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1 **Compartmentalized antimicrobial defenses in response to flagellin**

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13

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

26 **(143 words)**

27 **Detection of bacterial flagellin and regulation of immune responses**

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

36

37 **Structural organization of flagellum and flagellin**

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

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

54

55 **Detection by pattern-recognition molecules**

56 Toll-like receptor 5 (TLR5), a mammalian PRM, senses extracellular flagellin monomers
57 [4]. Orthologues of TLR5 are distributed among vertebrates and invertebrates in addition to the
58 so-called flagellin-sensitive two receptor or FLS2, a TLR5-like PRM, in plants (**Box 1**). Both
59 human and mouse TLR5 are type I transmembrane proteins that recognize similar molecular
60 determinants of native flagellin and have a comparable effective concentration in cell-based
61 assays [5]. While the hypervariable part of the *S. Typhimurium* flagellin FliC is dispensable to
62 TLR5 signaling, the D1 domain, especially the conserved residues 89-96 (QRIRELAV), as well
63 as the D0 domain, are essential [6–10]. Most epsilon- and alpha-proteobacteria produce flagellins
64 with an alternative 89-96 motif (such as DTVKVKAT or DTIKTKAT) that does not activate
65 TLR5 and evades immune sensing. The 89-96 motif that is embedded in the flagellum core is
66 accessible to PRM only when flagellin is released as a monomer (**Figure 1B-C**). The
67 extracellular domain of TLR5 is composed of 22 leucine-rich repeats (LRR). Mutagenesis and
68 crystallographic studies (based on the zebrafish TLR5 and flagellin from *S. enterica* serovar
69 Dublin) provided clues on the receptor interaction with flagellin [9]. This model was validated
70 functionally on mammalian TLR5. Two regions of flagellin are essential for primary interaction
71 with TLR5 that lead to the establishment of TLR5-FliC 1:1 heterodimer: (1) the carboxy-
72 terminal α -helix of the D1 domain that is in close contact with the TLR5 LRRs 1-6, and (2) the

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

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

86 TLR5 is ubiquitously expressed at the surface of various cells including myeloid cells
87 (macrophages, dendritic cells [DC], or monocytes), lymphoid cells (lymphocytes, or natural
88 killer cells), structural cells (epithelial cells [EC] from mucosa, keratinocytes, or fibroblasts) or
89 neurons (reviewed in [13,14]). It is important to stress out that TLR5 expression has mainly been
90 studied by RT-qPCR and knockout models but has not been validated at the protein levels due to
91 the lack of quality controlled TLR5-specific antibodies. Interestingly, the Unc-93 homolog B1 of
92 *Caenorhabditis elegans* (UNC93B1), an essential protein for intracellular traffic of PRM,
93 promote the addressing of TLR5 to the plasma membrane, thereby regulating TLR5 signaling
94 [15]. TLR5 signaling requires the myeloid differentiation primary response 88 (MyD88), an
95 adaptor protein essential for signaling by most TLRs as well as interleukin receptors including

96 IL-1R, IL-18R, IL-33R, and IL-36R (**Figure 1B**) [4,16]. TLR5 and MyD88 assemble into a high
97 scaffold-signaling complex (the so-called myddosome) that ultimately activates nuclear factor
98 (NF)- κ B, mitogen-activated protein kinase (MAPK), and interferon (IFN)-regulatory factor
99 (IRF) pathways [17]. This cascade upregulates transcription of genes coding for immune
100 mediators as described later in this review. The flagellin-mediated response is short-lived due to
101 the strong feedback regulatory mechanisms involving transcriptional and post-transcriptional
102 regulators such as tumor necrosis factor alpha-induced protein 3 or TNFAIP3, mRNA decay
103 activator protein ZFP36 or tristetraprolin, or the NF- κ B inhibitors I κ B ζ and I κ B α [18–20].
104 Several studies in TLR5-deficient animals stressed on flagellin's role in antibacterial defenses
105 [21–23]. Thus, TLR5 signaling is associated with the regulation of cytokine/chemokine
106 synthesis, the recruitment of phagocytes such as neutrophils, and the production of antimicrobial
107 molecules.

