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Human and mouse NAIP/NLRC4 inflammasome responses to bacterial infection

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Abstract

Intracellular immune complexes known as inflammasomes sense breaches of cytosolic sanctity. Inflammasomes promote downstream proinflammatory events, including IL-1 family cytokine release and pyroptotic cell death. The NAIP/NLRC4 inflammasome is involved in a range of pathogenic and protective inflammatory processes in mammalian hosts. In particular, the NAIP/NLRC4 inflammasome responds to flagellin and components of the virulence-associated type III secretion apparatus in the host cytosol, thereby allowing it to be a critical mediator of host defense during bacterial infection. Notable species- and cell type-specific differences exist in NAIP/NLRC4 inflammasome responses to bacterial pathogens. With a focus on *Salmonella enterica* serovar Typhimurium as a model pathogen, we review differences between murine and human NAIP/NLRC4 inflammasome responses. Differences in NAIP/NLRC4 inflammasome responses across species and cell types may have arisen in part due to evolutionary pressures.

Keywords

NAIP; NLRC4; inflammasome; pyroptosis; Salmonella Typhimurium; Legionella pneumophila; macrophage; intestinal epithelial cell

Introduction

The mammalian innate immune system harbors pattern recognition receptors (PRRs) that sense and respond to pathogens by detecting pathogen-associated molecular patterns (PAMPs) [1,2]. A subset of cytosolic PRRs oligomerize into multiprotein structures termed inflammasomes upon sensing an insult within the cytosol, such as PAMPs or pathogenic

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disruption of host processes [3–7]. After assembly, inflammasomes recruit and activate inflammatory caspases, such as caspase-1 [3–7]. Active caspase-1 cleaves and activates downstream substrates, including interleukin-1 (IL-1) family cytokines and the pore-forming protein gasdermin D, resulting in the maturation and release of IL-1 family cytokines and other alarmins and an inflammatory form of cell death termed pyroptosis [8–13].

Many inflammasomes are composed of nucleotide-binding leucine-rich repeat (NLR) family proteins. One such inflammasome is the NLR family, apoptosis inhibitory protein (**NAIP**)/NLR family, CARD domain-containing protein 4 (**NLRC4**) inflammasome. The NAIP/NLRC4 inflammasome is a key mediator of host defense against several bacterial pathogens. This inflammasome has also been implicated in pathological inflammation resulting from gain-of-function NLRC4 mutations in humans and a sepsis-like disease triggered by pathobionts in mice [14–20]. NAIP senses the cytosolic presence of evolutionarily related virulence-associated bacterial proteins, including type III secretion system (T3SS) structural proteins and flagellin, during infection [21–29]. Upon ligand binding, NAIP recruits the adaptor protein, NLRC4, which oligomerizes to form the active NAIP/NLRC4 inflammasome [30–32].

There are species- and cell type-specific differences in NAIP/NLRC4 inflammasome responses. Mice express several different NAIPs (mNAIPs), which have arisen as a result of gene duplication events, and each mNAIP recognizes a specific bacterial ligand [22–29,33]. In contrast, humans express only one functional NAIP (hNAIP), recently demonstrated to promiscuously recognize the various bacterial ligands detected by the individual mNAIPs [23,26,27,34–39] (Figure 1). In this review, we will discuss recent findings on NAIP/NLRC4 inflammasome responses to bacterial pathogens in both murine and human cells, focusing on species- and cell type-specific differences in NAIP/NLRC4 inflammasome responses.

