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TLR5 or NLRC4 is necessary and sufficient for promotion of humoral immunity by flagellin

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Abstract
That TLR-based vaccine adjuvants maintain function in TLR-deficient hosts highlights that their mechanism of function remains incompletely understood. Thus, we examined the ability of flagellin to induce cytokines and elicit/promote murine antibody responses upon deletion of the flagellin receptors TLR5 and/or NLRC4 (also referred to as IPAF) using a prime/boost regimen. In TLR5-KO mice, flagellin failed to induce NF-κB-regulated cytokines such as KC (CXCL1) but induced WT levels of the inflammasome cytokine IL-18 (IL-1F4). Conversely, in NLRC4-KO mice, flagellin induced KC, but not IL-18, while TLR5/NLRC4-DKO lacked induction of all cytokines measured. Flagellin/ovalbumin treatment resulted in high antibody titers to both flagellin and ovalbumin in WT, TLR5-KO and NLRC4-KO mice but did not elicit antibodies to either in TLR5/NLRC4-DKO mice. Thus, flagellin’s ability to elicit/promote humoral immunity requires a germ-line encoded receptor capable of recognizing this molecule. Such promotion of adaptive immunity can be effectively driven robustly by either TLR5-mediated activation of NF-κB or NLRC4-mediated activation of the inflammasome.

Keywords
Innate immunity; antibodies; NF-κB; NLRC4; IL-18

INTRODUCTION
Microbial extracts and their purified products have long been recognized to possess the potent ability to promote development of antibody responses to bystander antigens thus underlying the rationale employing such products as vaccine adjuvants [1]. The discovery and characterization of toll-like receptors (TLR) appeared to provide a molecular mechanism for such adjuvant activity [2]. Specifically, TLR-mediated recognition of a variety of microbial products results in production of cytokines and upregulation of dendritic cell (DC) costimulatory molecules that, together, allow for stronger activation of T-cells that were concomitantly recognizing the bystander antigen. However, Nemazee and colleagues showed that for adjuvants based on either complex microbial extracts or a synthetic TLR4 ligand, genetic ablation of TLR signaling did not significantly impact adjuvant function [3]. Moreover, we recently showed that while loss of the flagellin receptor TLR5 nearly eliminated the ability of bacterial flagellin to induce cytokine production and promote DC maturation, it did not substantially impact upon flagellin’s ability to elicit antibodies or

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CONFLICT OF INTEREST
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promote an antibody response to a bystander antigen [4]. Thus, at least some microbial-based adjuvants can function independently of the TLR by which such products induce innate immune responses.

Identification of the TLR-independent mechanisms by which some microbial-based adjuvants function is germane to the basic understanding of regulation of adaptive immunity and may allow for development of strategies to boost vaccine efficacy without the risk of adverse events. Thus, the goal of this study was to define the innate immune determinants by which flagellin promotes adaptive immunity in the absence of TLR5 signaling. We hypothesized that, in the absence of TLR5, recognition of intracellular flagellin by NLRC4, which results in processing of pro IL-1β and IL-18 to their mature bioactive forms, provides a sufficient “signal 2” to drive the T-cell proliferation necessary to generate antibodies to both flagellin itself and a coadministered antigen. Herein, we report that, in vivo, NLRC4-dependent recognition of flagellin drove rapid generation of inflammasome cytokines, which, for IL-18, did not require TLR5. Moreover, in the absence of TLR5 signaling, NLRC4 expression was required for flagellin’s promotion of humoral immunity. Thus, flagellin’s promotion of adaptive immunity requires innate immune recognition of this molecule and such recognition can be mediated by either TLR5 or NLRC4.

