



Flagellin-Stimulated Production of Interferon- β Promotes Anti-Flagellin IgG2c and IgA Responses

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Flagellin, a major structural protein of the flagellum found in all motile bacteria, activates the TLR5- or NLRC4 inflammasome-dependent signaling pathway to induce innate immune responses. Flagellin can also serve as a specific antigen for the adaptive immune system and stimulate anti-flagellin antibody responses. Failure to recognize commensal-derived flagellin in TLR5-deficient mice leads to the reduction in anti-flagellin IgA antibodies at steady state and causes microbial dysbiosis and mucosal barrier breach by flagellated bacteria to promote chronic intestinal inflammation. Despite the important role of anti-flagellin antibodies in maintaining the intestinal homeostasis, regulatory mechanisms underlying the flagellin-specific antibody responses are not well understood. In this study, we show that flagellin induces interferon- β (IFN- β) production and subsequently activates type I IFN receptor signaling in a TLR5- and MyD88-dependent manner *in vitro* and *in vivo*. Internalization of TLR5 from the plasma membrane to the acidic environment of endolysosomes was required for the production of IFN- β , but not for other pro-inflammatory cytokines. In addition, we found that anti-flagellin IgG2c and IgA responses were severely impaired in interferon-alpha receptor 1 (IFNAR1)-deficient mice, suggesting that IFN- β produced by the flagellin stimulation regulates anti-flagellin antibody class switching. Our findings shed a new light on the regulation of flagellin-mediated immune activation and may help find new strategies to

promote the intestinal health and develop mucosal vaccines.

Keywords: anti-flagellin antibody, flagellin, IgA, interferon- β , Toll-like receptor 5

INTRODUCTION

Flagellin is a major structural protein which polymerizes to form a flagellum of bacteria (Lowy and McDonough, 1964). Having a highly conserved structure and being widely expressed in nearly all motile bacteria, flagellin efficiently acts as a pathogen-associated molecular pattern (PAMP) to activate innate immune responses of animals and plants (Ciacci-Woolwine et al., 1998; Felix et al., 1999; Gomez-Gomez and Boller, 2000). Accordingly, a large number of studies have tested flagellin for its adjuvant activity and showed that it can be utilized as a highly potent vaccine adjuvant with minimal safety concerns (Hajam et al., 2017).

The most studied mammalian receptor for flagellin is TLR5 (Gewirtz et al., 2001; Hayashi et al., 2001). Recognition of extracellular flagellin by TLR5 on the cell surface leads to dimerization of the receptor and the subsequent recruitment of the adaptor molecule MyD88 (Yoon et al., 2012). Immune cells expressing TLR5 include monocytes, neutrophils, splenic CD4⁺ dendritic cells (DCs), and intestinal CD103⁺CD11b⁺ lam-

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ina propria DCs (LP-DCs) (Shibata et al., 2012). Upon stimulation with flagellin, these cells secrete pro-inflammatory cytokines such as interleukin (IL)-6, IL-12, tumor necrosis factor α (TNF- α), and various chemokines via a classical MyD88-IRAK1/4-TRAF6-IKK-NF- κ B signaling pathway (Vijay-Kumar et al., 2008). In addition, TLR5 is also highly expressed in intestinal epithelial cells and mediates secretion of pro-inflammatory cytokines and antimicrobial peptides (Gewirtz et al., 2001). Expression of TLR5 in the intestinal epithelium is limited to the basolateral surface, which prevents constitutive stimulation by flagellated commensal bacteria abundant on the apical side of the gut epithelium (Gewirtz et al., 2001). Unlike other cell types, intestinal epithelial cells were reported to require the adaptor molecule TRIF in addition to MyD88 for TLR5-mediated activation of NF- κ B, despite the *in vivo* relevance of this finding being unclear (Choi et al., 2010). Certain bacteria such as *Salmonella* can deliver flagellin into the host cell cytoplasm via a type III secretion system. The intracellular flagellin is recognized by NLR (NOD-like receptor) family proteins NAIP5 and NAIP6 (Kofoed and Vance, 2011; Zhao et al., 2011). Flagellin-bound NAIP5/6 then recruits another NLR family protein NLRC4 (also called Ipaf) and induces its polymerization, resulting in the formation of NLRC4 inflammasome, subsequent activation of caspase-1, maturation of pro-IL-1 β into IL-1 β , and eventual cell death via pyroptosis (Franchi et al., 2006; Halff et al., 2012; Hu et al., 2015; Miao et al., 2006; Zhang et al., 2015).

Not only can flagellin stimulate innate immune responses via TLR5 and NLRs, it can also effectively induce specific CD4⁺ T cell responses and a high affinity, class-switched antibody production due to its proteinaceous nature, a characteristic which is generally absent in other bacteria-derived PAMPs such as LPS and peptidoglycans (McSorley et al., 2002; Sanders et al., 2006). Though flagellin-specific CD4⁺ T cell activation can occur in the absence of TLR5, the endocytosis of flagellin-bound TLR5 enhances MHC class II-mediated presentation of flagellin epitopes and promotes the optimal activation of flagellin-specific CD4⁺ T cells (Letran et al., 2011). Interestingly, MyD88 was not required for robust induction of flagellin-specific CD4⁺ T cells. Instead, Syk activity in DCs was essential for the optimal presentation of flagellin to CD4⁺ T cells both *in vitro* and *in vivo* (Atif et al., 2015). These results suggest the possibility that internalization of TLR5—similar to the LPS-induced TLR4 endocytosis (Zanoni et al., 2011)—may be more dependent on the Syk-mediated signaling pathway than the classical MyD88-mediated signaling. However, this remains to be formally tested.

In the case of flagellin-specific antibody responses, anti-flagellin IgG2c and IgA responses were TLR5- and MyD88-dependent, whereas the IgG1 isotype was induced in the absence of TLR5 and MyD88 (Lopez-Yglesias et al., 2014). Accordingly, deficiency of TLR5 results in the reduced levels of anti-flagellin IgA antibodies in the gut at the steady state and causes microbial dysbiosis as well as mucosal barrier breach by flagellated bacteria (Cullender et al., 2013). Especially, the inability to control *Proteobacteria* was shown to promote chronic intestinal inflammation in TLR5-deficient mice (Carvalho et al., 2012). Despite the important roles of anti-flagellin antibodies in maintaining the intestinal homeo-

stasis, regulatory mechanisms underlying the flagellin-specific antibody responses are not well understood.

In this study, we found that the flagellin-mediated activation of TLR5 leads to interferon- β (IFN- β) production in a MyD88-dependent manner and the subsequent type I IFN receptor signaling is necessary for anti-flagellin IgG2c and IgA responses.

MATERIALS AND METHODS

Mice

Wild-type (WT) C57BL/6 mice were purchased from Jackson Laboratory. UNC93B1 knock-out (KO) mice were obtained from the Knockout Mouse Project Repository (University of California at Davis, USA). TLR4 KO (Hoshino et al., 1999), TLR5 KO (Uematsu et al., 2006), MyD88 KO (Adachi et al., 1998), TRIF KO (Yamamoto et al., 2003), IFNAR1 KO (Muller et al., 1994), and IFN- β -YFP reporter (*mob*) mice (Scheu et al., 2008) were previously described. All mice were bred and housed in specific pathogen-free facilities at Pohang University of Science and Technology (POSTECH, Korea) and Korea Advanced Institute of Science and Technology (KAIST, Korea). For the flagellin immunization experiments, mice at 6 to 8 weeks of age and the littermate controls were used. All animal experiments were approved by the Institutional Animal Care and Research Committees of POSTECH (POSTECH-2016-0072-R1) and KAIST (KA2018-10).

Reagents

High purity flagellin (isolated from *Salmonella typhimurium* strain 14028) and MALP-2 were purchased from Enzo Life Sciences (USA). For use in ELISA of anti-flagellin antibodies, flagellin was purchased from AdipoGen Life Sciences (Switzerland). LPS from *Escherichia coli* (O26:B6), bafilomycin A1, depleted zymosan, and ovalbumin were purchased from Sigma-Aldrich (USA). CpG-ODN (1826) was from TIB Molbiol (Germany). Biotinylated mouse anti-TLR5 monoclonal antibody (clone ACT5) was a gift from Kensuke Miyake (University of Tokyo, Japan). For flow cytometry, following fluorophore-conjugated antibodies were used: Ly6C-PB (clone HK1.4), Ly6G-APC (clone RB6-8C5) from eBioscience (USA); CD11b-PECy7 (clone M1/70), CD11c-PE (clone HL3) from BD Biosciences (USA); B220-PB (clone RA3-6B2) from TONBO Biosciences (USA); mPDCA1-APC (clone JF05-1C2.4.1) from Miltenyi Biotec (Germany). Following antibodies were used for immunoblotting: IRF3 (clone FL-425) from Santa Cruz Biotechnology (USA); IRF7 (clone EPR4718) from Abcam (UK); phospho-IRF3 (clone 4D4G), phospho-IRF7 (polyclonal) from Cell Signaling Technology (USA). Anti-mouse CD16/32 antibody was purchased from BioLegend (USA). Accudenz was from Accurate Chemical and Scientific Corporation (USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig antibodies were purchased from Southern Biotech (USA).

