Chapter 2

Cytokine Regulation of Teleost Inflammatory Responses

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Additional information is available at the end of the chapter

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1. Introduction

The inflammatory response is a highly regulated process initiated by tissue damage, infiltrating pathogens or both. The primary role of inflammation is the resolution of tissue damage, including the elimination of damaged or dead cells and any infiltrating pathogens, and restoration of homeostasis. The initial recognition of tissue damage and/or pathogens is mediated by tissue resident macrophages primarily through various sentinel pattern recognition receptors such as toll-like receptors. In response to and in accordance with distinct stimuli, macrophages become activated to produce a wide range of bioactive molecules, some of which attract other cells to the site of inflammation, and others that dictate the course of an inflammatory response and eventual tissue repair. Recent evidence suggests that there are at least two activation states of monocytes/ macrophages [4, 130, 211, 214]. The classically activated monocytes/macrophages possess significant antimicrobial armamentarium, secrete a plethora of factors that propagate and enhance the microbicidal activities and in general mediate pathogen clearance. The non-classically or alternatively activated monocytes/macrophages secrete factors that ablate the destructive components of the inflammatory response and promote tissue healing, repair and angiogenesis and will not be addressed further here.

The processes involved in the onset, progression and resolution of inflammation are complex and remain to be fully elucidated in vertebrates. However, it is widely believed that myeloid lineage cells are intimately involved in inflammatory reactions and their function is controlled by cytokines. This review focuses on the recent advancements in the understanding of the biology of hallmark fish pro-inflammatory cytokines, tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ) and interleukin-1 beta (IL-1β) and their receptors.

2. Antimicrobial responses of fish phagocytes

It is well established that fish phagocytes possess oxidative burst responses, comparable to those of mammals. Absence of readily available fish cytokines limited the early research of fish phagocytes to employing pathogen products and/or crude activated cell supernatants, presumed to contain hallmark "activating" agents. This fundemental work of fish phagocyte-mediated inflammatory processes has been comprehensively reviewed [138]. Since then the specific genes encoding the components of the fish NADPH oxidase complex (Fig. 1) have been cloned in various fish species [13, 84, 119] and their expression correlated with reactive oxygen radical production [13, 63, 141]. The priming of the fish phagocyte ROI responses by recombinant fish cytokines such as TNFα [67, 133, 217], IFNγ [62, 63, 215] and IL-1β [54, 97, 144] has also been reported. Similar to the mammalian monocyte/macrophage paradigm [22], fish monocytes have greater ability to generate reactive oxygen intermediates (ROI) following short stimulation [67, 136, 152], whereas mature fish macrophages require relatively prolonged immune stimulations to achieve comparable magnitudes of this response [58, 59, 136, 171].

Phagocytes (primarily mature macrophages) also produce microbicidal/tumoricidal reactive nitrogen intermediates in a stimulus-specific manner. This response, catalyzed by the inducible nitric oxide synthase enzyme (iNOS, Fig. 2), involves the conversion of arginine to citruline and results in the production of nitric oxide (NO) and other products including nitrite, nitrate, and nitrosamines [86, 134, 184]. The NADPH oxidase produced superoxide anion may also react with NO to form the peroxynitrite intermediate [$ONOO^-$] that also has potent microbiacidal activity [35, 156, 191, 213]. The biology of the iNOS enzyme has been reviewed in references [2, 111, 126].

The ability of fish phagocytes to produce microbicidal NOs has been well established (reviewed in reference [138]). The iNOS gene transcript has been identified in several fish species [101, 102, 163, 200] and fish macrophages have been demonstrated to up-regulate iNOS expression and produce copious amounts of NO in response to a plethora of immune stimuli [62-64, 66, 67, 85, 88, 140, 158, 180, 210]. The inflammatory cytokine regulation of fish iNOS and NO production is described below.

3. Cytokine regulation of inflammatory responses

The onset, progression and resolution of the inflammatory responses are tightly regultaed through soluble mediators (cytokines, monokines, chemokines) that orchestrate inflammation. Pro-inflammatory cytokines such as TNFα, IFNγ, and IL-1β enhance antimicrobial functions of immune cells and facilitate the pathogen clearance, while anti-inflammatory cytokines such as TGFβ and IL-10 down-regulate inflammatory processes and skew cell functions towards tissue repair mechanisms. It is important to note that in addition to the hallmark cytokines discussed in this review, other cytokines such as chemokines and growth factors also participate in the regulation of an inflammatory response.

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The last decade has yeilded significant advances in the understanding of inflammatory responses of lower vertebrates, such as bony fish. The genes encoding hallmark cytokines have been identified and characterized in a number of fish species. Interestingly, many of these exhibit structural similarities and gene synteny organization comparable to their higher vertebrate counterparts. Conversely, multiple isoforms of certain cytokines are present in distinct fish species.

3.1. Tumor necrosis factor alpha (TNFα)

Tumor necrosis factor alpha is a central inflammatory mediator, initially identifed as a serum component capable of eliciting "hemorrhagic necrosis" of certain tumors [20]. Since discovery, TNFα has been found to be produced by many cell-types and confer an increadible range of immune processess [44, 203, 209]. In the context of inflammatory responses, TNFα promotes the chemotaxis of neutrophils and monocytes/macrophages [123, 207], enhances their phagocitic capacity [95, 105, 194], primes ROI and NO reposnses [42, 135], chemoattracts fibroblasts [168] and elicits platelet activating factor production [19, 73, 104].

The mammaian TNFα functions as a 26 kDa type II trans-membrane protein as well as a 17 kDa soluble moiety, released by the TNFα cleaving metalloproteinase enzyme (TACE)-mediated cleavage [98, 128, 148]. A homotrimerized TNFα (soluble or membrane bound) engages one of two cognate receptors, TNF-R1 or TNF-R2, which in turn trimerize around the ligand [7, 45]. Currently, there is no consensus as to the respective contribution of these receptors to the biological effects caused by TNFα. Some evidence suggests that TNF-R1 propagates the signal from the soluble TNFα, while the membrane-bound TNFα acts exclusively through TNF-R2 [70]. Other evidence suggest that TNF-R1 is primarily involved in induction of apoptosis while the TNF-R2 functions in proliferation and cell survival [129], while other contributions suggest cooperation between the two receptors [204]. The prevailing theory proposes that TNF-R1 confers signal propagation, while TNF-R2 binds and redistributes TNFα to TNF-R1 in a process coined "ligand passing" [28, 43, 197]. Despite this, more recent literature suggests that TNF-R2 is directly involved in many inflammatory processes including the activation of T lymphocytes [93, 94], stimulation of myofibroblasts [190], as well as tumor suppression [212]. The TNF-R1 and TNF-R2 utilize largely non-overlapping signaling mechanisms, relying on recruitment of numerous downstream signaling molecules, the relative abundance of which ultimately dictates signaling outcomes (for a current review of TNFα signal transduction see recent review [143]). It is likely that the roles of respective TNF receptors in the biological outcomes of TNFα stimulation are cell type and cell activation state dependent.

3.1.1. Identification of TNFα in fish

Presence of an endogenous bony fish TNF system was first suggested in the early 1990s where the human recombinant TNFα elicited ROI production in trout leukocytes while administration of a monoclonal anti-TNF-R1 antibody blocked this response [75, 87]. Hirono *et al.* [76] identified and characterized the first cDNA transcript encoding a Japanese flounder TNFα, which had only 20-30% amino acid identity with the mammalian TNFs, but had very

similar intron/exon organization. The expression of this flounder TNFα gene increased in PBLs following LPS, ConA or PMA stimulation, suggesting a conserved role for this cytokine in fish inflammatory responses. Shortly thereafter, the rainbow trout TNFα was identified, shown to be constitutively expressed in the gill and kidney tissues, and upregulated in LPS-stimulated or IL-1β-treated kidney leukocytes and in the trout macrophage cell line, RTS11 [103]. The catfish TNFα was also constitutively expressed in healthy fish and several catfish immune cell lines including macrophage and T cell lines, but not in B cell or fibroblast cell lines [218]. Notably, all fish TNFα proteins possess the TNF family signature, [LV]-x-[LIVM]-x_3-G-[LIVMF]-Y-[LIMVMFY]$_2$-x_2-[QEKHL] [103], underlining the evolutionary conservation of this cytokine.

