NOD-like receptors in fish: evolution, structure, immune signaling, and targeting for aquaculture vaccine adjuvants

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Teleost fish possess a highly diverse innate immune system, which is well-adapted to the pathogen-rich aquatic environment in which they reside. NOD-like receptors (NLRs), a conserved family of cytosolic pattern recognition receptors, are at the center of this defense mechanism, activating immune responses, recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Here, we present an integrative overview of the current state of fish NLRs in terms of their evolutionary diversification, structural framework, signaling pathways, and functional roles in the context of bacterial, viral, and parasitic pathogens. We discuss six principal NLRs: nucleotide-binding oligomerization domain-containing protein 1 (NOD1), NOD2, NLRC3, NLRC5, NLR family member X1 (NLRX1), and NLR family pyrin domain-containing 1 (NLRP1), highlighting their domain structures, 3D conformations, and downstream signal chains. We focused on the immune regulatory roles of NLR family acidic transactivation domain-containing (NLRA) and NLR family CARD domain-containing (NLRC) subfamily components, the formation of the NLRP1 inflammasome, and the new roles of mitochondrial-specific NLRs in antiviral immunity. We discuss future directions for NLRs as immunological targets in aquaculture, referencing known NLR-activating adjuvants, exploring their ligand specificity, and highlighting challenges like functional redundancy. Much of the insight into the fish NLRs in this review comes from their well-researched mammalian counterparts. NLR-based immune modulation represents the ability of these receptors to detect microbial or danger signals and regulate key signaling pathways, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1), interferon regulatory factors (IRFs), and inflammasome activation. These pathways help shape the immune response by negatively or positively altering cytokine production and improving antigen presentation. By bringing together what we know about NLR evolution, structure, and function, this review aims to support new ideas and research into how fish defend themselves from disease and how we might strengthen that defense through improved vaccine and adjuvant design.

1 Introduction

Fish in aquatic environments constantly face challenges that trigger innate immunity, such as the introduction of pathogens and stressors. Within this first line of defense, components of pattern recognition receptors (PRRs) trigger downstream signaling cascades that lead to inflammation, cytokine production, apoptosis, and pathogen clearance (1). This occurs in the presence of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), such as flagellin, nucleic acids, lipopolysaccharides (LPS), γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), lipoproteins, muramyl dipeptide (MDP), glucans, N-formylmethionine, and toxins (2, 3). The sources of these elements originate from bacterial, viral, and parasitic infections that activate various PRRs in fish, including Toll-like receptors (TLRs), the most heavily investigated, as well as retinoic acid-inducible gene-I-like receptors (RLRs), C-type lectin receptors (CLRs), and the subject of this review, NOD-like receptors (NLRs) (4).

NLRs are emerging as key sensors in pattern-triggered immunity (PTI) and are located intracellularly in the cytoplasm, unlike the majority of TLRs, which are located either on the cell surface or within intracellular compartments. Depending on the subfamily of the protein, NLRs act as positive or negative regulators of pro-inflammatory mediated responses through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein-1 (AP-1) via mitogen-activated protein kinase (MAPK) signaling, and interferon regulatory factors (IRFs) (3, 5, 6). Upon activation, NF-κB is a transcription factor that activates pro-inflammatory and cell survival genes, producing cytokines (7–9). At the same time, MAPK is a highly conserved family of serine/threonine kinases that transmits PAMPs/DAMPs from the cell membrane to the nucleus through a phosphorylation cascade, ultimately producing AP-1, which regulates immune responses, apoptosis, development, and stress adaptation in fish (5, 10). In addition, specific subfamilies of NLRs are activated as inflammasomes via pro-caspase-1 signaling, making them modulators and negative regulators of innate immunity to maintain a stable immune response through various pathways (2, 3, 5, 11).

NLRs consist of an N-terminal effector domain, a central nucleotide-binding oligomerization domain (NBD), and a C-terminal leucine-rich repeat (LRR) domain, forming a tripartite structural arrangement (3, 12, 13). Each of these three domains plays a distinct role, which is vital within the PTI. The effector domain participates in signal transduction through protein interactions (13). This results in the distinction of five key subfamilies of NLR proteins: NLR family acidic transactivation domain-containing (NLRA), NLR family baculoviral inhibitor of apoptosis repeat (BIR)-containing (NLRB), NLR family caspase activation and recruitment domain-containing (NLRC), NLR family pyrin domain-containing (NLRP), and NLR family proteins with alternative effector domains (NLRX) (3, 12, 14, 15). Further distinctions across these classes will be detailed in section 2.1. NBD is responsible for oligomerization mediation and contains ATPases associated with diverse cellular activity (AAA+) subdomains for ATPase activity (3, 16, 17). This is also known as the NACHT domain, whose name originates from the four proteins that it was first identified in, NAIP, CIITA, HET-E, and TP1 (17). Along with this, the

C-terminal segment, consisting of the LRR domain, plays a vital role in recognizing PAMPs/DAMPs upon entering the cell by binding to the ligands present (13). These domains are present in most NLRs, with evolutionary variations leading to exceptions (18). Further functional domains may also be present as a result of evolutionary adaptations across various taxa or factors of divergence within NLR subfamilies. Given this, NLRs are structurally conserved across all known vertebrates, with fish displaying a unique expansion, structurally and functionally, most likely shaped by pathogen-driven selective pressures (18, 19). Unlike vertebrates, non-vertebrates lack an adaptive immune system, resulting in an evolutionary expansion of PRRs, such as in corals and sea urchins (18, 20). NLRs were initially discovered in mammalian species and later began to be researched in plants, reptiles, amphibians, birds, and most importantly, fish — the taxonomic class that will be expanded on in this review.

The role of fish NLRs in disease resistance is of increasing interest, especially in aquaculture, where infectious diseases threaten global fish production. Upon activation following ligand binding, NLRs can act as innate immunity pathogen receptors and regulate MHC gene expression depending on their classification (13). Along with this, some NLRs display inflammasome function, boosting cytokine release, antigen presentation, and adaptive immunity, allowing for their implementation in vaccine development as targets for adjuvant strategies (21).

In this review, we focus on six key members of the NLR family: NOD1, NOD2, NLRC3, NLRC5, NLRX1, and NLRP1. We base our selection on four main criteria: (i) their presence in North American and well-studied fish, (ii) the availability of full-length, annotated amino acid sequences in public databases like NCBI and Ensembl, and (iii) the existing or emerging functional evidence from studies on gene expression, domain structure, or immune responses. (iv) frequency of co-study between NLRs presented in this review. While NLRP3 has been studied extensively in cyprinids such as zebrafish (10.1074/jbc.RA119.011751) and grass carp (https://doi.org/10.1016/j.fsi.2024.109367), many available sequences are partial or not verified in these species. Also, NLRP3 fails criterion iv, as it is independently identified in experimental assays of current literature. On the other hand, NLRP1, though less functionally studied, is found in multiple genomes with complete sequences, which allows for comparative analysis.

This review explores the historical overview of NLR discovery, evolutionary classification, domain structure, and 3D modeling analysis (generated by AlphaFold), signaling mechanisms, functional roles in various pathogen defenses, and implications for vaccine or adjuvant development in aquaculture. The functional basis of these proteins offers key future directions that can be applied in other aspects of veterinary medicine.

2 Nomenclature, history, evolution of NLRs

The NLR family is an evolutionarily conserved family of PRRs located intracellularly (22). Identification of this class of proteins began in mammals in the 1990s as a key player in the activation of

innate immune responses via inflammation, host defense, and autoimmune diseases. Despite sharing common roles in immunity, the classification, evolutionary patterns, and structural features in mammals and teleosts differ.

This section will discuss the diversity of nomenclature systems used for NLRs across species, highlighting the inconsistency in teleost classification and the requirement for a uniform nomenclature based on phylogenetic relationships. We will then outline the historical timeline of key milestones in NLR discovery from early mammalian research to the more specialized work in fish (Figure 1). Following this, the evolutionary diversification of NLRs in teleosts and mammals will be considered, drawing on phylogenetic analyses that reveal conserved patterns and species-specific adaptations. Lastly, the functional domain elements will be detailed, followed by 2D and 3D structural characterizations. Together, these topics provide a foundation for understanding the diversity and rationale of NLRs across piscine species for subsequent sections on the functional investigation in immune signaling.

2.1 Nomenclature

The discovery of a majority of NLRs was initially done in mammals due to their accessibility and significance to human health. This resulted in delayed research in fish that have displayed a unique characterization of NLRs, which varies in gene expansion, domain architecture, and evolutionary lineage. Due to this complexity, a majority of fish species have been referred to with nomenclature that varies from the standardized nomenclature system established by the HUGO Gene Nomenclature Committee (HGNC) (23–25).

As mentioned, variations in the N-terminal segment constitute the distinction between these five subfamilies. In mammals, NLRA consists of an acidic activation domain, NLRB includes a baculovirus inhibitor of apoptosis repeat (BIR) domain, NLRC includes a caspase activation and recruitment domain (CARD), and NLRP has a pyrin domain (PYD) (3, 12, 14, 24). The NLRX subfamily varies, however, as it lacks an effector domain with a mitochondrial targeting sequence (MTS) near its N-terminal segment (26). In contrast, teleost fish exhibit a much more complex NLR repertoire, leading to distinct naming schemes. For example, zebrafish NLRs are grouped into NLR-A (NOD-like), NLR-B (NALP and NLRP-like), and the expansive, teleost-specific NLR-C subfamily, which includes hundreds of genes, many containing a C-terminal B30.2/PRY-SPRY domain (25, 27, 28). The B30.2 subfamily, also found in grass carp and other teleosts, likely evolved from a NOD3-like ancestor and does not exist in mammals. Likewise, grass carp categorize some NLRs as NLR-B30.2 as well (24). In contrast to these teleost-specific naming systems, species like channel catfish adopt a mostly mammalian-style nomenclature, using names such as NOD1, NOD2, NLRC3, NLRC5, and NLRX1, reflecting their structural similarity and simplifying cross-species comparison (24). Given this, even channel catfish have NLR proteins that are novel to teleosts

(24, 27, 29). However, despite the shared names, proteins like "NLRC3" and "NLRC5" in zebrafish and grass carp may not be direct orthologs to their mammalian counterparts. This underscoresxthe broader issue: NLR nomenclature varies significantly between fish and mammals, and even among fish species due to species-specific gene duplication, structural divergence, and varying domain compositions, complicating orthology and functional inference.

Chuphal et al. provide a comprehensive naming comparison of these NLRs in several teleost fish to their mammalian orthologs (27). The teleosts mentioned in the study include: zebrafish, channel catfish, common carp, goldfish, Japanese flounder, olive flounder, rohu, grass carp, miiuy croaker, mrigal, orange-spotted grouper, Nile tilapia, catla, Atlantic salmon, rainbow trout, Asian seabass, point snout bream, turbot, Japanese pufferfish, spotted snakehead, and Ya-fish (27).

In this review, we standardize the naming of NOD1, NOD2, NLRC3, NLRC5, NLRX1, and NLRP1 for all species for clarity. Although subfamily classification is historically based on the presence of specific N-terminal domains (e.g., CARD in NLRCs, PYD in NLRPs), evolutionary divergence has resulted in the loss or modification of these domains in some orthologs, particularly across vertebrate lineages. Furthermore, we do not introduce new nomenclature but rather adhere to standardized protein names provided by NCBI and Ensembl, along with recent literature conventions that correlated to the homology and clade of the protein leading to evolutionary conservation, rather than strict structural criteria as represented in the phylogenetic tree (Figure 2) and structural representations (Figure 3). For example, genes such as NLRC3 or NLRC5 may retain their designation despite lacking characteristic domains.