Cell lines

RAW264.7 (RAW) mouse macrophage cell lines were cultured in DMEM media supplemented with 5% fetal bovine serum (FBS). KBM7 human chronic myeloid leukemia cell line was cultured in IMDM media supplemented with 10%

FBS and 55 μ M 2-mercaptoethanol. Following stable cell lines expressing fluorescent protein-tagged TLR5, CD63, IFN- β reporter (IFN- β -GFP) or interferon-stimulated response element (ISRE) reporter (ISRE-GFP) were generated by transduction of RAW and KBM7 cells with retrovirus or lentivirus encoding the corresponding genes: RAW/TLR5-Cherry, KBM7/TLR5-Cherry, RAW/TLR5-GFP, RAW/IFN- β -GFP, RAW/IFN- β -GFP/TLR5-Cherry, KBM7/IFN- β -GFP, KBM7/IFN- β -GFP/TLR5-Cherry, KBM7/ISRE-GFP, KBM7/ISRE-GFP/TLR5-Cherry. Syk KO cells were generated by sequential transduction of RAW cells with Cas9-expressing retrovirus (pMSCV-flag-NLS-Cas9) and guide RNA-expressing lentivirus (pLX-sgRNA-mSyk). The target sequence in the exon 2 of mouse Syk is 5'-CCGGCCCCGGGAGTACAGCCCA-3'. Single cell clones were tested for Syk expression by immunoblotting and the mutation of the targeted sequence in the selected Syk KO clone was confirmed by sequencing.

Reporter constructs

The ISRE-GFP reporter construct (pTRH1-ISRE-dscGFP) was prepared by replacing the NF- κ B-binding sequence in pTRH1-NF- κ B-dscGFP with the ISRE sequence in pISRE-Luc. The IFN- β -GFP reporter construct (pTRH1-IFN- β -dscGFP) was generated by inserting the endogenous 5'-upstream sequence (-596 to -97) of human IFN- β gene in place of the PRD3 sequence in pTRH1-PRD3-dscGFP.

Retroviral and lentiviral transduction

Preparation of retroviruses was previously described (Kim et al, 2013). Lentiviruses were generated in HEK293T cells by co-transfection of pTRH1 or pHAGE plasmid encoding the reporter constructs or proteins of interest along with the packaging plasmids for Tet, Rev, gag/pol and VSV-G. At 30 h and 50 h post-transfection, medium containing viral particles was harvested and added to RAW or KBM7 cells together with 8 μ g/ml polybrene. Cells were centrifuged at 2,200 rpm for 90 min and were given fresh media on the following day.

Quantitative real-time polymerase chain reaction (PCR)

Total cellular RNA was isolated with Trizol (Qiagen, Germany) and mRNAs were reverse-transcribed into cDNAs by using a GoScript Reverse Transcription System (Promega, USA). Quantitative PCR (qPCR) analyses were performed using the SYBR Green PCR Kit (Qiagen) on Viia7 or StepOnePlus system (Applied Biosystems, USA). The results were analyzed by the $\Delta\Delta C_t$ method and normalized to the HPRT expression. The following primers were used: mouse HPRT_(s) 5'-CAGACTGAAGAGCTACTG-TAATGATCA-3', mouse HPRT_(as) 5'-TCAACAATCAAGACATTCTTCCA-3', mouse IFN- β _(s) 5'-CCCTATGGAGATGACGGAGA-3', mouse IFN- β _(as) 5'-TCCCACGTCATCTTTCCTC-3', mouse IL-6_(s) 5'-GAGGATACCACTCCCAACAGACC-3', mouse IL-6_(as) 5'-AAGTG-CATCATCGTTGTTACATA-3', mouse TNF- α _(s) 5'-GCTCTGTGAAGGGAATGGGT-3', mouse TNF- α _(as) 5'-CAGGTCAGTCCAGCATC-3', mouse Mx1_(s) 5'-CTCAGGGTGTGCGATGAGGTC-3', mouse Mx1_(as) 5'-TCTGAGG-AGAGCCAGACGAT-3', mouse ISG15_(s) 5'-CCCCAGCATCTCACCTTTA-3', mouse ISG15_(as) 5'-TGACTGTGAGAGCAAGCAGC-3', mouse ISG56_(s) 5'-CAGAAGCACACATTGAAGAA-3',

mouse ISG56_(as) 5'-TGTAAGTAGCCAGAGGAAGG-3', mouse IRF7_(s) 5'-CACCCCATCTTCGACTTCA-3', mouse IRF7_(as) 5'-CCAAAACCCAGGTAGATGGTGTA-3', human HPRT_(s) 5'-GCAGTATAATCC-AAAGATGGTCAA-3', human HPRT_(as) 5'-TGGAATTTCAAATCCAACAAAGT-3', human IFN- β _(s) 5'-AAACTCATGAGCAGTCTGCA-3', human IFN- β _(as) 5'-AGGAGATCTTCAGTTTCGGAGG-3', human Mx1_(s) 5'-GGTGGCTGAGAACAACCTGT-3', human Mx1_(as) 5'-GGTCCTGCTCCACACCTAGA-3', human ISG15_(s) 5'-GAGAGGCAGCGAACTCATCT-3', human ISG15_(as) 5'-CTTCAGCTCTGACACCGACA-3', human ISG56_(s) 5'-AAGGCAGGCTGTCCGCTTA-3', human ISG56_(as) 5'-TCCTGTCTTCATCCTGAAGCT-3', human IRF7_(s) 5'-TGCTCCTGGTGAAGCTGGAA-3', human IRF7_(as) 5'-GATGTCGTCATAGAGGCTGTTGG-3'.

Ex vivo IFN- β -YFP reporter assay

Bone marrow (BM) and spleens were isolated from *mob* mice. Red blood cells were removed from BM cells by using ACK lysis buffer. Spleens were minced and incubated with collagenase D and DNase I at 37°C for 30 min with stirring. After stopping the enzyme reaction with EDTA, red blood cells were removed with ACK lysis buffer. Isolated BM or spleen cells were stimulated with flagellin or LPS at 37°C for indicated time periods, washed, incubated with the Fc block on ice for 15 min, and then labeled with fluorophore-conjugated antibodies for cell surface marker staining on ice for 20 min. Following the wash with FACS buffer, cells were analyzed for the YFP expression on the LSR Fortessa flow cytometer (BD Biosciences).

Isolation of immune cells

After the ACK lysis, CD3-negative BM cells were enriched by magnetic-activated cell sorting with CD3 MicroBeads (Miltenyi Biotec). Cells were then incubated with the Fc block on ice for 15 min and labeled with fluorophore-conjugated antibodies on ice for 15 min. Neutrophils (CD11b⁺Ly6C⁻Ly6G⁺) and monocytes (CD11b⁺Ly6C⁺Ly6G⁻) were sorted on the MoFlo XDP cell sorter (Beckman Coulter, USA). LP-DCs were isolated as previously described (Jang et al., 2006).

Analysis of in vivo flagellin-induced cytokine production

WT, TLR4 KO, TLR5 KO, MyD88 KO, TRIF KO, UNC93B1 KO, or IFNAR1 KO mice were injected with flagellin (2 μ g/mouse) via an intraperitoneal (*i.p.*) or intravenous (*i.v.*) route. After indicated time periods, blood was collected and serum cytokine levels were measured with ELISA kits (IFN- β kit from PBL Assay Science [USA], TNF- α and IL-6 kits from R&D Systems [USA]) according to the manufacturer's protocol.

Detection of IRF3 and IRF7 by immunoblotting

RAW/TLR5-Cherry cells were stimulated with 100 ng/ml flagellin at 37°C for 2 h or 12 h. The total cell lysates were prepared by lysing the cells in the lysis buffer (50 mM Tris, 150 mM NaCl, 1% SDS, 1% Triton X-100, 5 mM EDTA, protease inhibitors and phosphatase inhibitors). For detection of phosphorylated IRF3 and IRF7 in the nuclear fraction, cells were resuspended in the ice-cold hypotonic lysis buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.25% NP-40, 0.5

mM dithiothreitol, protease inhibitors and phosphatase inhibitors) and incubated for 10 min on ice. The cytosolic fraction was removed by centrifugation at 1,200g for 5 min. The nuclei in the pellet were washed with the hypotonic buffer three times, resuspended in the ice-cold extraction buffer (10 mM HEPES, 450 mM NaCl, 0.2 mM EDTA, protease inhibitors and phosphatase inhibitors), and ruptured by 5 cycles of freezing/thawing. The nuclear extracts were obtained from the supernatant after centrifugation at 16,000g for 20 min. After protein quantification with the BCA protein assay kit (Pierce, USA), the nuclear extracts and the total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies for IRF3, IRF7, phospho-IRF3, and phospho-IRF7. Following the incubation with HRP-conjugated anti-rabbit IgG and the subsequent incubation with an ECL solution (DoGenBio, Korea), signals were visualized on ImageQuant LAS 4000 (GE Healthcare Life Science, USA).

TLR5 internalization assay

RAW/TLR5-GFP cells were stimulated with 100 ng/ml flagellin at 37°C for indicated time periods. TLR5 on the cell surface was labeled with biotinylated anti-TLR5 monoclonal antibody and Alexa647-conjugated streptavidin on ice and cells were analyzed on the LSR Fortessa flow cytometer (BD Biosciences). The extent of flagellin-induced TLR5 internalization was determined by comparing the cell surface TLR5 level with that of unstimulated cells.

Confocal imaging of TLR5 internalization

BM-derived dendritic cells (BM-DCs) were prepared from WT mice as previously described (Kim et al., 2013) and retrovirally transduced to express TLR5-GFP and CD63-Cherry. Cells were grown in 8 well chambered coverglass (Nunc, Denmark) and incubated with 100 ng/ml flagellin at 37°C. After indicated time periods, cells were imaged with a spinning-disk confocal microscope as previously described (Kim et al., 2013).

