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Flagellin Promotes Myeloid Differentiation Factor 88-Dependent Development of Th2-Type Response

Arnaud Didierlaurent,*† Isabel Ferrero,‡ Luc A. Otten,† Bertrand Dubois,§ Monique Reinhardt,* Harald Carlsten,¶ Rune Blomhoff,¶ Shikuo Akira,‖ Jean-Pierre Kraehenbuhl,*‡ and Jean-Claude Sirard2*§

Activation of dendritic cells (DC) by microbial products via Toll-like receptors (TLR) is instrumental in the induction of immunity. In particular, TLR signaling plays a major role in the instruction of Th1 responses. The development of Th2 responses has been proposed to be independent of the adapter molecule myeloid differentiation factor 88 (MyD88) involved in signal transduction by TLRs. In this study we show that flagellin, the bacterial stimulus for TLR5, drives MyD88-dependent Th2-type immunity in mice. Flagellin promotes the secretion of IL-4 and IL-13 by Ag-specific CD4+ T cells as well as IgG1 responses. The Th2-biased responses are associated with the maturation of DCs, which are shown to express TLR5. Flagellin-mediated DC activation requires MyD88 and induces NF-κB-dependent transcription and the production of low levels of proinflammatory cytokines. In addition, the flagellin-specific response is characterized by the lack of secretion of the Th1-promoting cytokine IL-12 p70. In conclusion, this study suggests that flagellin and, more generally, TLR ligands can control Th2 responses in a MyD88-dependent manner. The Journal of Immunology, 2004, 172: 6922–6930.

Dendritic cells (DC) play a key role in immune surveillance and have the unique capacity among APCs to stimulate naive T cells and differentiation of Th1- and Th2-type CD4+ T lymphocytes (1–3). DC activation is controlled by conserved signature molecules of microbes, called pathogen-associated molecular patterns (PAMP) (4). PAMP interaction with pattern recognition receptors (PRR), such as members of the Toll-like receptor (TLR) family, triggers the signal transduction required for coordinated activation of innate and adaptive immunocytes. In particular, recognition of PAMPs by PRRs can configure DCs to instruct the most appropriate Th cell response for protection (5). TLRs (TLR1 to TLR10) share common characteristics in the signal transduction machinery, including the myeloid differentiation factor 88 (MyD88) adapter molecule and the NF-κB transcriptional activator (4). MyD88-deficient mice develop a Th2-biased response after immunization with adjuvants containing TLR activators, such as CFA or the soluble Toxoplasma Ag (6–8). Thus, MyD88-dependent TLR signaling is probably dedicated to the induction of Th1-biased responses (5). In contrast, Th2 commitment is assumed to depend on DC activation via PRR unrelated to TLR, such as Schistosoma egg Ags. However, recent studies propose that TLR signaling contributes to the induction of Th2-type differentiation by control of DC activation (9–13). In particular, Kai-sho et al. (11) showed that MyD88-independent rather that TLR-independent DC activation seems to be involved in Th2 development.

Monomeric flagellin is a PAMP that mediates signal transduction in mammals via TLR5 and MyD88 (14, 15). Flagellin is the structural protein subunit of the flagellum, the motility apparatus of bacteria. Flagellin is known to elicit, in the absence of any adjuvant, strong and sustained Ab response, which is a hallmark of Th2-type immune responses (for review, see Ref. 16). These pioneer studies of flagellin were particularly instrumental to establish the paradigm of Th1/Th2 differentiation. More recent work in mice has reported that flagellin can potentiate the priming of Th1-type CD4+ T cells specific for the OVA Th peptide, OVA323–339 (17). Flagellin was also shown to induce human DCs to stimulate Th1 responses in an in vitro model (13). The development of Th1 immunity was correlated with the secretion of IL-12 p70 by activated DC. However, these data were not supported by the study by Means et al. (18), which showed that flagellin-activated human DCs do not produce IL-12 p70. Similarly, activation of mouse DC by flagellin was not observed in all conditions (17, 18). In contrast, the potency of flagellin as an adjuvant has been established in rats and mice (16). In this study we assessed both in vitro and in vivo the effects of flagellin on DC maturation and T cell differentiation using conventional mice and mice deficient for MyD88. We show that flagellin from Salmonella typhimurium induces MyD88-dependent development of Th2 responses. Although flagellin induces NF-κB-dependent transcription and production of IL-12 p40, flagellin-mediated DC activation is not associated with the secretion of the Th1-polarizing cytokine IL-12 p70 or high levels of proinflammatory cytokines. Our observations suggest that flagellin...
has a specific, MyD88-dependent role in adaptive immunity by activating DC to instruct the development of Th2-biased responses.

