Flagellins of *Salmonella Typhi* and Nonpathogenic *Escherichia coli* Are Differentially Recognized through the NLRC4 Pathway in Macrophages

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Key Words

Flagellin · Toll-like receptor 5 · NLRC4 · Macrophage · Immune response

Abstract

Flagellin is recognized by both Toll-like receptor (TLR)5 and NAIP5/NLRC4 inflammasome receptors. We hypothesized that the flagellins derived from different bacteria might differentially activate TLR5 and/or NAIP5/NLRC4 signal pathways. To test this, the immune recognition of recombinant flagellins derived from pathogenic *Salmonella Typhi* (SF) and the non-pathogenic *Escherichia coli* K12 strain MG1655 (KF) were examined by the activation of TLR5 and NLRC4 pathways in various cell types. While flagellins SF and KF were not distinguishable in activating the TLR5 pathway, KF induced significantly less interleukin-1 β production and pyroptotic cell death in peritoneal macrophages than SF, and showed markedly lower efficiency in activating caspase-1 through the NLRC4 pathway than SF. Macrophages may differentially recognize flagellins by intracellular sensors and thereby initiate the immune response to invading pathogenic bacteria. Our findings suggest an active role of flagellin as an important determinant in host differential immune recognition and for the control of bacteria infection.

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Introduction

A set of distinct microbial structures referred to as pathogen-associated molecular patterns (PAMPs) are usually sensed by the host innate immune system through germline-encoded pattern-recognition receptors (PRRs) [1–4]. These PRRs are localized on the cell surface or in intracellular compartments to sense PAMPs extracellularly or intracellularly [1, 5].

Bacterial flagellin acts as a typical PAMP and could be recognized by two different PRRs – the cell surface Toll-like receptor (TLR)5 [6] and the cytosolic NLRC4 inflammasome receptor NAIP5 [7, 8], which appear to have evolved independently [9]. TLR5 detects flagellin at the cell surface and signals through MyD88, resulting in the induction of proinflammatory cytokines, chemokines, antimicrobial defenses and antiapoptotic components [10–12]. NAIP5/NLRC4 senses flagellin within the cytosol and assembles the inflammasome to activate the cysteine protease caspase-1, leading to the proteolytic processing and secretion of the proinflammatory cytokine interleukin (IL)-1 β [13] and pyroptotic cell death [14]. This extracellular and intracellular dual sensor strategy

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implies that the host immune system can recognize flagellated pathogens and initiate immune responses based not only on whether PAMPs are present, but also on where the PAMPs are presented [1].

Flagellin is a major structural protein of bacteria flagella essential for the motility of bacteria. The conserved domains at its amino and carboxyl termini harbor the key motifs crucial for TLR5 and NAIP5 recognition [9, 15]. The flagellin molecule also contains a hypervariable domain in its middle part [16]. We and others have previously demonstrated that deletion of the hypervariable domain of *Salmonella* flagellins changes the profile of pathogenic *Salmonella* Typhi (SF)-induced innate responses and even alters the systemic activation of TLR5-dependent immunity [17, 18]. These findings hint that the non-conserved region of flagellins might also play a role in TLR5 recognition.

The vast diversity of flagellins in size and composition in different bacteria strains and species have raised the question of whether the host immune system could differentially recognize flagellated pathogens and selectively initiate the immune response based on the nature of flagellins. As one of the major PAMPs, the flagellin is detected by the innate immune system to maintain an immune balance between large amounts of commensal bacteria and host, and to initiate potent immune defense against possible invading pathogenic bacteria. Previous studies have shown that free flagellin from *Salmonella* strains alone is able to activate the NLRC4 pathway to a certain extent and induce production of the highly potent proinflammatory cytokine IL-1 β in peritoneal macrophages (M Φ s) [19], implying that peritoneal M Φ s could detect free flagellin before the pathogenic bacteria invade cells. However, the question is raised whether PRRs could differentially recognize nonpathogenic flagellins, since it is possible to induce undesired excess immune responses to commensal bacteria. Then, we hypothesized that the flagellins derived from pathogenic or nonpathogenic bacteria might differentially activate TLR5 and/or NAIP5/NLRC4 signal pathways [9]. In this study, we selected two recombinant flagellins derived from SF and the well-known nonpathogenic bacteria strain *Escherichia coli* K12 strain MG1655 (KF), and tested their ability to activate TLR5 or NLRC4 pathways. These two flagellins differ in their amino acid sequences. For example, SF and KF have 20.2% sequence divergence in C-terminal 35 amino acids which are critical for the NLRC4 pathway [9]. We found no significant differences in the activation of the TLR5 pathway by SF and KF. However, KF induced significantly lower IL-1 β production than SF in M Φ s, and