2.2 Discovery of NLRs in mammals

The majority of experimentation to characterize vital NLRs in mammals occurred in human and mouse species starting from the 1990s. Figure 1 provides a condensed timeline of key mammalian discoveries, together with fish discoveries detailed in the following section, summarizing the findings discussed in the text. The founding member of the NLR protein family is CIITA, an MHC class II gene expression regulator, which was first discovered in 1993 in mice through complementation cloning (39). Its tripartite structure is similar to today's known NLRs; however, the presence of a Proline, Serine, and Threonine (PST) rich domain located at the N-terminus contributes to functional differences as a transcriptional co-activator via nuclear localization and export signals in most antigen-presenting cells (APCs) (22, 40–43). This finding was followed by the discovery of nucleotide-binding oligomerization domain-containing protein 1 (NOD1) in mice as an Apoptotic protease activating factor-1 (Apaf-1)-like molecule containing CARD, nucleotide-binding domain (NBD), and LRR domains, the basis of NLRs (44). Apaf-1 is a cytosolic adaptor protein consisting of WD40 repeats, which is similar to that of LRRs in NOD1 (44–46). NOD1 was also found to perform homophilic CARD–CARD interactions to activate NF-κB by
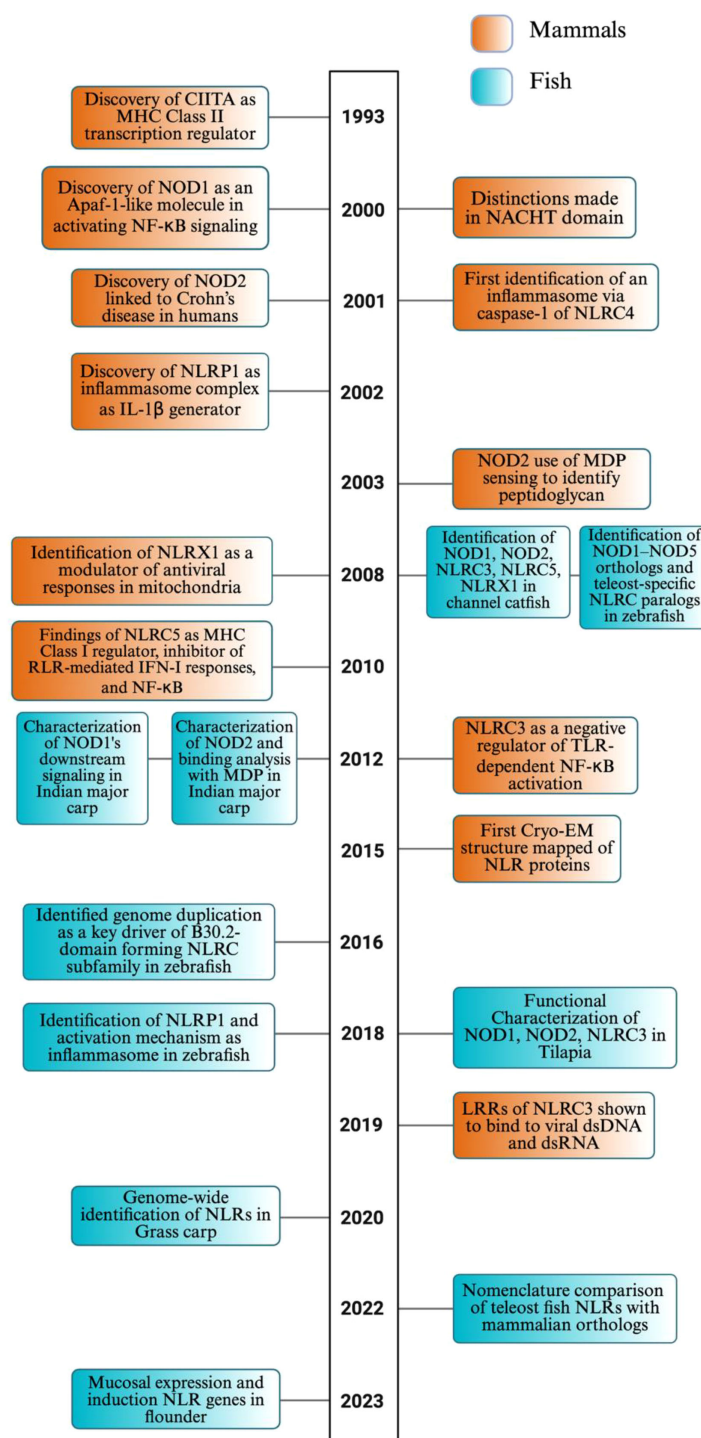
FIGURE 1
Timeline of NLR discovery and evolution in mammals and fish. This timeline highlights pivotal discoveries and major conceptual advances in the study of NOD-like receptors (NLRs) in both mammals (orange) and fish (teal), spanning from the early characterization of mammalian NLR functions in immunity to the growing body of research focused on teleost-specific genes, functions, and evolutionary mechanisms. The timeline underscores the increasing contribution of fish studies in recent years, revealing both conserved and lineage-specific insights into NLR biology. While this timeline captures several defining milestones in NLR research, we recognize that it does not include many additional contributions to the field due to space constraints. The image was created using BioRender.com.

binding to Receptor–Interacting Serine/Threonine–Protein Kinase 2 (RIP2 or RICK), a kinase containing a CARD domain (44, 46). This activation is driven by NOD1's ability to self-associate, which enables the recruitment of RIP2 to the γ subunit of the IκB kinase (IKKγ). Along with this, the NBD region or NACHT domain was further distinguished as a central ATPase core characterized by nucleotide binding and hydrolysis (16, 47, 48). These structural features allow the NACHT domain to regulate conformational changes essential for NLR oligomerization and activation in the presence of PAMPs/ DAMPs (16). The discovery of NOD2 in 2001 was especially important as a frameshift mutation in the tenth LRR of NOD2 was found to impair its responsiveness to bacterial lipopolysaccharides by inhibiting NF-κB and altering the region of linkage on chromosome 16, resulting in increased susceptibility to Crohn's disease in humans (49–53). In 2003, this was expanded on when NOD2 was shown to detect muramyl dipeptide (MDP), a conserved component of bacterial peptidoglycan, gram-negative but primarily gram-positive bacteria, due to their abundance of peptidoglycan (54, 55). NOD1, however, was shown to only detect meso-diaminopimelic acid (meso-DAP) in gram-negative bacteria, activating NF-κB (56, 57).

In a further human study in 2001, NLRC4 (now known as Ipaf) was identified to directly interact with and activate procaspase-1 via CARD–CARD interactions, establishing it as a specific and direct activator of caspase-1 (58, 59). Truncation of NLRC4's LRR domain led to constitutive activation of caspase–1–dependent apoptosis, marking the first functional identification of an inflammasome-like platform in human cells (58, 59). The discovery of NLRP1 added to the class of inflammasome complex proteins, involving Apoptosis-associated speck-like protein containing CARD (ASC) or Pycard, caspase-1, caspase-5, and NALP1, a pyrin-domain protein homologous to NODs (60–64). Here, the ASC/Pycard is essential to mediate the cleavage of pro-IL-1β into its active form through the activity of caspase-1, resulting in a pro-inflammatory response (60–63).

The following NLRs have been shown to act as negative regulators of innate immunity to maintain immune homeostasis and protect tissues from damage. In 2008, researchers identified NLRX1 as a noncanonical mitochondrial NLR that acts as a modulator rather than a receptor of PAMPs (65). The protein interacted with the mitochondrial antiviral signal protein (MAVS), also known as Cardif, VISA, and IPS-1, at the mitochondrial membrane, disrupting its association with RIG-I and MDA5 (RLRs), negatively regulating IFN-I transcription, interferon regulatory factor 3 (IRF3), NF-κB, and generating reactive oxygen species (ROS), resulting in a unique function from the previously mentioned NLRs (65–71). Two years following this study, NLRC5 was shown to result in similar inhibitions of antiviral signaling with varied mechanisms from NLRX1. NLRC5 interacts with the same cytosolic sensor RLRs, resulting in the upstream inhibition of MAVS, suppressing IFN-I responses. Along with this, NLRC5 performed inhibitory binding to IKKα and IKKβ, preventing NF-κB signaling (72–74).

Apart from antiviral signaling, in response to cytoplasmic protein degradation peptides (type of DAMP), NLRC5 promotes MHC class I expression by acting as a type II interferon gamma

(IFN-γ)–inducible transcriptional regulator that binds to MHC class I gene promoters such as HLA-A, HLA-B, and HLA-C to present antigens for CD8$^+$ T cell docking and apoptosis (75, 76). This expression is vital, as although CIITA can transactivate MHC class I genes, its function as such is not widespread, limiting its expression specifically to lymphocytes and APCs and primarily functioning as a novel MHC class II regulator (75, 77–82).

NLRC3 acts as a negative regulator of innate immunity by binding to TRAF6, preventing its K63-linked ubiquitination (7). This, along with the upstream signaling interference via MyD88, resulted in the suppression of NF-κB activation, similar to several other NLRs and TLR signaling (7, 83). Unlike NLRC5 and NLRX1, which suppress MAVS-mediated type I interferon (IFN-I) signaling, NLRC3 specifically dampens TLR-driven inflammation as a distinct but complementary immunomodulator (7). NLRC3 was shown to directly bind to the ligand of viral double-stranded DNA (dsDNA) and, to a lesser extent, double-stranded RNA (dsRNA) via its LRR domain (84). This interaction enhances NLRC3's ATPase activity and triggers a conformational change that causes it to release a stimulator of interferon genes (STING) and TANK-binding kinase 1 (TBK1) (84, 85). Freed TBK1 phosphorylates IRF3, lifting its suppression of the IFN-I pathway (84). NLRC3 functions to suppress CD4$^+$ T cell activation, proliferation, and cytokine production, which dampens Th1 and Th17 responses through inhibition of NF-κB and extracellular signal-regulated kinase (ERK) signaling pathways (86). In both viral (LCMV) and bacterial (*Mycobacterium tuberculosis*) infections, NLRC3 deficiency enhanced protective immunity by promoting robust CD4$^+$ T cell responses, positioning NLRC3 as a key modulator of adaptive immunity to prevent possible autoimmunity and chronic inflammation (86, 87). The modulators mentioned act as an immune "brake" to prevent overreaction of immunity, despite promoting pathogen survival in some cases due to this suppression of protective immunity.

In 2015, the first cryo-EM structure of an activated NLR inflammasome was presented, revealing that a single ligand-activated NAIP2 molecule results in conformational changes that nucleate the polymerization of multiple NLRC4 molecules (59). This was shown to form a large oligomeric complex, resembling a wheel, that activates downstream caspases and initiates the inflammatory response (59, 88–91). Prior to cryo-EM's structuring, X-ray crystallography was used to map the 3D structure of these proteins, but this method required crystallized proteins, which were often difficult to obtain, and lacked the ability to capture multiple conformational states (92). Protein mapping has advanced with tools like AlphaFold in 2018, which uses neural networks to predict 3D structures from amino acid sequences by leveraging evolutionary data and structural templates (37).

2.3 Discovery of NLRs in fish

With the rising economic value of aquaculture globally, immunological studies on NLRs in fish have gained traction, with the first classification occurring in 2008. In channel catfish,

structural, phylogenetic, and genomic analyses characterized NOD1, NOD2, NLRC3, NLRC5, and NLRX1 (93). These PRRs were constitutively expressed in cell lines of leukocytes and other tissues in catfish with enteric septicemia caused by *Edwardsiella ictaluri* (93). With this, zebrafish orthologs for all five mammalian NOD proteins (NOD1–NOD5) were identified as well as over 200 teleost-specific NLRC genes, many encoding a B30.2 domain (25). Phylogenetic analysis showed that NLRC likely evolved from a NOD3-like ancestor, and RT-PCR confirmed expression of NLRA, NLRB, and NLRC genes in the intestine, liver, and spleen (25). The naming of these zebrafish NLRs varies from what will be used in this review, as detailed in section 2.1.

In 2012, the first characterization of downstream signaling by NOD1 in Indian major carp (rohu) was performed by Swain et al. The full-length NOD1 cDNA was mapped, and its domains were displayed (94). Exposure to LPS and poly I:C led to robust, tissue-specific upregulation of NOD1 (up to 80-fold in blood) and its adaptor RIP2, indicating activation of downstream NF-κB and MAPK pathways (94). Several other studies have used LPS and poly I:C to elicit varied NLR responses in fish species. Furthermore, pathogen challenge with *A. hydrophila*, *E. tarda*, and *S. flexneri* confirmed inducible NOD1–RIP2 signaling *in vivo* (94). Soon after, the first structural insights into ligand recognition by fish NOD1 identified LRR1–2, LRR3–7, and LRR8–9 as critical binding motifs for poly I:C, LPS, and γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), respectively, and confirmed conserved NOD1 signaling activation in rohu (95). This was performed through methods of molecular docking and 6-ns molecular dynamics simulation (95). Swain et al. also characterized NOD2 in rohu, and observed upregulation of the protein (~4-fold at 4 hours and ~7-fold at 24 hours) in peripheral blood leukocytes upon muramyl dipeptide (MDP) stimulation, confirming MDP as a potent ligand for rohu NOD2 (95). Much like NOD1, NOD2 displayed concurrent upregulation of the downstream adaptor protein RIP2 (~6.3-fold at 4 hours), establishing both NODs as key cytosolic sensors in teleost innate immunity (95, 96). In mrigal, similar RIP2 upregulation (~5.6-fold in 6 hours) was seen upon MDP stimulation *in vivo* along with upregulation of IL-1β (~6-fold in 6 hours), resulting in a pro-inflammatory response (96). Similar upregulation of these NODs was discovered in Nile tilapia, goldfish, miiuy croaker, Japanese flounder, and catfish (previously mentioned) upon ligand and pathogen introduction, possibly indicating conserved signaling pathways across teleost fish (93, 97–100).

In 2016, Howe et al. identified the origins of these teleost-specific NLRC genes in zebrafish, B30.2 domains that underwent extensive tandem and segmental duplications, especially on chromosome 4 (18). Similar genomic duplications and expansions were shown in other fish species, contributing to their naming discrepancies as mentioned. Following this, NOD1, NOD2, and NLRC3 were functionally characterized in Nile tilapia and shown to be upregulated at the mRNA and protein levels in response to *Streptococcus agalactiae*, with distinct tissue-specific expression patterns (97). They demonstrated that NOD1 activates NF-κB in a ligand-independent manner, whereas NOD2 requires MDP, and NLRC3 requires either MDP or iE-DAP to enhance NF-κB signaling (97). This upregulation of NF-κB signaling represents

the context-dependent role as a regulator in fish, whereas in mammals, NLRC3 is known to inhibit the NF-κB pathway. Around this same time, the first activation mechanism of an inflammasome was identified in zebrafish as an NLRP1 homolog (DrNLRP1) (28). Here, two pro-inflammatory caspases, DrCaspase-A and DrCaspase-B, are activated in an ASC-dependent manner where homotypic interactions occur. Interestingly, the study was the first to identify the teleost-specific presence of PYD instead of CARD in DrCaspase-A/B, which was later replicated in channel catfish (28, 101). DrCaspase-A is activated first to initiate IL-1β processing, followed by DrCaspase-B to complete its maturation (28).

A genome-wide study in 2020 systematically identified 65 NLR genes in grass carp (24). These subfamilies, along with most other fish NLRs that have been characterized genomically, have been organized with their mammalian orthologs in 2022, bringing clarity to the field as mentioned in 2.1 (27). Recently, flounder NLR genes were highly expressed in mucosal tissues such as gills, skin, and hindgut following intraperitoneal and immersion vaccination with inactivated *Vibrio anguillarum* (13). Notably, the teleost-specific NLR-C subfamily members, especially those with B30.2 domains, showed the most pronounced immune responses, emphasizing their potential role in mucosal immunity (13).