Materials and Methods
Flagellin purification and reagents
*S. typhimurium* produces two flagellins: FliC and FljB. Isogenic FliC-producing strain SIN22 (fliB5001::MudJ) and flagellin-deficient SIN41 (fliC5050::MudJ fliB5001::MudCam) were obtained by phage transduction using strains TH714 and TH7925 as donors and ATCC14102 (American Type Culture Collection, Manassas, VA) as recipient (19). Flagellin FliC was prepared from SIN22. Briefly, flagella were sheared from surface, concentrated by ultracentrifugation, and heated for 30 min at 65°C or e - FliC was prepared from SIN22. Brie flican Type Culture Collection, Manassas, VA) as recipient (19). Flagellin fication using strains TH714 and TH2795 as donors and ATCC14028 (Amer-

Mice
Six- to 16-week-old mice (BALB/c (H-2b), C57BL/6 (H-2b), C3H/HeJ (H-2k), Naval Medical Research Institute (NMRI) outbred mice, DO11.10 SCID transgenic for OVA-specific TCR, Mxy88+/− on C57Bl/6 background, and (C57Bl/6 × CBAF1)F1 transgenic for a transcriptional fusion between promoter containing NF-κB sites from the Igκ L chain and the firefly luciferase (3′-κb-luc) were purchased from Harlan (Indianapolis, IN) or were bred in our animal facility (6, 20).

DC preparation
Cell cultures were performed in RPMI 1640 containing 5% FCS (RPMI-5) or in IMDM containing 10% FCS (IMDM-10) and supplements (Life Technologies). Bone marrow (BM) DC precursors were obtained from femoral and tibial bones (21). Briefly, BM cells were plated at 2 × 10^6 cells/ml with 10 ng/ml GM-CSF (BioSource International, Camarillo, CA) in IMDM-10 and supplemented 3 days later with GM-CSF-containing medium. On day 6, BM-derived DCs (BMDCs) were stimulated for 18 h and analyzed. Splenic DCs were prepared as previously described (22). Briefly, spleens were incubated with 0.5 mg/ml collagenase D and 40 μg/ml DNase I (BD Biosciences, San Jose, CA) in RPMI-5 for 10 min at 37°C. After mechanical dissociation, splenic DCs were enriched by centrifugation in cold isosmotic OptiPrep (Nycomed, Oslo, Norway).

Immunizations
OVA-specific CD4+ T cells were isolated from spleen and lymph nodes of DO11.10 mice using MACS CD4 beads (Miltenyi Biotech, Auburn, CA) with a purity >98%. The cells were stained with 5 μM CFSE (Molecular Probes, Eugene, OR), and 4–5 × 10^6 cells were injected i.v. into recipient BALB/c mice. One day later, mice were injected i.v. with OVA with or without various stimuli. CD4+ Vβ8+ splenocytes or lymph node cells were analyzed 72 h later. OVA-specific T cell responses were analyzed in conventional mice immunized s.c. Ten days later, 2–5 × 10^6 lymph node cells mixed with 2–5 × 10^6 irradiated splenocytes were incubated for 72 h with RPMI-5 with or without OVA to measure the secretion of IL-4, IL-13, and IFN-γ. Animals were immunized s.c. once or twice on days 0 and 21 with FliC and/or OVA, and the systemic Ab response was analyzed on day 28.

Luciferase assays
BMDC or CD11c+ cells sorted from BMDC derived from 3×-κB-lac mice were stimulated for 30 min to 3 h. Luciferase activity was measured in cell extracts using the luciferase assay system (Promega, Madison, WI). HeLa S3 cells were transfected with ELAM-lac with or without pEF6/V5-TOPO-hTLRS (1 μg of each plasmid) using Lipofectin (Invitrogen, Carlsbad, CA) and incubated for 6 h with 100 ng/ml flagellin, and cell lysates were tested in luciferase assays.

