

REVIEW

Bacterial flagellin—a potent immunomodulatory agent

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Flagellin is a subunit protein of the flagellum, a whip-like appendage that enables bacterial motility. Traditionally, flagellin was viewed as a virulence factor that contributes to the adhesion and invasion of host cells, but now it has emerged as a potent immune activator, shaping both the innate and adaptive arms of immunity during microbial infections. In this review, we summarize our understanding of bacterial flagellin and host immune system interactions and the role flagellin as an adjuvant, anti-tumor and radioprotective agent, and we address important areas of future research interests.

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INTRODUCTION

The immune system has evolved to fight off microbial invasion through the coordinated action of the innate and adaptive arms of the immunity. Innate immune cells respond to a variety of stimuli, including bacterial, viral, parasitic or fungal infections, via members of structurally related receptors termed toll-like receptors (TLRs). TLRs are evolutionarily conserved type I transmembrane receptors that provide a critical link between innate and adaptive immunity. TLRs do not possess fine specificity, such as that of BCRs, TCRs or adaptive immune receptors, but they can individually respond to a limited but specific number of microbial pathogen-associated molecular patterns (PAMPs). The interaction of PAMPs with the TLRs on innate immune cells regulates the induction of more efficient adaptive immune responses.¹ TLRs mostly function as homologous or heterologous dimers, acquiring the shape of a horseshoe, which helps in the direct recognition of PAMPs.^{2,3} TLRs sense bacterial cell wall components, such as lipopolysaccharide (LPS) (TLR-2/4), lipoteichoic acids (TLR-2/4), CpG DNA (TLR9), flagellin (TLR5) and others (reviewed in refs 4–6). This sensing initiates an intracellular signaling cascade that culminates in the activation of a variety of proinflammatory and immune response genes.^{1,6} Proinflammatory cytokines provide augmentary signals through the upregulation of co-stimulatory and adhesion molecules, which are essential for the activation of adaptive immune cells and the subsequent development of protective immune responses against the infectious nonself antigens.^{7,8} In recent years, a number of microbial components have been used as adjuvants to augment the immune responses of poorly immunogenic

vaccines. Even though all the adjuvants studied so far have proven to be effective, flagellin, a TLR5 agonist, has been shown more promising results without any major side effects.

Flagellin is the structural component of the flagellum, a locomotory organ that is mostly associated with Gram-negative bacteria. It is characterized by highly conserved N- and C-terminal domains (D1 and D2 domains) with an intervening hypervariable region (D3).⁹ Earlier, flagellin was viewed as a virulence factor in the context of motility rather than having a role in immune stimulation. The first report of the proinflammatory role of flagellin came from the *in vitro* studies of Ciacci-Woolwine *et al.*¹⁰ and Wyant *et al.*,¹¹ and their studies demonstrated that *Salmonella* flagellin is a potent inducer of cytokines in a pro-monocytic cell line at sub-nanomolar concentrations. Later, McDermott *et al.*¹² reported that induction of the proinflammatory response in monocytes involves a high-affinity interaction between flagellin and the surface receptor present on innate immune cells. This surface receptor, which is responsible for the inflammatory and innate immune activity of flagellin, was demonstrated to be TLR5 by Hayashi *et al.*,¹³ and it was subsequently confirmed by many other researchers. Further studies showed that recombinant proteins lacking the hypervariable region did not present the compromised adjuvant activity of flagellin; thus, these results indicate that the D1 and D2 domains are essential for the recognition of TLR5 and are sufficient to induce a proinflammatory response.^{14,15} The adjuvant potential of flagellin was first reported by Arnon and co-workers,^{16–18} and it was accordingly tested for adjuvant activity in combination with a number of bacterial, viral or parasitic antigens over the past

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Table 1 Flagellin as an adjuvant

