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Structural basis of bacterial flagellin for NAIP5 binding and NLRC4 inflammasome activation and the mechanism of flagellin induced release of cytokines \textit{in vivo}

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Structural basis of bacterial flagellin for NAIP5 binding and NLRC4 inflammasome activation and the mechanism of flagellin induced release of cytokines in vivo

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By
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2022

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ABSTRACT OF DISSERTATION

Structural basis of bacterial flagellin for NAIP5 binding and NLRC4 inflammasome activation and the mechanism of flagellin induced release of cytokines \textit{in vivo}

The bacterial flagellum is a whip-like structure that protrudes from the cell membrane and is one of the most complex and dynamic biological molecular machines that propels bacteria to swim toward beneficial environments and the sites of infection. It is composed of a basal body, a hook, and a long filament. The flagellar filament contains thousands of copies of the protein flagellin (FliC) monomer arranged helically and ending with a filament cap composed of oligomer protein FliD. The overall structure of the filament core is preserved across bacterial species, while the outer domains exhibit high variability, and in some cases are even completely absent. Apart from its role in locomotion, the filament is critically important in several other aspects of bacterial survival, reproduction, and pathogenicity, such as adhesion to surfaces, secretion of effector molecules, penetration through tissue structures, and biofilms formation.

Bacterial flagellin is an important pathogen-associated molecular pattern (PAMP), which can activate both innate and adaptive immunity. Previous \textit{in vitro} studies indicate that TLR5 is a major extracellular receptor for flagellin that mediates flagellin-induced production of proinflammatory cytokines, including interleukin-6 (IL-6), IL-12, and tumor necrosis factor $\alpha$ (TNF$\alpha$). Flagellin can also induce inflammasome activation through its intracellular receptor, the NLR family apoptosis inhibitory protein (NAIP) 5 and 6, leading to the generation of cytokines IL-1$\beta$ and IL-18, as well as pyroptosis. Here, we found that inflammasome activation and subsequent pyroptosis, but not TLR5-mediated signal transduction, is responsible for flagellin-induced IL-6 and TNF$\alpha$ generation \textit{in vivo}. Flagellin was fused to the cytosolic translocation domain of anthrax lethal factor (LFn) to enable efficient cytosolic delivery. LFn binds to anthrax protein protective antigen (PA), which delivers the LFn-flagellin fusion protein into the cytoplasm through receptor-mediated endocytosis. Injection of LFn-flagellin with PA, but not LFn-flagellin alone by \textit{i. v.}, increased plasma concentrations of IL-1$\beta$, IL-6, and TNF$\alpha$. LFn-flagellin/PA induced IL-1$\beta$, IL-6, and TNF$\alpha$ release was abolished in mice deficient in NAIPs, caspase-1, or...
GSDMD, but not TLR5. Depletion of monocytes and macrophages using clodronate inhibited LFn-flagellin/PA induced cytokine release. In addition, injection of the LFn fusion of another virulent factor, the T3SS rod protein EprJ from *E. coli*, together with PA also induced generation of IL-1β, IL-6, and TNFα in a caspase-1 and GSDMD dependent manner. Our data indicate that inflamasome activation leads to the generation of a broad range of inflammatory cytokines in vivo through pyroptosis, suggesting an important role of pyroptosis in cytokine storm.

Flagellin is a widespread bacterial virulence factor sensed by the membrane-bound Toll-like receptor 5 (TLR5) and by the intracellular NAIP5/NLRC4 inflammasome receptor. Bacterial flagellin is composed of highly conserved D0 and D1 domain, as well as hypervariable D2 and D3 domain. It has been reported that deletion of the D0 domain of flagellin completely abrogates the activation of TLR5. D0 domain of flagellin alone can bind NAIP5/6, leading to activation of the NLRC4 inflammasome, while whether the D1 domain of flagellin plays any functional role in NAIP5/NLRC4 inflammasome activation remains elusive. Besides, flagellins from *S. typhimurium*, *Yersiniosis enterocolitica*, and *Pseudomonas aeruginosa* can bind to NAIP5/6 and activate inflammasome NLRC4, those from *enteropathogenic E. coli*, *enterohaemorrhagic E. coli*, *Shigella flexneri*, and *Burkholderia thailandensis* cannot interact with NAIP5/6 and are unable to activate the inflammasome NLRC4. Replacement of the C-terminal D1 domain of flagellin from *P. aeruginosa* with the C-terminal D1 domain of *E. coli* flagellin diminished inflammasome activation. These data reveal that the D1 domain also plays an important role in flagellin-induced NAIP/NLRC4 inflammasome activation.

KEYWORDS: bacteria flagellin, virulence factors, PAMP, TLR5, NAIP/NLRC4 inflammasome activation, pyroptosis, cytokines

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05/05/2022
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LIST OF ABBREVIATIONS

PAMP: Pathogen-associated molecular pattern

DAMP: Damage-associated molecular pattern

TLR5: Toll-like receptor 5

NAIP: NLR family apoptosis inhibitory protein

NLR: NOD-like receptor

NOD: Nucleotide-binding and oligomerization domain

NLRC4: NLR containing a CARD domain 4

CARD: Caspase activation and recruitment domain

IL-1β: Interleukine 1β

TNF: Tumor necrosis factor

MD-2: Myeloid Differentiation factor 2

LPS: Lipopolysaccharide

LRRs: Leucine-rich repeats

TIR: Toll/Interleukin (IL)-1 receptor

MyD88: Myeloid differentiation 88

IRAK: IL-1 receptor associated kinase
TRAF6: TNF receptor-associated factor 6

NF-κB: nuclear factor κB

dsRNA: double-stranded RNA

PRR: Pattern recognition receptor

T3SS: Type 3 secretion system

EPEC: Enteropathogenic E. coli

EHEC: Enterohemorrhagic E. coli

NBD: Nucleotide binding domain

HD1: Helical domain 1

WHD: Winged-helix domain

HD2: Helical domain 2

BIR: Baculovirus inhibitor of apoptosis protein repeats

APAF-1: Apoptotic protease activating factor 1

IgG: Immunoglobulin G

IECs: Intestinal epithelial cells

DCs: Dendritic cells

MIP-3a: Macrophage inflammatory protein-3a

iNOS: Inducible nitric oxide synthase
BAL: Bronchoalveolar lavage fluid

AP-1: Activator protein-1

GRO: Growth-related oncogene

hBD: Human beta defensins

IRF3: Interferon response factor 3

IRAK: IL-1 receptor associated kinase

MAPK: Mitogen-activated protein kinase

MCP: monocyte chemoattractant protein

MIP: macrophage inflammatory protein

PYR: Pyrin domain

TAK1: Transforming growth factor-beta-activated kinase 1

TBK1: TANK-binding-kinase-1

TRIF: TIR domain containing adapter inducing interferon-b

STAT1: Signal transducer and activator of transcription 1

JAK: Janus kinase

LFn: Lethal factor N-terminal

PA: Protective antigen

GSDMD: Gasdermin D
ELISA: Enzyme-linked immunosorbent assay

IL-1RA: IL-1 receptor antagonist

BMDM: Bone marrow derived macrophage

LCM: L929-cell conditioned medium

FBS: Fetal bovine serum

LDH: Lactate dehydrogenase

PBS: Phosphate buffered saline

SDS: Sodium dodecyl sulfate

TCA: Trichloroacetic acid
CHAPTER 1. INTRODUCTION

1.1 Introduction

Human health is constantly threatened by various infectious diseases and large-scale epidemics caused by bacteria. Bacterial infections have been threatening mankind since its first existence. Infectious diseases are the second leading cause of death worldwide and the most significant cause of death in children, causing about 5 million children die from infectious diseases every year.[1-4] Sepsis is the body’s extreme response to an infection and a life-threatening condition that affects more than 1 million patients a year in the United States.[5, 6] It is a leading cause of death in ICU, with an incidence of about 2.5 per 1,000 people in the western world. Over the past two decades, the annual growth rate of sepsis is almost 10%.[7] About 20% of people with sepsis die in hospitals, and severe sepsis can cause about 40% of deaths.[8] Massive and sudden production of inflammatory cytokines is called cytokine storm, which is dysregulated acute inflammation response triggered by infections and other stress signals such as trauma.[9] Cytokine storm has been associated frequently with detrimental clinical outcomes. Pro-inflammatory cytokines IL-1β, IL-6, and TNFα play important roles in cytokine storm and are popular biomarkers used in monitoring cytokine release during inflammatory responses as their concentrations in blood correlates with the severity of infection.[10-13] IL-1β binds to the IL-1 receptor on the surface of immune cells and triggers various processes and expression of cytokines and chemokines.[14, 15] IL-6 interacts with its membrane receptor IL-6R (composed of receptor IL-6Rα and signal transducer gp130) expressed in lymphocytes, myeloid cells, and hepatocytes to activate Janus kinase (JAK) family tyrosine kinases, leading to the
activation of signal transducer and activator of transcription (STAT) family transcription factors.[16] Later it was found that when complexed with the soluble form of IL-6Rα, IL-6 can activate non-immune cells expressing only gp130.[17] Production of TNFα is triggered under various pathophysiological conditions including infection.[18, 19] TNFα exists in two forms, a membrane bound form and a soluble form, which corresponding to the extracellular domain of the former. Activity of TNFα is mediated by two receptors, TNFR1 (expressed on the surface of most human cell types) and TNFR2 (located mainly on immune and endothelial cells).[20] TNFR1 stimulation leads to inflammation or cell death, while TNFR2 signaling supports cell activation, migration, and proliferation.[12] Virulence factors are molecules that assist bacteria to colonize the host at the cellular level. The membrane-associated virulence factors aid the bacterium in adhesion and evasion of the host cell. The investigation of the structural basis and mechanism of virulence factors in infectious diseases is essential for the development of new therapeutic methods. As a common virulence factor from flagellated bacteria, flagellin promotes pathogens to adhere, invade, and proliferate in host cells and stimulates innate immune responses, which contributes to the immediate clearance of pathogens from the host.[21-25]

