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Commentary

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Potassium efflux fires the canon: Potassium efflux as a common trigger for canonical and noncanonical NLRP3 pathways

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Murine caspase-11 and its human orthologues, caspase-4 and caspase-5, activate an inflammatory response following cytoplasmic recognition of cell wall constituents from Gram-negative bacteria, such as LPS. This inflammatory response involves pyroptotic cell death and the concomitant release of IL-1α, as well as the production of IL-1β and IL-18 through the noncanonical NLR family, pyrin domain containing 3 (NLRP3) pathway. This commentary discusses three papers in this issue of the *European Journal of Immunology* that advance our understanding of the roles of caspase-11, -4, and -5 in the noncanonical pathway. By utilizing the new gene editing technique, clustered regularly interspaced short palindromic repeats (CRISPR), as well as sensitive cell imaging techniques, these papers establish that cytoplasmic LPS-dependent IL-1β production requires the NLRP3 inflammasome and that its activation is dependent on K^+ efflux, whereas IL-1α release and pyroptotic cell death pathways are NLRP3-independent. These findings expand on previous research implicating K^+ efflux as the principal trigger for NLRP3 activation and suggest that canonical and noncanonical NLRP3 pathways are not as dissimilar as first thought.

Keywords: Caspase-4 · Caspase-5 · Caspase-11 · LPS · NLRP3 inflammasome · Noncanonical · Pyroptosis

See accompanying articles by Schmid-Burgk et al., Baker et al., and Rühl and Broz.

Recently, murine caspase-11 and its human orthologues, caspase-4 and caspase-5, have been the subjects of intense research interest [1]. Currently there is a particular focus on the role these caspases play in directly sensing Gram-negative infections and in the induction of interleukin 1β (IL-1β) via a noncanonical NLR family, pyrin domain (PYD) containing 3 (NLRP3) pathway. This issue of the *European Journal of Immunology* features three papers by Schmid-Burgk et al., Baker et al., and, Ruhl and Broz [2–4] that, together, provide a significant step forward in our understanding of the role of the noncanonical NLRP3 pathway in IL-1β processing and pyroptotic cell death.

NLRP3 is a cytoplasmic protein sensor in inflammatory cells, which forms a large multimolecular complex, called the inflammasome, in response to injury or infection (reviewed in [5]). NLRP3 has a tripartite domain structure with a C-terminal leucine-rich repeat, a central NACHT domain and an N-terminal PYD [5]. In the canonical NLRP3 activation pathway, NLRP3 indirectly senses an insult, such as extracellular ATP [6], or uric acid crystals [7], and undergoes a conformational change, which leads to binding to the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) via a homotypic interaction of PYD domains [8]. This then nucleates the oligomerization of further ASC molecules based on a prion-like aggregation [9, 10]. The ASC molecule also

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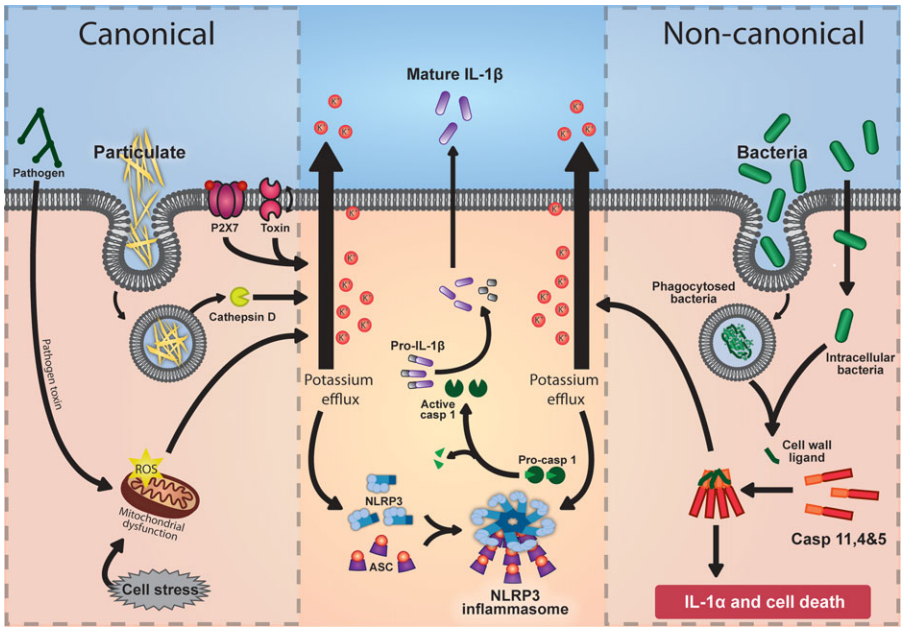