Antigen/disease agent	Model/route	Immune response	References
<i>Bacterial pathogens</i>			
<i>Salmonella dublin</i> flagellin- <i>Schistosoma mansoni</i> 9B-surface antigen	Mice/i.n.	Humoral response, significant protection against <i>in vivo</i> challenge	138
Maltose-binding protein (MBP) of <i>C. jejuni</i> -flagellin	Mice/i.n.	Systemic and mucosal Ab production, protection against heterologous challenge	139
<i>E. coli</i> heat stable enterotoxin- flagellin	Mice/oral	Ab production	73
<i>Y. pestis</i> F1 antigen+flagellin or flagellin+ <i>Y. pestis</i> F1-V fusion protein	Mice, non- human primate/i. n., i.m., intratracheally	Robust antigen-specific IgG Ab response, Th2 type immune response, protection against <i>in vivo</i> virulent <i>Y. pestis</i> challenge	45
Flagellin+tetanus toxoid	Mice/i.n.	Systemic and mucosal Ab production (IgG and IgA), protection against lethal dose of tetanus toxin	75
Flagellin-p27 antigen of <i>Mycobac-</i> <i>terium tuberculosis</i>	Mice/i.m.	Ab production, Th1 immune response	140
<i>Pseudomonas aeruginosa</i> OprF epitope 8-OprI-flagellin fusion proteins	Mice/i.m.	Humoral response, protection against challenge	141
Recombinant Pneumococcal sur- face protein A (PspA)-flagellin (FlaB–PspA)	Mice/i.n.	Systemic IgG and mucosal IgA response, protection against lethal challenge, enhances cross-protective immunity against <i>Streptococcus pneumoniae</i> infection	77
Recombinant FimH-flagellin	Mice/s.c.	Ab production, Th2-biased immune response, significant reduction in coloniza- tion of bladder by uropathogenic <i>Escherichia coli</i> (UPEC)	142
DNA encoded flagellin+Ag85B of <i>M. tuberculosis</i>	Mice/i.m.	Enhanced T-cell responses to Ag85B, significant protection against pathogenic aerosolized <i>M. tuberculosis</i> challenge, boosting through intranasal route resulted in pulmonary inflammation and transient weight loss	143
Flagellin-Lumazine synthetase (BSL)	Mice/i.p.	Efficient anti-BSL humoral immune responses	144
<i>Viral diseases</i>			
Flagellin-Influenza epitopes	Human-mice radiation chi- mera/i.n.	Ab production, protection against lethal challenge	16
Flagellin (STF2D)-EIII domain of WNV envelope protein	Mice/s.c., i.p.	IgG response, protection against lethal challenge	72
Flagellin-Influenza Virus-Like Particles	Mice/i.m.	Th1 immune response, protection against both homologous and heterologous viral challenge	131
Vaccinia virus-flagellin fusion pro- teins; L1R-flagellin and B5R- flagellin	Mice/i.m.	Humoral response and protection against challenge	145
Flagellin+whole inactivated A/PR/ 8/34 (PR8) virus	Mice/i.n.	Ab protection (IgG2a, IgG2b, IgA), protection against homologous lethal challenge	43
Flagellin-VLPs of HIV	Guinea pig/i.m., i.n.	Enhanced systemic and mucosal immune response, increased neutralized antibodies	146
Inactivated FMDV antigen +flagellin	Guinea pig/i.d.	Protected the animals against homotypic viral challenge, Th1-biased immune response	42
Flagellin-VLPs of rabies	Mice, dog/i.m.	Faster and enhanced virus-neutralizing Ab induction, increased CD4 ⁺ and CD8 ⁺ T-cell responses, production of strong IgG2a-specific response, protection against virus challenge	147
<i>Parasitic pathogens</i>			
<i>Plasmodium vivax</i> Merozoite Sur- face Protein-1 (MSP1) either admixed or fused to flagellin	Mice/s.c., i.n.	Elicited strong and long-lasting MSP-1-specific systemic Ab responses	148

Table 1 (Continued)

Antigen/disease agent	Model/route	Immune response	References
Recombinant Flagellin-circumsporozoite (CS) peptide of <i>Plasmodium yoelii</i> or flagellin +synthetic peptide	Mice/oral	Elicitation of CS peptide-specific CD8 ⁺ T cells	149
Recombinant malarial CS protein (MSP-119)-C-terminal end of FliC	Mice, rabbit/s.c.	Systemic Ab production, Th2-biased immune response, antibodies inhibit <i>in vitro</i> growth of the parasite	79
Recombinant immune mapped protein-1 (IMP1) of <i>Eimeria tenella</i> -truncated FliC	Poultry/i.m.	Stronger cellular immune responses, reduced oocyst output and cecal lesions	150
<i>Miscellaneous agents</i>			
Flagellin-EGFP fusion protein	Mice/s.c.	Antigen-specific T-cell responses	151
Flagellin-OVA fusion protein (STF2.OVA)	Mice/s.c.	IgG1 and IgG2a responses, CD8 ⁺ T-cell response	152
Chitosan-DNA nanoparticles carrying flaA gene of <i>C. jejuni</i>	White Leghorn/i.n.	Systemic and mucosal Ab production, protection against homologous challenge, significantly reduced bacterial load in intestines	76
Lyophilized plant powder containing flagellin +OVA	Mice/oral	Systemic and mucosal Ab response, mixed Th1 and Th2 response	93
DNA encoded flagellin+OVA	Mice/i.d., i.m., i.n.	Systemic and mucosal antibody production and Th1 response	153
Flagellin displaying cocaine hapten, GNE	Mice/s.c.	Dose-dependent stimulation of anti-GNE Ab production	154

