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Signaling cascades and inflammasome activation in microbial infections

Abstract: Recognition of extracellular pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) results in activation of host defense signaling pathways. Some virulent microbes can attenuate and escape antimicrobial immunity by manipulating these signaling pathways. However, impairment of the primary innate response may potentiate the activation of secondary defense program, centered around Nucleotide-binding domain and Leucine-rich repeat containing Receptor (NLRs) for inflammasome formation and IL-1β production. This review analyzes the current knowledge regarding association of innate immune signaling pathways with inflammasome activation in response to bacterial infection.

Keywords: inflammasome, signaling, NLR, p38, AKT, Bcl2, cIAP, MAPK, pathogen, anthrax

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1 Inflammasome activation and IL-1β production

It is generally accepted that two distinct signals are required for IL-1β production. "Signal 1" controls the expression of pro-IL-1β gene, and is mostly delivered by pathogen associated molecular patterns (PAMPs). PAMPS activate pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) leading to changes in the host transcriptional program via several pathways including NF-κB, MAPK and AKT. "Signal 2" is generated by microbial virulence factors including *Bacillus anthracis* lethal toxin, *Shigella* toxin, *Staphylococcus aureus* alpha hemolysin, group A streptococcal (GAS) streptolysin O (SLO) andflagellin, or by danger associated molecular patterns (DAMPs) including ATP, uric acid, alums and silica [1-4] "Signal 2" activates inflammasome formation for proteolytic cleavage of caspase-1, which controls the processing and secretion of IL-1β and IL-18 and induction of a "proinflammatory niche" [4,5]. Pathogen-induced NLR activation may also lead to host pyroptosis, which is marked by increases in cell membrane porosity, cell death and release of DAMPs [1-4].

Inflammasome complexes contain a unique sensor protein belonging to either the NLR (Nucleotide-binding domain and Leucine-rich repeat-containing receptors) or the PYHIN (pyrin and HIN domain-containing proteins) family [5]. More than 22 inflammasome sensors are known and their structure and activation have been reviewed elsewhere [1-5]. These sensors respond to specific ligands; for instance NALP1 is activated by *B. anthracis* lethal toxin, NALP3 responds to GAS SLO and Staphylococcus *aureus* alpha-hemolysin, NLRC4 is induced by Salmonella *typhimurium* flagellin and NLRP12 is stimulated by the plague *Yersinia pestis* [1,6-8].

Most "Signal 2" agonists do not directly associate with NLRs to activate inflammasomes. NLR inducers such as bacterial pore-forming toxins induce lysosomal destabilization, plasma membrane disruption, K^+ efflux and generation of DAMPs, *Shigella* toxin triggers mitochondrial reactive oxygen species (ROS) production, and anthrax lethal toxin induces ATP and K^+ efflux [1,6,9]. Events such as ROS induction, mitochondrial membrane permeabilization, or K^+ efflux play important roles in inflammasome activation [1,6,9]. However, the exact sequence of events leading to caspase-1 activation remains largely unknown and the exact functions of individual inflammasome sensor proteins are still nebulous. Emerging data suggest that several signaling pathways may also participate in transmitting effects of the "Signal 2"

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agonists to NLRs. In this context, the signaling components may induce the expression of inflammasome regulators or may directly affect inflammasome formation through modification of its components. Here we briefly review the signaling molecules demonstrated experimentally to participate in inflammasome activation. The result of our analysis supports the notion that impairment of signaling components that are required for generation of basal immunity may precipitate the nucleation of secondary defense programs including NLR activation.

2 Role of MAPK pathways in NLR activation in murine and plant infection models

Mitogen-activated protein kinases (MAPK) are a family of Ser/Thr protein kinases involved in many cellular processes such as cell proliferation, differentiation, motility and survival [10]. MAPKs are activated during most bacterial infections and induce a proinflammatory niche [11]; however, certain bacteria have evolved means to inhibit MAPK signaling. For example GP63 protease of *Leishmania,* YopJ from *Yersinia*, SpvC from *S. enterica* strains, OspF from *Shigella* spp. and lethal toxin from *B. anthracis* can inhibit MAPK signaling by distinct mechanisms [2, 6, 11]. While many of these pathogens induce inflammasome activation, in most cases the role of MAPK inhibition in inflammasome activation has not been thoroughly investigated.