showed a lower efficiency in activating caspase-1 through the NLRC4 pathway. Our results collectively show that the host immune system could recognize and respond to different flagellins differentially, implying an active role of flagellin as an important determinant for host immune recognition and the control of bacterial infection.

Materials and Methods

Purification and Verification of Recombinant Protein

Recombinant KF, SF and the control protein, HIV-1 p24, were cloned, expressed, and purified as described previously [17, 20]. The purified proteins were treated with Acrodisc syringe filters (Pall, Port Washington, N.Y., USA) and preserved at -80°C . Quantification of the purified proteins was performed using the Bradford assay. RAW 264.7 cells which could sensitively respond to lipopolysaccharide (LPS) and bacteria DNA but not flagellin [21, 22] were used to exclude the presence of residual bacteria DNA and LPS contaminations.

Animals

Female C57BL/6 and BALB/c mice at 8–10 weeks of age were either obtained from Harlan Laboratories and kept at the animal facilities of the University Hospital of Essen or obtained from Beijing Laboratory Animal Research Center and kept in the Animal Center of the Wuhan Institute of Virology (WIV), Chinese Academy of Sciences, under specific pathogen-free conditions. Animal studies were performed according to Regulations for the Administration of Affairs Concerning Experimental Animals in China (1988), the Guideline for Animal Care and Use, WIV, Chinese Academy of Sciences, and the Guide for the Care and Use of Laboratory Animals. The protocols were reviewed and approved by the Laboratory Animal Care and Use Committee of WIV, Chinese Academy of Sciences, permit No. WIVA09201105, and by the local Animal Care and Use Committee (Animal Care Center, University of Duisburg-Essen, Essen, Germany, and the district government of Düsseldorf, Germany).

Caco-2 Cell Culture and Stimulation with Recombinant Flagellins

The human intestinal epithelial Caco-2 cells were grown in DMEM supplemented with 10% FBS (Gibco, Life Technologies Corp., Carlsbad, Calif., USA) and 100 U/ml of penicillin/streptomycin at 37°C in 5% CO_2 . For the cytokine release assays, cells were seeded at a density of 200,000 cells per well into 24-well polystyrene plates (Costar) and used 5 days later. After overnight culturing in serum-free medium, cells were stimulated with flagellins at the indicated concentrations for 6 h. Culture supernatants were then collected and tested for cytokine levels by specific enzyme immunoassays.

The Response of Splenocytes to Flagellin

The spleens were smashed using syringe pistons in PBS and filtrated through strainers (BD Biosciences, San Jose, Calif., USA). After washing with PBS and RPMI 1640 medium containing 10% FBS and 100 U/ml of penicillin/streptomycin, single cells were seeded into 24-well plates (0.8×10^7 cells/well, 0.4 ml). The cells and supernatants were collected separately to assess the levels of

costimulatory molecules and cytokines after stimulation. For detection of costimulatory molecules, cells were blocked with anti-Fc receptor antibody (BD Biosciences), stained with APC-labeled anti-CD11c, FITC-labeled anti-CD86, PE-labeled anti-CD80 and 7-Amino-actinomycin D (BD Biosciences), and analyzed using FACSCalibur (Becton Dickinson, Heidelberg, Germany). For detection of TLR5 expression, the cells were stained with APC-labeled anti-CD11c, FITC-labeled anti-F4/80, PE-labeled anti-CD80/PE-labeled isotype control (Rat IgG2a κ ; BD Biosciences)/PE-labeled anti-TLR5 (IMGENEX) and 7-Amino-actinomycin D.

