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Alternative inflammasome activation enables IL-1 β release from living cells

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Abstract

Classical modes of NLRP3 activation entail a priming step that enables its activation (signal 1) and a potassium efflux-dependent activation signal (signal 2) that triggers pyroptosome formation and pyroptosis, a lytic cell death necessary for IL-1 β release. Opposing to that, human monocytes engage an alternative NLRP3 inflammasome pathway in response to LPS that proceeds in the absence of signal 2 activation and enables IL-1 β secretion without pyroptosis. This specifically relies on Caspase-8 to propagate signaling to NLRP3, leading to inflammasome activation in absence of pyroptosome formation. Here, we summarize the current knowledge about alternative inflammasome activation, discuss potential extensions of this signaling entity beyond LPS-dependent activation, speculate about its role in tissue homeostasis and sterile inflammation and highlight the implications of pyroptosis-independent IL-1 β secretion.

Introduction

The pleiotropic cytokine IL-1 β serves instrumental functions in initiation and orchestration of innate and adaptive immune responses. Its receptor IL-1R shares the intracellular TIR (Toll IL-1 receptor) signaling-domain with the well-characterized PRR-family of TLRs, thereby enabling TLR-like transcriptional responses in cells not expressing TLRs. This homology in signaling explains why the biological effects of IL-1 β closely resemble activation of the innate immune system. IL-1 β is a cytokine of numerous functions, exerting diverse effects on multiple cells types to culminate in broadly inflammatory events. Systemic consequences of IL-1 β signaling include fever, vasodilatation, hypotension and acute phase response. Locally, IL-1 signaling enables mesenchymal and endothelial cells to express adhesion molecules facilitating recruitment of lymphocytes and myeloid cells. Consequently, the infiltrating immune cells are activated and triggered to further amplify inflammation. Besides its crucial roles in the induction and orchestration of innate immune responses, IL-1 β has been implicated in adaptive responses by influencing Th1 and Th17-mediated immune activation. Aberrant IL-1 β signaling has been associated with rare heritable monogenetic auto-inflammatory diseases like Cryopyrin-Associated Periodic Syndromes (CAPS) or Familial Mediterranean Fever (FMF), yet also with common multifactorial diseases, such as Type 2 diabetes or gout [1–3].

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IL-1 β belongs to the family of IL-1 cytokines, of which several members, including IL-1 β and IL-18, are synthesized as inactive precursors within the cytosol, where they await proteolytic cleavage to gain bioactivity. This maturation step is most commonly executed by the cysteine protease Caspase-1, whose activity is controlled by a cytosolic activation platform called the inflammasome (Figure 1). At the same time, other enzymes, such as Caspase-8 or neutrophil proteases have been described to mature IL-1 β . Inflammasome signaling entails a sensor molecule (e.g. NLRP1, NLRP3, NLRC4 or AIM2) that seeds the assembly of the adapter molecule ASC into helically structured filaments forming a high-molecular weight complex that is called the pyroptosome [4]. In turn, Caspase-1 becomes recruited to the pyroptosome to also form helical structures, which enable its proximity-induced proteolytical auto-activation. Once Caspase-1 has become matured into the active p10²:p20² hetero-tetramer it is licensed to cleave the cytokine-precursors pro-IL-1 β /pro-IL-18. Besides cytokine maturation, induction of a lytic type of cell death called pyroptosis constitutes a hallmark of inflammasome activation. This programmed cell death is mediated by Caspase-1-dependent cleavage of the pyroptosis-effector molecule GSDMD (Gasdermin-D). Caspase-1 maturation of GSDMD abolishes the intra-molecular auto-inhibition of the C-terminal domain [5•,6•,7•]. Subsequently, phospholipid binding specificities of the matured N-terminal fragment of GSDMD enable its translocation to the inner leaflet of the plasma membrane, where it forms round, pore-like structures of approximately 15 nm in diameter [8,9,10•,11]. Consequently, breakdown of ion gradients over the plasma membrane leads to water influx driven by oncotic pressure, inducing the characteristic cell swelling and rupture of the plasma membrane. Although soluble cytosolic proteins are released during pyroptotic lysis, it has been shown that so called pore-induced intracellular traps (PITs) maintain larger fragments of the pyroptosed cell (including previously phagocytosed bacteria) within the cellular debris [12]. The previously noted correlation of pyroptosis and IL-1 β release [13,14] has been genetically supported by the defect in IL-1 β secretion but not in maturation in murine macrophages deficient for GSDMD [5•,6•,7•]. To this end, the picture emerges that IL-1 β secretion critically depends on GSDMD in the course of classical inflammasome pathways, either directly through the nascent pore or in the course of plasma membrane disintegration.