RESULTS AND DISCUSSION

Bacterial flagellin is a potent activator of NF-κB mediated pro-inflammatory gene expression in epithelial cells but is considered a modest activator of this pathway in murine macrophages [5]. For example, intestinal epithelial cell (IEC) lines secrete IL-8 in response to ng/ml concentrations of flagellin but lack responsiveness to the TLR4 agonist LPS [6] while macrophage-like J774A.1 cells produce TNFα in response to exposure to LPS but do not to flagellin (Fig. 1A). However, we observed that this relative hyporesponsiveness of J774A.1 cells to flagellin is less dramatic when inflammasome cytokines IL-1β and IL-18, whose production is largely regulated by caspase-1 mediated processing, are used as a readout. Specifically, exposure to relatively high concentrations of flagellin (1–10 μg/ml), about 1000-fold higher than that needed to robustly activate epithelial cells, induced clearly detectable levels of IL-1β and IL-18 (Fig. 1B, C). In contrast, IEC did not produce detectable levels of inflammasome cytokines in response to flagellin or any other agonist tested (for both HT29 cells and T84 cells, level of IL-1β following treatment with 0.1–10 μg/ml flagellin or 100 ng/ml TNFα was below the 15 pg/ml minimum detection limit of assay). In support of the notion that flagellin-induced IL-1β production in J774A.1 cells reflected inflammasome activation, we observed that flagellin induced caspase-1 activity (Fig. 1C) while flagellin-induced IL-1β release was blocked by a caspase-1 inhibitor (Fig. 1D). The high concentration-dependence of the macrophage response to flagellin likely reflected a requirement for flagellin to be internalized as studies showing NLRC4-mediated activation of the inflammasome at low flagellin concentrations used synthetic or bacterial-derived products to promote flagellin uptake [7,8]. In accordance, SDS-PAGE immunoblotting revealed flagellin to be readily detectable in cell lysates of flagellin-treated J774A.1 cells (Fig. 1F) while confocal microscopy revealed the presence of flagellin in 1 μM center slices of these cells (Fig. 1G). In contrast, exposure of IEC to flagellin did not result in the presence of detectable intracellular flagellin by either of these approaches.

We next examined flagellin-induced responses in murine cells ex vivo to determine whether the response might be mediated by either of the two known pathways that signal in response to flagellin, namely TLR5 or NLRC4. Peritoneal exudate cells (PEC) of mice engineered to lack TLR5 (TLR5KO), NLRC4 (NLRC4-KO) or both (DKO) were exposed to 10 μg/ml of flagellin for 24h. As shown in Fig. 2, loss of NLRC4 by itself or in combination with TLR5 eliminated flagellin-induced PEC production of these inflammasome cytokines while loss of
TLR5 was without effect. PEC are known to be enriched in macrophages, which are known to have a functional inflammasome pathways suggesting that the NLRC4-dependent production of IL-1β and IL-18 may have come from macrophages present in PEC. In accordance, we observed a similar pattern of responsiveness in bone marrow-derived macrophages although the magnitude of the response was about 5-fold less (data not shown) supporting the notion that, although the local environment may modulate their responsiveness, NLRC4 mediates flagellin-induced production of inflammasome cytokines by macrophages. That absence of TLR5 did not affect this response is in accord with studies that most commonly studied populations of murine macrophages lack TLR5 [5,9] but raises the question of what signaling mechanism drives the synthesis of pro IL-1β and IL-18, which are necessary for NLRC4 activation to result in processing/secretion of these cytokines. We speculate that these cells contain a small amount of pro IL-1β and/or IL-18 that can be processed by NLRC4 activation which can then activate transcription of these genes in an autocrine manner. Accordingly, we note that when assayed at 1–10 h following flagellin treatment, levels of both IL-1β and IL-8 were undetectable (i.e. less than the assay’s 15 pg/ml minimum detection limit).