Macrophage Collection and Incubation

Resident peritoneal M Φ s were collected from naïve animals by RPMI 1640 lavage as described previously [19], resuspended in RPMI 1640 containing 10% FBS, and seeded at a density of 1×10^5 cells/well in 96-well plates. After overnight incubation, nonadherent cells were removed by washing. Bone marrow-derived M Φ s (BMMs) were prepared from the femurs of mice by culture in RPMI 1640 containing 10% FBS with the addition of recombinant mouse macrophage colony-stimulating factor (25 ng/ml; eBioscience, San Diego, Calif., USA) and nonessential amino acids. After 7 days of induction, BMMs were seeded at the density of 1×10^5 cells/well in 96-well plates for cytokine and cell death assays or 8×10^5 cells/well in 12-well plates for mRNA and Western blotting analysis.

Stimulation of Macrophages with Flagellins

The M Φ s were pretreated with or without LPS at a concentration of 50 ng/ml for 3 h and stimulated with the indicated concentrations of recombinant flagellins. Alternatively, the cells were transfected with flagellins using DOTAP (Roche, Basel, Switzerland) [19]. Briefly, 15 μ l of DOTAP Liposomal Transfection Reagent was incubated with 10 μ l of purified flagellin for 20 min in serum-free media. After incubation, 1 ml of RPMI 1640 medium was added. An aliquot of 100 μ l was added to M Φ s in a 96-well plate. Supernatants were collected at the indicated time points. For the lactate dehydrogenase (LDH) release assay, cell lysis buffer was added 1 h before the collection of the supernatant samples. For the quantification of mRNA levels, the cells were collected 3 h after flagellin transfection.

LDH Release and Detection of Cytokines

The release of LDH was measured by the Cytotoxicity Detection Kit^{PLUS} (Roche). The cytokine concentrations were measured using ELISA kits (human IL-8 and MCP-1; BD Biosciences; mouse IL-6, IFN- γ and IL-1 β ; eBioscience). To exclude the release of pro-IL-1 β from dead cells, the IL-1 β values shown were normalized to the release of pro-IL-1 β from lysed M Φ s (mature IL-1 β = total IL-1 β signal – pro-IL-1 β lysis $\times$ percent release of LDH) as previously described [23].

Silencing of NLRC4

Small interfering RNAs (siRNA) were delivered into BMMs from BALB/c mice by electroporation as described previously [24]. 4×10^6 BMMs were mixed with 0.6 nmol siRNA (siControl: siGENOME Nontargeting siRNA No. 2; Thermo Scientific, Waltham, Mass., USA; siNLRC4: Mm_Card12_2 FlexiTube siRNA; Qiagen, Hilden, Germany) in 400 μ l and transferred to an electroporation cuvette (4-mm gap width, Bio-Rad, Hercules, Calif., USA) and incubated on ice for 10 min. After electroporation

(Gene Pulser X cell apparatus; Bio-Rad), the cells were seeded and subjected to the following assays 72 h after electroporation.

Western Blotting Analysis

8×10^5 BMMs seeded into 12-well plates were pretreated with or without LPS and then transfected with flagellins. In some cultures, the endogenous caspase-1 was blocked with the specific inhibitor z-YVAD-fmk (Calbiochem) at a final concentration of 2–8 μ M. Supernatants were collected and cells were lysed in 50 mM pH 8.0 Tris-HCl, 5 mM pH 8.0 EDTA, 150 mM NaCl, 1% Triton X-100 in the presence of a protease inhibitor cocktail (Pierce, Rockford, Ill., USA). After centrifugation, cytoplasmic lysates were used alone or combined with the supernatants. Proteins were precipitated with 10% trichloroacetic acid, washed with ethanol and subjected to SDS gel electrophoresis and Western blotting analysis with anti-caspase-1 p10 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), anti-mouse NLRC4 (Millipore, Billerica, Mass., USA), anti-mouse IL-1 β (R&D, Minneapolis, Minn., USA), anti- β actin (Sigma, St. Louis, Mo., USA), or rabbit polyclone antibody to both KF and SF. Signal intensities were quantified and analyzed with a chemiluminescence imaging system software (FluorChem HD2; Alpha Innotech) as described previously [25, 26]. To determine the relative amount of protein/ β -actin in each sample, bands were digitized into pixel densities and were exported into an Excel program for analysis.