Abs and flow cytometry
The following mAbs conjugated to FITC, PE, CyChrome, or biotin were used: CD11c (HL3), MHC class II (HB11.54.3), B220 (RA3.6B2), CD8a (53.6.7), CD4 (LT4), CD80 (16-10A1), CD86 (GL-1), CD40 (3/23 and FGG-45), CD62L (MEL-14), CD44 (IM-7), F4/80 (F4/80), HEL5/6-46 loaded in 1-A (C4H3), and mAb isotype controls (from BD PharMingen, San Diego, CA; or homemade) (23). Biotinylated Ab were revealed with streptavidin conjugated to CyChrome (BD PharMingen) or allophycocya-

Quantitative RT-PCR
Total RNA was treated with DNase I (Qiagen, Chatsworth, CA) and reverse transcribed using Superscript II (Invitrogen). Quantitative PCR was then performed with an ABI 5700 thermocycler (Applied Biosystems, Foster City, CA) using the SYBR Green PCR assay and the primers specific for TLR5 (GCCACGGCTTTATCCTTCC and GCGGAACTGACATCTTCA) and 18SribosomalRNA(TGGGCAAATGTTTGCTGTandGCGCCCTGATA

Results
Flagellin triggers DC activation
The regulation of adaptive immunity by TLR agonists is dependent on stimulation of DCs (4). TLRs are expressed by DCs, and their contribution to DC activation is established for TLR2, -3, -4, -6, -7, and -9 (4). The expression of TLR5 in BMDCs was previously detected in spleen or splenic macrophages (18, 25, 26). To determine whether mouse splenic DCs can respond to flagellin as previously suggested (17), the expression of TLR5 was assessed by real-time RT-PCR and normalized to TLR5 expression in total splenocytes (Fig. 1a). The specificity of the assay was checked by analysis of melting curves of various samples and by sequencing of the PCR product. TLR5 transcript levels in splenic DCs were ~10-fold higher than those in whole splenic cells. We confirmed that TLR5 transcripts are found in splenic macrophages at a lower level than in splenic DCs, but not at all in B lymphocytes (data not shown). These data identified DCs as the main in vivo TLR5-expressing splenic APCs and are in agreement with recent observations (27). The expression of TLR5 in BMDCs was consistently ~10-fold less than that in splenic DCs (i.e., 0.6–1.6 relative expression level compared with total splenocytes; Fig. 1a).