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

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

123

124 **Epithelial responses to flagellin at mucosal surfaces**

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

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

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

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

165 of EC signals that contribute to DC maturation remains to be defined. This indirect effect on the
166 cDC is essential for the TLR5-dependent mucosal adjuvant effect of flagellin. Indeed, the
167 flagellin-mediated mucosal adjuvant effect on antigen-specific CD4⁺ T-cell responses is a
168 process dependent on cDC2 [20]. Garont *et al.* recently demonstrated that human cDC2
169 preferentially home to mucosal tissues, suggesting that these cells can be a prime target for
170 mucosal adjuvants [40]. In conclusion, flagellin is an efficient mucosal adjuvant for nasal,
171 respiratory and oral vaccinations [41,42]. The application of flagellin by mucosal route is
172 associated with mixed Th1 and Th2 responses, systemic IgG1 production, and secretory IgA in
173 the mucosa lumen. Interestingly, IgA class switching in gut B cells is partly mediated by
174 flagellin-dependent secretion of a proliferation-inducing ligand (APRIL) by ECs [43].
175 Respiratory administration of flagellin was suggested to be associated with the priming of allergy
176 [44]. However, a recent study demonstrated that flagellin downregulates allergic response
177 [45]. This discrepancy may be due to the quantity and purity of flagellin that allows the
178 development of Tregs involved in the desensitization to allergens.

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

181

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

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

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

211

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

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

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

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

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

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

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

273

274 **Concluding remarks**

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

291

292

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300

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543

545 **Figure Legends**

546

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

548 Organization of flagellum and flagellin. Motile bacteria produce flagella composed mainly of
549 polymerized flagellin. *Salmonella enterica* serovar Typhimurium flagellin consists of 4 domains:

550 the terminal α -helixes (D0), the central α -helixes (D1), and the hypervariable β -sheets and turns
551 (D2 and D3). The α -helixes regions (D0+D1) are required for filament architecture and motility

552 functions and are embedded in the filament core. When released from the flagellum, the structure

553 of D0 domain of flagellin is disordered. The exposed D0 and D1 domains on monomeric

554 flagellin are mandatory for immune signaling. (B) The detection of flagellin by TLR5 triggers

555 signaling cascade involving the universal TLR-specific adapter molecule MyD88, and

556 downstream regulators such as NF- κ B, MAPK, thereby activating transcription of genes

557 involved in innate and adaptive immunity. (C) Schematic view of TLR5-flagellin structure. The

558 interactions of the D1 domain of flagellin with TLR5 at the cell surface is highlighted. This view

559 is based on the protein databank structure 3v47.

560

561 **Figure 2: Response of epithelial cells to flagellin.** The epithelium forms the interface between

562 the internal and the external environment. Epithelial cells of the mucosa are the main sentinel

563 cells that sense bacterial flagellin and stimulates TLR5 signaling. The epithelial cell-driven

564 immediate innate response is characterized by (1) secretion of various anti-microbial factors that

565 limit bacterial growth, and (2) production of pro-inflammatory cytokines and chemokines that

566 recruit phagocytes, mainly the circulating neutrophils and monocytes. Epithelial cells also have a

567 significant imprint on adaptive immunity by activating immature tissue-resident cDCs. Mature

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

575

576 **Figure 3. Direct activation of tissue-resident dendritic cells: from type 3 innate lymphoid**
577 **cells to conventional lymphocytes.** Flagellin can directly activate sentinel cells present in
578 mucosal tissues, a condition that appears when motile pathogenic bacteria breach epithelium.
579 The dark-shaded cells represent damaged epithelium. The tissue-resident conventional dendritic
580 cells (cDCs) are key players in the process of detection and integration of danger signals. Thus,
581 TLR5 signaling in cDCs induces the secretion of cytokines secretion including IL-12p40, IL-23
582 (IL-12p40 + IL-23p19) and IL-6 that are essential in the differentiation/activation of lymphoid
583 cells, the expression of co-stimulatory molecules such as CD80 and CD86 and the upregulation
584 of antigen presentation. The immediate consequences are the swift activation of mucosal type 3
585 innate lymphoid cells (ILC3) into IL-22 producing cells. Notably, IL-22R is only expressed by
586 structural cells, especially ECs that, in turn, switch on a program dedicated to microbial
587 clearance and tissue repair to promote recovery and homeostasis. Flagellin-activated ILC3 might
588 represent essential effectors for protection of mucosa against invasive pathogenic bacteria. The
589 mid-term effect of cDC maturation is the priming of naïve T and B lymphocytes into effector
590 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.