1.2 Structure and function of flagellin

The bacterial flagellum is known as one of the most complex and dynamic biological nanomachines and has attracted attention since its discovery in the late nineteenth century. The flagellum is a whip-like filament attached to the surface of bacteria, providing the major force for bacterial motility. The number of flagella per cell varies depending on the species. Monotrichous specie contains only one flagellum at the pole
(e.g., *Pseudomonas aeruginosa*); lophotrichous specie has multiple flagella form a bundle at the same pole on the cell (e.g., *Helicobacter pylori*); amphitrichous bacterium has one flagellum at each pole (e.g., *Campylobacter jejuni*); and peritrichous bacterium contains multiple flagella (e.g., *Escherichia coli, Salmonella enterica*).[26] The flagella polymer is composed of repeated helix stacking of flagellin monomers. Early biophysical characterization showed that the N- and C-termini of monomeric flagellin are mainly disordered and critical for polymerization, although they become highly structured in the filament.[27, 28] Monomeric flagellin (30-60kDa, depending on the species of bacteria) is secreted by the bacteria through the flagella export system. Structurally, the carboxyl (C) terminus of flagellin folds back to the amino terminus (N); the whole molecule contains four different globular domains, D0, D1, D2, and D3, forming a "boomerang".[29] (Figure 1.1) There are about 40 amino acids at each terminus of the flagellin molecule that makes up the D0 domain. The D1 domain contains approximately 100 residues from the N-terminus and 50 residues from the C-terminus. Flagella are stacked longitudinally to form a profilament unit through the intermolecular contact between two adjacent monomer D1 domains. A total of 11 profilaments are assembled into flagella filaments. (Figure 1.2) The flagella filament is a hollow tube about 15 microns long, with an external diameter and an inner diameter of about 240 Å and 20 Å, respectively.[30] The D0 and adjacent D1 domains constitute the inner and outer core of the hollow filament; the D2 and D3 domains come from the central segment of the flagellin polypeptide and are derived from the projections on the outer surface of the flagellin.[29, 30] Both D0 and D1 domains are primarily α-helical structures. The D2 and D3 domains are composed mainly of β-sheets. The D0 and D1 domains are essential for the assembly of helical filamentous structures, so they are
highly conserved in different bacteria species, while the other two domains show a high degree of sequence diversity. The highly conserved N-terminal and C-terminal D0 and D1 domains of flagellin are crucial for its immunostimulatory function.[24, 31, 32] While the middle hypervariable D2 and D3 domains are essential for the antigenicity of flagellin and may cause undesirable toxicity in flagellin-associated treatments.[33, 34] The D2 and D3 domains have vast diversity in their sequence, size, and composition in different bacterial strains and are even absent in some bacterial species, such as *T. pallidum.* As an activator in shaping both innate and adaptive immunity, flagellin as a vaccine carrier protein or a vaccine adjuvant has been shown to enhance the cross-protective response of related antigens against a variety of infectious diseases, and some of the flagellin-related vaccines are investigated in human preclinical and clinical trials.[35-37] More significantly, flagellin exhibits anti-tumor, anti-metastatic immunotherapy, and radioprotective properties through inducing TLR5 pathway activation.[34, 38-40] The flagellum filament is anchored into the bacterial membrane by a basal body.[41] The filaments, hook, and basal body together constitute complete flagellar machinery, which allows bacteria to swim through the rotation of the filaments. Flagella-mediated motility is very important for bacteria to find nutrients and avoid harmful substances in the environment. Counterclockwise rotation of the motor provides smooth forward swimming, while clockwise rotation of some of the flagella causes unraveling of the bundle and specific tumbling movement.[42] Filaments are much longer than the cell body and during smooth swimming, they adopt a left-handed supercoiled corkscrew shape, while clockwise rotation changes the handedness.[43, 44] Flagella is also important in promoting bacterial adhesion and invasion of host cells.[45] For enteric bacteria, flagella-mediated motility is conducive
to the rapid renewal of epithelial cells, the tight intercellular junctions, and the viscous mucus present in gastrointestinal tissues. For example, *Spiral Campylobacter jejuni* (a common cause of gastroenteritis) and *Helicobacter pylori* (the main pathogen of chronic gastric ulcer) both have flagella with polarity orientation; mutations that disrupt the flagella prevent these two bacteria from establishing a replication niche in the gastrointestinal tract.[46] Other bacterial pathogens such as *Pseudomonas aeruginosa* and *Vibrio cholera* also rely on an intact flagellum for colonization of their hosts, respectively.[47] Flagellated bacteria support the critical functions of flagella and are the main cause of many serious skin, soft tissue, lung, and urinary tract infections.
Figure 1.1. Organization of flagellum and flagellin. Motile bacteria produce flagella composed mainly of polymerized flagellin.

Flagellin consists of four domains: the terminal α-helixes (D0), the central α-helixes (D1), and the hypervariable β-sheets and turns (D2 and D3). The α-helix regions are required for filament architecture and motility functions.[48]

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While all flagellin structures known so far exhibit a high degree of structural similarity in the D0 and D1 region, the structures of the variable regions differ extensively. The structure of the *P. aeruginosa* type A FliC lacking the D0 domain showed that the D1 domain highly resembles its counterpart in *S. Typhimurium.[49]* Conversely, the D2 domain adopts a different fold with two β-sheets and one α-helix between them forming a less flexible cup-like structure positioned parallel to the D1 domain, instead of pointing away from it as in *Salmonella.* In the case of *Campylobacter,* the variable region of the
flagellin FlaA is larger and forms three domains (D2, D3, and D4) (Figure 1.3).[50] The D2 and D3 domains of the *Campylobacter* FlaA are structural homologs of each other and the *Pseudomonas* FliC D2. D4 is inserted between the C-terminal moieties of D2 and D1, with most of the FlaA glycans located on this domain. It is the most exposed outer domain resembling a shield for both D2 and D3 and, in contrast, D4 does not have structural homologs.

Figure 1.2. The structure of flagellin and the cross-sectional and top views of the flagellar filament.

The flagellar filament is composed of a single protein, flagellin.

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1.3 Flagellin sensing by immune and non-immune cells

Flagella elicit the activation of host inflammatory responses via flagellin interaction with specific molecules through a variety of signaling pathways. Flagellin has been identified as a key virulence factor across pathogenic bacterial genus and species, including *Helicobacter pylori, Pseudomonas aeruginosa,* and *Vibrio cholerae.*[52-54] Deletion of the gene encoding flagellin in these bacteria greatly impacts the pathogenesis of the bacterial infection.[52-54] Flagellins act as potent agonists of the innate immune system inducing proinflammatory responses, given their ability to stimulate the extracellular Toll-
like receptor 5 (TLR5), the intracellular NOD-like receptor (NLR) family 4 (NLRC4)-
NAIP 5/6 inflammasome, and an unknown additional third pathway recently proposed that
is independent of TLR5, Casp1/11, and MyD88.[55-60] Lack of flagella correlates with a
reduced ability of triggering inflammasome activation in macrophage and reduced
inflammation in mice infection models.[52-54]

1.3.1 Structural interactions between flagellin and TLRs

The Toll-like receptor (TLR) family in the mammalian genome was first described
decades ago as innate immunosensors that recognize evolutionarily conserved PAMPs.[24,
61] Since then, searching for the structural basis of TLRs recognition ligands and
understanding the molecular mechanisms of their activation have become the focus of
many structural biology studies. About 10 years ago, the structures have been reported for
TLR2-TLR1 and TLR2-TLR6 heterodimers in complex with lipopeptides,[62, 63] TLR3
in complex with double-stranded RNA molecule,[64] and TLR4 in complex with MD-2
with or without lipopolysaccharide (LPS).[65, 66] Although TLRs exhibit a conserved
horseshoe-shaped leucine-rich repeats (LRRs) structural fold, the region where each
receptor binds to the ligand depends on the nature of the ligand. The lack of a common
ligand binding mode in the published TLR ligand complexes makes it difficult to predict
the ligand recognition mode of the remaining TLRs. Yoon and his colleagues determined
the ligand complex crystal structure of TLR5, another member of the TLR family, adding
an important clue to how TLR mediates ligand recognition and immune activation.[67]
Human TLR5 recognizes bacterial flagellin, a major component of bacterial motor flagella
found in Gram-positive and Gram-negative bacteria.[55] Flagellin binding leads to the
accumulation of the cytoplasmic TIR [Toll/Interleukin (IL)-1 receptor] domain of TLR5 and the activation of the MyD88 (myeloid differentiation 88) pathway, which includes the recruitment of IRAK (IL-1 receptor associated kinase) and TRAF6 (TNF receptor-associated factor 6) and activation of the transcription factor nuclear factor (NF) κB, leading to the production of the pro-inflammatory cytokine IL-6 (Figure 1.4).[68]

Figure 1.4. Detection of flagellin and TLR5 signaling.

TLR5 utilizes the universal TLR-specific adapter molecule MyD88, which activates downstream molecules nuclear factor-kB, mitogen-activated protein kinases, and interferon regulatory factors, which turn on the transcription of genes involved in innate and adaptive immunity.[48]

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The crystallization of TLR receptors has proven to be challenging, mainly due to poor recombinant expression of the extracellular ligand-binding domain and the low crystallization tendency of natural TLRs. Two technical strategies were developed for TLRs crystallization: (i) screening human TLR5 homologues with different extracellular truncations to find a structure with abundant expression; (ii) fusing the hagfish variable lymphocyte receptor (VLR) at the C-terminus domain to promote crystallization. Both techniques had been applied to the structural determination of TLRs.[62] In this case, Yoon et al. succeeded in expressing several truncated forms of the extracellular domain of TLR5 from zebrafish and validated that a human TLR5 ligand functionally activated zebrafish TLR5 using a reporter system for transcriptional activation of NF-κB. They crystalized a construct containing the 14 N-terminal LRRs from zebrafish TLR5 fused to a hagfish VLR in complex with a fragment of *Salmonella* flagellin. They also solved the structure of a shorter construct of the TLR5 alone. The 2.5 Å resolution structure of the TLR5-flagellin (*Salmonella*) complex revealed both the unique aspects of ligand binding to TLR5 and the common dimerization feature shared between TLR5 and other TLRs.[62]

Yoon et al. described how TLR5 forms 2 : 2 dimers with *Salmonella* flagellin, similar to TLR4 : MD-2-LPS, but different from the 2:1 dimer observed in TLR1-TLR2: lipopeptides or TLR3 : dsRNA (double-stranded RNA) complexes. The binding interface of flagellin in TLR5 is rather extensive and involves the N-terminal and the first 10 LRR regions. The focal region is centered at the LRR9 loop, which mediates many important interactions. The LRRs for flagellin binding to TLR5 is different from the LRRs used by TLR2/1 in lipopeptide binding, which requires the central LRR 9 to 12 repeats, and from those involves in dsRNA recognition by TLR3, which requires N-terminal to third repeats
(NT to LRR3) and repeats 19 to 21. Although TLR4 uses similar regions [N-terminal to fifth repeats (LRR NT to LRR5) and between the eighth and tenth repeats (LRR8-10)] for MD-2 recognition, the detailed interface residues are different.[66] Therefore, depending on the ligands, different TLR uses different LRRs to recognize the ligand. This allows a limited number of TLRs to recognize a diverse collection of ligands. In terms of ligands, the recognition of TLR5 involves a set of relatively conserved residues among bacteria on the N-terminal and C-terminal helices in the D1 domain of flagellin. The TLR5 interface residues of flagellin are usually involved in the formation of bacterial flagellin oligomers and therefore will not be exposed in live bacteria.[56] Like other TLR-ligand complexes, the ligand-bound TLR5 forms a symmetric m-shaped dimer. The C-terminal LRR regions are juxtaposed with each other. This conformation is conducive to the dimerization of the intracellular TIR domains which will then activate downstream signaling pathways. The dimer interface induced by flagellin binding involves residues of LRRs 12 and 13 of TLR5. The authors demonstrated that TLR5 mutants containing mutations at these sites were unable to transcriptionally activate the NF-κB reporter system in response to flagellin. Besides TLR5 homodimer, extracellular flagellin binds to the TLR4/5 heterodimer and the dimerization of their TIR domains recruits adaptor TRIF (TIR-domain-containing adapter-inducing interferon-β), which activates the IRF3 pathway, leading to the production of interferon β.[69]

TLR5 is expressed by epithelial cells, monocytes, and immature dendritic cells.[70, 71] The initial identification of flagellin as a ligand for TLR5 came from a study that used HPLC to isolate stimulating components from *Listeria* culture supernatants.[55] Parallel studies investigating the activation of epithelial cells caused by *Salmonella* also confirmed
that flagellin is a stimulating ligand for TLR5.[72] Therefore, TLR5 can recognize the flagellin produced by gram-negative bacteria and provides a simple strategy for the host response to flagellar bacteria.