3.1.2. Isoforms of TNFα in fish

Trout were reported to possess an additional TNFα isoform [219] and the presence of multiple bony fish TNFs was confirmed in common carp, in which first two [162], and soon thereafter a third carp TNFα isoform [167] were identified. Interestingly, polymorphisms in the gene encoding the carp TNFα2 isoform were linked to carp resistance to the protozoan parasite, *T. borreli* [162].

3.1.3. TNFα receptors of fish

Tumor necrosis factor alpha has now been identified in a number of fish species including flounder [76], trout [103, 114], catfish [218], carp [162, 167], sea bream [18, 56], tilapia [151], turbot [142], ayu [193], fugu [165], zebrafish [165], sea bass [133], goldfish [67] and tuna [89]. Additionally, novel TNF-like molecules with unique intron/exon organization have been identified in zebrafish and flounder [165]. Despite this, knowledge regarding the cognate receptors for the teleost TNF proteins has been relatively limited.

A death domain containing TNF receptor was identified in zebrafish ovarian tissues and coined the ovarian TNF receptor (OTR) [12]. Predicted zebrafish TNF-R1 and TNF-R2 sequences are in the NCBI database, with zebrafish TNF-R1 showing high sequence homology to the OTR. The goldfish TNF-R1 and TNF-R2 cDNAs were identified based on the zebrafish sequences [61] and display conserved regions included cysteine residues and predicted docking sites for downstream signaling. Also, the goldfish TNF-R1 possesses a conserved death domain including the highly conserved motif (W/E)-X_{31}-L-X_2-W-X_{12}-L-X_3-L (with R in the W/E position) and six conserved or semi-conserved residues, crucial for the mammalian TNF-R1-mediated cytotoxicity [189]. Interestingly, while the mammalian TNF-R1 and TNF-R2 and the fish TNF-R2 contain four TNF homology domains (THD, defined by specific cysteine residues), the fish TNF-R1 proteins exhibit only 3 full THDs [61].

Recombinant extracellular domains of goldfish TNF-R1 and TNF-R2 bound to both goldfish recombinant TNFα1 or TNFα2 in *in vitro* binding assays [61]. As with the sea bream TNFα [56], the goldfish recombinant TNFα ligands and receptors all adopted dimeric conformations and interacted as dimers rather than trimers *in vitro*. Interetsingly, there have also been several reports indicateding dimerizion of the mammalian TNF-R1 [131, 132, 147]. Further-

more, the mammalian p75/NTR neurotrophin receptor, a member of the TNF superfamily of proteins with many structural similarities to teleost and mammalian TNF-R1, binds to its cognate NTR ligand as a dimer [26, 57].

By examining the TNF system in lower vertebrates such as teleost fish, we can gain insight into the evolutionary origins of our own immune systems and the selective pressures that shaped them. As in mammals, TNFα appears to be central to the regulation of inflammatory responses of bony fish. Further examination of biological effects of this molecule using different lineages of fish immune cells will yield a more concrete understanding of this system in teleosts.

3.1.4. Inflammatory roles of the fish TNFα

The first functional characterization of a fish TNFα was reported in 2003 when Zou et al. [217] demonstrated that recombinant trout TNFα1 and TNFα2 (isoforms) both induced the IL1β, TNFα1, TNFα2, IL-8 and COX-2 gene expression in primary kidney leukocytes and in the RTS11 trout macrophage cell line [217]. These recombinant TNFα isoforms also elicited dose-dependent chemotaxis of trout kidney leukocytes and phagocytosis of yeast particles. SDS-PAGE analysis of the recombinant TNFα1 and TNFα2 suggested that both of the recombinant molecules existed in monomeric, dimeric and trimeric states. Together, these findings suggested conservation in the pro-inflammatory roles for teleost TNFα.

Intraperitoneal administration of a mature recombinant (cleaved) form of gilthead sea bream TNFα to fish resulted in rapid recruitment of phagocytic granulocytes to the sites of injection, granulopoeisis and the priming for enhanced ROI of peritoneal and primary kidney leukocytes [56]. Intriguingly, size exclusion chromatography of this recombinant sea bream TNFα suggested that this protein existed primarily in a dimeric state [56], in contrast to the trimeric state of the mammalian TNFα.

Subsequent reports indicated that the pro-inflammatory effects of sea bream and zebrafish TNFαs were not direct but resulted from the stimulation of endothelial cells [155]. When sea bream peritoneal and head kidney leukocytes were primed with recombinant TNFα (100 ng/mL) or bacterial DNA (*Vibrio anguillarum*, 50 μg/mL) for 16 hours, TNFα elicited significant ROI responses, albeit modest when compared to those induced by *V. anguillarum* DNA. Intraperitoneal injections of the recombinant TNFα resulted in increased expression of several pro-inflammatory genes of sea bream peritoneal leukocytes, while TNFα treatments of sea bream endocardium endothelial cells (EECs) *in vitro* also increased their immune gene expression. Notably, while the *in vitro* TNFα stimulation of EECs and macrophages resulted in substantially elevated pro-inflammatory gene expression in both cell types, the stimulated macrophages exhibited significantly more robust transcriptional responses. Although this TNFα failed to chemoattract leukocytes, TNFα-stimulated EEC-conditioned medium and supernatants from TNFα-injected peritoneal exudate cells elicited leukocyte chemoattraction. Furthermore, the zebrafish TNFα conferred neutrophil recruitment but also increased fish susceptibility to bacterial and viral infections. Accordingly, the authors of these studies proposed that unlike the mammalian cytokine, the fish TNFα elicits inflammatory functions indirectly, through non-immune cells.

In similar studies, supernatants from recombinant carp TNFα-stimulated cardiac endothelial cells primed kidney phagocyte ROI responses while recombinant carp TNFα1 and 2 did not enhance phagocyte antimicrobial functions [155]. Notably, the recombinant forms of the zebrafish, trout, sea bream and carp TNFα proteins exhibited lytic activity towards *Trypanoplasma borreli*, akin to the trypanolytic capacity of the mammalian TNFs [52], where the membrane form of the fish TNFα was thought to be responsible for these effects. In summation of the above observations, Florenza *et al.* (2009) suggested that while the trypanolyitic roles of TNFα are evolutionarily conserved, the pro-inflammatory mechanisms elicited by this molecule were not [155].

Contrary to the sea bream and carp studies, other literature suggests that akin to the mammalian cytokine, teleost TNFα also directly elicits pro-inflammatory functions. The tilapia non-specific cytotoxic cells (NCCs) constitutively express membrane bound as well as soluble forms of TNFα (in addition to granzymes and Fas ligand) to confer cytolytic activity towards target cells, and when stimulated with recombinant tilapia TNFα, become protected from activation-induced apoptosis [151]. While the turbot recombinant TNFα did not enhance macrophage ROI, it elicited *in vitro* NO production and *in vivo* inflammatory cell recruitment and activation [142]. The Ayu fish, recombinant TNFα induced ROI production by kidney cells [193] and the blue fin tuna recombinant TNFα1 and 2 enhanced the phagocytic responses of tuna PBLs [89].