Flagellin-specific antibody measurement

TLR5 KO, IFNAR1 KO and the littermate WT mice were *i.p.* injected with flagellin (5 μ g/mouse) at day 0 and 15. Serum and feces were collected before the first immunization and at day 7, 14, and 22. The fecal extracts were prepared by suspending the fecal pellets in phosphate-buffered saline (PBS) supplemented with 0.01% sodium azide and protease inhibitors and taking the supernatant after the centrifugation at 13,000 rpm for 10 min. Flagellin-specific antibody levels in the serum and the fecal extracts were measured by ELISA. Briefly, high binding polystyrene half-area 96-well plates (CORNING) were coated with 1 μ g/ml flagellin. Diluted serum and fecal samples were added to wells and incubated for 1 h at room temperature (RT). After wash, the plates were incubated with HRP-conjugated anti-mouse Ig antibodies (anti-IgM, anti-IgG, anti-IgG1, anti-IgG2c, and anti-IgA) for 1 h at RT, washed again, and developed with TMB substrate solution (SurModics, USA). After stopping the reaction

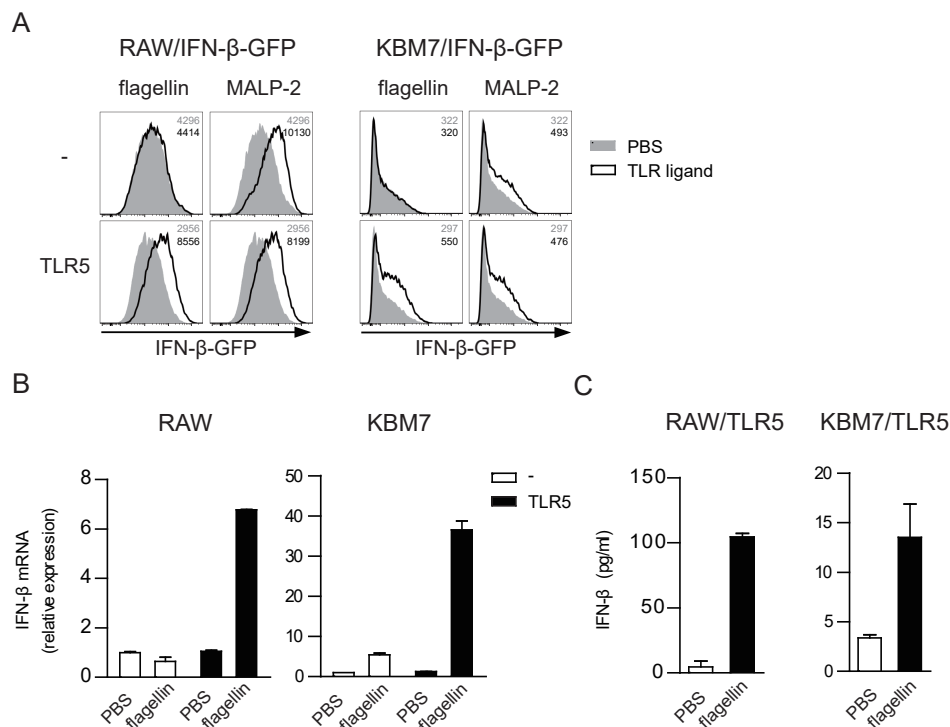


Fig. 1. Flagellin induces TLR5-dependent IFN- β production in mouse and human cell lines. (A) RAW/IFN- β -GFP and KBM7/IFN- β -GFP cells expressing or not expressing TLR5 were stimulated with flagellin (100 ng/ml) or MALP-2 (100 ng/ml) for 24 h, and GFP expression was measured by flow cytometry. (B) RAW and KBM7 cells expressing or not expressing TLR5 were stimulated with PBS or flagellin (100 ng/ml) for 2 h. IFN- β mRNA expression was measured by qPCR. (C) RAW and KBM7 cells expressing TLR5 were stimulated with PBS or flagellin (100 ng/ml) for 12 h (RAW/TLR5) or 24 h (KBM7/TLR5). IFN- β in the culture supernatant was measured by ELISA.

with 0.5 M H₂SO₄, the absorbance was read at 450 nm with SPECTROstar Nano (BMG Labtech, Germany).

Statistical analysis

Results are shown as the mean \pm SEM. Statistical significance was evaluated with an unpaired Student's *t*-test using GraphPad Prism 5 software (GraphPad Software, USA). Differences were noted as significant when $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

RESULTS

Flagellin induces IFN- β production and subsequent type I IFN receptor signaling via TLR5

Systemic immunization of flagellin induces production of anti-flagellin IgA antibodies (Flores-Langarica et al., 2012). Because the type I IFN signaling is implicated in the IgA class switching and several bacterial PAMPs such as LPS and MALP-2 induce IFN- β production via TLR activation, we tested whether flagellin can induce IFN- β production via TLR5. First, we used the mouse macrophage cell line RAW264.7 (RAW) and the human myeloid leukemia cell line KBM7, both of which express various TLRs but not TLR5. Using the IFN- β -GFP reporter assay, we found that flagellin can induce the IFN- β promoter activation in these cells only when TLR5 is ectopically expressed (Fig. 1A). As expected, IFN- β responses to MALP-2 (TLR2 ligand) were not affected by the TLR5 expression. Similarly, flagellin-stimulated induction of IFN- β mRNA was observed in the TLR5-expressing cells (Fig. 1B). We also detected secretion of IFN- β proteins from TLR5-expressing cells by ELISA (Fig. 1C).

Next, we evaluated whether IFN- β , produced upon flagellin stimulation, can promote the type I interferon receptor signaling. Binding of IFN- α and - β to the heterodimeric receptor IFNAR1/2 activates the JAK/STAT pathway and results in the subsequent induction of interferon-stimulated genes (ISGs). Many of ISGs have the interferon-stimulated response element (ISRE) in their promoter region. Thus, we first measured the flagellin-stimulated ISRE activation in KBM7 cells using the ISRE-GFP reporter assay. In accordance with the IFN- β production results shown in Fig. 1, flagellin induced the ISRE activation only when TLR5 was expressed (Fig. 2A). MALP-2 as well as recombinant IFN- β (used as a positive control for the ISRE activation) stimulated the ISRE activation irrespective of the TLR5 expression. Next, we confirmed the flagellin-induced activation of the interferon receptor downstream signaling by measuring mRNAs of several ISGs. Flagellin increased the expression of Mx1, ISG15, ISG56, and IRF7 mRNAs in a time-dependent manner in both RAW and KBM7 cells expressing TLR5 (Fig. 2B).

Flagellin induces IFN- β production in mouse primary cells

To test whether flagellin can induce IFN- β production in primary cells expressing endogenous TLR5, we used the IFN- β -YFP reporter (*mob*) mice in which the bi-cistronic IFN- β -IRES-YFP sequence was inserted in the endogenous IFN- β locus and therefore cells expressing IFN- β also express YFP (Scheu et al., 2008). Neutrophils and monocytes constitute the majority of TLR5-expressing cell populations in the BM

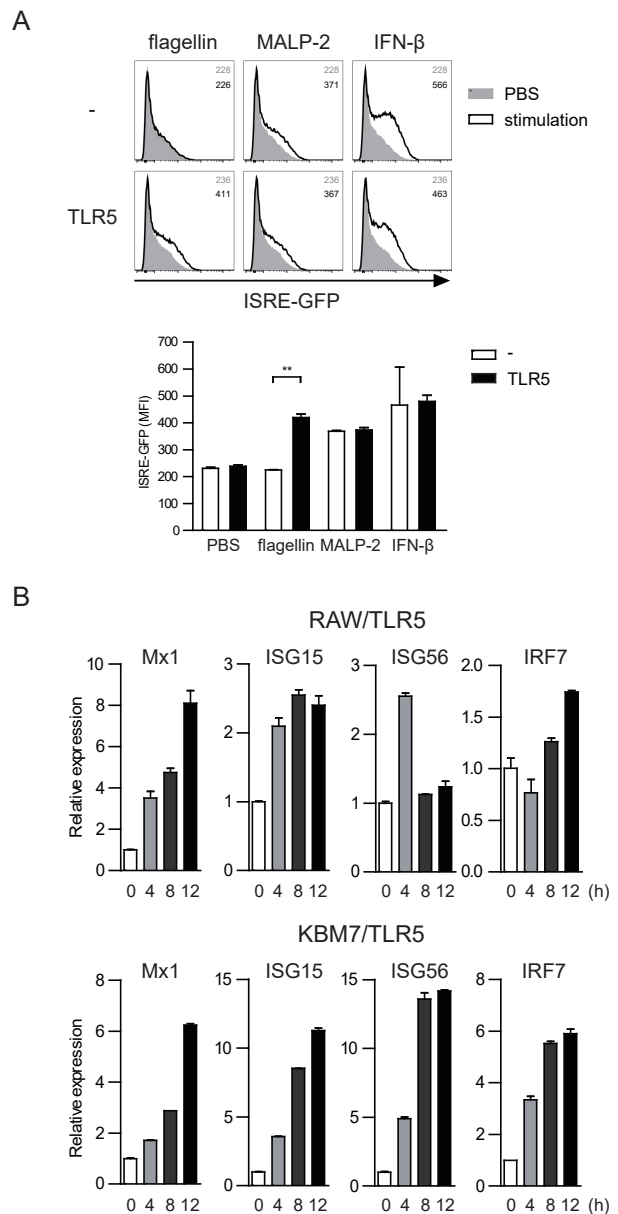


Fig. 2. Flagellin promotes type I IFN receptor signaling in a TLR5-dependent manner. (A) KBM7/ISRE-GFP cells expressing or not expressing TLR5 were stimulated with flagellin (100 ng/ml), MALP-2 (100 ng/ml), or IFN- β (20 U/ml) for 12 h and GFP expression was measured by flow cytometry. (B) RAW and KBM7 cells expressing TLR5 were stimulated with flagellin (100 ng/ml) up to 12 h. Expression of Mx1, ISG15, ISG56, and IRF7 mRNA was measured by qPCR.