A major concern in the analysis of bacterial product activity on mammalian cells, in particular on DC, is contamination by a low concentration of stimulatory signals, such as LPS or lipopeptides. To investigate the effect of flagellin on DCs, monomeric flagellin FliC was purified from *S. typhimurium*, and endotoxin contaminants were removed. Coomassie Blue staining and immunoblot revealed a unique 52-kDa band corresponding to FliC (Fig. 1b). To
FIGURE 1. Flagellin-mediated activation of DC. a, Splenic DCs from naive mice were isolated by FACS sorting as F4/80<sup>−/−</sup> CD11c<sup>+</sup> cells population. BMDC were isolated by CD11c-specific MACS sorting. TLR5 RNA levels were quantified by real-time RT-PCR, normalized with respect to 18S RNA and presented as the fold increase compared with values observed in total splenocytes. b, Flagellin preparations from S. typhimurium (2 µg or equivalent/ lane) were analyzed on SDS-PAGE, followed by gel staining with Coomassie Blue (left) or immunoblotting using FliC-specific Ab (right). Lane 1, Flagellin FliC; lane 2, preparation from flagellin-deficient strain; lane 3, trypsin-treated FliC. Preparation purity was assayed by immunoblot with FliC-specific serum generated in C57BL/6 and BALB/c mice immunized with 40 µg FliC and CFA, followed by 20 µg FliC and IFA. Coomassie Blue staining and immunoblot revealed a unique 52-kDa band corresponding to FliC. c, The effect of FliC on DCs was investigated in C3H/HeJ mice injected i.v. with PBS (dotted line), a preparation of flagellin-deficient S. typhimurium (thin line; equivalent to 20 µg of FliC), or 1 µg of FliC (bold line). Six hours later splenic DCs were analyzed by flow cytometry for the indicated activation markers. Histograms represent data for F4/80<sup>−/−</sup> CD11c<sup>+</sup> gated cells. Similar results were obtained in C57BL/6 mice. d, C3H/HeJ BMDCs were incubated for 20 h with medium alone (dotted line), 1 µg/ml FliC (bold line), or trypsin-treated FliC (thin line; equivalent to 1 µg/ml FliC). Diagrams represent flow cytometric analysis of CD11c<sup>+</sup> cells. Results are representative of five experiments. The gray diagrams correspond to staining with an isotype control antibody. e, BMDCs from C3H/HeJ mice were incubated for 20 h with 0.5 mg/ml HEL, 1 µg/ml FliC, and trypsin-treated FliC (equivalent to 1 µg/ml FliC), alone or in combination. The median fluorescence of CD11c<sup>+</sup> cells was measured by staining with C4H3 and MHC II-specific Abs (left). C4H3-specific median fluorescence was normalized to MHC II-specific fluorescence and was expressed as a percentage of the ratio for mock-treated DCs (right). The results shown are representative of two experiments.
rule out any activity of protein and nonprotein contaminants, two controls were included: trypsin-treated detoxified FltC and preparation from flagellin-deficient *S. typhimurium* (Fig. 1b). We first verified that FltC preparations were able to activate mammalian cells responding exclusively to flagellin. All flagellin FltC preparations used in the study, in contrast to controls, up-regulated the transcription of IL-8 and CCL20 genes in epithelial intestinal cell lines as described previously (data not shown) (19, 28, 29). Similarly, NF-κB luciferase fusion was up-regulated in HeLa cells transfected with human TLR5 expression plasmid upon incubation with flagellin (data not shown). Therefore, our preparations induce signaling in mammalian cells via flagellin, the TLR5-stimulating ligand. We then investigated DC activation in vivo 6 h after i.v. injection of mice with flagellin (Fig. 1c). Flagellin enhanced the surface expression of the costimulatory molecules CD40, CD80, and CD86 as well as the expression of MHC class II in splenic DCs. Activation was effective at FltC doses as low as 100 ng (i.e., 2 pmol), but was not observed after injection of trypsin-treated FltC or preparation from flagellin-deficient *S. typhimurium* (Fig. 1c and data not shown). A flagellin-specific effect on splenic DCs was observed in LPS-hyporesponsive (C3H/HeJ), TLR2−/−, or conventional inbred animals (C57BL/6 and BALB/c; Figs. 1c and 2a) (A. Didierlaurent and T. Roger, unpublished observations). In addition, splenic B lymphocytes did not proliferate or induce MHC class II expression upon incubation with flagellin, in contrast to other TLR activators such as peptidoglycan (TLR2 and TLR6), poly(I-C) (TLR3), LPS (TLR4), or unmethylated CpG DNA (TLR9) (A. Didierlaurent, K. Burns, and B. Brissoni, unpublished observations). Thus, our findings indicate that the stimulatory activity of flagellin preparations is independent of contaminants signaling through TLR2, TLR3, TLR4, TLR6, and TLR9. These observations also show that responsiveness to flagellin correlates with TLR5 expression at the transcriptional level. Together, our data show that splenic DCs, a major source of TLR5 in the spleen, are specifically stimulated by flagellin.

To determine whether flagellin was able to induce DC maturation in the absence of other cell types, DCs were derived from BM precursors. As observed for splenic DCs, flagellin induced the up-regulation of MHC class II and costimulatory molecules on BMDCs (Fig. 1d). Trypsin-treated FltC had no effect, and a dose-response analysis showed that flagellin was active at concentrations as low as 10 ng/ml (data not shown). These experiments demonstrate that flagellin induces a direct activation of DC. zsn increase in Ag processing and presentation functions is also an important feature of DC activation. The capacity of flagellin to potentiate processing and presentation of whole HEL was evaluated using C4H3 mAb specific for I-Ak loaded with HEL11-25 peptide (Fig. 1e) (23). As described previously, HEL alone was presented by BMDCs, in contrast to mock-treated cells. Incubation of BMDCs with HEL and FltC resulted in 2-fold enhancement of HEL presentation. Together these data indicate that flagellin induces the functional maturation of DC.

