fish was IL-1β, found in rainbow trout by homology cloning (Zou *et al.*, 1999a,b) and one of the key pro-inflammatory cytokines known from mammalian studies. This quickly led to the discovery of IL-1β in many other teleost fish species, including carp (Fujiki *et al.*, 2000), sea bass (Scapigliati *et al.*, 2001), sea bream (Pelegrin *et al.*, 2001), goldfish (Bird, 2001), turbot (Low *et al.*, 2003), Japanese flounder (Emmadi *et al.*, 2005), zebrafish (Pressley *et al.*, 2005), crocodile ice fish (Buonocore *et al.*, 2006), Atlantic salmon (Ingerslev *et al.*, 2006), Nile tilapia (Lee *et al.*, 2006), Japanese sea perch (Qiu *et al.*, 2006), channel catfish (Wang *et al.*, 2006) and haddock (Corripio-Miyar *et al.*, 2007). Homology cloning of this molecule in cartilaginous fish was also possible following this initial discovery (Bird *et al.*, 2002a; Inoue *et al.*, 2003a). Further analysis has shown that in some species a second IL-1β gene exists, especially in the salmonids (Pleguezuelos *et al.*, 2000) and cyprinids (Engelsma *et al.*, 2003; Wang *et al.*, 2006), and that even allelic variation is detectable in fish (Wang *et al.*, 2004). Curiously, the exon-intron organization varies between different fish groups, with the classical sevon exon arrangement seen in mammals present in cyprinids (Engelsma *et al.*, 2001), with six exons present in salmonids (Zou *et al.*, 1999b) and five present in more advanced acanthopterygian teleosts (Buonocore *et al.*, 2003a; Lee *et al.*, 2006).

Most conservation of the molecule, when translated into the amino acid sequence, can be seen within the beta sheet regions that form the secondary structure of IL-1β, that is a β-trefoil cytokine (Koussounadis *et al.*, 2004). Modelling of the receptor binding sites across species reveals a high level of variability in terms of the positions involved but indicates conservation of the overall shape of the ligand-receptor complex (Koussounadis *et al.*, 2004). However, analysis of the protein sequence fails to find an obvious processing site for cleavage by interleukin converting enzyme (ICE), required for generation of the bioactive mature peptide in mammals. Nevertheless, evidence to date suggests that fish IL-1β is processed (Hong *et al.*, 2004).

A second major pro-inflammatory cytokine discovered in fish was TNF-α. It was discovered during EST analysis of PMA/ConA stimulated blood leucocytes from Japanese flounder (Hirono *et al.*, 2000), and was the first non-mammalian TNF discovered. Again, this was quickly followed with the cloning of TNF by homology in several teleost fish species, including brook trout (Bobe and Goetz, 2001), rainbow trout (Laing *et al.*, 2001), sea bream (Garcia-Castillo *et al.*, 2002), carp (Saeij *et al.*, 2003), catfish (Zou *et al.*, 2003a), pufferfish and zebrafish (Savan *et al.*, 2005), Atlantic salmon (Ingerslev *et al.*, 2006), turbot (Ordás *et al.*, 2007), ayu (Uenobe *et al.*, 2007) and sea bass (Nascimento *et al.*, 2007a). As with IL-1β, in certain teleost groups multiple isoforms were found to exist (Zou *et al.*, 2002; Savan and Sakai, 2003).

Subsequently, the teleost TNF-α gene was found to be adjacent to another TNF family member in the genomes of pufferfish and zebrafish (Savan *et al.*, 2005), although the exact relationship to other known TNFs, namely TNF-β and lymphotoxin-β (LT-β), was not entirely clear. This allowed the cloning of two isoforms of the equivalent molecule from rainbow trout (Kono *et al.*, 2006), and further analysis revealed that this was the teleost LT-β molecule. Thus, it is apparent that either TNF-β does not exist in fish, as it is not adjacent to the other TNF family members in fish genomes, or it resides in a different part of the genome to mammals.

Chemokines, or chemotactic cytokines, are a family of cytokines that serve to attract leucocytes to particular sites (Laing and Secombes, 2004a). They are subdivided into two main groups, those that are involved in lymphocyte trafficking and immune surveillance as part of homeostatic mechanisms within the immune system, and those that are released during an infection and direct leucocyte migration to an injured or infected site.

Based on the arrangement of the first two cysteines in the molecule they are classified as CXC chemokines (α), CC chemokines (β), C chemokines (γ) or CX_3C chemokines (δ). Only members of these first two families have been found in fish to date.

