

Response to Boyle et al.

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We appreciate the comments by Boyle et al. to our recent work where we described the activation of caspase-1 in response to changes in extracellular osmolarity in human, mouse, and fish macrophages (Compan et al., 2012). In macrophages derived from mice deficient for *Nlrp3*, this response was abolished, suggesting an active role for the NLRP3 inflammasome in the sensing of restorative cell volume decrease and the activation of caspase-1 in mammals (Compan et al., 2012). We speculated that the similarities found among mammalian and fish macrophages may reflect the fact that cellular volume regulation could be an evolutionary conserved danger signal to activate caspase-1. In their letter, Boyle et al. comment on (1) the absence of direct NLRP3 orthologs in fish and (2) the lack of classical NLRP3 activation in teleost fish to argue against the evolutionary and mechanistic basis for the inflammatory response to cell swelling.

We completely agree with Boyle et al.'s first point that various NLR-specific expansions have occurred in fish and that no full orthologs of mammalian NLRs have been identified and functionally characterized in fish (Laing et al., 2008). However, BLASTP searches using the amino acid sequence of human NLRP3 (NCBI accession number NP_004886) identified a gene on zebrafish (*Danio rerio*) chromosome 6 (XM_690068) which, upon reciprocal BLASTP analysis against human protein database, returned NLRP3 as the most significant hit (2×10^{-106}). This zebrafish putative NLR shows 46.7% amino acid sequence similarity (29.8% amino acid identity) with human NLRP3 and identical domain organization; that is, an N-terminal PYD domain followed by NTPase domain and leucine-rich repeats (LRR) (44%, 52%, and 51% similarity,

respectively, with human NLRP3 amino acid domain sequences). In addition, BLASTP searches using the amino acid sequence of human NLRP3 also identified several hundred putative zebrafish NLRP genes with the same domain organization, including the N-terminal PYD, but with an additional C-terminal PRY-SPRY domain (the so-called NLR-C fish subfamily described by Laing et al., 2008). The most significant hit identified from the NLR-C fish family (ENSDARG00000078620) returned NLRP12 (with a 43% similarity) followed by NLRP3 (with a 44% similarity) as the most significant hits upon reciprocal BLASTP analysis against human protein database. The data obtained from similar analysis with other fish genomes are difficult to interpret, because most of them are not completed and returned truncated proteins, but we were able to find putative NLRP3 orthologs (harboring PYD, NTPase, and LRR domains but lacking PRY-SPRY domain) in the genomes of coelacanth (*Latimeria chalumnae*; ENSLACG00000001733; 55% amino acid similarity with human NLRP3) and platyfish (*Xiphophorus maculatus*; ENSXMAG00000006698; 44% amino acid similarity with human NLRP3).

Regarding the second point made by Boyle et al., we found seabream macrophages unable to respond to classical NLRP3 activators, such as ATP, nigericin, and alum and monosodium urate crystals (Angosto et al., 2012; Compan et al., 2012). However, we want to highlight that in mammalian macrophages the classical danger signals are not direct ligands for the NLRP3 inflammasome; rather, they activate surface receptors (i.e., P2X7 receptors) and involve intracellular organelles (i.e., lysosomes or mitochondria) to induce an appropriate intracellular environment and a not fully characterized

signaling cascade that specifically activates the NLRP3 inflammasome. Therefore, the lack of caspase-1 activity upon classical danger signal stimulation in fish macrophages could be due to a diversification and differential function of surface receptors and intracellular signaling cascades between mammalian and fish rather than a specific lack of NLRP3 orthologs. Supporting this idea, our functional studies showed that activation of caspase-1 induced by cell swelling in both fish and mammalian macrophages were sensitive to Bay11-7085 (Figures S2B and S2C in Compan et al., 2012), a compound directly blocking mammalian NLRP3 activation by targeting its ATPase activity, but for example not affecting NLRC4 inflammasome activation (Juliana et al., 2010). Furthermore, in our study we characterized TRPM7 and TRPV2 channel signaling during cell volume regulation in mammalian macrophages to activate the NLRP3 inflammasome, and the use of La^{3+} , as a broad TRP channel inhibitor, was able to impair caspase-1 activation in seabream macrophages in response to hypotonic solutions (Figure S4K in Compan et al., 2012).