**Maturation of DC by flagellin requires MyD88**

To provide evidence that TLR5 may participate in flagellin effect, experiments were conducted in MyD88-deficient mice (6, 14). LPS was used as a control for MyD88-independent DC activation (30). Splenic DCs and BMDCs from MyD88−−/− mice were not activated by flagellin or CpG in contrast to LPS (Fig. 2, a and b). Flagellin-mediated signaling in DC is therefore strictly dependent on the adapter molecule MyD88. TLR signaling pathways are invariably associated with transcriptional activation of NF-κB-dependent genes. BMDC derived from transgenic mice harboring a NF-κB-responsive luciferase fusion were incubated with LPS.
CpG, and FltC at various concentrations before monitoring luciferase activity (Fig. 2c) (20). The kinetics of NF-κB activation were similar for all TLRs tested, with maximal activity 2 h after addition of stimuli. Flagellin at concentrations as low as 1 ng/ml enhanced NF-κB transcription by ~10-fold, whereas the increase with CpG and LPS was significantly higher. These observations establish that DC activation by flagellin is mediated by MyD88 and results in NF-κB induction, as expected for a TLR5-dependent mechanism.

We next investigated the pattern of cytokine secretion in flagellin-activated DCs. The heterodimeric cytokine IL-12 p70 (formed of IL-12 p40 and IL-12 p35) is produced upon stimulation of DCs via various TLRs (31). Interestingly, flagellin did not stimulate any IL-12 p70 secretion in BMDCs in contrast to CpG (Fig. 3a). However, flagellin induced a significant increase in IL-12 p40 production that is fully dependent on MyD88. Only small quantities of the proinflammatory cytokines IL-6 and TNF-α were produced by DCs upon incubation with flagellin compared with CpG (Fig. 3a). In addition, CpG, but not flagellin, triggered systemic IL-12 p70 production after i.v. injection, whereas significant levels of IL-12 p40 were measured with flagellin (Fig. 3b). This cytokine pattern was not modified by flagellin dose or costimulation, as assessed by treatment with anti-CD40 mAb (Fig. 3b and data not shown). Although flagellin induces MyD88-specific signaling, mouse DC activation both in vitro and in vivo is not associated with secretion of the Th1-polarizing cytokine IL-12 p70 and a strong proinflammatory response.

**Flagellin induces the development of Th2-type responses**

The cytokine IL-12 p70 plays an important role in the development of Th1 responses (31). The TLR5 ligand flagellin has been proposed to elicit either Th1- or Th2-type immunity in mice (16, 17). Based on the observations that mouse DCs did not produce any IL-12 p70 upon flagellin stimulation, we investigated whether flagellin would promote Th2-biased responses. Mice were immunized s.c. with FltC, OVA, and OVA supplemented with FltC or CpG oligonucleotide as a Th1-promoting TLR9-dependent adjuvant (32). Ten days later the secretion of IL-4, IL-13, and IFN-γ was measured in OVA-stimulated lymph node cells (Fig. 4a). T cells from FltC-immunized animals did not induce any cytokine release when incubated in vitro with OVA. As expected, immunization with CpG resulted in Th1 polarization, characterized by the secretion of IFN-γ, but not IL-4 or IL-13. The production of IL-4 and IL-13, cytokines specific for Th2 responses, was only detected in T cells from animals immunized with FltC and OVA (Fig. 4a). As reported for other Th2-type inducing stimuli (33), significant production of IFN-γ was found in the group treated with FltC and OVA. The mouse genotype did not influence the T cell response, as C57BL/6 mice behaved similarly to BALB/c mice (Fig. 5a).