Murine NAIP/NLRC4 inflammasome responses to bacterial ligands

In mice, NAIP/NLRC4 inflammasome responses to bacterial pathogens have been extensively characterized. The first findings focused on understanding the genetic basis for the susceptibility or resistance of inbred mouse strains to the intracellular Gram-negative bacterial pathogen *Legionella pneumophila*, causative agent of the severe pneumonia Legionnaires' disease. mNAIP5 contributed to restricting *Legionella* replication in mice and murine macrophages [40,41], and this restriction was dependent on caspase-1 and the *Legionella* Dot/Icm type IV secretion system, which delivers bacterial effectors into the host cell cytosol [42]. Separate studies showed that NLRC4 was required for inflammasome responses to the intracellular enteric pathogen *Salmonella enterica* serovar Typhimurium (*Salmonella*) in murine macrophages [43]. Interestingly, inflammasome responses to *Salmonella* required its T3SS, which delivers virulence factors into the host cell cytosol [43]. These results suggested a potential role for specific T3SS- or T4SS-translocated bacterial ligands in NAIP or NLRC4 inflammasome activation.

When investigating bacterial components that potentially activate murine NAIP or NLRC4, multiple groups independently found that flagellin, the major subunit of flagella, from both *Legionella* (FlaA) and *Salmonella* (FliC) induced inflammasome activation in murine

macrophages [21,24,25,44,45]. During infection, flagellin is thought to be injected into the host cell cytosol via bacterial secretion systems, as has been shown for *Salmonella*'s T3SS [46]. Still, a direct observation of endogenous flagellin being translocated into the host cell cytosol has yet to be reported and represents an important gap in knowledge. T3SS components themselves also induced inflammasome activation. T3SS inner rod proteins from several bacteria, including *Salmonella* (PrgJ) and *Escherichia coli* (EprJ and EscI), activated the NLRC4 inflammasome when delivered to the cytosol of murine bone marrow-derived macrophages (BMDMs) [21]. *Salmonella*'s T3SS needle protein (PrgI) was also detected, albeit poorly, in BMDMs in an NLRC4-dependent manner [21,26]. Non-immune cells can also detect bacterial ligands and mount inflammasome responses. Intestinal epithelial cells (IECs), the primary site of infection for enteric pathogens, undergo NAIP/NLRC4 inflammasome activation followed by pyroptosis and luminal expulsion in response to cytosolic delivery of *Legionella*'s flagellin using an anthrax-toxin based system (FlaTox) [47].

While NLRC4 was previously mischaracterized as the "sensor" of T3SS-related ligands, further studies found that NAIPs are generally the sensors that dictate ligand specificity, whereas NLRC4 is an adaptor protein [21–23,26–29,48,49]. mNAIP5 and mNAIP6 detect flagellin, while mNAIP2 detects T3SS inner rod proteins and mNAIP1 detects T3SS needle proteins [21–23,26–29,48] (Figure 2). Expression of the mNAIPs varies amongst different cell types, potentially yielding differential ligand detection. For example, expression of *Naip1* is low in BMDMs, which poorly detect T3SS needle proteins, and is upregulated with IFN priming [26]. In contrast, peritoneal cavity (PerC) macrophages express higher *Naip1* levels and readily detect cytosolic PrgI [26]. Intriguingly, a functional NLRC4 inflammasome can assemble in the absence of the NAIPs in response to *Anaplasma phagocytophilum* infection through a mechanism involving prostaglandin E2 signaling [50].

In agreement with *in vitro* findings, bacterial ligands activate the NAIP/NLRC4 inflammasome *in vivo,* with individual mNAIPs detecting their cognate ligands [15,28,29]. Intraperitoneal administration of FlaTox to mice resulted in NAIP/NLRC4-dependent diarrhea, circulatory collapse and ultimately mortality at high doses [15,28]. Furthermore, restricting *Nlrc4* expression to either macrophages or IECs still resulted in mortality and circulatory collapse following FlaTox administration, due to caspase-1 activation and pathological release of eicosanoids [15,47]. These results highlight specific cell-intrinsic roles for NLRC4 *in vivo* (Figure 2).