Abbreviations: Ab, antibody; i.d., intradermal; i.n., intranasal; i.m., intramuscular; i.p., intraperitoneal; s.c., subcutaneous.

two decades (Table 1). The incorporation of flagellin as an adjuvant has produced safe, potent vaccines, and some of the vaccines eventually made their way into human clinical trials.^{19,20} More significantly, flagellin has also been examined for anti-tumor and radioprotective activities and has shown tremendous potential in combating tumor growth and radiation-associated tissue damage; some flagellin-based anti-tumor vaccines have successfully entered into human clinical trials (Table 2).

FLAGELLIN SENSING BY IMMUNE AND NON-IMMUNE CELLS

Flagellin activates immune and non-immune cells via the germ line-encoded pattern recognition receptor TLR5.^{15,21} TLR5 possesses three structural domains, namely, extracellular leucine-rich repeats, transmembrane region and TIR domain.³ The TLR5 recognition of flagellin involves a set of relatively conserved amino-acid residues present on both the N- and C-terminal domains of flagellin.²² Like other TLR-bound complexes, the flagellin-bound TLR5 forms a symmetric m-shaped dimer that mediates dimerization of the intracellular TIR domains, which is essential for the activation of downstream pathways. Flagellin-induced dimer formation involves amino-acid residues 12 and 13 from the LLRs of TLR5, which are critical for TLR5 activity, as mutations in these LLRs result in forms of TLR5 that fail to transcriptionally activate the NF- κ B reporter system.²² Furthermore, the TIR domain is important for downstream signaling, as a single point mutation in this domain abolishes TLR5 activity.²³

TLR5 is present on a variety of cells, including monocytes, macrophages, neutrophils, lymphocytes, NK cells, dendritic cells (DCs), epithelial cells and lymph node (LN) stromal cells.^{21,24,25} It is highly expressed in gut, especially in lamina propria DCs,²⁶ where it controls the composition of the microbiota.²⁷ The interaction of flagellin with TLR5 results in the induction of a variety of gene expression, including proinflammatory cytokines, nitric oxide (NO), H₂O₂, chemokines and host-defense proteins.^{3,27,28} Flagellin activates TLR5-expressing cells either through MyD88-dependent or -independent pathways. MyD88-dependent stimulation recruits downstream adapter molecules that activate the MAP and I κ B kinase pathways, leading to the induction of transcription factors AP-1 and NF- κ B, respectively, which subsequently activates a variety of genes that are important for host-defense (Figure 1).^{27,29} The MyD88-independent pathway involves the formation of a TLR5/TLR4 heterodimeric complex that activates cells through the TRIF-mediated pathway instead of the MyD88 adapter molecule. TRIF activation induces the production of antiviral cytokine IFN- β via the IRF3 transcription factor. Subsequently, IFN- β results in the activation of the STAT1 transcription factor, which promotes inducible NO synthase (iNOS) gene transcription and NO production (Figure 1).³⁰

Certain Gram-negative bacteria, such as *Salmonella*, translocate flagellin into the host cell cytoplasm via a type III secretory system.³¹ Type III secretory system is a very common virulence factor utilized by Gram-negative bacteria to facilitate the translocation of effector proteins, such as flagellin, into the cytoplasm of host cells to exert control over distinct host cell