These data demonstrate that flagellin promotes the development of Th2 cytokine-producing T cells in vivo. To further characterize the T cell response, CFSE-labeled, OVA-specific, transgenic CD4+ T cells were transferred into naive animals and then injected i.v. with PBS, FltC, OVA alone, or OVA in combination with FltC or CpG (Fig. 4b). Three days later the proliferation of CFSE-positive cells in spleen or lymph nodes was analyzed by flow cytometry. Neither FltC nor PBS induced proliferation of transferred CD4+ T cells, indicating that flagellin has no direct effect on OVA-specific T cells. A limited number of cells underwent divisions in animals treated with OVA alone in contrast to the study performed with OVA, CpG, and FltC, but not CpG alone (Fig. 5b).

Flagellin adjuvant effect requires MyD88

The importance of TLR signaling in Th1 induction is mostly based on the observation that MyD88-deficient mice are unable to mount Th1 responses when CFA, which contains TLR agonists, is used as adjuvant (7). Experiments were conducted to define the role of MyD88 in the adjuvant properties of flagellin (Fig. 5). MyD88-deficient animals and their wild-type counterparts, C57BL/6, were immunized as described above. As observed with BALB/c mice, flagellin triggered OVA-specific Th2-biased responses, whereas CpG elicited Th1 responses (Fig. 5a). In MyD88 animals, flagellin, like CpG, did not trigger any response in knockout animals (Fig. 5b). In contrast, CFA stimulated the secretion of IL-4 and IL-13, thereby inducing Th2-type responses (Fig. 5b). We then investigated the Ab response associated with T cell priming in immunized animals. Indeed, when flagellin was coinjected with OVA,

![FIGURE 3. Flagellin-mediated cytokine secretion in BMDCs and in vivo. a, BMDCs derived from C57BL/6 or MyD88−/− mice were incubated for 20 h with medium alone, FltC, or CpG (1 and 10 μg/ml, respectively). The secretion of IL-12 p40, IL-12 p70, TNF-α, or IL-6 was measured by ELISA. Similar results were obtained with BMDC from 3×-κB-luc transgenic and C3H/HeJ mice. b, C3H/HeJ mice (n = 4) were injected i.v. with PBS, FltC (10 μg), and CpG (50 μg), supplemented or not with anti-CD40 mAb (50 μg) as indicated. Serum concentrations of IL-12 p40 and IL-12 p70 were determined 6 h later.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)
the OVA-specific serum IgG response increased >20-fold (Fig. 4c). This effect was effective at doses of flagellin ranging from 100 ng to 30 μg/animal, but was totally abolished when flagellin was digested by trypsin before injection (Figs. 5 and 6). In addition, flagellin caused a marked increase in anti-OVA IgG1 titers without significantly increasing the anti-OVA IgG2a response in various mouse genetic backgrounds, such as C57BL/6, BALB/c, C3H/HeJ, and NMRI (Fig. 6). In MyD88 animals, flagellin did not potentate any OVA-specific responses compared with CFA (Fig. 5d). As reported for innate immunity (14), the effect of flagellin on adaptive immunity, especially on the development of Th2-biased responses, was therefore strictly dependent on the adapter molecule MyD88.

**Discussion**

DCs are instrumental for the development of Th cell responses (2, 3). In particular, it has been postulated that MyD88-dependent TLR activators promote Th1 responses (7, 8). In this study we provide evidence that flagellin induces Th2-biased responses in a
MyD88-dependent maturation of DCs both in vivo and in vitro. In vivo, flagellin induces the development of IL-4-producing CD4+ T cells, a hallmark of Th2 polarization. This effect is associated with the lack of Th1-polarizing cytokine IL-12 p70 production by flagellin-stimulated DCs.

A major concern in analysis of PRR stimulatory activity is contamination of the PAMP preparation by unrelated ligands, especially for in vivo studies or studies with DCs. Our flagellin preparations were first characterized for the presence of bioactive flagellin using intestinal epithelial cells as reported previously (19, 28). Although gangliosides have been reported to contribute to signaling, the flagellin-mediated effect is essentially dependent on TLR5 stimulation and MyD88 (14, 29, 34, 35). To rule out any direct contribution of ligands for TLR2, TLR3, TLR4, TLR6, and TLR9 in the flagellin preparation, we used endotoxin-depleted purified flagellin combined with B cell activation assays, which can detect these elicitors (data not shown). In addition, flagellin was fully effective at inducing maturation of splenic DC in mice with impaired TLR2 and TLR4 signaling. Together these data strongly support the idea that the bacterial product responsible for biological activity on DCs and adaptive immunity is flagellin, the TLR5 ligand. Although we determined that flagellin promotes Th2 responses in a MyD88-dependent manner, TLR5-deficient mice should be assayed to define the unique and specific role of TLR5 in this process.

