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ENIGMATIC INFLAMMASOMES: LESSER-STUDIED NLRs SERIES
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NLRP9 in innate immunity and inflammation

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Summary

The nucleotide-binding domain leucine-rich repeat containing receptors (NLRs) are a family of evolutionarily conserved proteins. Several members of NLRs, notably NLRP1, NLRP3 and NLRC4, are able to form cytosolic oligomeric signalling platforms termed inflammasomes to mediate immune response towards pathogens, damage and stress. However, the functions of many NLRs still remain elusive. In the past few years, a couple of less-characterized NLR members are emerging as important signalling molecules with fundamental functions in host defence and inflammation. Among them, NLRP9 is an NLR originally proposed to be expressed and function solely in the reproductive system. Recent evidence has suggested that NLRP9 is also capable of initiating inflammasome formation in the intestine to restrict replication and damage brought by rotavirus infection. Here, we highlight the latest progress in characterization of the role of NLRP9 in infectious and inflammatory diseases, as well as the newest crystallographic and biochemical studies on NLRP9. Finally, we discuss some important questions remained to be answered regarding the molecular and cellular mechanisms governing NLRP9's function in innate immunity and inflammation.

Keywords: inflammasome; intestine; NLR; NLRP9; rotavirus.

INTRODUCTION

The nucleotide-binding domain leucine-rich repeat containing receptors (NLRs), also known as nucleotide-binding oligomerization domain-like receptors (NOD-like receptors), are an ancestral group of germline-encoded intracellular proteins, of which many serve as pattern recognition receptors (PRRs) to detect a wide range of pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and other varieties of environmental stress.[1] NLRs have been discovered in a broad spectrum of species including those in the plant kingdom, all of which evolved from ancient nucleotide-binding-domain (NBD)-containing proteins.[2] Plant and animal NLRs are heavily involved in intracellular immune surveillance of invading pathogens via conserved principles of activation shared by domains commonly found in this family. It has been reported that at least 22 and 34 NLR genes are encoded in the human and mouse genome, respectively.[3] NLR proteins are characterized by a centrally located NAIP, CIITA, HET-E and TP1 (NACHT) domain involved in nucleotide-binding and oligomerization. The NLR protein family can be further divided into several subfamilies based on the different amino-terminal effecter domains. The ones containing a pyrin domain (PYD) at the N-terminus are named NLR family pyrin domain containing (NLRP) proteins.[4] To date, 14 human and 20 murine NLRPs have

Abbreviations: NLR, nucleotide-binding domain leucine-rich repeat containing receptors; NOD, nucleotide-binding oligomerization domain; PRR, pattern recognition receptors; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; NBD, nucleotide-binding-domain; NLRP, NLR family pyrin domain containing; TLR, Toll-like receptor; RLR, RIG-I-like receptor; ALR, AIM2-like receptor; CLRs, C-type lectin receptors; PYD, pyrin domain; CARD, carboxy-terminal caspase recruitment domain; IL, interleukin; dsRNA, double-stranded RNA; DHX, DExH-box helicase

been identified,[5,6] yet only a few such as NLRP1 and NLRP3 have been extensively studied. Recent years have witnessed an intensive effort in elucidating the functions and characteristics of the less well-known NLRs. In this review, we focus on the recent advances in our understanding of NLRP9, one of the least studied NLRs, in innate immune and inflammatory responses, as well as the implications in health and disease.

NLRP9 ISOFORMS AND REPRODUCTIVE FUNCTIONS

NLRP9 is one of the three NLRPs (together with NLRP1 and NLRP4) that have undergone lineage-specific duplications in rodents. While humans only have one NLRP9 gene (hNLRP9), mice harbour three isoforms (mNLRP9a, mNLRP9b and mNLRP9c).[7] Together with NLRP4, NLRP5, NLRP8 and NLRP14, NLRP9 belongs to a subgroup of reproduction-related NLRPs exclusively or mainly expressed in reproductive organs.[7,8] With the exception of NLRP14, all the human NLRPs with implicated functions in reproductive systems are tandemly distributed on chromosome 19, indicating that a series of tandem duplication events may have given rise to these NLR genes, and their functions may be closely related yet specified.[4,7]