RNA Isolation and Real-Time Quantitative PCR for Analysis of mRNA Levels

Total RNA was isolated from BMMs using the Trizol Reagent (Invitrogen, Carlsbad, Calif., USA) and diluted to 100 ng/ μ l in RNase-free water. For quantification of IL-6 and IL-1 β mRNAs, real-time reverse transcription (RT)-PCR was performed using the QuantiFast SYBR Green RT-PCR kit (Qiagen). The following specific primers for real-time RT-PCR were purchased from Qiagen: Mm_Il6_1_SG QuantiTect Primer Assay (QT00098875), Mm_Il1b_2_SG QuantiTect Primer Assay (QT01048355) and Mm_Actb_2_SG QuantiTect Primer Assay (QT01136772). For NAIP5 quantification, NAIP5 sense primer: TGCCAAACCTACAAG AGCTGA and antisense primer: CAAGCGTTTAGACTGGGG ATG were used [7]. Two-step RT-PCR was performed: using NAIP5-specific antisense primer plus the Revert Aid First strand cDNA Synthesis Kit (Thermo Scientific) for NAIP5-specific mRNA Revert; DyNamo Flash SYBR Green qPCR kit (Thermo Scientific) for real-time PCR of the cDNA. Amplification was carried out as recommended by the manufacturer. The relative cDNA concentrations were established by a standard curve using sequential dilutions of corresponding PCR fragments and normalized to β -actin. After amplification, melting curves were acquired and used to determine the specificity of the PCR products.

Stimulation with Flagellin Expressing Bacteria

Similarly to that described previously [27], the *E. coli* BL21 StarTM (DE3) strains expressing recombinant flagellins were grown in the log phase and pretreated with 0.5 mM isopropyl β -D-thiogalactoside plus 5 mM lactose. The numbers of bacteria were calculated as $1.0 \text{ OD}_{600} = 1.8 \times 10^9$ bacteria per ml. The bacteria were washed with PBS and added to Caco-2 cells or M Φ s for 1 h. After washing twice with RPMI 1640, 100 μ l of RPMI 1640 supplemented with 3% FBS and 50 μ g/ml of gentamicin were added to prevent extracellular bacterial growth. The culture supernatants

Fig. 1. The stimulatory effects of KF and SF through the TLR5 pathway on ECs and splenocytes. **a, b** IL-8 and MCP-1 in the cell culture supernatants of Caco-2 cells stimulated with control, KF and SF. **c, d** Dose-dependence of IL-6 and IFN- γ secreted in the

culture supernatant from splenocytes of naïve BALB/c mice. The cytokines were tested by ELISA. Data are represented as the mean $\pm$ SEM from triplicates of one representative of three experiments.

were harvested 6 h later and tested for cytokine levels by specific enzyme-linked immunoassays.

Statistical Analysis

All of the results are presented as the means $\pm$ standard error of the mean (SEM) from triplicates of one experiment that was repeated at least three times and were analyzed by means of nonparametric two-tailed Student *t* tests using GraphPad Prism 5. Statistical significance is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. A p value < 0.05 was considered statistically significant.

Results

Flagellins KF and SF Act Similarly to TLR5 Agonists

Caco-2 epithelial cells (ECs) constitutively express TLR5, a receptor for flagellin, and have been adopted as an in vitro cell model for studying the TLR5 pathway [17, 20]. Different concentrations of recombinant flagellins KF and SF were used to treat Caco-2 ECs, and the IL-8 and MCP-1 production was determined after stimulation. Compared with irrelevant recombinant HIV-1 p24 protein (labeled as control), SF and KF induced similar levels of IL-8 and MCP-1 secretion in a concentration-dependent manner (fig. 1a, b), demonstrating that the

TLR5 pathway in Caco-2 ECs was similarly activated by both flagellins.

We then investigated the effects of recombinant flagellins SF and KF on antigen-presenting cells (APCs) and lymphocytes. The activation of splenocytes by flagellins was monitored by ELISA assay of secreted cytokines IL-6 and IFN- γ . The production of IL-6 and IFN- γ also increased similarly with the duration of stimulation in a dose-dependent manner (fig. 1c, d). Finally, APCs such as splenic dendritic cells (DCs) were also tested ex vivo for expression levels of the costimulatory molecules CD80 and CD86 after flagellin stimulation [28], and responded similarly to KF and SF stimulation, as tested so far (online suppl. fig. S1; for all online supplementary material, see www.karger.com/doi/10.1159/000351476).

