FOCUS: IMMUNOLOGY AND IMMUNOTHERAPEUTICS

Regulating Caspase-1 During Infection: Roles of NLRs, AIM2, and ASC

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Pathogens are detected by a variety of innate immune sensors in host cells leading to rapid induction of cell autonomous responses. Proinflammatory cytokine secretion and a specialized form of inflammatory cell death called pyroptosis are induced during infection through activation of caspase-1. Pathogen-induced caspase-1 activation is regulated in large part by a vast array of cytosolic sensor proteins, including NLRs and AIM2, and an adaptor protein called ASC. Together, these proteins cooperate in forming caspase-1 activation platforms and, more importantly, direct caspase-1 toward cytokine secretion or cell death.

INTRODUCTION

Caspase-1 is a key mediator of inflammation in response to pathogen-derived molecules and endogenous danger signals. This cysteine protease cleaves a large repertoire of substrates leading to diverse downstream activities, including proinflammatory cytokine activation and secretion [1], and induction of cell death [2]. Activation of caspase-1 is regulated by upstream sensor proteins and adaptors, including the nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs†), absent in melanoma 2 (AIM2), and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). These proteins are responsible for

†Abbreviations: NLR, nucleotide-binding domain, leucine-rich repeat containing protein; LRR, leucine-rich repeat; CARD, caspase recruitment domain; NBD, nucleotide binding domain; PYR, pyrin domain; BIR, baculovirus inhibitory domain; IAP, inhibitor of apoptosis protein; TLR, Toll-like receptor; ROS, reactive oxygen species; T3SS, type III secretion system; MDP, muramyl dipeptide; LF, lethal factor; MEK, mitogen-activated protein kinase kinase.

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coordinating caspase-1 activation complexes in response to endogenous and foreign molecules associated with cellular danger.

Understanding the regulation of caspase-1 activation is of particular importance, as improper activation of caspase-1 in response to endogenous molecules is associated with a number of inflammatory diseases. The hereditary disorders familial Mediterranean fever (FMF), familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile cutaneous neurological articular syndrome (CINCA) are each associated with excessive activation of caspase-1-mediated proinflammatory cytokine production. Similarly, diseases such as Type 2 diabetes, gout, atherosclerosis, and Alzheimer’s disease are associated with increased caspase-1 activation and inflammation. Recent reviews have discussed in detail the relationship between caspase-1 activation and inflammatory disorders [3,4].

Major advances toward understanding caspase-1 activation and regulation have come from examining the role of caspase-1 during the innate immune response to microbial pathogens. One major class of foreign agonists leading to caspase-1 activation is composed of pathogen-derived molecules. Pathogens including bacteria, viruses, and protozoans have proven to be useful tools in dissecting the regulation of caspase-1 and the upstream proteins involved in activation. This review will discuss the current understanding of caspase-1 activation and highlight studies with pathogens that have uncovered new details in the regulation of caspase-1-associated activities.

NLRs, AIM2 and ASC: REGULATORS OF CASPASE-1

Caspase-1 activation is regulated in part by cytosolic sensor proteins comprised of the NLRs and AIM2. NLRs are characterized primarily by their shared domain architecture. The C-terminal region typically contains a leucine-rich repeat domain (LRR), and the region immediately upstream contains a nucleotide-binding domain (NBD). The N-terminal domains of NLRs further subdivide these proteins into distinct subfamily members. Members of the NLRP subfamily contain a N-terminal pyrin domain (PYR), while the NLRC proteins contain a caspase recruitment domain (CARD) at this position. The NAIP subfamily members contain baculovirus inhibitory domains (BIR) at their N-termini. Separate from the NLRs, AIM2 is a member of the PYHIN family of proteins and lacks both an NBD and LRR region. Instead, this protein contains a C-terminal HIN200 domain and an N-terminal PYR domain.

