Compartmentalized antimicrobial defenses in response to flagellin

Aneesh VIJAYAN¹, Martin RUMBO², Christophe CARNOY^{1#}, and Jean-Claude SIRARD^{1#}

¹ Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR8204 - CIIL - Center for Infection and Immunity of Lille, F-59000 Lille, France

² Instituto de Estudios Inmunológicos y Fisiopatológicos – CONICET- National University of La Plata, 1900 La Plata, Argentina

Correspondence: christophe.carnoy@univ-lille2.fr; jean-claude.sirard@inserm.fr

Motility is often a pathogenicity determinant of bacteria targeting mucosal tissues. Flagella constitute the machinery that propels bacteria into appropriate niches. Besides motility, the structural component flagellin, which forms flagella, targets Toll-like receptor 5 (TLR5) to activate innate immunity. The compartmentalization of flagellin-mediated immunity and the contribution of epithelial cells, and dendritic cells in detecting flagellin within luminal and basal sides are highlighted here, respectively. While a direct stimulation of the epithelium mainly results in recruitment of immune cells and production of antimicrobial molecules, TLR5 engagement on parenchymal dendritic cells can contribute to the stimulation of innate lymphocytes such as type 3 innate lymphoid cells, as well as T helper cells. This review, therefore, illustrates how the innate and adaptive immunity to flagellin are differentially regulated by the epithelium and the dendritic cells in response to pathogens that either colonize or invade mucosa.

(143 words)

Detection of bacterial flagellin and regulation of immune responses

Bacterial infection of mammalian hosts triggers pro-inflammatory responses, i.e., innate immune responses, through detection of microbe-associated molecular patterns (MAMP) by pattern recognition molecules (PRM). MAMPs are conserved molecules of microorganisms that bind to PRM initiating a signaling cascade that culminates in the pro-inflammatory response. The main feature of these events involves the activation of sentinel cells that are the cornerstone of innate and adaptive antibacterial defenses. This review describes how flagellins, significant MAMPs of flagellated bacteria, contribute to immunity with emphasis on the mucosal tissues and compartmentalization of responses.

Structural organization of flagellum and flagellin

Flagellin is the structural protein of flagellum, a surface filament dedicated to bacterial motility. The filament can be formed of as many as 20,000 subunits of flagellin. In pathogenic bacteria, flagella and the chemotaxis machinery can contribute to virulence [1]. The amino-acid composition of flagellin ranges between 250 to 1250 residues that include a conserved region flanking a central hypervariable region. The 170 residues at the N-terminal end and 100 residues at the C-terminal end are highly conserved sequences among different genera of bacteria. The structure of *Salmonella enterica* serovar Typhimurium flagellin FliC, a 495 amino-acid long protein, has been defined (**Figure 1A**). The terminal chains that form the packed helical structures, i.e., the D0 and D1 domains, are essential for polymerization of flagellin into the flagellum. The central hypervariable region (i.e., the D2 and D3 domains) that determines the flagellin serovar (H typing) is exposed as beta-sheet and turn-folded structures on the outer surface of the filament [2]. Thus, the D2 and D3 sequences are under intense selective pressure

and vary to escape the protective/neutralizing antibody responses (through phase variation for example), thus allowing colonization and infection by new bacterial serotypes. All domains are compactly folded in flagellum whereas the D0 domain is disordered in solution, i.e., when flagellin is released as free monomers [3].

Detection by pattern-recognition molecules

Toll-like receptor 5 (TLR5), a mammalian PRM, senses extracellular flagellin monomers [4]. Orthologues of TLR5 are distributed among vertebrates and invertebrates in addition to the so-called flagellin-sensitive two receptor or FLS2, a TLR5-like PRM, in plants (**Box 1**). Both human and mouse TLR5 are type I transmembrane proteins that recognize similar molecular determinants of native flagellin and have a comparable effective concentration in cell-based assays [5]. While the hypervariable part of the *S. Typhimurium* flagellin FliC is dispensable to TLR5 signaling, the D1 domain, especially the conserved residues 89-96 (QRIRELAV), as well as the D0 domain, are essential [6–10]. Most epsilon- and alpha-proteobacteria produce flagellins with an alternative 89-96 motif (such as DTVKVKAT or DTIKTKAT) that does not activate TLR5 and evades immune sensing. The 89-96 motif that is embedded in the flagellum core is accessible to PRM only when flagellin is released as a monomer (**Figure 1B-C**). The extracellular domain of TLR5 is composed of 22 leucine-rich repeats (LRR). Mutagenesis and crystallographic studies (based on the zebrafish TLR5 and flagellin from *S. enterica* serovar Dublin) provided clues on the receptor interaction with flagellin [9]. This model was validated functionally on mammalian TLR5. Two regions of flagellin are essential for primary interaction with TLR5 that lead to the establishment of TLR5-FliC 1:1 heterodimer: (1) the carboxy-terminal α -helix of the D1 domain that is in close contact with the TLR5 LRRs 1-6, and (2) the