(Shibata et al., 2012). Accordingly, we found that a significant proportion of neutrophils (CD11b⁺Ly6G⁺) and monocytes (CD11b⁺Ly6C⁺), but not other cells (CD11b⁻), expressed YFP when we stimulated BM cells with flagellin *ex vivo* (Fig. 3A). The IFN- β promoter activation by flagellin was also detected in splenic macrophages (R3) and to a lesser extent in CD11c^{hi} DC subsets (R1 and R2), but not in plasmacytoid

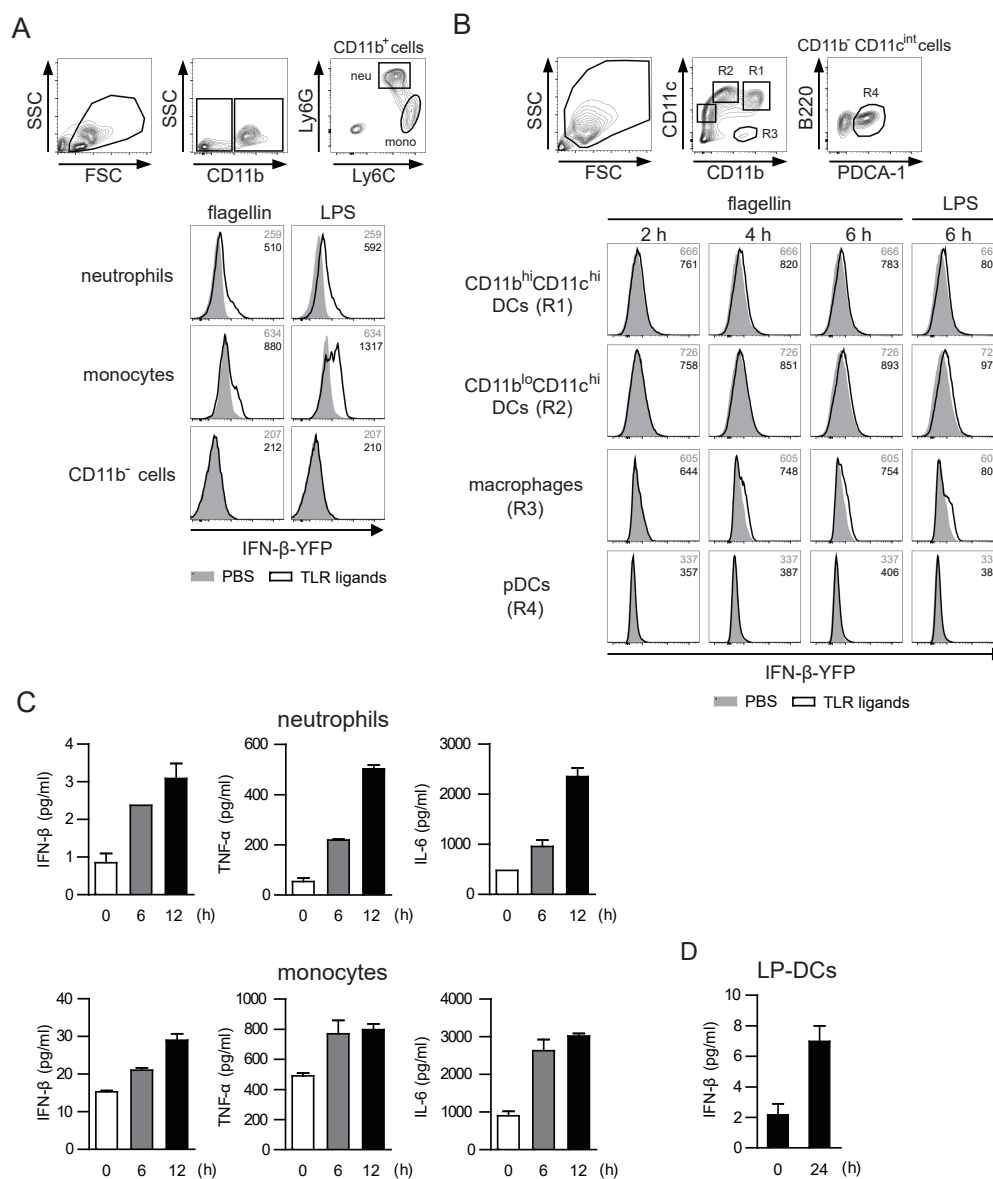


Fig. 3. Flagellin induces IFN- β production in mouse primary cells. (A) BM cells were isolated from the IFN- β -YFP (*mob*) mice and stimulated with PBS, flagellin (100 ng/ml) or LPS (100 ng/ml) for 6 h. YFP expression was measured in CD11b⁺Ly6G⁺ neutrophils, CD11b⁺Ly6C⁺ monocytes, and CD11b⁻ cells by flow cytometry. (B) *Mob* mice were injected with Flt3L-expressing B16 cells to expand the DC subsets. Twenty days after injection, splenocytes were isolated and stimulated with PBS, flagellin (100 ng/ml) or LPS (100 ng/ml) for 2, 4, or 6 h. YFP expression was measured in DC subsets and macrophages by flow cytometry. (C) BM neutrophils and monocytes were sorted by FACS and stimulated with PBS or flagellin (100 ng/ml) for 6 h and 12 h. IFN- β levels in the culture supernatant were measured by ELISA. (D) Small intestinal LP-DCs were stimulated with PBS or flagellin (1 μ g/ml) for 24 h, and IFN- β levels in the culture supernatant were measured by ELISA.

DCs (R4) (Fig. 3B). Furthermore, we were able to confirm the flagellin-induced secretion of IFN- β proteins in purified BM neutrophils and monocytes as well as in small intestinal LP-DCs (Figs. 3C and 3D).

Flagellin induces IFN- β production in a TLR5- and MyD88-dependent manner *in vivo*

Next, we examined whether we could detect flagellin-stimulated IFN- β secretion *in vivo*. Upon *i.p.* or *i.v.* injection of

WT mice with flagellin, serum IFN- β levels peaked after an hour and then rapidly decreased (Fig. 4A). Serum TNF- α levels also showed a similar kinetics of induction while the peak induction of IL-6 was delayed compared to IFN- β and TNF- α (Fig. 4A). Flagellin did not increase serum IFN- α levels (Supplementary Fig. S1). Consistent with the fast increase in the serum IFN- β , we detected YFP expression in splenic neutrophils, monocytes, and macrophages after an hour of flagellin injection to *mob* mice (Supplementary Fig. S2). Type