The most striking finding is that Th2-biased differentiation is associated with MyD88-dependent activation of DCs. Although CpG and flagellin both induce the production of IFN-γ by OVA-specific T cells, only flagellin promotes the secretion of Th2-specific cytokines, IL-4 and IL-13. How can signaling in DC via distinct TLRs give rise to different CD4+ T cell polarizations? Several mechanisms, including cell subsets, state of maturation, and cytokine release, could account for such a difference. CD8α+ DCs have been proposed to be involved in Th2 priming (36). Our experiments suggest that flagellin-programmed Th2-type differentiation is not controlled by a specific DC subset, as the whole DC population is activated in vivo (Figs. 1c and 2a). Th2 development has been associated with strong costimulatory signals provided by activated DCs (3). After injection of flagellin, splenic DCs were activated, with the level of costimulatory molecules higher than that after CpG injection (data not shown). CpG and flagellin have similar capacities to prime OVA-specific T cells, but have distinct effects on T cell polarization (Fig. 5). The role of costimulatory molecules in flagellin-mediated, Th2-type responses is therefore not conclusive and will require further investigations. Th1/Th2 development can be influenced by the Ag dose provided to DCs. In the study by McSorley et al. (17), flagellin administration with the Th peptide OVA123–339 also resulted in IFN-γ production by CD4+ T cells, but IL-4 production was not reported. This Th1-type promoting effect of flagellin, which diverges from our data, suggests that either the dose of Ag or the processing and presentation of whole OVA provide essential information for T cell polarization. The doses of whole OVA used in our work are 220-fold lower than the dose of peptide used by McSorley et al. (17), assuming that each OVA molecule will generate a Th peptide. We also observed a lower activation of DO11.10 cells with OVA compared with that obtained with OVA123–339 peptide, which alone triggers a strong proliferation without any microbial stimuli. This discrepancy in Th1/Th2 balance could therefore be attributed to an Ag dose effect in which a high Ag dose favors Th1 responses. Alternatively, flagellin activity on Ag processing and presentation in DCs such as occurs during immunization with a complete Ag may change the context in which T cell polarization occurs. Finally, the commitment to a Th1/Th2 phenotype depends on a specific pattern of cytokines released by DCs. The TLR stimuli tested to date (i.e., LPS, peptidoglycan, dsRNA, and CpG) promote and/or enhance the development of Th1-type responses as well as IL-12 p70 secretion by DCs (31, 32, 37, 38). The lack of IL-12 p70 secretion and the low levels of IL-6 and TNF-α appear to be unique to flagellin signaling and probably to TLR5 (Fig. 3a). Interestingly, flagellin-mediated activation of human DCs is also characterized by a distinct cytokine pattern compared with LPS-mediated activation, including lack of IL-12 p70 secretion (18). This singularity was already suggested by the moderate signs of inflammation triggered by flagellin compared with other TLR ligands (26, 39). The failure of DC to produce IL-12 p70 or other Th1-promoting cytokines, such as the cytokines IL-23 and IL-27, during T cell sensitization could directly impair Th1 development, expansion, or maintenance. Flagellin-dependent Th2 commitment could also rely on Th1-suppressive mechanisms, such as secretion of IL-10 or TGF-β or production of Th2-inducing cytokines (31).
This study challenges the observations of Means et al. (18) concerning stimulation of isolated splenic mouse DC. Nevertheless, we also found that splenic DCs incubated ex vivo with flagellin were not stimulated. Sorted DCs undergo spontaneous maturation that masks subsequent flagellin activation. It is known that any treatment of DCs is sufficient to induce their spontaneous maturation, in particular magnetic beads or FACS sorting, collagenase treatment, movement of culture dishes, or dislodgment of DC clusters (40, 41). Therefore, DC activation was analyzed directly in vivo after i.v. injection of flagellin or on BMDCs (Figs. 1 and 2). In our study splenic DCs as well as BMDCs express TLR5 and are fully activated by flagellin in a MyD88-dependent process. In addition, B lymphocytes that do not have any TLR5 transcripts are totally unresponsive to flagellin (data not shown). The recent study by Agrawal et al. (13) proposes that flagellin-activated human DC express IL-12-p70, in contrast to Means’s work (18). The cellular and molecular mechanisms of these differences remain to be elucidated. To shed light on the controversial studies of flagellin activity on Th responses, several cues should be explored, including the purity of flagellin preparations, the influence of Ag (OVA peptide, self-Ag for mixed lymphocyte reactions, or OVA protein), the relevance of readouts (in vivo or in vitro), the phenotype of DCs (in vitro- or in vivo-derived DCs, as well as the level of TLR5 expression), and host specificity (rodent vs human).