Together, these findings display the earliest discoveries of evolutionary innovation and functional diversification of NLRs in teleosts, driven by gene duplication events and selective pressures from aquatic pathogens. As immunological tools and genomic resources continue to expand, fish NLRs, especially teleost members, are becoming increasingly valuable for guiding vaccine development in aquaculture.

2.4 Evolution and diversity of fish NLRs

A circular phylogenetic tree was generated, based on full-length protein sequences for six NLR subtypes (NOD1, NOD2, NLRC3, NLRC5, NLRX1, and NLRP1) across diverse vertebrate species, emphasizing teleost fish (Figure 2). The amino acid sequences used to construct this phylogenetic tree, along with their accession numbers, are provided in Supplementary Table S1. The teleost fish selected for this phylogenetic analysis were chosen for their commercial importance and representation across diverse aquatic environments, ranging from freshwater to saltwater habitats. The selection criteria used to generate the tree dictated which NLRs were included in this review; because NLRP3 failed to meet most of those criteria, it was omitted. Each NLR subtype formed separate clades, color-coded to show their evolutionary relationships. NOD1 (pink) and NOD2 (blue) are presented to be highly conserved across teleosts, forming sister groups that underscore their shared evolutionary origin. NOD2 is ubiquitous in fish species; however, it is not present in some of the presented vertebrates.

The NLRC3 and NLRC clades appeared across fish and non-fish species, forming closely related sister clades that suggest a common ancestral origin and divergence through gene duplication. NLRP1 (green clade) clustered compactly and basally with fewer teleost
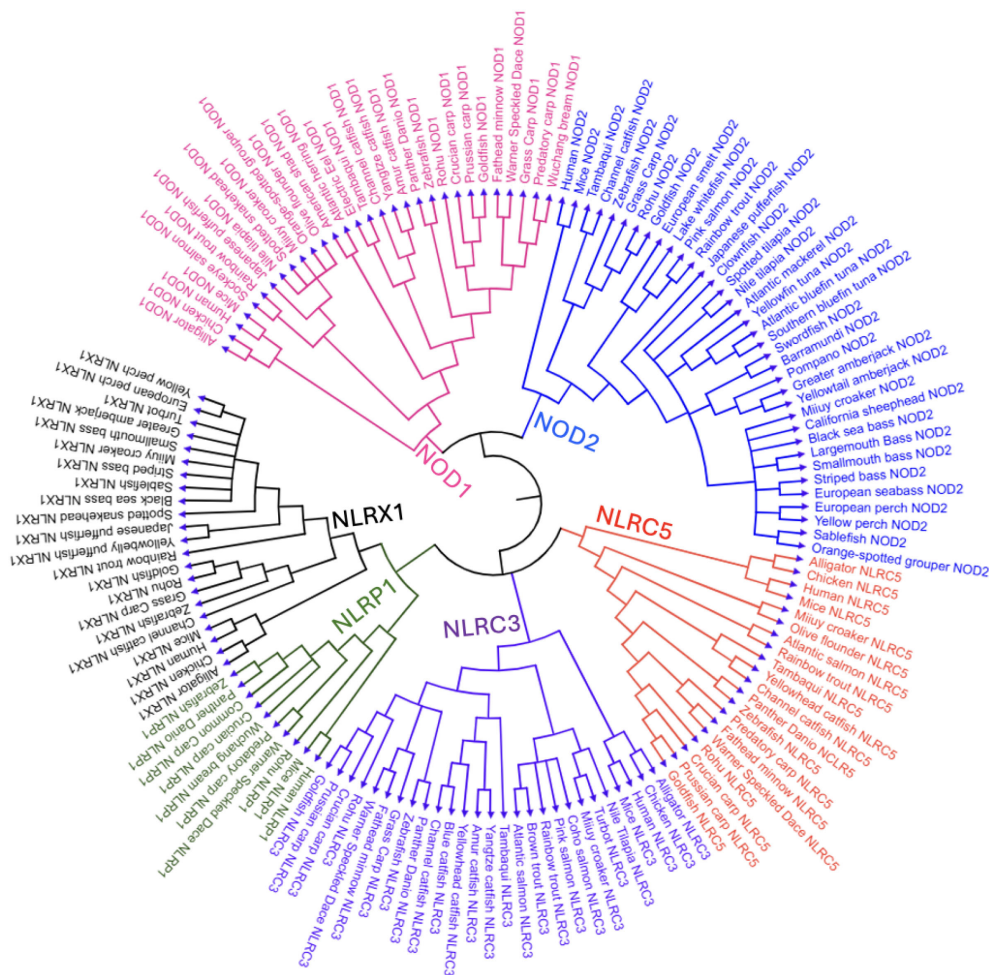
FIGURE 2
Phylogenetic tree of NLR proteins across vertebrate species, focusing on teleost fish.This circular phylogenetic tree shows the evolutionary relationships of various NLR subtypes—NOD1, NOD2, NLRC3, NLRC5, NLRX1, and NLRP1—from vertebrates such as teleost fish, amphibians (hourglass tree frog), reptiles (American alligator), birds (chicken), and mammals (mouse and human). Clades are color-coded: pink (NOD1), blue (NOD2), purple (NLRC3), orange (NLRC5), green (NLRP1), and black (NLRX1). We conducted a phylogenetic analysis of 57 freshwater and marine teleost fish—selected for their status as well-studied model organisms, evolutionary conservation across lineages, broad recognition, or representation of North American fauna—and five higher vertebrates, revealing both conserved and divergent patterns of NLR evolution within and across species. The phylogenetic tree was constructed using complete, full-length protein sequences of NLRs obtained from the NCBI's protein database (GenPept). BLAST searches were conducted to identify proteins in other species containing >90% query coverage and an E value of 0 (30–33). FASTA sequences were aligned and analyzed using MEGA12 software, applying the neighbor-joining method to infer evolutionary relationships (34, 35).

members, implying limited duplication and elements of conservation. It was more closely related to NLRX1 than to other NLRs, suggesting a deeper shared ancestor. Likewise, NOD1 and NOD2 formed a sister clade, highlighting duplication-driven diversification. Catfish, tuna, and bass each formed species-specific clades, indicating within-group evolutionary relationships, while NOD2 was uniquely observed in tuna species among the taxa represented.

Overall, the phylogenetic tree highlights a clear divergence between teleosts, likely in response to aquatic pathogen diversity and environmental pressures. In addition to the diverse representation of teleost fish, the phylogenetic tree also includes key higher vertebrates, such as mammals (human, mouse), birds (chicken), reptiles (alligator), and amphibians (hourglass tree frog), which serve as reference points for evolutionary comparison. These species consistently form distinct, well-supported clades from teleost branches within each NLR subtype. Given that teleosts represent an early-diverging lineage among vertebrates, the observed clade structure supports the inference that fish are ancestral to these higher vertebrate lineages. The driving force of evolutionary divergence in these non-aquatic vertebrate NLRs likely reflects adaptation to terrestrial environments, including exposure to novel pathogens and immune challenges.

2.5 Key functional domains of Fish NLRs

The CARD is a conserved protein–protein interaction module commonly found at the N-terminus of NLRs and was found on NOD1, NOD2, NLRC3, and NLRP1 in the proteins we mapped (Figure 3). The CARD domain is a part of the death domain (DD) superfamily, which also includes pyrin and death effector domains. Members of this superfamily typically adopt a six-helix bundle fold with a bent or disrupted H1 helix along with a hydrophobic core that stabilizes the structure (102–104). The functional interactions are generally driven by charge complementarity across helices H1–H4, often forming filament-like assemblies that serve as signaling platforms (105, 106). As a result, CARDs mediate homotypic interactions, enabling recruitment of adaptor proteins and downstream effectors involved in apoptotic and inflammatory signaling pathways, including caspase activation and NF-κB regulation (107–109). Although CARD sequences show low identity, their structural conservation allows for interaction diversity and pathway specificity (110, 111). Noncanonical variants like the untypical CARD (uCARD) domain of NLRC5 also exist, which have been shown to remain solvent-exposed in both open and closed states and contain a nuclear localization signal (NLS), implicating a potential dual role in both signaling and nuclear import (112).

The NACHT (NAIP, CIITA, HET-E, and TP1) domain belongs to the NB-ARC superfamily of signal-transducing modules and was found in all mapped NLRs (16, 104). Structurally, the NACHT domain consists of several subregions: NBD (Walker A and Walker B motifs), helical domain 1 (HD1), HD2, and winged-helix domain (WHD), which coordinate ATP binding and hydrolysis as mentioned (16, 47, 48, 113). These subdomains form a tightly packed core, with the NBD centrally located and flanked by regulatory helices and a winged-helix fold that stabilizes the domain structure and promotes conformational changes necessary for oligomerization and downstream signaling (102, 114, 115). The domain has since been identified to drive the assembly of multimeric complexes such as inflammasomes and apoptosomes in NLRs. Despite variability in adjacent domains, NACHT-mediated ATP binding is broadly required for the activation, autoinhibition release, and structural reorganization of these signaling platforms (16, 104).

LRR domains are found in all NLRs that were mapped across species, which emphasizes their functional necessity (Figure 3). LRRs are conserved motifs typically forming tandem arrays (two or more repeats), forming curved solenoid architecture ideal for protein-protein interactions (116, 117). This hook-type shape can be seen in the 3D display of the LRR region, colored blue (Figure 3). Each motif is typically around 20–30 amino acids and is characterized by a hydrophobic core and is made of an 11-residue pattern of LxxLxL where "L" is a hydrophobic amino acid such as leucine, isoleucine, valine, or phenylalanine, and "x" is any amino acid (118–121). This pattern supports a parallel β-sheet along the concave surface, represented as arrows on the 3D cartoon display indicating the polypeptide chain's N-terminus to C-terminus direction (Figure 3)

(118, 119). These β-sheets can only be seen in some angles, which were not completely displayed in all the NLRs in Figure 3 to allow visualization of the other domains. However, the β-sheet surface is key for its ligand recognition properties, while other surfaces are also less commonly involved (116, 122–127). Also, this concave surface interacts with other domains, such as CARD, to maintain an inactive state, but this interaction is disrupted upon ligand binding, triggering activation through conformational changes (128, 129). With this, the truncation of the C-terminal LRR region often causes NLRs to become constitutively active as well (128, 130). The convex surface, however, is composed of α-helices, polyproline II helices, 3_{10} helices, β-turns, and β-strands that are interwoven (116, 118, 119, 131).

These three mentioned domains are the basis of NLRs, and at least two of these elements are present in every NLR that we mapped. The order of these domains consistently falls in the order of CARD, NACHT, and lastly the LRR region from the N- to C-terminus, except in NLRP1, which will be expanded on in the following section. Also, section 3.1 will display these domains' binding affinities and their functional role in downstream signaling.

2.6 Structure and classification of fish NLRs

Understanding the structural organization of NLRs across diverse vertebrate species is critical for unraveling how innate immune recognition has evolved and diversified. Displaying both the 2D domain architecture and 3D protein conformation may offer insight into the conservation and divergence of key functional motifs such as CARD (caspase activation and recruitment domain), NACHT, LRR, transmembrane, coiled-coil, PYD (pyrin), and FIIND (function to find domain) (132). These regions of the protein collectively mediate pathogen recognition and signal transduction. By comparing representative species of teleost fish and other previously mentioned vertebrates, these structural visualizations represent shared evolutionary origins and lineage-specific adaptations that bridge sequence-based annotations with spatial protein dynamics, allowing for the interpretation of how structure relates to immune function across vertebrates. Some vertebrates and fish species lacked some of these NLRs, which is why there is no consistent display of the same species and a limited display of NLRP1.