The involvement of p38 MAPK in *B. anthracis*-induced inflammasome activation has been studied in some detail [12]. Anthrax lethal toxin (LT), secreted by *B. anthracis*, is a virulence factor that inhibits MAPK signaling [13]. LT is a protease that reaches the host cell cytoplasm and cleaves and inactivates MAPK kinases (MKKs) [10, 12]. Early work demonstrated that in mouse macrophages, LT mediated inhibition of MEK-3 and MEK-6 attenuates p38 activity and promotes cell death [10]. Anthrax LT also induces inflammasome activation in mouse macrophages [7, 14]. Interestingly, inhibition of the p38 pathway induced inflammasome formation, as overexpression of non-hydrolysable MEK-3 or MEK-6 proteins inhibited inflammasome activity and IL-1β release [12]. Furthermore, Mogridge and colleagues isolated a *B. anthracis* LT mutant that was defective in its ability to induce pyroptosis in Nlrp1b^{s}-expressing macrophages [15]. This mutant LT could not induce pro-IL-1β cleavage in fibroblasts. Moreover, the mutant LT cleaved MEK-1 and MEK-2 but not MEK-6 proteins, abrogating ERK activity but not the p38 activation, suggesting that inactivation of p38 pathway may contribute to NLR induction [14, 15]. Activated p38 phosphorylates CREB (c-AMP response element binding transcription factor), whose activity is required for transcriptional regulation of several genes including plasminogen activator inhibitor-2 (PAI-2), cyclooxygenase-2 (COX-2), and IL-1β [16]. Interestingly, depletion of PAI-2 resulted in NLRP3- inflammasome activation that was dependent on autophagy and mitochondrial ROS [17]. Additionally, p38 MAPK inhibition may induce ATP extrusion, which is required for P2X7-mediated NLR activation [12].

NOD2 associates with NLRP1 and NLRP3 inflammasomes for caspase-1 cleavage and IL-1β release in response to muramyl dipeptide (MDP) [7, 18]. MEKK4 also binds to RIP2 to sequester RIP2 from the NOD2 signaling pathway [19]. This MEKK4:RIP2 complex dissociates upon exposure to MDP, allowing more RIP2 to bind NOD2; however, caspase-1 cleavage was not observed in this study [19].

On note, a similar paradigm was observed in plant defense against bacterial infection, where disruption of a protein kinase-dependent pathway by *Pseudomonas syringae* led to activation of immunity mediated by SUMM2, an NLR protein [20]. In plant defense responses, mitogen-activated protein kinase (MAPK) cascades play important roles in transducing signals from upstream receptors to the downstream targets [21]. *P. syringae* pathogenic effector HopAI1 inhibits MPK4 kinase activity and results in activation of SUMM2-mediated host defense [20].

Thus, MAPK cascades can negatively regulate inflammasome functions directly or indirectly. However, inhibition of MAPK can also induce cell death in association with inflammasome activation [2].

3 Effects of NF-κB and PKR on inflammasome activation

Two signaling pathways can lead to the activation of NF-κB transcription factor, the canonical (or classical) pathway and the non-canonical (or alternative) pathway [22]. Certain pathogens have devised efficient strategies to disrupt NF-κB pathways. For example, *S. typhimurium* homologue AvrA, EPEC effector NleE, and *S. flexneri*, IpaH9.8 all block NF-κB activation by inhibition of IKKβ phosphorylation [11,23,24]. Many of these pathogens induce inflammasome activation [1,4], however the linkages between NF-κB inhibition and inflammasome activation have not been completely articulated.

Interestingly, in IKKβ-deficient macrophages various inflammasome activators including *E. coli and L. monocytogenes* lead to caspase-1 dependent IL-1β secretion [25]. Furthermore, in an endotoxin-induced shock model, IKKβ deletion in myeloid cells was deleterious due to elevated plasma IL-1β concentration [25]. In contrast, IKKβ deficient mice that cannot respond to IL-1β are hypersusceptible to GAS infection [26].

Yersinia YopJ is an acetyltransferase that inactivates MEK and IKKβ to cause TLR4-dependent apoptosis in naive macrophages. A YopJ isoform from *Y. pestis* KIM ($YopJ^{KIM}$) with an enhanced capacity to inhibit NF-κB signaling also led to increased caspase-1 activation in macrophages [27]. TLR4-induced apoptosis is dependent upon PKR (protein kinase RNA activated) activity [28]. Interestingly PKR inactivation by genetic deletion or pharmacological inhibition severely impaired inflammasome activation in response to various NLR agonists including double-stranded RNA, ATP, *E. coli* and *S. typhimurium* infection [29]. Moreover, PKR was found to physically interact with several inflammasome components including NLRP3, NLRP1, NLRC4 and AIM2 [29, 30]. Together, the accumulated evidence presented above suggests that NF-κB impairment in certain infection models may potentiates inflammasome.

3.1 AKT activation in inflammasome induction

AKT is a serine-threonine protein kinase with important roles in multiple cellular processes. Most bacterial infections stimulate the AKT pathway in a manner that contributes to cell survival and host defense [11]. We observed that *Bacillus anthracis* LT-can inhibit of AKT activation in macrophages, as evidenced by elevated AKT S473 phosphorylation in *Bacillus anthracis* ΔLT-infected cells relative to cells infected with WT *B. anthracis* [12]. Importantly, overexpression of Myr-AKT (myristoylated-AKT, a constitutively active variant of AKT) in mouse macrophages inhibited *B. anthracis*-induced cell death and inflammasome activation. AKT negatively regulated ATP extrusion by connexin-43 phosphorylation [12] and extracellular ATP is a well-known NLR agonist. Another mechanism of AKT-NALP1 cross-talk was elucidated by Cheng and colleagues, wherein AKT phosphorylates NALP1 at four serine/threonine sites, leading to inhibition of NALP1 functions [31]. Although inflammasome activation was not analyzed in that study, inactivation of NALP1 activity may affect inflammasome formation in other contexts.