The most well-characterized NLR involved in mammalian reproduction is NLRP5 (also known as MATER). Female NLRP5-deficient mice were unable to support embryonic development beyond the two-cell stage and therefore were sterile, indicating that NLRP5 is a maternal effect gene essential for early embryogenesis.[9] In contrast, the exact functions of NLRP9 in reproductive systems have remained mysterious until recently. Early studies have proposed that human, murine and bovine NLRP9 were selectively expressed in oocytes, ovaries and testes,[10-15] and as a result, NLRP9 has been implicated to function in preimplantation embryo development.[16] While neither simultaneous knockout of NLRP9a and NLRP9c nor single knockdown of NLRP9b affected early embryonic development in mice,[17,18] a recent study discovered that female mice deficient in all three isoforms of NLRP9 had defective blastocyst development and increased lethality in the preimplantation embryos.[19] In vitro culture also showed that fertilized eggs from these female mice had developmental arrest at the two-cell stage. In contrast, those from female mice with two NLRP9 isoforms depleted were able to develop into blastocysts, although they all exhibited varying degrees of developmental delay.[19] This indicates functional redundancy for NLRP9a, NLRP9b and NLRP9c in embryogenesis of mice. It remains to be determined how exactly NLRP9 isoforms affect cell division during embryonic development and, importantly, whether mutations in human NLRP9 are linked to infertility.

NLRP9 INFLAMMASOME ASSEMBLY AND ITS INDUCTION BY ROTAVIRUS INFECTION

The mammalian innate immune system comprises five major families of PRRs including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), C-type lectin receptors (CLRs) and NLRs.[20] Certain members of ALRs (e.g. AIM2) and NLRs (e.g. NLRP1, NLRP3 and NLRC4) oligomerize upon ligand stimulation, before recruiting downstream components to assemble intracellular multimeric protein complexes known as inflammasomes. While NLRP1 and NLRC4 can directly recruit the effector cysteine protease caspase-1, most inflammasome sensors first recruit the adaptor protein ASC (also known as PYCARD).[21] ASC is a bipartite protein consisting of an amino-terminal PYD and a carboxy-terminal caspase recruitment domain (CARD). ASC binds to the upstream inflammasome sensors through homotypic PYD-PYD interaction and to the downstream effector caspase-1 via CARD–CARD interaction. Besides serving as a bridge, ASC also amplifies the signal through prion-like polymerization to assemble filaments with fast kinetics.[22,23] After recruitment, caspase-1 undergoes proximity-induced autoproteolytic cleavage, releasing the catalytic subunits p20 and p10 to assemble into mature caspase-1. The biologically active form of caspase-1 not only processes and induces the secretion of proinflammatory cytokines including interleukin (IL)-1β and IL-18, but also cleaves the pore-forming protein gasdermin D (GSDMD) to trigger a specific form of proinflammatory cell death termed pyroptosis.[24] With the exceptions of NLRP1 and NLRP10, all the other 12 NLRP members including NLRP9 share similar domain structure: an amino-terminal PYD, a middle NACHT domain and a carboxy-terminal leucine-rich repeat (LRR) region. However, for a long time it has remained unclear whether NLRP9 can initiate inflammasome formation similar to NLRP3, especially as NLRP9 was previously reported to express predominantly in reproductive organs.[12-15]

Recent evidence, however, found that NLRP9b in mice, but not its closely related isoforms NLRP9a or NLRP9c, was one of the only two NLRPs (the other being NLRP6) highly expressed in the ileum.[25] In addition, caspase-1 cleavage was detected in ileal tissues from mice challenged with rotavirus.[25] Rotavirus is a genus of double-stranded RNA viruses that infect the small intestine and cause diarrhoeal disease, resulting in over 200,000 deaths annually worldwide.[26] Supporting the hypothesis that NLRP9b initiates inflammasome activation upon rotavirus infection, mice deficient in NLRP9b, ASC or caspase-1 showed elevated viral load and more severe pathogenesis compared with wild-type mice.[25] Unlike NLRP3, which is mainly expressed in immune cells of the myeloid lineage,[27] NLRP9b expression was enriched in intestinal epithelial cells but not the neighbouring lymphocytes.[25]

In concordance with this observation, conditional knockout of NLRP9b in intestinal epithelial cells also caused higher susceptibility to rotavirus infection,[25] indicating the protective function of NLRP9b is executed in epithelial cells that line the intestine. Together, these results established NLRP9b as an inflammasome sensor in response to rotavirus in the intestine (Figure 1).