motif 89-86 of the D1 domain that binds to LRR7-10. Moreover, LRRs 12-13 promotes the dimerization of TLR5-FliC 1:1 heterodimer to build up a 2:2 functional complex (**Figure 1C**). Interestingly, deletion of the C-terminal region (from LRR14 to C-terminal) results in a secreted and soluble TLR5 form that still binds flagellin (**Box 2**). While the D0 domain of flagellin is essential to TLR5 signaling the molecular interactions between D0 and TLR5 are not entirely understood [10]. A recent study demonstrated that the C-terminal part of the D0 domain of flagellin is essential to TLR5 signaling whereas the N-terminal one is dispensable [11]. The dissection of molecular mechanisms of D0 and TLR5 will be of major interest to unravel how interaction is integrated into the activation of signaling cascade.

Recently, the high mobility group box 1 (HMGB1), an alarmin released from necrotic cells, has been found to depend on TLR5 signaling [12]. Even though it was determined that the carboxy-terminal region of HMGB1 is involved in TLR5 interaction, further studies will be required to understand the molecular mechanisms involved.

TLR5 is ubiquitously expressed at the surface of various cells including myeloid cells (macrophages, dendritic cells [DC], or monocytes), lymphoid cells (lymphocytes, or natural killer cells), structural cells (epithelial cells [EC] from mucosa, keratinocytes, or fibroblasts) or neurons (reviewed in [13,14]). It is important to stress out that TLR5 expression has mainly been studied by RT-qPCR and knockout models but has not been validated at the protein levels due to the lack of quality controlled TLR5-specific antibodies. Interestingly, the Unc-93 homolog B1 of *Caenorhabditis elegans* (UNC93B1), an essential protein for intracellular traffic of PRM, promote the addressing of TLR5 to the plasma membrane, thereby regulating TLR5 signaling [15]. TLR5 signaling requires the myeloid differentiation primary response 88 (MyD88), an adaptor protein essential for signaling by most TLRs as well as interleukin receptors including

IL-1R, IL-18R, IL-33R, and IL-36R (**Figure 1B**) [4,16]. TLR5 and MyD88 assemble into a high scaffold-signaling complex (the so-called myddosome) that ultimately activates nuclear factor (NF)- κ B, mitogen-activated protein kinase (MAPK), and interferon (IFN)-regulatory factor (IRF) pathways [17]. This cascade upregulates transcription of genes coding for immune mediators as described later in this review. The flagellin-mediated response is short-lived due to the strong feedback regulatory mechanisms involving transcriptional and post-transcriptional regulators such as tumor necrosis factor alpha-induced protein 3 or TNFAIP3, mRNA decay activator protein ZFP36 or tristetraprolin, or the NF- κ B inhibitors I κ B ζ and I κ B α [18–20]. Several studies in TLR5-deficient animals stressed on flagellin's role in antibacterial defenses [21–23]. Thus, TLR5 signaling is associated with the regulation of cytokine/chemokine synthesis, the recruitment of phagocytes such as neutrophils, and the production of antimicrobial molecules.

TLR11 was proposed to sense flagellin in the gut using the same motifs as TLR5 that in turn protect against pathogenic intestinal bacteria in mice [24]. While TLR11 is not functional in humans, in mice, it was initially shown to detect uropathogenic *Escherichia coli* MAMPs in kidneys as well as the *Toxoplasma gondii* protein profilin [25,26]. Remarkably, TLR5 and TLR11 belong to different phylogenic families in contrast to TLRs that perceive related compounds like nucleic acids [27]. Recent findings pointed out that flagellin-mediated stimulation of immunity and antimicrobial defenses do not require TLR11 signaling [28]. Moreover, most observations in TLR5-deficient animals support that TLR11 cannot substitute for TLR5 and, therefore, is not required for immunostimulatory activity of flagellin in various mouse tissues including the gut. Taken as a whole, TLR11 is unlikely to be involved in detecting flagellin.