The first CXC chemokine discovered in fish was IL-8 (CXCL8), initially found in the lamprey (Najakshin *et al.*, 1999) and, subsequently, cloned in Japanese flounder (Lee *et al.*, 2001), rainbow trout (Laing *et al.*, 2002a), carp (Huising *et al.*, 2003—referred to as CXCa by these authors), banded dogfish (Inoue *et al.*, 2003b), silver chimaera (Inoue *et al.*, 2003c), catfish (Chen *et al.*, 2005) and haddock (Corripio-Miyar *et al.*, 2007). An important feature of the IL-8 molecule is the so-called ELR motif immediately upstream of the CXC residues, and responsible for the ability to attract and activate neutrophils. This motif has conservative substitutions in most fish species (e.g., DLR in trout), although the haddock molecule possesses an unsubstituted motif. The gene expression is markedly induced upon stimulation with pro-inflammatory stimuli such as LPS or IL-1β (Fig. 1.1). A second CXC chemokine discovered in fish has clear homology to the gamma interferon-induced chemokines CXCL9, CXCL10 and CXCL11, that all share a common receptor (CXCR3) and are all ELR^- CXC chemokines. This molecule was initially discovered in trout (Laing *et al.*, 2002b; O'Farrell *et al.*, 2002) and, subsequently, discovered in carp (Savan *et al.*, 2003a—referred to as CXCb by Huising *et al.*, 2003) and channel catfish (Baoprasertkul *et al.*, 2004). The trout molecule is more CXCL10-like (based on analyses of individual exon sequences) and is induced by trout recombinant interferon gamma

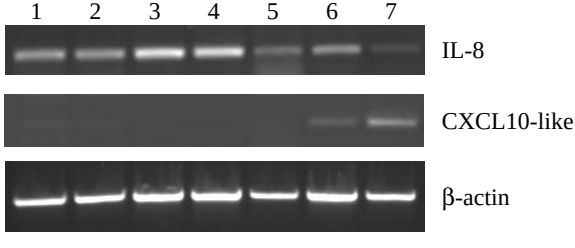

Fig. 1.1 Modulation of chemokine expression by the proinflammatory cytokines IL-1β and IFN-γ. Freshly isolated trout head kidney leucocytes were stimulated for 4 h with trout rIL-1β (lanes 2-4) and rIFN-γ (lanes 5-7) at doses of 1, 10 and 100 ng/ml respectively. Control cells (lane 1) were incubated with an equal volume of elution buffer (20 mM Tris, 100 mM KCl, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1% NP40, 20% glycerol, 250 mM imidazole) used to dissolve the recombinant proteins. Total RNA was extracted for semi-quantitative RT-PCR analysis of gene expression for IL-8 and CXCL10-like chemokines.

(IFN-γ), as expected (Zou *et al.*, 2005a; Fig. 1.1) and is, thus, a useful marker of IFN-γ action. CXC chemokines homologous to CXCL12 and 14 have also been identified in fish (Long *et al.*, 2000; Huising *et al.*, 2004; Baoprasertkul *et al.*, 2005) and are particularly highly conserved, suggesting they have critical roles, potentially in development of the organism (see section on **Activities** below). Two further CXC chemokines have been reported that have less clear homology to known genes, a so-called CXCd gene in trout (Wiens *et al.*, 2006) and a CXCL2-like molecule (17-24% amino acid identity to mammalian molecules) in catfish (Baoprasertkul *et al.*, 2005). CXCL2 is an ELR-containing chemokine in mammals, but like the catfish CXCL8 molecule, the CXCL2-like gene does not possess an ELR motif although, again, a related sequence (PDR) is present. A recent study in zebrafish and pufferfish suggests that molecules with homology to CXCL5, CXCL9 and CXCL11 may also exist in fish (DeVries *et al.*, 2006), although further work is needed to verify this. In some species multiple isoforms of CXC chemokines are present, as seen in carp (Huising *et al.*, 2004), trout (Wiens *et al.*, 2006) and zebrafish (DeVries *et al.*, 2006). This appears to be related to ancient genome duplication events or more recent tandem gene duplications, including the duplication of multiple genes by duplication of chromosomal segments in the case of zebrafish.

Lastly, many CC chemokines have been identified in fish, especially in trout (Dixon *et al.*, 1998; Laing and Secombes, 2004b), catfish (Bao *et al.*, 2006; Peatman *et al.*, 2006) and zebrafish (DeVries *et al.*, 2006; Peatman and Liu, 2006), where numbers can exceed those seen in mammals (e.g., 24 genes known in humans versus 26 in catfish and 46 in zebrafish). A CC chemokine gene has also been reported in a cartilaginous fish (Kuroda *et al.*, 2003). In trout, where 18 unique transcripts (including 6 pairs of closely related genes) were found by interrogation of EST sequences, phylogenetic analysis revealed some genes grouped with known mammalian clades, whilst others had no obvious homology to mammalian genes but were clustered with other fish sequences. This suggested the possibility that fish specific and species-specific gene duplications of CC chemokines have occurred, and that CC chemokines have high divergence rates. Similarly, in catfish, numerous genes were discovered and were mapped to BAC clones. This revealed that the genes were highly clustered in the genome, with 2-8 identified chemokines within individual assembled contigs. Between contigs, the genes were often very similar, suggesting that segmental gene duplication was involved in generating the

