

# PANoptosis: new insights in regulated cell death in ischemia/reperfusion models

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The first study describing the cell death and destruction of tissues, organs, and organ systems as programmed events during the development of multicellular organisms was performed by JW Saunders Jr in 1966. The term apoptosis was introduced by Kerr, Wyllie, and Currie in 1972 to describe a programmed phenomenon opposite to mitosis in the regulation of animal cell populations. Later, a description of ectodermal cell lineages as programmed cell death in the worm *Caenorhabditis elegans* was published by JE Sulston and HR Horvitz in 1976. These studies led to exponential growth into the research of cell death, which revealed many different types of death. The complexity of cell death led to the formation of the Nomenclature Committee on Cell Death (NCCD) to standardize the criteria and define the different types of death. The most recent NCCD guideline, published in 2018, has updated the criteria necessary for defining and interpreting cell death, including morphological, biochemical, and functional perspectives (Galluzzi et al., 2018). In 2012, the NCCD proposed classifying cell death based on quantifiable biochemical parameters instead of the classic morphologic criteria and suggested using the specific terms of cell death subroutines. Since defining cell death is essential, some criteria of cell death were abandoned, for example, the engulfment of a cell which can, in some cases, preserve its viability. In 2015, the NCCD recommended only using two criteria to describe cell death: irreversible plasma membrane permeabilization and complete fragmentation. The NCCD recently redefined the term regulated cell death (RCD) to include the types of death that involve genetically encoded molecular machinery, which can be altered by pharmacological or genetic interventions. This is in contrast to accidental cell death, represented by the instantaneous and catastrophic demise of cells as a consequence of chemical (e.g., extreme pH) or physical (high pressure, temperature, shear stress) insults. The NCCD also defined programmed cell death as only the RCD in physiologic instances. Currently, the NCCD has designated the following cell death subroutines: intrinsic apoptosis, extrinsic apoptosis, mitochondrial permeability transition-driven necrosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic cell death, neutrophil extracellular trap otic cell death, lysosome-dependent cell death, autophagy-dependent cell death, immunogenic cell death, cellular senescence, and mitotic catastrophe (Galluzzi et al., 2018).

In the last few years, the growing evidence of extensive crosstalk between RCDs has led to the unified concept of PANoptosis, defined as an inflammatory RCD pathway driven by the PANoptosome complex with the key features of apoptosis, pyroptosis, and necroptosis (Wang and Kanneganti, 2021). A prototypical Z-DNA binding protein 1 (ZBP1) PANoptosome

induced by influenza A virus (IAV), formed by a large protein complex, was initially reported to contain the receptor-interacting serine/threonine kinase 1 (RIPK1), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3 (NLRP3), caspase 8 (CASP8), and ZBP1 (Malireddi et al., 2019). However, new proteins such as RIPK3, CASP6, and the Fas-associated protein with death domain (FADD) have been found to play a role in the PANoptosome complex. A second AIM2 PANoptosome induced by herpes simplex virus type 1 and *Francisella novicida* infections has also been reported (Lee et al., 2021). The current models are based on the interactions of conserved domains involved in specific RCDs (Wang and Kanneganti, 2021).

Three classes of proteins have been suggested to be part of the PANoptosome: 1) sensors, such as ZBP1, NLRP3, the putative sensor for damage-associated molecular patterns and pathogen-associated molecular patterns; 2) adaptors, such as ASC and FADD; and 3) catalytic effectors, such as RIPK1, RIPK3, CASP1, and CASP8 (Samir et al., 2020). CASP6 has also been included as a component of the PANoptosome, but this caspase can have apoptotic or anti-apoptotic activities, and currently it is uncertain if CASP6 has roles in the PANoptosome complex other than PANoptosome assembly (Zheng and Kanneganti, 2020).

Apoptosis is subdivided into two different subroutines: extrinsic apoptosis, whose hallmark is CASP8 activation induced by external stimuli and subsequent activation of the effector caspase CASP3, and intrinsic apoptosis characterized by mitochondrial outer membrane permeabilization followed by activation of CASP9, which cleaves and activates executioner caspases CASP6, CASP7 and mainly CASP3 (Galluzzi et al., 2018). CASP8 contains two death effect domains (DED) allowing its recruitment by different inflammasomes, including the PANoptosome complex (Wang and Kanneganti, 2021).