The N-terminal domains of NLRs and AIM2 determine their ability to directly or indirectly interact with caspase-1. Proteins with CARD domains, such as NLRC4 and NLRP1, are able to directly interact with caspase-1 [5,6]. In contrast, AIM2 and the NLRP proteins, with the exception of NLRP1, require an adaptor protein in order to interact with caspase-1. This adaptor, known as ASC, is comprised of a PYD and a CARD domain. This bipartite architecture enables ASC to bridge PYD-containing proteins with the CARD of caspase-1. Although lacking PYD or CARD domains, NAIP proteins were initially thought to interact with caspase-1 through their BIR domains, which share homology with BIR domains of inhibitor of apoptosis proteins (IAPs) that bind to apoptotic caspases [7]. However, recent studies indicate that NAIP proteins signal to caspase-1 indirectly through binding interactions with NLRC4 [8,9]. Through these various routes of association, NLRs, AIM2, and ASC interact with caspase-1, resulting in formation of a caspase-1 activation complex called an inflammasome [10]. The role of inflammasome formation in caspase-1 activities will be discussed in more detail in a subsequent section.

Genetic and biochemical evidence have provided useful insight into the mechanistic details behind NLR activation. The LRR domains of NLRs are thought to act in a manner similar to LRRs found in Toll-like receptors (TLRs) and likely are important for protein-protein or receptor-ligand inter-
actions resulting from exposure to an appropriate agonist. Furthermore, LRRs are thought to be autoinhibitory to NLR function [5,8]. Until sensing of an appropriate signal by the LRRs, it is believed that the LRRs maintain the NLR in a conformational state that prevents downstream activation events. The central NBD found in NLRs has been shown to bind nucleotides and promote oligomerization of the NLR with itself and other components in signaling complexes [6,8]. Upon relief of autoinhibition by the LRRs, it is thought that oligomerization is initiated through the NBD while simultaneously exposing the N-terminal PYD, BIR, or CARD domain in order to recruit downstream molecules such as ASC, caspase-1, or another NLR. Ongoing studies continue to examine the interplay between NLRs, ASC, and caspase-1.

One of the most heavily studied proteins involved in caspase-1 activation is NLRP3. The detailed mechanism for activation of this NLR is a major area of research, and progress has been made in recent years. NLRP3 activation requires two distinct signals prior to activation. The first signal, typically a TLR agonist, is known as a priming event and involves transcriptional upregulation of NLRP3 [11]. The second signal involves stimulation with a NLRP3 agonist. A diverse group of pathogen-derived and endogenous molecules signal through NLRP3 to induce caspase-1 activation, suggesting that NLRP3 is responding to an intermediate signal rather than directly sensing any one of these microbial or host-derived agonists. Studies thus far have led to multiple models, each involving the sensing of a general change in the cellular environment following stimulation with a given agonist. One proposed model for activation involves reactive oxygen species (ROS) production. ROS production is a common event following exposure to several NLRP3 agonists and is required for caspase-1 activation in multiple instances [12-15]. However, recent data suggest that the role of ROS may be further upstream in the signaling pathway than previously thought and may play a role solely for priming, or transcriptional upregulation of NLRP3 [16]. Furthermore, macrophages derived from mice deficient in NOX2, a major producer of ROS in these cells, are still competent for activation of caspase-1 in response to a subset of NLRP3 agonists, suggesting that ROS production is not the sole player in this pathway [17]. A second model argues that lysosomal leakage as a result of membrane perturbation results in caspase-1 activation. This model proposes that proteases, which are normally active only in lysosomal compartments, are released into the cytosol following perturbation of the phagosomal membrane during endocytosis. The strongest evidence for this model are data indicating that the lysosomal protease cathepsin B plays an important role in NLRP3-dependent caspase-1 activation in response to several agonists [15,17-19]. However, much like the ROS model, the requirement for cathepsin B does not appear to be absolute as macrophages deficient for this protease are still competent for induction of caspase-1 activation in response to a subset of agonists, suggesting that other proteases or activities are sufficient for induction [18,19]. Finally, the most common feature of NLRP3-induced inflammasome activation is the requirement for potassium efflux. Potassium efflux occurs in response to a number of NLRP3 agonists, and many NLRP3-mediated pathways for caspase-1 activation are efficiently inhibited by high levels of extracellular potassium [12,15,20-23]. Although the connection between potassium efflux and NLRP3-mediated activation of caspase-1 remains unclear, a role for the adaptor ASC has been proposed. Biochemical data suggest that under conditions of low potassium, ASC oligomerizes more efficiently and provides a better scaffold for the recruitment and activation of caspase-1 [24]. Therefore, in the presence of an NLRP3 agonist, a resulting loss of potassium may render the cytosol more favorable for oligomerization of ASC and promote more robust caspase-1 activation. However, it remains unclear if NLRP3 and sensing of potassium efflux are directly linked since oligomerization of purified recombinant ASC and subsequent caspase-1 cleavage was found to occur in the absence
of NLRP3 when potassium levels were reduced in vitro [24]. In addition, potassium efflux has been shown to be important for NLRP3-independent but ASC-dependent pathways leading to caspase-1 activation, arguing that potassium efflux may be a more general requirement for ASC-dependent pathways [25-27]. Regardless, it cannot be ruled out that each of these hypotheses may contribute at least partially to the overall mechanism of activation for NLRP3. It is possible that these three events — ROS production, lysosomal leakage, and potassium efflux — each result in a distinct signal that is capable of signaling to NLRP3 either directly or through an intermediate molecule. In support of this latter possibility, it has been shown that ROS production promotes association of thioredoxin interacting protein (TXNIP) with NLRP3 and that TXNIP is required for activation of caspase-1 in response to NLRP3 agonists [28]. Future work identifying upstream components involved in sensing of NLRP3 agonists should further clarify the mechanistic details behind activation of this sensor molecule.