Murine NAIP/NLRC4 inflammasome responses to *Salmonella* infection

While the findings highlighted above focused on NAIP/NLRC4 inflammasome activation by specific bacterial ligands, different bacterial components can activate distinct arms of the immune response. In turn, some bacterial pathogens downregulate expression of particular ligands to evade immune detection during infection. Thus, a number of studies have extensively characterized the role of the NAIP/NLRC4 inflammasome during bacterial infection. For this review, we will focus on *Salmonella*, which, as highlighted above, was recognized early on to activate the NLRC4 inflammasome in murine macrophages [43].

Salmonella harbors two distinct T3SSs, known as the *Salmonella* pathogenicity island (SPI)-1 and SPI-2 T3SSs. While the SPI-1 T3SS is expressed early during infection to mediate invasion into host cells, the SPI-2 T3SS is expressed later to promote intracellular replication [51–59]. Interestingly, *Salmonella* grown under SPI-1-inducing conditions activates robust inflammasome responses in BMDMs in an NLRC4-dependent manner; however, *Salmonella* grown under SPI-2 inducing conditions does not [21]. While these data suggest that the SPI-2 T3SS evades inflammasome detection, *Salmonella* can induce SPI-1-independent inflammasome responses at later time points, namely between 17 and 20 hours post-infection (hpi) [60]. Broz *et al.* characterized these late responses in BMDMs and found that *Salmonella* activates both the NAIP/NLRC4 inflammasome and the NLRP3 inflammasome [61] (Figure 2), which responds to perturbations of cell physiology, such as potassium efflux as a result of plasma membrane damage [62–66]. Distinct *Salmonella* signals appear to activate these inflammasomes [61]. Additional studies corroborated these findings, suggesting that NAIP/NLRC4 and NLRP3 coordinate the inflammasome response to *Salmonella* by associating in the same macromolecular complex [67,68].

During *in vivo Salmonella* infection, the NAIP/NLRC4 inflammasome promotes inflammation to ultimately clear infection. Wild type (WT) and *Nlrc4*$^{-/-}$ mice on a BALB/c or C57BL/6 background displayed differences in mortality during orogastric challenge with *Salmonella* [69,70], in part due to NLRC4-promoted clearance of infection via neutrophil recruitment [69]. In contrast, C57BL/6 mice lacking NLRC4 displayed no differences in mortality compared to their WT counterparts during intraperitoneal *Salmonella* infection [21,69]. This may be in part due to differential *Salmonella* ligand expression during gastrointestinal versus systemic infection. *Salmonella* expresses PAMPs that activate the NAIP/NLRC4 inflammasome, including flagellin and the SPI-1 T3SS, most highly in the cecal epithelium and downregulates expression as it invades deeper tissues [71]. *Salmonella* ectopically expressing flagellin or PrgJ during systemic infection were attenuated compared to WT bacteria, and this was due to NLRC4 inflammasome activation [29,72]. These studies indicate an important role for the NAIP/NLRC4 inflammasome in responding to these ligands to control *Salmonella in vivo*.

Murine IECs, the primary site of gastrointestinal *Salmonella* infection, express high levels of *Naip* and *Nlrc4* [71,73–76]. The NAIP/NLRC4 inflammasome limits *Salmonella* accumulation within IECs, in part by facilitating expulsion of infected IECs into the lumen and limiting cellular spread of bacteria [47,71,76]. This restriction mechanism is independent of IL-1 release. Furthermore, IEC-intrinsic *Naip* or *Nlrc4* expression was both necessary and sufficient to restrict intraepithelial *Salmonella* loads, suggesting an intestinal epithelium-intrinsic role for the NAIP/NLRC4 inflammasome in restricting *Salmonella* [47,76]. Subsequent studies using intestinal enteroid models further elucidated this epithelium-intrinsic mechanism of restriction. Activation of epithelial NAIP/NLRC4, either via *Salmonella* infection or treatment with FlaTox, initiated focal contractions at the site of activation [77]. In addition to restricting intraepithelial bacterial loads, IEC-specific NAIP/NLRC4 inflammasome activation prevents bacterial dissemination to systemic sites, supporting an important role for the inflammasome in intestinal barrier defense [71] (Figure 2). Collectively, these findings in the murine system elucidating NAIP/NLRC4 recognition of specific ligands and response to bacterial infection form a strong foundation

