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INVITED REVIEW

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Molecular mechanisms activating the NAIP-NLRC4 inflammasome: Implications in infectious disease, autoinflammation, and cancer

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Abstract

Cytosolic innate immune sensing is a cornerstone of innate immunity in mammalian cells and provides a surveillance system for invading pathogens and endogenous danger signals. The NAIP-NLRC4 inflammasome responds to cytosolic flagellin, and the inner rod and needle proteins of the type 3 secretion system of bacteria. This complex induces caspase-1-dependent proteolytic cleavage of the proinflammatory cytokines IL-1β and IL-18, and the pore-forming protein gasdermin D, leading to inflammation and pyroptosis, respectively. Localized responses triggered by the NAIP-NLRC4 inflammasome are largely protective against bacterial pathogens, owing to several mechanisms, including the release of inflammatory mediators, liberation of concealed intracellular pathogens for killing by other immune mechanisms, activation of apoptotic caspases, caspase-7, and caspase-8, and expulsion of an entire infected cell from the mammalian host. In contrast, aberrant activation of the NAIP-NLRC4 inflammasome caused by *de novo* gain-of-function mutations in the gene encoding NLRC4 can lead to macrophage activation syndrome, neonatal enterocolitis, fetal thrombotic vasculopathy, familial cold autoinflammatory syndrome, and even death. Some of these clinical manifestations could be treated by therapeutics targeting inflammasome-associated cytokines. In addition, the NAIP-NLRC4 inflammasome has been implicated in the pathogenesis of colorectal cancer, melanoma, glioma, and breast cancer. However, no consensus has been reached on its function in the development of any cancer types. In this review, we highlight the latest advances in the activation mechanisms and structural assembly of the NAIP-NLRC4 inflammasome, and the functions of this inflammasome in different cell types. We also describe progress toward understanding the role of the NAIP-NLRC4 inflammasome in infectious diseases, autoinflammatory diseases, and cancer.

KEYWORDS

assembly, autoimmunity, cell death , epithelial cells, expulsion, gasdermin, *Helicobacter pylori*, inflammatory caspases, *Legionella pneumophila*, necrosis, neutrophils, pattern-recognition receptors, phosphorylation, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, structure

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1 | INTRODUCTION

The innate immune system is the first line of defense in the host response against microbial infection. Innate immune recognition is initiated by pattern-recognition receptors (PRRs), which include inflammasome sensors. Inflammasomes are multicomponent innate immune signaling complexes that can assemble in response to PRR-mediated recognition of pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), and homeostasis-altering molecular processes (HAMPs).[1-3] Inflammasome sensors include the nucleotide-binding domain and leucine-rich repeat receptors (NLRs) NLRP1, NLRP3, NLR apoptosis inhibitory protein (NAIP), NLRC4, NLRP6, and NLRP9. In addition, AIM2 from the AIM2-like receptor family and Pyrin from the Tripartite motif family can also initiate inflammasome complex formation.[4-6]

Activation of inflammasome sensors leads to the recruitment of the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC). ASC is a bipartite protein, containing a pyrin domain (PYD) and a caspase activation and recruitment domain (CARD). In this way, ASC couples activation of the upstream PRR to the activation of the effector cysteine protease caspase-1.[7] Recruitment of caspase-1 induces its self-dimerization, activating its catalytic activity. Active caspase-1 then proteolytically cleaves the proinflammatory cytokines pro-interleukin (IL)-1β and pro-IL-18 into their mature forms. Human caspase-4 and caspase-5 and mouse caspase-11 also form inflammasome complexes following binding to cytosolic LPS.[8-12] Inflammasome caspases induce proteolytic cleavage of the pore-forming protein gasdermin D (GSDMD).[13-15] Cleavage of GSDMD relieves the N-terminal domain from the autoinhibitory C-terminal domain, allowing the N-terminal domain to oligomerize in the host cell membrane, resulting in pore formation that causes a lytic and inflammatory form of cell death known as pyroptosis.[16,17]

This review will discuss recent progress in the biology of the NAIP-NLRC4 inflammasome. Specifically, we will focus on mechanisms of ligand detection and recognition by NAIP proteins, the structural and regulatory features of the NAIP-NLRC4 inflammasome, the distinct cell types expressing NLRC4 and its cell type-specific roles, and the role of this inflammasome in infectious and autoinflammatory diseases. We also discuss the emerging but often contentious role of this inflammasome in cancer. Finally, we identify some unanswered questions and highlight areas, which would be of interest to investigate in future studies on the NAIP-NLRC4 inflammasome biology in human health and disease.

2 | DISCOVERY AND EARLY CHARACTERIZATION OF NLRC4

NLRC4 was first identified by a search of genes with sequence similarity to caspase-1, and proteins with structural homology to the apoptotic protease activating factor-1 (APAF-1) protein.[18] This early study identified a cytoplasmic protein capable of inducing cleavage and activation of the interleukin-1 converting enzyme (ICE, now known as caspase-1), and it was hence named ICE-protease activating factor, or IPAF.[18] IPAF carries a nucleotide-binding domain (NBD, also known as a NACHT), a leucine-rich repeats domain (LRRs) and a caspase activation and recruitment domain (CARD). These domain features give rise to its current nomenclature within the NBD and LRRs (NLR) family, NLRC4 (Figure 1A).[19]

The first genetic evidence that NLRC4 might assemble an endogenous inflammasome complex was provided in 2004 by experiments showing that wildtype (WT), but not NLRC4-deficient (*Nlrc4*$^{-/-}$) mouse bone marrow-derived macrophages (BMDMs) underwent activation of caspase-1, release of IL-1β, and pyroptosis following infection with the foodborne bacterium *Salmonella enterica* serovar Typhimurium (herein referred to as *S* Typhimurium).[20] Moreover, ATP induced activation of caspase-1, release of IL-1β, and pyroptosis in both WT and *Nlrc4*$^{-/-}$ BMDMs, indicating a level of specificity of NLRC4 toward cytosolic sensing of *S* Typhimurium.[20]

Two studies in 2006 further identified flagellin, a monomeric component of the bacterial flagellum apparatus involved in motility and chemotaxis, as an activator of the NLRC4 inflammasome.[21,22] These studies showed that *S* Typhimurium strains lacking components of the flagellar apparatus, such as *fliC* and *fljB*, failed to activate NLRC4 in WT BMDMs and that WT but not *Nlrc4*$^{-/-}$ BMDMs, responded to the transfection of *S* Typhimurium flagellin into the cytoplasm. A further study showed that the flagellin of the Gram-negative bacterium *Legionella pneumophila* induced activation of the NLRC4 inflammasome in BMDMs.[23] These findings collectively demonstrated for the first time that flagellin is a key activator of NLRC4 and extended the frontiers of our understanding of innate immune recognition (Figure 1B). The concept that the same microbial ligand can be detected in mammalian immune cells in a compartmentalized manner is realized: Toll-like receptor 5 (TLR5) recognizes flagellin from the surface of the cell,[24] whereas NLRC4 recognizes flagellin from within the cytoplasm.

Subsequent studies observed that, in some cases, the NLRC4 inflammasome could be activated by bacteria without flagellin.[22] Indeed, further work revealed that the NLRC4 inflammasome is activated by virulence factors with a similar structure and/or function to flagellin, such as certain proteins of the type 3 secretion system (T3SS) derived from many bacterial pathogens (Figure 1B). These pathogens and their associated proteins include *S* Typhimurium (PrgJ), *Burkholderia pseudomallei* (BsaK), *Escherichia coli* (EprJ and EscI), *Shigella flexneri* (MxiI), and *Pseudomonas aeruginosa* (PscI).[25] These findings position NLRC4 as an innate immune sensor capable of exploiting the structural and functional conservation of bacterial virulence factors.