Table 2 Flagellin as an anti-tumor agent

Treatment	Model/ route	Effect	References
Flagellin+D2F2 tumor cells	Mice/s.c.	Increased IFN- γ /IL-4 ratio, decreased frequency of Treg cells, significant reduction of tumor growth, CD8 ⁺ cytotoxic immune response	103
Flagellin+CpG motifs	Mice/s.c.	Promoted Th1 polarization, synergism between TLR5 and TLR9, complete remission of tumor growth	103
Flagellin treatment	Mice/i.p.	Protection against radiation, induction of radioprotective genes	27
TLR5 activation on breast cancer cells	Mice/i.v., mammary fat pads	Neutrophil infiltration <i>in vivo</i> , increased autophagy protein MAP1S in cancer cells, autophagy of cancer cells, inhibited tumor growth <i>in vivo</i>	155,156
<i>Salmonella enteritidis</i> serovar Typhimurium infection	<i>In vitro</i>	Apoptosis of adenocarcinomic human alveolar basal epithelial cells A549 <i>in vitro</i>	157
Flagella-based MUC1 vaccines	Balb/c, or human MUC1 transgenic mice	Flagellin increased higher efficiency of therapeutic activity of MUC1-based vaccines, significant reduction in size and growth rate of the tumor, lowered number of metastases, expanded life span of vaccinated mice	158
Flagellin+glucose-regulated protein 170 (Grp170)	Mice	Protection against melanoma, colon and prostate cancer, induction of potent CD8 ⁺ T-cell responses	109
Flagellin+E6/E7 peptide of papillomavirus	Mice/s.c.	Elicitation of tumor-specific IFN- γ producing CD8 ⁺ T cells, retarded <i>in vivo</i> tumor growth, prolonged survival	117
Flagellin-derived TLR5 agonist, CBLB502	Mice/i.v.	Protected mice from tumor death, increased NK cell and CD8 ⁺ cytotoxic activities, clinical trial in patients with advanced solid tumors is currently ongoing	111
Flagellin+P10 peptide of <i>Paracoccidioides brasiliensis</i>	Mice/i.n.	Activation of tumor-specific CD4 ⁺ T lymphocytes, marked reduction of lung nodules, significant increase in survival, protection against metastatic melanoma growth after adoptive transfer	159

Abbreviations: i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous.

signaling pathways.³¹ The delivery of flagellin in the host cell cytoplasm is first recognized by the NAIP (NLR family, apoptosis inhibitory protein) family proteins NAIP5 and NAIP6.³² Subsequently, the flagellin-bound NAIP5/6 complexes interact with NOD-like receptor NLRC4, resulting in the activation of caspase-1, which cleaves pro-interleukin-1 β into active IL-1 β , a potent proinflammatory cytokine that is crucial for host–defense responses to infection and injury.^{33,34} While the conserved N- and C-terminal domains of flagellin are required for TLR-binding activity, the recognition of flagellin by NLRC4 seems to involve the 35 C-terminal amino acids of flagellin, as mutations of specific leucine residues in the C-terminal domain abrogate NAIP5/NLRC4-mediated inflammasome formation.^{35,36}

Flagellin is also recognized by plant cells via flagellin sensing 2 (FLS2), which is a leucine-rich repeats receptor kinase and a homolog of TLR5.³⁷ FLS2 interacts only with the linear motif in the N-terminal of flagellin, whereas TLR5 interacts with

both the N- and C-terminal domains. The recognition of flagellin by FLS2 results in the rapid phosphorylation of MAP kinase pathway, which subsequently culminates in the production of host–defense proteins that mediate protection against fungal and bacterial infections^{37,38} (Figure 2). Bacterial flagellin is also recognized by the FLS3 receptor in certain solanaceous plants, including tomato, potato and pepper.³⁹ FLS3 recognizes flgII-28, a region of bacterial flagellin that is distinct from the region perceived by the FLS2 receptor, and enhances immune responses that protect leaf tissues against bacterial colonization.³⁹ Certain plant pathogens have evolved to evade FLS2-mediated immunity; thus, transfer of the FLS3 receptor to other crop plants might offer the potential to mediate protection against pathogens that have evolved to evade FLS2-mediated immunity.³⁹

In addition to TLR5, flagellin is also recognized by TLR11, a recently discovered PRR present in mice.⁴⁰ The interaction of flagellin with TLR11 is more restricted compared to its

interaction with TLR5. A study by Hatai *et al.*⁴⁰ shows that TLR5 strongly interacts with flagellin at both acidic and neutral pH conditions, whereas acidic conditions are essential for TLR11–flagellin interactions. Furthermore, this study shows that both the N- and C-terminal domains of flagellin can independently interact with TLR11, whereas the TLR5–flagellin interaction requires both domains to mediate a proinflammatory cytokine response and thus adjuvant activity.¹⁴ TLR11 is highly expressed in epithelial cells in various organs, such as intestine, lung and skin, and it has a role in the prevention of *Salmonella* invasion in mice.⁴¹ This result is in contrast to TLR5-deficient mice, which show enhanced resistance against oral *Salmonella* infection.²⁶ These findings clearly suggest that flagellin–TLR11 signaling mechanisms differ from flagellin–TLR5 interactions, and therefore, active research is needed in this regard.