The acidic activation domain, B30.2/PRY-SPRY domain, AAA+ domain, uCARD domain, and others could not be displayed in Figure 3 as they did not reach the threshold to be mapped in the SMART database, despite possibly playing a role in immunogenicity (36). The AAA+ domain is especially important as it is a conserved subregion within the larger NACHT domain, responsible for ATP binding and oligomerization for inflammasome formation of NLRP1 following the cleavage within the FIIND domain, which will be discussed (115, 133). The nomenclature used in this paper is based on evolutionary history rather than the presence of specific domains, as not all proteins within a given subfamily consistently share the same domain architecture (Figure 3). Also, each NLR type maintains a relatively similar LRR range across species, though not often the
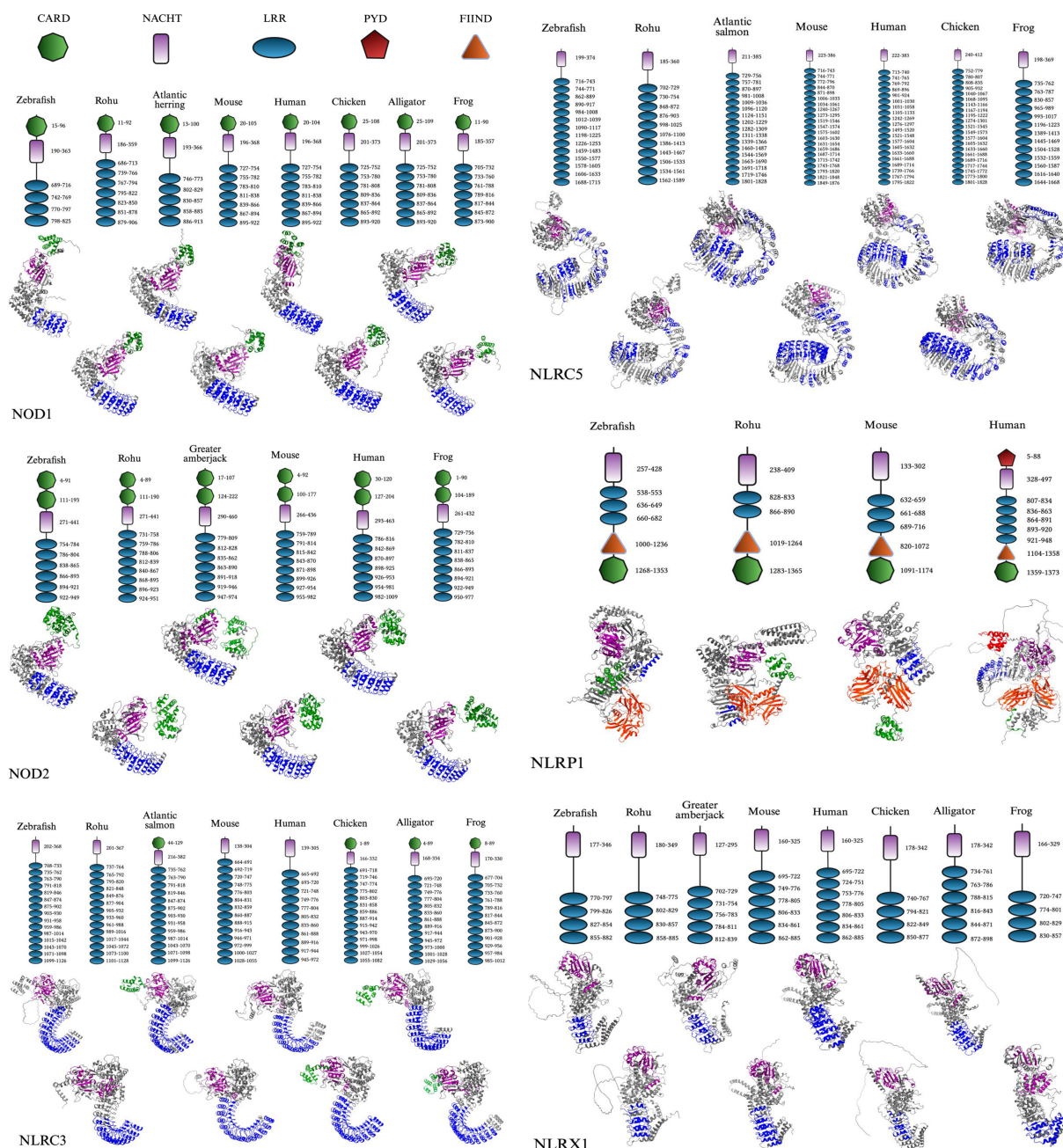
FIGURE 3

Comparative domain architecture and 3D structures of vertebrate NLR proteins. This figure displays the domain organization (top panels) and AlphaFold-predicted 3D structures (bottom panels) of six NOD-like receptors (NLRs): NOD1, NOD2, NLRC3, NLRC5, NLRX1, and NLRP1, across a diverse set of vertebrates including fish (e.g., zebrafish, rohu, Atlantic salmon, Atlantic herring, and greater amberjack) and representative tetrapods (e.g., mouse, human, chicken, American alligator, and Hourglass tree frog). Each protein's domain architecture includes the CARD (green), NACHT (purple), LRR (blue), PYD (red), and FIIND (orange) domains, with the domain key shown in the final panel. The same structural domain legend also applies to all pathway figures referencing these proteins. FASTA sequences were obtained from the NCBI protein database, and domain predictions were made using the SMART (Simple Modular Architecture Research Tool) database (36). 3D structures were generated using AlphaFold, and final visualizations were created with BioRender.com (37, 38).

same, while different NLR types display substantial variation (Figure 3). These patterns are likely driven by evolutionary pressures to recognize diverse PAMPs.

The 3D models reveal that the CARD domain appears to be surface-exposed and spatially distinct, supporting its role in initiating downstream signaling through homotypic interactions.

For NOD1 and NOD2, this is especially important for binding to RIP2, while in NLRP1, ASC binding occurs to form the inflammasome. Likewise, the LRRs display a stereotypical bent architecture in the form of a curved solenoid projecting outward from the NACHT domain. Several of the mapped LRRs contain identical amino acid regions to other species mapped. These

observations only apply to the applicable NLRs containing these domains. The NACHT domain, however, is present in every mapped NLR and forms the structural and functional core that connects upstream sensing to downstream effector activation through ATP-driven conformational changes. The AAA+ domain that controls this was not displayed in Figure 3, but is present in most NACHT domains displayed.

Several NLR-specific observations were made from the species in Figure 3. NOD1 displays a conserved domain organization across all examined vertebrate species, consisting of a single N-terminal CARD domain, a central NACHT domain, and a C-terminal array of LRR motifs. Regarding the 3D structure, functional studies have shown that NOD1's CARD domain must present a negatively charged surface to effectively engage in the RIP2-CARD formation (3). It can also be observed that several of the displayed species have seven LRRs for iE-DAP and other ligand binding. NOD2 contains tandem CARD domains, which are conserved across both teleost and non-teleost species, indicating functional importance. Also, the number of LRRs ranged from six to eight, which likely preserves its function to recognize MDP. This consistent structural organization of domains can only be seen in these two NLRs, suggesting that selective pressures have maintained the signaling framework while permitting mild interspecies variation in domain repeat number and sequence.

NLRC3 exhibits more variability at the N-terminus, where only some species—including Atlantic salmon, chicken, alligator, and frog, retain a CARD domain. However, in NLRC5 and NLRX1, no CARD domains are present. This suggests an evolutionary gain or loss of the CARD domain, with its presence in some teleosts indicating functional divergence. The NLRC subfamily also seems to display the first instance of a considerably expanded LRR region, often over ten repeats, with even more in NLRC5, possibly indicating a varied capacity for ligand discrimination. NLRC5 is unique as the LRR domains have notable discontinuities in the 3D models, appearing as structural gaps, contributing to a much longer amino acid sequence with possible flexible loop regions within the LRR segment. The protein's structural features likely enable its role in controlling nuclear trafficking, where histone acetyltransferase-mediated retention in the nucleus enhances its ability to activate MHC class I expression (134). Much like NOD1, NLRX1 consists of four to seven LRRs, with a straighter solenoid structure.

NLRP1 shows notable divergence from other NLRs in both domain architecture and species distribution. Structurally, NLRP1 is distinct in 3D conformation, with a more compact, globular fold and a unique arrangement of domains involved in inflammasome activation. In humans, NLRP1 contains an N-terminal PYD, which differs from the C-terminal CARD domain displayed in fish species of rohu and zebrafish. This is also unique, as the other NLR subfamilies have a CARD at the N-terminus. Also, all mapped NLRP1 proteins contain a conserved FIIND domain, along with NACHT and LRR domains. The functional role of these domains will be further detailed in section 3.3. The order of these domains is as follows: PYD (if present), NACHT, LRR, Function to Find Domain (FIIND), and CARD. Despite slight structural variability, domain conservation in these NLRs allows for the preservation of

function in microbial sensing, inflammasome formation, and pro-inflammatory effects throughout vertebrate evolution.

3 NLR signaling pathways in fish

This section details how NOD1, NOD2, NLRC3, NLRC5, NLRX1, and NLRP1 in fish activate immune responses through several key signaling cascades. NOD1 and NOD2 recognize bacterial peptidoglycan fragments, iE-DAP and MDP, respectively, triggering the recruitment of RIP2 and subsequent activation of inflammation. NOD1, NOD2, NLRC5, and in some teleosts, NLRC3 display characteristics of adaptive immune response through the upregulation of IFN-Is, cytokines, and MHC-I transcription, which will be detailed.
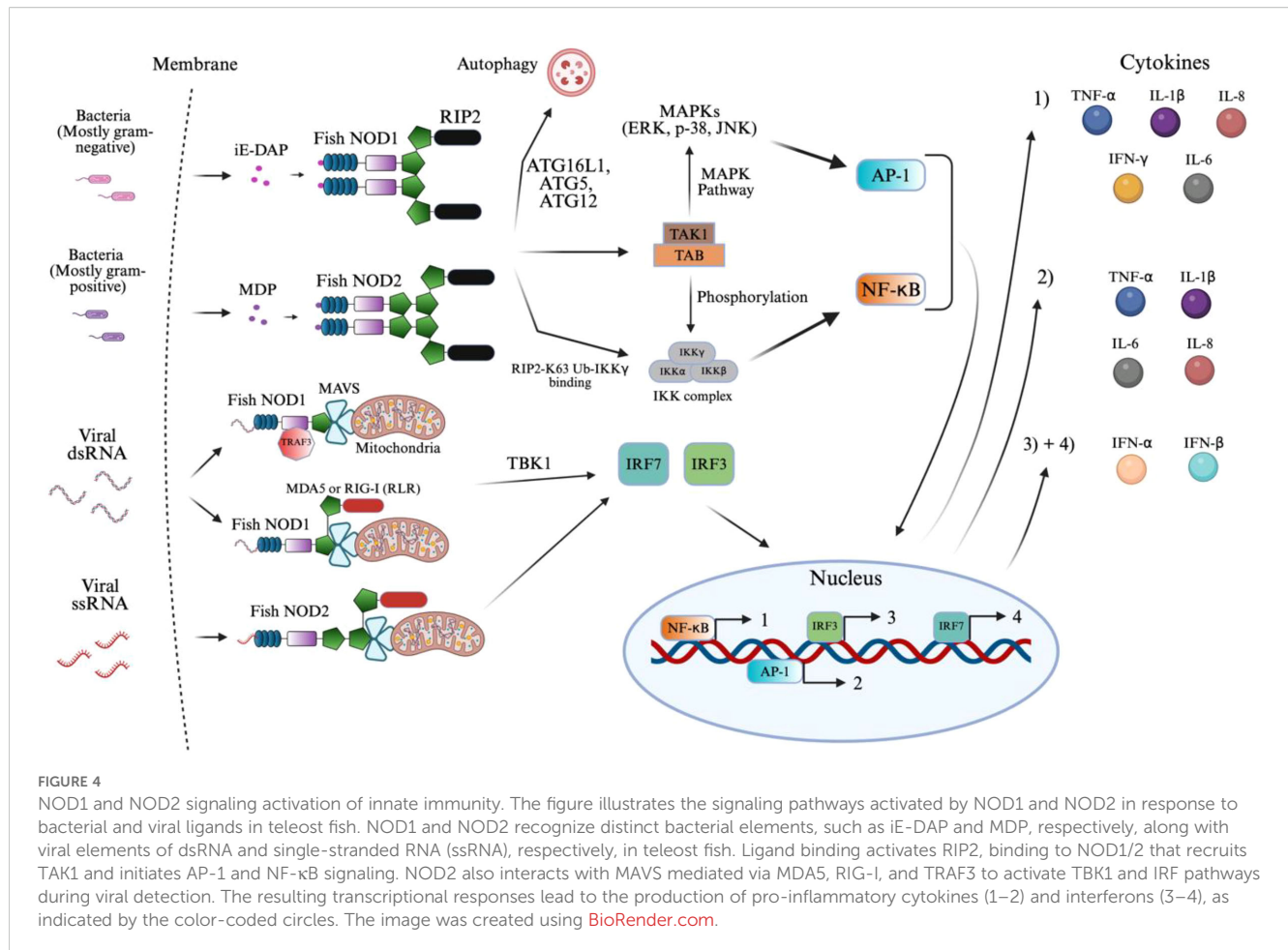
NLRC3 and NLRC5 are recognized as negative regulators of immune signaling, acting to constrain excessive inflammation, and at times can promote pathogen survival. Moreover, NLRC3 was shown to act as a positive regulator at times, specifically in some teleosts. While both belong to the NLR family, they engage in distinct inhibitory interactions with signaling hubs involved in innate immunity, particularly those tied to IFN-1 and cytokine expression, along with MHC Class I promotion.

NLRX1 localizes to mitochondria and negatively regulates antiviral signaling by disrupting STING and MAVS interactions, thereby reducing IFN-I responses, as well as negative regulation of cytokines in a similar mechanism to NLRC3. Meanwhile, NLRP1 varies by contributing to inflammasome formation by interacting with ASC and caspase proteins. These proteins are structurally different in piscine species, resulting in varied homotypic interactions, which will be expanded on.

Several other pathways may also be present, but will not be displayed in the signaling pathway figures due to their relative rarity and space constraints. Currently, research on parasite-induced NLR expression has been lacking, which is why it is not shown with the bacterial and viral pathogenic components in the following signaling pathways. However, it should be noted that NLRs are vital in resistance to parasitic infection in teleosts.

3.1 NOD1 and NOD2

Figure 4 summarizes the established signaling pathways of NOD1 and NOD2 in fish, which are described in detail below. NOD1's LRR domain binds to iE-DAP, a PAMP released by gram-negative and, to a lesser extent, gram-positive bacteria and acid-fast bacteria such as Mycobacterium (56). This binding triggers a conformational change that allows NOD1 to recruit RIP2 (135–137). Similar to iE-DAP, MDP is released from the peptidoglycan of primarily gram-positive bacteria, due to its abundance, which is recognized by NOD2, similarly recruiting RIP2 (54, 135–137). It should be noted that gram-negative bacteria also release MDP, but in trace amounts, resulting in variability of NOD2 signaling (54). The RIP2 signaling complex is formed when two NOD1/NOD2 molecules dimerize upon ligand binding and recruit two RIP2 adaptors through

FIGURE 4

NOD1 and NOD2 signaling activation of innate immunity. The figure illustrates the signaling pathways activated by NOD1 and NOD2 in response to bacterial and viral ligands in teleost fish. NOD1 and NOD2 recognize distinct bacterial elements, such as iE-DAP and MDP, respectively, along with viral elements of dsRNA and single-stranded RNA (ssRNA), respectively, in teleost fish. Ligand binding activates RIP2, binding to NOD1/2 that recruits TAK1 and initiates AP-1 and NF-κB signaling. NOD2 also interacts with MAVS mediated via MDA5, RIG-I, and TRAF3 to activate TBK1 and IRF pathways during viral detection. The resulting transcriptional responses lead to the production of pro-inflammatory cytokines (1−2) and interferons (3−4), as indicated by the color-coded circles. The image was created using BioRender.com.