The NLRP3 inflammasome

NLRP3 is the most studied inflammasome sensor that has attracted much attention due to its role in anti-microbial defense, but also in sterile inflammatory conditions. Initially identified by its association with monogenetic, IL-1-mediated auto-inflammatory diseases (CAPS) [15], it has also been shown to play a central role in multi-factorial, widespread diseases like Type 2 diabetes, Alzheimer's disease, gout and atherosclerosis. A recently identified pharmacological inhibitor of NLRP3 [16] has proven its efficacy in several murine inflammation models, including exacerbated inflammation caused by influenza infection and myocardial infarction [17–19]. Opposing to other inflammasome sensors, whose ligands have been identified (e.g. AIM2 or NLRC4/NAIP), the molecular mechanism of NLRP3 activation remains elusive. The current dogma proposes a two-step activation model, in which a first signal mediates transcriptional and post-translational priming of NLRP3. Only after signal 1-induced licensing, murine macrophages become sensitive to signal 2-

dependent inflammasome activation. The plethora and diverse physiochemical nature of signal 2 agonists of NLRP3 reason against direct activation of NLRP3 by any of these components. Instead, efflux of potassium along its chemical gradient across the plasma membrane has been identified as the common denominator of most known NLRP3 signal 2 agonists [20,21]. This establishes NLRP3 as a sensor of plasma membrane integrity, whose perturbation results in inflammasome activation, thereby alerting the immune system. The molecular mechanisms of potassium efflux being sensed by NLRP3 have not been understood completely, yet recent reports have implicated the cell cycle kinase Nek7 to exert a kinase-independent function in NLRP3 activation [22–24]. As such it has been proposed that potassium efflux enables NLRP3-Nek7 interaction to drive inflammasome activation [23]. Apart from that, both mitochondrial and phagosomal ROS production have been proposed to be causally connected to NLRP3 activation. Since the relationship to potassium efflux has not always been investigated in these reports, the relevance of these models remains to be determined. Recently, the other pro-inflammatory Caspases, Caspase-4, Caspase-5 and Caspase-11, have been implicated in the so called non-canonical pathway of NLRP3-inflammasome activation [25,26]. In fact, it has been demonstrated that recognition of cytosolic LPS by the CARD of respective Caspases leads to their activation [27]. Although these Caspases are capable of maturing GSDMD to induce pyroptosis, they are not able to directly mature IL-1 β . Instead, pyroptosis-linked efflux of potassium has been found to drive non-canonical NLRP3 signaling to activate Caspase-1 [28–30], thereby enabling cytokine maturation.

LPS triggers alternative inflammasome activation in human monocytes in the absence of pyroptosis

In the course of inflammasome activation, the induction of pyroptosis and IL-1 β secretion is strictly correlated and the requirement of lytic cell death for IL-1 β secretion is well supported by genetic means [5•,6•,7•]. Apart from that, the proposed two-step model of NLRP3 activation has mainly been inferred from studies in murine macrophages that critically depend on signal 2 agonists for NLRP3 signaling. To this effect, not much attention has been paid to the fact that human monocytes can secrete mature IL-1 β solely in response to LPS without the requirement of classical signal 2 activation, a feature that had been reported early after the discovery of IL-1 cytokines. We have recently characterized this response as a unique NLRP3 signaling entity that substantially differs from signal 2-mediated activation. To clearly discriminate this signaling modality from ‘classical inflammasome activation’ (canonical + non-canonical), we have coined the term ‘alternative inflammasome activation’ to define this signaling entity as such [31•]. The major differences discriminating both pathways are the absence of pyroptosis, the absence of pyroptosome formation and the independency of potassium efflux as an upstream activation mechanism in the course of alternative inflammasome activation. Instead, signaling relies on TLR4-TRIF-RIPK1-FADD-CASP8 to propagate activation to NLRP3, while the exact molecular mechanism of Caspase-8 activating NLRP3 remains unknown. In concordance with the absence of pyroptosis, the release of IL-1 β during alternative inflammasome activation does not require the pyroptosis effector GSDMD (own unpublished observations). Of note, LPS-mediated alternative inflammasome activation constitutes a species-specific