Compared with many other TLR ligands, the proteinaceous nature of flagellin allows us to study in detail the structural basis of flagellin-TLR5 interaction. Two different groups have demonstrated that flagellin produces biological activity in eukaryotic expression systems, demonstrating that the stimulation ability of flagellin is independent of other bacterial proteins or prokaryotic post-translational modifications.[56, 73] Using the defined deletion mutants of flagellin, two different groups initially identified two different sites required for biological activity. The hypervariable region is located in D3 and the conserved N- and C-termini of the protein.[31, 74] This discrepancy was resolved by more detailed mapping, which defined the TLR5 stimulatory activity in N-terminal residues 79-117 and 408-439.[56] Therefore, TLR5 is likely to recognize the spatial area of flagellin, comprising areas of the N- and C-terminus.

The TLR5 binding site of flagellin is likely to be located at the central base of the flagellum. This observation is interesting because these residues are necessary for the inherent structure of flagellum and bacterial movement. Therefore, bacteria cannot escape detection by the host through mutations in the flagellin sequence without compromising the beneficial aspects of bacterial motility. However, it is currently unclear how the internal structure of flagellar filaments interacts with TLR5, thereby mediating pro-inflammatory activity in vivo. One possibility is that monomers or oligomers of flagellin are released from the filaments in the harsh environment of the phagosome or exposed during bacterial replication.[75, 76]
In addition to TLR5, another recently discovered pattern recognition receptors (PRRs) present in mice, TLR11, was identified as a receptor of flagellin.[77] The interaction of flagellin with TLR11 is more restricted compared to its interaction with TLR5. A study by Hatai et al. [77] shows that TLR5 strongly interacts with flagellin at both acidic and neutral pH conditions, whereas acidic conditions are essential for TLR11-flagellin interaction. Furthermore, this study shows that both the N- and C-terminal domains of flagellin can independently interact with TLR11, whereas the TLR5-flagellin interaction requires both domains to mediate a proinflammatory cytokine response and thus adjuvant activity.[31] TLR11 is highly expressed in epithelial cells in various organs, such as the intestine, lung, and skin, and it has a role in the prevention of Salmonella invasion in mice.[78] This result contrasts with TLR5-deficient mice, which show enhanced resistance against oral Salmonella infection.[79] These findings suggest that flagellin-TLR11 signaling mechanisms differ from flagellin-TLR5 interaction, and therefore, more research are needed to clarify it.

1.3.2 structural interactions with intracellular receptor NAIP5/6

The cytosolic flagellin is detected through NOD-like receptors (NLR) in mice. Two of these, NAIP5 and NAIP6, are involved in the interaction with flagellin in the cytosol. Upon flagellin binding, NAIP5 associates with NLRC4 to form an inflammasome that activates caspase-1, which, in turn, induces the inflammatory response by cleaving and activating interleukins IL-1β and IL-18, and triggers the subsequent lytic form of cell death named pyroptosis.
In animals, several NLRs have been reported to function as PRRs, detecting PAMPs or host-derived damage-associated molecular patterns (DAMPs) in the cytosol and consequently initiating innate immune responses.[25, 80] In mice, the specificity of the inflammasomes is conferred by NAIPs, with NAIP5/6 and NAIP2 recognizing bacterial flagellin and the T3SS components, respectively.[21, 59] NAIP1 from mice and its human ortholog of hNAIP are considered as receptors for T3SS needle proteins.[81, 82] Like NAIP2/5, NAIP1 and hNAIP form ligand-induced NAIP-NLRC4 inflammasomes for caspase-1 activation as well. It is suggested that the NBD-associated central domains other than the LRR of NAIPs are critical for their specific recognition of the bacterial PAMPs.[83] It has also been reported that the BIRs of NAIP5 are critical for flagellin-induced NAIP5-NLRC4 inflammasome activation, although it remains unclear how they contribute to the activation of NAIP5-NLRC4 inflammasome.[59] Despite the important roles of NLRs in the detection of the invasion of pathogens in both animals and plants,[84] the mechanisms of how NLRs detect and interact with pathogen-associated ligands remain poorly understood.

Structural and mechanistic studies have provided profound insights into the process of NAIP-NLRC4 inflammasome assembly. (Figure 1.5) 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 structure of the flagellin-NAIP5-NLRC4 inflammasome complex shows that NAIP5 alone interacts with flagellin, making numerous contacts with both helices of the D0 domain.[85] Six different regions of NAIP5 (N-terminal helix, BIR1, HD1, HD2, ID, and
LRR) form a flagellin-binding pocket and confer ligand specificity. Based on two-hybrid assays, flagellins from five pathogenic bacteria (*L. pneumophila, S. Typhimurium, Y. enterocolitica, Photobacterium luminescens*, and *P. aeruginosa*) showed positive results while those from five others including *EPEC, EHEC, Shigella flexneri, Chromobacterium violaceum*, and *Burkholderia thailandensis* were unable to interact with NAIP5. Additionally, those flagellins that interacted with NAIP5 also induced inflammasome activation in bone marrow-derived macrophages. These studies confirmed that the ability of flagellins to bind NAIP5 correlates with their ability to induce an immune response and that NLRC4 acts as an adaptor through which inflammasome activation signals generated from different NAIP receptors are transduced to caspase-1.[59] 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. The recognition of flagellin by NAIP seems to involve the C-terminal D0 domain of flagellin and later N-terminal D0 domain of flagellin was proved to contribute to the recognition by NAIP.[86, 87] Mutagenic studies found that while mutation of these key recognition motifs in the ligand enabled immune evasion by NAIP5, it also disrupted flagellar motility.[85] As mutations of specific leucine residues in the C-terminal domain abrogate NAIP5/NLRC4-mediated inflammasome formation.[87, 88] 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, limits pathogen immune evasion.[56, 85] Interestingly, while the LRRs has been proposed to act as a sensor for NLR ligands, 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.\[83, 89, 90\]

Figure 1.5. Structure of the NAIP5-NLRC4 inflammasome.

(A) Schematic of domain architecture for NAIP5 and NLRC4. (B) Proposed events of inflammasome assembly. The flagellin D0 domain (purple) binds to NAIP5 and unfurls the protein for subsequent NLRC4 recruitment and activation. Active NLRC4 recruits further NLRC4 protomers for self-propagating oligomerization and completion of a caspase-1 recruitment platform.\[85\]

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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
The same study revealed that the C-terminal LRRs were positioned to sterically occlude one surface of the NBD and sequester NLRC4 in a monomeric state. The authors also demonstrated that mutagenesis abolishing crucial inhibitory interactions lead to constitutive NLRC4 inflammasome activation and bypassed the requirement for flagellin.

Once a ligand-bound NAIP complex interacts with NLRC4, it is sufficient to trigger conformational changes in NLRC4, driving it toward an active conformation. 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. 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. Unlike apoptosome oligomerization, which requires one cytochrome c ligand per APAF-1 monomer, 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.
1.3.3 The third potential pathway

It was recently reported that with the absence of TLR5, the inflammasome, and MyD88, flagellin can induce potent IgG1 anti-flagellin responses. Based on this observation, a third pathway that promotes anti-flagellin antibodies production was proposed, independent of TLR5 and inflammasome, and requiring the presence of all four domains of flagellin. Nempont et al. illustrated different flagellin domain deletions that had reduced immunogenicity and indicated that the D2/D3 domains may be the most critical for MyD88-independent IgG1 anti-flagellin responses.[94] Mice immunized with flagellin and different truncated versions made antibodies that showed reactivity to the D0/D1 and D2/D3 domains, indicating that both components of the molecule are antigenic. The D3 domain of flagellin influences immunogenicity independently of the known innate recognition sites in the D0/D1 domains to augment antibody production, suggesting that full-length flagellin and the preservation of all four domains were critical for optimal immunogenicity and primary and secondary antibody responses in flagellin-based vaccines.[60] The nature of the third pathway is poorly understood and requires further investigation.

1.4 Pro-inflammatory activity of flagellin

Preliminary studies with human blood mononuclear cells have shown that flagellin from different bacteria species can stimulate the production of cytokines, such as TNF-α and IL-1β.[75, 95] In addition, the flagellin produced by Salmonella typhi can induce the synthesis of IL-6 and the anti-inflammatory cytokine IL-10.[75, 95-97]
Recent studies by Gewirtz et al. determined that *Salmonella* flagellin stimulates the production of IL-8 when exposed to human intestinal epithelial cells.[98] Interestingly, the activation of this inflammatory response depends on the transfer of flagellin to the basolateral surface of epithelial cells, where TLR5 is expressed.[72, 98] Other researchers have tested the activation of human intestinal epithelial cell lines by flagellin and noted the production of the chemokine CCL-20 and subsequent migration of immature human dendritic cells (DCs) under this stimulus.[99] It was suggested in this report that the apical expression of TLR5 is related to the secretion of chemokines.[99] Consistent with this view, TLR5 is constitutively expressed on the top surface of human primary intestinal epithelial cells (IECs), and another recent study has shown that flagellin from commensal bacteria can induce TLR5 activation on the apical surface of freshly isolated murine IECs.[70, 100] Further research is needed to solve the problem of TLR5 expression on the surface of epithelial cells and flagellin translocation. If TLR5 is expressed on the apical surface of epithelial cells, why flagellar symbiotic bacteria cannot induce a permanent inflammatory state in the intestinal mucosa is still unclear. One possibility is that symbiotic microorganisms can also induce anti-inflammatory signaling pathways in epithelial cells.[101, 102]

TLR5 is also highly expressed in the lungs and seems to play an important role in combating respiratory pathogens.[103-105] Interestingly, there is a correlation between common human TLR5 polymorphisms and susceptibility to Legionnaires disease.[106] A single point mutation to a stop codon at position 392 resulted in an impaired response to flagellin in these patients.[106] In summary, the existing evidence shows that the
expression of TLR5 in the intestinal mucosa and respiratory mucosa is an important innate immunosensor for flagellate pathogens.