To examine the significance of these observations on innate and adaptive immune responses in vivo, mice (WT, TLR5KO, NLRC4KO and DKO) were subjected to a typical prime/boost immunization regimen that we used in our recent work [4]. Specifically, mice were intraperitoneally injected with ovalbumin (50 μg) alone or ovalbumin mixed with flagellin (10 μg) on day 0 and day 28. Mice were bled at 2h following the initial injection to assess acute production of serum cytokines and 14 days following the second injection (day 42) to assess antibody responses. Our previous use of this regimen indicated that loss of TLR5 dramatically attenuated flagellin-induced serum elevations of KC, IL-6, G-CSF, MCP-1, IL-17, IL-12 (p40), etoxacin, TNFα, RANTES, and MIP-1β but did not reduce flagellin-induced elevation in serum IL-18. In accordance, we verified that both TLR5-KO and TLR5/NLRC4-DKO lacked flagellin-induced elevations in serum KC (Fig. 3A). In contrast, relative to WT mice, NLRC4-KO mice had only a slight reduction in flagellin-induced levels of serum KC (similar results were seen for IL-6 – not shown). In contrast to the case for these cytokines, which are thought to be primarily regulated at the transcriptional level, flagellin elicted elevations in serum IL-18 in a manner dependent upon NLRC4 but not TLR5. Specifically, in contrast to WT and TLR5KO mice both NLRC4-KO and TLR5/NLRC4-DKO mice lacked detectable IL-18 production in response to flagellin (Fig. 3B). Such flagellin-induced TLR5-independent IL-18 production was also observed in mice injected with flagellin only (i.e. without ovalbumin) eliminating the possibility that the ovalbumin, or a contaminant therein drives the production of pro-IL-18 that serves as a precursor for IL-18 production [4]. Rather, since significant levels of pro-IL-18, but not pro IL-1β, are stored in vivo [10], we hypothesize that preexisting pro-IL-18 permits NLRC4 activation to elicit IL-18 secretion without a requirement for a TLR-mediated signal to activate transcription of this cytokine. In accordance with this notion, we observed that intraperitoneal injection of flagellin induced intestinal production of IL-18, which also required NLRC4 but not TLR5, was not associated with an increase in IL-18 mRNA (Fig. 3C, D). In contrast, flagellin-induced intestinal production of IL-1β has a substantial requirement for TLR5 and is associated with a TLR5-dependent increase in IL-1β mRNA ([11] and Fig. 3D). IL-1β was not detected in sera of any of our mice in accordance with observations made by numerous investigators that IL-1β is often difficult to detect in serum. To determine if the deficiency of the TLR5/NLRC4-DKO mice to respond to flagellin was specific for this agonist, WT and DKO mice were injected with LPS (10 μg) mixed with ovalbumin (50 μg) and bled 2h later. In response to LPS, DKO mice exhibited WT induction of serum IL-6 (153 ± 11.6 vs. 171 ± 10.2 ng/ml for WT and DKO respectively, p>0.05) and only modest impairment in induction of serum KC (504 ± 37.8 vs. 332 ± 92.2 ng/ml for WT and DKO respectively, p<0.05) – basal levels were undetectable in both strains – indicating
that flagellin-independent pathways of innate immunity remain functional. Thus, although relatively high concentrations of flagellin were required to activate NLRC4-dependent signaling \textit{in vitro}, this pathway is capable of recognizing flagellin \textit{in vivo} in experimental vaccine scenarios.

We next examined the extent to which flagellin-induced NLRC4-mediated activation of the inflammasome might play a role in promoting the humoral immune responses to flagellin and co-administered ovalbumin. We recently reported that, although loss of TLR5 dramatically reduced flagellin-induced cytokine production (except for IL-18) and DC activation, it did not substantially reduce flagellin’s ability to elicit anti-flagellin Ig nor promote generation of antibodies to co-administered ovalbumin in a prime-boost regimen and had only a modest effect in a primary immune response [4]. Such maintenance of flagellin’s ability to promote a humoral immune response to flagellin (observed in response to flagellin alone) or co-administered ovalbumin in TLR5-KO mice was not dependent upon a non-proteinaceous contaminant nor TLRs 2 or 4 but was partially dependent upon MyD88 suggesting a possible role for another TLR or IL-1β or IL-18, which signal in a MyD88-dependent manner. In light of our observation herein that soluble flagellin can elicit NLRC4-mediated generation of inflammasome cytokines, we hypothesized that, perhaps, either TLR5-mediated NF-κB activation or NLRC4-mediated activation of the inflammasome might be sufficient for flagellin to promote humoral immunity. In accord with our recent study, loss of TLR5 by itself did not markedly reduce ability of mice to generate antibodies to both flagellin and ovalbumin (Fig. 3E,F). Nor did loss of NLRC4, by itself, alter generation of antibodies in response to flagellin/ovalbumin treatment. However, strikingly, loss of both TLR5 and NLRC4 together markedly reduced antibody response to flagellin and completely abrogated the ability of flagellin to promote antibody responses to ovalbumin. Importantly, promotion of antibodies to ovalbumin by LPS was not significantly affected by loss of TLR5 and NLRC4 indicating that these mice are not globally defective in antibody production (antibody titers to ovalbumin and ovalbumin/LPS were 2667 ± 1159 and 48500 ± 10504 vs. 5050 ± 1732 and 59167 ± 17355 for WT and DKO respectively, n=3–6, p>0.05 for WT and DKO) Thus, while either TLR5 or NLRC4 is dispensable for flagellin to act as an adjuvant, one of these innate immune receptors is absolutely necessary for its promotion of humoral immunity. We speculate that the reduced, but not eliminated ability of flagellin to function as an adjuvant in MyD88-KO mice, in which signaling by TLR5, IL-1β and IL-18 is thought to be ablated, may reflect that some as yet undefined component of TLR5 or IL-18 receptor signaling is MyD88-independent analogous to previous observations by Re and colleagues for alum-induced signaling [12]. Given that, \textit{in vivo}, we detected IL-18 but not IL-1β in TLR5KO mice, it seems reasonable to speculate this cytokine plays an important role in mediating flagellin’s adjuvanticity in TLR5-deficient mice while it is likely redundant for promoting this function in WT mice. This specific mechanism we propose for explaining flagellin’s adjuvanticity in TLR5-deficient mice likely differs from that by which MyD88/Trif-DKO generate antibodies in response to complex and synthetic adjuvants [3]. Yet, we envisage that the concept of redundancy in the innate immune system may explain how such adjuvants maintained function in TLR-deficient mice.