Finally, bacterial flagellin is a unique MAMP, in the sense that it can trigger immune signaling in the extracellular medium via TLR5 as well as in the cytoplasmic compartment. The cytosolic detection of flagellin, at least in mice, is associated with the triggering of the inflammasome (reviewed in [29]) (**Box 3**).

Epithelial responses to flagellin at mucosal surfaces

Apical detection of the bacterial danger signal is the first event that explains how flagellated bacteria are sensed in the lumen by the impermeable mucosa in the absence of any injury. At the mucosal surfaces, ECs are the first cells that sense flagellin and modulate immune responses. Evidence from invertebrate species indicates that TLR-mediated flagellin recognition at epithelial surfaces is an old feature that has its evolutionary origin in the appearance of a stable association of microbiota to metazoans (**Box 1**). Intestinal, respiratory, and urogenital ECs, as well as keratinocytes derived from cell lines or primary cultures, are highly responsive to flagellin stimulation through TLR5 (reviewed in [13,14]). Initially, the interaction of flagellin with TLR5 was proposed to be exclusively at the basolateral compartment of the epithelial layer as exemplified by polarized human intestinal cell line T84 [30]. Currently, there is evidence that TLR5 is also expressed at the apical pole of ECs from the follicle-associated epithelium (FAE) of Peyer's patches, the small intestine or the respiratory mucosa [18,31–34].

Flagellin-stimulated ECs rapidly induce pro-inflammatory mediators such as chemokines CXCL1, CXCL2, CXCL5, CXCL8, CCL2, CCL20 or CXCL10 that in turn attract immune cells belonging to the myeloid and lymphoid origin (reviewed in [13,14]). This process is essential for recruiting cells that participate in innate response, thereby shaping antimicrobial defenses against flagellated bacteria (**Figure 2**). For instance, in wild-type animals that were reconstituted with

Tlr5^{-/-} bone marrow, radioresistant cells, mainly respiratory ECs produce chemokines in response to flagellin, leading to the infiltration of polymorphonuclear neutrophils (PMNs) in the conducting airways [18,35,36]. Moreover, TLR5 signaling elicits production of cytokines and cell growth factors (such as IL-6, IL-1 β , granulocyte colony-stimulating factor or G-CSF) that further contribute to activation and survival of recruited cells. Finally, stimulation of ECs by flagellin is characterized by the polarized secretion of various antimicrobial peptides or molecules including mucins and β -defensins in the mucosal lumen that can contribute to host defense (reviewed in [13,14]). Using mice deficient for MyD88 in lung epithelial cells, flagellin-deficient, and proficient bacteria, and *Tlr5*^{-/-} bone marrow chimera, a recent study further highlights the role of ECs in orchestrating TLR5-dependent innate defenses against bacterial respiratory infections [35].

TLR5-dependent epithelium signaling also impacts the adaptive response by regulating antigen transport in the lamina propria of mucosa and DC activation. First, TLR5 signaling in FAE facilitates transportation and antigen delivery to immature DCs via M cells [37]. Remarkably, the chemokine CCL20, a ligand for CCR6⁺ DCs, is constitutively expressed by FAE and can be upregulated by flagellin in a TLR5-dependent manner [32,38]. Upon maturation, by danger signals including MAMPs or by cytokines, DCs migrate to draining lymphoid tissues, present antigens to naïve lymphocytes and polarize the adaptive responses. Indeed, DCs provide appropriate signals for the development of CD4⁺ T helper cell (Th), regulatory T cell (Treg), CD8⁺ T cell, and B cell responses. Immature DCs in tissues are mostly conventional DCs (cDCs) expressing CD103 (cDC1) or CD11b/CD172 α (cDC2) [39]. Flagellin administration by the respiratory route induces, via ECs, the maturation of cDC1 and cDC2, as monitored by upregulation of MHCII, co-stimulatory molecules and antigen presentation [19,20]. The nature

