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The zebrafish: A research model to understand the evolution of vertebrate immunity

Diana García-Moreno[a,b,*], Sylwia D. Tyrkalska[a,b], Ana Valera-Pérez[a,b], Elena Gómez-Abenza[a,b], Ana B. Pérez-Oliva[a,b,**], Victoriano Mulero[a,b,***]

[a] *Departmento de Biología Celular e Histología, Facultad de Biología, Universidad de Murcia, Spain*
[b] *Instituto Murciano de Investigación Biosanitaria (IMIB)-Arrixaca, Murcia, Spain*

ABSTRACT

The zebrafish has unique advantages for understanding the evolution of vertebrate immunity and to model human diseases. In this review, we will firstly give an overview of the current knowledge on vertebrate innate immune receptors with special emphasis on the inflammasome and then summarize the main contribution of the zebrafish model to this field, including to the identification of novel inflammasome components and to the mechanisms involved in its activation, assembly and clearance of intracellular bacteria.

1. Receptors of the innate immunity arm

The innate immune system detects the presence of microbes and initiates mechanisms to eliminate potentially infectious threats. Microbial detection is achieved through germline-encoded pattern-recognition receptors (PRRs) that survey both the extracellular and intracellular space for conserved microbial determinants that work as indicators of the infection [1]. Most PRRs can be classified into two main classes: membrane-bound receptors and unbound intracellular receptors. The first class consists mainly of the TLRs, which are found at the cell surface or on endocytic compartments, hence they survey for the presence of microbial ligands in the extracellular space and within endosomes. The second class consists of leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), RIG-1 like receptors (RLRs), the AIM2-like receptors (ALRs), which are located in the cytoplasm, where they sense the presence of intracellular pathogens [1].

2. Toll-like receptors (TLRs)

It is known that cells are able to detect the presence of pathogens by means of the toll like receptors (TLR) present in the cell membrane. As mentioned before the TLRs belong to the PRRs [2], that can recognize pathogen-associated molecular patterns (PAMPs) which are shared by many pathogens but not by the host cells. That mechanism triggers the intracellular signaling pathways, which results in releasing of pro-inflammatory cytokines, chemokines, interferons (IFNs), and prostanoids, as well as leads to the expression of co-stimulatory molecules [3,4].

TLRs are single, membrane-spanning, non-catalytic receptors usually expressed in “guardian cells”, such as macrophages and dendritic cells. They are believed to function as dimers. Though most TLRs appear to function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6; each dimer having different ligand specificity. The recognition of structurally conserved molecules derived from microbes occurs through leucine-rich repeats (LRRs) in the extracellular domains that are involved in ligand binding and auto-regulation [5]. PAMPs represent vital molecules important for microbial survival and are, therefore, unlikely to vary in their structures because any major changes would be disadvantageous. Such molecules include bacterial structural components, such as LPS, peptidoglycans (PGN) or viral RNA, and are specifically recognized by their corresponding TLR [6]. Recognition of PAMPs by TLRs results in the activation of different intracellular signaling cascades, generally leading to the activation of NF-κB, activator protein-1 (AP-1) and type I IFN. Moreover, TLRs are key inducers of the pro-inflammatory cytokines IL-1β and IL-18, but do not directly contribute to the activation of inflammatory caspases [7].

3. NOD-like receptors (NLRs) and inflammasomes

Apart from TLRs, other PRR families have been identified. A very important one is the NLR receptor family, which was described 20 years ago [8–12]. NLRs include 22 members identified in humans and more than 30 in mice [13,14]. The NLR family is a group of intracellular

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[*] Corresponding author. Departmento de Biología Celular e Histología, Facultad de Biología, Universidad de Murcia, Spain.
[**] Corresponding author. Departmento de Biología Celular e Histología, Facultad de Biología, Universidad de Murcia, Spain.
[***] Corresponding author. Departmento de Biología Celular e Histología, Facultad de Biología, Universidad de Murcia, Spain.
E-mail addresses: dianagm@um.es (D. García-Moreno), anabpo@um.es (A.B. Pérez-Oliva), vmulero@um.es (V. Mulero).