Recent reports proposed that TLR2 and TLR4 play a role in Th2 differentiation (9–12). These studies propose that MyD88-independent mechanisms and the dose of TLR agonists contribute to Th2 polarization. Thus, the difference in T cell differentiation induced by flagellin compared with TLR stimuli may reside in distinct quantitative and qualitative signals. Our data show that in DC the factors that constitute the core TLR-specific signaling pathway, i.e., MyD88, NF-κB, and IL-12 p40 expression, are conserved in TLR5 if we assume that flagellin-mediated signaling totally depends on TLR5 activation as described by others (14, 29, 42, 43). We assessed whether the level of DC activation could explain the difference between CpG and flagellin. Upon flagellin injection, DCs are activated, and IL-12 p40 is secreted as for TLR-activating signals, suggesting a similar intensity of signaling. In addition, the outcome of activation is independent of flagellin dose, suggesting that TLR5 per se may be sufficient for instructing Th2-biased differentiation. The unique property of flagellin is probably due to a qualitative difference in the signaling machinery. For instance, TLRs have individual specificities, characterized by specific signaling complexes composed of MyD88 and accessory adapter molecules (such as Toll/IL-1R domain-containing adapter protein (TIRAP), Toll-interacting protein, and Toll/IL-1R domain-containing adapter-inducing IFN-β), which define the specificity of TLR2, TLR3, and TLR4 signaling (44–46). TLR5 does not require TIRAP (45), but whether other adaptors can modulate signal transduction through TLR5 is yet not known. The host cell glycolipid asialoganglioside monosialic acid 1 can bind flagellin and induce specific signaling, including mobilization of calcium and activation of mitogen-activated protein kinase (extracellular signal-regulated kinase 1/2) (34). We show that MyD88-dependent processes are essential for the development of flagellin-promoted Th2-type immunity, but TLR5 cooperation with PRRs such as asialo-GM1 may modulate the outcome of the response, as described recently for TLR2 and dectin-1 (47). Flagellin therefore represents a unique model to dissect TLR-associated signaling machinery specialized in development of Th2-biased responses.

How pathogens influence Th2 differentiation is poorly characterized and has consequently been classified as a default pathway. In this study flagellin was unambiguously shown to be the PAMP responsible for Th2 commitment. Usually, products used to study Th1/Th2 balance are composed of a mixture of microbial structures. For instance, CFA, Toxoplasma extract, or Schistosoma egg Ags are probably composed of several PAMPs that individually signal through PRRs promoting Th1 or Th2 responses. Similarly, commercial Ags or PAMP preparations that are considered pure PAMPs undoubtedly contain microbial contaminants, especially LPS. Under these conditions, a hierarchy between the various PRR signals may account for the preferential instruction of Th1 or Th2 responses. Such a model is supported by recent observations showing that Th1-promoting TLR-specific signals appear to predominate over MyD88-independent Th2-promoting signals (8).
In conclusion, our observations support a broader importance of TLRs in immune responses against pathogens. The combination of TLRs stimulated by the various PAMPs displayed by microbes might result in the fine-tuning of adaptive immune responses.

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