of EC signals that contribute to DC maturation remains to be defined. This indirect effect on the cDC is essential for the TLR5-dependent mucosal adjuvant effect of flagellin. Indeed, the flagellin-mediated mucosal adjuvant effect on antigen-specific CD4⁺ T-cell responses is a process dependent on cDC2 [20]. Garont *et al.* recently demonstrated that human cDC2 preferentially home to mucosal tissues, suggesting that these cells can be a prime target for mucosal adjuvants [40]. In conclusion, flagellin is an efficient mucosal adjuvant for nasal, respiratory and oral vaccinations [41,42]. The application of flagellin by mucosal route is associated with mixed Th1 and Th2 responses, systemic IgG1 production, and secretory IgA in the mucosa lumen. Interestingly, IgA class switching in gut B cells is partly mediated by flagellin-dependent secretion of a proliferation-inducing ligand (APRIL) by ECs [43]. Respiratory administration of flagellin was suggested to be associated with the priming of allergy [44]. However, a recent study demonstrated that flagellin downregulates allergic response [45]. This discrepancy may be due to the quantity and purity of flagellin that allows the development of Tregs involved in the desensitization to allergens.

Taken together, the sensing of flagellin by ECs is a major process for shaping the immune response against flagellin and flagellated bacteria.

Direct activation of dendritic cells: key mechanism of systemic response to flagellin

Innate immune sensors, such as TLRs, are essential for the direct activation of innate and adaptive immunity by DCs. This section discusses the TLR5-mediated effect of flagellin monomer on DC and in turn on adaptive immunity (**Figure 3**). This mode of action can have a significant impact when flagellated bacteria invade mucosal tissues and deliver flagellin in the parenchyma compartment or deeper tissues.

Flagellin was initially reported to stimulate Th1-type responses when administered by systemic route [46,47]. However, our observations and studies from Cunningham *et al.* also support induction of Th2 responses against *S. Typhimurium* flagellin [16,48]. Thus, flagellin promotes the development of TLR5- and MyD88-dependent mixed Th1-Th2 responses when co-administered with protein antigens. It is characterized by the production of interferon- γ , IL-4, and IL-13 by antigen-specific CD4⁺ T lymphocytes, and prominent IgG1 antibody isotype. Indeed, systemic administration of flagellin induces DC maturation and up-regulation of co-stimulatory molecules and antigen-presenting capacity in mucosal tissues as well as in secondary lymphoid tissues such as the spleen or lymph nodes (**Figure 3**). Notably, DCs are a primary source of TLR5 within the myeloid compartment. In DCs from mouse or human, flagellin triggers secretion of IL-12p40 (but not IL-12p70), that is pivotal for instructing and maintaining Th2 cells and to a certain extent Th1 cells [49,50]. Recently, gut cDC2 cells were found to respond to flagellin by producing IL-23 (an heterodimer of IL-12p40 and IL-23p19), IL-6, and Transforming Growth Factor β (TGF β) [51–54]. This TLR5-dependent activity led to the induction of the Th17-type cytokine IL-22 by the type 3 innate lymphoid cells (ILC3) and the development of Th17 responses [19,51,53–55]. In contrast to the lymphocyte activation in lymphoid tissue due to migratory DCs, ILC3 activation is immediate and depends heavily on tissue-resident DCs. Moreover, CD103⁺ DCs (likely cDC1s) recruited to mesenteric lymph nodes, upon systemic flagellin administration, can prime Treg expressing FoxP3 and IgA-producing B cells [56]. How different subsets of cDCs that are stimulated by flagellin/TLR5 influence innate and adaptive immunity remain to be investigated with regards to the route of administration, the amount of flagellin, and the mucosal tissue environment (see Outstanding Questions).