response that is absent from murine but present in human and porcine PBMCs [31••]. Interestingly, Caspase-8 mediated alternative inflammasome activation in human monocytes can also be induced in vitro by co-culture with iNKT-cells utilizing an unknown upstream signaling pathway [32•]. It is tempting to speculate that a membrane-borne TNFR-family ligand mediates activation in this setting, leading to TRIF-independent Caspase-8 activation to drive the alternative inflammasome. Importantly, the role for Caspase-8 upstream of NLRP3 during alternative inflammasome activation is distinct from roles for Caspase-8 in classical inflammasome activation and IL-1 β secretion. To this end, Caspase-8 exerts ill-defined roles in the NF κ B-signaling-pathway [33,34] and thus critically contributes to signal 1-mediated inflammasome priming [35–37]. Furthermore, in absence of Caspase-1, recruitment of Caspase-8 to the pyroptosome can result in apoptotic cell death and IL-1 β maturation, a reaction that involves heterotypic PYD-DED interaction [38,39]. Moreover, Caspase-8 can also function as a *bona fide* IL-1 β maturing enzyme, whose activation in multiple (pro-apoptotic) circumstances leads to release of mature IL-1 β [40]. Lastly, NLRP3 can be activated in the course of Caspase-8-mediated cell death induced by IAP depletion [41]. Mechanistically ill defined, it remains to be determined if classical or alternative inflammasome is at play, however the absence of pro-apoptotic activation of Caspase-8 in the course of alternative inflammasome activation might argue that classical NLRP3 activation in response to perturbed cellular integrity is at play during IAP depletion. In summary the upstream engagement of Caspase-8 during alternative inflammasome activation is different from reported role for Caspase-8 in IL-1 β secretion.

Broadening the concept of alternative inflammasome signaling

Notably, two additional studies have characterized NLRP3 activation patterns with striking similarities to alternative inflammasome activation, as observed in LPS-stimulated monocytes [42••,43••] (Table 1). The first study investigated the role of the known immune-modulator oxPAPC, an endogenous oxidized membrane lipid, in inflammasome activation. In TLR-primed murine dendritic cells (DCs) oxPAPC is sufficient to activate a Caspase-11-NLRP3 inflammasome pathway that drives IL-1 β secretion. For this, oxPAPC binds to the catalytic domain of Pro-Caspase-11, which does not lead to its auto-processing not even does it require its catalytic activity, yet it results in IL-1 β secretion. Of note, oxPAPC also binds to Caspase-1, indicating that the signaling epistasis has to be further clarified. Although its genetic association with Caspase-11 theoretically marks this pathway as non-canonical, the fact that the catalytic activity of Caspase-11 is not required for the former clearly discriminates the two. Indeed, oxPAPC-stimulated DCs activate the NLRP3 inflammasome independently of potassium efflux and independently of pyroptosis. Nevertheless, this pathway involves pyroptosome formation [42••]. The second report investigates the relationship of proximal metabolic perturbation of glycolysis and subsequent inflammasome activation. Inhibition of hexokinase and subsequent translocation into the cytoplasm is sufficient to activate the NLRP3 inflammasome. The phenotypic characteristics linking this pathway to alternative inflammasome activation include independence of potassium efflux, absence of pyroptosome formation and absence of pyroptosis. However, at this point it is not clear if the detachment of hexokinase from the mitochondria or its

presence in the cytosol triggers inflammasome activation and in addition, insights into the molecular mechanism of NLRP3 activation are missing [43••].