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receptors that are able to detect pathogens that enter to the cell via phagocytosis or pores, and DAMPs that are associated with cell stress or danger signals occurring in the cytosol. The NLRs present three characteristic structural domains: a central nucleotide-binding and oligomerization (NACHT) domain, which is commonly flanked by C-terminal LRRs and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains. The C-terminal LRR domain is considered to be the sensing motif able to recognize different ligands, as in TLRs. The intermediary NACHT domain is essential for the oligomerization and activation of NLRs. Oligomerization of the NACHT domain is a pre-requisite for transduction of the signal mediated by the third N-terminal effector domain, which may be PYD or CARD [15,16]. The diversity of effector domains allows the NLRs to interact with a wide variety of binding partners and to activate multiple signalling pathways. The most studied receptors belonging to this family are NOD1, NOD2, NLRP1, NLRP3, NLRC4 (formerly IPAF) and AIM2.

The NLR proteins are normally present in the cytoplasm in an inactive, auto-repressed form. The LRRs fold intramolecularly back onto the NACHT domain, thereby inhibiting spontaneous oligomerization and activation of the NLR protein. Upon direct or indirect binding of a PAMP to the LRR, the molecule undergoes a conformational rearrangement, exposing the NACHT domain and thereby triggering oligomerization. In turn NLRs expose the effector domains. Through a homotypic interaction, CARDs and PYDs recruit CARD- and PYD- containing effector molecules, bringing them into close proximity with each other and leading to their activation [17]. These proteins are the ideal molecular platform needed for the activation of inflammatory caspases.

Based on the domains present in N-terminal region, the NLRs are classified in two subgroups: NLR*C*s (*C*ARD), and NLR*P*s (*P*YRIN). The NLR members NOD1 and NOD2 belong to the NLRC subgroup as they contain an amino-terminal CARD domain. NOD1 contains a single CARD domain, whereas NOD2 has two of them [18,19]. Activating ligands for NOD1 and NOD2 are subcomponents of PGN, namely D-γ-glutamyl-meso-DAP(mDAP) and muramyl dipeptide (MDP), respectively [20,21]. NOD1 and NOD2 activate NF-κB through the recruitment and oligomerization of receptor-interacting protein 2 (RIP2), resulting in the activation of the IκB kinase complex [18,22]. NOD2 has an essential role as an intracellular sensor of PGN in the intestinal immunity, since it has been found that mutations in NOD2 correlate with Crohn's disease (CD) [23]. At present, there are no clear links between NOD1 and NOD2 and caspase-1 activation. It seems however that despite the independent activation of NLR and TLR, the signalling cascades triggered by their activation might be similar and possibly involved in redundant functions.

In contrast, other members of the NLR family, such as NLRPs and NLRCs, are involved in caspase-1 activation and the formation of the inflammasome, a multiprotein complex [24–26]. One of the characteristics shared by NLRPs proteins is their ability to recruit the adaptor protein ASC with a C-terminal CARD and an N-terminal PYD domain. ASC is able to form PYD-PYD interactions with the define NLR, recruiting caspase-1 through CARD-CARD interactions, leading to its activation [23,27]. NLRC4, however, can interact directly with caspase-1 through its CARD domain and, therefore, ASC is probably not required in this process [28].

Moreover, based on previously published results, a model for unified prion-like polymerization mechanism for the assembly of ASC-dependent inflammasome was proposed. Stimulation of NLRP3 or AIM2 induces a conformational change that result in the oligomerization or close apposition of their individual PYRIN domains (PYD). Subsequently, these oligomers, through PYRIN-PYRIN interactions recruit multiple ASC proteins resulting in prion nucleation, which is otherwise prevented from occurring spontaneously due to a high-energy barrier. ASC prions rapidly template other ASC molecules resulting in the formation of large polymers and through CARD-CARD interactions, the ASC polymers recruit multiple caspase-1 molecules, bringing them into close proximity to induce their autocleavage and activation [29,30]. The PYRIN domains of ASC acts as the building block of the filaments and the CARDs are flexibly linked to induce pro-caspase-1 polymerization and activation. Relative few sensor molecules are sufficient to induce polymerization of relatively large numbers of ASC and caspase-1 molecules and also the process is self-propagating, allowing robust signal amplification.