Manipulation of flagellin-specific signaling for prophylaxis or treatment of diseases

The ability of flagellin to elicit a prompt and robust innate response that in turn primes the adaptive arm of the immune system makes it attractive as an immunomodulatory adjuvant for control of various diseases. Studies in animals and non-human primates show flagellin as an effective vaccine adjuvant when administered with an antigen or produced as a fusion protein with antigen [41,42,57]. Interestingly, the potency of flagellin to induce strong adaptive immune responses, in a low-dose regimen, is appealing for flagellin-based vaccines. Recombinant flagellins derived from *Salmonella* species have already been tested in some clinical trials that are ongoing or are completed (for review see: clinicaltrials.gov). The safety of recombinant flagellins, as well as their capacity to promote an immuno-adjuvant effect, was validated in human volunteers using intramuscular or subcutaneous routes of administration with the effective doses of flagellin ranging from 1 ng/kg to 300 ng/kg. Furthermore, the adjuvant properties of flagellin have been demonstrated in the context of influenza vaccine candidates in neonatal non-human primates and clinical studies with older subjects, indicating that the efficiency of flagellin is independent of the age [58,59]. The flagellin's adjuvant activity is extensively associated with TLR5 signaling (**Box 2 and Box 3**). However, some studies suggest that neither TLR5 nor NAIP5/6 is required for immune signaling, but a pathway that remains to be identified [60–62]; The dose and the quality of flagellin, as well as the route of administration, might explain this discrepancy. Further studies are still required to evaluate flagellin's crosstalk with the immune system and how this, in turn, influences its adjuvant activity.

TLR5-deficient mice are more susceptible to intestinal, respiratory or urinary tract bacterial infection, indicating the central role of flagellin in innate antibacterial defenses [21–23].

Flagellin-based interventions have demonstrated protective activity in many infectious diseases. Mucosal or systemic administration of standalone flagellin protects mice against intestinal, respiratory or cutaneous infections caused by either Gram-negative (*Salmonella sp.*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Yersinia pseudotuberculosis*) or Gram-positive bacteria (*Enterococcus faecalis*, *Clostridium difficile*, or *Streptococcus pneumoniae*) [63–69]. The antibacterial effects of flagellin correlate with the epithelial production of antimicrobial compounds such as RegIII γ , cathelicidin, β -defensin-2 or calgranulins [64,67,70–73] (**Figure 2**). The protection also depends on the recruitment of neutrophils in the mucosal tissues through TLR5-dependent activation of ECs or the DC/ILC3 axis. The contribution of the epithelial compartment to neutrophil infiltration was supported by recent studies [35,69,74]. Protective effects of flagellin treatment were mainly defined in the prophylactic regimen, i.e., when administered prior or at the time of infection. Interestingly, the combination of flagellin with antibiotics was very effective for therapeutic intervention against acute bacterial respiratory infection [74]. Such combinatory treatments were more effective than standalone antibiotic therapy in post-influenza pneumococcal infection, a pathophysiological context where the lung is actively inflamed and infected, and tissue architecture is altered [74].

Flagellin is also reported to promote antiviral activity. When delivered 48h to 72h before challenge, the TLR5 agonist CBLB502 or Entolimod (a recombinant truncated flagellin from *S. enterica* serovar Dublin) protects mice from lethal infection by cytomegalovirus in an NK cell-dependent manner [75]. Flagellin also protects against intestinal viral infection as daily systemic administration of flagellin prevents mice from rotavirus infection and cures chronically infected mice [63,76]. The resistance mediated by flagellin administration requires TLR5 and NLRC4,

which are responsible for the production of IL-22 and IL-18, respectively [76]. Combined administration of both IL-22 and IL-18 recapitulates antiviral defense.

The therapeutic effect of flagellin goes beyond protection against infections. Indeed, a single dose of Entolimod, administered before lethal radiation, improves the survival probability of mice and non-human primates [63,77]. Flagellin induces irradiation resistance of the radiosensitive intestinal mucosa through NF- κ B-mediated anti-apoptotic properties. The liver is an essential target of flagellin treatment, participating in Entolimod-mediated radioprotection; However, the molecular and cellular mechanisms remain unknown [78]. Flagellin also demonstrated efficacy in experimental cancer prevention either by a direct effect on TLR5-expressing tumor cells or by activating bystander immune and non-immune cells [78–80]. For instance, tumor-specific T cells engineered to produce flagellin contributes to antitumor efficacy by increasing T cell infiltration and reducing the recruitment/activation of Treg cells and myeloid-derived suppressor cells [81]. More recently, injection of attenuated *S. Typhimurium* designed to secrete heterologous flagellin decreases tumor volume and improves survival of mice through tumor-suppressor M1-type macrophages [82]. Altogether, flagellin via TLR5 signaling provides a unique opportunity to manipulate immunity for vaccine adjuvants, infection treatment, radioprotective countermeasures, or cancer immunotherapy.