3 | NAIPS CONFER LIGAND SPECIFICITY

It was speculated that NLRC4 might bind flagellin and certain virulence factors of the T3SS; however, no study had been able to show a direct interaction between these ligands and NLRC4. Another

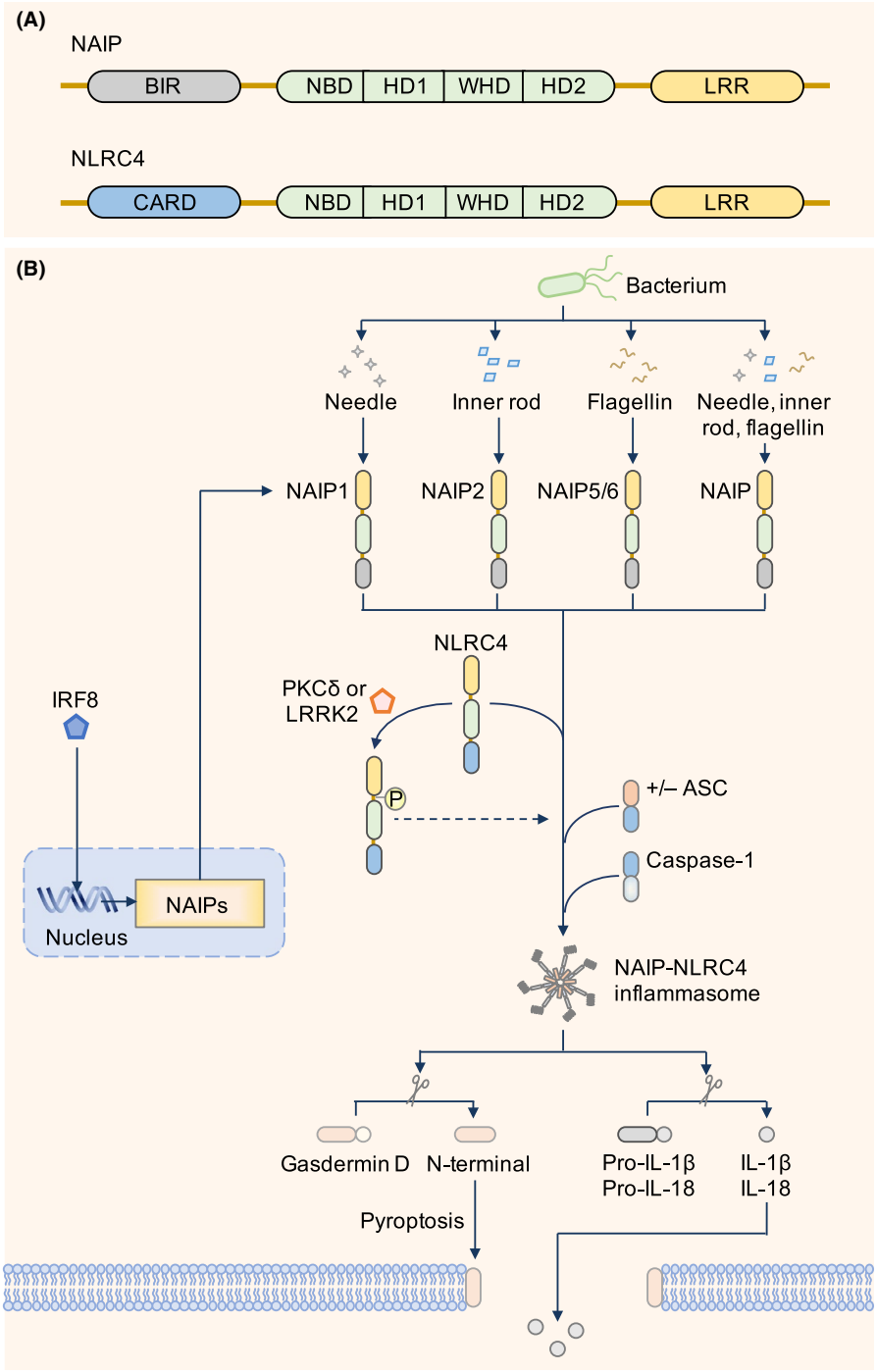

FIGURE 1 Mechanisms of NAIP-NLRC4 inflammasome activation. (A) Both NAIPs and NLRC4 contain a central NBD domain with associated domains (HD1, WHD, HD2) and a C-terminal LRR. In addition, NAIPs contain an N-terminal BIR domain and NLRC4 contains an N-terminal CARD. The domain architecture is conserved between mice and humans. (B) Bacterial ligands including the needle and inner rod protein of the Type 3 secretion system and the flagellin monomer of the flagellar apparatus can be recognized by NAIP proteins, which associate with NLRC4 to induce activation of the NAIP-NLRC4 inflammasome. This pathway results in the cleavage and activation of caspase-1, which mediates cleavage of Gasdermin D and the immature cytokines pro-IL-1β and pro-IL-18. Cleavage of Gasdermin D induces pyroptosis via the formation of membrane pores. Cleavage of the immature cytokines enables their secretion via Gasdermin D pores and induces inflammation. NAIPs are transcriptionally induced by IRF8. Phosphorylation of NLRC4 by the kinase PKCδ or LRRK2 might, in part, contribute to the activation of the inflammasome (indicated by the dashed arrow)

possibility was that NLRC4 might recruit an adaptor protein for ligand recognition. Indeed, a study suggested that NLRC4 could form heterotypic complexes with other NACHT-containing proteins.[26] One such NACHT-containing protein already implicated in inflammasome activation was NAIP5 in mice, since it was required for flagellin-mediated inflammasome activation[27] but not for flagellin-independent NLRC4 inflammasome activation.[28]

Indeed, two studies published in 2011 identified that NAIP proteins acted as upstream sensors of NLRC4-activating ligands.[29,30] Specifically, they demonstrated that mouse NAIP2 and mouse NAIP5 directly and specifically interacted with the T3SS inner rod protein and flagellin, respectively (Figure 1B). Further work demonstrated that mouse NAIP1 recognized the T3SS needle protein[31,32] and that mouse NAIP6 sensed flagellin, redundantly to mouse NAIP5.[30] The identification of mouse NAIP paralogues as distinct ligand sensors provides a convincing explanation for how NLRC4 could detect multiple bacterial ligands (Figure 1B). Further, genetic deletion of NAIPs in mice confirmed their importance in mediating ligand specificity.[33-35]

The murine genome encodes seven NAIP proteins, while the human genome only encodes a single functional NAIP protein (hNAIP).[36] Initially, it was shown that hNAIP was functionally

analogous to murine NAIP1, sensing the T3SS needle protein.[31,32] Additional studies have revealed that hNAIP can mediate NLRC4 inflammasome activation in response to both T3SS components and flagellin.[37,38] Notably, it has been suggested that a longer hNAIP isoform confers flagellin sensitivity,[37] but ectopic expression of this longer isoform in HEK293 cells sensitized them to NLRC4 inflammasome activation by flagellin and T3SS needle and inner rod proteins.[38] The presence of a single NAIP in humans that can sense multiple distinct ligands is intriguing and indicates differential division of labor for recognition of bacterial ligands compared with murine NAIPs. It should be noted that only the needle protein of the Gram-negative bacterium *Chromobacterium violaceum* (CprI) has been shown to directly interact with hNAIP.[29] It remains unclear whether flagellin and T3SS inner rod protein are also directly bound by hNAIP, and how a single sensor might recognize these distinct ligands.

4 | STRUCTURAL INSIGHTS INTO NAIP-NLRC4 INFLAMMASOME ASSEMBLY

Inflammasome assembly must be tightly regulated to avoid inappropriate activation that would cause inflammatory damage to the host.[39] Inflammasome activation by NLRs is initiated by sensor activation and oligomerization, which provides a site for ASC and/or caspase-1 recruitment.[40] In the case of the NAIP-NLRC4 inflammasome, activation requires (a) ligand interaction with an inactive NAIP protein, (b) ligand-induced activation of the NAIP protein, (c) ligand-NAIP induced interaction with an inactive NLRC4 monomer, (d) activation of an NLRC4 monomer, (e) active NLRC4 interaction with an inactive NLRC4 monomer, and (f) the successive addition of NLRC4 monomers.[41,42]