### 1.5 Flagellin and inflammatory disease

Flagellin can induce the expression of a variety of pro-inflammatory mediators, such as TNF-α, IL-1, IL-6, MIP-3a, and iNOS, so it is likely involved in the development of bacterial-related pathology. (Figure 1.6) To support this hypothesis, direct instillation of 1 μg of flagellin into the mouse trachea caused acute inflammation in the lungs, including infiltration of neutrophils and macrophages, and presence of inflammatory cytokines in bronchoalveolar lavage fluid (BAL).[107] Surprisingly, flagellin is more effective than lipopolysaccharide in the induction of lung inflammation, which may be due to lower levels of TLR4 in the lung, as compared in the intestinal epithelium.[70, 107] Therefore, the chronic lung pathology caused by flagellar pathogens such as Pseudomonas or Legionella may be partly due to the host response to flagellin.[105, 106]

Similar to endotoxin, flagellin is an effective mediator of systemic inflammation. Intravenous injection of flagellin in mice can rapidly produce typical cytokines, chemokines, and NO production, and can cause clinical symptoms such as septic shock, hypotension, respiratory distress, cyanosis, organ damage, and death.[76, 108] For LPS and flagellin induced infection, although it is not clear which one is more effective in causing toxic shock in mice, these data suggest that flagellin may be an important factor in the inflammatory process of bacterial sepsis.[76, 108, 109]

Inflammasome is a large cytoplasmic complex that can sense microbial infections/dangerous molecules and induce the production of caspase-1 activation-
dependent cytokines and the inflammatory death of macrophages.[110] The inflammasome assembled by the NOD-like receptor (NLR) protein NLRC4 responds to bacterial flagellin and a conserved type III secretion system (T3SS) rod component.[111-113] Certain bacteria such as S. typhimurium can directly deliver flagellin into the cytoplasm of host cells through T3SS.[114, 115] Another route of flagellin entry into cytoplasm is endocytosis. How the NLRC4 inflammasome detects the two bacterial products and the molecular mechanism of NLRC4 inflammasome activation are not understood. It is reported that NAIP5, a BIR-domain NLR protein required for Legionella pneumophila replication in mouse macrophages, is a universal component of the flagellin-NLRC4 pathway.[21, 59, 87, 116, 117] The binding of flagellin to NAIP5 promotes the physical binding of NAIP5-NLRC4 and completely reconstructs the flagellin-responsive NLRC4 inflammasome in non-macrophage cells.[59] This finding further indicates that the remaining NAIP family members may recognize other unidentified microbial products to activate NLRC4 inflammasome-mediated innate immunity.
Figure 1.6. Signal transduction by flagellin in mammalian cells.

Immune cells respond to extracellular monomeric flagellin through either TLR5 homodimer or heterodimer complexes, resulting in the transcription of a variety of genes that are important for the proper stimulation of immune cells. The intracellular flagellin is detected by NAIP5/6 which recruits and phosphorylates NLRC4, which induces caspase-1 activation and subsequent pyroptosis.

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CHAPTER 2. BACTERIAL FLAGELLIN INDUCED IL-6 AND TNFα RELEASE IN VIVO

DEPENDS ON INFLAMMASOME ACTIVATION AND PYROPTOSIS

2.1 Introduction

As we mentioned previously, cytokine storm is a massive and sudden production of inflammatory cytokines, and it has been associated frequently with detrimental clinical outcomes. Flagellin as a common virulence factor which can induce inflammation was used to explore the mechanism of cytokine generation and release in vivo in this study.

We used flagellin from L. pneumophila to investigate flagellin-induced inflammation in vivo. Flagellin was fused to the cytosolic translocation domain of anthrax lethal factor (LFn) to enable efficient cytosolic delivery. LFn binds to anthrax protein protective antigen (PA), which delivers LFn-flagellin fusion protein into cytoplasm through receptor-mediated endocytosis.[59] We found that flagellin-induced release of the pro-inflammatory cytokines in vivo depends on inflammasome activation and pyroptosis. Deficiency of the flagellin intracellular receptor NAIPs, caspase-1, or GSDMD, but not the cell surface receptor TLR5, abolished LFn-flagellin/PA induced cytokines. Depletion of macrophages and monocytes by clodronate significantly reduced LFn-flagellin/PA induced cytokine release. Our findings illustrate the important role of inflammasome activation and pyroptosis in inflammation during bacterial infection.
2.2 Material and methods

2.2.1 Mice.

Wild-type C57BL/6J, Naip<sup>+/−</sup>, Casp1<sup>+/−</sup>, Tlr4<sup>+/−</sup>, Tlr5<sup>+/−</sup>, Gsdmd<sup>+/−</sup>, mice were housed in the University of Kentucky Animal Care Facility, following institutional and National Institutes of Health guidelines after approval by the Institutional Animal Care and Use Committee. Male mice at 8-12 weeks were used in all experiments.

2.2.2 LFn fusion protein expression and purification

As described previously,[119] proteins were expressed using LPS free *E. coli* strain ClearColi BL21(DE3) (Lucigen Corporation, Cat#60810) at 37 °C for 4 hours with 500 μM IPTG after OD<sub>600</sub> reached 0.6-0.8. Bacteria were collected and lysed in 50 mM Tris-HCL and 300 mM NaCl. Proteins containing a His-tag were purified by affinity chromatography using HisPur Ni-NTA resin (Thermo scientific, Cat#88222). Proteins were then eluted with 250 mM imidazole in 50 mM Tris-HCL and 300 mM NaCl, and subsequently dialyzed against PBS to remove imidazole. Protein concentrations were determined by measuring their absorption at 280 nm before sterile filtration.

2.2.3 In vivo study

C57BL6 and the corresponding gene deficient mice caspase 1<sup>+/−</sup>, Tlr4<sup>+/−</sup>, Tlr5<sup>+/−</sup>, Gsdmd<sup>+/−</sup> and Naip<sup>+/−</sup> (10-12 weeks old) were injected with 3 μg of LFn-fla with or without
PA, or 1.5 μg of LFn-EprJ with or without PA, as indicated. Blood samples were collected before or at various time (90 min, 4 h, and 8 h) following injection of the bacterial proteins. To determine the inflammasome activation, blood samples were collected via retro-orbital bleeding. Blood samples were centrifuged at 10,000 g for 1 minute at room temperature to obtain plasma. IL-1β, IL-6, and TNFα were measured using ELISA following manufacturer’s instruction.

2.2.4 Monocytes/macrophage depletion

To deplete monocytes/macrophages, C57BL6 mice were injected intravenously with 40 mg/kg of clodronate-loaded liposome suspension (Encapsula NanoSciences, Nashville, TN) 24 hours prior to injection with LFn-fla/PA. Control mice were injected with PBS-loaded liposomes instead.

2.2.5 IL-1R blocking experiment

C57BL6 mice were injected intravenously with 1 mg/kg of recombinant human IL-1 receptor antagonist (IL-1RA, PeproTech, Cat# 200-01RA) 10 min before the injection with 3 μg of LFn-fla and 3 μg of PA. Blood samples were collected before or at various time points (90 min, 4 h, and 8 h) after the injection with LFn-fla/PA.
2.2.6 Bone marrow derived macrophage (BMDM) culture and stimulation

BMDMs were prepared as described previously,[120] and seeded into 12-well cell culture plate (1 mL/well) or 96-well cell culture plate (100 µL/well) at a density of 1 x 10^6 cells/mL of RPMI-1640 medium containing 15% L929-cell conditioned medium (LCM). BMDMs were allowed to settle overnight and refreshed with Opti-MEM (Life Technologies, Cat#31985-070) before purified protein were added. For study of cytokine release, supernatant samples were collected 6 h after stimulation. For study of cytotoxicity, supernatant samples were collected 90 min after stimulation.

2.2.7 Peritoneal macrophage culture and stimulation

Peritoneal macrophages were harvested from C57BL/6J and Tlr4-/- mice following the protocol published by Zhang et al.[121] Cells were seeded into a 96-well cell culture plate at a density of 1×10^6 cells/ml in DMEM/F12 medium supplemented with 10 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% (v/v) fetal bovine serum (FBS). Peritoneal macrophages were allowed to settle overnight and refreshed with 100 µL of Opti-MEM before purified proteins were added (LFn-Fla with or without PA at 1 µg/ml each, or LPS 200 ng/mL). For study of flagellin induced cytokine release, the supernatants were collected 6 h after stimulation.
2.2.8 Kupffer cell isolation, culture, and stimulation

Kupffer cells were isolated from C57BL/6J and Tlr4−/− mice following the protocol published by Zeng et al.[122] Cells were cultured on a 10 cm cell culture dish in DMEM supplement with 10 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and 10% (v/v) FBS at 37 °C. The culture medium was then changed every two days until the cells reached 80% confluence. The cells were then seeded into a 96-well cell culture plate at a density of 1×10^6 cells/ml of culture medium. Kupffer cells were allowed to settle overnight and refreshed with 100μL of Opti-MEM before the indicated proteins were added.

2.2.9 Monocytes isolation and stimulation

Monocytes were isolated from C57BL/6J and Tlr4−/− mice using the Monocytes Isolation Kit (BM) (Miltenyi Biotec, cat# 130-100-629). The isolated cells were seeded into a 96-well cell culture plate at a density of 1×10^6 cells/ml in DMEM medium supplemented with 10 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal bovine serum (FBS). Monocytes were allowed to settle for 2 hours and refreshed with 100 μL of Opti-MEM before purified proteins were added (LFn-Fla with or without PA at 1 μg/ml each). For study of flagellin induced cytokine release, the supernatants were collected 6 h after stimulation.
2.2.10 ELISA analysis

To determine IL-1β, IL-6, and TNFα cytokine levels in culture supernatants, ELISAs were performed using the mouse IL-1β, IL-6, and TNFα Invitrogen ELISA kits from ThermoFisher Scientific. ELISA assays were performed according to manufacturer instructions. To determine IL-1β, IL-6, and TNFα levels, supernatants or plasma samples were diluted appropriately. Plates were read on a Cytation 5 at 450 and 580 nm. Cytokine concentration was determined by extrapolation from the standard curve.

2.2.11 Lactate dehydrogenase cytotoxicity assay

To determine cell viability, the lactate dehydrogenase (LDH) CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Cat#G1780) was used. 50 μl of CytoTox 96 Reagent was added to each well of 96-well plate followed by 50 μl of supernatant samples and incubate for 10min at room temperature or until a sufficient colorimetric change was visible. The reaction was stopped using the LDH stop solution, and the plate was read on a Cytation 5 at 490 nm.

2.2.12 Western blotting

For detection of active caspase-1 and IL-1β cleavage by western blotting, cells were washed with cold PBS and lysed with an SDS sample buffer. Culture supernatants were precipitated with 1/10 volume of 2% sodium cholate and 1/10 volume of 100% trichloroacetic acid (TCA), and then dissolved in the SDS sample buffer. Total protein
from lysates and supernatants (equivalent to 1 × 10^6 cells) was analyzed by western blotting for multiplex detection. Both pro-caspase-1 and p20 caspase-1 were determined using anti-caspase-1(p20) (Adipogen, Cat#AG-20B-0042) at 1:1000 dilution. Pro-IL-1β and IL-1β (p17) was detected using anti-IL-1β (GeneTex Cat#GTX74034) at 1:1000 dilution. Blots were imaged using BIO-RAD ChemiDoc MP imaging system.

2.2.13 Statistical analysis

Statistical analysis was performed in Prism GraphPad 9.0.0. Statistical analysis was performed using one-way ANOVA with Dunnett’s multiple comparisons for multiple groups and two-way ANOVA repeated measures with Holm-Sidak multiple comparisons for time point studies. A p value <0.05 was considered significant.