Flagellin KF Induces Much Less IL-1 β Production and Pyroptotic Cell Death than SF in Peritoneal Macrophages

M Φ s are another type of professional APCs, which can also respond to PAMPs, linking innate and adaptive immunities. Peritoneal M Φ s isolated from naïve mice were selected firstly as an ex vivo M Φ s model. Unlike LPS, neither KF nor SF, even at the high concentration of 100 nM,

Fig. 2. The stimulatory effects of KF and SF on peritoneal MΦs. **a** IL-6 secretion 20 h after stimulation with 0.01–100 nM flagellin or 50 ng/ml LPS. **b** IL-1 β secretion 20 h after stimulation with 100 nM flagellin directly. **c** IL-1 β secretion 20 h after 10 and 100 nM flagellin stimulation on LPS-pretreated peritoneal MΦs. **d** IL-1 β secretion 20 h after transfection of 100 nM flagellin using DOTAP. **e, f** LPS-pre-

treated peritoneal MΦs were stimulated with flagellin transfection using DOTAP: dose dependence of IL-1 β secretion 20 h after flagellin transfection (**e**), percentage of cell death calculated by LDH release 20 h after 100 nM flagellin transfection (**f**). Data are presented as the means $\pm$ SEM from triplicates of one representative of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

induced significant IL-6 production, which is a hallmark of TLR5 activation (fig. 2a). Flow cytometry analysis showed that TLR5 expression was absent in MΦs (online suppl. fig. S2), explaining the fact that peritoneal MΦs did not significantly respond to flagellin through the TLR5 signaling pathway.

As a proinflammatory cytokine and highly potent endogenous pyrogen, IL-1 β has long been considered a key

mediator in a variety of disease states associated with inflammation [29]. Surprisingly, 100 nM of flagellin SF but not KF significantly induced IL-1 β secretion in peritoneal MΦs from BALB/c mice (fig. 2b) and from C57BL/6 mice (online suppl. fig. S3A). These results suggested that a pathway other than the TLR5 pathway might be involved in the response to flagellin in peritoneal MΦs.

The production of IL-1 β requires two steps – the synthesis of pro-IL-1 β and IL-1 β processing [14]. There was no significant difference in pro-IL-1 β mRNA levels in KF- and SF-stimulated peritoneal M Φ s (online suppl. fig. S4A). In contrast, pretreatment with 50 ng/ml of LPS induced higher transcription of pro-IL-1 β (online suppl. fig. S4B), as described previously [30, 31]. As shown in figure 2c, LPS pretreatment enhanced SF- and KF-induced IL-1 β secretion. However, SF still showed much higher efficiency of inducing IL-1 β in LPS-pretreated peritoneal M Φ s than KF (fig. 2c). These results indicated that KF and SF did not influence the pro-IL-1 β synthesis, but rather the IL-1 β processing differentially.

Once in the cytosol, flagellin triggers the NAIP5/NLRC4 inflammasome and activates caspase-1, leading to processing pro-IL-1 β into mature IL-1 β and pyroptotic cell death [1, 5, 9, 23, 32–34]. To confirm the different efficiencies of IL-1 β processing triggered by KF and SF, flagellin was transfected into the cytosol of M Φ s using DOTAP [19, 34]. The Western blotting examination showed that DOTAP transfection of KF and SF in M Φ s was of similar efficiency (online suppl. fig. S4C). Under this condition, IL-1 β secretion increased about 4 times for both KF and SF (fig. 2d; online suppl. fig. S3B) compared with that without DOTAP transfection (fig. 2b; online suppl. fig. S3A). LPS pretreatment also enhanced flagellin-induced IL-1 β secretions in transfected M Φ s in a dose-dependent manner (fig. 2e; online suppl. fig. S3C). Cytosolic flagellin also leads to pyroptotic cell death via caspase-1 activation. In parallel to IL-1 β secretion, SF-induced cell death as detected by the LDH assay, while KF was significantly less efficient in this assay (fig. 2f; online suppl. fig. S3D). The difference between KF and SF in inducing IL-1 β secretion could also be observed in bone marrow-derived DCs (BM-DCs; online suppl. fig. S5A), hepatic Kupffer cells (online suppl. fig. S5B) and alveolar M Φ s (online suppl. fig. S5C).