The composition of microbiota, which also colonize human intestines, was not significantly altered in mice lacking NLRP9b, suggesting that NLRP9b does not execute its antiviral role indirectly through influencing microbiota.[25] The authors then investigated which downstream signalling of inflammasome is critical for defence against rotavirus. IL-18 but not IL-1β was detected in intestinal epithelial cells, prompting the authors to examine the contribution of IL-18 in restricting rotavirus infection. Surprisingly, although IL-18 secretion induced by rotavirus challenge was impaired by NLRP9b deficiency, IL-18-deficient mice did not exhibit increased susceptibility compared with control mice,[25] contrary to the previous report that the presence of IL-18 helped eliminate rotavirus infection.[28] On the other hand, it remains to be examined whether IL-1β level in intestines could be induced upon rotavirus infection, as has been shown in NLRP3 inflammasome activation[27] and whether this proinflammatory cytokine contributes to NLPR9b-mediated protection against enteric viruses.

In contrast, mice lacking GSDMD were more vulnerable to rotavirus infection than wild-type mice,[25] implicating that pyroptosis plays a predominant role in rotavirus surveillance, likely by eliminating infected cells. Because rotavirus infection is known to cause diarrhoeal disease through epithelial damage,[29] further studies are required to understand how cells killed or damaged by NLRP9b-dependent pyroptosis can be repaired or replenished in a timely manner to prevent enhanced pathogenesis.

MOLECULAR AND STRUCTURAL MECHANISMS OF NLRP9 ACTIVATION

Although NLRP9b did not bind to viral RNA directly, it co-precipitated with short double-stranded RNA (dsRNA) stretches.[25] DExH-box helicase (DHX)9, an RNA helicase that preferentially bound to short dsRNA, was proposed as the intermediate protein bringing NLRP9b and viral RNA into the same complex.[25] How DHX9 distinguishes between viral RNA and host RNA to avoid triggering autoinflammatory response in this context is still not fully understood.[30] Moreover, the exact molecular mechanisms underlying NLRP9b inflammasome formation still require further dissection. Identifying the regions/domains in DHX9 and NLRP9b that mediate the complex formation, as well as examining the subcellular localization of NLRP9b inflammasome in rotavirus-infected intestinal cells or organoids, may unravel the exact molecular and cellular mechanisms governing the initiation of this inflammasome.

Two recent studies[31,32] have solved the crystal structure of PYD derived from human NLRP9 (hNLRP9^{PYD}), highlighting the similarities and differences of NLRP9 with other NLRs. As the common module shared by all NLR proteins, PYD is not only indispensable for the interaction between NLRs and ASC, but is also crucial for signal amplification of inflammasome pathways through its nucleation-driven polymerization. The overall structure of hNLRP9^{PYD} assumes an antiparallel six-helical bundle fold[31,32] that is typical for the death domain superfamily that PYD belongs to.[33] Surprisingly, hNLRP9^{PYD} exited as monomers instead of a higher oligomeric filament structure in solution.[31,32] Consistently, hNLRP9^{PYD} did not self-polymerize or promote speck formation of ASC when overexpressed in HEK293 T cells.[31] This is in great contrast to PYDs in other inflammasome sensors including NLRP3, NLRP6 and AIM2, all of which could oligomerize to act as the nucleation seed for ASC filament assembly.[22,34-36] Further analysis suggested that monomeric hNLRP9^{PYD} already exists in a conformation compatible with higher oligomeric filament formation,[31,32] suggesting that certain autoinhibitory mechanism may prevent its oligomerization and activation. Two hypothetical mechanisms have been proposed to explain the inability of hNLRP9^{PYD} to self-polymerize. First, a kinked N-terminal loop is oriented towards the interior of the helical bundle and may block interactions between hNLRP9^{PYD} molecules.[32] Second, charge inversions in the proposed interfaces of hNLRP9^{PYD} molecules may cause repulsive effect for intra- and inter-strand interactions.[31] Nevertheless, it cannot be ruled out that other domains of NLRP9 help drive the polymerization of PYD to recruit ASC. Although it still remains extremely difficult to solve structures of full-length NLRs, rapid technology advances on structural biology may eventually overcome the challenges and fuel our understanding of how exactly NLRs, including NLRP9, co-ordinate their different domains in triggering innate immune responses. In addition, the presence of the DHX9-RNA complex may license NLRP9 to assemble an inflammasome complex, and whether the structure of murine NLRP9b may differ from its human homolog has yet to be fully investigated. Indeed, overexpressed full-length human NLRP9 was shown to interact with ASC when reconstituted in HEK293 T cells in a rotavirus infection-dependent manner.[25]