2.3 Results

2.3.1 Injection of LFn-fla together with PA, but not LFn-fla alone, increased plasma TNFα, IL-6 and IL-1β concentrations

Flagellin induces cytokine production through its receptors TLR5 and NAIPs. While induction of IL-1β release by flagellin requires caspase-1 activation, previous in vitro studies have shown that flagellin mediates TNFα and IL-6 release through TLR5 in vitro.[123-128] We used mouse models to investigate the mechanism by which flagellin induces inflammation in vivo. Recombinant flagellin of L. pneumophila was fused to LFn to create LFn-fla. C57BL/6J mice were injected with LFn-fla with or without PA by i.v.,
and blood was collected at various time points thereafter. Cytokine concentrations in plasma were measured using ELISA assays. Surprisingly, injection of LFn-fla alone into C57BL/6J mice did not increase plasma concentrations of TNFα and IL-6 (Figure 2.1A-C). In contrast, injection of LFn-fla plus PA dramatically increased plasma concentrations of these cytokines, as well as IL-1β (Figure 2.1A-C). These data suggest that inflammasome activation mediates flagellin-induced inflammation in vivo.

Figure 2.1. Administration of LFn-fla/PA, but not LFn-fla alone, induced proinflammatory cytokine release. (A-C) C57BL/6J mice were injected intravenously with LFn-fla (3 μg) or LFn-fl plus PA (3 μg). Blood samples were collected right before and at 90 min, 4 h, 8 h after injection. IL-1β (A), IL-6 (B), and TNFα (C) concentrations in plasma were measured by ELISA assay. Error bars denote SEM, n=4 for each group. One asterisk, p<0.05; two asterisks, P<0.01; three asterisks, P<0.001; two-way ANOVA with Holm-Sidak multiple comparisons.

2.3.2 Deficiency of NAIPs but not TLR5 protected against flagellin-induced inflammation

To verify that inflammasome activation is responsible for flagellin-induced inflammation in vivo, we examined LFn-fla/PA induced cytokine release in mice deficient
of the flagellin intracellular receptor NAIPs. While humans have only one subtype of
NAIP, mice have 6 subtypes of NAIPs (NAIP1-6) that respond to different kinds of
PAMPs.[59] We used a mouse model in which all NAIPs are deleted.[129] Injection of
LFn-fla plus PA did not significantly increase plasma concentrations of TNFα, IL-6, and
IL-1β in the NAIP deficient mice (Figure 2.2A-C). In contrast, LFn-fla/PA induced release
of the proinflammatory cytokines in the TLR5 deficient mice to levels similar as in the WT
mice. These data demonstrate that inflammasome activation, but not TLR5, is responsible
for flagellin-induced inflammation in vivo.

It has been reported previously that while the flagellins from S. typhimurium, Y.
enterocolitica, and P. aeruginosa could bind to NAIP5 and activate NLRC4
inflammasome, those from enteropathogenic E. coli, enterohaemorrhagic E. coli, Shigella
flexneri, and Burkholderia thailandensis could not interact with NAIP5 and were unable to
activate inflammasome NLRC4.[59] To further test whether flagellin induces
inflammation in vivo dependent on inflammasome activation, we purified LFn fusion of
flagellins from P. aeruginosa and enterohaemorrhagic E. coli. As expected, LFn-flagellin
containing P. aeruginosa flagellin induced pyroptosis of wild-type mouse BMDMs as
evident by release of LDH into the supernatant, and caspase-1 activation and IL-1β
cleavage (Figure 2.2D-F). In contrast, flagellin from enterohaemorrhagic E. coli failed to
induce pyroptosis of BMDMs, caspase-1 activation or IL-1β cleavage (Figure 2.2D-F).
Injection of LFn-Flagellin from P. aeruginosa, but not the flagellin from
enterohaemorrhagic E. coli, in the presence of PA, increased plasma concentrations of
TNFα, IL-6, and IL-1β in C57BL/6J mice (Figure 2.2G-I). Taken together, these data
suggest that inflammasome activation, but not TLR5, mediates flagellin-induced inflammation in vivo.

Figure 2.2. Flagellin induced cytokine release in vivo depended on its intracellular receptor NAIPs, but not TLR5.

(A-C) C57BL/6J (WT), Naip^5^−/−, or Tlr^5^−/− mice were injected intravenously with LFn-Fla/PA (3 μg each). Blood samples were collected right before and at 90 min, 4 h, and 8 h
after injection. IL-1β (A), IL-6 (B), and TNFα (C) concentrations in plasma were measured by ELISA assay (n=4 for each experimental group). (D-F) BMDMs from C57BL/6J WT were incubated with 1 μg/ml of LFn fused flagellin from *P. aeruginosa* or *E. coli* with PA for 90 min. Cell viability (D) was assessed by measuring LDH release into supernatant, and caspase-1 activation (E) and IL-1β cleavage (F) were detected by western blot. (G-I) C57BL/6J mice were injected intravenously with LFn-fla from *P. aeruginosa* or *E. coli* with PA (n=5 for all experimental group). Error bars denote SEM. One asterisk, p<0.05; two asterisks, P < 0.01; three asterisks, P<0.001; two-way ANOVA with Holm-Sidak multiple comparisons. ### p < 0.001, one-way ANOVA with Dunnett’s multiple comparisons.

2.3.3 LFn-fla/PA-induced cytokine release depended on caspase-1 and GSDMD

In consistent with results from the NAIP deficient mice, LFn-fla/PA-induced cytokine release was abolished in the caspase-1 deficient mice (*Figure 2.3A-C*). Flagellin induces pyroptosis through GSDMD-dependent pore formation on cell membrane.[130-132] To determine whether flagellin induces inflammation through pyroptosis, we injected GSDMD deficient mice with LFn-fla plus PA, and monitored cytokine concentrations in these mice. LFn-fla/PA-induced cytokine release was significantly reduced in the GSDMD deficient mice (*Figure 2.3A-C*). These data indicate that pyroptosis following inflammasome activation is responsible for LFn-fla/PA-induced cytokine release *in vivo.*
Figure 2.3. Caspase 1 and GSDMD dependent pyroptosis is essential to LFn-Fla/PA-induced IL-1β (A), IL-6 (B), and TNFα (C) release.

(A-C) C57BL/6J (WT), Caspase 1−/−, Gsdmd−/− mice were injected intravenously with LFn-Fla/PA (3 μg each). Blood samples were collected right before and at 90 min, 4 h, and 8 h after injection. IL-1β (A), IL-6 (B), and TNFα (C) concentrations in plasma were measured by ELISA assay (n=4 for each group). Error bars denote SEM. one asterisk, p<0.05; two asterisks, P < 0.01; three asterisks, P<0.001; two-way ANOVA with Holm-Sidak multiple comparisons.

2.3.4 Depletion of monocytes/macrophages by chlodronate significantly reduced flagellin-induced TNFα release

Monocytes and macrophages are major sources of TNFα in vivo.[120] To determine whether monocytes and macrophages are the major sources of LFn-fla/PA-induced TNFα release, we depleted monocytes and macrophages by pre-treating mice with clodronate following published protocol.[120] Depletion of monocytes and macrophages indeed dramatically reduced LFn-fla/PA-induced TNFα release (Figure 2.4A-C). Depletion of monocytes and macrophages also significantly reduced LFn-fla/PA-induced IL-6 and IL-1β release.
Figure 2.4. LFn-Fla/PA-induced cytokine release \textit{in vivo} required macrophages/monocytes.

C57BL/6J mice were injected intravenously with control liposomes (Lipo) or clodronate-containing liposomes (Cldn) were given 24 hours prior to intravenous injection of LFn-Fla/PA (3 μg each). Mice (C57BL/6J with or without macrophage depletion) were orbital sinus injected with LFn-Fla/PA (3 μg each). Blood samples were collected right before and at 90 min, 4 h, and 8 h after injection. IL-1β (A), IL-6 (B), and TNFα (C) concentrations in plasma were measured by ELISA assay (n=4 for each group). Error bars denote SEM. One asterisk, p<0.05; two asterisks, P < 0.01; three asterisks, P<0.001; two-way ANOVA with Holm-Sidak multiple comparisons.

2.3.5 LFn-EprJ/PA induced pro-inflammatory cytokine release in mice was inflammasome activation dependent

Flagellin and conserved type III secretion system (T3SS) rod components of the Gram-negative bacteria are potent activators of NLRC4.\cite{59, 113} To determine whether inflammasome activation is a common mechanism for cytokine release, C57BL/6J mice were challenged with the \textit{E. coli} T3SS rod protein EprJ, which is a potent activator of the NLRC4 inflammasome. Injection of LFn-EprJ plus PA, but not LFn-EprJ alone, increased plasma concentrations of IL-1β, IL-6, and TNFα (Figure 2.5A-C). It has been reported that EprJ can induce caspase-1 dependent inflammasome activation and pyroptosis.\cite{119} LFn-EprJ/PA-induced cytokine release was abolished in caspase-1 deficient mice, and also
significantly reduced in the GSDMD deficient mice (Figure 2.5D-F). Thus, EprJ-induced IL-1β, IL-6, and TNFα release depends on inflammasome activation and pyroptosis. These data demonstrate that inflammasome activation in vivo induces release of multiple proinflammatory cytokines through direct or indirect mechanisms.

Figure 2.5. E. coli T3SS rod protein EprJ induced proinflammatory cytokine release in vivo through inflammasome activation.

(A-C) C57BL/6J mice were injected intravenously with LFn-EprJ (1.5 μg) and LFn-EprJ plus PA (1.5 μg), respectively. Blood samples were collected right before and at 90 min, 4 h, and 8 h after injection. IL-1β (A), IL-6 (B), and TNFα (C) concentrations in plasma were measured by ELISA assay (n=3 for each group). (D-F) C57BL/6J (WT), Caspase 1\(^{-/-}\), Gsdmd\(^{-/-}\) mice were injected intravenously with LFn-EprJ (1.5 μg) and LFn-EprJ plus PA (1.5 μg), respectively. Blood samples were collected right before and at 90 min, 4 h, and 8 h after injection. IL-1β (D), IL-6 (E), and TNFα (F) concentrations in plasma were measured by ELISA assay (n=4 for each group). Error bars denote SEM. One asterisk,
p<0.05; two asterisks, P < 0.01; three asterisks, P<0.001; two-way ANOVA with Holm-Sidak multiple comparisons.

2.3.6 LFn-fla/PA induced IL-6 and TNFα release in TLR4 deficient mice in vivo but not in macrophages isolated from TLR4 deficient mice in vitro

LPS can induce cytokine production through a TLR4-dependent mechanism.[133] Therefore, both LFn-fla and PA utilized was purified from the ClearColi E. coli strain, which produces a mutant form of LPS that does not trigger the endotoxic response to minimize the potential effect from LPS.[134] In addition, we used TLR4 deficient mice to further exclude the possible effect of LPS contamination. We verified that injection of LFn-fla/PA into TLR4 deficient mice induced release of IL-1β, IL-6, and TNFα (Figure 2.6A-C). As expected, incubation of TLR4 deficient BMDMs with LFn-fla/PA induced IL-1β release (Figure 2.6D). However, LFn-fla/PA failed to induce IL-6 and TNFα release in the TLR4 deficient BMDMs (Figure 2.6E, F). We also tested primary macrophages from peritoneal cavity and liver. LFn-fla/PA failed to induce IL-6 and TNFα release in these primary macrophages either (Figure 2.7). Since depletion of macrophages and monocytes by clodronate drastically reduced LFn-fla/PA-induced IL-6 and TNFα release, we examined whether monocytes might be responsible for the cytokine production. LFn-fla/PA failed to induce IL-1β, IL-6, and TNFα release from monocytes (Figure 2.8).
Figure 2.6. LFn-Fla/PA induced proinflammatory IL-6 and TNFα release in vivo, but not in vitro.