It is now generally accepted that activation and release of IL-1β, in mammals, requires two distinct signals [31]: a first signal leading to the synthesis of the proIL-1β by activation of the transcription factor NF-κB [28], and a second one involved in caspase-1 activation and IL-1β release. What constitutes these signals *in vivo* during an infection or an autoinflammatory response is not known for certain. However, *in vitro* studies suggest that the first signal responsible for the proIL-1β accumulation in the cytosol can be triggered by TLR activation. Different investigations indicate that the second signal, which leads to the activation of caspase-1 and inflammasome, being a physiological signal is extracellular ATP (eATP), since eATP is able to induce the rapid processing and the massive release of IL-1β by binding the purinergic receptors P2X7 [32–37]. The channel model proposes that extracellular ATP activates the P2X7 receptor and allows the efflux of intracellular potassium ions (K^+) resulting in NLRP3 activation [38]. Also, phagocytosis of crystals leads to lysosomal swelling and damage. The lysosomal perturbation, together with the release of cathepsin B, a lysosomal cysteine protease, results in the activation of the NLRP3 inflammasome [39]. Even ROS generated during mitochondrial damage and oxidized mitochondrial DNA (mtDNA) produced during apoptosis lead to activation of NLRP3 [40].

The relevance of the NLR proteins, NLRP or NLRC, lies in the fact that infection with bacterial pathogens, both *in vitro* and *in vivo*, does not require ATP to trigger activation of the inflammasome. This finding might reflect the possibility that bacterial infection simultaneously induces the signals that are individually activated by eATP and TLR stimulation. In fact, it might be that pore formation in cell membranes caused by bacterial secretion systems triggers an ion flux in the cell that is mimicked by the activity of eATP [41,42].

4. Inflammatory caspases: Caspase-1

Caspases are a family of cysteine proteases that cleave after aspartate residues (Asp) [43,44]. Although caspase-mediated processing can result in substrate inactivation it may also generate active signaling that participates in ordered processes [45] and develop key roles in the apoptosis and proteolytic activation of cytokines [46–48]. In humans, the caspase family includes 13 members, whose functions seem to correlate with their phylogenetic relationship [49]. Cell death caspases are initiators (caspase-2, -8, -9, and -10) and executioners (caspase-3, -6, and -7) of apoptosis [48]. Initiator caspases sense death signals, and activate more downstream executioner caspases, which cleave cellular substrates, mediating the changes associated with apoptosis. Human inflammatory caspases include caspase-1, -4, -5, and -12. In mice, caspase-5 is absent but they have an additional inflammatory caspase, caspase-11, which has probably arisen by tandem gene duplication of caspase-4 [48]. The arrangement of the exon-intron structure of the inflammatory caspases, suggest that they originated from the same ancestral gene. Both inflammatory and apoptotic caspases are synthesized as inactive zymogens, situated in the cytosol and share a common conserved structure composed of a prodomain and a catalytic region [44,48,49].

Caspase-1 is one of the best characterized inflammatory caspases. Originally, caspase-1 was found in an attempt to purify the enzyme responsible for IL-1β processing [50], although later it was also shown to be able to activate IL-18 [23,51]. Caspase-1, like other pro-inflammatory caspases, contains an N-terminal caspase-recruitment domain (CARD), and a catalytic region composed of both a large (p20) and a small (p10) subunits with a conserved Gln-Ala-Cys-X-Gly active site sequence (where X is Arg, Gln or Gly) found in the large subunit. There are other structural proteins that present this CARD domain, like the adaptor protein ASC that binds to caspase-1, producing the oligomerization of pro-caspase-1 within the inflammasome, facilitating the caspase auto-cleavage into the active p20 and p10 subunits [52,53]. However, this dogma has recently been challenged by an elegant study [54]. Thus, in the cell, the dominant species of active caspase-1 dimers elicited by inflammasomes are in fact full-length p46 and a transient species, p33/p10. In addition, p33/p10 autoprocessing occurs with kinetics specified by inflammasome size and cell type, and this releases p20/p10 from the inflammasome, which becomes unstable and protease activity is terminated.

Although pro-caspase-1 is constitutively expressed in resting cells, it remains inactive in the cytoplasm until inflammatory cells, like monocytes or macrophages, receive the appropriate stimulus [55]. In these cells, K^+ release induces a rapid and strong activation of caspase-1, triggering the processing and release of the mature IL-1β [56]. In addition, it has recently been shown that caspase-11 (also known as caspase-4) is critical for caspase-1 activation and IL-1β production in macrophages infected with *Escherichia coli*, *Citrobacter rodentium* or *Vibrio cholerae* [57].