Legionella pneumophila inhibits AKT activity by inducing AKT ubiquitination via a Dot/Icm dependent pathway, a type IV bacterial secretory system [32]. Curiously, Dot/Icm also plays a key role in *L. pneumophila*-induced inflammasome activation [33]. The precise effects of AKT ubiquitination on inflammasome nucleation remain unknown.

PI3Ks also activate Rho GTPases that control processes like cell motility, growth and phagocytosis [34]. Rho GTPases can be inhibited or activated by specific pathogens leading to different consequences; inhibition of Rho GTPases prevents cell motility and bacterial phagocytosis, whereas their activation facilitates the movement of pathogens into the cytosol. *Clostridium difficile* toxin inactivates Rho GTPases; these inactivated GTPases are then detected by pyrin proteins to trigger inflammasome activation [34]. *Salmonella* activation of Rho GTPases is detected by NOD1, another NLR protein [35]. Hence the same signaling cascade may have varying effects on inflammasome activation in different infections; however the central theme is preserved wherein inflammasome is activated when the infected cell is in distress.

3.2 cIAP proteins in inflammasome activation

Inflammasome activation and cell death are often observed during bacterial infections [1, 2]. Moreover, inhibition of MAPK, NF-κB or AKT signaling may lead to apoptosis [11]. We researched the literature on the possible roles of cell death regulators in inflammasome activation. The cellular Inhibitor of apoptosis proteins (cIAPs) negatively regulate cell death [36]. cIAP1 and cIAP2 contain a C-terminal RING-finger domain with E3 ubiquitin ligase activity. They along with TRAF2 associate with caspase-1 and promote its K63-linked polyubiquitination, which is essential for NLRP3 activation. The deletion of the gene encoding cIAP2 ($Birc3^{-/-}$) results in impaired NLRP3 activation in response to NLR agonists [36]. In contrast, Vince et al., showed that deletion of all three IAPs (XIAP, cIAP1, and cIAP2) led to RIP3- and ROS- dependent NLRP3-inflammasome activation [37]. In another study linking cIAPs to NLR signaling, a physical interaction was demonstrated between RIP2 and cIAP-1, cIAP-2 or XIAP. Furthermore, macrophages derived from $Birc2^{-/-}$ or $Birc3^{-/-}$ mice exhibited impaired NOD2 signaling in response to MDP [38, 39]. Together, the results from published studies suggest a role of cIAPs in inflammasome activation.

3.3 BCL-2 family members in inflammasome activation

BCL-2 family proteins can either induce (pro-apoptotic functions) or inhibit (anti-apoptotic functions) apoptosis. These key regulators of cell survival operate by controlling mitochondrial outer membrane permeabilization (MOMP) and subsequent release of apoptotic mitochondrial proteins. Reed and colleagues have demonstrated that BCL-2 and BCL-XL, which inhibit apoptosis, negatively regulate the NLRP1 inflammasome [40]. BCL-2 and BCL-XL inhibit ATP binding to NLRP1, which is required for oligomerization of NLRP1 [40, 41]. Another BCL-2 family member, BID, a cytosolic BH3-only protein, interacts directly with NOD1 and NOD2, and regulates their downstream signaling [42]. These BCL-2 activities are reminiscent of events observed in *C. elegans*. where the BCL-2 homolog CED-9 inhibits apoptosis by directly interacting with CED4, an NLR-related protein [43]. Furthermore, induction of mitochondrial dysfunction and subsequent release of oxidized mitochondrial DNA into the cytosol potentiates inflammasome activation. Interestingly, BCL-2 inhibits mitochondrial dysfunction [44].

Thus, cell death-regulating components like cIAPs and BCL-2 that may function in conditions in which survival signaling pathways are impaired, are shown to influence NLR activation.

4 Conclusions and future perspectives

Recent evidence suggests that several cell signaling pathways have strong modulatory effects on the different steps of inflammasome activation. Interestingly, certain virulent microbes inhibit central pathways including NF-κB, MAPK and AKT and thereby enhance inflammasome activation. These pathways are linked to cell survival, and hence their impairment sends a distress signal whereupon NLR activation appears to serve as the key responder. This strategy to activate NLR in cells with impaired primary defense can be productive or detrimental to the host depending on the site, stage and magnitude of infection, as well as the infection model chosen.

Although both host-derived DAMPs and bacterial PAMPs activate inflammasomes, the common factors tying together these different processes remain obscure. ROS activation, membrane destabilization, K^+ efflux are shown to participate in different inflammasome activation events, however the precise downstream molecular events must still be elucidated further. These cellular perturbations may influence cell survival pathways that can trigger NLR stimulation. NLR activation induces IL-1β release that alerts neighboring cells about the ensuing danger and primes them for effective response. Recent reports on extracellular inflammasome formation supports the notion of importance of NLR activation to the neighboring cells [45, 46], however the role of signaling pathways in release and activation of extracellular NLRs remains unknown. Finally, identification of the central molecular machinery under distress conditions may help to reveal the key signatures linking signals from various NLR agonists to inflammasomes.

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