Zebrafish earns its stripes for in vivo ASC speck dynamics

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Assembly of the ASC speck is critical for signaling by the inflammasome. In this issue, Kuri et al. (2017. *J. Cell Biol.* https://doi.org/10.1083/jcb.201703103) use live microscopy to track fluorescently tagged endogenous ASC in the zebrafish, describing the molecular domains driving ASC speck assembly and identifying a key role for macrophages in ASC speck removal in vivo.

Inflammasomes are intracellular signaling complexes assembled and activated in response to the loss of cellular homeostasis. Inflammasome activation is triggered by pathogens (e.g., influenza and candida) or noninfectious insults (insoluble crystals and irritants such as β-amyloid fibrils, asbestos, or silica). Inflammasome activation is critical for host protection against infection, but activating mutations in inflammasome signaling molecules, or the persistent presence of inflammasome-activating agents, cause pathogenic inflammasome signaling to drive inflammatory diseases such as hereditary fever syndromes, atherosclerosis, gout, Alzheimer's disease, asbestosis, and silicosis. It is thus important to understand how inflammasome complexes are activated, assembled, and removed in vivo (Broz and Dixit, 2016).

Mammalian inflammasome complexes are usually comprised of a sensor protein connected to the protease, caspase-1, via the common inflammasome adaptor, ASC. The sensor protein is frequently a member of the Nod-like receptor (NLR) family, but non-NLR inflammasome sensors also exist (e.g., AIM2 and Pyrin). When inflammasome sensor proteins encounter their activating stimulus, they recruit ASC, which then polymerizes to form large intra- and interconnected filaments called ASC specks. ASC polymerization and speck formation creates a signaling platform with multiple caspase-1 binding sites, allowing for caspase-1 clustering and activation and signal amplification. Activated caspase-1 then cleaves its substrates. In mouse and human cells, these substrates include IL-1β and IL-18, which are secreted to recruit and activate phagocytes, and gasdermin-D, which inserts into the plasma membrane to form pores and drive an inflammatory form of lytic cell death called pyroptosis (Broz and Dixit, 2016).

In this issue, Kuri et al. fluorescently tagged endogenous ASC in the zebrafish and used this novel genetic system to examine ASC speck dynamics using live imaging in vivo. Zebrafish is an attractive model organism for such studies because it is optically accessible and allows protein behavior to be monitored using live microscopy in the intact organism. This is particularly pertinent for studies of the inflammasome. End point analysis, typically used to capture inflammasome activation in tissues, can be challenging to interpret as inflammasome-activated cells can undergo pyroptosis within minutes of ASC speck formation, and so there is a limited window of opportunity to image ASC specks in intact cells before the cell lyses.

The major technical advance of this study is the fluorescent tagging of endogenous ASC and its examination in an intact, optically transparent organism. Kuri et al. (2017) used CRISPR/Cas9 gene editing to knock in an enhanced GFP (eGFP) tag into the endogenous zebrafish ASC locus. Previous studies ectopically overexpressed tagged ASC as a transgene (Tzeng et al., 2016) or via viral infection (Sagoo et al., 2016), which renders signal-induced ASC speck formation difficult to study because ASC overexpression tends to trigger its spontaneous polymerization even in the absence of an inflammatory stimulus. Not all experiments by Kuri et al. (2017) used their ASC-EGFP knock-in zebrafish line, as some relied on the more conventional transgenic approach. One transgenic line expressed an mKate2-tagged ASC transgene under the control of a heat-shock promoter, such that zebrafish exposure to heat-shock drives ASC expression and speck assembly, whereas another transgenic line was engineered to allow inducible NLR expression, NLR–ASC interaction, and NLR-driven ASC speck assembly (Kuri et al., 2017). Studies with these transgenic models supported the observations in experiments with the zebrafish ASC-EGFP knock-in line.

Kuri et al. (2017) studied ASC behavior in keratinocytes of intact skin tissue during larval development or upon application of an agent toxic to larvae—$CuSO_4$ (Fig. 1). Skin is a good choice of organ model for these studies, because it is an understudied but highly relevant tissue for inflammasome signaling: skin is the first port of entry for many pathogens and it is frequently the site of tissue injury by noninfectious insults (e.g., toxins and UV). Keratinocytes in skin express ASC and assemble inflammasomes in response to dysregulated homeostasis (e.g., $CuSO_4$ in this study and UV irradiation in Feldmeyer et al. [2007]).