Despite the importance of caspase-1 in inflammation, the information on the presence and activity of this enzyme in fish is scant. Inflammatory caspases have so far only been found in vertebrates [23]. The first caspase-1 homologue of fish showing the N-terminal CARD domains has been identified in gilthead seabream [58] but this caspase-1 is unable to process IL-1β [59].

5. Inflammasome effectors: IL-1β

IL-1 is the common name for a diverse family of proteins (11 cytokines), of which IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra) and IL-18 are the most representative and studied, although several newly discovered molecules show a clear homology to this group [60–66]. Members of that family induce a complex network of pro-inflammatory cytokines and via expression of integrins on the surface of leukocytes and endothelial cells, regulate and initiate inflammatory responses (Dinarello, 2011).

The IL-1 family signature is conserved in the IL-1β sequences of mammals, birds and amphibians, but is only partially conserved in fish. So, a new family signature has been proposed taking in count all IL-1β sequences: [FCL]-x-S-[ASLV]-x(2)-[PSR]-x(2)-[FYLIV]-[LIV]-[SCAT]-T-x(7)-[LIVMK]. Although the sequences of IL-1β share the family signature with IL-1 and probably show the presence of β-sheets, the homology of the nucleotide or amino acid sequence among vertebrate molecules is quite low and varies considerably between fish species [67], due to the great phylogenetic diversity existing within this group of animals.

The role of IL-1β in the immune response has been partly elucidated by expression studies. In mammals, IL-1β is produced in response to many stimuli, including bacterial LPS, numerous microbial products, cytokines (TNF, IFN-γ, GM-CSF and IL-2), T-cell/antigen-presenting cell interactions and immune complexes [68]. Similar studies have revealed that bird, amphibian and fish IL-1β show a similar expression pattern to its mammalian counterpart. In chicken, IL-1β is quickly induced in blood monocyte-derived macrophages, reaching optimal levels within 1 h after LPS treatment [69]. The *Xenopus* IL-1β transcript was inducible *in vivo* following injection with LPS [70]. In fish, IL-1β expression studies have usually been performed by RT-PCR or Northern blot and it has been shown that *in vivo* and *in vitro* treatment with LPS is able to induce IL-1β mRNA in all the species tested, including carp, trout, seabass and catshark [71–75]. In trout many expression studies have been developed. Thus, it is known that stimulation with 5 μg/ml LPS induces the expression of IL-1β 1 h after stimulation, reaching maximum levels after 4 h. In addition, culture temperature has a marked effect on IL-1β expression, because a temperature increase acts as a positive regulator in IL-1β synthesis [73]. Moreover, IL-1β expression levels of the trout mononuclear phagocyte cell line RTS-11 are up-regulated after LPS treatment [76]. In seabream, IL-1β mRNA accumulation is induced in leukocytes after bacterial challenge [77] or *in vitro* stimulation with different PAMPs [78]. The protein is also accumulated in leukocytes activated with LPS or bacterial DNA, as detected by western blot using a polyclonal Ab to seabream IL-1β [79].

6. Pyroptosis, a caspase-1 dependent form of programmed cell death

Many forms of the programmed cell death pathways are critical for organogenesis, development, immunity or even the maintenance of homeostasis in multicellular organisms. Pyroptosis, a highly pro-inflammatory form of cell death, is an essential innate immune response to prevent the spread of the intracellular infection. Pyroptosis is induced by the activation of pro-inflammatory caspases within inflammasomes [54].

The existence of this unusual cell death was first seen in 1992, when the Sansonetti laboratory observed that murine macrophages infected with the Gram-negative bacterium, *Shigella flexneri*, were undergoing a form of cell death which was similar to apoptosis [80]. Afterwards, the Falkow laboratory made similar observations in cells infected with a closely related pathogen, *Salmonella enterica* serovar Typhimurium, reporting the presence of DNA degradation, changes in nuclear morphology and finally vacuole formation [81]. In addition to these apoptosis-like features, Cookson and co-workers reported that cell death by such infected cells also presented features similar to other death type: necrosis [82]. These cells formed membrane pores of 1–2.5 nm and displayed swelling and Ca^{2+} influx, leading to membrane rupture and the extracellular release of cellular contents. This unusual form of cell death induced by pathogens, containing hallmarks of both: apoptosis and necrosis [82], was found to be dependent on the proteolytic activity of caspase-1 [81].

The term ‘pyroptosis’ was coined by Cookson and Brennan in 2001 to simply distinguish this form of cell death from apoptosis and necrosis [83]. It has only been described in macrophages and dendritic cells [84,85], although there is some evidence of caspase-1 activity in other cell types [86].