Although the upstream signaling pathways of LPS, oxPAPC and hexokinase detachment-mediated NLRP3 activation significantly differ, distal signaling events display striking similarities. Since the molecular details of the respective activation pathways are not known, it remains speculative whether all three pathways converge on a presumably common platform of alternative inflammasome activation. In this scenario, multiple, at least three, different upstream signaling events are able to activate the alternative NLRP3 inflammasome to induce IL-1 β secretion without the requirement for potassium efflux and pyroptosis. Addressing the role of Caspase-8 in metabolic disturbance-mediated inflammasome activation and the translocation of hexokinase in LPS-induced inflammasome activation may help clarify convergence or divergence of upstream signaling cascades. To this effect, it is noteworthy that both Caspase-8 and hexokinase detachment have been associated with apoptosis [44,45]. Since no apoptotic signaling cascade has been observed in LPS-stimulated monocytes, sub-apoptotic activation may comprise a common trigger of the alternative inflammasome.

In summary, the alternative inflammasome comprises a distinct signaling entity that engages the hardwiring of the classical NLRP3 inflammasome pathway to mature and secrete IL-1 β , yet does not engage pyroptosis. As outlined above, the upstream activation pathways of alternative inflammasome signaling are likely to be diverse, while phenotypic criteria are not stringent enough to define alternative inflammasome signaling as such. Therefore, we would currently consider GSDMD-independent, inflammasome-mediated IL-1 β secretion in pyroptosis-competent cells as the most appropriate definition for alternative inflammasome activation. Nonetheless, identification of additional molecules that specifically regulate alternative inflammasome activation may lead to better, non-exclusion-based genetic criteria. This could involve both upstream regulators that are required for Caspase-8 to activate NLRP3 or downstream mediators involved in GSDMD-independent IL-1 β release.

Implications of alternative inflammasome activation

Ever since its discovery, the unconventional mode of secretion of IL-1 β has been subject of intense research efforts. The notion that IL-1 β is synthesized as an inactive precursor in the cytosol had already implied Golgi-independent secretion mechanisms. With the discovery of GSDMD it became clear that passive release either directly through the GSDMD pore or in the course of plasma membrane rupture constitutes the main mechanism of unconventional secretion of IL-1 β during classical inflammasome activation. However, the absence of pyroptosis from alternative inflammasome activation argues for another, GSDMD-independent, active secretion pathway for IL-1 β . Several mechanisms have already been suggested to explain the unconventional secretion of IL-1 β , including autophagy-mediated engulfment into vesicles and subsequent exocytosis as well as shedding of mature IL-1 β in vesicles from the plasma membrane [46]. Although some of these models have been proposed in the course of studying LPS-stimulated monocytes (and thus alternative inflammasome activation), others arose from characterizing classical inflammasome activation and thus have been falsified by our current knowledge of GSDMD biology. Most

appealingly, autophagy-assisted entry into the exocytotic pathway may constitute a key mechanistic feature of IL-1 β secretion, as suggested from studies in LPS-stimulated monocytes [47] and pyroptosis-incompetent non-myeloid cells [48•].