Structural and mechanistic studies have provided profound insights into the process of NAIP-NLRC4 inflammasome assembly. In the first step, an activating ligand (flagellin or T3SS needle or inner rod proteins) interacts with an inactive NAIP protein and causes a conformational change in the NAIP protein that relieves autoinhibition. This step is illustrated by the example of the flagellin-NAIP5 interaction: the D0 domain of flagellin is able to make contact with six domains of NAIP5, forcing it to convert into an active conformation.[43] While structural insights are lacking for the other ligand-NAIP partners, it is assumed that they proceed via a similar mechanism of ligand-induced NAIP activation. It has been reported that multisurface ligand recognition is common to NAIPs. Mutagenic studies found that while mutation of these key recognition motifs in the ligand enabled immune evasion by NAIP5, it also disrupted flagellar motility.[43] This observation, coupled with the fact that the membrane-localized flagellin sensor TLR5 senses a conserved and functionally important site in the D1 domain of flagellin,[44] limits pathogen immune evasion.[43] Interestingly, while the LRRs has been proposed to act as a sensor for NLR ligands,[45,46] studies have revealed that ligand specificity by NAIPs is mediated by an internal region containing NBD-associated α-helices (in particular the helical domain 1 or HD1, winged-helix domain or WHD, and helical domain 2 or HD2) rather than the LRRs.[47]

Once a NAIP protein has become activated by its respective ligand, it can interact with an inactive NLRC4 monomer. The inactive NLRC4 monomer has been structurally characterized by X-ray crystallography and revealed that an ADP-mediated interaction between the central NBD and WHD domains stabilized the closed conformation of NLRC4.[48] The same study found that the C-terminal LRRs was positioned to sterically occlude one surface of the NBD and sequester NLRC4 in a monomeric state.[48] The authors also demonstrated that mutagenesis abolishing crucial inhibitory interactions lead to constitutive NLRC4 inflammasome activation and bypassed the requirement for flagellin.[48] Indeed, we will discuss below the clinical importance and consequences of mutations in humans leading to constitutive activation of the NLRC4 inflammasome.

Once a ligand-bound NAIP complex interacts with NLRC4, it is sufficient to trigger conformational changes in NLRC4, driving it toward an active conformation.[41,42] Activation of an NLRC4 monomer is mediated by a 90° conformational rotation in the hinge region between HD1 and WHD. This conformational change exposes a largely basic "catalytic surface" on the active NLRC4 that can interact with the largely acidic "receptor surface" on an incoming inactive NLRC4 monomer. This interaction activates the second NLRC4 monomer, which can recruit additional NLRC4 monomers and leads to a self-propagation mechanism resulting in the formation of a 10-12 subunit wheel-like structure.[41,42] Importantly, NAIPs also possess a "catalytic surface" that matches the "receptor surface" of NLRC4 and enables initiation of oligomerization, but they do not possess a "receptor surface" and therefore only a single NAIP is found per NAIP-NLRC4 inflammasome complex.[41,42] Unlike apoptosome oligomerization, which requires one cytochrome *c* ligand per APAF-1 monomer,[49] the NAIP-NLRC4 inflammasome only requires a single bound ligand to initiate oligomerization. This point of difference suggests that the NAIP-NLRC4 inflammasome can respond to a lower concentration of activating ligand and that different cell death pathways have different thresholds for initiation.

5 | NLRC4 ACTIVATION BY PHOSPHORYLATION

Inflammasome components, including NLRC4, are subject to post-translational modifications that can regulate their assembly and function.[50-53] Earlier work in murine BMDMs identified that NLRC4 was phosphorylated at position S533 by Protein Kinase C delta (PKC-δ) in response to *S* Typhimurium infection and suggested that this modification was required for NLRC4 inflammasome assembly (Figure 1B).[54] The importance of PKC-δ and phosphorylation at S533 in NLRC4 inflammasome activation has been questioned in subsequent work. For example, using an independently generated mouse strain lacking PKC-δ, both *S* Typhimurium and *S flexneri* were found to induce activation of the NLRC4 inflammasome independently of PKC-δ.[55] It is important to note that additional kinases can

phosphorylate NLRC4 besides PKC-δ. Indeed, leucine-rich repeat kinase 2 (LRRK2) has also been shown to phosphorylate NLRC4 at S533,[56] suggesting possible functional redundancy between kinases inducing phosphorylation of NLRC4. However, *Helicobacter pylori* flagellin can induce NLRC4 phosphorylation but does not lead to inflammasome activation,[57] suggesting that NLRC4 phosphorylation is insufficient for inflammasome activation. These findings were supported by the observation that S533 is phosphorylated in the inactive NLRC4 monomer.[48]

The requirement for phosphorylation of NLRC4 at S533 in the activation of the NLRC4 inflammasome was initially supported by at least two lines of evidence. First, immortalized macrophages from *Nlrc4*$^{-/-}$ mice were reconstituted either with WT NLRC4 or a non-phosphorylatable S533A NLRC4 mutant. This experiment revealed that macrophages carrying the mutant S533A NLRC4 had an impaired ability to induce activation of the inflammasome in response to *S* Typhimurium infection compared with macrophages carrying WT NLRC4.[54] Second, the same group generated a mouse strain carrying a non-phosphorylatable S533A NLRC4 (*Nlrc4*$^{S533A/S533A}$). Using BMDMs from this mouse strain, the authors showed that *Nlrc4*$^{S533A/S533A}$ BMDMs induced activation of the NLRC4 inflammasome with a delayed kinetics following infection with *S* Typhimurium compared with WT BMDMs.[58] Given that previous studies showed that both NLRC4 and NLRP3 collectively induce activation of the inflammasome in response to *S* Typhimurium infection,[59,60] the authors generated a *Nlrc4*$^{S533A/S533A}$ mouse strain lacking NLRP3. BMDMs from *Nlrc4*$^{S533A/S533A}$ *Nlrp3*$^{-/-}$ mice had a substantially impaired ability to induce activation of the inflammasome in response to *S* Typhimurium infection.[58] Therefore, the authors concluded that phosphorylation of NLRC4 at S533 is important for activation of the NLRC4 inflammasome.

A further study generated several new mouse strains to clarify the role of S533 phosphorylation and NLRC4 inflammasome activation. These mouse strains included a *Nlrc4*$^{S533A/S533A}$ mouse strain, and a *Nlrc4*$^{S533A/S533A}$ *Nlrp3*$^{-/-}$ mouse strain. They reported no difference in inflammasome activation between WT and *Nlrc4*$^{S533A/S533A}$ BMDMs when a higher dose of *S* Typhimurium was used (multiplicity of infection of 5), but a minor decrease in the activation of the inflammasome was observed in *Nlrc4*$^{S533A/S533A}$ BMDMs when a lower dose of *S* Typhimurium was used (multiplicity of infection of 1).[61] Further, the use of WT BMDMs vs *Nlrc4*$^{S533A/S533A}$ *Nlrp3*$^{-/-}$ BMDMs did not reveal a greater level of difference.[61] Differences in the CB57BL/6 substrains used between studies are a potential explanation for these discrepancies, given that the latest study used the C57BL/6J substrain,[61] whereas the previous studies presumably used the C57BL/6N substrain.[20,54,58] Regardless, the subtle contribution of S533 phosphorylation in NLRC4 inflammasome activation might suggest that phosphorylation of NLRC4 is not required for robust NLRC4 inflammasome activation, or that post-translational modifications at multiple sites of NLRC4, rather than only at S533, would be required for optimal activation. Further, perhaps the subtlety in the requirement for S533 phosphorylation is mouse-specific and that a more prominent role for phosphorylation would be observed in cells from humans or other mammalian species.

6 | OTHER REGULATORY MECHANISMS

Evidence suggests that NLRC4 is regulated by ubiquitination. Notably, an interaction between NLRC4 and the suppressor of Gal 1 (SUG1) has been reported, which leads to NLRC4 ubiquitination and caspase-8-mediated cell death in human lung carcinoma A549 cells.[62] This finding is supported by another study showing that an autoinflammatory mutant of NLRC4 (site His433Pro) enabled a stronger interaction with SUG1, leading to enhanced caspase-8-mediated cell death even in the absence of S533 phosphorylation.[63] It is possible that modifications such as ubiquitination of NLRC4 might compensate for a lack of phosphorylation; however, this is yet to be validated.