593 **Text Boxes**

594

595 BOX 1

596 **Evolution TLR5 and flagellin recognition.**

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

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

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

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

617

618

619 BOX 2

620 **TLR5 polymorphism in infection and chronic diseases**

621 Human TLR5 deletion starting at LRR14 (TLR5_{392-stop}, also known as 1174C>T, Arg392Ter or
622 rs5744168) is associated with the loss of responsiveness to flagellin [89]. The variant TLR5₃₉₂₋
623 stop is dominant negative and is present in about 5% in the global human populations (2.3% in
624 African, 3.0% in American, 3.9% in East Asian, 6.1% in European, and 10.3% in South Asian
625 individuals; [www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=1:223111358-](http://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=1:223111358-223112358;v=rs5744168;vdb=variation;vf=3234911)
626 [223112358;v=rs5744168;vdb=variation;vf=3234911](http://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
627 mouse TLR5 molecule (mTLR5_{393-stop}) abrogates the response to flagellin [90]. In humans, the
628 TLR5_{392-stop} mutation is associated with susceptibility to lung infections with *Legionella*
629 *pneumophila* [89], and to urinary tract infections with uropathogenic *E. coli* [91]. Whether other
630 polymorphisms associated with a reduced TLR5 signaling impact on diseases remains to be
631 defined [92]. Moreover, similar vulnerability is found in TLR5-deficient animals, highlighting a
632 major contribution of TLR5 to innate antimicrobial defenses.

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

638 composition and trigger low-grade inflammation and metabolic abnormalities [97]. Furthermore,
639 the changes of short-chain fatty acids' composition due to TLR5-associated dysbiosis promotes
640 hepatic lipogenesis [98] whereas hepatic TLR5 deficiency increases the hepatic abnormalities
641 induced by a high-fat diet [99]. Recently, the Ly6/ PLaur domain containing 8 (Lypd8) was
642 found to interact with flagella and regulate the segregation of motile bacteria within gut lumen
643 [100]. Unraveling how Lypd8 and TLR5 complement, synergize or antagonize activity on
644 immune signaling to flagellated bacteria is a major issue for future investigations.

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

653

654

655 BOX 3

656 **NLRC4-dependent inflammasome activation**

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

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

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

677 NLRC4 activation can significantly hamper the TLR5-mediated adjuvant activity of flagellin
678 [60]. Adaptive immune responses raised against a flagellin-antigen protein is influenced by the
679 site of fusion (N or C terminus of flagellin), an effect that is dependent on differential TLR5 and
680 NLRC4 signaling [105]. Additional investigations are needed to characterize the molecular
681 determinant of flagellin and antigen that might control TLR5 and NLRC4 signaling pathways.

682

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.

