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Flagellin Induces Antibody Responses through a TLR5- and Inflammasome-Independent Pathway

Américo Harry López-Yglesias,* Xiaodan Zhao,* Ellen K. Quarles,* Marvin A. Lai,* Tim VandenBos,† Roland K. Strong,† and Kelly D. Smith*

Flagellin is a potent immunogen that activates the innate immune system via TLR5 and Naip5/6, and generates strong T and B cell responses. The adaptor protein MyD88 is critical for signaling by TLR5, as well as IL-1Rs and IL-18Rs, major downstream mediators of the Naip5/6 Nlrc4-inflammasome. In this study, we define roles of known flagellin receptors and MyD88 in Ab responses generated toward flagellin. We used mice genetically deficient in flagellin recognition pathways to characterize innate immune components that regulate isotype-specific Ab responses. Using purified flagellin from Salmonella, we dissected the contribution of innate flagellin recognition pathways to promote Ab responses toward flagellin and coadministered OVA in C57BL/6 mice. We demonstrate IgG2c responses toward flagellin were TLR5 and inflammasome dependent; IgG1 was the dominant isotype and partially TLR5 and inflammasome dependent. Our data indicate a substantial flagellin-specific IgG1 response was induced through a TLR5-, inflammasome-, and MyD88-independent pathway. IgA anti-FliC responses were TLR5 and MyD88 dependent and caspase-1 independent. Unlike C57BL/6 mice, flagellin-immunized A/J mice induced codominant IgG1 and IgG2a responses. Furthermore, MyD88-independent, flagellin-induced Ab responses were even more pronounced in A/J MyD88−/− mice, and IgA anti-FliC responses were suppressed by MyD88. Flagellin also worked as an adjuvant toward coadministered OVA, but it only promoted IgG1 anti-OVA responses. Our results demonstrate that a novel pathway for flagellin recognition contributes to Ab production. Characterization of this pathway will be useful for understanding immunity to flagellin and the rationale design of flagellin-based vaccines.

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Innate immunity is responsible for both sounding the alarm of pathogen invasion and directing cellular and humoral immunity. The innate immune system recognizes pathogens with germline-encoded pattern receptors that respond to conserved pathogen-associated molecular patterns (1–3). Two key groups of pattern recognition receptors are membrane-bound TLRs and cytosolic NOD-like receptors (NLRs). TLRs recognize structurally diverse pathogen-associated molecular patterns, including nucleic acids, glycolipids, lipoproteins, and proteins. The only known protein ligand for human TLRs is bacterial flagellin, which is recognized by TLR5 (4).

Flagellin is exposed on the surface of flagellated bacteria and is a major antigenic target of the immune system in a wide variety of hosts, ranging from plants and invertebrates to vertebrates (5–7). In Salmonella enterica serovar, Typhimurium (S. typhimurium) flagellin is encoded by the genes fliC and fljB, with fliC being the primary gene (8). FliC is a potent immunogen that is capable of inducing strong immune responses to itself (intrinsic adjuvancy) and coadministered Ags (extrinsic adjuvancy) (9–17). The intrinsic and extrinsic adjuvancy of flagellin has been attributed to conserved structures in its D0 domain, recognized by Naip5 and Naip6 (Naip5/6), and its D1 domain, recognized by TLR5 (4, 6, 18–24). Studies from several groups have established that recognition of FliC by the innate immune system leads to microbicidal activity, cytokine production, and dendritic cell (DC) activation (25–27). Immunization of mice with FliC elicits robust T cell activation and T cell–dependent Ab responses (14–16, 26, 28–33).

Flagellin, the ligand for TLR5, has been shown to induce a Th2-biased response (29, 30, 34), and it is currently being developed as a vaccine adjuvant (35, 36). Because flagellin is a protein, the molecule can be engineered for vaccine development to retain immunogenicity and display foreign epitopes of interest from pathogens such as influenza (hemagglutinin and matrix proteins), Yersinia pestis, and Helicobacter pylori (FlaA flagellin) (9, 37–41). Compared with the coadministration of flagellin with an Ag, flagellin fusion proteins elicit enhanced humoral responses and are therefore a more alluring alternative for vaccine design (35, 42–45). The greater efficacy of the flagellin–Ag fusions suggests that proximity of the Ag to the adjuvant allows for enhanced antigenicity.