NLRP9 IN OTHER INFLAMMATORY DISEASES

A recent study also implicated NLRP9b's role in a mouse model of acute lung injury.[37] NLRP9b-deficient mice showed lower neutrophilic inflammation, more protected alveolar architecture and subsequently higher survival rate

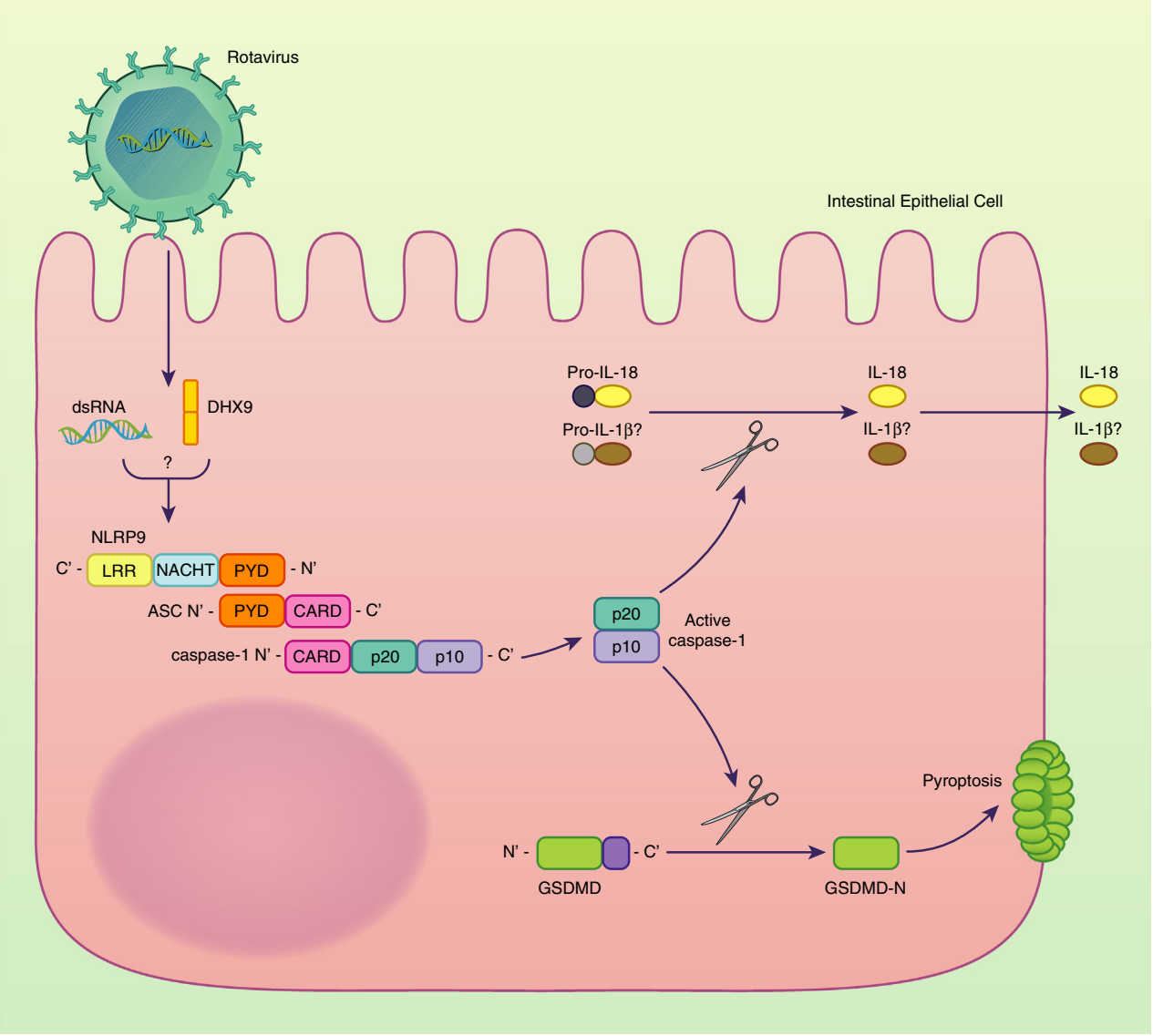

Figure 1. Rotavirus infection induces NLRP9 inflammasome activation. Upon rotavirus infection in the intestinal epithelial cells, the RNA helicase DHX9 binds to viral RNA derived from rotavirus, before forming a complex with NLRP9 via mechanisms still not fully understood. Activated NLRP9 then recruits the downstream adaptor ASC through PYD-PYD interaction. ASC in turn interacts with the cysteine protease caspase-1 through CARD–CARD interaction. Caspase-1 undergoes autocleavage to assemble into mature caspase-1 comprising p20 and p10 subunits. The active form of caspase-1 cleaves pro-IL-18 into IL-18 to facilitate its secretion, triggering proinflammatory responses. Whether caspase-1 also processes pro-IL-1β in the context of rotavirus infection is still unclear. Caspase-1 also leads to activation of GSDMD, which forms pores on the plasma membrane to induce the proinflammatory cell death mode termed pyroptosis.

compared with wild-type mice. Elevated levels of proinflammatory cytokines, apoptosis, NF-κB activation and oxidative stress have all been proposed as responsible for the detrimental role of NLRP9b,[37] although their exact contributions still require further studies.

NLRP9 is also associated with a number of other inflammatory diseases, including systemic-onset juvenile idiopathic arthritis,[38] multiple sclerosis,[39] familial late-onset Alzheimer's disease,[40] urothelial carcinoma[41] and *Helicobacter pylori* infection.[42] Genetic deletion of NLRP9 in animal models and further mechanistic studies are still underway for us to fully understand the roles of NLRP9 in these inflammatory disorders.

CONCLUSIONS AND PERSPECTIVES

Although the importance of NLR proteins in innate immunity and inflammation has been known for more than 15 years, only recently have we started to appreciate the roles of the less-studied NLRs in host defence and other inflammatory disorders. The discovery of NLRP9b as an inflammasome sensor in response to rotavirus

infection and the recent characterization of hNLRP9^{PYD} structure have raised a number of new questions, including the ones discussed above and a few highlighted below. Addressing these questions would provide substantial insights into the functions of NLRP9 and the future design of novel therapeutic approaches targeting related diseases.