Beside the formation of pores in the plasma membrane was directly linked to pyroptotic cell death, the exact mechanism underlying the formation of these pores remained unknown for a long time. In 2015, Gasdermin D (GDSMD) was simultaneously identified by different researchers as the main pore formation mediator during pyroptosis [87,88]. GSDMD acted downstream caspase-1 and caspase-11 and it was shown that GSDMD is a substrate of caspase-1 and its cleavage at the predicted site during inflammasome activation was required for pyroptosis and IL-1β secretion [89]. Once is cleaved GSDMD N-terminal domain assembles membrane pores to induce cytolysis, whereas its C-terminal domain inhibits cell death through intramolecular association with the N terminal domain [90].

Pyroptosis occurs mainly in innate immune cells, such as monocytes and macrophages [85,91]. Recent studies show that, in contrast to its activation in macrophages, GSDMD cleavage and activation in neutrophils was caspase independent and mediated by a neutrophil-specific serine protease, neutrophil elastase (ELANE), released from cytoplasmic granules into the cytosol in aging neutrophils. ELANE-mediated GSDMD cleavage was upstream of the caspase cleavage site and produced a fully active ELANE-derived NT fragment (GSDMD-eNT) that induced lytic cell death [92].

Several important human pathogens establish an intracellular niche within host cells, where they can proliferate and protect themselves from soluble immune mediators. Several studies have demonstrated clear functions for inflammasomes in controlling the replication of *Salmonella* inside of the host cell, through cytokine activation and pyroptosis induction [93–95]. Pyroptosis of infected cells is appearing as an important mechanism of microbial clearance [95], in addition to host defense mechanisms coordinated by inflammasome dependent IL-1β [93] and IL-18 [94,96]. Macrophage pyroptosis releases *Salmonella* from its intracellular replicative niche, and therefore increases its susceptibility to neutrophil-mediated extracellular destruction. This mechanism also appears to be very effective in limiting the replication of other intracellular pathogens, including *Legionella* and *Burkholderia* [95]. Additionally to eliminate the bacterial replicative niche, pyroptosis also releases multiple alarmins [97]) such as ATP, IL-33 [98] and HMGB1 [99] to alertabout the presence of the pathogen and recruit immune cells to the site of infection [100]. The oligomeric form of the adaptor protein - ASC can also act as an unusual alarmin released during pyroptosis that can propagate inflammasome signaling in surrounding macrophages [26,101]. Pyroptotic cells are also reported to undergo lysosomal exocytosis [102] to release lysosomal antimicrobial peptides [103] that facilitate the clearance of pathogens by recruiting neutrophils. The ability to undergo pyroptotic death is not unique to immune cells. For example, pyroptosis of the gut epithelium promotes the extrusion of infected cells into the gut lumen, preventing *Salmonella*, and probably other pathogens, from traversing the epithelial barrier to invade the host [104,105].

7. The zebrafish: description, distribution, taxonomy, ecology and reproduction

Zebrafish (*Danio rerio*) is a small shoaling cyprinid fish, which size reaches maximum 60 mm. Taxonomically, the zebrafish (*Danio rerio* H.) is a derived member of the genus *Danio*, of the family Cyprinidae, order Cypriniformes. For many years it was referred to, in scientific literature *Brachydanio rerio,* until its reassignment to the genus *Danio* [106]. Although details of the distribution are not entirely clear, *D. rerio* may be widely distributed in shallow, slow-flowing, tropical freshwaters on the Indian subcontinent. Their natural range is centered on the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh, and Nepal. They are most commonly encountered in shallow ponds and standing water bodies with visibility to a depth of approximately 30 cm, often connected to rice cultivation [107]. Zebrafish owes its name for the characteristic stripes running along the body and the fins.