NLRs are cytosolic sensors that oligomerize after ligand recognition and form multiprotein complexes termed inflammasomes (46, 47). A broad range of pathogen-derived and endogenous signals initiate inflammasome formation, and one of its triggers, alum, has been used for decades as an adjuvant that elicits Th2 type responses toward coadministered Ags (48). The best studied NLR, Nlrp3, is required for alum-induced activation of the inflammasome (49, 50), but alum also uses an inflammasome-independent pathway to induce Th2 immunity (51, 52). The Naip family of...
NLRS activates the inflammasome in an Nlr4-dependent manner (21, 22). Murine Naip2 recognizes the rod proteins of some bacterial type III secretory systems, whereas murine Naip5 and Naip6 recognize flagellin. Human NaIP recognizes the needle protein of some bacterial type III secretion systems (21) and has been reported to recognize flagellin (21, 53). Recognition of these protein ligands by the Naip proteins induces oligomerization with Nlr4, leading to recruitment and activation of caspase-1 (54).

Active caspase-1 processes pro–IL-1β and pro–IL-18 into mature forms for secretion, and initiates a form of cell-mediated death termed pyroptosis (55). The Nlr4 system has been recently found to contribute to flagellin-induced Ab production in mice, in a manner that is redundant with TLR5 (16). In the absence of TLR5, Nlr4 is required for flagellin’s immunogenicity (16). The isotype specificity of Nlr4 inflammasome-dependent Ab responses is unknown.

TLR5 is expressed on the surface of epithelial cells, neutrophils, monocytes, and DCs (4, 18, 56). Flagellin recognition by TLR5 induces its dimerization and signaling through adaptor protein MyD88 (57, 58). Activation of DCs via TLR5 leads to the upregulation of MHC class II, CD80, and CD86, and the secretion of cytokines, such as IL-23, IL-6, and Cxcl1 (14, 26, 29). TLR5 also promotes flagellin uptake and presentation that is required for efficient T cell activation (14, 33, 34, 45). Thus, TLR5 recognition also promotes flagellin uptake and presentation that is required for upregulation of MHC class II, CD80, and CD86, and the secretion of cytokines (33, 60). Flagellin uptake (33, 60), or this commonality, Ab responses toward flagellin are maintained in some, IL-1β-dependent cytokine responses in vivo, which are required for flagellin-induced Ab production in mice, in a manner that is redundant with TLR5 (16). In the absence of TLR5, Nlr4 is required for flagellin’s immunogenicity (16).

TLR5 and the major cytokine outputs of the Nlr4 inflammasome, IL-1β and IL-18, require MyD88 for signaling (59). Despite this commonality, Ab responses toward flagellin are maintained in MyD88-deficient mice (15). Thus, MyD88-independent pathways emanating from either TLR5, such as flagellin uptake (33, 60), or the Nlr4 inflammasome (61) may also contribute to flagellin-specific Ab responses. In this article, we dissect the innate immune components that are responsible for flagellin’s intrinsic and extrinsic adjuvant properties, as well as the production of isotype-specific Ab responses to flagellin and a model coadministered Ag, OVA. These studies define the innate immune components that are required to generate robust isotype-specific Ab responses toward FliC or coadministered Ags, and uncover a novel TLR5, inflammasome- and MyD88-independent flagellin recognition pathway that contributes to flagellin’s immunogenicity.

Materials and Methods

Protein isolation and purification

Wild-type (WT) flagellin monomers were isolated from S. typhimurium strain SL1344 (ΔfgfM); purity was verified as previously described (18, 62). OVA was purchased from Sigma and ultrafiltered (Amicon) to reduce endotoxin. Removal of residual endotoxin from isolated flagellin monomers and OVA (Sigma-Aldrich) was performed by using polymyxin B columns (Thermo Scientific). Endotoxin levels were <1 pg/μg protein, as measured using the limulus amebocyte lysis assay (Lonza). The purified flagellin was characterized biochemically, and its biological activity for TLR5 and Naip5 was determined before mouse studies (Supplementary Fig. 1).

NF-κB Luciferase Reporter Assay

CHO K1 cells were stably transfected with mouse TLR5 cDNA cloned into the pEF6 VS/His TOPO vector (Invitrogen) or the empty vector, plus the ELAM-LUC plasmid; luciferase assays were performed as previously described (18, 62).