for understanding NAIP/NLRC4-mediated immune amplification. Clear differences exist between different cell types and organ systems at large, begging the question of what drives the evolution of varying responses downstream of a common infectious or inflammatory signal. For example, it is possible that IEC responses differ from macrophage responses due to a necessity to maintain barrier integrity as a physical component of immune defense. Studying these questions in the murine system offers a powerful way to examine whole body consequences of NAIP/NLRC4 inflammasome responses and deepen understanding in the field.

Human NAIP/NLRC4 inflammasome responses to bacterial pathogens

In contrast to mice and rats, humans and other animals express a single functional NAIP (hNAIP) [34,35]. Early studies found that T3SS needle proteins from various bacteria, including *Salmonella* (PrgI) and enterohemorrhagic *Escherichia coli* (EHEC) (EprI), induced NAIP/NLRC4 inflammasome activation in the human monocytic cell lines U937 and THP-1 [23,26,27]. Strikingly, these same studies also found that U937 and THP-1 cells did not respond to cytosolic delivery of flagellin or certain T3SS inner rod proteins [23,26,27]. Thus, it appeared that hNAIP retained homologous function to mNAIP1, as it only detected the T3SS needle protein.

Subsequent studies found that hNAIP detects additional bacterial ligands. In primary human monocyte-derived macrophages (hMDMs), flagellin from *Salmonella* (FliC) or *Legionella* (FlaA) activated the NAIP inflammasome [36,37]. Subsequently, it was shown that hMDMs also undergo inflammasome responses to the T3SS inner rod protein from *Salmonella* (PrgJ) and other bacteria [37,39], and that this response was also dependent on hNAIP [37]. These apparent discrepancies compared to the earlier studies could be due to differences in ligand delivery systems and T3SS inner rod proteins used or lower expression of *NAIP* and *NLRC4* in U937 and THP-1 cells compared to primary human macrophages [36]. Importantly, the single hNAIP is sufficient to mediate inflammasome responses to the T3SS needle, inner rod, and flagellin proteins [37]. Furthermore, both NAIP and NLRC4 are required for inflammasome responses to these bacterial ligands [38,78]. These studies show that in contrast to mice, which have evolved multiple specialist NAIPs that each recognize only one ligand, humans express a generalist NAIP which has evolved to promiscuously recognize three structurally related but distinct bacterial ligands. The structural basis for this difference in ligand recognition by mNAIPs and hNAIP is unclear. Interestingly, rats, like mice, also retain multiple NAIPs that have arisen as a result of gene duplication events, suggesting that pathogen-imposed selective pressure in the rodent lineage resulted in the emergence of specialist NAIPs. In contrast, other mammals express a single functional NAIP, including non-human primates, cows, horses, and bats [34,35]. Future studies are needed to investigate whether the single NAIP in other mammals also behaves as a generalist like hNAIP and broadly recognizes multiple ligands.

While *Salmonella*'s SPI-1 T3SS structural components activate mNAIPs and hNAIP, *Salmonella*'s SPI-2 T3SS inner rod protein (SsaI) is not sensed by murine NAIP2 or hNAIP [21,37]. In addition, *Salmonella* SPI-2 activity suppresses SPI-1-induced inflammasome responses in human macrophages [79]. Thus, a prevailing model posits that *Salmonella*'s

SPI-2 T3SS evades inflammasome detection to promote *Salmonella*'s intracellular lifestyle. However, recent findings show that *Salmonella*'s SPI-2 T3SS needle protein (SsaG) is, in fact, sensed by hNAIP, leading to NAIP/NLRC4-dependent restriction of *Salmonella* replication within human macrophages [38] (Figure 3).