While NAIPs are critical in NLRC4 inflammasome activation, there is no report of their post-translational regulation. However, a study has demonstrated that both NAIPs and NLRC4 are transcriptionally induced by interferon regulatory factor (IRF) 8.[64] Mechanistically, IRF8 was reported to act as a transcriptional activator for murine NAIPs 1, 2, 5, and 6 and NLRC4. Interestingly, this study showed that NLRC4 inflammasome activation in *Irf8*$^{-/-}$ BMDMs was not completely abrogated, suggesting that additional factors contribute to the transcriptional regulation of NAIPs and NLRC4.[64] One such factor might be the transcription factor SPI1 (also known as PU.1), which interacts with another transcription factor IRF4 and is required to drive B cell transcriptional processes[65]; however, this requires further investigation. Additionally, IRF8 peaks were not found in chromatin immunoprecipitation sequencing data at the NAIP1 locus but were found at the NAIP2, NAIP5 and NAIP6 loci.[64] It is possible that NAIP1 may be regulated differently to the other NAIPs, which is supported by the observation that NAIP1 required additional priming signals, such as poly(I:C).[31] Lastly, negative regulators of the NLRC4 inflammasome have also been identified, including the B cell adaptor for phosphoinositide 3-kinase,[66] heat shock cognate protein 70,[67] the *S* Typhimurium SopB protein,[68] and the tick protein sialostatin L2.[69]

7 | NLRC4 IS EXPRESSED IN MULTIPLE CELL TYPES

Inflammasome complexes have been extensively studied in mouse macrophages; however, emerging evidence indicates that inflammasome components are expressed and functional in a variety of cell types in mice and humans, including those of hematopoietic and non-hematopoietic origin (Figures 2 and 3).

Earlier studies have demonstrated that components of the NAIP-NLRC4 inflammasome are all expressed in mouse BMDMs (Figure 2), and thus, many of the mechanistic studies were performed using this cell type. For example, BMDMs lacking NAIPs or NLRC4 have

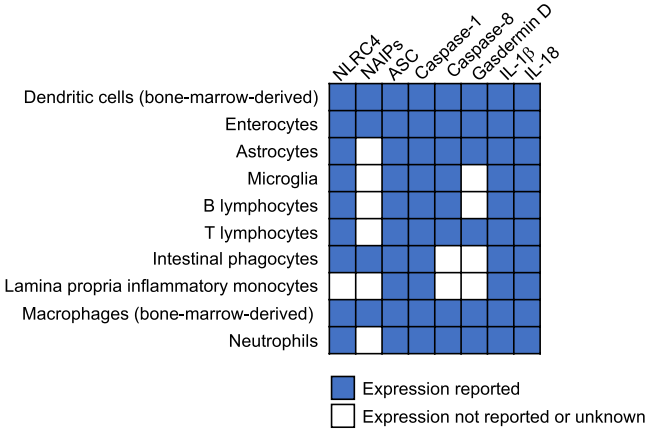

FIGURE 2 Expression of NAIP-NLRC4 inflammasome components in mouse cells. ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; IL-1β, interleukin-1β; IL-18, interleukin-18; NAIPs, NLR apoptosis inhibitory proteins; NLRC, nucleotide-binding domain, leucine-rich repeat containing protein containing a caspase activation and recruitment domain

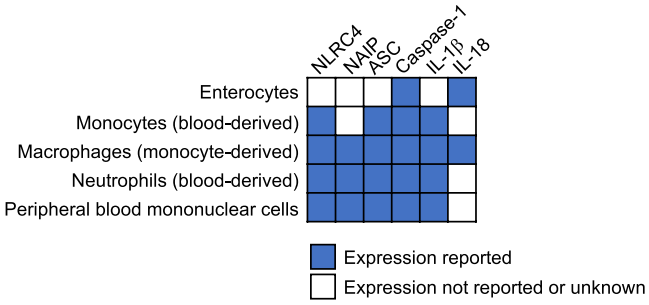

FIGURE 3 Expression of NAIP-NLRC4 inflammasome components in human cells. ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; IL-1β, interleukin-1β; IL-18, interleukin-18; NAIP, NLR apoptosis inhibitory protein; NLRC, nucleotide-binding domain, leucine-rich repeat containing protein containing a caspase activation and recruitment domain

an impaired ability to induce cleavage of caspase-1 and pro-IL-1β and undergo pyroptosis, in response to infection by bacteria such as *S* Typhimurium or *L pneumophila*.[21,22,70] Further work has revealed that the NAIP-NLRC4 inflammasome is activated in human monocytes and macrophages in response to *S* Typhimurium infection.[29,31,71] Additionally, there is increasing evidence that other immune cells, such as dendritic cells, also express NLRC4 and can form functional inflammasome complexes (Figures 2 and 3).[72-74]

In mouse neutrophils, the NLRC4 inflammasome can be activated to secrete IL-1β upon stimulation with *S* Typhimurium.[75] A study has shown that NLRC4 inflammasome activation in the cell type expressing the Myeloid-related protein 8, predominantly composed of neutrophils, causes systemic inflammation in mice.[76] Therefore, if left uncontrolled, activation of the NLRC4 inflammasome in neutrophils can be detrimental. Unlike macrophages, neutrophils resist pyroptosis in response to NLRC4 and other canonical inflammasome activation.[75] The lack of pyroptosis in neutrophils undergoing NLRC4 activation suggests that neutrophil extracellular traps, a network of chromatin and anti-microbial proteins, cannot be released. This is in contrast to neutrophils undergoing caspase-11 activation, which readily release neutrophil extracellular traps to kill bacteria.[77] This difference might suggest that the function of the NLRC4 inflammasome in neutrophils is more targeted toward sustaining inflammation at the site of infection. Indeed, neutrophils have been implicated as the major producers of IL-1β during acute bacterial infection.[75,78]

Bone marrow chimera experiments have revealed important anti-microbial functions for NLRC4 in non-hematopoietic cells in mice. These studies demonstrated that NLRC4 is highly expressed in intestinal epithelial cells and that NLRC4 has a protective role against the enteric pathogen *S* Typhimurium, via mechanisms such as expulsion of infected intestinal epithelial cells.[79-81] These mechanisms of action will be discussed in more detail below.

NLRC4 is also expressed in the brain, predominantly in astrocytes and microglia.[18,82] It has been shown that the NLRC4 inflammasome can respond to bacterial flagellin in both of these cell types.[82] Interestingly, emerging studies suggest that astrocytic NLRC4 can respond to additional stimuli, including the central nervous system-associated DAMP lysophosphatidylcholine (LPC) and the fatty acid palmitate.[82,83] Furthermore, microglia have been reportedly activated following ischemia, and NLRC4 is implicated in ischemic brain injury.[84,85] The potential of the NLRC4 inflammasome in glial cells to be activated by non-bacterial ligands puts forward the intriguing proposition that it may be activated in response to a novel endogenous danger-associated ligand or stimulus. This will be an exciting area of future research into NLRC4 inflammasome biology.

8 | NLRC4 AND NAIPS IN INFECTIOUS DISEASES

8.1 | Host defense against *S* Typhimurium

The physiological role of the NLRC4 inflammasome has been explored using several bacterial infection models, most notably with the foodborne bacterium *S* Typhimurium (Table 1). Studies have shown that mice lacking NLRC4 infected with *S* Typhimurium either had no difference in bacterial burden in the spleen, liver, or mesenteric lymph nodes compared to WT mice[59,86,87] or that they succumbed to infection more quickly and had increased bacterial loads in the cecum, liver and spleen compared with WT mice.[60,61,79-81,87] The subtlety of the effect of NLRC4 in response to *S* Typhimurium infection might be owing to its functional redundancies with another inflammasome sensor, NLRP3.[59,60] Indeed, *S* Typhimurium can activate the NLRC4 and NLRP3 inflammasomes in mouse BMDMs.[58-60] Mice lacking NLRC4 or NLRP3 individually were still capable of conferring some or little protection against *S* Typhimurium.[59,60,86,87] Mice lacking both NLRC4 and NLRP3 infected with *S* Typhimurium exhibited elevated bacterial loads in the spleen, liver and mesenteric lymph nodes and reduced circulating IL-18 similar to mice lacking

TABLE 1 Investigations into the roles of NLRC4 and NAIPs in salmonellosis (*S* Typhimurium) using mouse models