Protein transfection

Bone marrow–derived macrophages were prepared from femurs of C57BL6 mice, Naip5-deficient mice, caspase-1–deficient mice, or AJI mice, and cultured in RPMI 1640 supplemented with 10% FBS (Atlas Biologicals), 10% L-cell supernatant (CSF1 source), 2 mM l-glutamine, and 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies) (19, 63, 64). All assays were performed in triplicate and each experiment was repeated at least twice. Bone marrow–derived macrophages were primed with 10 ng/ml ultrapure LPS (List Biologicals) for 3 h to induce pro–IL-1β expression before protein transfection, using Profect-P1-lipid–based protein delivery reagent (Targeting Systems) as previously described (19, 65); IL-1β secretion was determined by ELISA (Duoset; R&D Systems).

Mice and immunizations

The University of Washington Institutional Animal Care and Use Committee approved all animal protocols. Mice were bred and housed in a specific pathogen-free facility at the University of Washington. AJI and C57BL/6 animals were purchased from Jackson Laboratories and bred in-house. Naip5−/− (66), caspase-1 (Casp1−/−) (67), MyD88−/− (68), and TLR5−/− (69) mice were all generated on the C57BL/6 background and bred in-house. TLR5−/−/Casp1−/− mice were generated and bred in our animal facility. AJI MyD88−/− mice were generated by backcrossing the MyD88 deletion onto the AJI background for nine generations and then crossing to generate homozygous MyD88−/− mice. Eight- to 14-wk-old matched animals were used in all experiments. retro-orbital bleeds were performed on all animals before immunization to obtain naive serum. Mice received two sequential i.p. immunizations with 30 μg FlilC and 30 μg OVA separated by 21 d. Blood was drawn 2 wk after each immunization.

Cytokine analysis

Mouse sera were evaluated for cytokine responses after i.p. injections of 30 μg FlilC or PBS using commercially sourced ELISA kits according to manufacturer’s instructions (Duoset). IL-18 cytokine analysis was determined by ELISA, using anti-mouse IL-18 (clone 74; R&D Systems) as a capture Ab and biotinylated anti-mouse IL-18 (clone 93-10C; R&D Systems) as a detection Ab.

Ab analysis

High binding capacity 96-well plates (COSTAR) were coated with 1 μg/ml monomeric FlilC or OVA diluted in PBS (OmniPure) and allowed to incubate overnight at room temperature (RT). Plates were washed three times with PBS containing 0.05% Tween 20 and blocked for 1 h at RT in PBS containing 1% BSA (Sigma). Plates were washed and serial dilutions of serum were added to the wells and incubated for 1 h at RT. Plates were washed again and HRP-conjugated secondary Abs (anti-IgG1, -IgG2a, -IgG2c-HRP [Jackson Immunoresearch]; or –IgA-HRP [Biolegend]) were added and incubated for another hour at RT. Plates were developed with TMB substrate (Thermo Scientific), stopped with H2SO4, and absorbance was read at 450 nm (Molecular Devices). Ab end-point titer was defined by the reciprocal of the maximal serum dilution that exceeded three times the SD above the mean background absorbance.

Statistical analysis

Significance was determined by one-way ANOVA with Bonferroni multiple-comparison posttest or Mann–Whitney U test, using GraphPad Prism 5 software. Differences were noted as significant when p < 0.05.

Results

TLR5 and the Naip5 inflammasome control distinct early cytokine responses in vivo

Innate recognition of FlilC leads to cytokine and chemokine production that contributes to host defense and adaptive immunity. To characterize similarities and differences in early phase of innate detection of flagellin, we defined the pathways necessary for triggering flagellin-dependent cytokine responses in vivo using WT, TLR5−/−, Naip5−/−, Casp1−/−, MyD88−/−, and TLR5−/−/Casp1−/− (double knockout [DKO]) mice. Mice were injected i.p. with 30 μg FlilC, isolated and purified from S. typhimurium (Supplementary Fig. 1), and sera were assessed at 2 and 4 h after flagellin injection. WT mice produced IL-6, Cxcl1, IL-12/23p40, and IL-18 (Fig. 1). At the 2-h time point, serum Cxcl1, IL-6, and IL-12/23p40 were TLR5 and MyD88 dependent (Fig. 1A, 1C, 1D). Serum IL-6 and IL-12/23p40 levels were also partially dependent on Naip5 and Casp1 (Fig. 1C, 1D). In contrast, flagellin induction of IL-18 was TLR5 and MyD88 independent, but entirely dependent on Naip5 and Casp1 (Fig. 1B). At 4 h postin-
Flagellin-induced cytokines are differentially regulated by TLR5, Naip5, Casp1, and MyD88. WT (n = 7–12), TLR5−/− (n = 4–8), Naip5−/− (n = 3), Casp1−/− (n = 4–11), DKO (n = 3–4), MyD88−/− (n = 3–6), and mock (n = 8–10) mice were injected i.p. with FliC (30 µg) or PBS (mock). Serum was collected 2 h after injections, and cytokine levels were determined by ELISA. IL-12/23p40 (A), IL-18 (B), IL-6 (C), and Cxcl1 (D). All groups have a minimum n = 3. Statistical analysis was done using one-way ANOVA with Bonferroni multiple-comparisons posttest: **p < 0.01, ***p < 0.001.