Using ASC-EGFP knock-in zebrafish, Kuri et al. (2017) report that keratinocytes generate ASC specks sporadically during zebrafish development and that these ASC specks are

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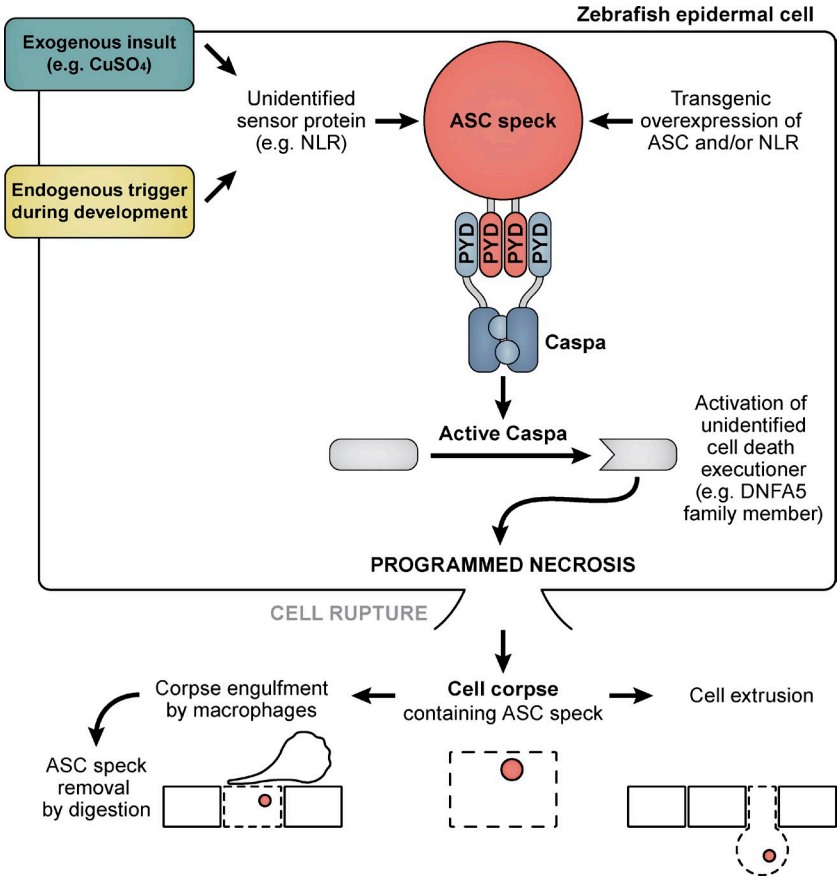

Figure 1. **Inflammasome signaling and dynamics of in vivo ASC specks in zebrafish.** See text for details.

generated within the dying cells of the epidermis of unchallenged animals (Fig. 1). When zebrafish were exposed to a $CuSO_4$, more keratinocytes generated ASC specks and died. When heat shock was used to drive mKate2-ASC transgene expression in vivo, muscle cells likewise assembled ASC specks. Each cell assembled a single ASC speck, composed of highly intercrossed filaments compacted into a large (700 nm) globular structure. These observations in zebrafish largely confirm previous in vitro findings in mammalian cells (Dick et al., 2016; Schmidt et al., 2016).

Speck formation required the ASC Y152 phosphorylation site that is conserved between zebrafish, mouse (Y144), and human (Y146), and that was previously reported to control ASC speck assembly in murine cells (Hara et al., 2013). Both studies support a model in which ASC phosphorylation at this site is a regulatory switch, promoting inflammasome assembly. Regulatory functions for the other conserved phospho-sites in ASC remain to be tested. Phospho-switches also regulate other components of the inflammasome. Notably, phosphorylation of NLRP3 at S5 provoked a signaling nonpermissive state in NLRP3, whereas dephosphorylation at this site converted NLRP3 to a signaling-permissive state (Stutz et al., 2017). It will be of interest in future studies to identify the relevant kinases, phosphatases, and external signals that regulate these phospho-switches as a means to control inflammasome activity. Identification of other posttranslational modifications (e.g., ubiquitination and acetylation) in inflammasome components is also of great interest and may yield insight into further regulatory mechanisms.

Similar to mammalian cells, ASC speck-positive keratinocytes in zebrafish in vivo died in a manner dependent on *Caspa*, the zebrafish orthologue of mammalian *Casp1* (Fig. 1). Cell death resembled the pyroptotic cell death of mammalian cells that requires caspase-1–dependent gasdermin-D cleavage, formation of gasdermin-D membrane pores, and lysis (Broz and Dixit, 2016). However, it is unclear how cell death is executed in zebrafish, as zebrafish appear not to encode orthologues of mammalian gasdermin family genes (Li et al., 2017), although they do encode orthologues of the related DFNA5 family (Kuri et al., 2017). Another difference in the inflammasome signaling pathways between mammals and zebrafish is in the protein interaction domains used for ASC–caspase interaction. In both zebrafish and mammals, ASC has pyrin (PYD) and caspase activation and recruitment (CARD) domains. In mammals, caspase-1 is recruited to the ASC speck via $CARD_{CASP1}$–$CARD_{ASC}$ interactions, whereas in zebrafish, Caspa does not contain a CARD domain and is instead recruited via PYD_{CASPA}–PYD_{ASC} interactions. Individual ASC domains (PYD or CARD), when expressed alone, could polymerize to form filaments, and PYD_{ASC}, when expressed alone, could induce slow cell death of keratinocytes, but the full-length ASC (containing PYD and CARD domains) was required for the formation of the compact ASC speck and rapid cell death. The requirement for full-length ASC to drive rapid cell death in zebrafish keratinocytes may reflect the necessity for a compact speck comprised of interconnected filaments, as mammalian ASC speck compaction requires $CARD_{ASC}$–$CARD_{ASC}$ interactions for intra- and interfilament cross-linking (Dick et al., 2016; Schmidt et al., 2016).