Zebrafish TNFα elicited cell signaling and conferred increased resistance to *Mycobacterium marinum* [30] while knockdown of the TNF-R1 led to enhanced mycobacterial disease progression, increased fish mortality, accelerated bacterial growth, granuloma breakdown and necrotic macrophage cell death [30]. Thus, it appears that the zebrafish TNFα is pivotal in the maintenance of encapsulated *M. marinum* granulomas and the restriction of the growth of this pathogen. Our recent work supports these findings, where the pre-treatment of goldfish macrophages with recombinant goldfish TNFα2 ablated the *M. marinum*-mediated down-regulation of NO production by these cells and reduced the survival of intracellular bacteria [65].

We recently identified two isoforms of the goldfish TNFα and functionally characterized a recombinant goldfish TNFα2 (rgTNFα2) in the context of primary kidney-derived goldfish macrophage cultures (PKMs) [67]. This rgTNFα2 induced dose-dependent chemotaxis of goldfish macrophages, enhanced their phagocytic abilities, NO production and primed the ROI responses of PKMs.

The extent of the conservation in the biology of teleost TNFα will become more evident with increased availability of tools, reagents and cell culture systems. While some literature proposes a lack of conservation of the inflammatory roles of teleost TNFα, others strongly implicate this molecule in the regulation of fish antimicrobial functions. It is also possible that the discrepancies in the above findings stem from the culture systems employed. For example, both the sea bream and carp studies that failed to observe direct effects of fish TNFα utilized freshly isolated adherent kidney phagocyte populations [52, 155]. Notably, freshly isolated mammalian myeloid populations are highly variable in their antimicrobial capabilities [116]. Similarly, goldfish PKMs exhibit temporal gain and loss of antimicrobial capabili-

ties after prolonged cvultivation [67, 136]. Specifically, ROI is primarily mediated by PKM-monocytes while NO is predominantly produced by mature macrophages [67, 136].

3.2. Interferon-gamma (IFNγ)

Interferon gamma is a pleiotropic, pro-inflammatory and anti-viral cytokine, identified in the supernatants of PHA-activated lymphocyes for its unique anti-viral properties [205]. IFNγ is primarily produced by activated Th1 phenotype CD4+ cells [127], CD8+ cells [161] and natural killer (NK) cells [149] and is of central importance in host defense against intracellular and extracellular pathogens [8, 91, 99, 179, 182]. For example, IFNγ gene knock-out mice are incapable of controlling infections with *Leishmania major* [202], *Listeria monocytogenes* [81], and *Mycobacterium* [32], underlining the importance of this cytokine in the regulation of antimicrobial responses [8, 10, 21, 51, 115].

The mammalian IFNγ dimer ligates the interferon gamma receptor 1 (IFNGR1), which then associates with IFNGR2, forming a signaling complex and activating the Janus kinases (Jak) 1 and 2, associated with the receptor chains 1 and 2, respectively [83]. Jak1 and Jak2 in turn activate the IFNGR1-associated signal transducer of activation-1 (Stat1) transcription factor [33]. The IFNGR ligation may also activate and utilize Stat2 [187], albeit to lesser extent than Stat1. Subsequent transcriptional regulation of several other genes then ensues through homodimeric Stat1, heterodimeric Stat1: Stat2, through the transcription factor complexes ISGF3 and Stat1-p48, composed of Stat1: Stat2:IRF-9 and Stat1: Stat1:IRF-9, respectively [11, 118, 187, 188]. These confer transcriptional changes through recognition of IFNγ-activated sequences (GAS) in the promoter regions of target genes [188]. Within 30 minutes of IFNγ receptor ligation, there are increased transcript levels for several interferon regulatory factors (IRFs), which then modulate subsequent waves of gene expression in the IFNγ signaling cascade [208].

3.2.1. Identification of IFNγ in bony fish

Trout mitogen-simulated leukocyte supernatants possess macrophage activating capabilities (MAFs) akin to the mammalian IFNγ [58, 59], suggesting the existence of an IFNγ counterpart(s) in fish. It was also established that downstream signaling factors employed by the mammalian IFNγ (Stats), were present in fish [160] where the antibody-purified fish Stat-like factor was shown to bind the mammalian IFNγ promoter [160].

The initial fish IFNγ homolog discovery came from examination of fugu gene scaffolds [220]. Fugu homologs of mammalian genes syntenic to IFNγ were also present on the same fugu gene scaffold. This fugu IFNγ had 4 exon / 3 intron organization similar to its mammalian counterpart and shared 32.3-32.7 % and 34.9-43.3% identities with bird and mammalian IFNγ sequences, respectively. An identified trout IFNγ sequence exhibited low sequence identity with other vertebrate IFNγ proteins, but possessed the conserved signature motif ([IV]-Q-X-[KQ]-A-X_2-E-[LF]-X_2-[IV]) and C-terminal nuclear localization signal (NLS), characteristic of the mammalian IFNγ proteins [215].

3.2.2. Inflammatory roles of the fish IFNγ

The recombinant trout IFNγ (rtIFNγ) elicited increased immune gene expression in the RTS-11 monocyte-macrophage cell line [199], while this response was pharmacologically abrogated with ERK (transcription factor) or protein kinase C (PKC) inhibitors as well as by deleting the C-terminal NLS on the rtIFNγ [215]. Also, rtIFNγ stimulation of trout kidney leukocytes primed their ROI production, suggesting functional similarities between the trout and mammalian type II IFNs [215].

Adult zebrafish microinjected with recombinant IFNγ did not exhibit increased immune gene expression or enhanced protection against *Streptococcus iniae* and the spring viremia carp virus (SVCV) [110]. However, this lack of response could stem from IFNγ being bound up by cells expressing only the IFNGR1 without eliciting a detectable level of response from the relatively few cells expressing both IFNGR1 and IFNGR2. If this is the case, these results underline the localized nature of the zebrafish IFNγ function rather then the efficacy of regulation of antimicrobial functions by this cytokine.

We [62] and others [3] have functionally characterized the cyprinid IFNγ. A recombinant goldfish IFNγ (rgIFNγ) primed goldfish monocytes ROI response in a concentration dependent manner [62] and, as in mammals [72, 157], at lower concentrations rgIFNγ conferred additive ROI priming effects. The rgIFNγ also enhanced the expression of the ROI enzyme, NADPH oxidase, catalytic subunits, $p67^{phox}$ and $gp91^{phox.}$ Similarly, the recombinant carp IFNγ (rcIFNγ) also primed carp kidney phagocytes for enhanced ROI production [3].

The goldfish rIFNγ elicited modest but significant enhancement of phagocytosis and NO production by goldfish monocytes and macrophages, respectively [62]. This was paralled with increased iNOS gene expression in rgIFNγ-stimulated macrophages. In contarst, carp kidney phagocytes only displayed significant iNOS gene expression and NO production when treated with a combination of carp recombinant IFNγ and 30 μg/mL LPS, but not following rcIFNγ treatments alone [3].

Since carp and goldfish are closely related species, the above discrepancies presumably stem from differences in experimental systems. Akin to bone-marrow-derived macrophages, which acquire antimicrobial capabilities with culture time, we have observed that the antimicrobial capabilities of goldfish kidney-derived phagocytes are dynamic [136, 137]. For this reason, we used culture-derived cells, while the carp studies used freshly isolated phagocytes. Additionally, it is well established that in mammals the production of ROIs is seen as early as 1 hour after immune stimulation, whereas the production RNIs is first detected approximately 24 hours post stimulation [169]. This is very similar to what we have observed with rgIFNγ-elicited iNOS expression and NO responses.