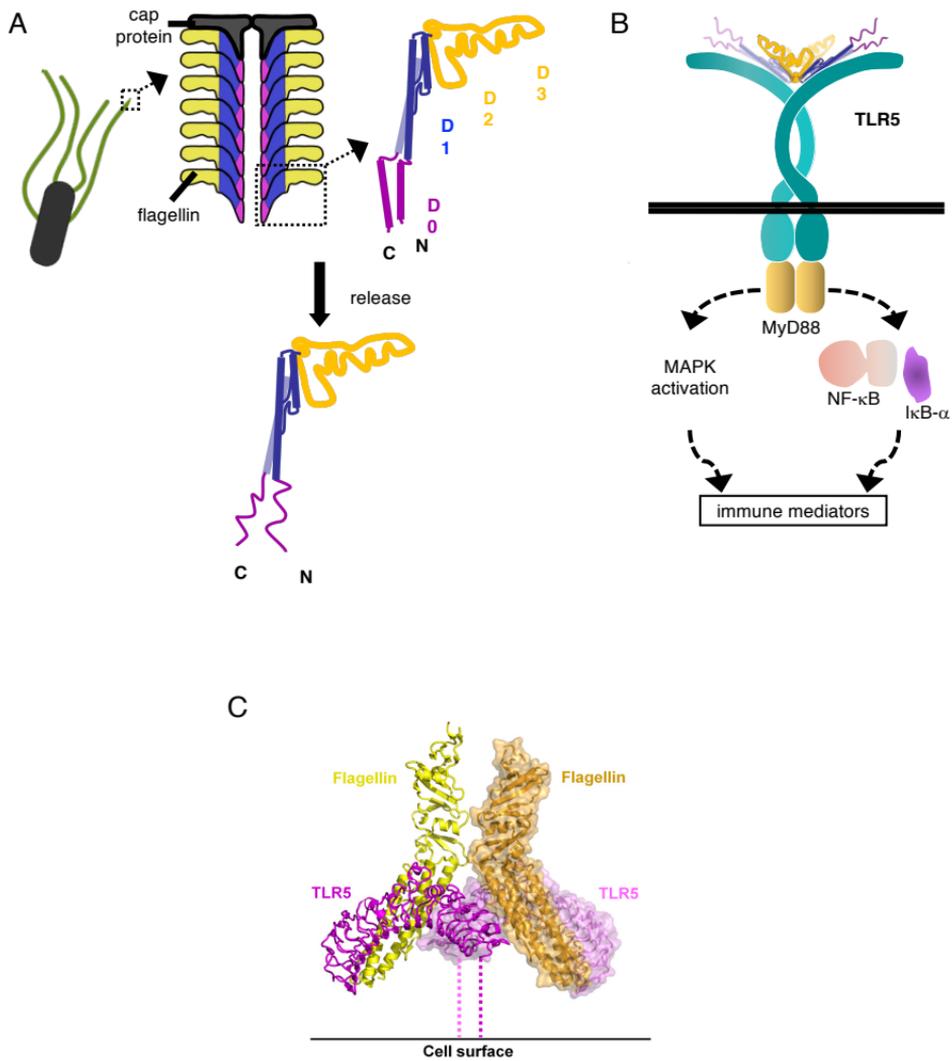


Figure 1

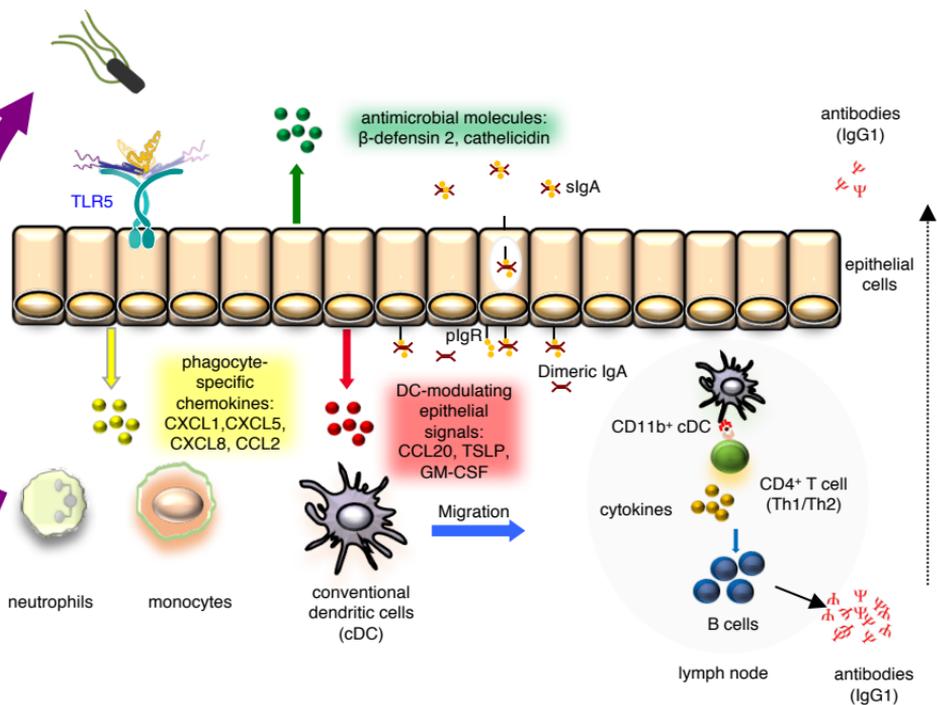


Figure 2