In summary, cytosolic KF induced much less IL-1 β production and pyroptotic cell death than SF, which might result from differential caspase-1 activation and pro-IL-1 β processing through the NAIP5/NLRC4 pathway.

Flagellin KF Activates Caspase-1 through the NLRC4 Pathway Much Less Efficiently than SF in Bone Marrow-Derived Macrophages

BMMs are relatively abundant in mice and widely used to assay the NLRC4 pathway activation. Based on the above data, the flagellin-induced activation of the NLRC4 pathway was further examined at the level of caspase-1 activation. It is well-known that activated cas-

pase-1 can cleave itself into the p10 and p20 subunits and assemble into a heterotetramer [35]. The level of caspase-1 cleavage was used as a marker of caspase-1 activation in our experiments [23]. As BMMs possess only low phagocytic activity, flagellin was transfected into BMMs by using DOTAP according to the published protocols. The activation of caspase-1 occurred within 0.5–1 h and declined at 2 h after SF transfection with DOTAP (fig. 3a). Thus, the relationship between NLRC4 and caspase-1 activation was examined at 1 h posttransfection in the following experiments. Compared with the mock and siRNA control, NLRC4-specific siRNA significantly reduced NLRC4 expression levels and decreased SF-induced caspase-1 cleavage by 73% (fig. 3b). Further assays indicated that SF possessed higher activity than KF in the induction of caspase-1 cleavage, independent of LPS pretreatment (fig. 3c, d). Moreover, the caspase-1 cleavage was dependent on the flagellin concentrations. Caspase-1 cleavage was induced by SF at a concentration of 10 nM but only to a low extent by 100 nM KF (fig. 3e). Quantitative analysis showed that about 21% of caspase-1 cleavage was induced by 100 nM of KF compared to that of 100 nM SF. Therefore, KF has a much lower efficiency in activating caspase-1 cleavage through the NLRC4 signaling pathway in BMMs than SF.

We then comparatively analyzed the downstream IL-1 β production and pyroptotic cell death in BMMs. The pro-IL-1 β mRNA levels, both at baseline and LPS induced, remained unchanged after the stimulation by DOTAP transfection with flagellins (fig. 4a), confirming again that SF and KF had no effect on pro-IL-1 β transcription. The baseline level of pro-IL-1 β mRNA was very low (fig. 4a, left side), thus, neither KF nor SF could induce detectable IL-1 β secretion in BMMs (fig. 4b, left side). As shown on the right sides of figure 4a and b, LPS pretreatment highly upregulated pro-IL-1 β and allowed the performance of IL-1 β secretion assay in BMMs. SF significantly increased IL-1 β secretion in LPS-pretreated BMMs, while KF did so only fractionally (fig. 4b, right side). In this experiment, the NAIP5 transcription level was unchanged (fig. 4c). As the cytosolic flagellin level and NLRC4 expressing level were comparable for KF and SF (fig. 4d), the different caspase-1 activation and IL-1 β processing could only be explained by the different NAIP5/NLRC4 activating efficacy. The silencing of NLRC4 by siRNA led to reduced IL-1 β secretion induced by SF and KF (fig. 4e), showing that IL-1 β secretion was initiated from the NLRC4 pathway activation. SF-induced IL-1 β secretion occurred rapidly within 3 h and did not increase significantly thereafter up to 24 h. Simi-

a

b

c

d

e

Fig. 3. Caspase-1 activation by transfected flagellin in BMMs. Caspase-1 p10 was detected by Western blotting in the cytoplasmic lysates from BMMs combined with the supernatants after flagellin transfection. The relative amounts of caspase-1 p10 to β-actin in each sample were also calculated according the signal intensities. **a** Time course of caspase-1 activation after transfection of 100 nM SF or control protein p24. **b** siRNA pretreated BMMs were trans-

fected with 100 nM SF or control protein p24, NLRC4 expression and caspase-1 activation were determined 1 h after transfection. **c, d** Caspase-1 activation 1 h after transfection of 100 nM flagellin without (**c**) or with (**d**) LPS pretreatment. **e** Caspase-1 activation 1 h after transfection of 1, 10 and 100 nM flagellin in LPS-pretreated BMMs. Data are presented as the means ± SEM from triplicates of one representative of three experiments.