Interestingly, the uncoupling of inflammasome activation and pyroptosis has also been reported in neutrophils. Indeed, this cell type appears to harbor a general pyroptosis defect in response to AIM2 and NLRC4 activation, despite normal IL-1 β secretion [49]. The protection from pyroptosis in the course of the alternative inflammasome is likely to differ both mechanistically and conceptually. In neutrophils a broad defect in the whole pyroptotic pathway may be due to perturbed GSDMD expression or function. However, in the course of alternative inflammasome activation, the precise omission of GSDMD-dependent pyroptosis alludes to the existence of a specific signaling event inhibiting or not inducing pyroptosis. It has been reasoned before that disabled neutrophilic pyroptosis may be beneficial to the host since most intracellular bacteria are not replication competent within neutrophils [50] and ‘premature’ pyroptosis may prevent neutrophilic anti-pathogenic functions. Opposing to that, the alternative inflammasome pathway is operational in potentially long-lived myeloid cells of the immune system, such as macrophages and dendritic cells. These cells may engage the alternative inflammasome in order to omit pyroptosis but to enable induction of IL-1 β dependent immune responses in circumstances where pathogen sensing and/or effector functions require temporal and or spatial separation. Indeed, alternative inflammasome activation may be instrumental in both antigen transport to secondary lymphoid organs and tissue homeostasis by resident myeloid cells. Notably, oxPAPC has already proven its efficacy in a vaccination setting [42••]. With regards to tissue homeostasis, it has to be noted the alternative inflammasome has mainly been studied in peripheral blood monocytes [31••,32•]. However, LPS-mediated IL-1 β secretion and thus alternative inflammasome activation has also been documented in human tissue macrophages [51,52], the immune cell type most instrumental for tissue homeostasis. Indeed, it is conceivable that alternative inflammasome activation is a major mediator of homeostatic, sterile inflammation. As such, activation of TLR4 and NLRP3 by endogenous sterile ligands is believed to play an important role in various sterile disease conditions. In this model TLR4-ligating DAMPs like HMGB1 [53], HSPs [54], Hyaluronan [55], Biglycan [56], Heparan sulphate [57] or S100-proteins [58] are thought to provide signal 1-mediated inflammasome priming to enable responsiveness to DAMPs such as ATP, uric acid or cholesterol crystals that activate the classical NLRP3 inflammasome via signal 2 [59]. With the epistatic relationship of TLR4 functioning directly upstream of NLRP3 in the alternative inflammasome pathway, DAMPs previously considered to provide only priming signals should be sufficient to drive alternative inflammasome activation. Considering that TLR-4-mediated alternative inflammasome activation constitutes a species-specific response, the importance of signal 2-stimuli for IL-1 driven sterile disease conditions should be revisited in appropriate conditions or models like the human or porcine system.

In summary the alternative inflammasome pathway constitutes a distinct signaling entity in the inflammasome repertoire of human monocytes and macrophages. Although mechanistic details of upstream signaling events remain ill-defined, Caspase-8 activation in response to LPS sensing or iNKT stimulation is integrated to activate the alternative NLRP3 inflammasome without the requirement for potassium efflux. Subsequent signaling engages

the inflammasome components ASC and Caspase-1 without inducing pyroptosome formation to drive IL-1 β secretion in absence of pyroptosis. This may enable spatio-temporal separation of sensing and effector function and may be instrumental in tissue homeostasis and sterile inflammation.

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Figure 1. The alternative inflammasome comprises a signaling entity distinct from classical inflammasome activation.

Classical activation of the NLRP3 inflammasome involves a two-step activation model (left side of figure): a PRR-derived signal (signal 1) is required to license NLRP3 activation via upregulation of its expression at the transcriptional level and/or its posttranslational modification. NLRP3 can now detect perturbations of cellular integrity by recognizing potassium efflux (signal 2). Consequently, Nek7–NLRP3 interaction leads to pyroptosome assembly and Caspase-1 activation to mature IL-1β and GSDMD. Pore formation of GSDMD induces pyroptosis, which is critically required for IL-1β secretion. Opposing to that alternative inflammasome activation can be triggered by one signal only, for example, LPS or an iNKT cell derived signal (right side of figure): LPS sensing induces a TLR4-TRIF-RIPK1-FADD-CASP8 signaling axis, resulting in activation of NLRP3 by cleavage of an unknown Caspase-8 substrate independently of potassium efflux. The alternative NLRP3 activation complex is different from the pyroptosome and possibly displays stoichiometric composition. Although Caspase-1 becomes matured and cleaves IL-1β, pyroptosis is not induced and IL-1β is secreted by an unconventional mechanism that functions independently of GSDMD.

Table 1
Key characteristics of classical and alternative inflammasome activation

Type of inflammasome activation	Classical (canonical + non-canonical)	Alternative	Presumably alternative	
Description	Multiple groups	Gaidt et al. [31••]	Wolf et al. [43••]	Zanoni et al. [42••]
NLRP3-ASC-Caspase 1 signaling	Yes	Yes	Yes	Yes
Caspase-1, IL-1 β maturation and secretion	Yes	Yes	Yes	Yes
Potassium efflux requirement	Yes	No	No	No
Pyroptosome formation	Yes	No	No	Yes
Pyroptosis	Yes	No	No	No
Upstream mechanism	Nek7	Caspase-8	Hexokinase detachment	Pro-Caspase-11
GSDMD requirement	Yes	No	Not known	Not known