The role of TLR5 and the Naip5 inflammasome in flagellin-induced cytokine production was more complex (Fig. 1). IL-12/23p40 and IL-18 continued to be dependent on the flagellin sensors TLR5 and Naip5 inflammasome, respectively (Fig. 1A, 1B). However, IL-6 and Cxcl1 were detected at 4 h in TLR5−/−, but not MyD88−/− or TLR5/Casp1 DKO mice, suggesting a delayed cytokine cascade, where flagellin-induced IL-18 or other Casp1-dependent factors induce Cxcl1 and IL-6 in an MyD88-dependent manner (Fig. 1C, 1D). Thus, the innate immune receptors TLR5 and Naip5 function both independently and in concert to regulate early cytokine production induced by flagellin. Flagellin has also been reported to induce low levels of TNF and IL-1β in mice (15, 16, 70). In our studies, we found no significant flagellin-dependent induction of IL-1β or TNF at 2 and 4 h postinjection in any of the mice (data not shown). For TNF, it is likely that the 2-h time point missed the early and low-level induction, which typically peaks around 1–1.5 h postinjection (data not shown) (70, 71). Other investigators have also had difficulty detecting IL-1β in mouse serum (15, 70), suggesting that technical issues may have precluded our ability to detect the low levels reported by Bedoui and colleagues (12).

**IgG1 isotype-specific responses are MyD88 independent**

Flagellin is a major antigenic target during bacterial infections and when injected as a purified protein also induces Abs against itself and coadministered Ags. Because flagellin is being developed as a platform for recombinant vaccines, we dissected the innate immune pathways needed to generate isotype-specific Ab responses against flagellin or a coadministered Ag, OVA. C57BL/6 and MyD88−/− mice were injected i.p. with 30 µg FliC and boosted with the same dose of flagellin 3 wk later. The immunized animals were bled before immunization (naive serum) and at 2 wk after primary and secondary immunizations. We tested the sera for IgG1 and IgG2c Abs against FliC. After primary immunizations, the IgG1 anti-FliC median titer was 3160 in WT mice and 316 in MyD88−/− mice (Fig. 2A). After the secondary immunization, IgG1 anti-FliC median titers increased ≥100-fold in both WT and MyD88−/− animals, and IgG1 titers remained significantly reduced in MyD88−/− compared with WT mice (Fig. 2A).

In contrast with IgG1, FliC-immunized C57BL/6 mice generated low titers of IgG2c anti-FliC, which were best detected after the secondary immunization (Fig. 2B). The IgG2c anti-FliC response was absolutely dependent on MyD88 (Fig. 2B). Our data demonstrate that IgG2c and a portion of IgG1 anti-flagellin responses are MyD88 dependent. Our results also support an MyD88-independent pathway for IgG1 anti-flagellin Abs in C57BL/6 mice.