Keratinocyte lysis released the ASC speck, alongside cell debris such as fragments of the ruptured plasma membrane, into the extracellular space. Kuri et al. (2017) observed that extracellular ASC specks were removed and digested by

neighboring tissue resident macrophages, likely through efferocytosis (Fig. 1). In mammals, inflammasome-signaling cells are not always destined to die by pyroptosis. For example, neutrophils can activate caspase-1 and release mature IL-1β, but resist cell death, whereas caspase-1–activated macrophages rapidly succumb to pyroptosis (Chen et al., 2014). In zebrafish, keratinocytes express endogenous *Asc* and *Caspa* and undergo cell death within minutes of speck formation, triggering their extrusion from the healthy epithelium. In contrast, zebrafish muscle cells do not express endogenous ASC, but transgenic ASC expression drives ASC speck formation without inducing changes in cell shape or viability. It is currently unclear why this happens. A likely explanation is that muscle cells do not express *Caspa* and/or the unidentified proteins that drive cell death execution. These data support research using mammalian systems that indicates that inflammasome signaling outcomes are specified by the cell type (Chen et al., 2014) and the nature of the stimulus (Zanoni et al., 2016), and serves as a caution against extrapolating inflammasome signaling pathways and outcomes between distinct cell types.

The ASC speck is a large insoluble aggregate and many questions remain open regarding precisely how ASC specks are cleared by the body during inflammatory resolution. Several studies have suggested different fates for ASC specks during in vitro and in vivo inflammation: (1) ASC specks are digested by autophagy to shut down inflammasome signaling while the cell is still alive (Shi et al., 2012); (2) ASC specks are released by inflammasome-signaling cells during cell lysis and are then engulfed by macrophages where they rupture the phagosome and trigger further inflammasome signaling in the recipient cell to provoke further inflammation (Baroja-Mazo et al., 2014; Franklin et al., 2014); and (3) ASC specks become entrapped within pore-induced intracellular traps (PITs), pyroptotic cell corpses comprised of ruptured plasma membrane and the insoluble contents of the cell, which are silently removed by macrophages through efferocytosis (Jorgensen et al., 2016). The current study in zebrafish supports the latter model for ASC speck removal, as its imaging localizes the ASC speck to within cell corpses and tracks their eventual removal via corpse ingestion and digestion by macrophages. Collectively, these studies raise the intriguing question of how the inflammatory consequences of ASC speck ingestion by macrophages is regulated to propagate further inflammation or, alternatively, to inhibit inflammasome signaling and ensure inflammatory resolution. Several regulatory mechanisms are possible and may not be mutually exclusive. One possibility is that macrophage ingestion of ASC specks may have different consequences depending on the nature of the engulfing macrophage (e.g., activation state and polarization). Another possibility is that the context of the ASC speck (e.g., extracellular ASC specks versus ASC speck–containing cell corpses) dictates the resultant response. For example, cell corpses may ligate inhibitory receptors on macrophages, preventing further inflammatory responses, akin to complement protein C1q that directs macrophage polarization and limits inflammasome activity during the uptake of apoptotic cells (Benoit et al., 2012). Another possibility suggested by Kuri et al. (2017) is that the propagation of inflammasome activity in recipient macrophages as reported by the Pelegrin and Latz laboratories (Baroja-Mazo et al., 2014; Franklin et al., 2014) requires additional signals present in inflamed tissues (e.g., inflammatory regulators or high concentrations of ASC specks). Such possible mechanisms specifying inflammatory outcomes during ASC speck clearance in vivo remain to be tested.

One surprising finding from this study was that ASC specks assembled sporadically within dying cells of the epidermis during development (Kuri et al., 2017). It is unclear whether this was a spontaneous, stochastic event or a specific response to an endogenous inflammasome agonist released during tissue development. It also raises the question of whether, like apoptosis, inflammasome-dependent cell death may contribute to tissue remodeling during development. In support of this hypothesis, morpholino knockdown of *Caspa* caused dysregulated morphogenesis of the cartilaginous pharyngeal skeleton in zebrafish (Masumoto et al., 2003). Whether ASC and caspase-1 have nonredundant functions in organ development in mammals is unclear, but $Asc^{-/-}$ and $Casp1^{-/-}$ mice are healthy and appear to develop normally.

In summary, the article from Kuri et al. (2017) reports the first characterization of endogenous ASC speck assembly and removal in an intact organism and progresses the field beyond the study of isolated cell types in vitro or ex vivo, or in ectopically expressed ASC in vivo. In studying in vivo ASC speck dynamics in zebrafish, the authors confirm that the phosphorylation events and molecular domains required for ASC polymerization and assembly into the speck are conserved between zebrafish and mammals, as is the structure of the fully formed ASC speck. The authors further demonstrate that neighboring tissue-resident macrophages silently cleared ASC specks without propagating inflammasome signaling and inflammatory responses. Surprisingly, ASC specks were also observed during normal zebrafish development, indicating that inflammasome signaling may contribute to tissue remodeling or other aspects of in vivo development, suggesting new avenues for future research.

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