CARD–CARD interactions, creating a stable 2:2 NOD1–RIP2 tetrameric signaling unit (135–137). This initiates K63-linked polyubiquitination, in which ubiquitin molecules are sequentially attached via their lysine-63 residues to form a flexible, linear chain that serves as a scaffold for recruiting signaling proteins rather than marking substrates for degradation by K48-linked chains (138). This structure and binding were not visually displayed in Figure 4 to avoid confusion with other signaling pathways. These ubiquitin chains then serve as docking sites for IKKγ (NEMO), by binding to its ubiquitin-binding domains (138). This interaction facilitates the spatial proximity required for the TAK1-TAB kinase complex to phosphorylate and activate the catalytic subunits IKKα and IKKβ within the IKK complex (138–140). Once activated, the IKK complex phosphorylates the cytoplasmic heterodimer of IκB proteins, leading to their degradation and the release of NF-κB (138, 141, 142). Freed from its inhibitor, NF-κB translocates into the nucleus, where it binds to consensus DNA-binding κB sites, located in the promoter regions of target genes (141). This promotes the transcription of genes encoding key pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, IL-1β, and IL-8, resulting in a robust innate immune response (96, 139, 143–145). NF-κB was also shown to modulate *ntl* expression, the zebrafish ortholog of the mammalian Brachyury gene, which is involved in mesoderm formation and notochord development during early embryogenesis in zebrafish (141). This represents influences in developmental gene expression. In parallel, TAK1 also activates MAPKs, driving AP-1-mediated transcription and releasing TNF-α, IL-1β, IL-6, and IL-8 (96, 144–148). The exact MAPKs found across teleost fish are unclear; however, one study in ayu (*Plecoglossus altivelis*) classified the phosphorylation of ERK, p38, and JNK through western blotting analysis, which may represent conserved MAPKs across other teleost fish (144).

The antiviral signaling pathway is kicked off through the binding of viral dsRNA or ssRNA (149–152). Here, cytosolic NOD1/2 collaborate with MDA5, a RIG-like receptor (RLR) through homotypic CARD interactions (149, 153). The RNA binding itself occurs in the helicase/CTD regions of MDA5, resulting in oligomerization that exposes its CARDs, docking onto MAVS and NOD1/2 (149, 153–157). Retinoic acid-inducible gene (RIG-I) in place of MDA5 has also been shown to display similar interactions in Zebrafish (149, 153–156). Much like these RLRs, an adaptor protein, TNF Receptor–Associated Factor 3 (TRAF3) recruits MAVS on NOD1 specifically (149, 156, 158). Following the formation of the MAVS complex, mediated by these MAVS recruiting proteins, TBK1 activates to phosphorylate interferon regulatory factors, IRF3 and IRF7, leading to their dimerization and translocation into the nucleus (154, 158). The result is a key driver in innate immunity through the formation of IFN-Is (IFN-α/β) as well as IFN-stimulated genes (ISGs) (149, 153–

156, 158). This integrated signaling network results in a vast transcriptional program coordinated by NF-κB, AP-1, IRF3, and IRF7 to counter both bacterial and viral infections. The proteins have not been found to detect viral DNA, suggesting their ligand specificity is biased toward RNA.

NOD1/2 have a clear role in innate immunity and the subsequent priming of adaptive immune components. Although not represented in Figure 4, the production of IFNα/β has been found to activate dendritic cell maturation, increasing antigen presentation (159, 160). Similarly, TNF-α, IL-1β, IL-6, and IFN-γ increase MHC and costimulatory molecule expression on antigen-presenting cells. These features support Th1 differentiation and create an inflammatory environment that improves CD4$^+$ and CD8$^+$ T cell priming (159–161).

A study on Grass carp (*Ctenopharyngodon idella*) demonstrated that NOD1 recruited autophagy-related genes to initiate autophagosome formation (162, 163). These autophagosomes subsequently fuse with lysosomes, enabling the lysosomal degradation of the gram-negative bacteria, highlighting a NOD1-dependent autophagy pathway in the presence of iE-DAP in teleost fish (162, 164). Autophagy is a highly conserved catabolic process where these cytoplasmic components, including intracellular pathogens, are sequestered into double-membraned autophagosomes formed through the conjugation activity of autophagy-related proteins

such as ATG16L1, ATG5, and ATG12 (162–164). This signaling pathway is similar in mammals as well (164).

Due to the complexity of Figure 4 and space constraints, the signaling pathway of ROS (reactive oxygen species) expression by NOD2 was not displayed. Following the previously mentioned NOD2 binding with MDP, the plasma membrane translocates, where it physically interacts with the dual oxidase (DUOX2) via its LRR domain (165). This interaction, aided by the DUOXA2 activator, stimulates DUOX2 to produce ROS, specifically hydrogen peroxide (165, 166). The resulting ROS contributes directly to bacterial killing and amplifies NOD2-mediated NF-κB signaling and cytokine production (165–167).

3.2 NLRC3 and NLRC5

Figure 5 summarizes the inhibitory signaling roles of NLRC3 and NLRC5, which are described in detail below. NLRC3 is broadly inhibitory across multiple inflammatory axes. In a key mechanism in large yellow croaker, NLRC3 directly binds to the adaptor protein, STING, inhibiting its binding to TBK1 in the presence of viral RNA or DNA (27, 168, 169). This attenuates their ability to activate IRF3/IRF7 promoters, which in turn limits IFN-I production (27, 168, 169). This mechanism parallels findings in
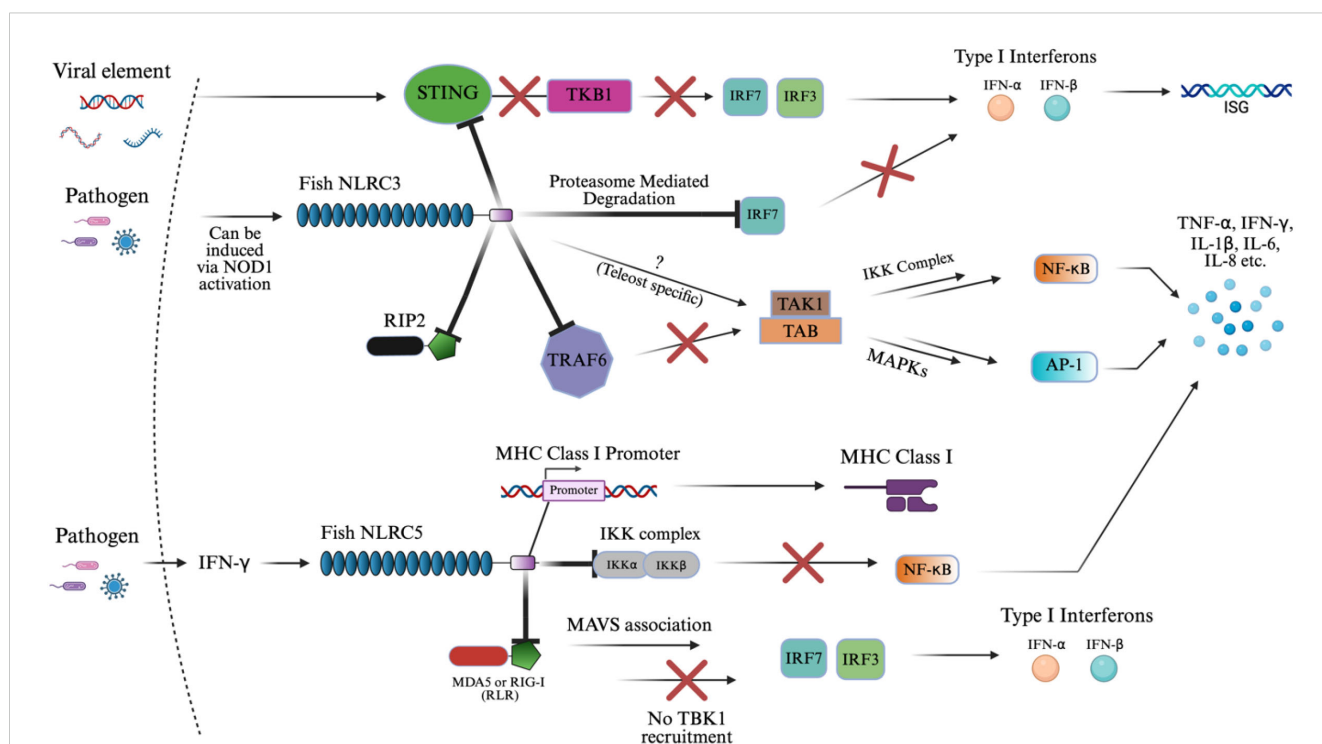
FIGURE 5

NLRC3 and NLRC5 signaling activation of innate immunity. The schematic representation of NLRC3 and NLRC5 signaling pathways in immune regulation. This diagram reflects the known inhibitory roles of NLRC3 in IFN-I, NF-κB, and AP1 signaling via suppression of STING-TBK1, TRAF6, RIP2, and IRF-mediated pathways. The "X" symbol in red represents some form of inhibition or suppression of the subsequent biomolecule. The "?" symbol represents an unknown pathway where teleost-specific interactions may differ by contributing to cytokine upregulation in fish species. Similarly, NLRC5 presents a similar result by downregulating cytokine production downstream of NF-κB and IFN-I response through inhibitory binding to the IKK complex and RLRs (MDA5 or RIG-I), while also directly promoting MHC class I expression via binding to the MHC class I promoter. The image was created using BioRender.com.

mammalian models and underscores a conserved inhibitory strategy within vertebrates. By blocking this pathway, NLRC3 reduces downstream expression of ISGs (169). In addition, NLRC3 promotes proteasome-mediated degradation of IRF7, further dampening IFN-I (IFN-α/β) response (170).

NLRC3 also acts on NF-κB and AP-1 signaling by binding to or interacting with critical upstream components such as RIP2 and TRAF6 (87, 137, 168, 169). In teleosts, overexpression of NLRC3 binding to TRAF6 selectively suppressed K63-linked ubiquitination, required for signaling, and enhanced K48-linked ubiquitination of TRAF6, promoting its proteasomal degradation. As a result, TRAF6 abundance in the cytoplasm was reduced, preventing the assembly of the TAK1–TAB complex, which is necessary for activating both the IKK complex (leading to NF-κB nuclear translocation) and MAPKs (activating AP-1) as outlined in **section 3.1** (87, 137, 168, 169). In addition to TRAF6, piscine NLRC3 also interacts directly with RIP2 via its NACHT domains (171). This binding interferes with the formation of the NOD1–RIP2 complex, thereby suppressing NOD1-mediated activation of NF-κB and MAPKs (171). Overexpression of zebrafish NLRC3 not only blocked this signaling axis but also downregulated the transcription of genes encoding key cytokines and chemokines during bacterial infection (137, 171). In contrast, in goldfish, NLRC3 interacts with RIP2 without binding to suppress NF-κB, AP-1, and IFN-I activity, suggesting species-specific variation in regulatory mechanisms (137, 172).

Interestingly, NOD1 and RIP2 can act as transcriptional regulators of NLRC3, downregulating these proteins in the presence of NLRC3 in zebrafish (137, 171, 173, 174). Also, the pathway may be modulated differently in teleost fish, where NLRC3 was associated with the upregulation of pro-inflammatory cytokines, indicating possible teleost and pathogen-specific pathways, which are not completely understood (137). This could result in a similar potential to NOD1/2 as a stimulator of adaptive immunity.

NLRC5 similarly inhibits the NF-κB and type I interferon pathways, but through distinct molecular interactions. Instead of acting upstream, NLRC5 binds directly to the IKK complex, blocking its ability to phosphorylate IκB and thereby preventing the release and nuclear translocation of NF-κB (72, 175, 176). This suppression of NF-κB results in the downregulation of several cytokines in teleost mucosal and systemic tissues. Additionally, NLRC5 inhibits RLRs such as MDA5 and RIG-I, resulting in MAVS association that interferes with TBK1's recruitment of IRF3 and IRF7, suppressing IFN-I production (72, 154, 175). In zebrafish, this function appears to be IFN-independent, as overexpression of NLRC5 reduced viral replication of SVCV (spring viremia of carp virus) without activating type I IFN promoters, distinguishing it from classical antiviral PRRs (175).

A unique feature of NLRC5 is its dual role in immune regulation. In addition to suppressing inflammation, it has a transcriptional regulatory capacity (27, 75, 175, 177). In mammals, NLRC5 translocates to the nucleus via a bipartite nuclear localization signal (NLS) and acts as a master transactivator of MHC class I genes through interaction with the

SXY module of the MHC I enhanceosome (175, 178–182). In contrast, zebrafish NLRC5 contains a monopartite NLS and shows a nuclear-cytoplasmic distribution. It was even found that microinjected larvae of zebrafish selectively activate MHC class II genes, substituting CIITA's function, resulting in a unique discovery (175, 178, 179). NLRC5 in teleosts can translocate into the nucleus and bind directly to the MHC class I promoter, facilitating its expression in response to IFN-γ (27, 75, 175, 177). This enhances antigen presentation and supports adaptive immune responses, distinguishing NLRC5 from other NLRs, which typically lack transcriptional regulatory functions.