HOW DOES FLAGELLIN CONTRIBUTE TO THE ADJUVANTICITY OF VACCINES?

Flagellin has shown tremendous potency as an adjuvant, either in the context of a fusion protein or by co-administration with antigens.^{19,20,42,43} The presence of TLR5 on a number of innate and adaptive immune cells might form the basis of its adjuvant activity (Figure 3). Most of the adjuvants, including TLR agonists, mediate their activity, in part, by activating the innate immune system, including DC activation, maturation and recruitment to T-cell areas in the LNs.⁴⁴ Flagellin is a potent activator of both the innate and adaptive immune system. Studies have demonstrated that the dose of flagellin required to promote a maximal antigen-specific antibody response is relatively less than the dose required to stimulate maximal innate immune responses,^{29,45} suggesting that the induction of an antibody response is not linearly dependent on the strength of an innate immune response. This result is most likely due to the ability of flagellin to stimulate the induction of proinflammatory cytokines and chemokines in a number of innate and non-immune cells, including DCs, NK cells, epithelial cells and LN stromal cells,^{24,25,46,47} which is critical for the activation and development of antigen-specific adaptive immune responses. The administration of flagellin or flagellin-based vaccines has also been shown to rapidly achieve a higher concentration in draining LNs, which therefore activates DCs and non-immune cells, such as epithelial and LN stromal cells.^{47,48} The activation of these cell types by flagellin or flagellin-based vaccines leads to the induction of cytokines and chemokines, which promote a marked recruitment in T and B lymphocytes to draining LNs,^{29,49} and thus maximizes the chances of antigen-specific lymphocytes encountering their cognate antigens. These mechanisms reasonably contribute to the overall potency of flagellin-based vaccines.

The interaction of flagellin with DCs is either direct or indirect, depending on the type of DC involved; for instance, myeloid-derived DCs respond directly to flagellin through TLR5, while splenic DCs respond indirectly to flagellin and are most likely activated through a bystander process that requires the stimulation of other TLR5-expressing cells.^{50–52}

There are also indications that the effect of flagellin is dependent on the immunological site involved as, unlike splenic DCs, DCs in lamina propria respond directly to flagellin.⁵³ There are many discrepancies in the literature regarding the interaction of flagellin and DCs in different species. Some investigators showed the stimulatory effect of flagellin on murine bone marrow-derived DCs,^{24,25,54,55} while others reported an effect on human myeloid-derived DCs, but not murine DCs.⁵⁶ This variation in responses might be due to a number of factors (reviewed in ref. 47). The quality of flagellin also influences the outcome of experimental results. Recombinant flagellin, if used as an adjuvant, should be free of any endotoxins or nucleic acids as they activate the DCs in a TLR5-independent manner. In addition, long-term storage of some fusion proteins causes molecular aggregation, which activates cells in a TLR5-independent manner, as has been reported with the polymeric flagellin that directly stimulates B cells.⁵⁷ The ability of cells to respond to flagellin also depends on the state of differentiation. The pretreatment of innate immune cells with GM-CSF and IL-4 makes them more responsive to flagellin as compared to untreated cells.^{10,47} Flagellin of different bacterial species also varies in immunogenicity,⁴³ and not all bacterial flagellins activate TLR5.⁵⁸ In contrast to the flagellins of γ -proteobacteria, flagellins from the ϵ -proteobacteria *Campylobacter jejuni* and *Helicobacter pylori* do not activate TLR5 signaling and thus can evade TLR5-mediated immune surveillance at mucosal surfaces.⁵⁸ This difference, in part, might be attributed to the structural differences among the flagella of different microbes; for instance, flagellin of *C. jejuni* consists of 7 protofilaments instead of the 11 found in the γ -proteobacteria *Salmonella*, which contains highly conserved residues between the protofilaments that are involved in the TLR5 interaction.^{59,60} The functional differences between TLR5-activating flagellins and non-activators might also be attributed to sequence variations among the flagellins of bacteria species. For instance, the hot spot residues (residues 89 and 114) on the flagellins of γ -proteobacteria that make complementary contacts with TLR5 are replaced with threonine and aspartate residues, respectively, in *C. jejuni* and *H. pylori* flagellins. Thus, *C. jejuni* and *H. pylori* flagellins might fail to make complementary contacts with TLR5 and subsequently fail at TLR5 activation.⁶¹