These critical differences between hNAIP and mNAIP recognition of bacterial ligands represent fascinating and fundamental distinctions between the two species, highlighting the importance of broadly conducting studies across different species. Much still remains unknown about hNAIP-mediated ligand detection, including the molecular determinants of hNAIP's broad ligand detection, hNAIP's binding affinity for each ligand, and the precise structure of ligand-bound hNAIP. These open questions present exciting opportunities for future expansion of the field of human inflammasome biology.

As noted previously, bacterial infections do not recapitulate purified ligand delivery, as they elicit more complex and nuanced inflammasome responses. As such, NAIP/NLRC4 inflammasome responses to bacterial infection have been studied in human cells. hNAIP is required to restrict intracellular *Legionella* replication in both THP-1 macrophages and A549 lung epithelial cells [80]. *Legionella* T4SS activity also activates the NLRP3 inflammasome and CASP4 inflammasome, which senses cytosolic LPS, in THP-1 cells and hMDMs [81]. Moreover, *Salmonella* infection induces robust inflammasome activation in human macrophages that requires SPI-1 T3SS or flagellin [27,36–38,78,79]. *Salmonella* elicits a multifactorial response in human macrophages, involving the NAIP/NLRC4, NLRP3, and CASP4/5 inflammasomes [38,78,79] (Figure 3). Furthermore, NLRC4 and NLRP3 co-localize to the same macromolecular complex upon *in vitro Salmonella* infection of human macrophages [68]. The recruitment of NLRC4 to punctate structures has also been reported in BMDMs during *in vitro Legionella* infection in an ASC-dependent manner [82]. Whether NLRC4 co-localizes with other inflammasome structures during *in vivo* infections remains unknown and requires further study. Finally, inflammasome activation mediates *Salmonella* control in human macrophages such that both the NAIP/NLRC4 and NLRP3 inflammasomes restrict *Salmonella* replication within human macrophages [38].

Like murine IECs, human IECs can mount cell-intrinsic inflammasome responses to bacterial pathogens, including *Salmonella*. In response to *Salmonella*, human IECs undergo caspase-4 inflammasome-dependent pyroptosis, IL-18 cytokine release, restriction of bacterial replication, and extrusion of infected cells [83–85] (Figure 3). However, the role of the NAIP/NLRC4 inflammasome in human IECs during infection remained unclear. Surprisingly, unlike in murine IECs where the NAIP/NLRC4 inflammasome is functional [28,29,47], delivery of bacterial ligands failed to induce NAIP/NLRC4 inflammasome activation in immortalized human IECs or human intestinal enteroids [86] (Figure 3). Furthermore, human $NAIP^{-/-}$ immortalized IECs had no defect in inflammasome responses to *Salmonella*. Immortalized human IECs and primary small intestinal enteroids express very low levels of *NAIP* and *NLRC4* compared to human peripheral blood mononuclear cells [86], which potentially contributes to the lack of a functional NAIP/NLRC4 inflammasome in human IECs in these *in vitro* models. Whether the NAIP/NLRC4 inflammasome functions in human intestinal epithelium *in vivo* is unknown. It is possible that *in vivo*, host or microbial signals may upregulate NAIP/NLRC4 expression

in human IECs, or NAIP/NLRC4 may be expressed in a rare IEC subset not represented in *in vitro* models. Intriguingly, human patients with gain-of-function NLRC4 mutations display enterocolitis early in infancy, although gastrointestinal disease no longer presents in patients that survive infancy [17–20]. Whether infantile enterocolitis is caused by NLRC4 activation in IECs or another cell type is unclear. Together, these findings in human macrophages and IECs underscore important cell type-specific and species-specific differences that exist between mice and humans and the importance of broadening these studies to include multiple cell types. Recent advances in *ex vivo* culture systems, including organoids, represent exciting new avenues with which to study these questions and deepen understanding of cellular roles