Zebrafish are omnivorous, feeding primarily on zooplankton and insects, although phytoplankton, filamentous algae and vascular plant material, spores and invertebrate eggs, fish scales, arachnids, detritus, sand, and mud have also been reported from gut content analyses [107]. Zebrafish are promiscuous and breed seasonally during monsoon season, which occur from April to August (spawning has also been recorded outside wet season, suggesting that breeding may be seasonal as a result of food availability). Mating behavior is also heavily influenced by photoperiod, as spawning begins immediately at first light during breeding season and continues for about an hour. In order to initiate courtship about 3–7 males chase a female and try to lead her towards a spawning site by nudging her and/or swimming around her in a tight circle or figure eight. Spawning sites consists of bare substrate that tends to be well vegetated. In captivity, gravel spawning sites are preferred to silt spawning sites. In the wild, zebrafish breed in silt-bottomed habitats. When a breeding pair reaches the spawning site, the male aligns his genital pore with the female's and begins to quiver, which causes the female to release her eggs and the male to release his sperm. The female releases 5 to 20 eggs at a time. This cycle repeats for about an hour. While the presence of female pheromones is required for initiation of courtship behavior in the male, male gonadal pheromones are required by the female for ovulation to occur. There is limited evidence for male-male competition and female mate preference [108].

Zebrafish lay non-adhesive eggs without preparing a nest, and are considered to be group spawners and egg scatterers. Although time to hatch depends on water temperature, most eggs hatch between 48 and 72 h after fertilization. Chorion thickness and embryo activity also impact incubation time. Zebrafish are approximately 3 mm upon hatching and are immediately independent. They are able to swim, feed, and exhibit active avoidance behaviors within 72 h of fertilization [109–111].

8. The zebrafish as a vertebrate research model

For many decades, zebrafish has been both a very popular aquarium fish and an important research model in several fields of biology (notably, toxicology and developmental biology). Since it was first used in a scientific laboratory 38 years ago, its popularity in biomedical research has significantly increased due to their unquestionable advantages respect to other vertebrate models. The development of zebrafish as a model organism for modern biological investigation began with the pioneering work of George Streisinger and colleagues at the University of Oregon [112–114]. The use and importance of zebrafish in biological research has exploded and diversified to the point that these fish are extremely important vertebrate models in an extraordinary array of research fields [115], due to multiple advantages, including small size; low maintenance cost and small space needed; high resistance to pathogens; high fecundity and large production of embryos (around 200 eggs/female/week) that make phenotype-based forward genetics feasible; short generation time (for a vertebrate); zebrafish eggs are large relative to other fish, optically transparent and externally developed following fertilization, making them easily accessible to embryonic manipulation and imaging; transparency of zebrafish embryos, together with the large availability of transgenic lines, let *in vivo* tracking of cells; rapid development, which is very similar to the embryogenesis in higher vertebrates including humans, the precursors develop to all major organs within 36 h; special value as a model of human disease and for the screening of therapeutic drugs [116] and is often more tractable for genetic and embryological manipulation and cost effective than other vertebrate models such as mice [117]; easy to transfer among different labs by transporting their eggs; genome has now been completely sequenced, making it an even more valuable research organism; considerable genetic sequence similarity with humans; relatively easy to knockdown specific genes by using morpholinos and overexpressing proteins by mRNA or plasmids; amenable for reverse genetics approaches using zinc finger nucleases (ZFNs) [118] and CRISPR strategy [119,120] for generating different transgenic and mutant lines, which help in analyzing new roles of additional genes in larval and adult zebrafish; and moreover the existence of a centralized online resource for the zebrafish research community (http://zfin.org) makes easier the work with this model.

All these advantages have led to the increased interest of scientists using zebrafish as an animal model research in the last years. In the past, it was a major vertebrate model especially in the developmental and genetic research [121], whereas now, the zebrafish gains also growing importance in other fields. Nowadays, it has been proposed as an excellent model for the study of the immune system [122], hematopoiesis [123], vascular development [124–126] and cancer research [127,128], among others. Moreover, zebrafish has become a popular model in pharmacological studies and drug screening [129].

9. The zebrafish model: shedding light into inflammasome evolution

Although the zebrafish has extensively been used as a biomedical model, it contribution to evolutionary immunity in general, and the inflammasome in particular, is been crucial. The NLR has been drastically expanded into very large families in fish, including a first subfamily (NLR-A) resembling mammalian NODs, a second (NLR-B) resembling mammalian NALPs and a third (NLR-C) unique to teleost fish [130]. A recent study has reported that 400 NLR proteins are encoded in the zebrafish genome and, interestingly, the NLR-B30.2 proteins represent a new family with diversity in the specific recognition module that is present in fish [131]. The diversification of the NLR family is out of the scope of this revision.