Figure 1. The canonical and noncanonical pathways of NLRP3 activation. The key features of NLRP3 activation (middle) are NLRP3 oligomerization, ASC recruitment, caspase-1 cleavage, and the production of the mature inflammatory cytokine IL-1β. The canonical pathway of NLRP3 activation (left) involves a diverse range of stimuli, including bacterial toxins, particulate matter, and extracellular ATP, indirectly activating NLRP3 through the common pathway feature of K^+ efflux. The noncanonical pathway (right) involves caspase-11 in mice and, caspase-4 and caspase-5 in human cells, and is activated on recognition of cell wall ligands, such as LPS, from phagocytosed and intracellular bacteria. Similar to activators of the canonical NLRP3 pathway, activation of NLRP3 through the noncanonical pathway also requires K^+ efflux. Additionally, caspase-11, -4, and -5 also induce IL-1α release and pyroptotic cell death through an NLRP3-independent pathway (right bottom).

contains a CARD (caspase activation and recruitment domain) that recruits procaspase-1, causing its activation; this leads to the direct cleavage of the proinflammatory cytokines pro-IL-1β and pro-IL-18 to their mature forms, which are then secreted [5] (Fig. 1). Although the closely related proinflammatory molecule pro-IL-1α is not a substrate for caspase-1, the activation of inflammasomes has also been shown to drive IL-1α release [11]. Inflammasome activation also regulates an inflammatory form of cell death called pyroptosis, which has been shown to be important for controlling the spread of intracellular pathogens such as *Salmonella* and *Shigella* (reviewed in [12]).

Although the number of known inflammasomes is growing [5], NLRP3 remains the best characterized and most studied thus far. A diverse range of pathogen-associated, endogenous, and particulate stimuli are known to trigger the "canonical" NLRP3 inflammasome pathway (Fig. 1 and reviewed in [13]). There is limited evidence supporting a direct physical interaction between such stimuli and NLRP3, and diverse host pathways have been suggested to integrate the noxious stimuli. These include lysosomal destabilization and cathepsin activity [14], reactive oxygen species (ROS) production and/or mitochondrial dysfunction [15], posttranslational modification such as deubiquitination of inflammasome components [16–18], and a universal requirement for K^+ efflux from the cell [19]. To date it has been the dependence of NLRP3 activation on one of these mechanisms that has defined the pathway as canonical.

The original caspase-1 knockout mouse [20, 21] also had caspase-11 knocked out from its genome, and when this was realized attempts were made to determine whether any of the roles ascribed to caspase-1 were in fact due to caspase-11. The seminal study performed by Kayagaki et al., [22] identified that NLRP3 and caspase-1-dependent IL-1β release in response to infection by *Escherichia coli*, *Citrobacter rodentium,* or *Vibrio cholera* requires caspase-11, while caspase-11 is dispensable for activation of the "canonical" NLRP3 inflammasome pathway, such as that triggered by exposure to ATP, leading to the identification of the "noncanonical" NLRP3 pathway. The noncanonical pathway also regulates NLRP3-dependent caspase-1 activation in response to other Gram-negative pathogens such as *Hemophilus influenzae, Klebsiella pneumoniae, Neisseria gonorrhea, Shigella flexneri, Enterobacter cloacae,* and *Proteus mirabilis* (Fig. 1) [23]. The active caspase-11 agonist was later shown to be LPS itself, and direct transfection of LPS into the cytoplasm of macrophages was shown to activate caspase-11 [24]. Subsequent work has suggested that LPS is a direct agonist to the CARD domain of caspase-11, and to its human orthologue caspase-4 (caspase-5 being another human orthologue of murine caspase-11) [25]. However, how exactly caspase-11/4 activates NLRP3-dependent inflammasome formation and IL-1β/IL-18 release remains unclear.