Another factor that contributes to the adjuvant activity of flagellin is the presence of TLR5 on the cells of the adaptive immune system, T and B cells. Flagellin directly influences the phenotype and functions of these cells, and thereby directly regulates the adaptive immune system. Most of the studies in humans have shown that flagellin directly activates T cells and is equivalent to anti-CD28 in stimulating the proliferation of T cells.^{62,63} A study by Means *et al.*⁵⁶ shows that flagellin-treated DCs have a slightly lower stimulatory T-cell effect than LPS-treated DCs, and flagellin stimulation induces the expression of chemokines GRO- α , GRO- β , GRO- γ , IL-8, MCP-1, MIP-1 α , MIP-1 β and RANTES in DCs. The induction of these chemokines is very rapid and occurs even before the expression of homing chemokine receptors, thereby recruiting

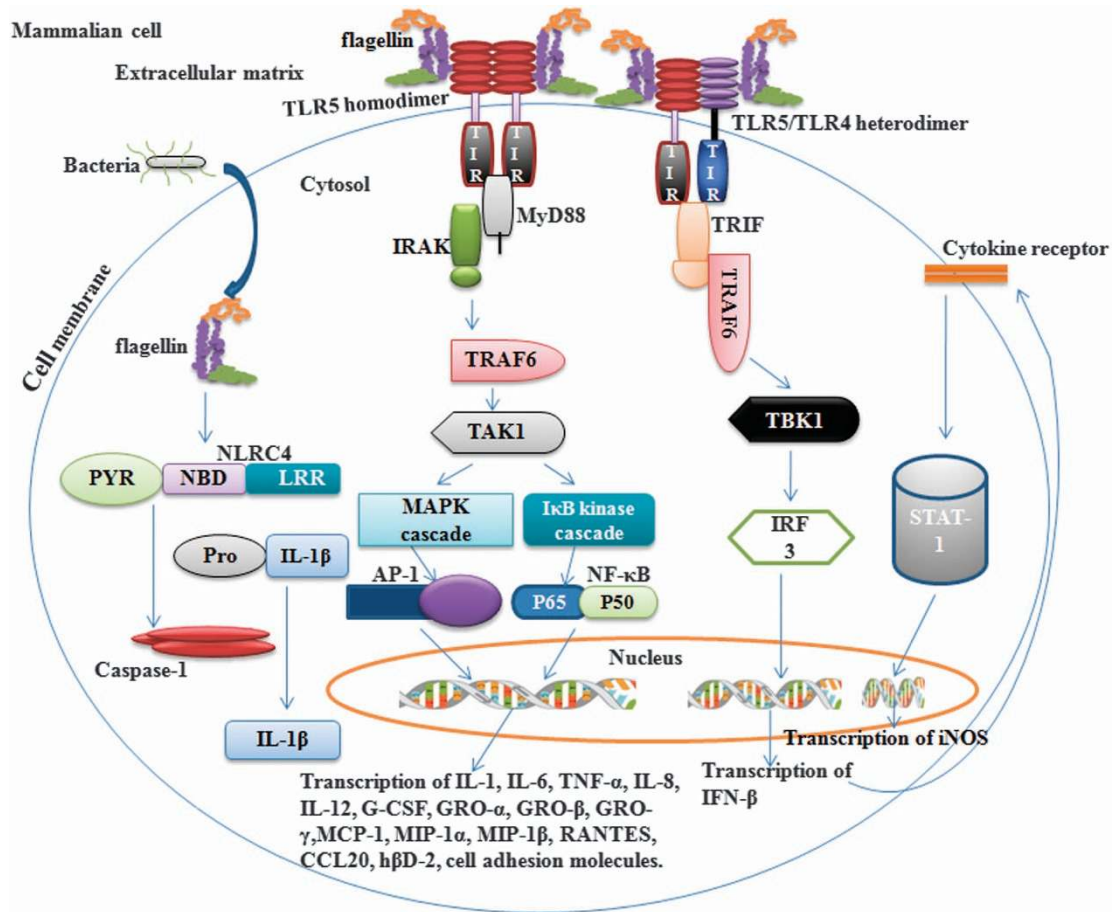


Figure 1 Signal transduction by flagellin in mammalian cells. Immune cells respond to extracellular monomeric flagellin through either TLR5 homodimer or heterodimer complexes, resulting in the transcription of a variety of genes that are important for the proper stimulation of immune cells. Signaling through TLR5 occurs through a MyD88-dependent adapter molecule that passes the signal to the MAPK and IκB cascades via IRAK, TRAF6 and TAK1. The MAPK and IκB cascades result in the induction of transcription factors AP-1 and NF-κB, respectively. These two transcription factors induce a variety of genes involved in innate and adaptive immunity. Signaling through TLR5/TLR4 is MyD88-independent and occurs via the IRF3 pathway, which results in the production of IFN-β. Subsequently, IFN-β induces iNOS gene transcription through the activation of STAT1, which culminates in the production of NO. However, when intracellular flagellin is introduced into the host cell cytoplasm via the type III secretory system by some bacteria, it is detected by NLRC4, which culminates in the secretion of IL-1 β via active caspase-1. AP-1, Activator protein-1; GRO, growth-related oncogene; hBD, human beta defensins; IRF3, interferon response factor 3; IRAK, IL-1 receptor associated kinase; LRR, leucine-rich repeats; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MyD88, myeloid differentiation factor 88; NBD, nucleotide-binding domain; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B cells; PYR, pyrin domain; TAK1, transforming growth factor-beta-activated kinase 1; TBK1, TANK-binding-kinase-1; TLR, Toll-like receptor; TRAF6, TNF receptor associated factor 6; TRIF, TIR domain containing adapter inducing interferon-β; STAT1, signal transducer and activator of transcription 1.