3.3 NLRX1 and NLRP1

Figure 6 outlines the distinct signaling mechanisms of NLRX1 and NLRP1, which are detailed below. NLRX1 was shown to act similarly to NLRC3 via its slightly varied inhibition of STING to TBK1 binding, and inhibition of NF-κB via TRAF6 (11, 169, 183). Upon viral infection, the zebrafish NLRX1 isoform was shown to downregulate IFN response by targeting STING for proteasome-dependent degradation (11). Mechanistically, NLRX1 binds to the N-terminal domain of STING and recruits the E3 ubiquitin ligase RNF5, which catalyzes K48-linked polyubiquitination of STING and marks the protein for proteasome degradation (11). As a result, STING protein levels are reduced before it can interact with and activate TBK1, blocking the phosphorylation of IRFs and the production of antiviral IFNs (11).

Much like NLRC5, antiviral signaling also begins with RIG-I or MDA5 recognizing viral RNA and activating MAVS (184, 185). Under normal conditions, MAVS oligomerizes and recruits downstream kinases TBK1 and IKKε, which phosphorylate IRF3/7, leading to their nuclear translocation and the induction of IFNs and ISGs (185). However, in black carp, this signaling is negatively regulated by the mitochondrial protein NLRX1, as the protein prevents MAVS oligomerization upon binding at the mitochondrial membrane and NACHT domain, blocking recruitment of TBK1 (185, 186). TUFM, another mitochondrial-associated protein, does not bind MAVS directly but instead associates with NLRX1 to enhance its inhibitory effect, collectively dampening the antiviral IFN response and promoting viral persistence (185).

During *Edwardsiella piscicida* infection in zebrafish, NLRX1 overexpression led to increased bacterial proliferation and decreased host survival (183). Mechanistically, NLRX1 interacts directly with TRAF6 at its NACHT domain, inhibiting TRAF6's ability to activate downstream NF-κB signaling (183). This resulted in the negative regulation of pro-inflammatory cytokines, IL-6, IL-8, TNF-α, and IL-1β, and specific antimicrobial peptides in a very similar manner to NLRC3 (169, 183).
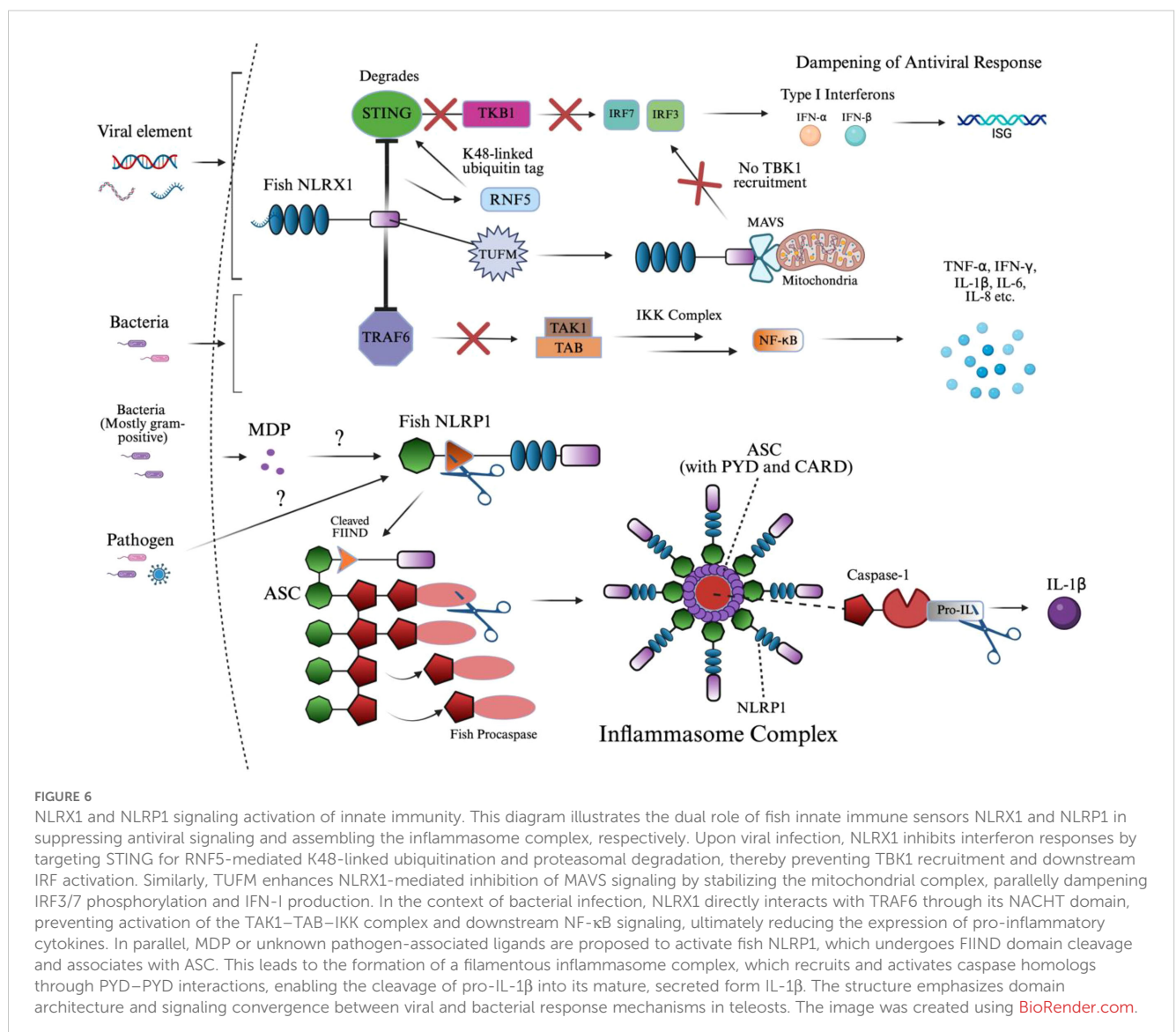
The ligand responsible for NLRP1 activation has been identified as MDP in fish, along with an imbalance of the redox state in humans (28, 101). The NLR uses its FIIND domain, which is involved in autolytic cleavage, to induce a conformational change of the CARD domain to interact with the CARD of ASC, as

referenced in section 2.6 (28, 187–191). This FIIND domain in NLRP1 consists of the ZU5 and UPA subdomains in humans, where ZU5 is cleaved, initiating the signaling cascade; however, in fish, the structure of the domain has not been identified (192). Moreover, these homotypic interactions of ASC in humans are replaced by PYD-PYD interactions, which fish lack (187–191). Due to the homology between these domains in signaling ASC, it was found that the PYD domain was dispensable in humans (132, 187). Both mechanisms of NLRP1-ASC binding result in nucleation of a filamentous platform for procaspase binding.

As mentioned, procaspase-A and procaspase-B were identified in zebrafish and are likely homologs of procaspase-1, which is the canonical NLRP1 inflammasome protein across most studied species (28, 193–196). Other, nonconventional proteins have been identified in humans, caspase-4 and caspase-5, and in mice, caspase-11. The specific type of procaspase or caspase will not be mentioned due to the complexity of species-specific variation involved in teleosts, and should be implied as caspase-1 or its homologs. In zebrafish and common carp, the caspase precursor,

procaspase, contains a PYD domain instead of the canonical CARD domain seen in mammals (28, 101, 187, 193). This domain substitution is critical, as it alters the recruitment mechanism: instead of CARD–CARD binding, teleost ASC filaments interact with procaspase via PYD–PYD binding, driving oligomerization to form the inflammasome (28, 101, 187, 193). This distinction in procaspase structure indicates that some or all teleosts may have undergone lineage-specific modifications to accommodate different innate immune contexts.

Structurally, the activated inflammasome in studied teleosts forms a three-dimensional ring-like complex following the cleavage of procaspase (28, 101, 193). This domain architecture is composed of multiple NLRP1 molecules branched to ASC filaments that form the core (28, 101, 193). Procaspase molecules are anchored to these ASC filaments, which cannot be visualized in a top-down view, allowing for efficient clustering and activation (28, 101, 193). The integrity of this oligomerized structure is crucial for the spatial coordination of signal transduction, ensuring that cytokine signaling is regulated and localized.

FIGURE 6
NLRX1 and NLRP1 signaling activation of innate immunity. This diagram illustrates the dual role of fish innate immune sensors NLRX1 and NLRP1 in suppressing antiviral signaling and assembling the inflammasome complex, respectively. Upon viral infection, NLRX1 inhibits interferon responses by targeting STING for RNF5-mediated K48-linked ubiquitination and proteasomal degradation, thereby preventing TBK1 recruitment and downstream IRF activation. Similarly, TUFM enhances NLRX1-mediated inhibition of MAVS signaling by stabilizing the mitochondrial complex, parallely dampening IRF3/7 phosphorylation and IFN-I production. In the context of bacterial infection, NLRX1 directly interacts with TRAF6 through its NACHT domain, preventing activation of the TAK1–TAB–IKK complex and downstream NF-κB signaling, ultimately reducing the expression of pro-inflammatory cytokines. In parallel, MDP or unknown pathogen-associated ligands are proposed to activate fish NLRP1, which undergoes FIIND domain cleavage and associates with ASC. This leads to the formation of a filamentous inflammasome complex, which recruits and activates caspase homologs through PYD–PYD interactions, enabling the cleavage of pro-IL-1β into its mature, secreted form IL-1β. The structure emphasizes domain architecture and signaling convergence between viral and bacterial response mechanisms in teleosts. The image was created using BioRender.com.

Procaspase is then cleaved within the inflammasome at conserved aspartate residues between its large (subunit varies) and small (often p10) catalytic subunits, forming the active caspase heterodimer, identified as p35 in zebrafish (28, 197). Once activated, caspase cleaves pro-inflammatory cytokine, pro-IL-1β, in zebrafish into its mature form, IL-1β (28). In mice, IL-1β and IL-18 are induced following NLRP1 inflammasome formation (198).

Although NLRP3 is not expanded on in this study, the protein has been well studied in several teleosts as an inflammasome that results in inflammatory caspase activation and interleukin-1β maturation, much like NLRP1, and has also been explored in zebrafish, common carp, Atlantic salmon, goldfish, and other teleosts (28, 172, 193, 199–202). NLRP3 can coordinate caspase activation in a two-step manner and release IL-18, unlike NLRP1, with both ASC-dependent and ASC-independent (via direct caspase-B triggering) routes (28, 199). The result is linked to gasdermin E-mediated pyroptosis, indicating broader or more flexible downstream effector engagement (199). Despite its importance, NLRP3 is not found in all teleost lineages, and most studies focus on cyprinid species. Still, its similarities to mammalian inflammasome responses make it a valuable model for studying inflammation and immune activation in fish.

4 Functional roles of fish NLRs in disease protection

NLR, along with other novel PRRs, can be induced by any pathogen that stimulates the innate immune system, such as bacterial, viral, and parasitic infections, due to the induction of signaling molecules from the presence of specific ligands. These infections have significant implications in aquaculture, where immune dysfunction or overactivation can lead to mortality and economic loss. Additionally, studying fish responses to these pathogens can provide broader insights into vertebrate immune evolution and adaptation.

In this section, bacterial infections will focus on gram-negative and gram-positive bacteria due to their association with iE-DAP and MDP. Regarding viral infections, ssRNA, dsRNA, and dsDNA viruses will also be expanded on. Although ssDNA viruses exist, they will not be discussed in this section due to limited research on their significance in NLR stimulation. For parasitic infections, the discussion will center on protozoan, ectoparasitic, and endoparasitic challenges that have been documented to modulate NLR responses in fish species. The phenotypic traits and genetic elements of these pathogens will be emphasized as they are key to determining the class of NLR stimulated via differences in ligands presented. Bacterial features like lipid A structure and motility, as well as TLR activation, lie beyond the scope of this analysis but warrant future investigation. Similarly, the location of tissue upregulation will not be a point of emphasis in terms of organization due to the variety of teleost species and NLRs covered. It should be noted that NOD1/2 are conserved across tissues, and are especially prominent in the mucosal barriers of gills, skin, and intestinal mucosa, where they provide cellular and humoral protection through

the release of cytokines and recruitment of T cells (203). Table 1 summarizes the diversity of NLR ligands and pathogen interactions across bacterial, viral, and parasitic infections in teleost fish.

4.1 Bacterial infections

The functional roles of NLRs upon bacterial infection in fish can be pro-inflammatory or regulatory, depending on the receptor type. Regarding pro-inflammatory defense, NOD1 was shown to elicit the expression of IL-1β, IL-8, and other antimicrobial effectors in zebrafish (*Danio rerio*) upon administration of *Edwardsiella piscicida* (edwardsiellosis), promoting NF-κB and MAPK pathway activation through RIP2 interaction (149). This same study showed that *Flavobacterium columnare* had a similar effect (149). Similarly, an *E. piscicida* vaccine was constructed that substituted the chromosomal *murA* promoter with the arabinose-dependent *araC* P$_{araBAD}$ cassette (205). Removal of this arabinose upon administration resulted in cell wall lysis and spread of the strain, upregulating TNF-α, IL-1β, IL-8, IL-6, and IFN-γ by stimulating both NOD1 and NOD2 in channel catfish (*Ictalurus punctatus*) (205). In this same fish species, NOD1 expression in the intestine increased ~6-fold at 3 days post-infection with *Edwardsiella ictaluri* (93). Similarly, three bacterial infections of *Aeromonas hydrophila*, *Shigella flexneri*, and *Edwardsiella tarda* upregulated NOD1 and RIP2, forming a complex for further signaling in rohu (94). It should be noted that *Shigella* spp. do not present virulence activity below 35°C, which limits its pathogenic potential in ectothermic hosts such as most fish, but it was worth mentioning (234). The mentioned bacterial species are all gram-negative bacterial infections, resulting in the release of the iE-DAP bacterial ligand in NOD1 specifically, as outlined in Figure 4 (56, 93, 96, 149, 205). Gram-positive bacteria can also upregulate NOD1 upon iE-DAP stimulation with *S. agalactiae* infection in Nile tilapia (*Oreochromis niloticus*) as well as *Streptococcus uberis* in mrigal (*Cirrhinus mrigala*), which increased NF-κB and subsequent pro-inflammatory cytokine production (96, 97).