(A-C) C57BL/6J WT or Tlr4−/− mice were injected intravenously with LFn-fla/PA (3 μg each). Blood samples were collected right before and at 90 min, 4 h, and 8 h after injection. IL-1β (A), IL-6 (B), and TNFα (C) concentrations in plasma were measured by ELISA assay (n=4 for each group). Error bars denote SEM. One asterisk, p<0.05; two asterisks, P < 0.01; three asterisks, P<0.001; two-way ANOVA with Holm-Sidak multiple comparisons. (D-F) BMDMs from TLR4 deficient mice were incubated with LPS (200
ng/ml), PA (1 μg/ml), LFn-fla (1 μg/ml), and LFn-fla plus PA, respectively. Concentrations of IL-1β (D), IL-6 (E), and TNFα (F) in supernatant were measured at 6 hours post-treatment. Mean with SD of three independent experiments (three replicates) are shown. (G-I) C57BL/6J mice were injected intravenously with IL-1RA (1 mg/kg body weight) 10 min prior to intravenous injection of LFn-Fla/PA (3 μg each). Blood samples were collected right before and at 90 min, 4 h, and 8 h after injection. IL-1β (G), IL-6 (H), and TNFα (I) concentrations in plasma were measured by ELISA assay (n=4 for each group). Error bars denote SEM. One asterisk, p<0.05; two asterisks, P < 0.01; three asterisks, P<0.001; two-way ANOVA with Holm-Sidak multiple comparisons.

2.3.7 LFn-fla/PA induced IL-6 and TNFα release was not inhibited by IL-1β receptor antagonist

Inflammasome activation leads to production and release of IL-1β and IL-18. It is known that IL-1β induces IL-6 and TNFα production and release in mice.[135, 136] Thus, we investigated whether LFn-fla/PA-induced IL-6 and TNFα release downstream of IL-1β. If that is the case, pretreatment of mice with the IL-1β receptor antagonist should inhibit IL-6 and TNFα release. We found pretreatment of mice with the IL-1β receptor antagonist did not significantly reduce the plasma concentrations of IL-6 and TNFα following LFn-fla/PA injection (Figure 2.6G-I).
Figure 2.7. LFn-fla/PA failed to induce IL-6 and TNFα release in primary *Tlr*4−/− macrophages.

(A-C) Peritoneal macrophages isolated from *Tlr*4−/− mice were incubated with LPS (200 ng/ml), PA (1 μg/ml), LFn-fla PA (1 μg/ml), and LFn-fla/PA, respectively for 6 h. Concentrations of IL-1β, IL-6, and TNFα in the supernatant were measured by ELISA assays.

(D-F) Kupffer cells isolated from *Tlr*4−/− mice were incubated with LPS (200 ng/ml), PA (1 μg/ml), LFn-fla PA (1 μg/ml), and LFn-fla/PA, respectively for 6 h. Concentrations of IL-1β, IL-6, and TNFα in the supernatant were measured by ELISA assays at 6 hours post-treatment. Mean with SD of three independent experiments (three replicates) are shown.
Figure 2.8. LFn-fla/PA failed to induce IL-6 and TNFα release in Tlr4-/- monocytes. (A-C) Monocytes from isolated from Tlr4-/- mice were incubated with LPS (200 ng/ml), PA (1 μg/ml), LFn-fla PA (1 μg/ml), and LFn-fla/PA, respectively for 6 h. Concentrations of IL-1β (A), IL-6 (B), and TNFα (C) in supernatant were measured at 6 hours post-treatment. Mean with SD of three independent experiments (three replicates) are shown.

2.4 Discussion

Flagellin is a potent activator of a broad range of cell types involved in innate and adaptive immunity. It is a potent inducer of cytokine production in a human promonocytic cell line, reaching maximal activity at the sub-nanomolar range.[96] Several studies established TLR5 as the receptor for extracellular flagellin.[55, 72, 137] When flagellin is present intracellularly, it signals via NAIP5 and NLRC4.[87, 111, 112] In our study, extracellular flagellin cannot induce proinflammatory cytokine release in mice in vivo, nor in macrophages in vitro. Generation and release of cytokines into blood in vivo was only observed when flagellin was translocated into the cytosol via PA/LFn mediated uptake. Consistent with this observation, deficiency of TLR5 did not influence plasma cytokine level upon treatment with LFn-fla/PA. In contrast, deficiency of pyroptosis-related genes including naips, caspase 1, and Gsdmd abolished the release of IL-1β, IL-6, and TNFα,
suggesting that flagellin induces proinflammatory cytokine release through inflammasome activation and pyroptosis. Experiments with another virulence factor, the T3SS inner rod protein EprJ from *E. coli*, support the idea that cytokine secretion depends on inflammasome activation and pyroptosis. To our best knowledge, no extracellular receptor has been reported for EprJ. LFn-EprJ/PA could lead to increased plasma cytokine concentrations in C57BL/6J mice but not in caspase 1 or GSDMD deficient mice. Two cytokines, IL-1β and IL-18, are generated directly from inflammasome activation. Our data indicate that inflammasome activation *in vivo* results in release of multiple proinflammatory cytokines including IL-6 and TNFα that are known to play important roles in the pathogenesis of infectious disease, thereby establish inflammasome activation and subsequent pyroptosis as an essential mechanism contributing to cytokine storm during infection.

Cytokines play critical roles in orchestrating a rapid and effective response of leukocytes and parenchymal cells upon detection of microorganisms or stress signals. The major acute innate cytokines, IL-1, TNF-α, IL-6, are used to activate endothelial cells and tissue leukocytes locally, triggering cytokine-mediated amplification loops to promote chemokine release and endothelial cell adhesion molecule expression, slowing down blood flow, and increasing vascular permeability.[138] A cohort of cytokines (IL-1, IL-6, and TNF-α) are released to prepare the organism for defense against pathogens by initiating the acute response syndrome and to recruit antigen-presenting cells (APCs) to initiate adaptive immunity.[139]
As innate immune cells, both monocytes and macrophages are critical for host defense against pathogens. Monocytes circulate in the blood and spleen. Once recruited to tissues, monocytes can differentiate into macrophages.[140] Both cells are capable of phagocytose, secreting chemokines, and proliferating and recruiting other immune cells in response to infection.[141] Depletion of monocytes and macrophages dramatically reduced flagellin-induced cytokine release. Although our data did not prove that monocytes and macrophages are the primary sources of these cytokines, they indicate that pyroptosis of monocytes and/or macrophages is required for the generation and release of these cytokines in vivo. The plasma levels of IL-1β and TNFα peaked at a similar time (90 min after treatment), while IL-6 peaked later (4 h after treatment). IL-1β has been shown to trigger the generation of other cytokines in vivo, and IL-1 and TNFα mutually stimulate the production of each other.[135, 136] In our study, we found IL-6 and TNFα secretion are not IL-1 dependent since blocking of IL-1 receptor did not influence the plasma level of IL-6 and TNFα, indicating that flagellin induces pro-inflammatory cytokines secretion through inflammasome activation and pyroptosis, not as a secondary effect mediated by IL-1.

Our results indicate that flagellin-induced IL-1β, IL-6, and TNFα release in vivo depends on inflammasome activation and subsequent pyroptosis. However, the origin of pro-inflammatory cytokines remains elusive. LFn-fla/PA induced IL-1β release from mouse BMDMs or primary macrophages, suggesting that macrophages as a major source of IL-1β release in vivo following inflammasome activation. Since LFn-fla/PA could not induce IL-6 or TNFα secretion in mouse BMDMs in vitro, nor in primary macrophages or monocytes, it is not clear whether macrophages/monocytes are the major sources of these
cytokines in vivo and if so, that release of these cytokines may require specific in vivo environment. Alternatively, these cytokines could be generated by other type of cells downstream of macrophage/monocyte pyroptosis. In this regard, it is known that many cell types can produce IL-6.[142] Although TNFα was thought to be produced primarily by macrophages,[143] it could also be produced by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neurons.[144] Additional studies are necessary to elucidate the origin and mechanism of pro-inflammatory cytokine production, which may provide new method to intercept cytokine storm and related diseases. Nevertheless, our study has shed light on how flagellin as an important virulence factor binds inflammasome receptors and initiates vigorous proinflammatory response through a previously unrecognized mechanism in mouse model, establishing novel therapeutic targets.
CHAPTER 3. STRUCTURE BASIS OF FLAGELLIN INDUCED INFLAMMASOME ACTIVATION

3.1 Introduction

As discussed in chapter 1, flagellin D0 and D1 domain are essential for the recognition by its extracellular and intracellular receptors. Structural and mechanistic studies have provided profound insights into the process of NAIP-NLRC4 inflammasome assembly. However, how flagellin is recognized by NAIPs remains unclear. In this study, we used flagellin from P. aeruginosa and E. coli to investigate flagellin-induced inflammasome activation. Flagellin was fused to the cytosolic translocation domain of anthrax lethal factor (LFn) to enable efficient cytosolic delivery. LFn binds to anthrax protein protective antigen (PA), which delivers LFn-flagellin fusion protein into the cytoplasm through receptor-mediated endocytosis.[59, 145] In this study, we explored the role of the C-terminal D1 domain of flagellin in NAIP5/NLRC4 inflammasome activation. We found that even though the flagellin C-terminal D0 domain is enough for NAIP/NLRC4 inflammasome activation for the full-length flagellin, the C-terminal D1 domain also participates in the interaction with NAIPs. Our findings indicate the important role flagellin C-terminal D1 domain in ligand recognition and inflammasome activation.
3.2 Materials and methods

3.2.1 Construction of wild-type and mutant flagellin plasmid DNAs

FliC was fused to the cytosolic translocation domain of anthrax lethal factor (LFn) to enable efficient cytosolic delivery. LFn binds to anthrax protein protective agent (PA), which delivers the LFn-FliC fusion protein into the cytoplasm through receptor-mediated endocytosis.[59, 145] *P. aeruginosa* flagellin gene and the constructed Pa-Ec-100aa, Ec-Pa100aa, and Pa-Ec-11aa gene were digested with BamHI and XhoI and ligated into similarly digested vector pET28a-LFn which was previously constructed in our lab. Site-directed mutagenesis was conducted using the QuickChange mutagenesis kit following the manufacturer’s instruction (Agilent Technologies) to construct point mutation flagellin. PaD1EcD0 and EcD1PaD0 were synthesized and fused to *E. coli* flagellin and *P. aeruginosa* flagellin, respectively. Myc-tag was then inserted to the N-terminal of each plasmid for the transfection and co-immunoprecipitation experiment. The constructed plasmids are shown in table 2.