CASPASE-1 ACTIVATION BY PATHOGENS

Bacterial, viral, and eukaryotic pathogens have all been shown to induce caspase-1 activation. The mechanisms for activation by these various pathogens share a number of features, suggesting that caspase-1 is activated in response to common activities or products of pathogenic organisms. One key determinant for the activation of caspase-1 is the escape of microbial products into the host cell cytosol and subsequent detection by NLRs and AIM2. Microbial products gain access to host cell cytosols through a number of mechanisms, including pore-forming activities of toxins, specialized protein secretion systems that inject microbial products into host cells, or phagosomal leakage resulting from endocytosis of microbial products.

NLRC4

A major implication for bacterial-induced caspase-1 activation came with the elucidation of NLRC4 as a key mediator in the response to bacterial flagellin. NLRC4 has been shown to be involved in the response to flagellin during infection of macrophages by Legionella pneumophila, Salmonella enterica serovar Typhimurium, and Listeria monocytogenes [29-35]. In addition, there exist flagellin-independent pathways leading to caspase-1 activation that require NLRC4. The type III secretion system (T3SS) rod protein is sensed in a manner dependent on NLRC4. Pseudomonas aeruginosa, Shigella flexneri, and S. Typhimurium are examples of organisms capable of inducing caspase-1 activation following detection of the T3SS rod protein [36]. In either case, flagellin and T3SS rod protein are thought to gain access to host cell cytosols through accidental release by the various secretion mechanisms or membrane disrupting activities required for survival of these organisms during infection. For instance, L. pneumophila deficient in type IV secretion, S. Typhimurium or P. aeruginosa deficient in type III secretion, and L. monocytogenes deficient in listeriolysin O production all fail to induce caspase-1 activation [33,35,37,38]. Thus, NLRC4 enables host cells to discriminate between organisms by specifically detecting activities associated with virulence.

Activation of NLRC4-dependent pathways for caspase-1 activation requires signaling from an upstream NAIP protein. Multiple NAIP proteins in mice and the lone human NAIP protein are able to directly bind to distinct agonists, resulting in formation of high molecular weight oligomers containing NLRC4 [8,9]. These NLRC4-containing oligomers are competent for caspase-1 recruitment and activation [8,9]. These data indicate that NLRC4 serves as an adaptor protein for the various NAIP proteins, rather than acting as a direct receptor for microbial agonists. Instead, it is evident that NAIPs serve as the primary receptors and enable caspase-1 activation through NLRC4 in response to multiple microbial-derived agonists. NLRC4-dependent caspase-1 activation mediated by bacterial flagellin has been shown to require NAIP5 in murine macrophages, while signaling in
response to the T3SS rod protein from various bacterial species has been shown to require NAIP2 [8,9]. Interestingly, in contrast to flagellin- and T3SS rod protein-mediated activation observed in murine cells, activation of NLRC4 in human macrophage-like cells by NAIP occurs in response to T3SS needle protein [9]. Together, these data indicate that NAIP proteins impart a level of specificity for the NLRC4 inflammasome. Future studies should elucidate if other ligands exist for the other NAIP proteins encoded in the mouse genome.