The treatment of mature goldfish macrophages with rgIFNγ resulted in increased expression of TNFα and IL-1β isoforms; IL-12 p35 and p40; IFNγ; IL-8 (CXCL-8]; CCL-1; and viperin (an anti-viral molecule) [62]. The treatment of carp phagocytes with a combination of carp IFNγ and LPS resulted in increased expression of TNFα; IL-1β; IL-12 subunits p35 and isoforms of IL-12 p40 subunit [3]. The carp IFNγ also induced the expression of the CXCL-10 like chemokine, CXCLb, while inhibiting the LPS-induced expression of CXCL-8 isoforms,

CXCL-8_L1 and CXCL-8_L2 [3]. The latter is reminiscent of the mammalian IFNγ and CXCL-8 relationship, where shorter treatments of mammalian granulocytes with IFNγ causes decreased CXCL-8 production [23, 90, 120], while prolonged treatments increase the CXCL-8 mRNA and protein levels [90]. Additionally, mammalian blood monocytes and macrophage cell lines stimulated with IFNγ up-regulate CXCL-8 mRNA transcripts and protein levels [14, 36] due to post-transcriptional stabilization of the CXCL-8 mRNAs rather than gene expression [14]. Together, these findings suggest that the pro-inflammatory roles of IFNγ, including synergism with LPS, are conserved in cyprinid fish, while it is probable that the discrepancies in gene expression profiles may be due to distinct immune cell model systems used (primary kidney phagocytes versus cultured mature macrophages).

3.2.3. Interferon-gamma-related (IFNγrel) cytokine

Using gene synteny analysis, Igawa *et al*. (2006) discovered two tandem IFNγ isoforms next to the fish IL-22 and IL-26 genes [82]. The corresponding IFNγ sequences, coined IFNγ1 and IFNγ2, were later renamed IFNγ-related (rel) and FNγ respectively [166]. These proteins share only 17 % amino acid identity, but exhibit exon/intron organization similar to that of mammalian and fugu IFNγ, and have the IFNγ signature motif ([IV]-Q-X-[KQ]-A-X_2-E-[LF]-X_2-[IV]). Interestingly, only IFNγ but not IFNγrel has a C-terminal nuclear localization signal (NLS).

The existence of two IFNγ isoforms, sharing all of the above characteristics was soon confirmed in siluriformes and other cypriniformes when IFNγ and IFNγrel were identified in the catfish [122] and common carp [183], respectively. Catfish and carp were both reported to possess two distinct, alternatively spliced IFNγ transcripts, as well as a single transcript of IFNγrel [122, 183]. The pattern of tissue gene expression of catfish IFNγ and IFNγrel differed while both cytokines were expressed in various cell lines and immune cells, with highest expression observed in macrophages, T cells and NK cells [122]. Interestingly, low transcript levels of IFNγrel but not IFNγ were also reported in a catfish B-cell line. Expression of the carp IFNγ increased in T cells after stimulation with PHA, while the mRNA levels of carp IFNγrel increased in IgM^+, B lymphocyte-enriched immune cell fractions, stimulated with high doses of LPS [183]. Furthermore, increased transcript levels of the carp IFNγ, but not IFNγrel, were observed in the head kidneys of fish infected with *T. borreli*. The goldfish IFNγ and IFNγrel exhibited similar mRNA levels across tissues and immune cell types [63].

3.2.4. Inflammatory roles of fish IFNγrel

While the siluriforme IFNγrel has yet to be functionally characterized, there has been some insight into the functions of cyprinid IFNγrel. Zebrafish IFNγrel mRNA is detectable in freshly laid eggs, suggesting maternal supply of this transcript [174]. The IFNγrel mRNA levels persist throughout embryonic development, while the expression of IFNγ is not detected until later stages of development. Embryos injected with *in vitro* transcribed IFNγ or

IFNγrel mRNAs individually, exhibited similar immune gene expression changes, while combined injections further increased certain expression profiles, suggesting non-overlapping roles for the respective IFNγ proteins.

Morpholino knock-downs of either IFNγ or IFNγrel alone had negligible effects on zebrafish embryo survival following *Escherichia coli* challenge, while knock-down of both IFNγs resulted in substantially diminished survival following infection [174]. Interestingly, individual morpholino knockdowns of IFNγ or IFNγrel caused decreased survival rates of embryos infected with *Yersinia ruckeri,* while double knockdowns had a further deleterious effect on embryo survival. Presumably IFNγ and IFNγrel elicit some overlapping and some distinct antimicrobial mechanisms such that the presence of one cytokine may be sufficient for dealing with certain pathogens but not others. It is noteworthy that while the *E. coli* (strain DH5α) is not a natural fish pathogen, the *Y. ruckeri* is [159].

A comprehensive functional investigation of recombinant goldfish (rg) IFNγrel and rgIFNγ revealed that these molecules differed in respective capacities to modulate pro-inflammatory responses of goldfish PKMs [63]. While rgIFNγ conferred long-lasting ROI priming effects, rgIFNγrel induced short-lived ROI priming, causing subsequent unresponsiveness to ROI priming by other recombinant cytokines (rgIFNγ or rgTNFα2). While rgIFNγ elicited modest phagocytosis and nitric oxide responses in goldfish monocytes and macrophages, respectively [62, 63], rgIFNγrel was a highly potent inducer of both responses. Interestingly, rgIFNγ and rgIFNγrel induced different gene expression profiles in goldfish monocytes, where rgIFNγrel elicited significantly greater expression of key inflammatory genes. Notably, while both cytokines induced the phosphorylation of Stat1, its nuclear translocation was only observed following rgIFNγ treatment. Together, these findings suggest a functional segregation of the goldfish type II interferons in the regulation macrophage antimicrobial functions.

Further confirmation of this functional dichotomy between the fish type II IFNs is warranted using *in vivo* and other *in vitro* fish models. Notably, the zebrafish IFNγrel has recently also been demonstrated to elicit more robust pro-infalmmatory gene expression than IFNγ in larvae microinjected with respective IFN expression constructs [110]. Furthermore, these zebrafish IFNγrel-mediated effects were dependent on the myeloid transcription factor SP1, underlying the specificity of this cytokine for macrophag-lineage cells.

3.2.5. IFNγ receptors in fish

Despite the growing knowledge regarding the teleost type II IFNs, the receptor systems employed by these fish molecules remain poorly understood. The IFNGR1 and IFNGR2 chains were recently identified in the rainbow trout [55]. The expression of the IFNGR1 was generally greater than that of IFNGR2 and was subject to decrease following rIFNγ or rIL1β stimulation. Furthermore RTG-2 trout fibroblast cells transfected with an IFNGR1 construct, or CHO cells transfected with constructs expressing IFNGR1 and IFNGR2, both bound rtIFNγ, where, as in mammals, the expression of the IFNGR2 chain was essential for the IFNγ-induced activity. It should be noted that a reliable trout IFNγ reporter cell line has been estab-

lished and was effectively demonstrated to specifically increase luciferase reporter expression following IFNγ stimulation but not in response to a range of other stimuli [24, 25]. Hence, a system is now in place to elucidate the distinct binding and signaling mechanisms involved in the salmonid IFNγ biological processes.