Earlier studies reported two inflammatory caspases with a highly conserved caspase domain, namely Caspa (Caspy-1) and Caspb (Caspy-2), with highly conserved catalytic domain, and with an N-terminal pyrin domain (PYD) rather than the typical CARD of inflammatory caspases [49,132–134]. The function of these fish caspases has been controversial: while the first study suggested that they were involved in apoptosis rather than inflammation, since they are able to induce apoptosis when transfected into mammalian cells and are essential for morphogenesis of the jaw and gill-bearing arches of fish larvae [135], more recent studies have unequivocally showed that they are the functional orthologs of mammalian CASP1 and CASP11/CASP4/CASP5, respectively. Thus, Caspa has been shown to interact with Asc and being the main effector of the canonical inflammasome upon its activation by bacterial infections [134,136,137]. However, Caspb directly interact with cytosolic LPS promoting pyroptosis via caspase-5-like activity [138].

The zebrafish is also contributing to the identification of novel inflammasome components. For example, using a zebrafish-*S.* Typhimurium model the IFNγ-inducible GTPase Gbp4 has been shown to be involved in inflammasome activation and ST clearance [136]. Gbp4 inflammasome activation occurs in two steps: first neutrophils are recruited to the infection site through the inflammasome independent production of IL-8 and leukotriene B4 and then the bacteria is cleared through the Gbp4 inflammasome-dependent biosynthesis of prostaglandin D2. In addition, the CARD domain of Gbp4 is required for the assembly into the inflammasome through Asc interaction, while its GTPase activity is indispensable for inflammasome assembly, Caspa activation and bacterial clearance. Notably, neither IL-1β processing nor pyroptosis are activated by Gbp4 inflammasome and are, therefore, dispensable for bacterial clearance. These results, together with the inability of classical inflammasome activator to promote IL-1β processing and release in phylogenetically distant zebrafish and gilthead seabream [59,136,139], support the idea that the use of the inflammasome as a molecular platform for the processing of pro-inflammatory cytokines arose after the divergence of fish and tetrapods more than 450 million years ago [59]. Furthermore, because of the relatively less elaborated and restrictive adaptive immune response of early vertebrates [140], fish would display a more sophisticated intracellular sensing system than mammals, strengthening their ability to clear intracellular pathogens through the induction of pyroptotic cell death. Nevertheless, it is currently unknown whether Casp1/Caspa might mediate the processing of other cytokines, such as IL-18, in fish.

The zebrafish model has also allowed the identification of an evolutionarily conserved inflammasome adaptor, called Caiap from CARD- and ANK-containing Inflammasome Adaptor Protein, which is also involved in the inflammasome-dependent resistance to *S.* Typhimurium in zebrafish [141]. This protein is highly conserved from cartilaginous fish to marsupials but, curiously, is absent in placental mammals. Caiap has an unprecedented combination of domains: an N-terminal CARD domain and 16 C-terminal ANK domains. Caiap acts downstream bacterial flagellin, as Gbp4, and interacts with catalytic active Caspa through its ANK domain, while its CARD domain promotes its self-oligomerization. Caiap is the first inflammasome adaptor protein that has been shown to directly interact with active effector caspase further promoting its activation. Strikingly from a phylogenetic point of view is that Caiap should have been lost in placental mammals after its split from marsupials and, therefore, other proteins should have replaced Caiap in these animals to carry out similar functions.

One of the main advantages of the zebrafish model is the transparency of its embryo and larval stages. This advantage has been used to study the dynamics of Asc oligomerization *in vivo* by generating a knock-in line Asc-eGFP [134]. This model showed that although the basic structural features that lead to Asc speck formation are well conserved from fish to mammals, Asc interacts with Caspa via its PYD domain. Notably, there is a correlation between the compaction of the Asc speck and its efficiency to promote pyroptosis and, more importantly, Asc specks persist after cell death and can be phagocytosed by macrophages without promoting their pyroptosis, in contrast to the results found *in vitro* with mouse macrophages [26,101].

10. Conclusions and future directions

The unique advantages of the zebrafish make this teleost species not only a fantastic model for biomedical research but also for understanding the evolution of immunity. The possibility of *in vivo* tracking of immune cells and real time imaging of any immunological processes in a whole vertebrate organism together with the amenability for genetic and chemical screening are unique advantages to this animal model and complimentary to other vertebrate species, such as the mouse. However, straightforward extrapolation from one species to another should be avoided and the long phylogenetic distances among teleost species and their different ecological niches have to be taken into account when developing prophylactic strategies for the aquaculture industry.

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