Mouse	Infection route	Genetic background	Littermate	Phenotype compared with wildtype mice (unless stated otherwise)	Reference
Nlrc4$^{-/-}$	Orogastric	C57BL/6	Yes	No difference in bacterial burden in the spleen and mesenteric lymph nodes 5 d postinfection	86
		C57BL/6N	No	No difference in bacterial burden in the spleen, liver, and mesenteric lymph nodes 5 d postinfection	59
		BALB/c	No	Decreased survival rate Increased bacterial burden in the liver and spleen	87
		C57BL/6 Charles River	Yes	Increased epithelial invasion 18 h postinfection, compared with heterozygous *Nlrc4*$^{+/-}$ mice	80
		C57BL/6J	Yes	Increased bacterial burden in the cecal tissues 18 h postinfection	81
		C57BL/6J	Yes	Increased bacterial burden in the cecum and mesenteric lymph nodes 18 h postinfection, compared with heterozygous *Nlrc4*$^{+/-}$ mice	61
	Intraperitoneal	BALB/c	No	No difference in survival rate No difference in bacterial burden in the liver and spleen 3 d postinfection	87
		C57BL/6	No	Increased bacterial burden in the spleen and liver 7 and 13 d postinfection	60
		C57BL/6	No	Decreased survival rate	34
		C57BL/6J	No	Decreased survival rate Increased bacterial burden in the spleen and liver Reduced IL-18 in the spleen and liver	64
	Intravenous	C57BL/6NJ	Yes	No difference in bacterial burden in the spleen and mesenteric lymph nodes 24 h postinfection	80
Naip2$^{-/-}$	Intraperitoneal	C57BL/6	No	Decreased survival rate No difference in bacterial burden in the spleen and liver	34
Naip5$^{-/-}$	Intraperitoneal	C57BL/6	No	Decreased survival rate No difference in bacterial burden in the spleen and liver	34
		C57BL/6	No	No difference in survival rate No difference in bacterial burden in the spleen and liver No difference in IL-18 in the spleen and liver	64
Naip1-6$^{\Delta/\Delta}$	Orogastric	C57BL/6 Charles River	Yes	Increased histopathology score Increased bacterial burden in both the cecum and mesenteric lymph nodes 18 and 36 h postinfection, compared with heterozygous *Naip1-6*$^{fl/\Delta}$ mice	80

both caspase-1 and caspase-11, with both groups of mice showing increased susceptibility to the infection compared with WT mice.[59]

Further, previous studies have established that the routes used for *S* Typhimurium infection and the genetic background of mice can determine the effectiveness of NLRC4 in preventing bacterial infection (Table 1). For example, difference in the bacterial burden in the spleen or liver was observed between WT mice and mice lacking NLRC4 on the BALB/c background following an orogastric infection, but not following an intraperitoneal infection.[87] Either an increase or a lack of difference in bacterial burden has been reported between WT mice and mice lacking NLRC4 on the C57BL/6 background following an orogastric infection.[59,88] In response to intravenous infection with *S* Typhimurium, studies have reported either an increase or a lack of difference in bacterial burden in mice lacking NLRC4 compared with WT mice.[60,88] These studies indicate that the subtle effect of NLRC4 in response to *S* Typhimurium in mice could be owing to the routes of administration, doses of bacteria used, functional redundancies between inflammasome sensors, and differences in background genetics and gut microbiota of mice housed in different facilities (Table 1).

NLRC4 is expressed in both immune and non-immune cells (Figures 2 and 3). Studies using bone marrow chimeric mice have shown that, in an orogastric infection model of *S* Typhimurium, NLRC4-deficient mice with a WT bone marrow reconstitution had elevated bacterial loads in the cecum compared with WT mice with a WT bone marrow reconstitution.[80] This finding argues that NLRC4 expressed in non-hematopoietic-derived cells is essential for protection against *S* Typhimurium. Indeed, transcriptional and protein analyses have revealed that the expression of NLRC4 is among the highest in the cecum and small intestine compared with other tissues

in mice.[75,87] This raised the question of whether NLRC4 expressed in intestinal cells may have a more dominant role in the protection against enteric pathogens infecting the lower regions of the gut, such as *S* Typhimurium. Subsequent studies showed that cre-driven expression of NLRC4 in intestinal epithelial cells reduced the bacterial burden in the cecum of NLRC4-deficient mice infected with *S* Typhimurium.[81] Further, tissue-specific knockout of NAIP1-6 in intestinal epithelial cells led to increased *S* Typhimurium colonization in the gut of mice following orogastric infection.[80]

The mechanisms of protection induced by the NAIP-NLRC4 inflammasome in the gut requires expulsion of infected intestinal epithelial cells.[80] While caspase-1 and GSDMD are considered to be the terminal effectors of NLRC4-driven activities,[89] a study has shown that both caspase-1 and caspase-8 are required for cell expulsion from the intestinal epithelium, whereas caspase-1 and GSDMD alone are only sufficient for IL-18 secretion but not cell expulsion.[81] This study adds to the growing body of literature indicating that NLRC4 inflammasome can recruit caspase-8, via an interaction between the PYD of ASC and the death effector domain of caspase-8.[90-92] The recruitment of multiple caspases diversifies the signaling and functional repertoire of the NLRC4 inflammasome, providing more ways to orchestrate host responses. Indeed, in response to infection by *S* Typhimurium, NLRC4 might activate multiple caspase-dependent and caspase-independent cell death pathways, including pyroptosis, apoptosis, and necroptosis, collectively known as PANoptosis.[93]

8.2 | Host defense against other gastrointestinal pathogens

In addition to *S* Typhimurium, NLRC4 defends against infection by several other gastrointestinal pathogens (Table 2).[94] For example, the Gram-negative bacterium *Citrobacter rodentium* is a mouse-adapted pathogen used to model the pathogenesis of enteropathogenic *Escherichia coli* and enterohaemorrhagic *E coli* (EHEC) in humans. NLRC4-deficient mice orogastrically infected with *C rodentium* sustained severe pathological damage to the cecum and colon and increased bacterial loads in the cecum.[79] Unlike for other pathogens, NLRC4 induces a detrimental outcome in response to infection with the Gram-negative bacterium *H pylori*, a causative agent of chronic gastritis and stomach

TABLE 2 The role of NLRC4 in the host defense against bacteria other than *S* Typhimurium using mouse models

Mouse	Infection route	Bacteria	Genetic background	Littermate	Phenotype compared with wildtype mice (unless stated otherwise)	Reference
Nlrc4$^{-/-}$	Orogastric	*C rodentium*	C57BL/6	No	Increased bacterial burden in the cecum 8 and 14 d postinfection Increased pathological score in the colon and cecum	79
		H pylori	C57BL/6N	Yes	Reduced bacterial burden in the stomach 1-mo postinfection Reduced IL-18 secretion and neutrophil recruitment to the stomach	95
	Nasal	*L pneumophila*	C57BL/6	Yes	Increased bacterial burden in the lung 48 and 96 h postinfection	97
			C57BL/6J	No	Increased bacterial burden in the lung 48 and 72 h postinfection	96
		L micdadei	C57BL/6	Yes	Increased bacterial burden in the lung 48 and 72 h postinfection	99
		L bozemanii	C57BL/6	Yes	Increased bacterial burden in the lung 48 and 72 h postinfection	99
		L gratiana	C57BL/6	Yes	Increased bacterial burden in the lung 48 and 72 h postinfection	97,99
		L rubrilucens	C57BL/6	Yes	Increased bacterial burden in the lung 48 and 72 h postinfection	99
		P aeruginosa	C57BL/6	No	Increased bacterial burden in the bronchoalveolar lavage 18 h postinfection	106
	Intraperitoneal	*P aeruginosa*	C57BL/6J	Yes	Increased bacterial burden in the peritoneal lavage 12 h postinfection Reduced circulating IL-1β and IL-18	105
Nlrc4 siRNA knockdown	Ocular	*P aeruginosa*	BALB/c	N/A	Increased bacterial burden in the cornea 5 d postinfection Severe pathological scoring Reduced neutrophil infiltration	104

cancer. Mice lacking NLRC4 infected with *H pylori* had lower bacterial loads and reduced neutrophil infiltration to the stomach compared with WT mice.[95] This enhanced resistance to *H pylori* colonization in mice lacking NLRC4 was thought to be owing to reduced IL-18 secretion, which led to increased expression of the anti-microbial peptide β-defensin 1 by epithelial cells.[95] The deleterious effect of NLRC4 in the host defense against *H pylori* is interesting and might reveal new information about the functionality of NLRC4 in the gastric lining. Other reasons for this detrimental effect might be that the NLRC4-dependent cell expulsion mechanism is not functional or effective in gastric cells lining the stomach and/or that production of inflammasome-associated cytokines is generally detrimental in that region of the gastrointestinal tract.