Concluding remarks and remaining questions

In summary, the NAIP/NLRC4 inflammasome senses bacterial components in the host cell cytosol and induces a cascade of inflammatory events to mediate host defense against bacterial pathogens. Recent studies have revealed species-specific NAIP/NLRC4 inflammasome responses. While mice express multiple NAIPs which each detect a specific ligand, humans harbor a single NAIP which broadly recognizes multiple ligands. We find this key difference between mice and humans compelling. It begs the question: is there a selective advantage or disadvantage to promiscuous ligand detection by human NAIP when compared to selective ligand detection by mouse NAIPs? Relative commensal and pathogen exposure by different species may have provided evolutionary pressures underlying selective versus promiscuous ligand detection. In addition, as previously highlighted, there are other non-human mammalian species that express a single NAIP; whether the single NAIP in these other species also promiscuously recognizes multiple ligands is unknown. Future studies more broadly comparing different species may help address these questions. Recent findings suggest cell type-specific differences in NAIP/NLRC4 inflammasome responses to bacteria that vary between mice and humans. For example, NAIP/NLRC4 inflammasome activation is both necessary and sufficient in IECs to control *Salmonella* infection in mice, whereas the NAIP/NLRC4 inflammasome appears to be nonfunctional in human IECs. In addition, there are also cell type-specific differences within a given species. While the NAIP/NLRC4 inflammasome senses and responds to *Salmonella* infection in human macrophages, the inflammasome appears dispensable in human IECs. Thus, there exists differential NAIP/NLRC4 inflammasome responses to pathogens that are species- and cell type-specific. We find the field of NAIP/NLRC4 inflammasome responses during bacterial infections to be an ever expanding and particularly fascinating one. While considerable work has been done to delineate these responses in mice and humans in various cell types, there are still many exciting unanswered questions in the realm of NAIP/NLRC4 biology that will be investigated for years to come.

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Highlights

- Mice express several different NAIPs, each recognizing a specific bacterial ligand
- Humans express one functional NAIP, which broadly detects multiple bacterial ligands
- *Salmonella* activation of the NAIP inflammasome in murine IECs promotes control of infection
- NAIP inflammasome is critical for controlling *Salmonella* in human macrophages but not in human IECs

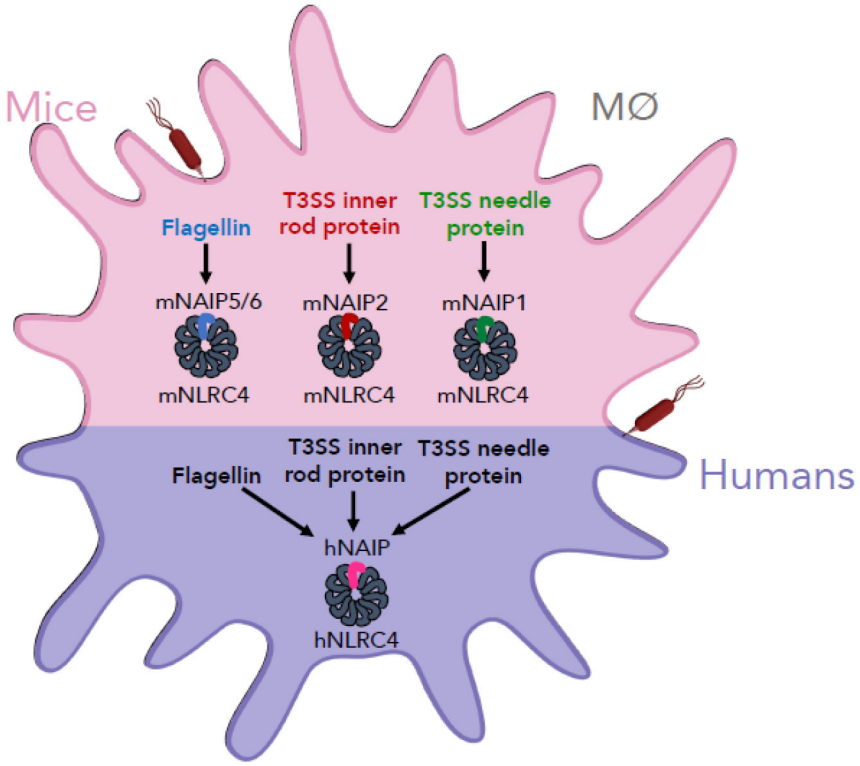

Figure 1: Species-specific NAIP/NLRC4 inflammasome responses to bacterial ligands