Concluding remarks

Given the nature of the mucosa and its continuous exposure to antigens and pathogens, the epithelial cells, that constitute the mucosa, are more than physical barriers between the internal and external environment. Because they express PRM, ECs cells represent sentinel cells able to sense danger signals such as MAMPs on the outer side. In contrast, when inside MAMPs directly impact the PRM signaling in mucosal myeloid cells. The immune responses induced by TLR5 and flagellin interaction in mucosa are profoundly influenced by the nature of sentinel cells. ECs and cDCs similarly turn on TLR5 signaling but differentially regulate the set of immune mediators expressed in luminal and tissue contexts and therefore compartmentalize the immune responses. Flagellin-stimulated ECs mainly produce chemokines and cytokines to promote recruitment and activation of myeloid cells, including neutrophils and cDCs. In contrast, direct stimulation of tissue-resident cDCs by flagellin transactivates the lymphoid components including the type 3 innate lymphoid cells. Given the central role that DCs play in shaping the adaptive responses, a direct or indirect action of flagellin on these cells holds great promise to understand the evolution of acquired immunity against mucosal microbial infections. In conclusion, formulating MAMPs, like flagellin, that specifically target mucosal compartments or sentinel cells offers the prospect of developing novel PRM-mediated host-directed therapies.

293 **Acknowledgments**

294 AV is supported by a Marie Skłodowska-Curie Actions Individual Fellowship from the European
295 Commission (H2020-MSCA-IF-2014 Grant Agreement No. 657107). The Argentinean National
296 Research Council (CONICET) supports MR. AV, JCS, and CC are supported by the Institut
297 Pasteur de Lille, Inserm, CNRS, the Université de Lille, the national fund agency ANR (ANR-
298 16-ASTR-0013-01) and the nonprofit organization Vaincre la Mucoviscidose
299 (RF20160501644/1/1/86).

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543

Figure Legends

Figure 1. Structure of flagellin and molecular mechanisms of TLR5-induced immunity. (A)

Organization of flagellum and flagellin. Motile bacteria produce flagella composed mainly of polymerized flagellin. *Salmonella enterica* serovar Typhimurium flagellin consists of 4 domains: the terminal α -helixes (D0), the central α -helixes (D1), and the hypervariable β -sheets and turns (D2 and D3). The α -helixes regions (D0+D1) are required for filament architecture and motility functions and are embedded in the filament core. When released from the flagellum, the structure of D0 domain of flagellin is disordered. The exposed D0 and D1 domains on monomeric flagellin are mandatory for immune signaling. (B) The detection of flagellin by TLR5 triggers signaling cascade involving the universal TLR-specific adapter molecule MyD88, and downstream regulators such as NF- κ B, MAPK, thereby activating transcription of genes involved in innate and adaptive immunity. (C) Schematic view of TLR5-flagellin structure. The interactions of the D1 domain of flagellin with TLR5 at the cell surface is highlighted. This view is based on the protein databank structure 3v47.

Figure 2: Response of epithelial cells to flagellin. The epithelium forms the interface between the internal and the external environment. Epithelial cells of the mucosa are the main sentinel cells that sense bacterial flagellin and stimulates TLR5 signaling. The epithelial cell-driven immediate innate response is characterized by (1) secretion of various anti-microbial factors that limit bacterial growth, and (2) production of pro-inflammatory cytokines and chemokines that recruit phagocytes, mainly the circulating neutrophils and monocytes. Epithelial cells also have a significant imprint on adaptive immunity by activating immature tissue-resident cDCs. Mature

cDCs later migrate through afferent lymphatic vessels into the lymph nodes where they stimulate differentiation of CD4⁺ lymphocytes into Th1 and Th2 cells as well as B lymphocytes into antibody-producing plasma cells, mainly IgA and IgG-producing cells. Polymeric immunoglobulin receptor (pIgR) that is expressed on the basolateral side of epithelial cells, binds to dimeric IgA and is transported to the apical side where it gets cleaved to release secretory IgA (SIgA). The crosstalk between epithelial cells and DCs shape the adaptive arm of the immune response.