8.3 | Host defense against extra-gastrointestinal pathogens

Bacteria can colonize and infect anatomical sites other than the gastrointestinal tract of the mammalian host, such as the lungs, skin, and eyes. Therefore, host cells in these sites must be able to detect pathogens, including the use the NAIP-NLRC4 inflammasome to respond to bacteria carrying flagellin and/or other NLRC4-activating ligands. The flagellated pneumonia-causing bacterium *L pneumophila* infects the lungs and can activate the NAIP-NLRC4 inflammasome (Table 2). Upon nasal infection with *L pneumophila*, mice lacking NLRC4 harbored substantially greater bacterial loads in the lungs compared with WT mice.[96-99] Additionally, mice lacking NLRC4 were more permissive to bacterial replication compared to mice lacking both caspase-1 and caspase-11.[96] These studies suggest that in addition to caspase-1 and caspase-11, NLRC4 might also activate other anti-microbial pathways to confer protection against *L pneumophila*. Similar results were also observed for other *Legionella* species, such as *L micdadei*, *L bozemanii*, *L gratiana*, and *L rubrilucens*, which all express flagellin.[98,100] These bacteria proliferated unrestricted in mice lacking NLRC4.[98,99] Further, mice lacking NLRC4 were more susceptible to *L micdadei*, *L bozemanii*, *L gratiana*, and *L rubrilucens* than mice lacking caspase-1 alone.[97,98]

Indeed, NLRC4 can trigger multiple pathways, including activation of apoptotic caspase-7 and caspase-8, phagolysosome maturation, and cell-autonomous immunity.[23,91,101-103] To address the role of caspases in the host defense against *L pneumophila* infection, caspase-1, caspase-8, and caspase-11 were genetically deleted in mice (*Casp1/8/11/Ripk3*$^{-/-}$ mice; note that this mouse strain also carries a genetic deletion of the kinase RIPK3 as it is required to rescue the embryonic lethality of caspase-8 deficiency).[96] These mice were as susceptible as mice lacking NLRC4 in response to *L pneumophila* infection.[96] Furthermore, caspase-7 was found to operate downstream of caspase-1/8/11 and that mice lacking caspase-7, caspase-1, and caspase-11 nasally infected with *L pneumophila* were as susceptible as mice lacking NLRC4.[96] These studies provide additional examples to show that NLRC4 orchestrates activation of multiple inflammatory and apoptotic caspases, allowing the convergence of these pathways to provide effective protection against *L pneumophila* infection.

Infection of the eye with *P aeruginosa*, a Gram-negative bacterium encoding flagellin and T3SS, is a frequent cause of microbial keratitis for contact lens users and immunocompromised individuals. The role of NLRC4 in the eye has been investigated using an siRNA approach to knockdown *Nlrc4* in the cornea of BALB/c mice, followed by an ocular infection with *P aeruginosa*.[104] The cornea treated with the siRNA against *Nlrc4* had higher bacterial loads, more severe disease, and reduced neutrophil infiltration compared with the cornea treated with a non-targeting scrambled siRNA.[104] Protein levels of IL-1β and IL-18 were also reduced in the cornea treated with the siRNA against *Nlrc4*.[104] In an intraperitoneal infection model of *P aeruginosa*, mice lacking NLRC4 harbored slightly more bacteria in the peritoneal lavage at 12 hours postinfection compared with WT mice.[105] Moreover, mice lacking NLRC4 had substantially less circulating IL-1β and IL-18 compared with WT mice,[105] suggesting an involvement from the NLRC4 inflammasome. In an acute pneumonia model, mice lacking NLRC4 nasally infected with *P aeruginosa* had an increased bacterial load in the bronchoalveolar lavage, but not in lung tissues, compared with WT mice.[106] Therefore, the biological effect of NLRC4 is largely protective against several bacteria across multiple anatomical sites.

8.4 | A double-edged sword

While activation of the NLRC4 inflammasome can elicit an inflammatory response to benefit the host, ensuing inflammation can also cause substantial collateral damage to the host. Indeed, the NLRC4 inflammasome can become "rogue" if it (a) is no longer effective at controlling bacterial replication/dissemination; and (b) causes sufficient damage in which the survival of the host is threatened. Bacterial infections capable of activating the NLRC4 inflammasome in mice are associated with two systemic symptoms: (a) hypothermia and (b) vascular changes characterized by increased hematocrit, diarrhea, and peripheral edema.[33,34,76,81,107,108] These systemic responses are often detrimental and can lead to death of a host. For example, WT mice intravenously infected with a lethal strain of *E coli* O21:H+ were found to have a substantially lower survival rate compared to mice lacking both NAIP5 and NLRC4.[107] Furthermore, WT mice also suffered severe small intestinal bleeding, multiple organ damage, hypothermia and had no additional protective advantage against bacterial dissemination compared with mice lacking both NAIP5 and NLRC4.[107]

An experimental approach to trigger systemic activation of the NAIP-NLRC4 inflammasome is to intravenously inject mice with anthrax toxin components protective antigen (PA) along with lethal factor-bound needle (LFn-needle), rod (LFn-rod), or flagellin (LFn-flagellin), such that these NLRC4 ligands are delivered to the cytoplasm of host cells systemically. WT mice undergoing this procedure

experienced rapid and substantial reduction in body temperature, elevated hematocrit, and diarrhea, and lower survival rate compared with mice lacking NAIP1-6 or NLRC4.[33,34,81,108]

Additionally, cre-induced flagellin expression to activate the NAIP-NLRC4 inflammasome specifically in myeloid cells caused severe limb swelling at the tibiotarsal joints of WT mice, but not in mice lacking NLRC4.[76] Severe damage sustained in the tibiotarsal joints, duodenum and the kidneys, and the presentation of leukocytosis can also be observed in these WT mice.[76] Increased vascular permeability that manifests in the form of diarrhea and peripheral edema is a hallmark of NLRC4 activation in mice. Further studies have demonstrated that NLRC4 activation led to a substantial increase in prostanoids production.[81,108] To assess the importance of prostanoids production for hypothermia and vascular changes observed following NLRC4 activation, WT mice and mice lacking cyclooxygenase-1 (COX-1) were treated with PA and LFn-flagellin intraperitoneally.[108] Hypothermia and elevated hematocrit were observed in WT mice but not in mice lacking COX-1,[108] showing that prostanoids are crucial for the induction of hypothermia and vascular changes following NLRC4 activation. In the context of systemic activation of NLRC4, both hematopoietic-derived and non-hematopoietic-derived cells are probably important for induction of hypothermia and vascular changes.[108] Based on findings from multiple models of infectious disease, it is reasonable to argue that a more localized activation of the NAIP-NLRC4 inflammasome is protective, whereas a systemic and widespread activation may cause substantial harm to the host. This concept is further explored in the context of autoinflammation discussed below.

9 | NLRC4 IN AUTOINFLAMMATORY DISEASES

Whole exome sequencing techniques have identified novel and *de novo* mutations in the gene encoding NLRC4 in humans. These mutations are heterozygotic gain-of-function mutations and can lead to autoinflammatory diseases, including early-onset macrophage activation syndrome (MAS), neonatal enterocolitis, fetal thrombotic vasculopathy, and familial cold autoinflammatory syndrome (FCAS) (Table 3).

Patients carrying one of several missense mutations of *NLRC4* in the HD1 domain all exhibit autoinflammatory clinical manifestation.[109-111] Studies have found that mutations within this domain may result in a disruption of interactions between the LRRs and NBD, promoting ATP for ADP exchange.[109] These alterations likely allow ligand-independent activation of the NLRC4 inflammasome and thus result in constitutive activation of caspase-1, and secretion of IL-1β and IL-18.[109] Mechanistically, disruption caused by the mutation, p.Val341Ala, may decrease residue hydrophobicity, reducing interactions in the HD1 domain and similarly promoting ATP for ADP exchange, likely manifesting in the observed cytokine production.[109] Interestingly, a different substitution of leucine at the identical location, p.Val341Leu, led to increased residue hydrophobicity and spontaneous NLRC4 activation evidenced by higher levels of IL-1β and IL-18 in comparison with healthy controls.[110] Both of these gain-of-function mutations have been associated with MAS (Table 3).