Fig. 4. NLRC4 pathway activation induced by KF and SF in BMMs. **a** Pro-IL-1 β mRNA levels in total RNA isolated from BMMs 3 h after transfection of 100 nM flagellin using DOTAP with or without LPS pretreatment. **b** IL-1 β secretion 20 h after transfection of 100 nM of flagellin using DOTAP with or without LPS pretreatment. **c–f** BMMs were pretreated with LPS before flagellin transfection: NAIP5 mRNA levels in BMMs 3 h after transfection of 100 nM of flagellin (**c**); the NLRC4 expressing level, caspase-1 levels,

pro-IL-1 β levels, mature IL-1 β levels and cytosolic flagellin quantification 1 h after transfection of 100 nM of flagellin (**d**); IL-1 β secretion 20 h after transfection in siRNA-pretreated BMMs (**e**); time course of IL-1 β secretion after transfection of 100 nM of flagellin using DOTAP (**f**). Data are presented as the means $\pm$ SEM from triplicates of one representative of three experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.

larly, KF only slightly increased IL-1 β secretion within the first 3 h (fig. 4f). As caspase-1 activation was detected mainly within 2 h after SF stimulation (fig. 3a), the time course of IL-1 β secretion (fig. 4f) was consistent with the concept that cytosolic SF activates caspase-1 through the NLRC4 pathway and induces IL-1 β processing and secretion. These results demonstrated that KF is less effi-

cient in activating caspase-1 through the NLRC4 pathway than SF.

KF-Expressing Bacteria Are Less Effective in Inducing IL-1 β than SF-Expressing Bacteria

The majority of the experiments on the intracellular actions of flagellin were performed by direct transfer of

Fig. 5. The stimulatory effects of nonpathogenic bacteria expressing KF and SF. Cytokines in cell culture supernatants were analyzed 6 h after removing bacteria. **a** Flagellin expressions in bacteria were examined by SDS-PAGE after isopropyl b-D-thiogalactoside induction. **b** Dose dependence of IL-8 secretion by Caco-2

cells. **c, d** Dose dependence of IL-1β secretion after bacteria stimulation of peritoneal MΦs (**c**) and BMMs (**d**). Data are presented as the means ± SEM from triplicates of one representative of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

purified recombinant flagellin into the cytosol or by transfer mediated by bacterial invasion systems, such as the type III secretion system (T3SS) or pore-forming toxins. We tried here to address whether the flagellins KF and SF have the ability to activate the NLRC4 pathway if the flagellins are expressed and carried by nonpathogenic bacteria. Further, we asked whether there will be a difference in inducing IL-1β secretion in peritoneal MΦs or BMMs by KF and SF. Thus, a nonflagellated *E. coli* BL21 StarTM (DE3) model was explored to express recombinant flagellins and then adopted for comparative analysis (fig. 5a). Compared with controls, the bacteria expressing either KF or SF significantly upregulated IL-8 secretion to similar levels in Caco-2 ECs (fig. 5b), consistent with the previous experiments using purified flagellins (fig. 1a). Thus, the TLR5 pathway could be equally activated by KF- and SF-expressing bacteria. For testing the NLRC4 activation, KF- and SF-expressing *E. coli* BL21 bacteria were incubated with peritoneal MΦs or BMMs. Bacteria expressing SF significantly enhanced IL-1β secretion in peritoneal MΦs (fig. 5c) as well as in BMMs (fig. 5d), compared with controls and KF-expressing bacteria. Incubation with dif-

ferent bacteria did not change the levels of the factors in the NLRC4 pathway including procaspase-1 and pro-IL-1β (online suppl. fig. S6). These results suggest that flagellin is an important bacterial determinant for inducing IL-1β secretion by peritoneal MΦs and BMMs.