3.2.2 LFn fusion protein expression and purification

As described previously,[119] proteins were expressed using LPS free *E. coli* strain ClearColi BL21(DE3) (Lucigen Corporation, Cat#60810) at 37 °C for 4 hours with 500 μM IPTG after OD₆₀₀ reached 0.6-0.8. Bacteria were collected and lysed in 50 mM Tris-HCl and 300 mM NaCl. Proteins containing a His-tag were purified by affinity chromatography using HisPur Ni-NTA resin (Thermo Scientific, Cat#88222). Proteins
were then eluted with 250 mM imidazole in 50 mM Tris-HCL and 300 mM NaCl, and subsequently dialyzed against PBS to remove imidazole. Protein concentrations were determined by measuring their absorption at 280 nm before sterile filtration.

3.2.3 In vivo study

C57BL6 mice (10-12 weeks old) were injected with 3 μg of LFn-fliC or LFn- Pa-R488Q without PA, as indicated. Blood samples were collected before or at various time (90 min, 4 h, and 8 h) following injection of the bacterial proteins. To determine the inflammasome activation, blood samples were collected via retro-orbital bleeding. Blood samples were centrifuged at 10,000 g for 1 minute at room temperature to obtain plasma. IL-1β, IL-6, and TNFα were measured using ELISA following the manufacturer’s instruction.

3.2.4 Bone marrow derived macrophage (BMDM) culture and stimulation

BMDMs were prepared as described previously,[120] and seeded into 12-well cell culture plate (1 mL/well) or 96-well cell culture plate (100 μL/well) at a density of 1 x 10^6 cells/mL of RPMI-1640 medium containing 15% L929-cell conditioned medium (LCM). BMDMs were allowed to settle overnight and refreshed with Opti-MEM (Life Technologies, Cat#31985-070) before purified proteins were added. For the study of cytotoxicity, supernatant samples and cell pellets were collected 90 min after stimulation.
3.2.5 Lactate dehydrogenase cytotoxicity assay

To determine cell viability, the lactate dehydrogenase (LDH) CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Cat#G1780) was used. 50 µl of CytoTox 96 Reagent was added to each well of 96-well plate followed by 50 µl of supernatant samples and incubated for 10 min at room temperature or until a sufficient colorimetric change was visible. The reaction was stopped using the LDH stop solution, and the plate was read on a Cytation 5 at 490 nm.

3.2.6 Western blotting

For detection of active caspase-1 and IL-1β cleavage by western blotting, cells were washed with cold PBS and lysed with SDS sample buffer. Culture supernatants were precipitated with 1/10 volume of 2% sodium cholate and 1/10 volume of 100% trichloroacetic acid (TCA) and then dissolved in the SDS sample buffer. Total protein from lysates and supernatants (equivalent to $1 \times 10^6$ cells) was analyzed by western blotting for multiplex detection. Both pro-caspase-1 and p20 caspase-1 were determined using anti-caspase-1(p20) (Adipogen, Cat#AG-20B-0042) at 1:1000 dilution. Pro-IL-1β and IL-1β (p17) were detected using anti-IL-1β (GeneTex Cat#GTX74034) at 1:1000 dilution. Blots were imaged using the BIO-RAD ChemiDoc MP imaging system.
3.2.7 Co-immunoprecipitation binding assay

HEK 293T cells expressing recombinant flagellin and NAIP5 were solubilized in modified RIPA Buffer (50 mM Tris, pH 7.4, 10 mM MgCl2, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM NaF), and complete protease inhibitor cocktail P8340 (Sigma). Cell lysates were incubated with 2 μg/ml of a polyclonal antibody against HA, Myc, or mouse IgG, and further incubated with protein G agarose beads (Sigma). After 3-6 washes with lysis buffer, immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis and immunoblotting with antibodies against Myc or HA.

3.2.8 Statistical analysis

Statistical analysis was performed in Prism GraphPad 9.0.0. Statistical analysis was performed using one-way ANOVA with Dunnett’s multiple comparisons for multiple groups and two-way ANOVA repeated measures with Holm-Sidak multiple comparisons for time point studies. A p value <0.05 was considered significant.
Table 1. Primers used in this study

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<td>Myc tag is added at N-terminal of Pa-Ec_D1</td>
</tr>
<tr>
<td>Myc-Ec-Pa_D1</td>
<td>Myc tag is added at N-terminal of Ec-Pa_D1</td>
</tr>
<tr>
<td>Myc-Ec-Pa_D1-Q497R</td>
<td>Myc tag is added at N-terminal of Ec-Pa_D1-Q497R</td>
</tr>
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</table>
3.3 Results

3.3.1 C-terminal domains of *P. aeruginosa* flagellin plays a more important role in NAIP/NLRC4 inflammasome activation

Flagellin is known to interact with NAIP through the D0 domain. To elucidate whether N-terminal or C-terminal D0 domain plays role in flagellin induced NAIP/NLRC4 inflammasome activation, *E. coli* and *P. aeruginosa* flagellin C-terminal D1 and D0 domain were exchanged with each other (Ec-Pa_100aa and Pa-Ec_100aa). NAIP/NLRC4 inflammasome activation by Ec-Pa_100aa was evident by the release of LDH into the supernatant, and caspase-1 activation and IL-1β cleavage. In contrast, Pa-Ec_100aa failed to induce pyroptosis of BMDMs, caspase-1 activation, or IL-1β cleavage. These results indicate that C-terminal D1 and D0 domain are more important than the N-terminal D1 and D0 domain in full-length *P. aeruginosa* flagellin induced NAIP/NLRC4 inflammasome activation. (Figure 3.1A, B) Further *in vitro* study of replacement of *P. aeruginosa* flagellin C-terminal 11 amino acid residues with *E. coli* flagellin (Pa-Ec_11aa) shows that the last 11 amino acid residues of *P. aeruginosa* flagellin are essential for flagellin induced NAIP/NLRC4 inflammasome activation. (Figure 3.1A) These data indicate that flagellin C-terminal domains play a more important role in NAIP/NLRC4 inflammasome activation than N-terminal domains.
Figure 3.1. *P. aeruginosa* flagellin C-terminal domains are critical in NAIP/NLRC4 inflammasome activation.

(A-B) BMDMs from C57BL/6J WT were incubated with 1 μg/ml of LFn fused recombinant flagellin from *P. aeruginosa* with PA for 90 min. Cell viability was assessed by measuring LDH release into the supernatant, and caspase-1 activation and IL-1β cleavage were detected by western blot.

3.3.2 *P. aeruginosa* flagellin C-terminal arginine is critical for inflammasome activation but not for NAIP recognition

To further explore the role of the C-terminus of flagellin in inflammasome activation, further mutagenesis experiments were performed to replace the flagellin C-terminal last 11 amino acids. There are three different amino acid residues between *P. aeruginosa* and *E. coli* flagellin among the last 11 residues. (Figure 3.2A) Site-direct point mutation of the three residues (Pa-L479V, Pa-A482Q, Pa-R488Q) *in vitro* study results indicate that the last arginine of *P. aeruginosa* flagellin is critical for the activation of
NAIP/NLRC4 inflammasome, (Figure 3.2B) which is consistent with a previous study.[146] While the *in vivo* study indicated that Pa-R488Q still induced the release of the proinflammatory cytokine, *in vitro* study was then performed and the result illustrated that Pa-R488Q can induce inflammasome activation and pyroptosis at a higher dose. (Figure 3.2C, D) These data indicate that the mutation of flagellin C-terminal arginine lowers the toxicity of flagellin, but not affects the interaction between flagellin and NAIP.
Figure 3.2. *P. aeruginosa* flagellin C-terminal arginine is essential for NAIP/NLRC4 inflammasome activation.

(A) The alignment of C-terminal last 11 amino acids from *E. coli* and *P. aeruginosa* flagellin. (B-C) BMDMs from C57BL/6J WT were incubated with 1 μg/ml of LFn fused recombinant flagellin from *P. aeruginosa* with PA for 90 min. Cell viability was assessed by measuring LDH release into the supernatant, and caspase-1 activation and IL-1β cleavage were detected by western blot. (D) C57BL/6J mice were injected intravenously with 3 μg of LFn-fliC and LFn-Pa-R488Q from *P. aeruginosa* with PA (n=5 for all experimental groups). One asterisk, p<0.05; two asterisks, P < 0.01; three asterisks, P<0.001; one-way ANOVA with Dunnett’s multiple comparisons.
3.3.3 C-terminal arginine mutation did not affect NLRC4 recruitment and phosphorylation

It has been reported that the phosphorylation of NLRC4 ser 533 is critical for the activation of NAIP/NLRC4 inflammasome.[147] To determine how the C-terminal arginine plays its role in NAIP/NLRC4 inflammasome activation, it was mutated to lysine and glutamine (Pa-R488K and PA-R488Q) to explore if the side chain length of amino acid matters in the inflammasome activation. *In vitro* study shows that with the treatment of mutant Pa-R488K, the cell viability was significantly increased, and the activation of caspase-1 and cleavage of IL-1β was significantly decreased. (Figure 3.3A) Mutation of arginine to glutamine eliminated the activation of caspase-1 and cleavage of IL-1β, the LDH release result indicates that Pa-R488Q mutation cannot induce cell death at a lower dose. (Figure 3.2B) However, the mutants still can interact with NAIP5 and induce phosphorylation of NLRC4. (Figure 3.3B, C) These results indicate that both the positive charge and length of the side chain are essential for the function of flagellin. To further explore the critical role of arginine played in flagellin induced NAIP/NLRC4 inflammasome activation, *E. coli* flagellin glutamine 497 was mutated to arginine (Ec-Q497R). *In vitro* study indicates that Ec-Q497R is not able to induce cell pyroptotic death, either the activation of caspase-1 and IL-1β cleavage. (Figure 3.3A) These results illustrated that even though *P. aeruginosa* flagellin C-terminal arginine is critical for its function, however, arginine alone is not enough to make *E. coli* flagellin virulent.
Figure 3.3. C-terminal arginine is essential for the function of *P. aeruginosa* flagellin, the mutation of it abrogates inflammasome activation but not affects NAIP binding or NLRC4 phosphorylation.

**(A)** BMDMs from C57BL/6J WT were incubated with 1 μg/ml of LFn fused recombinant flagellin from *P. aeruginosa* with PA for 90 min. Cell viability was assessed by measuring LDH release into the supernatant, and caspase-1 activation and IL-1β cleavage were detected by western blot. **(B)** Co-immunoprecipitation assays of *P. aeruginosa* flagellin and NAIP5. The mutation of arginine to glutamine doesn’t affect the interaction between flagellin and NAIP5. **(C)** NLRC4 phosphorylation with *P. aeruginosa* flagellin treatment. BMDMs from C57BL/6J WT were incubated with 1 μg/ml of LFn fused recombinant flagellin from *P. aeruginosa* with PA for 90 min. NLRC4 phosphorylation from the supernatant and cell lysate was detected by western blot.
3.3.4 *P. aeruginosa* flagellin C-terminal D1 domain plays a role in flagellin-induced inflammasome activation

It has been reported that the D0 domain alone can interact with NAIP and cause inflammasome activation. Besides, the D0 domain also participates in flagellin TLR5 interaction, while whether the D1 domain plays a role in flagellin NAIP interaction remains unknown. To explore whether the D1 domain of flagellin contributes to flagellin NAIP interaction, *P. aeruginosa* flagellin C-terminal D1 domain replaced by *E. coli* flagellin C-terminal D1 domain (Pa-Ec_CD1) was constructed and tested. It is interesting that Pa-Ec_CD1 neither induce cell pyroptotic death, nor inflammasome activation which depicted as caspase-1 activation and IL-1β cleavage. ([Figure 3.4 A](#)) These data indicate that *P. aeruginosa* flagellin C-terminal D1 domain plays a role in inflammasome activation and subsequent pyroptosis.