**TLR5 and the inflammasome play largely redundant roles in IgG1 anti-FliC responses**

We next examined the individual components of innate flagellin recognition for their contribution to the anti-FliC Ab responses. Compared with WT mice, TLR5−/−, Naip5−/−, and Casp1−/− mice produced similar IgG1 titers toward flagellin (Fig. 3A–C). In contrast, the IgG2c anti-FliC responses were significantly reduced in TLR5−/−, Naip5−/−, and Casp1−/− mice after secondary immunizations (Fig. 3D–F). Our results suggest that each individual innate recognition pathway for flagellin contributes to the generation of IgG2c anti-FliC responses. In contrast, loss of TLR5 or the individual inflammasome molecules, Naip5 or Casp1, did not affect robust IgG1 anti-FliC responses.
MyD88-independent IgG1 anti-FliC responses are also TLR5 and inflammasome independent

To determine whether TLR5 or the inflammasome contributes to MyD88-independent IgG1 anti-FliC responses, we generated TLR5 and Casp1 DKO mice. TLR5/Casp1 DKO mice had significantly reduced IgG1 anti-FliC titers but maintained moderate titers as seen in MyD88−/− mice (Fig. 4A). As expected, the DKO animals had significantly reduced IgG2c responses toward FliC (Fig. 4B). These results strongly suggest that the MyD88-independent anti-FliC IgG1 response is mediated by a novel pathway that is distinct from the known TLR5 and inflammasome pathways for flagellin recognition.

Serum IgA anti-FliC responses are TLR5 and MyD88 dependent and Casp1 independent

We tested naive and boost sera of C57BL/6 mice and knockout mice, and assessed IgA anti-FliC titers after secondary immunizations of FliC because previous in vitro and in vivo studies have shown that monomeric flagellin is capable of inducing IgA responses toward itself (72, 73). IgA anti-FliC responses were detected at low levels after the secondary immunization (Fig. 5A). Consistent with recent results of Cunningham and colleagues (72), our results with TLR5−/−, MyD88−/−, and DKO mice show IgA anti-FliC responses are TLR5 and MyD88 dependent (Fig. 5B, 5E, 5F). Furthermore, the IgA anti-FliC titers were detected in Naip5−/− and Casp1−/− mice, suggesting that the inflammasome is not required for IgA anti-FliC responses (Fig. 5C, 5D).

Flagellin’s adjuvancy toward extrinsic Ags is partially dependent on TLR5 and the inflammasome

Next, we examined how flagellin regulates Ab responses toward a coadministered (extrinsic) Ag, OVA. Mice immunized with OVA alone failed to generate anti-OVA responses, whereas mice coinjected with FliC and OVA generated anti-OVA Abs (Fig. 6A). We also tested the isotype specificity of the FliC-dependent anti-OVA Ab responses and found that in all animals tested, IgG1 was the only isotype detected against OVA; no IgG2c or IgA was generated against OVA (data not shown). The IgG1 anti-OVA responses were similar in WT, TLR5−/−, Naip5−/−, and Casp1−/− mice (Fig. 6B–D). The IgG1 anti-OVA titers were significantly reduced in MyD88−/− mice and approached, but did not reach, statistical significance in DKO animals (p = 0.07; Fig. 6E, 6F). Thus, the IgG1 and anti-OVA and IgG1 anti-FliC responses appear to be regulated by similar MyD88-dependent and -independent mechanisms.

Defective flagellin-induced IL-18 in A/J mice

A/J mice contain a hypofunctional Naip5 that is associated with susceptibility to Legionella pneumophila (21, 22, 47, 74). We assessed the serum cytokines in response to FliC 2 h after i.p. injection with 30 μg FliC. A/J and C57BL/6 mice produced equivalent amounts of IL-6, IL-12/23p40, and Cxcl1 (Fig. 7A–C). However, A/J mice failed to produce IL-18 (Fig. 7D). As with C57BL/6 mice, A/J mice did not produce detectable TNF or IL-1β (Fig. 7E, 7F). Thus, the flagellin-induced cytokine responses in A/J mice are consistent with intact TLR5 and impaired Naip5 recognition of flagellin due to the hypofunctional Naip5A/J allele.

Augmented IgG1 and IgG2a anti-FliC responses in A/J mice

We compared isotype-specific Ab responses against FliC in A/J and C57BL/6 mice and their respective MyD88 deficient strains. In contrast with C57BL/6 mice, A/J mice had more robust Ab responses to flagellin after primary and secondary immunization, with ~10-fold increased IgG1 anti-FliC titers after primary and secondary, and >100-fold increased IgG2a/c anti-FliC titers after
primary and secondary immunizations (Fig. 8A, 8B). The IgG2a responses in A/J mice were partially MyD88 dependent, whereas IgG1 anti-FliC titers were reduced in A/J MyD88<sup>−/−</sup> mice, trending toward significance (p = 0.08; Fig. 8A, 8B). In contrast with C57BL/6 mice, which have a strong IgG1 biased Ab response against flagellin, A/J mice had a balanced Ab response toward flagellin, with equivalent IgG1 and IgG2a anti-FliC titers.