chemokine gene clusters. Only five of the chemokine genes were present as a single copy, with 5 as two copies, 8 as three copies, 5 as four copies, 2 as five copies and one as six copies, giving some 75 genes in total. Many of the catfish genes shared highest identity to CCL3 or CCL14, perhaps members of the original CC repertoire in early vertebrates. In zebrafish, analysis of ESTs and the available genome allowed a more extensive evaluation of the CC chemokine repertoire in fish (Peatman and Liu, 2006). Forty-six genes were identified, again with evidence of extensive 'en bloc' duplication events. Many of the genes form species-specific clades in phylogenetic analysis, supporting the concept that species-specific duplication events of fish chemokine genes have occurred. A model of the evolutionary history of the chemokine system has been proposed by DeVries *et al*. (2006) based on analyses of fish, amphibian, bird and mammalian genomes.

ACTIVITIES

The recombinant IL-1β protein has been produced in *E. coli* and its bioactivity studied in trout (Hong *et al*., 2001; Peddie *et al*., 2001), carp (Yin and Kwang, 2000a; Matthew *et al*., 2002) and sea bass (Buonocore *et al*., 2005). With no clear ICE cut site, the recombinant proteins were started at various locations in the cut site region determined by multiple alignment. In trout, Ala^{95} was used as the starting point for the recombinant protein, whereas in carp Thr^{115} was used and in seabass Ala^{86} was used. As outlined below, in all the cases a bioactive protein was produced, although the exact start of the native mature protein remains unknown.

The trout rIL-1β was shown to have a number of effects on trout leucocytes. It increased its own expression in head-kidney leucocytes, with optimal rIL-1β dose and kinetics studied (Hong *et al*., 2001). It was also able to induce the expression of cyclooxgenase 2 (COX-2), a potent pro-inflammatory gene, and to increase the expression of the MHC class II β chain in a trout macrophage cell line. Lastly, the rIL-1β increased head-kidney leucocyte phagocytic activity for yeast particles (Hong *et al*., 2001), and induced leucocyte chemotaxis (Peddie *et al*., 2001). Since the recombinant protein was produced in bacteria, and LPS is known to be a potent stimulant for leucocytes, a number of controls were included in this study, such as the effect of pre-incubation with a specific polyclonal antiserum for 1 h at 4°C, or of heating the rIL-1β to 95°C for 20 min, and in each case the activity of the trout rIL-β was significantly decreased.

Similar results were found with sea bass rIL-1β produced in *E. coli*, which enhanced phagocytosis of head-kidney leucocytes and expression of IL-1β and COX-2 (Buonocore *et al.*, 2005). The seabass rIL-1β was also shown to induce the proliferation of sea bass thymocytes following stimulation with a sub-optimal dose of Con A. Carp rIL-1β was also shown to stimulate *in vitro* the proliferation of carp head-kidney and splenic leucocytes (Mathew *et al.*, 2002).

IL-1β derived peptides have also been produced, based upon the modelling of IL-1β with its receptor, to identify contiguous regions of the protein that are part of the receptor binding domain (Koussounadis *et al.*, 2004). *In vitro* studies showed that one of the peptides (called P3) was particularly potent, and was able to enhance the locomotory (Peddie *et al.*, 2001), phagocytic and bactericidal (Peddie *et al.*, 2002a) capacity of head-kidney leucocytes. The latter was not a result of enhanced oxygen radical production from the cells, which was not increased by P3. Interestingly, when combined with a second peptide (P1)—which alone had limited effects—a synergistic effect was seen on phagocytic and locomotory activity. Similarly, *in vivo* injection of P3 into the peritoneal cavity increased the number of peritoneal leucocytes harvested at days 1 and 3 post-injection, relative to control groups, including fish injected with P1 or an irrelevant peptide, and increased their phagocytic activity (Peddie *et al.*, 2003). One of the most fascinating findings from the *in vivo* studies, however, was that whilst P3 had clear effects on leucocyte activity, it failed to induce the normal activation of the hypothalamic-pituitary-adrenal (HPI) axis that results in the detectable release of cortisol into the blood stream (Holland *et al.*, 2002). Trout rIL-1β, on the other hand, does activate the HPI axis when administered intraperitoneally (ip), with elevated cortisol levels apparent 3h-8h post-injection.

As an alternative approach to producing the recombinant protein *in vitro* for bioactivity studies, in some experiments, plasmid DNA containing the cloned IL-1β gene has been administered. In carp, intramuscular injection of the construct resulted in increased macrophage respiratory burst and phagocytic activity, as well as increased leucocyte proliferation following PHA stimulation of the cells (Kono *et al.*, 2002a). In Japanese flounder, fish injected intramuscularly with a construct containing the full length IL-1β in the pCL-neo expression vector (Emmadi *et al.*, 2005) had kidney tissue isolated at days 1, 3 and 7 post-injection, and the samples used for expression profiling with a Japanese flounder cDNA micro array