In light of the functional differences between IFNγ and IFNγrel, we postulated that these cytokines might function through distinct receptors. When we performed gene synteny analysis of IFNGR1, we observed that while some genes were localized to the chromosome bearing the known zebrafish IFNGR1 gene, other syntenic neighbours of the mammalian IFNGR1 were present on a distinct zebrafish chromosome. Further analysis of the chromosomal region flanked by these genes revealed a second gene, encoding a distinct IFNGR1 protein [60], (these genes were denoted IFNGR1-1 and IFNGR1-2). The corresponding goldfish receptor cDNA transcripts were identified. The fish IFNGR1 sequences displayed putative Jak1 and Stat1 binding sites, pivotal for the biological functions of the mammalian IFNγ [49, 68, 69]. While the zebrafish receptors displayed comparable tissue expression, the goldfish IFNGR1-1 exhibited substantially greater mRNA levels than the IFNGR1-2 in all tissues and immune cell types examined. In order to elucidate possible binding partners for the goldfish IFNGR1-1 and IFNGR1-2, recombinant forms of their extracellular domains were produced and *in vitro* binding assays were performed. While IFNGR1-1 bound exclusively to IFNγrel (IFNγ1), IFNGR1-2 bound strictly to IFNγ (IFNγ2, receptors were named after the fact). It has recently been reported that morpholino knockdowns of IFNGR1-1, IFNGR1-2 or a putative IFNGR2 abolished zebrafish IFNγ-induced gene expression [1]. In contrast, only the knockdown of IFNGR1-1, but not the knockdown of IFNGR1-2 or IFNGR2, abrogated gene expression elicited by IFNγrel. It was suggested that IFNγ might signal through a heterodimer of IFNGR1-1 and IFNGR1-2 and a homodimer of IFNGR2 while the IFNγrel would ligate with a homodimeric IFNGR1-1 and an as of yet unidentified receptor 2 chains. Alternatively, since IFNγrel is present in cyprinids early in development, the knockdown of its putative receptor, IFNGR1-1 in embryos might effect development of the components required for IFNγ function. A biological relationship of such nature would be phenotypically manifest as a loss of IFNγ function (as seen in the above zebrafish study).

It would appear that certain teleost species possess receptor signaling systems to facilitate a dichotomy of type II IFN functions. Presumably, the cyprinid IFNGR1 genes arose from duplications of an ancestral IFNGR1 and subsequently diverged in respective signaling mechanisms used, where the IFNγrel-induced Stat1-phosphorylation might be an artifact, remnant of the ancestral gene. Indeed, the importance of the C-terminal NLS of fish (and mammalian) IFNγ has been demonstrated [217] while the lack of this NLS on IFNγrel proteins is the key distinguishing feature of the latter cytokine. The leading model for mammalian IFNγ signaling [185] suggests that after IFNγ receptor ligation, Stat1 is delivered into the nucleus via the IFNγ NLS in a complex consisting of Stat1:IFNGR1:IFNγ. Therefore, due to a lack of an NLS, IFNγrel may have evolved to utilize distinct signaling mechanism.

3.3. Interleukin-1 beta (IL-1β)

3.3.1. Interleukin-1 cytokine family

The interleukin-1 cytokine family is becoming increasingly diverse, with new family members being discovered and/or assigned to this family. In addition to the well characterized IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) and IL-18 the new members of the family now include the IL-1F5-10, and IL-1F11 [34, 192]. Accordingly, it has been proposed that IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) should be renamed IL-1F1, IL-1F2, IL-1F3 and IL-1F4, respectively [177]. The exact biological roles of these new IL-1 cytokines (IL-1F5-11) have not been fully elucidated, and it was reported that concentrations of 100-1000 fold greater than those of IL-1β are required to them to induce biological effects [177, 178]. These new family members will not be discussed further, instead the remainder of this section will deal with the classical IL-1 cytokines and primarily IL-1β.

The IL-1α [106] and IL-1β [6] were initially identified as monocyte transcripts with only 23% amino acid identitiy but with structurally similarities. Both of these cytokines are produced as leaderless 31 kDa pro-peptides that are cleaved to generate mature 17 kDa molecules, which mediated their respective effects by binding to the IL-1RI [37, 38, 40]. The synthesis of IL-1 has been reported in several cell types, including keratinocytes, Langerhan's cells, synovial fibroblasts, mesangial cells, astrocytes, microglia, corneal cells, gingival cells, thymic epithelial cells, in addition to myeloid and some lymphoid cell types (reviewed in references [38, 40]). Although IL-1α and IL-1β signal through the same receptor, they induce different biological functions. It has been suggested that IL-1α is produced primarily by epithelial cells and keratinocytes [125] and is involved in mediating local inflammatory processes. In contrast, IL-1β is synthesized by cells such as monocytes, macrophages, Langerhan's cells and dendritic cells [38], and mediates systemic inflammatory responses [37]. This is corroborated by the fact that while IL-1α may act as a membrane-bound pro-IL-1α through myristolation of the protein [100, 181], IL-1β requires intracellular processing for activation.

As mentioned, IL-1α and IL-1β are both produced as pro-peptides and while IL-1α can mediate biological effects as pro-IL-1α or as a mature IL-1α following enzymatic cleavage (e.g. with calpain) [96], only the proteolytically cleaved mature IL-1β, but not the pro-IL-1β can elicit immune functions. In fact, the release of IL-1β appears to be a two-step process, whereby the first stimulus such myeloid cell encounter of a pathogen results in increased synthesis and cytosolic accumulation of pro-IL-1β, while a second (as of yet poorly defined) stimulus induces the proteolytic processing of pro-IL-1β by caspase-1/IL-1β-converting enzyme (ICE), and the subsequent release of the biologically active mature IL-1β [39]. This processing event is further enhanced by the presence of extracellular ATP, which is recognized by the ATP receptor $P2X_7R$, causing an efflux of K^+ and a concomitant activation of ICE [50]. A number of mechanisms have been proposed for the release of the mature IL-1β into the extracellular milieu. These include: exocytosis of IL-1β containing lysosomes; through microvesicular budding of the plasma membrane; release in exosomes by fusion of multivesicular bodies with the plasma membrane; export through specific membrane transporters; and cell-lysis-mediated release (reviewed in [46]). Possibly, these mechanisms are mutually ex-

clusive and/or cell type dependent. Further research in multiple model organism and cell types is warranted before specific mechanism(s) can be defined.

Both IL-1α and IL-1β bind to IL-1RI, resulting in recruitment of the IL-1R associated protein (IL-1RAcP), which amplifies the signal transduction [176]. The IL-1RI is structurally related to the toll-like pattern recognition receptors where the signal propagation through the IL-1RI involves many of the same downstream signaling components (MyD88, IL-1R associated kinase (IRAK), and NFκB) as those employed in TLR signaling [139]. IL-1α and IL-1β also bind to the IL-1RII, but because this receptor lacks the intracellular signaling components of IL-1RI, it functionally serves as a "decoy" receptor by dampening the IL-1α/β signal transduction [31]. Additionally, the IL-1 receptor antagonist (IL-1Ra) exhibits competitive inhibition of the IL-1α/β signaling by interacting with the IL-1RI without eliciting the downstream signaling events [31].

Unlike most other cyokines and growth factors IL-1α and IL-1β (primarily IL-1β) target nearly every cell type and induce a range of biological processes (reviewed in references [37, 38, 41]). Some of the numerous pro-inflammatory roles of IL-1β include increases in collagen and pro-collagenase synthesis; increase of chondrocyte protease and proteoglycan release; increase in osteoclast-activating factor release and hence bone resorption; induction of the synthesis of lipid mediators such as PGE_2; and enhancement of the proliferation of fibroblasts, keratinocytes, mesangial, glial cells and smooth muscle cells. IL-1β also induces chemotaxis of T and B cells, the synthesis of thromboxane by neutrophils and monocytes, basophil histamine release and eosinophil degranulation. Additionally, IL-1β induces synthesis of type I IFNs, endothelial plasminogen activator inhibitors, and expression of leukocyte adherence receptors on endothelial cell surfaces. Based on the above, it is not surprising that IL-1β has been implicated in numerous disorders including cardiac disease [15], rheumatoid arthritis [92], and neurodegenerative diseases [71].

3.3.2. Identification of IL-1 in fish

Fish were first suspected of possessing an IL-1 homolog when the mammalian PBL-derived IL-1 was demonstrated to enhance the proliferation of catfish T-lymphocytes in response to ConA [74], while carp epithelial cells [175], carp macrophages and granulocytes [195] and catfish monocytes [47], were shown to produce factor(s) with properties similar to those of the mammalian IL-1. Gel filtration analysis of catfish monocyte revealed two distinct bands of approximately 70 kDa and 15 kDa, recognized by polyclonal antibodies raised against mammalian IL-1α and IL-1β. Paradoxically, the catfish 70 kDa molecule activated catfish (but not mouse) T cells, while the 15 kDa molecule activated mouse (but not catfish) T cells [47].