Disruption in NLRC4 interface interactions has also been reported within the LRRs leading to MAS-like autoinflammation.[112,113] For example, the mutation, p.Trp655Cys, induced a loss of NLRC4 autoinhibition.[112] Transduction of this mutant version of NLRC4 into THP-1 cells led to increased cell death and secretion of IL-1β and IL-18 compared with cells transduced with a WT NLRC4.[112] This mutant LRRs likely have enhanced interaction with the LRRs of an adjacent NLRC4, promoting NLRC4 oligomerization and increased inflammasome activation. Research into the NLRC4-MAS phenotype has further identified another disease-causing alteration, p.Gln657Leu, in the LRRs.[113] How this mutation affects the activation mechanism of the NLRC4 inflammasome is currently unknown; however, the patient displayed elevated IL-18 levels in line with other patients carrying an NLRC4 mutation.[113] These findings demonstrate that mutations occurring in different regions of NLRC4 can manifest in similar autoinflammatory characteristics (such as in MAS), likely via distinct mechanisms.

Gain-of-function mutations have also been observed in the NBD of NLRC4. Many of these mutations have similarly been reported to cause the destabilization of inactive NLRC4, increasing the propensity of constitutive inflammasome activation and autoinflammation (Table 3). For example, a patient carrying the mutation, p.Thr177Ala, in the NBD had neonatal-onset multisystem inflammatory disease (NOMID).[114] This alanine substitution, similar to mutations observed in HD1, caused a change in hydrogen bonding, subsequently disrupting ADP-mediated interaction at the WHD-NBD domain, leading to NLRC4 autoactivation.[114] Further, IPSC-derived macrophages from a patient carrying the heterozygous NLRC4 mutation, p.Thr177Ala, produced IL-1β, and IL-18 in response to LPS stimulation without a second signal, whereas IPSC-derived macrophages from a patient without NLRC4 mutation did not.[114] Mutations in the WHD of NLRC4 have also been associated with autoinflammation,[115] including a case with increased NLRC4-containing inflammasome formation in the absence of a secondary signal.[116] These studies, along with those presented in the HD1 and LRRs, demonstrate that while different molecular mechanisms may lead to overt NLRC4 activation, the inflammasome itself is likely to play a key role in autoinflammatory disease.

Indeed, therapy targeting IL-1β and IL-18 secretion has already shown promise in NLRC4-related autoinflammation. The use of mTOR inhibitor, rapamycin, in the treatment of an infant displaying neonatal MAS, reduced caspase-1 activation and IL-1β and IL-18 secretion within treated phagocytes, leading to weight gain, reduced cytokine serum levels, and overall clinical symptom improvement.[110] Treatment of a similar NLRC4-related case of MAS with a recombinant IL-18 binding protein (rhIL-18BP), to neutralize IL-18, has also shown clinical response.[117] In this case, reduced MAS severity was associated with stabilization of IL-18 serum levels, hypothesized to occur by inhibition of ongoing IL-18 signaling, demonstrating that therapeutic treatments targeting inflammasome-associated

TABLE 3 List of known NLRC4 mutations linked to an autoinflammatory phenotype

Nucleotide	Amino acid	NLRC4 domain	Predicted type of mutation	Resulting phenotype	Reference
c.512C>T	p.Ser171Phe	NBD	Gain-of-function	Perinatal autoinflammation with MAS-HLH and fetal thrombotic vasculopathy	132
c.529A>G	p.Thr177Ala	NBD	Unknown	Neonatal-onset multisystem inflammatory disease (NOMID)	114
c.1009A>T	p.Thr337Ser	HD1	Gain-of-function	Consistent autoinflammation with recurrent MAS-HLH in European child	111
c.1021G>C	p.Val341Leu	HD1	Gain-of-function	Neonatal MAS in male infant	110
c.1022T>C	p.Val341Ala	HD1	Gain-of-function	(1) Neonatal-onset enterocolitis, episodes of autoinflammation in a family in the USA (2) Infant with severe, refractory MAS	(1) 109 (2) 117
c.1333T>C	p.Ser445Pro	WHD	Unknown	Cutaneous erythematous nodes and urticarial rash, arthralgias, and late-onset enterocolitis in sample of 13 patients	115
c.1589A>C	p.His443Pro	WHD	Gain-of-function	FCAS in a family in Japan	116
c.1965G>C	p.Trp655Cys	LRR	Gain-of-function	Early-onset MAS in 2 patients	112
c.1970A>T	p.Gln657Leu	LRR	Gain-of-function	Recurrent fever, skinerythema, and inflammatory arthritis in 12-y-old Malay girl	113

Abbreviations: FCAS, familial cold autoinflammatory syndrome; HD1, helical domain 1; HLH, hemophagocytic lymphohistiocytosis; LRR, leucine-rich repeat domain; MAS, macrophage activation syndrome; NBD, nucleotide-binding domain; WHD, winged-helix domain.

cytokines may be useful in mitigating the effects of NLRC4-related autoinflammation.[117] Currently, no pharmacological inhibitors of NLRC4 have been identified. Drug screening programs accelerating the search for specific inhibitors of NLRC4 would help deliver a more targeted therapy for patients with NLRC4-related autoinflammatory diseases. Advances in such treatment, combined with the increased description of mechanisms underpinning inflammasome activation, will help to demystify the role of NLRC4 and improve patient care for those suffering autoinflammatory diseases.

10 | NLRC4 IN CANCER

The differential expression of NLRC4 has been observed in a variety of tumor tissue types. Reduced mRNA levels of NLRC4 are evident in the tumor tissues of colorectal cancer.[118] However, more recent studies have demonstrated normal levels in lung tumors[119] and hepatocellular carcinoma,[120] along with increased expression in breast cancer and glioma.[121,122] The role of NLRC4 in tumor regulation and suppression is not always consistent even in the same tumor model (Table 4), as discussed further below.

The role of NLRC4 in colorectal cancer has been investigated in mice intraperitoneally injected with the DNA-damage agent azoxymethane (AOM) and the chemical colitogen dextran sulfate sodium (DSS). This study showed that *Nlrc4*$^{-/-}$ mice displayed increased tumor formation, reduced apoptosis in tumors, and increased proliferation of colonic epithelial cells during the early-stage of the disease compared with WT mice.[123] A similar susceptibility to tumorigenesis was also observed in *Casp1/11*$^{-/-}$ mice,[123] suggesting that the NLRC4 inflammasome is mediating protection in this disease context. While these findings suggest that the NLRC4 inflammasome confers protection against colitis-associated tumorigenesis, another study using the same model showed that *Nlrc4*$^{-/-}$ mice had no increase in hyperplasia and tumor numbers in the colon compared with WT mice, suggesting that NLRC4 has no role in the protection against tumorigenesis.[124] Both studies did not use littermate controls to minimize differences in the gut microbiota profile and/or subtle genetic differences, such as acquired *de novo* mutations. A later study using littermate controls revealed that *Nlrc4*$^{-/-}$ mice were more susceptible to DSS-induced colitis compared to WT mice, potentially owing to reduced production of IL-18.[125] However, the role of NLRC4 on tumorigenesis, if any, was not investigated.