**NLRP3**

In addition to NLRC4, a major factor in the caspase-1-mediated response to bacterial-derived molecules is NLRP3. The group of agonists leading to NLRP3-dependent activation of caspase-1 is extensive and continues to grow. One class of activators contains a number of bacterial-derived pore-forming toxins. Examples of pore-forming toxins that induce NLRP3-dependent caspase-1 activation are streptolysin O (*Streptococcus pyogenes*) [39], listeriolysin O (*L. monocytogenes*) [40], toxin A (*Clostridium difficile*) [41], hemolysins (*Staphylococcus aureus*) [42], and aerolysin (*Aeromonas hydrophila*) [43]. Activation by pore-forming toxins is thought to occur as a result of potassium efflux. It has been demonstrated that caspase-1 activation induced by pore-forming toxins can be efficiently blocked by increasing extracellular potassium concentrations [40,42,43]. In addition to pore-forming toxins, other bacterial products appear to contribute to NLRP3-dependent caspase-1 activation. *S. Typhimurium* and *Yersinia spp.* induce caspase-1 activation through an NLRP3-mediated pathway [21,44,45]. The mechanism for NLRP3 activation by *S. Typhimurium* is unclear. Activation by *Y. pestis* is thought to occur through effector protein YopJ-mediated inhibition of NF-κB signaling, which has been shown previously to negatively regulate inflammasome activation [21].

NLRP3 also has been shown to be important for sensing of eukaryotic pathogens. The fungal pathogens *Candida albicans* and *Aspergillus fumigatus* are two examples of organisms capable of inducing NLRP3-dependent activation of caspase-1 [22,46]. The mechanism behind activation of NLRP3 in response to these fungal pathogens is relatively unclear. Studies with *C. albicans* indicate that uptake of the yeast form of this pathogen and subsequent differentiation into the hyphal form is required for efficient activation of caspase-1 [46]. One hypothesis is that transition into the hyphal form leads to phagosomal disruption or reactive oxygen species production, which in turn stimulates NLRP3 [46]. In addition to fungal pathogens, the protozoan genus *Plasmodium* induces NLRP3-dependent activation of caspase-1 [15,47,48]. The mode of activation involves a byproduct of hemoglobin metabolism produced by the protozoan known as hemozoin. During the detoxification of heme, which is a normal part of hemoglobin metabolism by *Plasmodium* parasites, hemozoin is produced as an insoluble waste product [49]. The mechanism for activation by hemozoin is relatively unclear, although data suggests that activation may take place indirectly through the release of uric acid, a well-studied NLRP3 agonist [48].

Viruses also are sensed by NLRP3-dependent mechanisms. Typically, the sensing of viruses involves detection of nucleic acid in the host cell cytosol. It has been demonstrated that RNA triggers an NLRP3-dependent inflammasome [50]. RNA viruses, including influenza and sendai virus, are capable of inducing activation of caspase-1 through NLRP3 [50,51]. Interestingly, in addition to sensing of viral RNA, NLRP3-dependent activation of caspase-1 occurs in response to the influenza virus M2 protein, which acts as a proton-selective ion channel during viral pathogenesis [52]. It is unclear how M2 leads to NLRP3 activation, although the ability of M2 to influence cellular ionic gradients is likely a factor [52].

**NLRP1**

Anthrax lethal toxin is another example of a bacterial toxin capable of inducing caspase-1 activation. This toxin is produced by the bacterium *Bacillus anthracis* and is ca-
pable of entering host macrophages through receptor mediated endocytosis, followed by translocation of the catalytic subunit of this protein complex, known as lethal factor (LF), into the host cell cytosol [53]. Once in the cytosol, LF is sensed by caspase-1 inflammasomes in a manner dependent on NLRP1 (NLRP1b in mice) [54,55]. LF has been shown previously to possess protease activity against mitogen-activated protein kinase kinases (MEKs) [56]. Protease activity was found to be critical for activation of caspase-1 by lethal factor in murine macrophages [26]. However, MEK cleavage alone was not sufficient to induce activation, suggesting that MEK cleavage per se may not be the signal for caspase-1 activation through NLRP1b [26]. Furthermore, activation of caspase-1 by anthrax lethal toxin was found to require proteasome activity and, similar to NLRP3, potassium efflux [26,57]. The role of the proteasome during caspase-1 activation is unclear. One hypothesis is that NLRP1 is kept in an inactive state by another protein that becomes degraded through a proteasome-dependent mechanism following the sensing of anthrax lethal toxin.