The first fish IL-1β cDNA sequence was identified in trout and exhibited 49-56% amino acid identity to the mammalian IL-1β [170]. Notably, the trout IL-1β did not possess a putative ICE cleavage site required for the maturation-cleavage of the mammalian IL-1β, while the gene expression of this trout cytokine could be induced in tissues and head kidney leukocytes following LPS stimulation [170, 216], suggesting its pro-inflammatory nature.

Although there have been no reports of a fish IL-1α, recently a novel IL-1 family member, nIL-1F has been identified in trout and *Tetraodon* spp. and was reported to have IL-1 family signature motif as well as a putative ICE cleavage site [198]. The expression of nIL-1F increased after activation of macrophages with LPS or recombinant IL-1β. Furthermore, a recombinant form of the C-terminal nIL-1F abrogated the rIL-1β-induced immune gene expression in RTS-11 trout macrophage-like cells, suggesting a possible competitive inhibition through the trout IL-1RI. This mechanism would be analogous to the mammalian IL-1Ra, which also competes with IL-1β for IL-1RI binding, without inducing downstream events.

3.3.3. Isoforms of IL-1β in fish

Because many fish species are tetraploid and have undergone genome duplication events, it is not surprising that additional isforms of IL-1β exist in certain fish species. An identified second trout IL-1β, denoted IL-1β2, also has the 6 exon/5 intron organization, 82% amino acid identity to the trout IL-1β1 and no putative ICE cleavage site [150]. In catfish, two IL-1β genes have been described and shown to undergo distinct expression patterns following challenge with *Edwardsiella ictaluri* [201]. Additionally identified cDNA of another carp IL-1β, IL-1β2, exhibited 74% identity with the carp IL-1β1 and 95-99% identity across individual IL-1β2 transcripts [48]. Since several distinct IL-1β2 sequences were identified in a homozygous individual, it was suggested that there might be multiple IL-1β2 genes. The expression of the carp IL-1β1 and IL-1β2 differed following immune stimuli where the expression of IL-1β1 gene was on average at least ten fold greater than that of IL-1β2. It was also reported that transcripts of IL-1β2 had high substitution numbers in the coding regions, including key areas predicted to be involved in receptor binding. In light of the above, it was suggested that the IL-1β2 may be a pseudogene [48]. Our observations in the goldfish, a close carp relative, support this theory. Notably, the predicted goldfish IL-1β2 protein is truncated compared to the IL-1β1, and while the expression of both IL-1β isoforms is subject to change following immune stimuli, specific treatments elicit more robust changes in the expression of IL-1β1 compared to IL-1β2 (unpublished observations).

3.3.4. Maturation cleavage of the fish IL-1β

As alluded to above, despite the functional similarities of the fish IL-1βs and their mammalian counterpart, all fish IL-1β proteins identified to date lack the typical ICE cleavage site necessary for the functional maturation of the mammalian IL-1β1. Despite this, evidence suggests that indeed the fish IL-1β proteins undergo cleavage. Early fish IL-1 studies using anti-mammalian IL-1α/β serum to surveys supernatants of activated fish cells demonstrated the recognition of distinct, multiple IL-1 protein species in these supernatants. While the catfish monocyte supernatants contained 70 kDa and 15 kDa molecules with IL-1-like activity [47], carp macrophages secreted 22 kDa and 15 kDa IL-1-like factors that were recognized by mammalian anti-IL-1 antibodies [195]. The immunoprecipitation experiments using the anti-mammalian IL-1 polyclonal antibodies recognized the 15kDa factor from the macrophage supernatants, suggesting possible maturation events of the carp IL-1 [195]. In an independent survey of PHA-stimulated carp leukocytes using a monoclonal anti-carp IL-1β antibody,

the 15 kDa protein was confirmed to be a mature carp IL-1β, with the putative carp IL-1β cleavage site situated approximately 15 amino acids downstream of the mammalian ICE site [117]. Analysis of the supernatants of the trout macrophage cell line, RTS-11, using an anti-trout IL-1β polyclonal antibody detected putative native, as well as potentially cleaved trout IL-1β proteins of 29 kDa and 24 kDa, respectively [79]. When the RTS-11 cells were transfected with a plasmid encoding a C-terminally His^6 tagged trout IL-1β, and the RTS-11 supernatants assayed using an Ni-NTA-column specific for the His^6 tagged IL-1β, the authors detected primarily a mature, 24 kDa IL-1β protein, confirming that the trout IL-1β underwent maturation cleavage.

In an elegant set of studies it was demonstrated that combined immune stimuli of sea bream head kidney leukocytes resulted in an accumulation of a 30 kDa, pro-IL-1β protein, which, unlike the mammalian counterpart [113], did not exhibit a maturation cleavage or secretion following stimulation of the cells with extra-cellular ATP [146]. Furthermore, sea bream peritoneal acidophilic granulocytes and peripheral blood leukocytes accumulated the 30 kDa form of IL-1β following challenge with *Vibrio anguillarum* [27]. In contrast, the sea bream SAF-1 fibroblast cell line shed a mature, 22 kDa IL-1β protein through microvesicular plasma membrane budding within 30 minutes of treatment with extracellular ATP [113]. Interestingly this IL-1β maturation/shedding process could be ablated with a pharmacological inhibitor of the mammalian ICE, suggesting a presence of an orthologous sea bream enzyme responsible for this process.

The $P2X_7R$ receptor is the primary receptor responsible for the recognition of extracellular ATP and the concomitant release of mature IL-1β [50]. Upon ATP treatment and activation of the HEK 293 mammalian cell line expressing the rat $P2X_7R$, human ICE and sea bream IL-1β, a non-cleaved (30 kDa) sea bream IL-1β was secreted by these cells [109]. Interestingly, neither sea bream nor zebrafish $P2X_7R$ expression in ATP-stimulated HEK 293 cells resulted in sea bream or mammalian IL-1β secretion. In contrast, the expression of a chimeric $P2X_7R$ bearing the sea bream ATP-binding and rat intracellular domains led to maturation/secretion of the mammalian IL-1β, while the outcomes of this combination on the sea bream IL-1β were not addressed [109]. The authors of this work suggest that the mechanisms involved in IL-1β secretion are conserved across vertebrates while the distinct stimuli that elicit the maturation events are not. We propose that alternatively, functional specificity may stem from the $P2X_7R$ intracellular signaling, activation of species specific ICE (or alternative maturation mechanisms), and the substrate specificity of the fish Caspase1/ICE. Notably, the above group also identified the sea bream Caspase 1 and demonstrated that the recombinant form of this fish enzyme cleaved a commercially available substrate, for which the mammalian counterpart holds specificity [107]. Additional studies are needed to establish the association between the sea bream Caspase I/ICE, $P2X_7R$ and IL-1β.

3.3.5. IL-1 receptors of fish

Majority of the IL-1 receptor signaling components have been described in fish. A fish IL-1RI was first described in Atlantic salmon, with 43-44% similarity to chicken and 31% similarity to the human IL-1RI, respectively [186]. The expression of this gene increased in fish tissues

following LPS injection. The identification of a carp IL-1R1 was also described and the receptor characterized in the context of acute stress conditions [121].

The trout IL-1RII was identified through a selective subtractive hybridization of genes upregulated following immune stimulation [164]. This receptor displayed low sequence identity with the mammalian IL-1RII, but exhibited surprisingly similar overall gene organization including a very short intracellular domain. The gene expression of the identified sea bream IL-1RII increased in stimulated macrophages to levels 15 times greater than IL-1β expression [108], suggesting a conservation in the roles of the sea bream IL-1RII as a "decoy" receptor. Furthermore, sea bream IL-1RII expressed on HEK 293 cells bound the recombinant IL-1β, confirming the specificity of this receptor-ligand pair.