lymphocytes to the secondary lymphoid sites. This outcome indicates that flagellin not only stimulates adaptive immune responses, but also recruits innate immune cells to the sites of infection or immunization. Thus, flagellin activates T cells in two ways: one is direct activation of T cells and the other is via presentation of the cognate antigen by antigen-presenting cells.

Flagellin also activates T regulatory cells (Tregs), which are potent suppressors of T-cell responses and possess higher levels of TLR5 than CD4⁺ CD25⁻ T cells.⁶³ However, flagellin may simultaneously inhibit TCR-mediated Treg activation via a suppressor of the cytokine signaling 1-dependent mechanism,⁶⁴ albeit these studies are currently lacking in the literature. Thus, the contribution of flagellin-mediated Treg activation and

suppressor of the cytokine signaling 1-dependent regulation in the overall adjuvant potential of flagellin-based vaccines warrants further studies.

B cells are directly activated by flagellin through either cross linking of BCRs or signaling through TLR5, depending on the form of flagellin that is involved. B cells require TLR5 signaling, in addition to T-cell help, for T-dependent antibody production⁶⁵ or production can occur in presence of cytokines, such as TNF-α.⁶⁶ Monomeric flagellin activates TLR5 signaling in B cells and promotes a long-lasting, T-dependent antibody response. In contrast, polymeric flagellin directly stimulates B cells by cross linking BCRs⁵⁷ and generates antibody responses of the IgM type without the help of T cells.

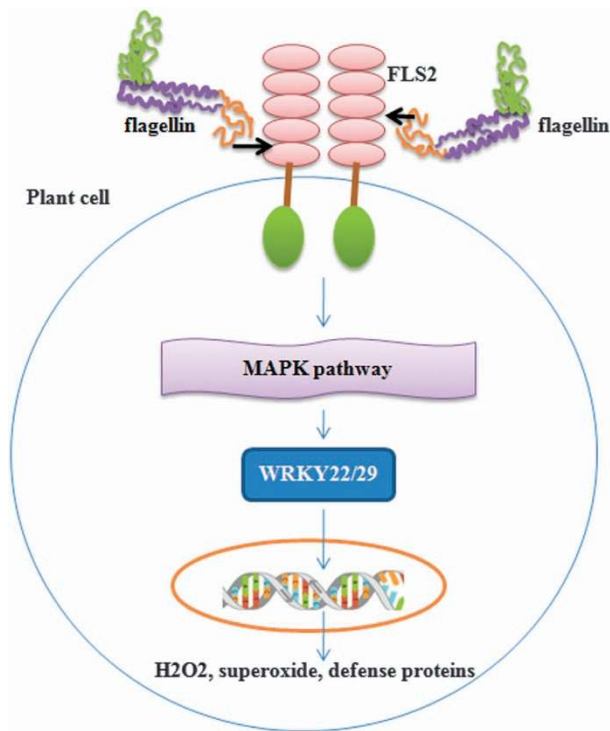


Figure 2 Signal transduction by flagellin in plant cells. Plant cells also possess receptors similar to TLR5 called flagellin sensing receptors (FLS2). FLS2 interact with the linear motif of flagellin in the N terminus (shown by arrow), which results in the rapid induction of a phosphorylation cascade of MAP kinases. MAPKs, in turn, activate the transcription factor WRKY22/29, which induces the transcription of genes providing resistance against fungal and bacterial infections. Due to differences in the homology of LLRs in TLR5 and FLS2, the flagellin-interacting motifs are different in these species.