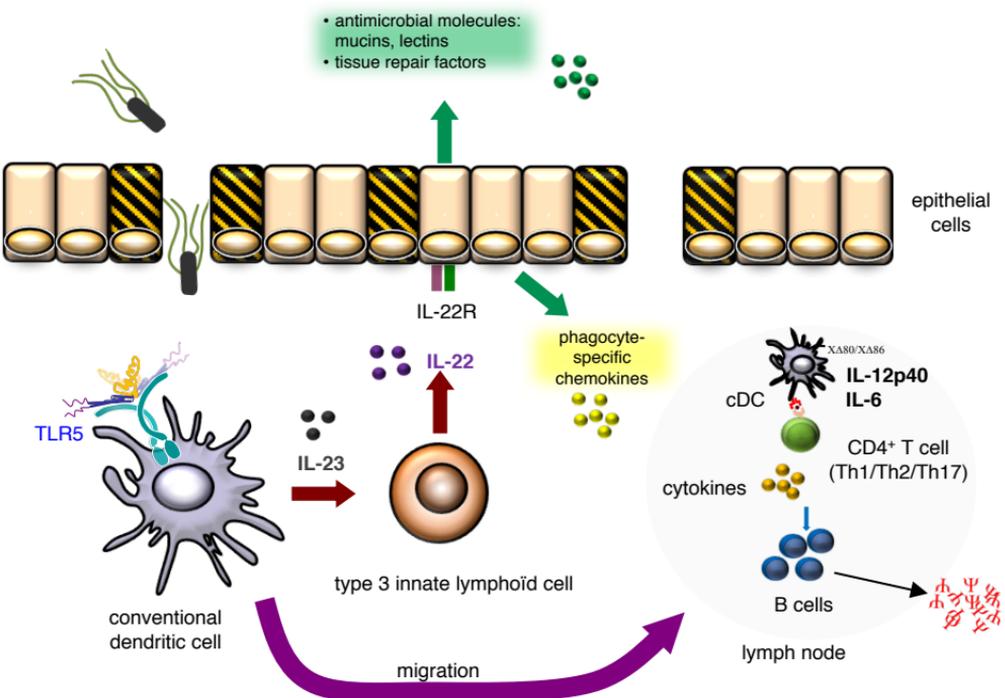


Figure 3

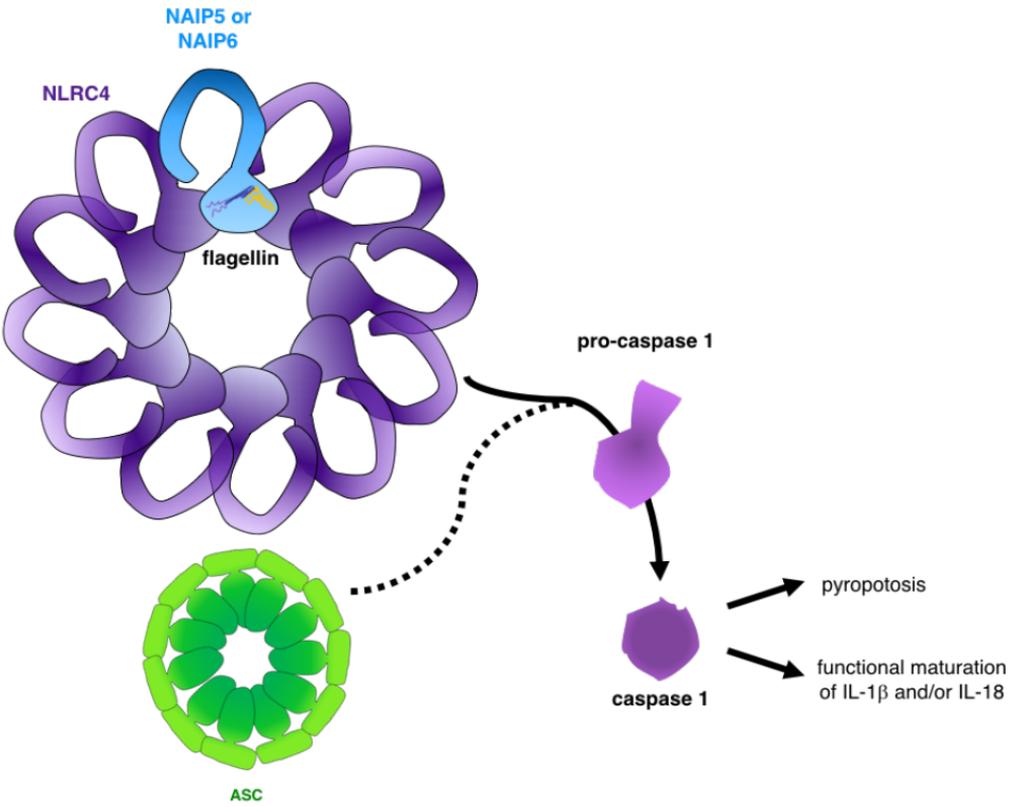


Figure I of Box 3