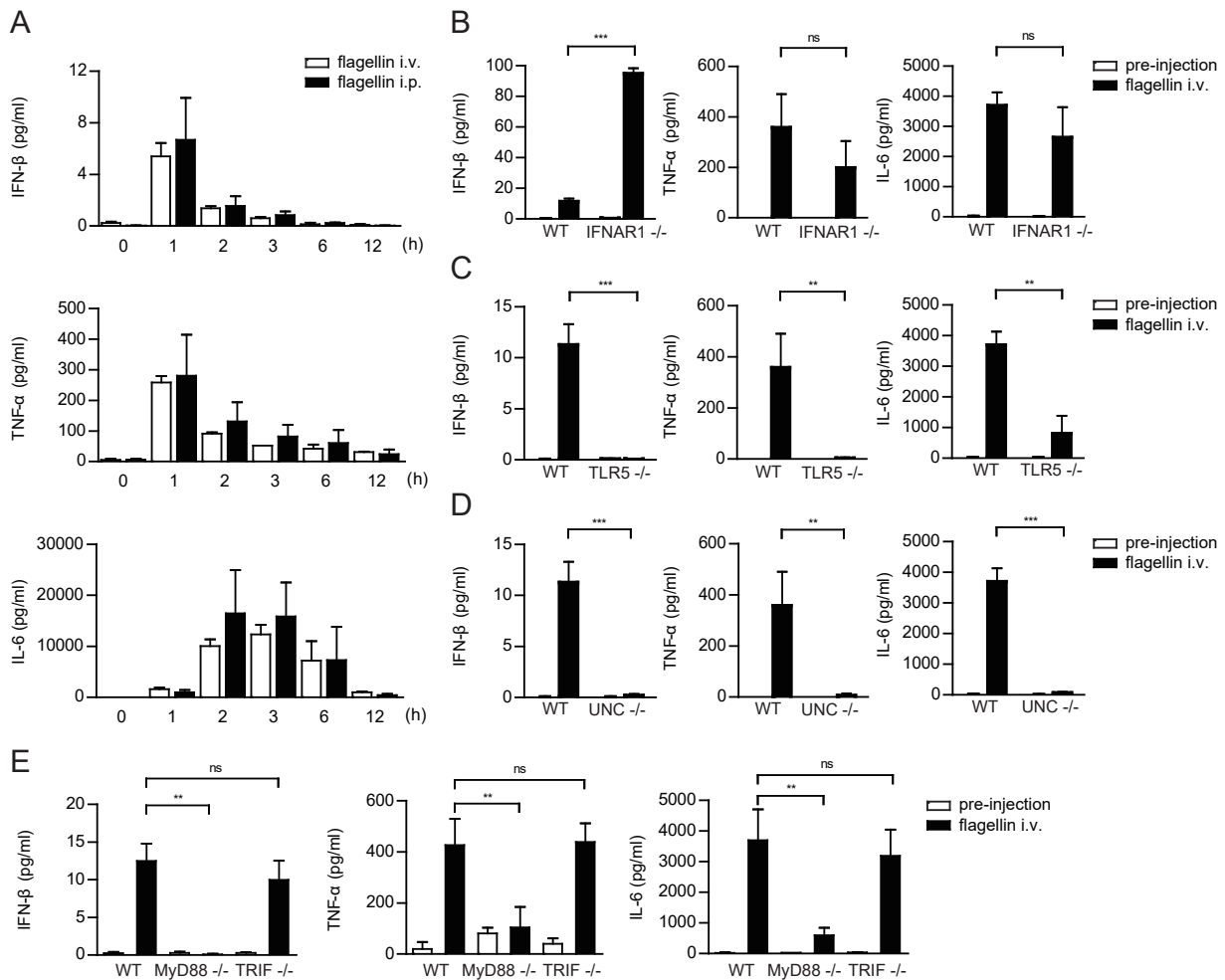


Fig. 4. Flagellin induces IFN- β production in a TLR5- and MyD88-dependent manner *in vivo*. (A) WT mice were injected *i.v.* or *i.p.* with flagellin (2 μ g/mouse). Sera were taken before and up to 12 h after injection. IFN- β , TNF- α , and IL-6 levels were measured by ELISA. (B-E) WT, IFNAR1 KO, TLR5 KO, UNC93B1 KO, MyD88 KO, and TRIF KO mice were *i.v.* injected with flagellin (2 μ g/mouse). Sera were taken before and 1 h after injection. IFN- β , TNF- α , and IL-6 levels were measured by ELISA. Data are presented as mean \pm SEM; ** P < 0.01, *** P < 0.001. ns, statistically not significant.

I IFN receptors (IFNARs) are ubiquitously expressed in various cell populations (Langer and Pestka, 1988). We hypothesized that the rapid decline of serum IFN- β levels might be due to the receptor-mediated consumption of IFN- β . Indeed, we found that IFNAR1 KO mice showed almost 10 times higher serum IFN- β levels compared to WT mice upon flagellin injection whereas serum TNF- α and IL-6 levels were not changed in IFNAR1 KO mice (Fig. 4B).

To test whether flagellin induces IFN- β secretion via TLR5, we compared WT and TLR5 KO mice. The flagellin-stimulated increases of serum IFN- β , TNF- α , and IL-6 were severely blunted in TLR5 KO mice compared to those of WT mice (Fig. 4C). In contrast, TLR4 KO mice showed no defects in the flagellin-induced IFN- β secretion (Supplementary Fig. S3). We previously showed that UNC93B1 is essential for the intracellular trafficking of TLR5 from the ER to the plasma membrane (Huh et al., 2014). In UNC93B1-deficient cells, TLR5 is retained in the ER and cannot transmit the flagellin-stimulated

signals. Accordingly, we found that flagellin-stimulated IFN- β secretion was defective in UNC93B1 KO mice, similar to TLR5 KO mice (Fig. 4D).

Bacteria-sensing TLRs such as TLR2 and TLR4 require both MyD88 and TRIF adaptor molecules to transmit activating signals for the IFN- β induction (Aubry et al., 2012; Fitzgerald et al., 2003; Kagan et al., 2008). In contrast, we found that the flagellin-stimulated induction of IFN- β , as well as TNF- α and IL-6, required MyD88 but not TRIF (Fig. 4E). The flagellin injection increased all three cytokines in both TRIF KO and WT mice in a similar manner, whereas almost no cytokine induction was found in MyD88 KO mice. Transcription factors IRF3, IRF5, and IRF7 become phosphorylated by TBK1 upon TLR activation and promote the type I IFN gene expression (Doyle et al., 2002; Honda et al., 2004; Kawai et al., 2004; Takaoka et al., 2005). Using the TLR5-expressing RAW cells, we found that flagellin also promotes the accumulation of phosphorylated IRF3 and IRF7 in the nucleus (Supplementary Fig. S4).

Therefore, our data indicates that flagellin induces IFN- β production via a TLR5-MyD88-TBK1-IRF3/7 signaling pathway.

Internalization and endolysosomal signaling of TLR5 is required for the flagellin-induced IFN- β production

At steady state, TLR5 is mostly localized at the plasma membrane where it recognizes extracellular flagellin and initiates signaling cascades leading to the production of pro-inflammatory cytokines (Huh et al., 2014). It was suggested that flagellin-bound TLR5 is subsequently internalized, resulting in the MHC class II-mediated presentation of flagellin peptides in antigen-presenting cells (Letran et al., 2011). TLR5-dependent internalization of flagellin is also observed in polarized intestinal epithelial cells (Eaves-Pyles et al., 2011). In addition, the TLR-mediated signaling for type I IFN production is believed to originate from endolysosomal compartments. For TLR3, TLR7, and TLR9, the receptors are constitutively localized in endolysosomes, and TLR2 and TLR4 enter them after the ligand-mediated endocytosis. However, the internalization of TLR5 from the cell surface has not been directly demonstrated yet. Thus, we examined if TLR5 is also internal-

ized into endolysosomal compartments upon flagellin stimulation. First, we measured changes in the cell surface level of TLR5 in RAW cells expressing TLR5-GFP using an anti-TLR5 monoclonal antibody. The cell surface TLR5 levels gradually decreased and became ~20% of the basal level after flagellin stimulation for 12 h. In comparison, the total TLR5 levels—measured by GFP intensities—were moderately reduced by flagellin stimulation, implying that the loss of cell surface TLR5 is mainly due to receptor internalization and sequestration inside cells rather than receptor degradation (Fig. 5A). Next, we visualized the subcellular localization of TLR5 in BM-DCs expressing TLR5-GFP and CD63-Cherry (as an endolysosomal marker) by confocal microscopy. Before flagellin stimulation, TLR5-GFP was uniformly distributed on the plasma membrane and was barely colocalized with CD63-Cherry (Fig. 5B). Upon stimulation with flagellin for 1 h, the decrease of TLR5-GFP signals from the plasma membrane became evident. Twelve hours after stimulation, TLR5-GFP was hardly seen on the plasma membrane and was extensively colocalized with CD63-Cherry. These findings demonstrate that flagellin induces the internalization of TLR5 from the plasma

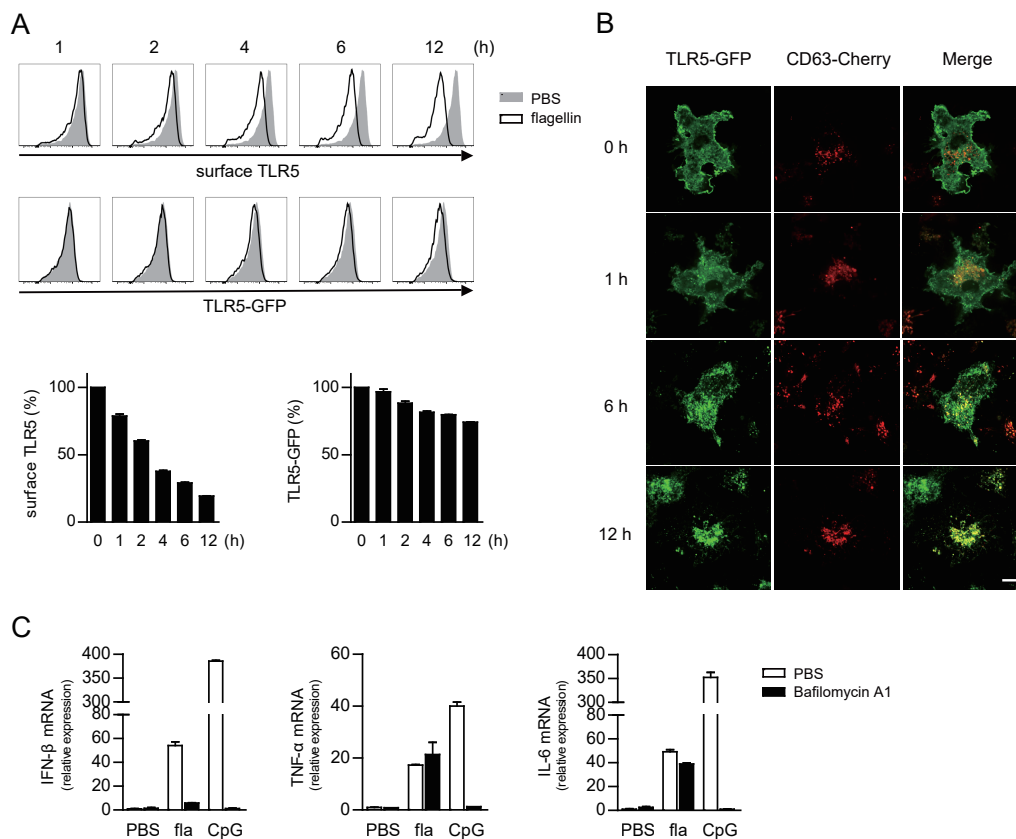


Fig. 5. Internalization and endolysosomal signaling of TLR5 is required for the flagellin-induced IFN- β induction. (A) RAW/TLR5-GFP cells were stimulated with flagellin (100 ng/ml) up to 12 h and stained with biotinylated mouse anti-TLR5 antibody and streptavidin-conjugated Alexa Fluor-647 on ice. The cell surface TLR5 (Alexa Fluor-647) and the total TLR5 (GFP) levels were analyzed by flow cytometry and compared to the ones in unstimulated cells. (B) BM-DCs expressing TLR5-GFP and CD63-Cherry were stimulated with flagellin (100 ng/ml) for 1, 6, or 12 h. TLR5 and CD63 localization was analyzed by confocal microscopy. Scale bar = 10 μ m. (C) RAW/TLR5-Cherry cells were treated with bafilomycin A1 (1 μ M) for 1 h, and then stimulated with flagellin (100 ng/ml) or CpG DNA1826 (1 μ M) for 12 h. Expression of IFN- β , TNF- α , and IL-6 mRNA was measured by qPCR.

membrane into endolysosomes.