The role of TLR signaling in promoting antibody responses for both classic and modern/synthetic vaccine adjuvants has recently been a very controversial area of immunology. Herein, we show that in the case of flagellin, recognition by either cell surface TLR5 or intracellular NLRC4 necessary and sufficient for promotion of adaptive immunity. This redundancy might be clinically important to the ongoing development of flagellin-based vaccines as 8–10% of humans carry a dominant-negative allele for TLR5 [13] and might otherwise not respond to flagellin-based vaccines in the absence of NLRC4. Moreover, by providing a molecular mechanism by which an apparent profound loss of innate immunity

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did not affect development of T-cell dependent antibody responses, these results reinforce a central tenet of immunology that adaptive immunity is indeed dependent upon innate immunity.

MATERIALS AND METHODS

Materials

Salmonella Typhimurium flagellin (FltC) was purified, and purity verified as previously described [4,6]. LPS and crystallized chicken egg white albumin (Grade VI) were purchased from Sigma. Our previous study indicated that antibody responses to this preparation of ovalbumin were not affected by loss of TLR4 indicating LPS does not play a significant role in antibody responses elicited by this reagent [4]. Caspase1 inhibitor II (Ac-YVAD-CMK) was purchased from Calbiochem. GFP coupled flagellin was a gift from Kanneganti Murthy, (Inotek Pharmaceuticals, Beverly MA).

Mice

TLR5KO and WT mice were previously described [9]. In this study, TLR5KO mice used were rederived by embryo transplant, which substantially reduced spontaneous colitis phenotype. Male mice lacking NLRC4 (NLRC4-KO), generated on a pure C57BL/6j background were kindly provided by Vishva Dixit (Genentech, Inc. South San Francisco, CA). These male NLRC4-KO mice were bred to TLR5-KO mice. The offspring of these mice (heterozygous for both TLR5 and NLRC4 were bred to each other to generate the 4 mice strains used herein (WT, TLR5KO, NLRC4-KO, and DKO). Thus, all the mice used herein were descendants of mice that were littermates of each other.

In vitro studies

Model intestinal epithelial cell lines (HT29) and mouse macrophage cell line J774A.1 (purchased from ATCC) were grown using DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin and streptomycin and non-essential amino acids. On the day of stimulation, cells were washed twice with PBS and stimulated with flagellin or indicated stimuli in serum-free DMEM for 24h at which time supernatants and lysates were collected and stored at −80 °C until analyzed.

Ex vivo studies

Peritoneal exudate cells—Resident peritoneal macrophages were collected from euthanized animals by DMEM lavage, resuspended in DMEM containing 10% FBS, and seeded at the density of 4×10^5 cells/mL.

Bone marrow derived macrophages—Bone marrow cells were differentiated in DMEM supplemented with 20% heat-inactivated FBS and 20% L929 supernatants containing macrophage-stimulating factor (M-CSF). Bone marrow cells were cultured at an initial density of 10^6 cells/ml for 5–7 days, and fresh medium was added at day 3. Cells were harvested with cold 0.2% EDTA and plated at the density of 4×10^5 cells/ml in DMEM supplemented with 10% FBS. Macrophages were cultured for at least 12 h before stimulation in serum-free DMEM.

ELISA—IL-1β, KC, and IL-6 were quantitated by Duoset kits from R & D Systems according to the manufacturer instructions. The minimum detectable dose (pg/mL) were KC (15.6), IL-6 (15.6), and IL-1β(15.6). IL-18 was quantitated with R &D systems quantikine kit (minimum detection was 25 pg/mL).
**Caspase 1 activity**—The caspase 1 activity in cell lysates were measured by a kit from Biovision according to the manufacturer instructions.