Anti-OVA responses in A/J mice are partially dependent on MyD88. A/J and A/J MyD88<sup>−/−</sup> mice were immunized with FliC and OVA. As with IgG2c responses in C57BL/6 mice, IgG2a anti-OVA Ab responses were undetectable in A/J mice (data not shown). After primary immunizations in both A/J and A/J MyD88<sup>−/−</sup> animals, IgG1 anti-OVA Abs were at titers of ≤100 in all mice (Fig. 8C). In A/J mice after secondary immunizations with FliC and OVA, IgG1 anti-OVA responses were robust, with titers reaching a median of 10<sup>5</sup> (Fig. 8C). As for C57BL/6, MyD88<sup>−/−</sup> and A/J MyD88<sup>−/−</sup> mice had reduced, but detectable, IgG1 anti-OVA titers compared with that of their WT counterparts (Fig. 8C).

MyD88 suppresses IgA anti-FliC responses in A/J mice. Sera from A/J and A/J MyD88<sup>−/−</sup> were also assessed for IgA anti-FliC responses. In contrast with C57BL/6 mice, A/J mice did not generate significant IgA anti-FliC after two immunizations (Fig. 9A). Conversely, the A/J MyD88<sup>−/−</sup> mice produced a substantial IgA anti-FliC titer after two immunizations (Fig. 9B). These results illuminate the fine intricacies between different strains of mice, resulting in quantitative and qualitative differences in isotype-specific responses generated against FliC.

Discussion

Dissecting the innate immune pathways that recognize flagellin’s structural properties and promote adaptive immune responses is vital for the rational design of flagellin-based vaccines. Flagellin-based fusion proteins are currently being developed as vaccines for infectious diseases (35, 36) and assessed in phase I and II clinical trials (38–40). Flagellin is also being developed as a therapeutic agent to treat infectious diseases, toxic exposures, and cancers (75–77). Several studies have shown that flagellin’s adjuvancy is dependent on TLR5 recognition (35–37). More recently, murine Naip5 and Naip6 have been demonstrated to recognize bacterial flagellin and activate the Nlrc4 inflammasome (21, 22). Additional studies have indicated that inflammasome-mediated detection of flagellin also contributes to its immunogenicity (15). Vijay-Kumar et al. (16) demonstrated that TLR5 and the Nlrc4 inflammasome play redundant roles in flagellin-induced adaptive immune responses.
Ab responses, with neither being necessary and either being sufficient for flagellin-induced Ab responses. This same group determined that MyD88 is not required for flagellin-induced IgG Ab responses, suggesting that an MyD88-independent pathway emanates from either TLR5 or the Nlrc4 inflammasome, and contributes to anti-flagellin IgG production.

In our study, we dissected the role of flagellin detection by the innate immune system in generating isotype-specific Ab responses to flagellin itself (intrinsic adjuvancy) and coadministered OVA (extrinsic adjuvancy). Although neither deletion of TLR5 nor the Naip5 inflammasome alone were sufficient to reduce IgG1 anti-FliC Abs, deletion of both TLR5 and Casp1 or elimination of MyD88 significantly reduced IgG1 responses toward flagellin. However, IgG1 anti-FliC responses were still detected in MyD88 and TLR5/Casp1 DKO mice, suggesting flagellin is recognized by a third uncharacterized pathway. This conclusion is supported by our studies in A/J MyD88\(^{-/-}\) mice, which are naturally impaired in Naip5 (74). The A/J MyD88\(^{-/-}\) mice produced reduced, but moderate, IgG1 and IgG2a anti-FliC responses. The combined data from immunizations performed in C57BL/6 and A/J mice

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Flagellin-induced IgG1 anti-OVA responses are partially MyD88 dependent. WT mice were immunized twice on days 1 and 21 with OVA alone \((n = 5)\) or OVA plus FliC \((n = 5)\), and sera were collected on days 14 and 35. (A) Naive, day 14, and day 35 sera were analyzed for IgG1-specific Ab responses against OVA by ELISA. (B–F) WT, TLR5\(^{-/-}\) \((n = 19)\), Naip5\(^{-/-}\) \((n = 9)\), Casp1\(^{-/-}\) \((n = 14)\), MyD88\(^{-/-}\) \((n = 12)\), and TLR5\(^{-/-}\)/Casp1\(^{-/-}\) DKO \((n = 9)\) mice were immunized twice on days 1 and 21 with OVA plus FliC, and sera were collected on days 14 and 35. Naive, day 14, and day 35 sera were analyzed for IgG1-specific Ab responses against OVA by ELISA. (B–F) Data are a combination of two to three independent experiments with \(n = 3–7\) per experiment. Statistical analyses were done using Mann–Whitney analysis of individual groups: *\(p < 0.05\), **\(p < 0.001\).