Figure 3. Direct activation of tissue-resident dendritic cells: from type 3 innate lymphoid cells to conventional lymphocytes. Flagellin can directly activate sentinel cells present in mucosal tissues, a condition that appears when motile pathogenic bacteria breach epithelium. The dark-shaded cells represent damaged epithelium. The tissue-resident conventional dendritic cells (cDCs) are key players in the process of detection and integration of danger signals. Thus, TLR5 signaling in cDCs induces the secretion of cytokines secretion including IL-12p40, IL-23 (IL-12p40 + IL-23p19) and IL-6 that are essential in the differentiation/activation of lymphoid cells, the expression of co-stimulatory molecules such as CD80 and CD86 and the upregulation of antigen presentation. The immediate consequences are the swift activation of mucosal type 3 innate lymphoid cells (ILC3) into IL-22 producing cells. Notably, IL-22R is only expressed by structural cells, especially ECs that, in turn, switch on a program dedicated to microbial clearance and tissue repair to promote recovery and homeostasis. Flagellin-activated ILC3 might represent essential effectors for protection of mucosa against invasive pathogenic bacteria. The mid-term effect of cDC maturation is the priming of naïve T and B lymphocytes into effector cells. There is evidence that flagellin-mediated signaling in cDC stimulates the differentiation

591 into Th1, Th2 and Th17 CD4⁺ Th cells and elicits a prominent systemic IgG1 response and local
592 IgA response. Such an adaptive response might help long-term protection against pathogens.

Text Boxes

BOX 1

Evolution TLR5 and flagellin recognition.

Genome sequence analysis of a large number of animal phyla shows that TLRs are present in virtually all eumetazoans, indicating that TLRs are ancient PRMs [83]. Concerning TLR5, orthologues of this receptor are found in most vertebrates, i.e., mammals, birds, reptiles, amphibians and fishes [84–86]. The extracellular LRRs of TLR5 orthologues are responsible for flagellin recognition as inferred from structure and sequence analysis performed with zebrafish TLR5 [9]. Interestingly, the most divergent sequences are found in fish with several species encoding a soluble form of TLR5 formed by LRRs. The function of soluble TLR5 is controversial with studies that suggest enhancement of NF- κ B signaling in cells expressing membrane-bound TLR5 or inhibition of cell activation by sequestration [84].

The genome of primitive metazoan hydra that emerged more than 500 million years ago, encodes, instead of the typical TLRs, four transmembrane proteins: two proteins constituted by TIR domains and short extracellular regions (HyTRR1 and HyTRR2), and two proteins with LRR-containing ectodomains and short cytoplasmic tails (HyLRR1 and HyLRR2) [87]. HyLRR2 with HyTRRs transduce activation signals upon flagellin stimulation. Thus, hydra ECs produce antimicrobial peptide expression, which in turn may modulate the microbiota associated with the endodermal layer.

Plants have also developed a unique TLR-like FLS2 to detect flagellin of pathogenic phytobacteria [88]. This process was also related to defense mechanisms to promote integrity of

plant cells upon infection. In conclusion, environmental monitoring of flagellin and the shaping of host-associated defense mechanisms were conserved during evolution.

BOX 2

TLR5 polymorphism in infection and chronic diseases

Human TLR5 deletion starting at LRR14 (TLR5_{392-stop}, also known as 1174C>T, Arg392Ter or rs5744168) is associated with the loss of responsiveness to flagellin [89]. The variant TLR5_{392-stop} is dominant negative and is present in about 5% in the global human populations (2.3% in African, 3.0% in American, 3.9% in East Asian, 6.1% in European, and 10.3% in South Asian individuals; www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=1:223111358-223112358;v=rs5744168;vdb=variation;vf=3234911). Remarkably, a similar mutation in the mouse TLR5 molecule (mTLR5_{393-stop}) abrogates the response to flagellin [90]. In humans, the TLR5_{392-stop} mutation is associated with susceptibility to lung infections with *Legionella pneumophila* [89], and to urinary tract infections with uropathogenic *E. coli* [91]. Whether other polymorphisms associated with a reduced TLR5 signaling impact on diseases remains to be defined [92]. Moreover, similar vulnerability is found in TLR5-deficient animals, highlighting a major contribution of TLR5 to innate antimicrobial defenses.