**Immunoblotting**—Flagellin immunoblotting was performed in supernatants and cell lysates using anti-rabbit flagellin antibodies as described in [14].

**Immunizations**—As previously described, 6–10 week old mice were immunized intraperitoneally with ovalbumin (50 μg) by itself or mixed with purified flagellin (10 μg) in PBS (100 μl total volume) and serum cytokines and antibody titers were measured by ELISA [4] with titer being defined as the reciprocal dilution of 3 times background. Because titer is a relative, rather than absolute measurement, data in each panel is from a single analysis. For assay of intestinal responses, supernatants and mRNA was harvested as recently described [11].

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**Abbreviations**

- **TLR** Toll-like receptor
- **NLR** Nod-like receptor. NLRC4 originally named IPAF for Interleukin converting enzyme Protease Activating Factor
- **FliC** flagellin
- **IEC** Intestinal epithelial cells
- **IL** Interleukin
- **KC** keratinocyte derived chemokine
- **T5KO** TLR5-KO
- **N4KO** NLRC4-KO
- **DKO** NLRC4-KO/TLR5-KO

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Macrophage-like J774A.1 cells produce inflammasome cytokines in response to purified flagellin

J774A.1 cells were treated with flagellin, FliC (10 μg/ml or indicated concentration), or 20 ng/ml LPS. (A) TNFα (B) IL-1β, or (C) IL-18 was measured 24h later. D, Cells were treated with 10 μg/ml flagellin and caspase-1 activity measured as described in the Materials and methods section. E, Cells were pre-treated with indicated concentration of caspase-1 inhibitor II (Ac-YVAD-CMK) 30 min before exposure to 10 μg/ml flagellin and IL-1β measured 24 h later. F, Lysates and supernatants were collected at indicated time and assayed for flagellin via immunoblotting. G, J774A.1 cells were exposed to 5μg/ml flagellin-GFP fusion protein. At indicated time point, cells were washed 3 times and subjected to confocal microscopy to assess intracellular flagellin-GFP. Images are center slices 1 μM in depth. The scale bar shown is applicable to all confocal images shown. Data in A–E show mean ± SEM of 3 side-by-side experiments. Data in F and G are representative of 3 independent experiments. *p<0.05, Student’s t-test) from PBS-treated negative control (for A–D) or flagellin-treated positive control (for E).
Figure 2. Macrophage production of inflammasome cytokines is dependent upon NLRC4 but not TLR5

Peritoneal exudate cells (4 × 10^5 cells/well) from WT, T5KO, N4KO, DKO mice were stimulated with 10μg/ml of flagellin in serum-free media and (A) IL-1β and (B) IL-18 were measured in the supernatants after 24h. Data show mean ± SEM of 3 side-by-side experiments. *p<0.05, Student’s t-test) from PBS-treated negative control.
Figure 3. Ability of flagellin to elicit and promote humoral immunity requires either TLR5 or NLRC4

A&B, Mice (WT, T5KO, N4KO, or DKO) were injected i.p. with 50μg of ovalbumin (n=3–4 mice per condition) or ovalbumin mixed with 10μg of purified flagellin (n=6 mice per condition). Two hours later, mice were bled via retrobulbar intraorbital capillary plexus. Levels of A, KC and B, IL-18 were measured in the sera by ELISA. Data show mean ± SEM of values obtained from individual mice. C&D, Mice (WT, T5KO, N4KO, or DKO) were injected i.p. with 10μg of purified flagellin (without ovalbumin). One hour later, mice were euthanized and colons isolated. (C) Colonic supernatants (n=6 mice per condition) were collected and IL-18 measured by ELISA. (D) Colons were subjected to mRNA extraction (n=3 mice per condition) followed by quantitation of relative levels of mRNA for IL-18 and IL-1β. E&F, Mice used in A and B were re-injected 28 days later with same materials (ova or ova and flagellin). Serum was collected on day 42. Anti-IgG titers were measured by ELISA for (E) ovalbumin and (F) flagellin. Data are the values obtained from individual mice (n=3–6 mice per condition as indicated by number of points). Line indicates arithmetic means (*p<0.05, Student’s t-test relative to mice of that genotype that received ovalbumin only).