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Defective flagellin-induced IL-18 in A/J mice. C57BL/6 \((n = 5)\) and A/J \((n = 5)\) mice were injected i.p. with FliC \((30 \mu g)\). Serum was collected 2 h after injections, and cytokine levels were determined by ELISA: IL-6 (A), IL-12/23p40 (B), Cxcl1 (C), IL-18 (D), IL-1β (E), and TNF (F). Statistical analysis was done using one-way ANOVA with Bonferroni multiple-comparisons posttest: *\(p < 0.05\).
support our working hypothesis that a novel third pathway contributes to Ab responses toward flagellin, and that this pathway is independent of MyD88 and known innate detection pathways for flagellin recognition, TLR5, and Naip5. Uncloaking this third pathway would help to understand the complexities of innate immune recognition of flagellated pathogens and will also be critical for the rationale design of flagellin-based vaccines. It will be critical to assess whether this third pathway is conserved in humans and how this pathway may influence the generation of robust cellular and humoral responses, and the establishment of long-term immunity.

Our data also demonstrate that, in C57BL/6 mice, TLR5 and the Naip5/Nlrc4/caspase-1 inflammasome work in parallel to drive IgG2c anti-FliC responses in an MyD88-dependent manner. The MyD88 dependency of the IgG2a/c Ab response is even more apparent in A/J mice, which have quantitatively greater Ab responses and markedly enhanced IgG2a response that is equivalent to the IgG1 response. The studies in A/J mice demonstrate that the IgG2a primary response has greater dependence on MyD88 than the IgG1 response, as seen in C57BL/6 mice. However, unlike C57BL/6, A/J mice also had an MyD88-independent component for the IgG2a response. This suggests that underlying genetic differences between C57BL/6 and A/J mice contribute to isotype specificity and overall quantity of anti-flagellin immune responses.

Although this MyD88-independent pathway did not contribute to IgG2c responses in C57BL/6 mice, this may be because of the low magnitude of the IgG2c responses toward flagellin observed in C57BL/6 mice. Thus, it will be important to further dissect the components of the immune systems in C57BL/6 and A/J mice that are responsible for MyD88-independent responses against flagellin. Similarly, the structural components of bacterial flagellin that dictate MyD88-independent Ab production are uncharacterized. Understanding this third pathway of flagellin is another piece to the puzzle that can be used to enhance flagellin-based vaccine design.

Our results from C57BL/6 mice demonstrate that IgA anti-FliC responses are TLR5 and MyD88 dependent, consistent with a recently published report (72). We add to this body of work by demonstrating that the inflammasome does not impact IgA anti-FliC titers, and thus does not compensate for TLR5 deficiency. Our results from the C57BL/6 mice are congruent with the data from Cunningham and colleagues (72), which indicate that lamina propria TLR5+ CD103+ DCs prime Foxp3+ regulatory T cells to induce flagellin-specific IgA in the mesenteric lymph node. Conversely, our data from A/J and A/J MyD88–/– mice indicate that IgA anti-FliC responses may be regulated differently in different strains of mice. In A/J mice, MyD88-dependent signals suppressed IgA production. The mechanism for suppression of IgA
anti-FliC responses in A/J mice is currently unknown and requires further investigation.

It is well publicized that flagellin, when used as an adjuvant, imparts an IgG1 isotype-specific response toward coadministered Ags, presumably because of TLR5 recognition (9, 15, 26, 30). Our data support the conclusion that TLR5 and the Naip5/Nlrc4 inflammasome play overlapping roles for IgG1 responses toward OVA when coadministered with FltC (extrinsic adjuvancy). Because the anti-OVA IgG1 is reduced, but detectable, in both MyD88−/− and DKO, our data also indicate that the MyD88-independent pathway contributes to the adjuvancy of FltC toward OVA. Therefore, our immunization studies support the existence of a third pathway for flagellin recognition that also contributes to flagellin’s adjuvancy.