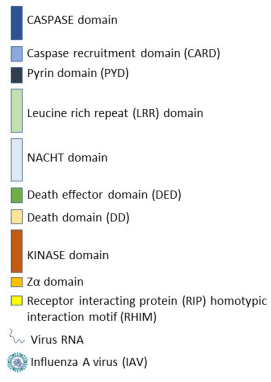
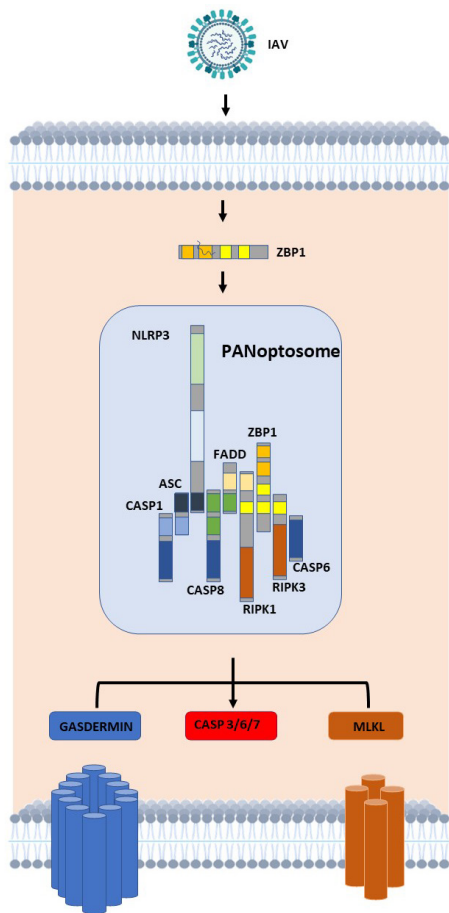
NLRP3 interacts with the adaptor protein ASC through its pyrin domain and, in turn, ASC binds to CASP1 through a caspase recruitment domain, promoting CASP1 cleavage and activation (Wang and Kanneganti, 2021). Activated CASP1 cleaves substrates, such as members of the gasdermin family, which form plasma membrane pores, a hallmark of pyroptosis (Galluzzi et al., 2018). Moreover, CASP1 can also elicit apoptosis. Other caspases, such as CASP8 or CASP3, can also elicit pyroptosis depending on the initiating stimulus (Wang et al., 2017). Pyroptotic and caspase mechanisms can be activated by the heterotypic interaction between pyrin domain (ASC) and DED (CASP8), which has been suggested as the backbone of the PANoptosome elicited by IAV infection. This IAV-induced PANoptosome (Figure 1) is the most studied and is characterized by the

activation of CASP1, CASP3, CASP8, and mixed lineage kinase domain-like phosphorylation (Wang and Kanneganti, 2021). The protein ZBP1 senses IAV RNA through its second domain Z $\alpha$  and recruits RIPK3 through the RIP homology interaction motif. RIPK3 phosphorylates the pseudokinase mixed lineage kinase domain-like, a pore-forming protein, which disrupts the nuclear envelope releasing DNA and promotes the disruption of the cell membrane leading to necroptotic cell death (Zhang et al., 2020). RIPK3 can also elicit apoptosis by binding to RIPK1 through interacting RIP homology interaction motifs. RIPK1 presents a death domain that interacts with the death domain of the adaptor protein FADD whose DED binds to the DED domain of CASP8 (Nogusa et al., 2016). CASP6 has been reported to contribute to the ZBP1-PANoptosome assembly by interacting with RIPK3 in the presence of RIPK1. In fact, RIPK1 and RIPK3 have been suggested to interact with ZBP1 (Zheng and Kanneganti, 2020).

The DED of CASP8 interacts with the pyrin domain of ASC whose caspase recruitment domain binds to the respective caspase recruitment domain of CASP1, allowing PANoptosome assembly and the activation of pyroptosis, apoptosis, and necroptosis. Figure 1 shows a model of the prototypical ZBP1-dependent PANoptosome (Wang and Kanneganti, 2021).

Interactions between the components of the PANoptosome are still under study, and different models exist. The ZBP1 PANoptosome has also been suggested to vary throughout the progression of IAV infection, increasing the complexity in the interactions of its components (Zheng and Kanneganti, 2020). Furthermore, PANoptosis, which is mainly associated with infection, can be elicited by different PANoptosome sensors depending on the pathogen. Thus, the protein absent in melanoma 2, a cytosolic DNA sensor, is involved in the PANoptosis mediated by *Francisella novicida*, forming the protein absent in melanoma 2-PANoptosome (Lee et al., 2021). Transforming growth factor beta-activated kinase 1, which phosphorylates RIPK1 and inhibits PANoptosome assembly, is another PANoptosome regulator inhibited by *Yersinia sp* leading to the activation of PANoptosis (Malireddi et al., 2019). PANoptosis has also been suggested to be present in sterile inflammation and cancer (Wang and Kanneganti, 2021). Thus, PANoptosis research opens a new frontier in the RCD field.