In addition to anthrax lethal toxin, a bacterial cell wall component, muramyl dipeptide (MDP), has been shown to activate caspase-1 through human NLRP1 [6,58]. In vitro reconstitution experiments suggest that MDP may be a direct ligand for NLRP1, as no other protein components are required to induce NLRP1 activation in this setting [6]. However, it remains unclear whether a direct interaction between this bacterial agonist and host sensor protein takes place in vivo. Furthermore, MDP-mediated activation of caspase-1 in murine cells occurs through a NOD2-dependent mechanism, and it remains unclear whether a NLRP1 homologue is required for activation in this system.

**AIM2**

DNA is also sensed in host cell cytosols and can induce caspase-1 activation. AIM2 has been shown to directly bind to DNA and induce inflammasome formation during infection by the intracellular bacterial pathogens, *Francisella tularensis* and *L. monocytogenes* [59-65]. In addition to bacterial infections, infections by DNA viruses such as vaccinia virus lead to potent AIM2-dependent activation of caspase-1 [60]. Similar to the NLRPs, activation of caspase-1 by AIM2 requires the adaptor protein ASC [59-62].

**NLRP6**

In addition to responding to virulent microorganisms, caspase-1 inflammasomes have been shown recently to be critical for maintaining balance of normal microbiota in the murine gut. Mice deficient for NLRP6 were found to be colonized more heavily relative to wild-type mice by several bacterial species, including members of the bacterial genus *Prevotella* [66]. Consequently, NLRP6-deficient mice were more susceptible to dextran sodium sulfate (DSS)-induced colitis [66]. Resistance to colitis was restored with antibiotic treatment, suggesting a possible link between increased inflammation and the expansion of *Prevotella* or other bacterial species in the gut [66]. Mice deficient for caspase-1 or the caspase-1-dependent cytokine IL-18 displayed phenotypes similar to NLRP6-deficient mice, suggesting that NLRP6/caspase-1-mediated production of IL-18 helps to maintain balance of normal gut microbiota [66]. It is unclear whether NLRP6 is directly sensing and responding to *Prevotella* or whether the absence of NLRP6-dependent signaling enables *Prevotella* persistence and expansion. These data suggest that inflammasomes may not only serve as a primary responder to pathogenic organisms, but also as regulators of homeostasis among microbial communities in the host gut.

**REGULATION OF INFLAMMASOME FORMATION, CYTOKINE PROCESSING AND CELL DEATH**

Microbe-induced signaling through NLRs or AIM2 ultimately leads to recruitment and activation of caspase-1 via inflammasome formation, followed by the action of
this protease on its downstream targets. Two heavily studied activities of caspase-1 include cleavage of the pro-inflammatory cytokines IL-1β and IL-18 and the induction of a cell death pathway termed pyroptosis. IL-1β and IL-18 are synthesized as inactive precursor proteins (pro-IL-1β and pro-IL-18). Caspase-1-mediated cleavage of these proteins results in production of the bioactive form of the cytokines, which are then secreted through an unconventional protein secretion pathway [1,67]. The targets of caspase-1 that lead to cell death are less clear. Following caspase-1 activation, pores are formed in host cell membranes that disrupt ion fluxes, resulting in osmotic lysis and death of the cell [68].