To further prove whether C-terminal D1 domain of flagellin participates in flagellin induced inflammasome activation. *E. coli* flagellin C-terminal D1 domain was then replaced by *P. aeruginosa* flagellin C-terminal D1 domain (Ec-Pa_CD1). As our expected, Ec-Pa_CD1 cannot induce NAIP/NLRC4 inflammasome activation. While the mutation of C-terminal glutamine to arginine (Ec-Pa_CD1-Q497R) make the flagellin virulent and can induce inflammasome activation and subsequent pyroptotic cell death in a dose dependent manner. ([Figure 3.4 A, B](#))
Figure 3.4. Flagellin C-terminal D1 domain contributes to NAIP/NLRC4 inflammasome activation.

(A) BMDMs from C57BL/6J WT were incubated with 1 μg/ml of LFn fused recombinant flagellin Pa-Ec_CD1, Ec-Pa_CD1, and Ec-Pa_CD1 Q497R with PA for 90 min, respectively. Cell viability was assessed by measuring LDH release into the supernatant, and caspase-1 activation and IL-1β cleavage were detected by western blot. (B) BMDMs from C57BL/6J WT were incubated with 1 μg/ml, 5 μg/ml, and 10 μg/ml of LFn fused recombinant flagellin Ec-Pa_CD1 Q497R with PA for 90 min, respectively. Cell viability was assessed by measuring LDH release into the supernatant, and caspase-1 activation and IL-1β cleavage were detected by western blot.

3.4 Discussion

The bacterial flagellar system is one of the most complex and dynamic biological proteinaceous structures which provide mobility of flagellated bacteria. Flagellin monomer is also known as a virulence factor that can be recognized by extracellular receptor TLR5 and intracellular receptor NAIP of host cells. However, the mechanisms for how flagellin interact with NAIP remains elusive.
Previous studies indicated that the flagellin C-terminal D1 domain is essential for TLR5 recognition, and the C-terminal D0 domain participates in TLR5 recognition as well. Besides, the D0 domain is critical for NAIP5/6 recognition and interaction. Several studies have illustrated the amino acid residues on the flagellin C-terminal that are essential for NAIP recognition. In this study, we identified the role of the flagellin C-terminal D1 domain in flagellin NAIP recognition and interaction. *P. aeruginosa* flagellin can interact with NAIP5, while the exchange of the C-terminal D1 domain with the corresponding sequence from *E. coli* flagellin, which cannot be recognized by NAIP5, lost its interaction with NAIP5 and subsequent inflammasome activation. It is interesting that when *E. coli* flagellin C-terminal D1 domain exchanged with corresponding sequence from *P. aeruginosa* flagellin together with the glutamine 497 mutated to arginine made *E. coli* flagellin virulent and can induce inflammasome activation and subsequent pyroptosis in a dose dependent manner. We propose this would be related to the conformational change due to the difference of C-terminal D1 domain between virulent and non-virulent flagellin. The slightly change of C-terminal D0 domain direction pointed out that flagellin C-terminal D1 domain plays its role in NAIP recognition through affecting the folding direction of C-terminal D0 domain. Flagellin C-terminal D0 domain cannot bind to NAIP binding pocket because of the slightly change in its folding direction.

Structural study of flagellin unveiled the mechanisms of flagellin-NAIP interaction. It has been reported that the flagellin D0 domain is essential for flagellin-TLR5 interaction, however, whether the D1 domain plays a role in flagellin-NAIP interaction remains elusive. Even though we proved that the flagellin C-terminal D1 domain participates in interaction between flagellin and NAIP, how does the C-terminal D1 domain contribute to
the interaction with NAIP and NLRC4 inflammasome activation remain elusive. It has been reported that the NAIP/NLRC4 inflammasome can be activated by the D0 domain alone.[86] More studies are needed to prove whether the C-terminal D1 domain exchange caused conformation change of flagellin and thus indirectly affected the interaction between the D0 domain and NAIP5, or it indeed participates in the interaction of flagellin with NAIP. Besides, it is proved the single C-terminal arginine mutation abrogates the flagellin induced NAIP/NLRC4 inflammasome activation since the mutant still can interact with NAIP5 and induce NLRC4 phosphorylation. How does it work and why the arginine is critical remain elusive. However, whether the length or the positive charge of the arginine side chain contributes more to flagellin-NAIP interaction, or both are essential for the interaction remains to be proved. In our study, the C-terminal arginine mutation did not affect the interaction between flagellin and NAIP and the mutant can still lead to phosphorylation of NLRC4 but is not capable of activating inflammasome at a lower dosage. In vivo study showed that mutants without the C-terminal arginine still induced proinflammatory cytokine release. The result further indicated that interaction between flagellin and NAIP and downstream NLRC4 phosphorylation is essential but not enough for inflammasome activation. We are proposing if any other mediators participate in the NLRC4 inflammasome activation, either between NAIP and NLRC4 or between NLRC4 and downstream factors such as ASC and caspase 1.

In conclusion, our structural and biochemical data showed that (i) the last arginine from tested flagellin is essential for NAIP/NLRC4 inflammasome activation. Both the positive charge and length of mutated residue are critical for the function of flagellin. (ii) The C-terminal D1 domain plays a role in the function of full-length flagellin through
affecting the folding direction of C-terminal D0 domain. (iii) In contrast to the C-terminal three leucine residues mutation will make flagellin lose its interaction with NAIP,[59] the mutation of arginine to other residues did not affect its binding to NAIP. Flagellin has been widely used in vaccines investigation and some of them are in preclinical trials; it also has been applied in cancer therapy. To get a thorough understanding of the structure function relationship would be helpful for its using in vaccine development and any other therapy methods.
CHAPTER 4. DISCUSSION AND FUTURE WORK

The bacteria flagellar system is one of the most complex and dynamic biological proteinaceous structures which provide motility of flagellated bacterial. While our knowledge of the filament and its component flagellin monomer has increased in the past few decades, many open questions remain. There is still a long way to go to fully understand the process of filament growth and its pathogenesis.

Despite unprecedented insights into the structure and activation mechanisms of the NAIP-NLRC4 inflammasome, there are still gaps in our knowledge. In 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*. Structural study of flagellin unveiled the mechanisms of flagellin-NAIP interaction.[85] It has been reported that the flagellin D0 domain is essential for flagellin-TLR5 interaction, however, whether the D1 domain plays a role in flagellin-NAIP interaction remains elusive.[56] Even though we proved that the flagellin C-terminal D1 domain participates in interaction between flagellin and NAIP, how the D1 domain contributes to the interaction with NAIP and NLRC4 inflammasome activation needs further exploration. More studies are needed to explore whether the D1 domain caused conformation change of flagellin, or if it indeed participated in the interaction between flagellin and NAIP. Besides, it is reported the single C-terminal arginine mutation abrogates the flagellin induced NAIP/NLRC4 inflammasome activation,[146] since the mutant still can interact with NAIP5 and induce NLRC4 phosphorylation, how it works and why the arginine is critical remain elusive. However, whether the length or the positive charge of the arginine side chain contributes more to
flagellin-NAIP interaction, or both are essential for the interaction remains to be proved. In our study, C-terminal arginine mutation did not affect the interaction between flagellin and NAIP, it can also cause the phosphorylation of NLRC4, but not for inflammasome activation in a lower dosage. *In vivo* study showed that the mutation of C-terminal arginine can still induce proinflammatory cytokine release. The result further indicated that interaction between flagellin and NAIP and downstream NLRC4 phosphorylation is essential but not enough for inflammasome activation. We are proposing if any other mediators participate in the NLRC4 inflammasome activation, either between NAIP and NLRC4 or between NLRC4 and downstream factors such as ASC and caspase 1.

Generally, flagellin induced immune response causes the release of proinflammatory cytokines. The studies also illustrated that flagellin induces transcription of proinflammatory cytokines through interaction with TLR5.[55, 72, 137] However, our study showed that flagellin induced cytokine release *in vivo* is inflammasome activation and subsequent pyroptosis dependent, but not TLR5 dependent. Interestingly, extracellular flagellin cannot induce cytokine release either *in vivo* or *in vitro*. While cytosol flagellin induces cytokine release only with the presence of inflammasome activation and subsequent pyroptosis. How inflammasome activation induces the transcription of proinflammatory cytokines remains elusive. The potential connection between inflammasome activation and TLRs induced transcription pathway still need to be explored. Whether inflammasome activation directly or indirectly induce proinflammatory cytokine release is still a mystery. It is interesting that in our study, flagellin cannot induce primary macrophage release cytokine except IL-1β even with the presence of inflammasome activation and pyroptosis, which contradicts with previous research. While
the depletion of macrophages and monocytes in vivo eliminated the release of proinflammatory cytokines induced by flagellin which indicates that macrophages and monocytes are the major sources for proinflammatory cytokines expression and release in vivo. Whether the in vivo environment is essential for the expression and transcription of proinflammatory cytokines remains unknown.

At least 4 mouse NAIPs are required to recognize multiple bacterial ligands whereas a single human NAIP and its isoforms succeed to do so. It remains unclear how hNAIP binds multiple ligands and if additional upstream sensors are required for hNAIP or if it binds directly to those bacterial ligands. Answers to these questions would shed light on the field of innate immune recognition and inflammasome activation. 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 backgrounds.

NAIP-NLRC4 inflammasome limits bacterial proliferation and dissemination through the activation of multiple pathways, including secretion of signaling molecules such as IL-1β and IL-18, pyroptosis, and clearance of infected cells from the host. Mice with NLRC4 deficiency do not readily succumb to infection in a specific-pathogen-free environment, indicating that lacking NLRC4 in mice does not lead to hyper-susceptibility to environmental microbiota. Besides, some mammalian species, such as pigs, lack functional NLRC4, and NAIPs. NAIP-NLRC4 inflammasome activation might protect against certain pathogens invasion in mice. However, over activation of NAIP-NLRC4 would cause autoinflammatory conditions, such as systemic inflammation, sepsis,
and death in humans and mice. Therefore, the evolutionary advantage of encoding NAIP-NLRC4, against a backdrop of the inherent risk of triggering overt inflammation, probably extends beyond protection against bacterial infection.

In autoinflammatory conditions associated with gain-of-function mutations of NLRC4, progress has been made to transfer fundamental discoveries to clinical practice. Patients with macrophage activation syndrome have been successfully treated by targeting cytokines production caused by constitutive NAIP-NLRC4 inflammasome activation.[148] Another issue that requires attention is the consequences of sustained inhibition of inflammasome-associated cytokines in patients since these proinflammatory cytokines are critical for the host to control infection. Would these complications be alleviated if NAIP or NLRC4 is specifically inhibited instead? Unfortunately, no pharmacological inhibitors have been discovered. Identifying safe and efficacious compounds that specifically inhibit NAIP or NLRC4 could provide 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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