Endolysosomal TLR signaling is dependent on the acidic environment of the organelles and can be blocked by lysosomotropic agents or vacuolar-type H⁺-ATPase inhibitors such as bafilomycin A1. To test if TLR5 transmits IFN- β -inducing signals from the endolysosomes, we pretreated the TLR5-expressing RAW cells with bafilomycin A1 and then stimulated them with either flagellin or CpG DNA (a TLR9 agonist). TLR9 is constitutively localized in the endolysosomes and the TLR9-mediated production of pro-inflammatory cytokines as well as type I IFNs is sensitive to the bafilomycin A1 treatment (Hacker et al., 1998; Lund et al., 2003). As expected, we observed that the bafilomycin A1 pretreatment completely inhibited the CpG DNA-induced production of IFN- β , TNF- α , and IL-6 (Fig. 5C). In contrast, when the cells were stimulated with flagellin, only IFN- β induction was inhibited by bafilomycin A1. These data support the notion that TLR5 initiates

signaling for the IFN- β induction from the endolysosomes in a pH-dependent manner, whereas TLR5-mediated signaling for the pro-inflammatory cytokine production happens on the plasma membrane and is not regulated by acidity of intracellular organelles.

Syk is not required for the flagellin-stimulated TLR5 internalization and IFN- β production

For LPS-induced endocytosis of TLR4, the signaling adaptors MyD88 and TRIF were not required, and instead the Syk tyrosine kinase was shown to be essential (Zanoni et al., 2011). Therefore, we tested if Syk is also involved in the flagellin-stimulated TLR5 endocytosis using RAW cells where Syk was deleted by the CRISPR/Cas9 system. The extent of flagellin-stimulated TLR5 internalization in Syk KO cells was same as in WT cells (Supplementary Fig. S5A). Furthermore, flagellin-induced IFN- β production was also normal in the absence

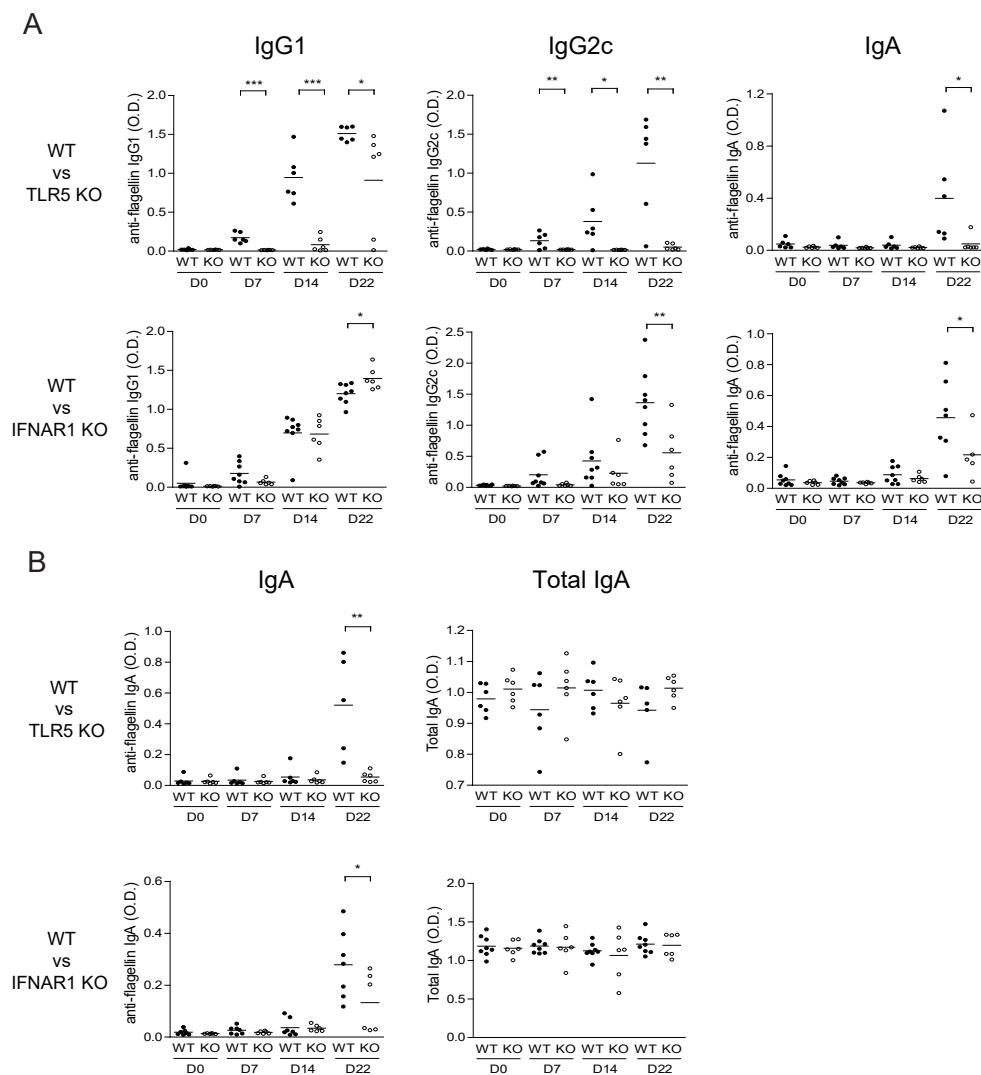


Fig. 6. Flagellin-specific IgG2c and IgA responses require the type I IFN receptor signaling. TLR5, IFNAR1 KO, and the respective littermate control mice were *i.p.* immunized with flagellin (5 μ g) at day 0 and 15. Sera and feces were collected at day 0, 7, 14, and 22, and flagellin-specific antibodies in the sera (A) and fecal extracts (B) were measured by ELISA. * P < 0.05, ** P < 0.01, *** P < 0.001.

of Syk. As a control, we also stimulated cells with depleted zymosan, which signals via the Dectin-1/Syk pathway, and found that both IFN- β and IL-6 production were significantly blunted in Syk KO cells compared to WT cells. Therefore, we concluded that the flagellin-stimulated TLR5 endocytosis and IFN- β production does not require Syk activation, unlike the LPS-induced TLR4 endocytosis and IFN- β production.

Type I IFN signaling is required for flagellin-specific IgG2c and IgA antibody responses

Previous studies showed that systemic immunization with soluble flagellin induces not only IgG responses in the spleen but also IgA responses in the mesenteric lymph nodes (mLNs) (Flores-Langarica et al., 2012). Interestingly, anti-flagellin IgG2c and IgA responses were highly dependent on TLR5 and MyD88, whereas both TLR5 and NLRC4 inflammasome pathways seem to activate anti-flagellin IgG1 response in a partly redundant manner (Lopez-Yglesias et al., 2014). Because type I IFN signaling was implicated in antigen-specific antibody class switching (Swanson et al., 2010; Thompson et al., 2008) and we found that flagellin induces IFN- β production via the TLR5/MyD88 pathway, we tested whether type I IFN signaling regulates anti-flagellin antibody responses.

WT, TLR5 KO, and IFNAR1 KO mice were immunized intraperitoneally with flagellin, and anti-flagellin antibodies in the serum and feces were analyzed 7 and 14 days later. For detection of IgA responses, mice were boost-immunized with flagellin at day 15 and serum and fecal antibodies were measured 7 days later. Consistent with previous studies, we did not observe induction of anti-flagellin IgG2c and IgA antibodies in the serum of TLR5 KO mice compared to littermate control mice. Anti-flagellin IgG1 antibodies were inhibited in the primary responses but partially recovered after the boost immunization (Fig. 6A). In the case of IFNAR1 KO mice, anti-flagellin IgG1 responses were normal and even seem to be slightly higher after the boost immunization compared to littermate control mice. In contrast, anti-flagellin IgG2c and IgA responses were significantly inhibited in IFNAR1 KO mice, especially after the boost immunization (Fig. 6A). Similarly, we found that fecal anti-flagellin IgA responses were severely defective in both TLR5 and IFNAR1 KO mice, whereas the total fecal IgA levels were not affected by the deficiency of either TLR5 or IFNAR1 (Fig. 6B). In contrast to the flagellin immunization, we found that IgG2c and IgA responses to ovalbumin immunization was not impaired in IFNAR1 KO mice (Supplementary Fig. S6). Taken together, these data suggest that IFN- β , produced upon flagellin stimulation in a TLR5-dependent manner, specifically promotes the flagellin-specific IgG2c and IgA class switching via type I IFN receptor signaling.