Mice and humans exhibit differences in NAIP/NLRC4 inflammasome responses to bacterial ligands. Mice express several different NAIPs (mNAIPs). Each mNAIP recognizes a distinct bacterial ligand: mNAIP5 and mNAIP6 detect flagellin, mNAIP2 detects T3SS inner rod proteins, and mNAIP1 detects T3SS needle proteins. Unlike mice, humans express a single NAIP (hNAIP), and this single hNAIP is capable of recognizing *all* three bacterial ligands that are individually recognized by the different mNAIPs. Upon ligand detection, NAIP recruits its adaptor, NLRC4 (murine NLRC4, mNRLC4; human NLRC4, hNLRC4), thereby forming the active NAIP/NLRC4 inflammasome.

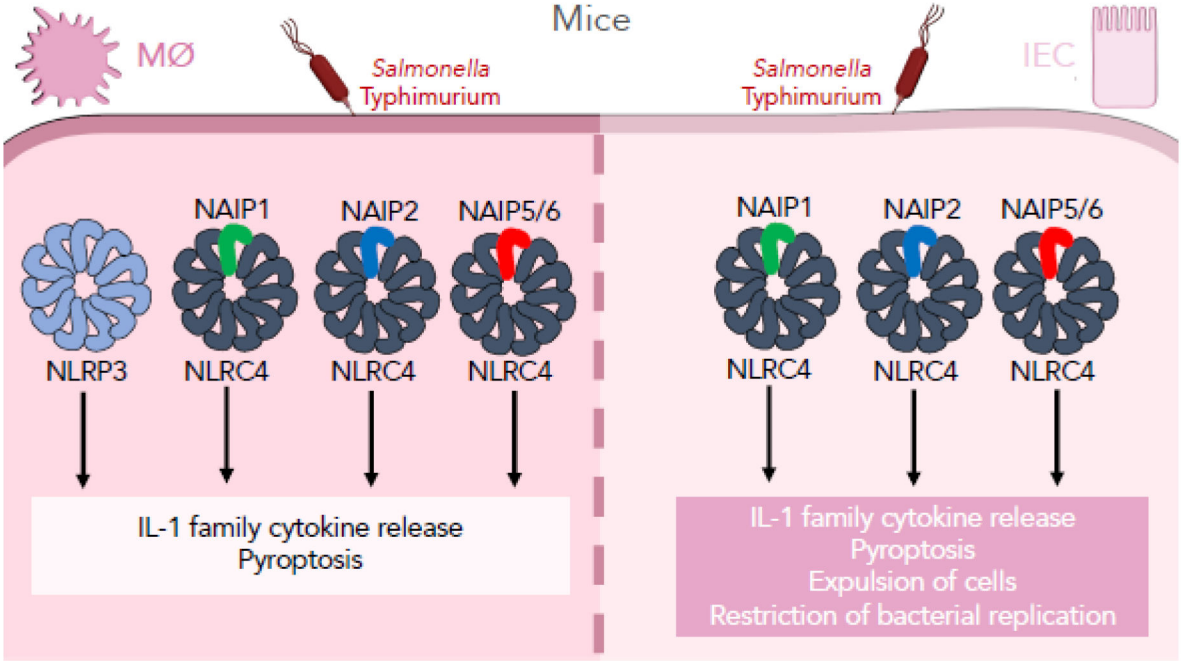

Figure 2: Murine cell type-specific inflammasome responses to *Salmonella* infection

Murine macrophages and intestinal epithelial cells (IECs) display distinct inflammasome responses to *Salmonella* infection. In murine macrophages, *Salmonella* infection induces both NLRP3 and NAIP/NLRC4 inflammasome responses, leading to IL-1 cytokine release and an inflammatory form of cell death, pyroptosis. In murine IECs, the NAIP/NLRC4 inflammasome senses and responds to *Salmonella* infection, mediating IL-1 cytokine release, pyroptosis, and expulsion of cells, which ultimately leads to control of infection *in vivo*.