Gram-negative bacteria still contain a peptidoglycan, releasing MDP in some cases for NOD2 ligand binding (54). As a result, in rohu (*Labeo rohita*), *E. tarda* infections upregulated NOD2 ~5-6-fold, inducing IFN-γ production up to ~10-fold (55). This same study found rohu infected with *A. hydrophila* upregulated NOD2 from ~2 to ~5-fold, while infected mrigal (*Cirrhinus mrigala*) upregulated both NOD1 and NOD2, conferring the production of IL-8, IL-1β, and IFN-γ, primarily in the liver, kidney, and spleen (96). Similarly, miiuy croaker (*Miichthys miiuy*) infected with *Vibrio anguillarum* resulted in varying expression levels of NOD1 and NOD2 in these same organs (99). Concerning the same ligand pathway in gram-positive bacteria, *S. uberis* infection in migral resulted in an even greater NOD2 upregulation than the previously mentioned gram-negative bacteria due to the increased presence of MDP (96).

Bacterial infections also activate other NLRs, either individually or in combination, which vary depending on fish and bacterial species. *E. tarda* and *Streptococcus iniae*, which are Gram-negative and Gram-positive bacteria, respectively, resulted in the expression

TABLE 1 Pathogen and ligand diversity driving NLR signaling pathways in teleost fish.

NLR	Known ligands	Bacterial fish pathogens activation	Viral fish pathogens activation	Protozoan fish pathogens activation
NOD1	iE-DAP (95), LPS (204), viral dsRNA (143)	*Edwardsiella piscicida* (149, 205), *Flavobacterium columnare* (149), *Aeromonas hydrophila* (94, 96, 206), *Streptococcus agalactiae* (97), *Edwardsiella ictalurid* (93), *Shigella flexneri* (55), *Edwardsiella tarda* (94, 206), *Streptococcus uberis* (96), *Vibrio anguillarum* (99), *Mycobacterium marinum* (98), *Aeromonas salmonicida* (98), *Streptococcus iniae* (206)	Nervous necrosis virus (NNV) (207), Tilapia lake virus (TiLV) (97), Grass carp reovirus (GCRV) (208–211), catfish hemorrhage reovirus (CCRV) (206), Poly (I:C) (143)	*Cryptocaryon irritans* (212)
NOD2	MDP (Muramyl dipeptide) (213), viral ssRNA (153)	*Edwardsiella piscicida* (205), *Aeromonas hydrophila* (96, 206), *Edwardsiella tarda* (55, 206), *Vibrio anguillarum* (99), *Streptococcus agalactiae* (97), *Edwardsiella tarda* (55, 206), *Streptococcus uberis* (96), *Vibrio anguillarum* (99), *Mycobacterium marinum* (98), *Aeromonas salmonicida* (98), *Streptococcus iniae* (206)	TiLV (97), GCRV (208–211), CCRV (206)	NA
NLRC3	Viral RNA (214), viral DNA (84), LPS (214)	*Aeromonas hydrophila* (206), *Edwardsiella tarda* (100, 206, 215), *Streptococcus agalactiae* (97), *Streptococcus iniae* (100, 206, 215)	Hirame novirhabdovirus (HIRRV) (216), hematopoietic necrosis virus (IHNV) (217), spring viremia of carp virus (SVCV) (137, 170), Salmon Anemia Virus (ISAV) (218), Piscine orthoreovirus-1 (PRV-1) (219), CCRV (219), Poly (I:C) (214)	*Ichthyophthirius multifiliis* (220)
NLRC5	Viral RNA (221), LPS (175)	*Aeromonas hydrophila* (206), *Edwardsiella tarda* (206), *Streptococcus iniae* (101, 206)	IHNV (217), ISAV (218), CCRV (206), Poly (I:C) (221)	*Cryptocaryon irritans* (212)
NLRP1	Viral RNA and DNA (28), MDP (28, 101)	*Aeromonas hydrophila* (101), *Edwardsiella tarda* (28)	ISAV (218)	NA
NLRX1	Viral RNA and DNA (11, 185), LPS (186)	*Aeromonas hydrophila* (98, 206), *Aeromonas salmonicida* (98), *Mycobacterium marinum* (98), *Streptococcus iniae* (206), *Edwardsiella tarda* (206)	SVCV (11), CCRV (206)	*Paramoeba perurans* (222)
NLRs (not identified)	Unknown	NA	TiLV (223, 224), Piscine myocarditis virus (PMCV) (225, 226), Megalocytivirus (MCV) (227–230), CCRV (206)	*Cryptocaryon irritans* (231), *Ichthyophthirius multifiliis* (220), *Amyloodinium ocellatum* (232, 233)

of NLRC3 in Japanese flounder (*Paralichthys olivaceus*), subsequently increasing IL-1β mRNA expression (100, 215). Likewise, a similar study induced *E. tarda, A. hydrophila, and S. iniae* infections in channel catfish, resulting in the upregulation of NOD1, NOD2, NLRC3, NLRC5, and NLRX1 in the intestine, liver, and head kidney, along with varied instances of NLR downregulation in the spleen (206). NOD1, NOD2, and NLRX1 were induced in goldfish (*Carassius auratus* L.) upon heat-killed *Aeromonas salmonicida* and the acid-fast bacterium *Mycobacterium marinum* challenges, indicating functional conservation of NLRs in teleost fish (98). In a previously mentioned study, *S. agalactiae* underwent MDP-stimulation, overexpressing NOD2 and NLRC3, which peaked in the spleen, kidney, gill, and blood of Nile tilapia, enhancing NF-κB signaling (97). *E. tarda* and *A. hydrophila* infections were also shown to upregulate NLRP1 expression in common carp (*Cyprinus carpio*) (101). *E. tarda* had the same effect in zebrafish, activating caspase-A/B, functional homologs of caspase-1 in mammals, and IL-1β, indicating inflammasome activation (28). The further signaling pathway of pro-inflammatory or regulatory response was not always performed;

however, it should be noted that NLRC3, NLRC5, and NLRX1 have been shown to result in inhibitory effects on innate immune signaling pathways in fish, as mentioned (11, 137, 235). Direct LPS (Lipopolysaccharide), peptidoglycan (PGN), MDP, and iE-DAP have been administered *in vitro* and *in vivo*, resulting in similar NLR production, further backing these mechanisms (96, 204). Several other studies using other bacterial and fish species combinations have been performed, with the general principles of NLR signaling applied upon infection.

4.2 Viral infections

Devastation to aquaculture has been largely driven by viral infections. As a result, extensive research has been done in teleosts to find downstream immunogenic signaling proteins and cytokines. Their variability in ssRNA, dsRNA, and dsDNA forms makes it especially challenging for the novel prediction of immune response and elicits a wide range of NLRs, though distinctions between positive-sense and negative-sense RNA viruses are beyond the scope of this review.

Several ssRNA viruses have induced NLR response in various teleosts. One study in orange-spotted grouper (*Epinephelus coioides*) found that NOD1 was slightly upregulated following infection with the nervous necrosis virus (NNV), but acts as a negative modulator of IFN signaling at the RNA sensor level by suppressing RIG-I- and MDA5-mediated IFN promoter activation (207). In Nile tilapia, Tilapia lake virus (TiLV) infection activated NOD1 and NOD2, such as TRAF-mediated NF-κB activation, which induces pro-inflammatory cytokines, particularly IL-1β, which, together with viral viroporins, promotes NLRP3 inflammasome formation (97, 223, 224, 236–238). Also, the Hirame novirhabdovirus (HIRRV) in Japanese flounder upregulated NLRC3 and several interferon-stimulated genes, including IRF3, IRF7, IKKβ, and TBK1 (216). Signaling became broader when rainbow trout infected with hematopoietic necrosis virus (IHNV) significantly upregulated NOD1, NLRC3, and NLRC5 expression in their skin, alongside pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α, and IFN-I (217). NLR studies of spring viremia of carp virus (SVCV) were tested in several teleosts where species-specific immune modulation occurred (137, 170). In zebrafish, NLRC3-like proteins act as either positive or negative regulators during pathogen infection, while in grass carp, NLRC3 acts as a negative regulator by degrading IRF7 and suppressing the RLR-mediated interferon response, ultimately enhancing viral replication (137, 170). Along with this, SVCV upregulates NLRX1 in zebrafish and suppresses the IFN response by degrading and disrupting STING–TBK1 signaling (11). In Atlantic salmon infected with Infectious Salmon Anemia Virus (ISAV), NLRC3, NLRC5, and NLRP1 were upregulated in gills and head kidney alongside interferon-stimulated genes and pro-inflammatory cytokines (218). The specific regulatory effects on innate immunity of these three NLRs were not further explored in this study (218).

Similarly, the effects of dsRNA viruses have also been observed in grass carp infected with grass carp reovirus (GCRV), where NOD1 and NOD2 expression was significantly upregulated in the spleen and trunk kidney (208–211). In Atlantic salmon red blood cells exposed to Piscine orthoreovirus-1 (PRV-1), NLRC3-like receptors were primarily expressed (219). Likewise, a broader study focused on the outcomes of Atlantic salmon suffering from cardiomyopathy syndrome (CMS) following intraparietal injection of piscine myocarditis virus (PMCV) in the heart, displayed the general upregulation of NLRs (225, 226). The last notable finding was the production of NOD1, NOD2, NLRC3, NLRC5, and NLRX1 from a previously mentioned bacterial study that also introduced a channel catfish hemorrhage reovirus (CCRV) challenge (206). None of these dsRNA virus studies explored any form of downstream immune regulation mediated by these NLRs. Also, upon polyinosinic:polycytidylic acid (poly (I:C)) challenge, NLRC5 was upregulated, and failed to activate IFN-Is (239).

The last classification of piscine viruses is composed of dsDNA, such as megalocytivirus (MCV) administered in spotted knifejaw (*Oplegnathus punctatus*), which presented general NLR expression, while channel catfish virus (CCV) elicited NLR, RIG-I, and MDA5, constitutively expressed across different tissues (227–230). There are several other ssRNA, dsRNA, and dsDNA viruses studied or are yet to be studied that may play a pivotal role in identifying a canonical ligand or signaling pathways to predict NLR expression.

4.3 Parasitic infections

In contrast with bacterial and viral pathogens, parasites typically develop long-term relationships with their hosts, testing the immune system in novel ways. Interactions with NLRs in response to infection vary with patterns of fish and parasite species but may act as a key driver for resistance in teleosts. Research on NLR response following parasitic infection in fish has been sparsely addressed, primarily focusing on protozoan, ectoparasite, and endoparasitic challenges *in vivo*.

Cryptocaryon irritans is an obligate ciliate parasite that embeds itself in the epithelial tissue of marine fish species, leading to white spot lesions and secondary infections (240). Exposure to *C. irritans* in golden pompano *(Trachinotus ovatus)* led to elevated expression of APAF1 and NOD1 in nearby skin regions (NRS), while NLRC5 was downregulated in that region, suggesting increased apoptotic and inflammatory activity (212). Another study in Japanese pufferfish (*Takifugu rubripes*) infected with the same parasite also revealed NLR induction (231). On the other hand, the ciliate protozoan parasite, *Paramoeba perurans*, makes surprising attempts to possibly even evade innate immune responses in Atlantic salmon by downregulating NLRX1 along with IL-1β, TNF-α, IFNα3, and IRFs (222).

In grass carp infected with *Ichthyophthirius multifiliis*, a bacterium causing white spot disease in freshwater fish, significantly upregulated NLRC3 and NLRP3 in resistant individuals, contributing to pathogen recognition and resistance by suppressing NF-κB signaling to prevent excessive inflammation (220). Another study found that following an *Amyloodinium ocellatum* infection, the inflammasome, NLRP12, and other NLR components, including CARD9 and Proline-Serine-Threonine Phosphatase Interacting Protein 1 (PSTPIP1), were significantly upregulated in the skin of golden pompano (232, 233). While studies on parasite-induced NLR activation in fish remain limited, mammalian research has demonstrated a wide range of NLRs, such as NOD1, NOD2, NLRP1, NLRP3, and NLRP12, which are activated in response to protozoan infections of *Entamoeba histolytica, Leishmania* spp.*, Plasmodium* spp.*, Toxoplasma gondii*, and *Trypanosoma cruzi* leading to inflammasome assembly, cytokine production, and modulation of Th1/Th2 responses in mouse, human epithelial, monocytic cells, and in rats (201, 241–257).

5 Emerging role of NOD-like receptors in vaccine-induced immunity in aquaculture

As aquaculture continues to expand globally, effective vaccination strategies remain essential for disease control and fish health management. While many current vaccines rely on surface PRRs such as Toll-like receptors (TLRs) for immune activation, recent research suggests that intracellular sensors like NLRs may also play a role in modulating vaccine-induced innate and adaptive immunity, possibly through pathways outlined in section **3**. Disease outbreaks

pose a major threat to the rapidly growing aquaculture industry, and effective vaccination is crucial for sustainable production.