Enhanced understanding of the evolutionary mechanisms responsible for shaping the vertebrate IL-1 biological pathways will be achieved through studies that examine the IL-1 receptor-ligand relationships and biological outcomes of these interactions in teleosts.

3.3.6. Inflammatory roles of the fish IL-1β

Using the selective subtractive hybridization technique, a carp IL-1β was identified [53] and the C-terminus of the cytokine produced as a recombinant protein [206]. This recombinant carp IL-1β dimerized and enhanced carp antibody responses to *Aeromonas hydrophila*, where sera from carp co-injected with the IL-1β and *A. hydrophila* had a greater agglutinating capacity than the respective controls.

A number of studies have since utilized recombinant technology to investigate the functions of the fish IL-1β. A recombinant form of the mature trout IL-1β (rtIL-1β) was produced by Hong and co-workers [80] and shown to enhance the expression of the MHCII β chain, IL-1β and COX-2 genes in trout head kidney leukocytes and the macrophage cell line, RTS-11. Functionally, rtIL-1β elicited the proliferation of trout head kidney cells as well as the proliferation of a murine cell line, D10.G4.1, known for its dependence on the mammalian IL-1β [80]. Also, rtIL-1β enhanced the phagocytosis of yeast particles by trout head kidney cells [80] while peritoneal admininstrations of rtIL-1β induced migration of trout leukocytes to the site of injection, enhanced phagocytosis of peritoneal cells and increased the systemic expression of IL-1β, COX-2 and lysozyme II [77]. The injection of fish with rtIL-1β also enhanced trout resistance to infection with the fish pathogen *A. salmonicida*. Additionally, the rtIL-1β and the recombinant sea bass IL-1β were demonstrated to induce Ca^{2+} mediated downstream signaling events, abrogated by leukocyte trypsin-treaments and indicating a requirement for receptor engagement [9]. Interestingly, these authors reported that IL-1β of trout, sea bass and humans were highly species specific, which is in contradiction of the early fish IL-1β carp and catfish work, where cross-reactivity was observed [9, 47, 74, 175, 195].

The distinct biological roles of individual sub-domains of the trout IL-1β were also examined by generating appropriate peptides [78, 144, 145]. While a control scrambled peptide (P2) had no effect and the peptide corresponding to the putative trout IL-1β receptor binding region (P1) had little effect on its own, a peptide (P3) corresponding to an alternative receptor binding area of the mammalian IL-1β [5, 196], had chemotactic properties towards

head kidney cells alone or in combination with the P1 peptide (at low concentrations) [145]. Intraperitoneal injections of the rtIL-1β P3 peptide induced peritoneal leukocyte migration and enhanced phagocytosis and reactive oxygen production by peritoneal cells. Additionally, the rtIL-1β P3 conferred fish resistance to viral hemorrhagic septicemia virus (VHCV) early after injection. Surprisingly, injection of rtIL-1β P1 caused an increase in *in vivo* expression of the antiviral gene Mx and inhibition of trout TNFα expression, while having no effects on the expression of a panel of other pro-inflammatory genes [78]. The injection of the rtIL-1β P3 peptide resulted in a more robust, widespread pro-inflammatory response with increased expression in trout rtIL-1β, IL-8 and lysozyme. This work suggests that the fish IL-1β utilizes a highly complex receptor-ligand system, possibly unique to that of mammals.

The functional roles of the trout IL-1β have been corroborated in others fish species. For example, injection of carp with a plasmid encoding the carp IL-1β gene caused enhanced PHA-induced proliferation of carp lymphocytes, increased carp macrophage reactive oxygen production, enhanced phagocytosis and improved protection against *A. hydophila* challenge [97]. A sea bass recombinant IL-1β exhibited immuno-adjuvant properties when combined with rsIL-1β in immunization trials against the pathogen, *V. anguillarum*. The sea bass IL-1β also induced the proliferation of the murine IL-1β reporter cell line (D10.G4.1), sea bass thymocytes, enhanced kidney leukocyte phagocytosis and activated peritoneal macrophages when administered i.p. [16, 17, 29]. The orange spotted grouper rIL-1β stimulated the proliferation of grouper head kidney leukocytes and increased the gene expression of IL-1β and COX-2 through p38 MAPK and Jnk signaling pathways [112]. Together, the above findings suggest that the functions of IL-1β have been evolutionarily conserved in vertebrates.

4. Concluding remarks

A successful inflammatory response is defined by the presence and proficient coordination of cytokine networks consisting of hallmark mediators such as TNFα, IFNγ and IL-1β. The synchronized involvement of these pleiotropic yet functionally distinct agents in the recruitment, regulation and functional polarization of inflammatory cells dictates the outcome of the mounted response. Thus, it can be argued that the inflammatory processes are largely defined by the efficacy of the individual and interdependent cytokine pathways.

The regulation of the vertebrate inflammatory response is complex, involving numerous mechanisms, some of which are poorly understood while others remain to be identified. This is particularly true for the teleost model systems, where lack of specific reagents for different fish species hampers our ability to examine different aspects of the regulation of inflammation at a mechanistic level. However, there is growing evidence that the key immune components required for effective inflammatory responses are present in teleosts. Notably, certain fish species possess additional pathways that regulate inflammatory processes (for example IFNγrel and its receptor IFNGR1-1, novel chemokines and PRRs) that are distinct from those reported in mammals. The elucidation of the coordination of inflammatory responses by these factors may shed new light on the evolution of innate host defense mechanisms in lower vertebrates.