Discussion

In the present report, we showed that flagellins from SF and nonpathogenic *E. coli* are not distinguishable in activating the TLR5 pathway in various cell types but are different in activating the cytosolic NLRC4 pathway in MΦs. KF induced significantly less IL-1β than SF in peritoneal MΦs, BM-DCs, Kupffer cells and alveolar MΦs. Lower IL-1β production and pyroptotic cell death were induced by KF in LPS-primed BMMs due to its inefficiency in activating caspase-1 through the NLRC4 pathway. Finally, KF and SF expressed in bacteria showed different abilities to induce IL-1β production in MΦs. Thus, NAIP5/NLRC4 might recognize different flagellin molecules more accurately and initiate differential responses, which could con-

tribute in host immune recognition for discriminating different types of bacteria. Recently, intestinal mononuclear phagocytes were found to distinguish between pathogenic and commensal bacteria in the gut, eliciting substantial amounts of mature IL-1 β by the NLRC4 pathway [36]. Our data are in concordance with the new finding and provide further insights into how this selective recognition of invading pathogenic bacteria is achieved.

Some bacteria evade TLR5 sensing and recognition by downregulating flagellin expression or mutating flagellin molecules [37], indicating the importance of TLR5 recognition in innate immune response. TLR5 deficiency could also lead to spontaneous colon inflammation by high bacteria loads and some other disorders [38]. The recognition of commensal flagellated bacteria-derived flagellin KF with high efficiency through TLR5 consists with the concept that TLR5 is important for the maintenance of the balance between bacterial symbionts and the mucosa and inhibition of excessive inflammation [38].

To activate the NAIP5/NLRC4 pathway and the ensuing IL-1 β secretion, at least 10 nM of flagellin was required, which is nearly 1,000 times greater than that for TLR5 activation. These different flagellin concentration requirements of TLR5 and NLRC4 activation suggested that the dual sensors work separately and initiate responses not only based on whether and where flagellin is present, but also on how many flagellin molecules are presented. It was believed that activation of the NLRC4 inflammasome requires the presence of an intact T3SS or type IV secretion system (T4SS) that mediates the translocation of bacterial virulence factors to the cytosol [34]. Our study showed that flagellin did not change the expression levels of NAIP5, NLRC4, procaspase-1 or pro-IL-1 β in BMMs (fig. 4). Therefore, the flagellin-enhanced IL-1 β secretion solely depends on pro-IL-1 β processing, via caspase-1 activation through NAIP5/NLRC4 recognition. KF and SF differed in their abilities to activate NAIP5/NLRC4.

Actually, peritoneal M Φ s are different from BMMs in the response to flagellin in some aspects. Firstly, peritoneal M Φ s but not BMMs express much higher pro-IL-1 β without LPS pretreatment (data not shown). In this regard, peritoneal M Φ s are similar to intestinal mononuclear phagocytes described recently [36]. Secondly, peritoneal M Φ s could efficiently take up flagellin molecules (data not shown), leading to the NLRC4 activation and followed by caspase-1-mediated production of mature IL-1 β independently on T3SS or T4SS (fig. 2b). This is consistent with the results showing that intraperitoneal application of flagellins could activate the host immune system through the NLRC4 pathway [39]. However, once

BMMs were exposed to LPS and transfected with flagellin, NLRC4 could be activated and IL-1 β was produced and released (fig. 4d–f). These further suggested that primary macrophage cells may be functionally different to recognize and respond to bacteria flagellins as well as to other bacterial virulence factors [30].

Bacteria expressing flagellin KF and SF activated TLR5 responses in ECs to a similar extent (fig. 5b) but induced IL-1 β production in peritoneal M Φ s and BMMs differently (fig. 5c, d). The biological significance of the differential recognition of the flagellins of *E. coli* and *Salmonella* Typhi by the NLRC4 pathway remains to be explained. The flagellin molecule contains a hypervariable domain, which varies in size and composition in different bacteria strains and species. Considering most commensal *E. coli* being a benefit to hosts, the host immune system may become tolerant to commensal *E. coli* to some extent, for example, ignored by the NLRC4 pathway. Thereby, M Φ s might be able to discriminate commensal and invading pathogenic bacteria by the differential recognition of flagellins and initiate innate immune responses only to pathogenic bacteria. Future studies will be helpful to gain more insight into how the immune system discriminates between commensal and pathogenic bacteria.

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