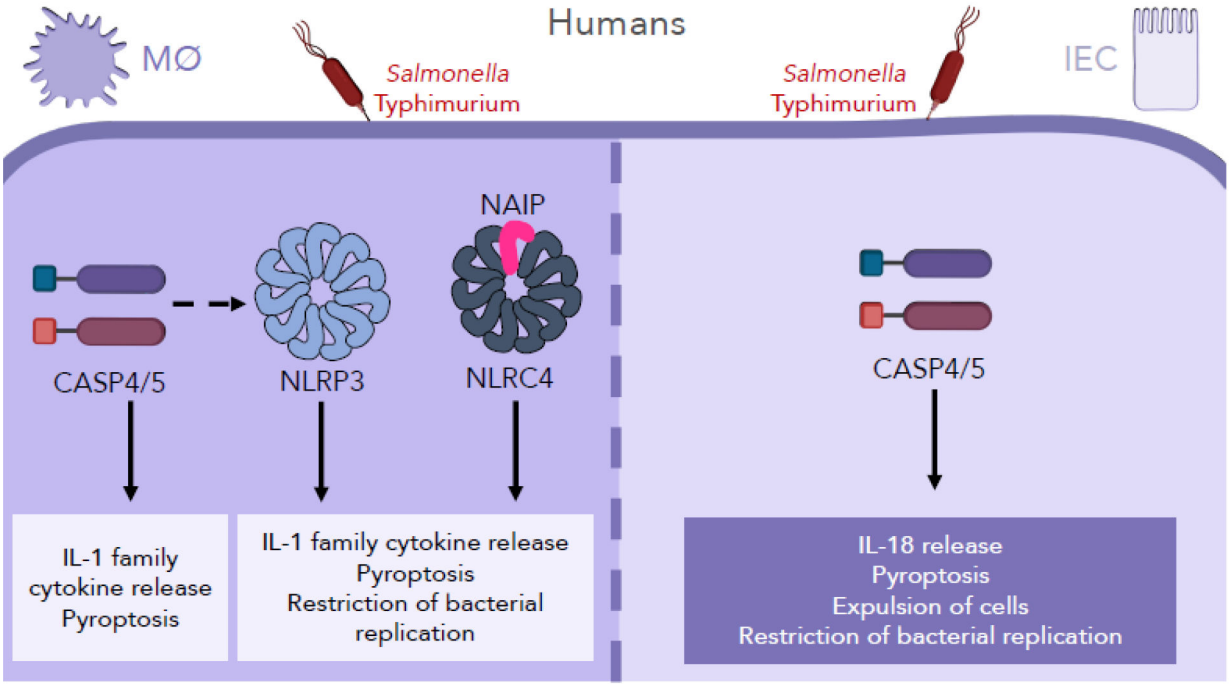

Figure 3: Human cell type-specific inflammasome responses to *Salmonella* infection

While human macrophages employ a multifactorial inflammasome response to *Salmonella* infection, human intestinal epithelial cells (IECs) rely on a single inflammasome, the caspase-4/5 inflammasome, during *Salmonella* infection. In human macrophages, *Salmonella* infection induces NAIP/NLRC4, NLRP3, and caspase-4/5 inflammasome responses, facilitating IL-1 cytokine release, pyroptosis, and ultimately restriction of bacterial replication. In contrast, in human IECs undergo a caspase-4-dependent, NAIP/NLRC4- and NLRP3-independent inflammasome response to *Salmonella* infection.