Vaccination exposes fish to antigenic components of a pathogen to trigger a protective immune response to prevent disease upon subsequent exposure. They are often coupled with an adjuvant to boost innate immune activation and antigen presentation, and with a delivery vector to enhance cellular uptake, stability, and targeting. Adjuvants often contain known bacterial ligands which have the potential to engage with fish NLRs, especially NOD1 and NOD2 to encourage the previously mentioned pathways of immune response.

5.1 Adjuvants and vaccines as NLR agonists

NLRs, like other PRRs, are not used as adjuvants themselves due to several challenges. This includes their intracellular, cytosolic presence, risk of hyperinflammation, as there may be overexpression of cytokine response, and impracticality, as activation through known NLR activating ligands is more ideal. Mammalian studies have shown that NLR upregulation follows the administration of adjuvants and vaccines serving as analogs of canonical ligands, such as iE-DAP and MDP, eliciting NOD1 and NOD2 activation, respectively. This can trigger signaling pathways inside cells that influence cytokine production and antigen presentation. These systems are better characterized in mammals due to more extensive immune cell profiling and transgenic animal availability. Although there is currently no direct evidence that NLRs serve as immune targets in developing aquaculture adjuvants in fish, new studies hint at their possible role in shaping host immune responses. Therefore, we suggest NLRs as promising, though unverified, candidates for immunomodulation in aquaculture.

In mammals, MDP-containing adjuvants such as Complete Freund's Adjuvant (CFA), Murabutide, and Muramyl-Tripeptide Phosphatidylethanolamine (MTP-PE) have been shown to elicit NOD2, and even in some cases NOD1 (258–264). Notably, MDP was identified as a key component of CFA's adjuvancy, where NOD2 is required for effective CD4+ T cell priming, resulting in immunoglobulin G (IgG1 and IgG2c) production (258, 260, 265). The MDP synthetic derivative, Murabutide, was shown to activate HIV-infected antigen-presenting cells and induce cytokines that suppress viral replication, further demonstrating NOD2's immunomodulatory potential (258, 261, 262). Similarly, MTP-PE reduced pyrogenicity and significantly enhanced cellular immunity (258, 263, 266). It should be noted that N-acetyl MDP is produced by most bacteria and activates NOD2; however, N-glycolyl MDP is produced by the NamH enzyme in *Mycobacterium* and has been found to evade NOD2 signaling in mammals, which most likely also applies to fish (260). DAP containing adjuvants also exist, signaling NOD1, such as CFA and the synthetic peptides, FK-156 and FK-565. In CFA, DAP-type PGN fragments exist, promoting Th1 polarization and class switching to IgG2b, IgG2c, and IgG3 antibody isotypes, while FK-156/565 induced a Th2-biased immune response in human dendritic cells (264, 265, 267). To effectively introduce NOD ligands, encapsulation using poly(lactic acid) nanoparticles has been used and added with the antigenic

components of a vaccine to produce NOD1 and NOD2 activation (258, 268). In fish, these adjuvants have also been explored extensively and have been clearly shown to elicit cytokine and immune responses. While direct demonstration of NOD1/2 activation remains limited in fish, the conservation of NLR signaling in fish studies from other methods of iE-DAP and MDP-based challenges suggests similar effects by these adjuvants (269, 270). In mammals, several adjuvants were also shown to act as NLRP3 agonists, resulting in a similar inflammasome cascade as NLRP1, producing IL-1β and IL-18 (258, 271).

Although the role of NLRs in fish immunity is still emerging, their potential as adjuvant targets spans several vaccine platforms. Live attenuated and DNA vaccines may naturally engage NLRs through intracellular PAMPs, while inactivated and subunit vaccines could benefit from co-administered NLR agonists, like the ones mentioned, potentially enhancing cytokine production, APC maturation, and T or B cell responses. However, studies directly assessing NLR activation by these different vaccine platforms are limited. Recent literature has demonstrated that recombinant attenuated *Edwardsiella piscicida* vaccines (RAEVs) activate NOD1- and NOD2-mediated signaling pathways in teleost fish, resulting in the upregulation of pro-inflammatory cytokines and robust innate immune responses that confer protection against wild-type *E. piscicida* challenge (205). These findings align with recent reviews highlighting the importance of NOD-like receptor pathways in modulating mucosal and systemic immune responses to *Aeromonas hydrophila* vaccines in fish, particularly under immersion and oral delivery conditions (272). Some notable previously mentioned studies include a heat-killed bacterial challenge in goldfish and a live-attenuated, recombinant, *Edwardsiella piscicida* vaccine in channel catfish (98, 205). The first study resulted in the upregulation of NOD1, NOD2, and NLRX1, as mentioned, with no further downstream analysis, while the second produced IL-8, IL-1β, TNF-α, IL-6, and IFN-γ following the NF-κB pathway from NOD1 and NOD2 activation (98, 205). Along with these, a formalin-inactivated *Vibrio anguillarum* vaccine tested in Japanese flounder resulted in upregulation of NOD1, NOD2, NLRC3, and NLRC5, along with key proteins involved in the cascade to produce cytokines (13). In this same species, strictly NLRC5 was tested upon formalin-killed *E. tarda* and *S. iniae* infections, and was significantly upregulated from only the *E. tarda* vaccine (239).

6 Challenges and future directions in aquaculture

As the aquaculture industry continues to face evolving pathogen challenges and production demands, recent innovations in fish vaccination have focused on improving antigen design, adjuvant efficacy, and mucosal delivery strategies (273). These advances underscore the importance of integrating host-pathogen interaction studies with practical immunization platforms to achieve sustainable disease management (273). Despite the advances in the knowledge about fish NLRs, several gaps continue to restrict their use in

aquaculture. One such challenge is the partial functional characterization of many teleost NLRs. The unique structural domains and context-dependent processes of mammalian homologs in fish make it difficult to make direct comparisons. In this regard, teleost-specific subfamilies such as within NLRC have resulted in inconsistent nomenclature of the protein. Also, evolutionary divergence regarding gene duplications and structural diversity results in species-specific mechanisms for signaling. Notably, adaptations such as the teleost-specific modification of NLRC3, acting as a positive regulator of downstream pro-inflammatory responses, indicate functional divergence from the mammalian counterpart. This highlights the need for both comparative genomics and functional verification in commercially relevant aquaculture models.

Another key obstacle is ligand specificity. While some ligands' functional roles have been displayed, such as iE-DAP and MDP, many other ligands remain unidentified or untested in fish species. Mapping these ligand-receptor relationships is critical for understanding how different antigenic elements trigger innate responses through NLRs. These efforts would also inform vaccine development, as interspecies variation in NLR expression and signaling presents challenges for adjuvant design. Also, certain NLR agonists may be ineffective or incompatible with specific vaccine formulations, stressing the importance of determining optimal dosages and delivery systems to minimize inflammation or toxicity.

Approaches such as nanoparticle-mediated delivery could also idealize target effects. Along with this, the development of multi-component adjuvant systems combining NLR agonists with ligands for other PRRs may yield synergistic effects, amplifying protective immunity. However, these combinations must be empirically tested in fish models, detailing the presence of cross-talk and downstream signaling cascades in the presence of TLRs, RLRs, etc., to confirm compatibility and efficacy. One of the major challenges in advancing fish vaccine development is the limited understanding of mucosal immune mechanisms and the intracellular PRRs that regulate them. Despite promising data on the involvement of NLRs in pathogen sensing, few studies have systematically explored their role in mucosal immunization or adjuvant response (272).

The central focus of subsequent studies should be the discovery of novel NLR agonists that are safe, efficient, and applicable for aquaculture. Currently, fish studies use several known mammalian NLR agonists as adjuvants; however, these studies lack findings of NLR induction for vaccine use other than the use of the ligand itself. Integrating high-throughput transcriptomic and proteomic approaches may aid in uncovering the implications of these vaccine components, uncharacterized NLR pathways, and their regulators in fish.

7 Conclusion

The study of NLRs in fish reveals a remarkably intricate and evolutionarily diverse family of intracellular immune regulators, first identified in mammals. These proteins expanded and diversified structurally in teleosts, likely in response to the unique selective pressures imposed by aquatic pathogens, preceding further divergence in terrestrial vertebrates. Through phylogenetic mapping, structural modeling, and functional characterization, this review illustrates how NLRs in fish retain universal signaling motifs, CARD, NACHT, and LRR domains, which form their nomenclature, while also possessing lineage-specific adaptations that affect ligand recognition, downstream signaling, and immune modulation.

NLRs of fish coordinate a diverse array of activities in response to bacterial, viral, and parasitic infections. NOD1 and NOD2 act as pro-inflammatory sensors by recruiting RIP2 to trigger canonical NF-κB and MAPK cascades. Others, including NLRC3, NLRC5, and NLRX1, serve as immunological brakes, inhibiting IFN-I and inflammatory signaling to avert immune overactivation. Piscine NLRC3 has been shown to also upregulate these cascades in some species, while NLRC5 has a bifunctional role as an MHC-expressional transcriptional regulator and a cytoplasmic inflammatory pathway suppressor. The NLRP1 inflammasome formation displays teleost-specialized adaptations, including unique domain substitutions and mechanisms of caspase activation, further reflecting the structural and functional plasticity of NLR's gene family in fish.

Even though NLR signaling in parasite and vaccine response has been comparatively less well-explored, new information indicates responsiveness of NLRs to pathogen types and potential to be key determinants of immune resistance. Conservation of central signaling pathways like NF-κB and IRF-mediated cascades, combined with variations in ligand specificity and tissue expression, argues for the application of fish NLRs as targets for immunomodulation to benefit aquaculture. A range of mammalian NLR-agonist adjuvants, with MDP- and iE-DAP–derived compounds leading the list, hold potential in fish. However, the species specificity of NLR expression, localization, and signaling response remains the central challenge.

Future work must prioritize the identification of novel fish-specific NLR ligands, high-throughput screening of NLR-adjuvant interactions, and the development of delivery platforms that target these intracellular pathways without provoking deleterious inflammation. It will be vital to standardize nomenclature, extend functional analysis across commercial teleosts, and combine transcriptomic and proteomic tools such as molecular docking to close the gap between evolutionary insight and vaccine production. Collectively, these endeavors put fish NLRs at the center stage, not only as key innate immunity mediators but potential molecular levers to modulate disease and vaccine efficacy in today's aquaculture.

Author contributions

BS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing –

original draft, Writing – review & editing. KM: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

Acknowledgments

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

NLRA	NLR family acidic transactivation domain containing
NLRB	NLR family BIR domain containing
NLRC	NLR family CARD domain containing
NLRP	NLR family PYD domain containing
NLRX	NLR family with alternative effector domains
NOD1	Nucleotide Binding Oligomerization Domain Containing Protein 1
NOD2	Nucleotide Binding Oligomerization Domain Containing Protein 2
NLRC3	NLR family CARD domain containing protein 3
NLRC4	NLR family CARD domain containing protein 4
NLRC5	NLR family CARD domain containing protein 5
NLRP1	NLR family PYD domain containing protein 1
NLRX1	NLR family member X1
CARD	Caspase Activation and Recruitment Domain
PYD	Pyrin Domain
NB ARC	Nucleotide Binding Apaf 1, R proteins, CED 4 domain
NACHT	Domain named after NAIP, CIITA, HET E, and TP1
LRR	Leucine Rich Repeat
BIR	Baculoviral Inhibitor of Apoptosis Repeat
FIIND	Function to Find Domain
uCARD	Untypical CARD Domain
PRY SPRY	Protein Interaction Domain
AAA+	ATPases Associated with diverse cellular Activities
WHD	Winged Helix Domain
PST	Proline, Serine, and Threonine–rich region
iE DAP	γ D glutamyl meso diaminopimelic Acid
MDP	Muramyl Dipeptide
DAP	Diaminopimelic Acid
LPS	Lipopolysaccharide
PGN	Peptidoglycan
poly I:C	Polyinosinic:polycytidylic acid
Murabutide	Synthetic MDP derivative
MTP PE	Muramyl Tripeptide Phosphatidylethanolamine
FK 156 and FK 565	synthetic diaminopimelic acid–derived peptides

PLA	Polylactic acid nanoparticle delivery
TLR	Toll Like Receptor
RLR	RIG I Like Receptor
CLR	C type Lectin Receptor
PRR	Pattern Recognition Receptor
NF κB	Nuclear Factor kappa light chain enhancer of activated B cells
AP 1	Activator Protein 1
MAPK	Mitogen Activated Protein Kinase
IRF3 and IRF7	Interferon Regulatory Factors 3 and 7
IFN I/IFN α/β	Type I Interferon, alpha/beta
IFN γ	Type II Interferon Gamma
IL 1β	IL 6, IL 8, IL-18, Interleukins 1β, 6, 8
TNF α	Tumor Necrosis Factor Alpha
RIP2	Receptor Interacting Serine/Threonine Protein Kinase 2
TRAF3 and TRAF6	TNF Receptor Associated Factors 3 and 6
MAVS	Mitochondrial Antiviral Signaling Protein
STING	Stimulator of Interferon Genes
TBK1	TANK Binding Kinase 1
TAK1	Transforming Growth Factor Beta Activated Kinase 1
IKKα	IKKβ, IKKγ, IκB Kinase subunits α, β, γ
NEMO	NF κB Essential Modulator
MyD88	Myeloid Differentiation Primary Response 88
MHC	Major Histocompatibility Complex
ROS	Reactive Oxygen Species
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
SMART	Simple Modular Architecture Research Tool
Cryo EM	Cryo Electron Microscopy
RT PCR	Reverse Transcription Polymerase Chain Reaction
ATG	Autophagy Related Gene family
TUFM	Tu Translation Elongation Factor, Mitochondrial
RNF5	Ring Finger Protein 5
CIITA	Class II Transactivator.