DISCUSSION

Although flagellin is known to efficiently promote induction of many pro-inflammatory cytokines, several studies reported its inability to induce type I IFNs (Choi et al., 2010; Hemont et al., 2013; Means et al., 2003). However, one previous study noted that flagellin exhibited an anti-osteoclastogenic effect via IFN- β secretion during osteoclast differentiation from BM-derived macrophages (Ha et al., 2008). Another study

showed that flagellin induces type III IFNs via TLR5 (Oden-dall et al., 2017). In the present study, we directly demonstrate that flagellin can induce IFN- β production in various TLR5-expressing cell lines and primary cells, as well as in live mice. Notably, IFNAR1 KO mice showed a greater increase in serum IFN- β levels upon flagellin injection compared to WT mice, suggesting that IFN- β is rapidly bound and sequestered by IFNAR1/2 which are ubiquitously present in many cell types. Another interpretation of the result is a possible negative feedback regulation of IFN- β production via the IFNAR1/2-mediated signaling. However, the TLR-mediated type I IFN production is usually positively regulated by the IFNAR1/2-mediated signaling (Marie et al., 1998; Sato et al., 1998). In fact, when we measured IFN- β production from purified LP-DCs *ex vivo*, flagellin-stimulated IFN- β production was partially inhibited in IFNAR1 KO cells compared to WT cells (data not shown). Therefore, we believe that the higher serum IFN- β levels found in flagellin-stimulated IFNAR1 KO mice is likely due to the lack of IFN- β consumption by neighboring cells. Although we showed that flagellin injection up-regulates the IFN- β promoter activities in splenic neutrophils, monocytes, and macrophages, further studies are required to identify the major cells types that produce and consume IFN- β in response to flagellin stimulation *in vivo*.

Our study also demonstrates that flagellin stimulates IFN- β production via a TLR5-MyD88-IRF3/7 signaling pathway. Similar to other bacteria-sensing TLRs such as TLR2 and TLR4, we found that flagellin-stimulated TLR5 needs to be internalized into endolysosomes to transmit signals for IFN- β induction in an acidic environment. Nonetheless, there are a few differences between TLR4- and TLR5-mediated IFN- β production. In case of TLR4, the receptor endocytosis from the plasma membrane requires Syk kinase activity and the subsequent signaling for IFN- β induction depends on the adaptor molecule TRIF (Zanoni et al., 2011). However, we found that flagellin-induced TLR5 endocytosis and IFN- β production occurs normally in the absence of Syk and TRIF. In this aspect, TLR5 behaves similarly to TLR7 and TLR9, inducing type I IFNs in a MyD88-, but not TRIF-, dependent manner. Of note, TLR5 also shares a similarity with TLR7 and TLR9 in that they all require UNC93B1 for the trafficking out of the ER and the proper localization inside cells.

Among various potential implications of flagellin-stimulated IFN- β induction, we focused on the anti-flagellin antibody production and found that anti-flagellin IgG2c and IgA—but not IgG1—responses are dependent on the type I IFN signaling. In IFNAR1 KO mice, induction of flagellin-specific IgG2c and IgA antibodies was severely impaired whereas ovalbumin-specific antibody induction was normal. Therefore, the requirement of the type I IFN signaling is specific to flagellin-specific antibody responses.

Upon systemic flagellin immunization, intestinal CD103⁺ DCs accumulate in the mLNs and mediate IgA responses (Flores-Langarica et al., 2012). Type I IFN promotes CCR7 expression in DCs for lymph node homing (Jang et al., 2006; Parlato et al., 2001). Because we found that intestinal LP-DCs produce IFN- β upon flagellin stimulation, we hypothesized that the type I IFN signaling may regulate the accumulation of CD103⁺ DCs in mLNs, thereby promoting anti-flagellin IgA

responses. However, we found that flagellin-stimulated accumulation of CD103⁺ DCs in mLNs was normal in IFNAR1 KO mice (data not shown). Flagellin-stimulated intestinal CD103⁺ DCs were also shown to promote IgA class switching by directly acting on B cells in an *in vitro* DC-B cell co-culture assay (Uematsu et al., 2008). However, we found that the *in vitro* IgA induction was not defective in IFNAR1 KO B cells when co-cultured with intestinal CD103⁺ DCs and flagellin equally promoted the differentiation of IgA-secreting cells from both WT and IFNAR1 KO B cells (data not shown). Therefore, molecular mechanisms underlying the type I IFN-mediated regulation of anti-flagellin IgG2c and IgA responses are still unclear and further studies are needed. Especially, it needs to be examined whether the IFN- β signaling modulates the production of cytokines known to regulate the antibody class switching, such as IL-4, IL-10, IL-21, TGF- β , APRIL, and BAFF.

Anti-flagellin antibodies, especially that of the IgA isotype, are essential for maintaining the intestinal homeostasis and keeping the epithelial barrier functions intact by limiting the motility of flagellated bacteria in the gut (Cullender et al., 2013). In addition, TLR5-mediated sensing of commensal bacteria is shown to be necessary for effective antibody responses to seasonal influenza vaccination (Oh et al., 2014). Therefore, our findings presented in this study may help develop strategies for promoting the intestinal health and more efficacious mucosal vaccine design.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

Disclosure

The authors have no potential conflicts of interest to disclose.

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REFERENCES

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998). Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9, 143-150.

Atif, S.M., Lee, S.J., Li, L.X., Uematsu, S., Akira, S., Gorjestani, S., Lin, X., Schweighoffer, E., Tybulewicz, V.L.J., and McSorley, S.J. (2015). Rapid CD4(+) T-cell responses to bacterial flagellin require dendritic cell expression of Syk and CARD9. *Eur. J. Immunol.* 45, 513-524.

Aubry, C., Corr, S.C., Wienerroither, S., Goulard, C., Jones, R., Jamieson,

A.M., Decker, T., O'Neill, L.A.J., Dussurget, O., and Cossart, P. (2012). Both TLR2 and TRIF contribute to interferon-beta production during *Listeria* infection. *PLoS One* 7, e33299.

Carvalho, F.A., Koren, O., Goodrich, J.K., Johansson, M.E., Nalbantoglu, I., Aitken, J.D., Su, Y., Chassaing, B., Walters, W.A., Gonzalez, A., et al. (2012). Transient inability to manage proteobacteria promotes chronic gut inflammation in TLR5-deficient mice. *Cell Host Microbe* 12, 139-152.

Choi, Y.J., Im, E., Chung, H.K., Pothoulakis, C., and Rhee, S.H. (2010). TRIF mediates toll-like receptor 5-induced signaling in intestinal epithelial cells. *J. Biol. Chem.* 285, 37570-37578.

Ciacci-Woolwine, F., Blomfield, I.C., Richardson, S.H., and Mizel, S.B. (1998). *Salmonella* flagellin induces tumor necrosis factor alpha in a human promonocytic cell line. *Infect. Immun.* 66, 1127-1134.

Cullender, T.C., Chassaing, B., Janzon, A., Kumar, K., Muller, C.E., Werner, J.J., Angenent, L.T., Bell, M.E., Hay, A.G., Peterson, D.A., et al. (2013). Innate and adaptive immunity interact to quench microbiome flagellar motility in the gut. *Cell Host Microbe* 14, 571-581.

Doyle, S., Vaidya, S., O'Connell, R., Dadgostar, H., Dempsey, P., Wu, T., Rao, G., Sun, R., Haberland, M., Modlin, R., et al. (2002). IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* 17, 251-263.

Eaves-Pyles, T., Bu, H.F., Tan, X.D., Cong, Y.Z., Patel, J., Davey, R.A., and Strasser, J.E. (2011). Luminal-applied flagellin is internalized by polarized intestinal epithelial cells and elicits immune responses via the TLR5 dependent mechanism. *PLoS One* 6, e24869.

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* 18, 265-276.

Fitzgerald, K.A., Rowe, D.C., Barnes, B.J., Caffrey, D.R., Visintin, A., Latz, E., Monks, B., Pitha, P.M., and Golenbock, D.T. (2003). LPS-TLR4 signaling to IRF-3/7 and NF-kappa B involves the toll adapters TRAM and TRIF. *J. Exp. Med.* 198, 1043-1055.

Flores-Langarica, A., Marshall, J.L., Hitchcock, J., Cook, C., Jobanputra, J., Bobat, S., Ross, E.A., Coughlan, R.E., Henderson, I.R., Uematsu, S., et al. (2012). Systemic flagellin immunization stimulates mucosal CD103(+) dendritic cells and drives Foxp3(+) regulatory T cell and IgA responses in the mesenteric lymph node. *J. Immunol.* 189, 5745-5754.

Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T.D., Ozoren, N., Jagirdar, R., Inohara, N., Vandenabeele, P., Bertin, J., Coyle, A., et al. (2006). Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat. Immunol.* 7, 576-582.

Gewirtz, A.T., Navas, T.A., Lyons, S., Godowski, P.J., and Madara, J.L. (2001). Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J. Immunol.* 167, 1882-1885.

Gomez-Gomez, L. and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell* 5, 1003-1011.

Ha, H., Lee, J.H., Kim, H.N., Kwak, H.B., Kim, H.M., Lee, S.E., Rhee, J.H., Kim, H.H., and Lee, Z.H. (2008). Stimulation by TLR5 modulates osteoclast differentiation through STAT1/IFN-beta. *J. Immunol.* 180, 1382-1389.

Hacker, H., Mischak, H., Miethke, T., Liptay, S., Schmid, R., Sparwasser, T., Heeg, K., Lipford, G.B., and Wagner, H. (1998). CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* 17, 6230-6240.