In this issue Schmid-Burgk et al., [2] sought to determine whether caspase-4 functions analogously in human cells to caspase-11 in murine cells. Using clustered regularly interspaced short palindromic repeats (CRISPR), the authors knocked out NLRP3, ASC, caspase-1, and caspase-4 in human THP-1 monocytes [2]. Activation of the noncanonical NLRP3 inflammasome pathway was induced by LPS transfection and cell death was measured by release of lactate dehydrogenase and high-mobility group box 1 (HMGB1). IL-1β release was also measured. In this system, they show that the cell death in response to cytoplasmic LPS is completely dependent upon caspase-4, while still occurs in THP-1 cells in which NLRP3, ASC, or caspase-1 have been knocked out [2]. However, LPS-dependent IL-1β production required the components of the NLRP3 inflammasome. Furthermore, they show that incubation of the cells in media containing high concentrations of K^+, to suppress the efflux of K^+ along a concentration gradient, suppressed NLRP3inflammasome dependent IL-1β release, but not caspase-4-dependent cell death, in response to LPS transfection (Fig. 1). These data therefore confirm the presence of the

noncanonical NLRP3 pathway in human cells and suggest that caspase-4 acts independently and upstream of the NLRP3 inflammasome.

In a second article in this issue, Baker et al., [3] also use CRISPR to knock out specific genes in THP-1 cells. These authors also show that caspase-4 is required for the noncanonical NLRP3 pathway in human cells in response to transfected LPS. Interestingly, they also investigated the role of caspase-5, the other human orthologue of murine caspase-11. Although knocking out caspase-5 has no effect on cell death or IL-1β release in response to LPS transfection alone, and does not enhance the protective effects of caspase-4 ablation in this model, a marked synergistic reduction in cell death and IL-1β release is observed in response to *Salmonella* Typhimurium infection when both caspase-4 and caspase-5 are deleted, suggesting that caspase-5 is functionally important for an appropriate response to infection. Baker et al. also used the recently described selective NLRP3 inflammasome inhibitor MCC950 [26] to show that IL-1β release in response to cytoplasmic LPS is NLRP3 inflammasome dependent, while MCC950 had no effect on cell death induced by LPS transfection. Furthermore, incubating the cells in high K^+ concentrations also inhibited IL-1β release, but not cell death, again suggesting that caspase-4 activation occurred upstream of NLRP3 inflammasome activation.

Together the above work confirms that caspase-4 regulates the noncanonical NLRP3 pathway in human cells, and suggests that caspase-4 activation occurs independently and upstream of the NLRP3 inflammasome, which is dependent upon K^+ efflux (Fig. 1). This mechanism is further elucidated in this issue by Ruhl and Broz [4], who show in murine cells that the link between caspase-11 and the NLRP3 inflammasome is a cell-intrinsic process. Using the cell permeable fluorescent K^+ indicator PBFI-AM, they show that caspase-11 activation induced by LPS transfection induces K^+ efflux, which in turn leads to NLRP3 inflammasome activation and IL-1β release (Fig. 1). Again, incubating cells in high concentrations of K^+ inhibits IL-1β release but not cell death [4]. Together these papers show that the noncanonical NLRP3 pathway is present in human cells, and strongly suggest that the link between caspase-4/11 activation and NLRP3 inflammasome formation is K^+ efflux (Fig. 1).

In light of these findings it seems reasonable to ask whether the "noncanonical" pathway is really noncanonical after all. Muñoz-Planillo et al. [19] show that diverse soluble and particulate activators of the canonical inflammasome, previously ascribed to utilize lysosomal disruption or mitochondrial ROS to activate NLRP3, ultimately depend upon K^+ efflux to induce NLRP3 inflammasome formation (Fig. 1). The work presented in this issue clearly supports the view that the noncanonical pathway leading to NLRP3 activation also requires K^+ efflux (Fig. 1). Thus, there may be one NLRP3 inflamamasome, activated by diverse stimuli that integrate at a point upstream of K^+ efflux (Fig. 1). Given that evidence for an interaction between NLRP3 and its activators is lacking, does the true sensor of these diverse inflammatory stimuli lie upstream of NLRP3 and does its activation cause K^+ efflux? The related protein NLR family CARD domain-containing protein 4is now recognized as an adaptor protein rather than a sensor, since the discovery that it does not sense bacterial ligands directly but does so through coreceptors called NLR family, apoptosis inhibitory proteins [27]. Is NLRP3 an adaptor for additional stress sensing proteins? Or does NLRP3 simply sense changes in cellular K^+ levels? These questions will surely be answered soon given the levels of interest in the NLRP3 inflammasome and its importance to disease [28], where uncovering the mechanisms of its activation may lead to the identification of new therapeutic targets for the treatment of inflammatory disease.

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Abbreviations: **ASC**: apoptosis-associated speck-like protein containing a CARD · **CARD**: caspase activation and recruitment domains · **CRISPR**: clustered regularly interspaced short palindromic repeats · **NLRC4**: NLR family CARD domain-containing protein 4 · **NLRP3**: NLR family, pyrin domain containing 3 · **PYD**: pyrin domain

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