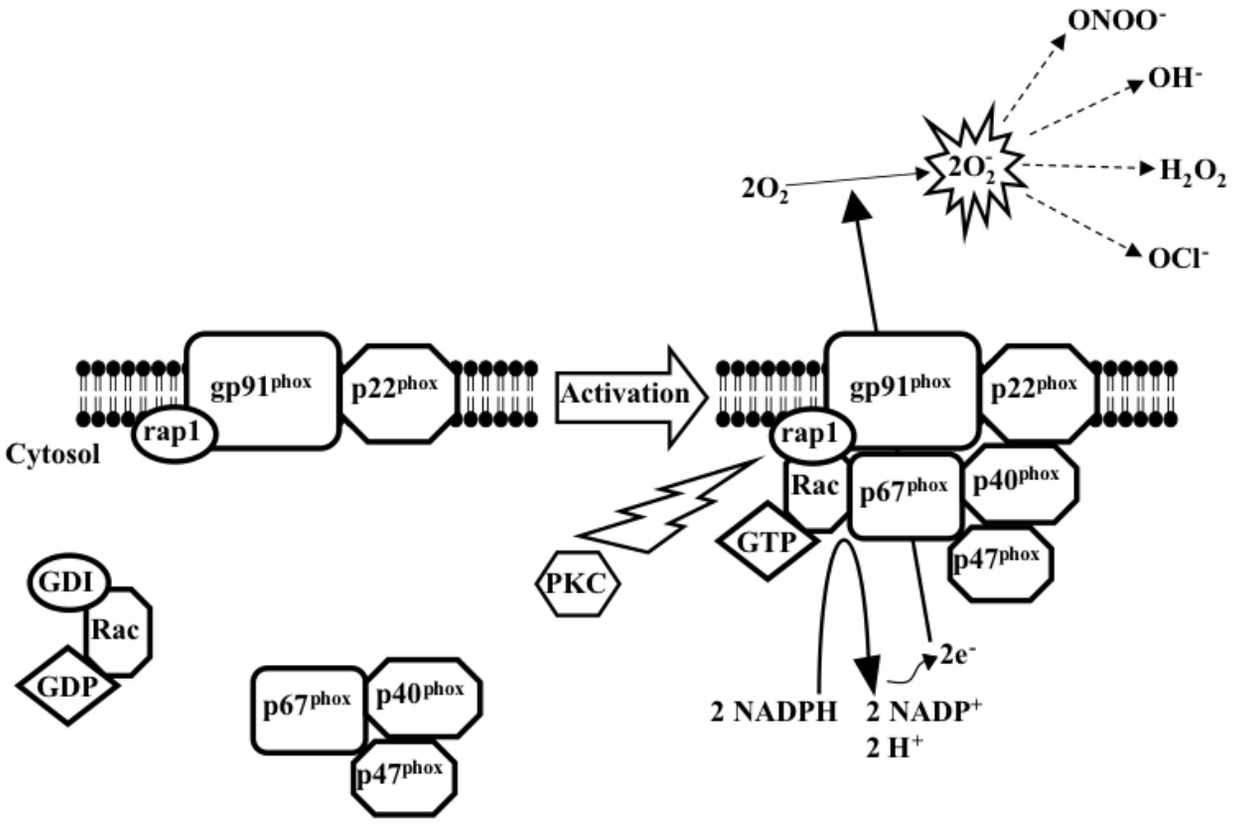

Figure 1. Schematic representation of the NADPH oxidase complex mobilization and reactive oxygen production following phagocyte activation. In a resting state, the gp91^{phox} and p22 ^{phox} components are membrane bound while the p40 ^{phox}, p47 ^{phox} and p67 ^{phox} components are located in the cytosol and the small G-protein Rac is GDP bound. Upon cell activation, Rac is rapidly converted from a GDP- to a GTP-bound state and facilitates the translocation and assembly of the cytosolic NADPH oxidase components at the cell membrane. Following PKC activation, Rap1 is thought to serve as the final switch in the activation of the NADPH complex. The activated NADPH oxidase complex accepts the electrons form the reduced NADPH and transfers these to molecular oxygen, forming the superoxide anion (O_2^-). The generated O_2^- can subsequently be converted to other reactive oxygen species. Reviewed in references [124, 153, 154, 173].

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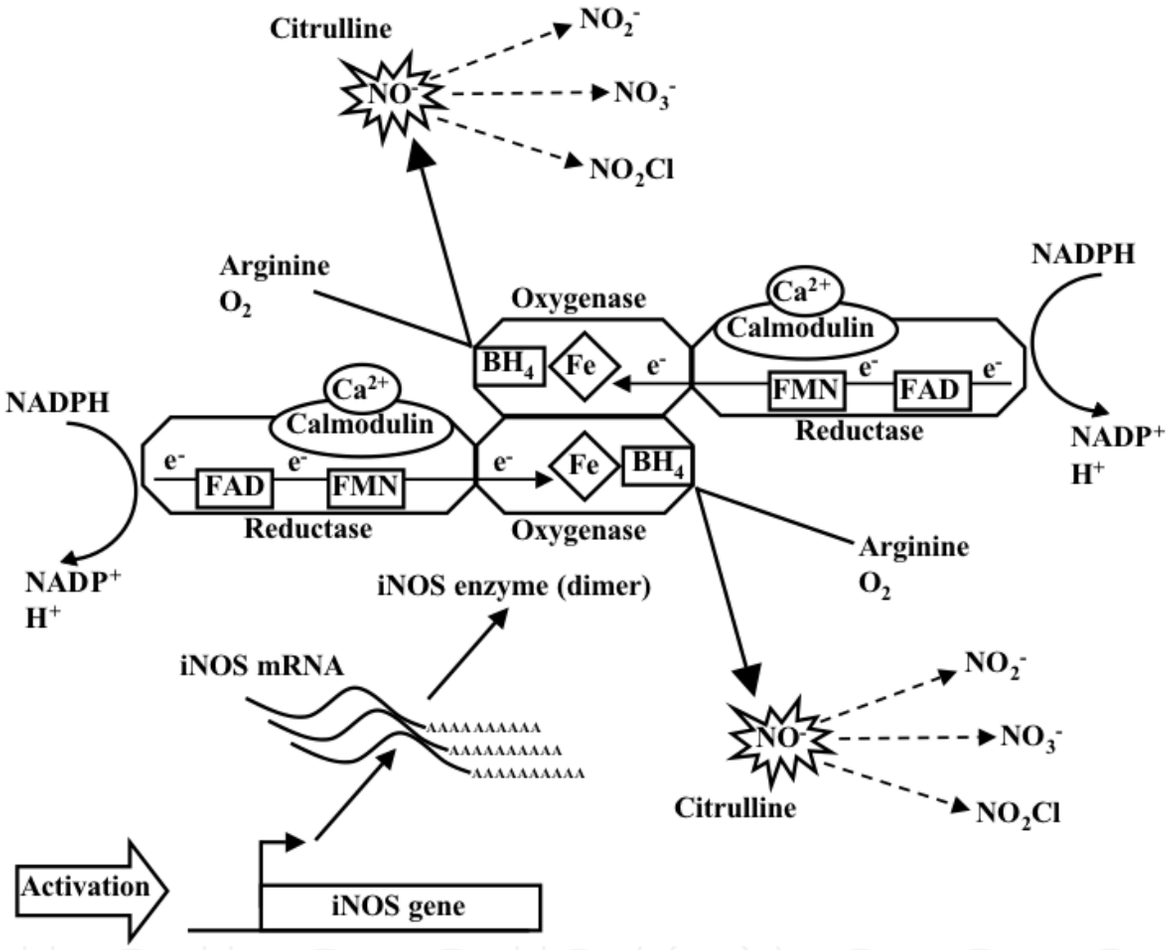

Figure 2. Schematic representation of iNOS gene expression, iNOS protein synthesis and enzymatic production of nitric oxide by the iNOS dimer complex. Upon phagocyte activation, there is a substantial increase in the expression from the iNOS gene and de novo synthesis of the iNOS enzyme. The iNOS enzyme forms a dimer and associates with a Ca^{2+} bound calmodulin, stabilizing the structure and facilitating the function of the enzymatic complex. The iNOS dimer catalyzes the transfer of electrons from a reduced NADPH, through a series of cofactors (FAD, FMN, Fe, BH_4) in the oxidation of L-Arginine to L-Citrulline with a concomitant production of nitric oxide (NO^-). The generated NO^- can subsequently be converted to other reactive nitrogen species. Reviewed in references [2, 111, 126, 172].

Abbreviations

ConA: concanavalin A; **GAS:** γ-IFN-activated sequence; **Jak:** janus activated kinase; **ICE:** IL-1 cleaving enzyme; **IFN:** interferon; **IFNGR:** interferon gamma receptor; **IL:** interleukin; **IL-1R:** interleukin-1 receptor; **IL-1RAcP:** IL-1R associated protein; **iNOS:** inucible nitric oxides synthase; **IRAK:** IL-1R associated kinase; **IRF:** interferon regulatory factor; **MAF:** macrophage activating factor(s); **NK:** natural killer; **NLS:** nuclear localization signal; **NO:** nitric oxide; **NTR:** neurotropin receptor; **PBL:** peripheral blood leukocyte; **PHA:** phytohemaglutanin; **PKC:** protein kinase C; **PKM:** primary kidney macrophage; **PMA:** phorbol myrystate acitate; **PRR:** pattern recognition receptor; **rg:** recombinant goldfish; **RNI:** reactive nitrogen intermediates; **ROI:** reactive oxygen intermediates; **Stat:** signal transducer of activation transcription factor; **TACE:** TNFα cleaving enzyme; **TLR:** toll-like receptor; **TNF:** tumor necrosis factor; **TNFR:** tumor necrosis factor receptor.

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