Does human NLRP9 also oligomerize to form an inflammasome upon rotavirus infection? Although hNLRP9 was also highly expressed in human intestinal epithelial cells,[25] whether rotavirus infection triggers inflammasome formation and caspase-1 cleavage is still unclear. Given that the amino acid composition of hNLRP9 only shares less than 50% similarity with that of mNLRP9 isoforms, further investigation is essential to test whether they are different in ligand recognition. Another intriguing question is whether mNLRP9a and mNLRP9c, which are not detected in intestines, are involved in inflammasome activation in other organs and tissues.

How does NLRP9b synergize with other PRRs in clearing rotavirus? NLRP6 is the only other NLRP highly enriched in intestine.[25] Interestingly, NLRP6-deficient mice exhibited increased viral loads in the gastrointestinal tract when challenged with rotavirus[25] or encephalomyocarditis virus.[43] Analogous to how NLRP9b works in concert with DHX9 to detect viral RNA, NLRP6 requires another RNA helicase DHX15 to interact with viral RNA.[43] Interestingly, while NLRP9b preferred to form complexes with low molecular weight RNA, NLRP6 favoured high molecular weight RNA.[25] It thus remains to be explored which type of viral RNA is predominantly present in rotavirus-infected epithelial cells in the small intestine, and whether NLRP9b, NLRP6 and other PRRs that were reported to respond to rotavirus,[26] work synergetically to elicit immune responses towards rotavirus invasion. Besides rotavirus, whether other pathogens in the gastrointestinal tract activate the NLRP9 inflammasome also requires deeper examination.

The recent breakthrough regarding NLRP9's role in inflammasome signalling came from a careful examination of its expression pattern.[25] Besides intestines, NLRP9 has been reported in lung tissue,[37] brain endothelial cells,[44] brain pericytes,[45] brain macrophages and microglia.[39] As the field moves forward, a thorough re-examination of the localization of NLRP9 and other less well-studied NLRs may provide hints on their potential functions in different tissues and organs, as well as how they contribute to infectious and inflammatory diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

No data have been generated.

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