Regardless of the above findings, the functions of the NLRC4 inflammasome in colitis-associated tumorigenesis might be distinct from how NAIPs function. For example, mice lacking all functional paralogues of NAIPs (called *Naip1-6*$^{\Delta/\Delta}$ mice) and mice lacking all functional paralogues of NAIPs specifically within the intestinal epithelial cells (called *Naip1-6*$^{\Delta/\Delta IEC}$ mice) had more tumors in the colon following administration of AOM and DSS compared with WT mice.[35] Mice lacking NAIPs did not have impaired inflammasome activation, but instead, had an impaired ability to attenuate hyperactivation of STAT3, a transcription factor which can function to promote tumor growth.[35] Since hyperactivation of STAT3 was not observed in mice lacking NLRC4, these results suggest that the protective effects of NAIPs could be independent of NLRC4.[35]

The role of NLRC4 in melanoma tumor suppression is also contradictory. *Nlrc4*$^{-/-}$ mice injected subcutaneously with mouse B16F10 melanoma were initially found to display enhanced tumor growth.[126] The presence of NLRC4 in tumor cells was important in modulating signaling pathways responsible for tumor growth control.[126] Interestingly,

TABLE 4 Investigation into the role of NLRC4 and NAIPs in cancer using mouse models

Cancer model	Mouse	Genetic background	Littermate	Phenotype compared with wildtype mice	Reference
Colorectal (induced by AOM + DSS)	*Nlrc4*$^{-/-}$	C57BL/6	No	Increased tumor formation Reduced apoptosis within tumors Increased proliferation of colonic epithelial cells	123
	Nlrc4$^{-/-}$	C57BL/6	No	No increase in hyperplasia or tumor numbers within the colon	124
	Naip1-6$^{\Delta/\Delta}$	C57BL/6	No	Increased tumors within colon compared with non-littermate *Naip1-6*$^{fl/fl}$ mice Impaired ability to attenuate STAT3 hyperactivation	35
	Naip1-6$^{\Delta/\Delta IEC}$	C57BL/6	Yes	Increased tumors within colon compared with littermate *Naip1-6*$^{fl/fl}$ mice Impaired ability to attenuate STAT3 hyperactivation	35
	Naip1-6$^{\Delta/\Delta LysM}$	C57BL/6	Yes	Similar tumor burden compared with littermate *Naip1-6*$^{fl/fl}$ mice	35
Colorectal (induced by AOM only)	*Naip1-6*$^{\Delta/\Delta}$	C57BL/6	No	Increased tumors within colon compared with non-littermate *Naip1-6*$^{fl/fl}$ mice	35
Melanoma (transplanted with B16F10 cells)	*Nlrc4*$^{-/-}$	C57BL/6N	No	Enhanced tumor growth Decreased cytokine and chemokine production	126
	Nlrc4$^{-/-}$	C57BL/6J	Yes	No difference in tumor incidence when compared with littermate WT mice	61
Breast (transplanted with Py8119 cells or E0771 cells)	*Nlrc4*$^{-/-}$	C57BL/6N	No	Mice on a high-fat diet: • Decreased tumor size (Py8119 or E0771) compared with non-littermate WT mice • Reduced CD45^{+} tumor-infiltrating leukocytes • Reduced angiogenesis and VEGF-A production On a normal diet: • Decreased tumor size (E0771 only) compared with non-littermate WT mice	128

Abbreviations: AOM, azoxymethane; DSS, Dextran Sodium Sulfate; VEGF-A, Vascular endothelial growth factor-A.

Nlrc4$^{-/-}$ mice also displayed decreased cytokine and chemokine production, such as CXCL9, CXCL10, CXCL16, and CCL5, used in T cell recruitment.[126] Similarly, the reduced production of IFN-γ by CD4^{+} and CD8^{+} T cells observed in *Nlrc4*$^{-/-}$ mice may contribute to tumor growth.[126] A more recent study using the same model found no role for NLRC4 in melanoma progression, observing no difference in tumor incidence between littermate WT and *Nlrc4*$^{-/-}$ mice.[61] The use of littermate mice in this study would have largely removed confounding factors such as gut microbiota and subtle genetic differences, arguing for the importance of using littermate controls in future studies. The generation of *Nlrc4*$^{-/-}$ mice on different substrains of C57BL/6, such as C57BL/6J vs C57BL/6N, may have also contributed to differences in tumor suppression between studies.[126,127] Further studies using littermate controls and mice of an identical genetic background are therefore warranted to demystify the role of NLRC4 in mouse models of cancer.

Research has also expanded into investigating the potential role of NLRC4 in other cancers. NLRC4 and IL-1β have been shown to promote breast tumor progression in diet-induced obese mice.[128] Macrophages were recruited by the obese tumor microenvironment, with activation of the NLRC4 inflammasome in these macrophages promoting the production of adipocyte-originated growth factor vascular endothelial growth factor-A (VEGF-A) and hence angiogenesis.[128] Cell expansion and VEGF-A production were also mediated by NLRC4 in metastases induced by fatty liver disease, similarly driven by IL-1β production.[129] Indeed, these mouse studies are supported by data from a cohort of human patients with breast cancer, showing that patients with higher levels of NLRC4 mRNA transcripts have a poorer survival rate.[128] These survival plots extend to glioma patients, demonstrating a similar relationship between higher NLRC4 expression and poor prognosis.[130] The study also confirmed increased IL-1β expression in glioma patients with a poorer outcome,[130] implicating a role of IL-1β secretion in driving glioma progression. Further studies are therefore required to investigate the tentative link between NLRC4-mediated IL-1β secretion and tumor progression, potentially positioning the NLRC4-IL-1β pathway as a potential therapeutic target for inhibition in glioma and other cancers.

11 | CONCLUSIONS AND FUTURE DIRECTIONS

Despite unprecedented insights into the structure and activation mechanisms of the NAIP-NLRC4 inflammasome, limitations

in our knowledge still exist. In the context of infectious diseases, NAIP-NLRC4 exploits the structural and functional conservation of virulence factors encoded by many pathogenic bacteria, such as *S* Typhimurium and *P aeruginosa*. A single human NAIP and its isoforms recognize multiple bacterial ligands whereas at least 4 mouse NAIPs are required to do so. How is human NAIP able to bind multiple ligands? Does human NAIP require additional upstream sensors or does it directly bind to these bacterial ligands? Answers to these questions would open new lines of enquiry in the field of innate immune recognition. Evidence that NLRC4 can induce sterile inflammation in the brain might even suggest that it can sense endogenous danger signals. Further, the role of the remaining murine NAIPs 3, 4, and 7 in NLRC4 inflammasome activation remains unexplored. Given that the C57BL/6 mice only express NAIPs 1, 2, 5, and 6, the function of the remaining murine NAIPs will likely require interrogation using mouse strains of other genetic background.

NAIP-NLRC4 activates multiple pathways to limit bacterial replication and dissemination, including secretion of IL-1β and IL-18, pyroptosis, and expulsion of infected cells from the host. So far, an association between loss-of-function mutations in either NAIP or NLRC4 and susceptibility to infections in humans has not been reported. Mice lacking NLRC4 do not readily succumb to infection in a specific-pathogen-free environment, suggesting that deficiency of NLRC4 in mice does not lead to hypersusceptibility to host or environmental microbiota. Further, some mammalian species, such as pigs, lack functional NLRC4, and NAIPs.[131] NAIP-NLRC4 might provide protection against certain bacterial pathogens in mice, but overt activation of NAIP-NLRC4 induces systemic inflammation, sepsis, and death in humans and mice. Therefore, the evolutionary advantage of encoding NAIP-NLRC4, against a backdrop of inherent risk of triggering overt inflammation, probably extends beyond protection against bacterial infection.

Indeed, emerging studies have shown that NLRC4 is differentially expressed in some tumor tissues compared with healthy tissues. Although no consensus on the role of NAIPs or NLRC4 using mouse models of cancer has been made, the recognized needs to use littermate controls in these experiments will increase the consistency and reproducibility of studies in the future. Further, the emerging inflammasome-independent roles of NAIPs or NLRC4 in tumorigenesis are exciting because these mechanistic insights would identify novel pathway-targets to be used in the development of immunotherapies.

In autoinflammatory conditions linked to gain-of-function mutations of NLRC4, progress has been made in translating fundamental discoveries to clinical practice. Patients with MAS have been successfully treated by targeting cytokines produced as a result of constitutive activation of the NLRC4 inflammasome. An important consideration is the consequences of sustained inhibition of inflammasome-associated cytokines in patients, given that these cytokines are critical for the control of infection. Would these complications be minimized if NLRC4 is specifically inhibited instead? Unfortunately, no pharmacological inhibitors of NLRC4 have been discovered. Identifying safe and efficacious compounds that specifically inhibit NLRC4 would provide a more targeted therapy for these patients. Future studies will yield insights into the role of NAIP-NLRC4 in clinical manifestations beyond infectious diseases and identify pharmacologic compounds and host targets to accelerate the development of therapies to improve patient care.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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