Hajam, I.A., Dar, P.A., Shah Nawaz, I., Jaume, J.C., and Lee, J.H. (2017). Bacterial flagellin-a potent immunomodulatory agent. *Exp. Mol. Med.* 49, e373.

Halff, E.F., Diebolder, C.A., Versteeg, M., Schouten, A., Brondijk, T.H., and Huizinga, E.G. (2012). Formation and structure of a NAIP5-NLRC4 inflammasome induced by direct interactions with conserved N- and C-terminal regions of flagellin. *J. Biol. Chem.* 287, 38460-38472.

- Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410, 1099-1103.
- Hemont, C., Neel, A., Heslan, M., Braudeau, C., and Josien, R. (2013). Human blood mDC subsets exhibit distinct TLR repertoire and responsiveness. *J. Leukoc. Biol.* 93, 599-609.
- Honda, K., Yanai, H., Mizutani, T., Negishi, H., Shimada, N., Suzuki, N., Ohba, Y., Takaoka, A., Yeh, W.C., and Taniguchi, T. (2004). Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. *Proc. Natl. Acad. Sci. U. S. A.* 101, 15416-15421.
- Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162, 3749-3752.
- Hu, Z., Zhou, Q., Zhang, C., Fan, S., Cheng, W., Zhao, Y., Shao, F., Wang, H.W., Sui, S.F., and Chai, J. (2015). Structural and biochemical basis for induced self-propagation of NLRC4. *Science* 350, 399-404.
- Huh, J.W., Shibata, T., Hwang, M., Kwon, E.H., Jang, M.S., Fukui, R., Kanno, A., Jung, D.J., Jang, M.H., Miyake, K., et al. (2014). UNC93B1 is essential for the plasma membrane localization and signaling of Toll-like receptor 5. *Proc. Natl. Acad. Sci. U. S. A.* 111, 7072-7077.
- Jang, M.H., Sougawa, N., Tanaka, T., Hirata, T., Hiroi, T., Tohya, K., Guo, Z., Umemoto, E., Ebisuno, Y., Yang, B.G., et al. (2006). CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J. Immunol.* 176, 803-810.
- Kagan, J.C., Su, T., Homg, T., Chow, A., Akira, S., and Medzhitov, R. (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat. Immunol.* 9, 361-368.
- Kawai, T., Sato, S., Ishii, K.J., Coban, C., Hemmi, H., Yamamoto, M., Terai, K., Matsuda, M., Inoue, J., Uematsu, S., et al. (2004). Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat. Immunol.* 5, 1061-1068.
- Kim, J., Huh, J., Hwang, M., Kwon, E.H., Jung, D.J., Brinkmann, M.M., Jang, M.H., Ploegh, H.L., and Kim, Y.M. (2013). Acidic amino acid residues in the juxtamembrane region of the nucleotide-sensing TLRs are important for UNC93B1 binding and signaling. *J. Immunol.* 190, 5287-5295.
- Kofoed, E.M. and Vance, R.E. (2011). Innate immune recognition of bacterial ligands by NAIps determines inflammasome specificity. *Nature* 477, 592-595.
- Langer, J.A. and Pestka, S. (1988). Interferon receptors. *Immunol. Today* 9, 393-400.
- Letran, S.E., Lee, S.J., Atif, S.M., Uematsu, S., Akira, S., and McSorley, S.J. (2011). TLR5 functions as an endocytic receptor to enhance flagellin-specific adaptive immunity. *Eur. J. Immunol.* 41, 29-38.
- Lopez-Yglesias, A.H., Zhao, X., Quarles, E.K., Lai, M.A., VandenBos, T., Strong, R.K., and Smith, K.D. (2014). Flagellin induces antibody responses through a TLR5-and inflammasome-independent pathway. *J. Immunol.* 192, 1587-1596.
- Lowy, J. and McDonough, M.W. (1964). Structure of filaments produced by re-aggregation of *Salmonella* flagellin. *Nature* 204, 125-127.
- Lund, J., Sato, A., Akira, S., Medzhitov, R., and Iwasaki, A. (2003). Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells. *J. Exp. Med.* 198, 513-520.
- Marie, I., Durbin, J.E., and Levy, D.E. (1998). Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. *EMBO J.* 17, 6660-6669.
- McSorley, S.J., Ehst, B.D., Yu, Y., and Gewirtz, A.T. (2002). Bacterial flagellin is an effective adjuvant for CD4+ T cells in vivo. *J. Immunol.* 169, 3914-3919.
- Means, T.K., Hayashi, F., Smith, K.D., Aderem, A., and Luster, A.D. (2003). The toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. *J. Immunol.* 170, 5165-5175.
- Miao, E.A., Alpuche-Aranda, C.M., Dors, M., Clark, A.E., Bader, M.W., Miller, S.I., and Aderem, A. (2006). Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat. Immunol.* 7, 569-575.
- Muller, U., Steinhoff, U., Reis, L.F., Hemmi, S., Pavlovic, J., Zinkernagel, R.M., and Aguet, M. (1994). Functional role of type I and type II interferons in antiviral defense. *Science* 264, 1918-1921.
- Odendall, C., Voak, A.A., and Kagan, J.C. (2017). Type III IFNs are commonly induced by bacteria-sensing TLRs and reinforce epithelial barriers during infection. *J. Immunol.* 199, 3270-3279.
- Oh, J.Z., Ravindran, R., Chassaing, B., Carvalho, F.A., Maddur, M.S., Bower, M., Hakimpour, P., Gill, K.P., Nakaya, H.I., Yarovsky, F., et al. (2014). TLR5-mediated sensing of gut microbiota is necessary for antibody responses to seasonal influenza vaccination. *Immunity* 41, 478-492.
- Parlato, S., Santini, S.M., Lapenta, C., Di Pucchio, T., Logozzi, M., Spada, M., Giammarioli, A.M., Malorni, W., Fais, S., and Belardelli, F. (2001). Expression of CCR-7, MIP-3 beta, and Th-1 chemokines in type IIFN-induced monocyte-derived dendritic cells: importance for the rapid acquisition of potent migratory and functional activities. *Blood* 98, 3022-3029.
- Sanders, C.J., Yu, Y., Moore, D.A., 3rd, Williams, I.R., and Gewirtz, A.T. (2006). Humoral immune response to flagellin requires T cells and activation of innate immunity. *J. Immunol.* 177, 2810-2818.
- Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., and Tanaka, N. (1998). Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Lett.* 441, 106-110.
- Scheu, S., Dresing, P., and Locksley, R.M. (2008). Visualization of IFNbeta production by plasmacytoid versus conventional dendritic cells under specific stimulation conditions in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 105, 20416-20421.
- Shibata, T., Takemura, N., Motoi, Y., Goto, Y., Karuppachamy, T., Izawa, K., Li, X., Akashi-Takamura, S., Tanimura, N., Kunisawa, J., et al. (2012). PRAT4A-dependent expression of cell surface TLR5 on neutrophils, classical monocytes and dendritic cells. *Int. Immunol.* 24, 613-623.
- Swanson, C.L., Wilson, T.J., Strauch, P., Colonna, M., Pelanda, R., and Torres, R.M. (2010). Type I IFN enhances follicular B cell contribution to the T cell-independent antibody response. *J. Exp. Med.* 207, 1485-1500.
- Takaoka, A., Yanai, H., Kondo, S., Duncan, G., Negishi, H., Mizutani, T., Kano, S., Honda, K., Ohba, Y., Mak, T.W., et al. (2005). Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* 434, 243-249.
- Thompson, J.M., Whitmore, A.C., Staats, H.F., and Johnston, R. (2008). The contribution of type I interferon signaling to immunity induced by alphavirus replicon vaccines. *Vaccine* 26, 4998-5003.
- Uematsu, S., Fujimoto, K., Jang, M.H., Yang, B.G., Jung, Y.J., Nishiyama, M., Sato, S., Tsujimura, T., Yamamoto, M., Yokota, Y., et al. (2008). Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat. Immunol.* 9, 769-776.
- Uematsu, S., Jang, M.H., Chevrier, N., Guo, Z.J., Kumagai, Y., Yamamoto, M., Kato, H., Sougawa, N., Matsui, H., Kuwata, H., et al. (2006). Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c(+) lamina propria cells. *Nat. Immunol.* 7, 868-874.
- Vijay-Kumar, M., Aitken, J.D., and Gewirtz, A.T. (2008). Toll like receptor-5: protecting the gut from enteric microbes. *Semin. Immunopathol.* 30, 11-21.
- Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., et al. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301, 640-643.
- Yoon, S.I., Kurnasov, O., Natarajan, V., Hong, M., Gudkov, A.V., Osterman, A.L., and Wilson, I.A. (2012). Structural basis of TLR5-flagellin recognition

and signaling. *Science* 335, 859-864.

Zanoni, I., Ostuni, R., Marek, L.R., Barresi, S., Barbalat, R., Barton, G.M., Granucci, F., and Kagan, J.C. (2011). CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* 147, 868-880.

Zhang, L., Chen, S., Ruan, J., Wu, J., Tong, A.B., Yin, Q., Li, Y., David, L., Lu, A.,

Wang, W.L., et al. (2015). Cryo-EM structure of the activated NAIP2-NLRC4 inflammasome reveals nucleated polymerization. *Science* 350, 404-409.

Zhao, Y., Yang, J., Shi, J., Gong, Y.N., Lu, Q., Xu, H., Liu, L., and Shao, F. (2011). The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* 477, 596-600.