Stroke is one of the leading causes of death worldwide and includes two categories: ischemic and hemorrhagic stroke. Ischemic stroke accounts for 87% of human strokes and it results from a restriction of the blood flow due to vessel occlusion (focal ischemia) or a generalized reduction of the blood flow in the brain (global ischemia). Hemorrhagic stroke results from bleeding either in the brain parenchyma (intracerebral hemorrhage) or in the meninges (subarachnoid hemorrhage). Different RCD subroutines have been described in stroke. However, PANoptosis in human stroke or in experimental *in vivo* or *in vitro* models has not been described so far. A recent publication based on data mining (Yan et al., 2022) suggests PANoptosis in ischemia/reperfusion injury, but no direct evidence exists. Studies on global cerebral ischemia looking for markers of apoptosis and necroptosis in the rat cerebral cortex and hippocampus failed in finding simultaneous markers of RCD in the cerebral



**Figure 1 | PANoptosis elicited by influenza A virus.** PANoptosis is a type of regulated cell death triggered by specific stimulation with key features of apoptosis, pyroptosis, and necroptosis but distinct of these subroutines considered independently. A large protein complex (PANoptosome) that includes sensors, adaptor, and catalytic effector bound by interacting domains assembly to elicit PANoptosis. Figure was modified from Wang and Kanneganti (2021) and CASP6 has been included based on the report of Zheng et al. (2020) who suggested that CASP6 could bind to RIPK3 through intrinsically disordered regions (IDRs) present in both molecules. These authors also indicate that interaction between CASP6 and RIPK3 enhances the RIPK3-ZP1 interaction. ASC: Apoptosis-associated speck-like protein containing a caspase recruitment domain; CASP: Caspase; FADD: Fas-associated protein with death domain; MLKL: mixed lineage kinase domain-like; NLRP3: nucleotide-binding oligomerization domain (NOD)-like receptor pyrin domain-containing 3; RIPK: receptor-interacting serine/threonine kinase; ZBP1: Z-DNA binding protein 1.

cortex and hippocampus, at least in the first days after the reperfusion (Font-Belmonte et al., 2019).

In a recent study, Yan et al. (2023) presented convincing evidence for PANoptosis-like cell death in R28 cells (retinal precursor cells) subjected to oxygen-glucose deprivation/recovery, a model used in cultured cells and *ex vivo* assays to mimic the ischemia-reperfusion process that characterizes ischemic stroke. Morphological results were based on identifying cells death in the presence and the absence of specific inhibitors of apoptosis, pyroptosis (disulfiram), and necroptosis (necrostatin 1). Simultaneous cell death was assessed by TUNEL staining for apoptosis, ethidium homodimer III for pyroptosis, and propidium iodide for necroptosis. As biochemical hallmarks of apoptosis, they report cleaved CASP3, increases in the pro-apoptotic protein BAX, and decreases in the anti-apoptotic protein BCL-2. These events are considered hallmarks of the apoptosis process (Galluzzi et al., 2018). For pyroptosis, they report CASP1 cleavage and increases in NLRP3, gasdermin D, and the pro-inflammatory interleukin-1 $\beta$  and interleukin-18. Upregulation of phosphorylation of RIPK3 and mixed lineage kinase domain-like are hallmarks of necroptosis. Different combinations of inhibitors for apoptosis, pyroptosis, and necroptosis reduced cell death. The combination of all inhibitors resulted in the best protective effect, thus providing additional evidence of PANoptosis in R28 cells.

Yan et al. (2023) also reported PANoptosis in rat retinal neurons using an acute high intraocular pressure model that induces retinal ischemia. They indicated the presence of apoptosis and necroptosis markers in the ganglion cell layer,

inner nuclear layer, and outer nuclear layer of the retina and pyroptosis markers in the ganglion cell layer and inner nuclear layer. These results support the idea of possible PANoptosis in the retina following acute high intraocular pressure. However, further evidence about the presence of PANoptosome and crosstalk between these types of RCD is required. The presence of PANoptosis does not rule out the presence of other RCD subroutines. PANoptosis is defined as a unique type of immunogenic cell death, triggered by specific stimulation and distinct from apoptosis, pyroptosis, and necroptosis (Wang and Kanneganti 2021). In this regard, autophagic death by autophagy has also been described in retinal neurons supporting that different RCD subroutines can coexist in the acute high intraocular pressure model.

In summary, the different subroutines of cell death elicited by PANoptosis seems to be addressed to assure the RCD following infections by virus (HVI or IAV) or bacteria (*F. novicida*), however, a role for PANoptosis beyond the context of infections has been suggested indicating the contribution of PANoptosis in development and immune responses (Samir et al, 2020). In addition, the excessive production of cytokines mediated by inflammatory cell death (cytokine storm) can also induce PANoptosis (Wang and Kanneganti, 2021). Thus, future studies characterizing the PANoptosis in processes other than infections will contribute to gaining insight into the homeostasis control mechanisms.

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