The role of the inflammasome and caspase-1 in fish is controversial. None of the nonmammalian vertebrate IL-1 β genes show conserved caspase-1 processing site. Furthermore, although it has recently been found that inflammatory caspases A and B can process zebrafish IL-1 β during *Francisella noatunensis* infection (Vojtech et al., 2012), caspase-1 is not involved (for example) in the processing of gilthead seabream (*Sparus aurata*) IL-1 β (Angosto et al., 2012). Also, the lack of IL-1 α orthologs in fish suggest a unique IL-1 cytokine in fish (the so-called IL-1 β) and further investigation warrants studies about the bioactivity of unprocessed fish pro-IL-1. We also expect novel downstream

inflammasome signaling effectors independent of IL-1 β in fish, which ultimately could also be functional in mammals.

In conclusion, although we agree with Boyle et al. that the specific fish NLR involved in the activation of the inflammasome by cell swelling remain to be elucidated, we want to stress the importance that the signaling able to activate the immune response, and in particular the inflammasome, diverge from fish to mammals. Therefore, the phylogenetic and functional studies support the idea

of hypotonic environments as an evolutionary alert mechanism with a signaling conserved from fish to mammalian macrophages to activate caspase-1, probably through the formation of an NLRP3-like inflammasome in teleost fish.

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Cell Swelling and the NLRP3 Inflammasome

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Multiple chemically and biologically diverse stimuli activate the NLRP3 inflammasome, but the precise mechanism for activation remains to be determined (Davis et al., 2011). Cellular perturbation and homeostatic destabilization also activate NLRP3. Consistent with this, in the September 2012 issue of *Immunity*, Compan et al. (2012) presented data suggesting that the NLRP3 inflammasome is activated by cell swelling. Cell swelling followed by a restorative reduction in volume resulted in caspase-1 activation in murine bone-marrow-derived macrophages and macrophages from the teleost fish *Sparus aurata* (gilthead seabream) (Figure 2 in Compan et al., 2012). Murine cells lacking NLRP3 (or ASC or caspase-1) did not process interleukin-1 β in response to cell swelling, leading to the conclusion that the NLRP3 inflammasome mediates the cellular response to cell swelling and that this is evolutionarily conserved from fish to mammals.

Various NLR-specific expansions have been identified in fish. Generally they cannot be identified as full orthologs of specific mammalian NLRs. However, a number show greatest similarity to the NACHT domain of NLRC3 (Huang et al., 2008; Laing et al., 2008). To date there are, to our knowledge, no published reports of direct NLRP3 orthologs in fish.

We have consequently performed a detailed search of the currently available fish genome assemblies in ENSEMBL for evidence of NLRP3 orthologs. These encompassed *Takifugu rubripes* (fugu), *Xiphophorus maculatus* (platyfish), *Gasterosteus aculeatus* (stickleback), *Gadus morhua* (cod), *Latimeria chalumnae* (coelacanth), *Tetraodon nigroviridis* (tetraodon), *Oreochromis niloticus* (tilapia), and *Danio rerio* (zebrafish).

BLASTP searches with the amino acid sequence of human NLRP3 (NCBI accession number NP_004886.3) identified a gene on zebrafish chromosome 17 (ENSEMBL gene ID ENSDARG00000090946) that, upon reciprocal BLASTP analysis against the nonredundant human protein database, returned NLRP3 as the most significant hit. However, the domain organization of the zebrafish protein differs from that of mammalian NLRP3, consisting of an NTPase domain, leucine-rich repeats, and a C-terminal PRY-SPRY domain. Homology searches failed to identify a specific N-terminal effector domain. Hence it is questionable whether this protein can be described as a true NLRP3 ortholog. Importantly, we were unable to identify any other putative NLRP3 orthologs in the other fish genomes with human NLRP3, or indeed the supposed zebrafish NLRP3, as search terms. Instead, NLR expansions with

greatest similarity to the NLRC3 NTPase were returned as hits. All the fish, except the tetraodon, possessed clear orthologs of ASC.

It is plausible that NLRP3-like functionality is provided by a fish-specific NLR expansion, such as NLR-B, the NACHT of which phylogenetically clusters with the mammalian NLRPs (Laing et al., 2008). However, *Sparus aurata* macrophages do not respond to classical NLRP3 activators including ATP, nigericin, alum crystals, and monosodium urate crystals (Figure 2E in Compan et al., 2012), suggesting that this may not be the case. The absence of direct NLRP3 orthologs and the lack of classical NLRP3 activity in the teleost indicate that reconsideration of the evolutionary and mechanistic basis for the observed inflammatory response to cell swelling is needed.

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