Besides, TLR5 polymorphism is also involved in various chronic diseases. In humans, TLR5 deficiency and TLR5 hypomorphic variants increase the risk of ulcerative colitis but promotes resistance to systemic lupus erythematosus or Crohn's disease [93–95]. In mice, TLR5 deficiency is associated with changes in microbiota that in turn contribute to spontaneous colitis and metabolic syndrome [60,96]. Loss of intestinal epithelial TLR5 seems to alter the microbiota

composition and trigger low-grade inflammation and metabolic abnormalities [97]. Furthermore, the changes of short-chain fatty acids' composition due to TLR5-associated dysbiosis promotes hepatic lipogenesis [98] whereas hepatic TLR5 deficiency increases the hepatic abnormalities induced by a high-fat diet [99]. Recently, the Ly6/ PLAU domain containing 8 (Lypd8) was found to interact with flagella and regulate the segregation of motile bacteria within gut lumen [100]. Unraveling how Lypd8 and TLR5 complement, synergize or antagonize activity on immune signaling to flagellated bacteria is a major issue for future investigations.

TLR5 signaling can impact the response to a vaccine. In humans, TLR5 expression positively correlates with response to vaccination [101]. TLR5-deficient mice have impaired antibody response after flu vaccination compared to wild-type animals. This effect is related to deficient TLR5 signaling in macrophages and short-lived plasma cells. Besides, Cullender *et al.* showed that flagellin-specific antibodies (and TLR5 signaling) are essential to regulate populations of flagellated bacteria from microbiota, thus having a global impact on immune responses [102]. Consistent with these findings, germ-free mice have impaired antibody response to a vaccine that can be rescued by colonization with flagellated bacteria [101].

BOX 3

NLRC4-dependent inflammasome activation

Bacterial flagellin is a unique MAMP, in the sense that it can trigger immune signaling in the extracellular medium via TLR5 as well as in the cytoplasmic compartment. Thus, in mouse phagocytes, the cytosolic detection of flagellin by PRM of the NAIP family (for nucleotide-binding and oligomerization domain-like receptor (NLR) apoptosis-inhibitory protein) promotes

the activation of inflammasome through the recruitment and the oligomerization of NLRC4 (NLR family caspase recruitment domain-containing 4) (reviewed in [29]) (**Figure I**). This gatekeeping mechanism provides alternative sensing for intracellular flagellated bacteria or intracellular injection of flagellin by bacterial secretion systems. Inflammasome activation is associated with caspase 1-dependent maturation of the pro-inflammatory cytokines IL-1 β and IL-18 and with pyroptosis, a cell death linked to cytokine release. Thus, NAIP/NLRC4 activation impacts the intracellular replication of flagellated bacteria and participates to host's defense. In mice, the PRM NAIP5 and NAIP6 bind flagellin carboxy-terminal amino acids [103]. In contrast, NAIP2 activates NLRC4-dependent inflammasome by sensing needle components of type 3 secretion systems from gram-negative bacteria [29,103].

Humans produce a unique NAIP molecule, but flagellin detection by this sensor remains controversial [29,104]. Remarkably, in most studies using flagellin to manipulate immune responses in animal models or clinical trials, formulations are made of soluble monomer that is poorly capable *per se* of entering mammalian cells. Further studies are required to determine whether flagellin activate the inflammasome machinery in humans as it does in mice and how relevant it is in immunity.

NLRC4 activation can significantly hamper the TLR5-mediated adjuvant activity of flagellin [60]. Adaptive immune responses raised against a flagellin-antigen protein is influenced by the site of fusion (N or C terminus of flagellin), an effect that is dependent on differential TLR5 and NLRC4 signaling [105]. Additional investigations are needed to characterize the molecular determinant of flagellin and antigen that might control TLR5 and NLRC4 signaling pathways.

683 **Figure I: Schematic view of flagellin-mediated inflammasome activation.** Upon detection of
684 cytosolic flagellin by NAIP5 or NAIP6, NLRC4 is recruited and oligomerizes. This process is
685 dependent on the apoptosis-associated speck-like protein containing a CARD (ASC) and results
686 in the proteolytic cleavage of the pro-caspase 1 into active caspase 1 that in turn mediates
687 inflammatory responses via interleukin activation.

Figure 1

Figure 2

Figure 3

Figure I of Box 3