Interestingly, humans contain one full-length Naip homolog, Naip (78, 79). Human Naip recognizes needle proteins from bacterial type three secretory systems (21); however, there are also reports indicating that Naip recognizes flagellin (53, 80) and that copy number variation for the human Naip gene may affect ligand detection (79, 81). Although flagellin recognition by human Naip has been implicated with cellular assays (53), it still remains unsubstantiated by biochemical or genetic analysis (21, 53, 82). Further studies are required to determine the ligand specificity of human Naip and the contribution of Naip to flagellin-induced immune responses in humans.

In addition, it has been reported that TLR11 can recognize flagellin in mice of the C57BL/6 background (83). It is possible that TLR11 contributed to Ab responses toward FltC. However, like all other TLRs, except TLR3 (84, 85), TLR11 is MyD88 dependent (86). Because FltC-immunized MyD88−/− and TLR5/Casp1 DKO mice have similar phenotypes, TLR11 does not appear to contribute significantly to flagellin-dependent Ab responses. In any case, the translational implications of TLR11 recognition of flagellin for vaccine development in humans are of little relevance because human TLR11 is a nonfunctional pseudogene (7).

Our data indicate that flagellin is a potent adjuvant for IgG1 and IgG2a/c isotype-specific responses toward itself. In addition, our data indicate that TLR5 and MyD88 contribute to the IgG2a/c immune responses in both C57BL/6 and A/J mice. Our studies revealed that the IgG2a/c versus IgG1 bias of the anti-flagellin humoral response was strongly influenced by mouse genetic background. Because C57BL/6 mice have a weaker and IgG1-biased anti-flagellin Ab response, A/J mice may be a more relevant model for deciphering the immunogenic characteristics of flagellin and the host innate immune pathways needed for robust anti-flagellin immune responses. Because A/J animals generate robust IgG1 and IgG2a anti-FliC responses in an Naip5-independent manner, studies in A/J mice may also be more relevant to human vaccine development.

In both A/J and C57BL/6 mice, Ab responses generated against OVA were solely IgG1, with no detectable IgG2a/c anti-OVA Abs. It is not clear why flagellin induces distinct isotype-specific responses against intrinsic (FltC) and extrinsic (OVA) Ags. It is conceivable that increased proximity of Ag to the immunogenic portions of flagellin (sites recognized by TLR5, inflammasome, and third pathway) dictates a more robust IgG2a/c Ab response to intrinsic Ags. Further dissection of the cellular and molecular pathways that distinguish and differentiate responses toward intrinsic and extrinsic Ags are needed.

Currently, the identity of this third pathway that promotes anti-FliC responses is unknown. The flagellin used in our immunization is a monodispersed species, as expected for flagellin monomers (Supplemental Fig. 1); therefore, it is unlikely that the third pathway represents a T cell–independent response to flagellin poly-
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FLAGELLIN INDUCES Ab RESPONSES THROUGH A NOVEL PATHWAY


Supplementary Figure 1. Highly purified flagellin is free of exogenous innate immune agonists, and is a robust agonist of TLR5 and the inflammasome. Highly purified FliC and OVA contained less than 0.1 pg endotoxin (0.001 EU) per µg of protein (data not shown), and consisted of a single protein of approximately 55 kDa (A). Flagellin did not activate RAW 264.7 cells, which do not express TLR5 and are unresponsive to flagellin (B). RAW 264.7 cells stimulated with either PBS, FliC, or OVA had less than 100 pg/mL TNF from collected supernatants, while cells given 10 ng/ml LPS produced greater than 4000 pg/mL in supernatants collected following overnight treatment (B). TLR5 biological activity was determined using CHO cells stably transfected with murine TLR5 and a NF-κB luciferase reporter (C). The purified FliC had an EC50 of ~8 ng/mL, similar to what we have reported previously (20, 64). Flagellin induced Naip5- and caspase-1-dependent IL-1β secretion in LPS primed BMDMs with an EC50 of approximately 100 ng/mL (D). Size exclusion chromatography demonstrated that FliC purified from S. Typhimurium (FliC) (E) or recombinant FliC purified from E. coli (FliC-C) (F) eluted at the same volume as a monodispersed species, consistent with flagellin monomers.