In murine macrophages, large inflammasome complexes have been observed in response to agonists of NLRC4, NLRP3, NLRP1b, and AIM2 [26,34,45,69], suggesting that these structures are a common feature of caspase-1 activation. These complexes are organized by the adaptor protein ASC, which as discussed previously undergoes oligomerization during caspase-1 activation. NLRs and AIM2 may participate in complex formation by directly associating with ASC and caspase-1. Recent evidence using pathogen models have indicated that caspase-1-mediated cytokine processing and cell death induction are influenced by the adaptor ASC. The intracellular pathogens S. Typhimurium and L. pneumophila induce NLRC4- and ASC-dependent pathways for caspase-1 activation in murine macrophages [25,45]. During infection with these bacteria, complex formation mediated by NLRC4 and ASC is critical for efficient processing of caspase-1 into its active subunits [69,70]. However, in the absence of ASC-dependent complex formation, caspase-1-mediated cell death via NLRC4 occurs normally [25,38,70,71]. These data suggest that while NLRs and AIM2 cooperate with ASC in inflammasome formation to induce cleavage of caspase-1, NLRC4 also can function independently of ASC and direct distinct inflammasome formation.

Recent studies have provided evidence in support of this hypothesis. Reconstitution studies using 293T cells suggest that NLRC4 is able to form large molecular weight complexes with NAIP5 or NAIP2 in response to flagellin or T3SS rod protein, respectively, and that formation of these complexes are sufficient for induction of cell death [8]. This complex formation presumably is independent of ASC, as 293T cells do not express detectable levels of this protein [72]. This suggests that NLRC4-mediated pathways of caspase-1 activation involve complex formation distinct from ASC-dependent pathways. Furthermore, formation of these distinct complexes significantly influences downstream caspase-1-associated activities. Although the ability of caspase-1 to induce cell death in the absence of ASC requires the catalytic activity of the protease, cleavage of caspase-1 is not required [70]. In fact, a variant of caspase-1 that is unable to undergo proteolytic processing is still able to induce pyroptosis. In contrast, this variant protein is unable to efficiently cleave pro-IL-1β into its active form, even in the presence of ASC [70]. These data suggest that inflammasome formation through ASC-dependent mechanisms leads to caspase-1 autoproteolysis and that this form of the active protease is critical for cytokine cleavage. In the absence of ASC, caspase-1 activation occurs without autoproteolysis, and this form of the protease targets a distinct subset of substrates critical for the induction of pyroptosis [70]. Together, these recent findings indicate that formation of distinct inflammasomes control the ability of caspase-1 to target specific substrates.

It remains unclear how proteins lacking a CARD domain, such as NLRP3 and AIM2, induce cell death through ASC. One possibility is that the main ASC complex formed in response to all caspase-1 agonists also promotes cell death. It is possible that within the ASC complex, a pool of caspase-1 is processed and acts on cytokines to promote their cleavage and secretion, and an alternate pool is activated but not processed which then targets substrates leading to pore formation and cell death. Alternatively, it cannot be ruled out that processed and non-processed
caspase-1 target the same substrates leading to cell death and that processed caspase-1 may simply be more efficient at cleavage of substrates than non-processed caspase-1. In this scenario, inefficient caspase-1 activity may have a more drastic consequence on the levels of cytokines, which are direct targets of caspase-1. In contrast, it is possible that caspase-1-mediated cell death may involve a signal amplification cascade that requires very little caspase-1 activity in order to initiate the pathway. Future studies examining the substrates of processed and non-processed caspase-1 will provide more insight into the ability of caspase-1 to differentially regulate cytokine processing and cell death.

CONCLUSIONS

Studies examining macrophage responses to microbial pathogens and their associated molecules have yielded significant mechanistic insight into caspase-1 activation and regulation. A diverse class of cytosolic sensor proteins comprised of the NLRs and AIM2 enables host cells to detect a range of microbial products that gain access to host cell cytosols. Sensing of microbial products by NLRs and AIM2 may occur through direct binding of an agonist to a sensor protein, production of intermediate signals that bind to a sensor protein, or detection of an overall change in the cellular environment. Ultimately, different signals are able to converge on caspase-1 through the adaptor ASC, which organizes specialized platforms leading to caspase-1 processing and cytokine cleavage. Independent of ASC-mediated complex formation, caspase-1 can be directly recruited and activated by NLRC4 and possibly other sensors without processing, leading to induction of cell death. Studies thus far have uncovered mechanisms for regulating caspase-1 specificity with respect to cytokine secretion and induction of cell death. Future work in this area will likely identify roles for previously uncharacterized NLRs and further elucidate mechanistic details behind NLR activation and regulation of caspase-1-mediated activities.

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