Furthermore, flagellin also promotes class switching in B cells, thus generating more potent and diverse antibody responses.⁶⁷ In addition to TLR5, the recognition of flagellin by inflammasomes (NLRC4) also plays an important role in the generation of antibody responses, and either TLR5 or inflammasome (NLRC4) is necessary and sufficient for the production of humoral immunity.⁶⁸ However, López-Yglesias *et al.*⁶⁹ reported that anti-flagellin IgG1 antibody responses develop through TLR5- and inflammasome-independent pathways, suggesting that other receptors or distinctive pathways are involved in the generation of humoral responses. The elucidation of other pathways of recognition will clearly contribute to a better understanding of flagellin–host interactions and will be critical for the rational design of flagellin-based vaccines.

FLAGELLIN AS A MUCOSAL ADJUVANT

The administration of vaccines through the mucosal route is an attractive idea, albeit the adjuvants that elicit robust immune responses at mucosal surfaces are lacking. The presence of TLR5 on epithelial cells, which are often the first major cell types to encounter infectious and non-infectious agents, might form the basis for prospects of flagellin as a mucosal

adjuvant.^{29,51} The elicitation of immune responses at mucosal surfaces through the use of flagellin has the potential to eradicate or at least prevent the bad outcome of diseases. During the past two decades, flagellin has been extensively tested as a mucosal adjuvant against epitope-based influenza vaccines,^{19,20,70,71} West Nile virus (WNV),⁷² *Escherichia coli*,⁷³ *Yersinia pestis*,^{45,74} *Clostridium tetani*,⁷⁵ *C. jejuni*,⁷⁶ *Streptococcus*⁷⁷ and *Plasmodium falciparum*.^{78,79}

The induction of cytokines and chemokines is key to the adjuvant potential of flagellin.^{21,29} Flagellin is a more potent activator of epithelial cells than LPS and polarizes the response toward IL-8 secretion at epithelial surfaces.^{80–82} Eaves-Pyles *et al.* showed that flagellin causes rapid degradation of I κ B α , the inhibitor of NF- κ B, in epithelial cells, and induces a variety of proinflammatory mediators, including NO, IL-6, IL-8, CXCL2 and cytoprotective genes.^{80,83,84} These mediators play an important role in the activation of innate and adaptive immune responses at mucosal surfaces,^{85,86} thus mediating protection against the intruding pathogens. NO enhances IgA class switching and production by upregulating TGF β RII expression on B cells and by inducing the expression of BAFF and APRIL in DCs.^{86,87} Moreover, NO and CXCL2 play an important role in the activation and recruitment of professional antigen-presenting cells at a vaccinal or tissue injury site,⁸⁵ where they pick up and transport the foreign antigens to the draining LNs for presentation of the processed antigens to the CD4⁺ T cells, which is essential for the development of effective antigen-specific immune responses.⁸⁸ Furthermore, these mediators recruit neutrophils and professional killer cells at mucosal surfaces,⁸⁵ thus providing nonspecific protection against the intruders.

The cytokine IL-6 helps in the development of effective and potent mucosal immune responses and protects the host against bacterial and viral infections.⁸⁹ Flagellin is the major determinant in Crohn's disease, a chronic intestinal disorder caused by inappropriate immune responses to commensal intestinal microflora generated by the interaction of TLR5 present on the epithelial cells and flagella in the intestinal milieu.⁹⁰ This implies that flagellin is a potent inducer of proinflammatory cytokines at mucosal surfaces and that it is sufficient to overcome oral tolerance, which is a major pitfall for the mucosal route delivery of vaccines.⁹¹ Flagellin-adjuvanted vaccines administered through a mucosal route have been shown to elicit a strong antibody and cell-mediated immune responses, both at mucosal surfaces and at systemic level.^{77,92}

Plant-expressed flagellin has proven potent adjuvant activity for orally administered antigens.⁹³ A study by Girad *et al.*⁹³ showed that orally administered, lyophilized plant powder containing plant-expressed flagellin and ovalbumin induced ovalbumin-specific humoral and cell-mediated immune responses and thus prevented the occurrence of oral tolerance. All these findings clearly indicate that flagellin has a substantial potential to act as a mucosal adjuvant. In addition to its role in mucosal adjuvanticity, TLR5 also plays a role in controlling bacterial infections at mucosal surfaces. Hawn *et al.*⁹⁴ reported

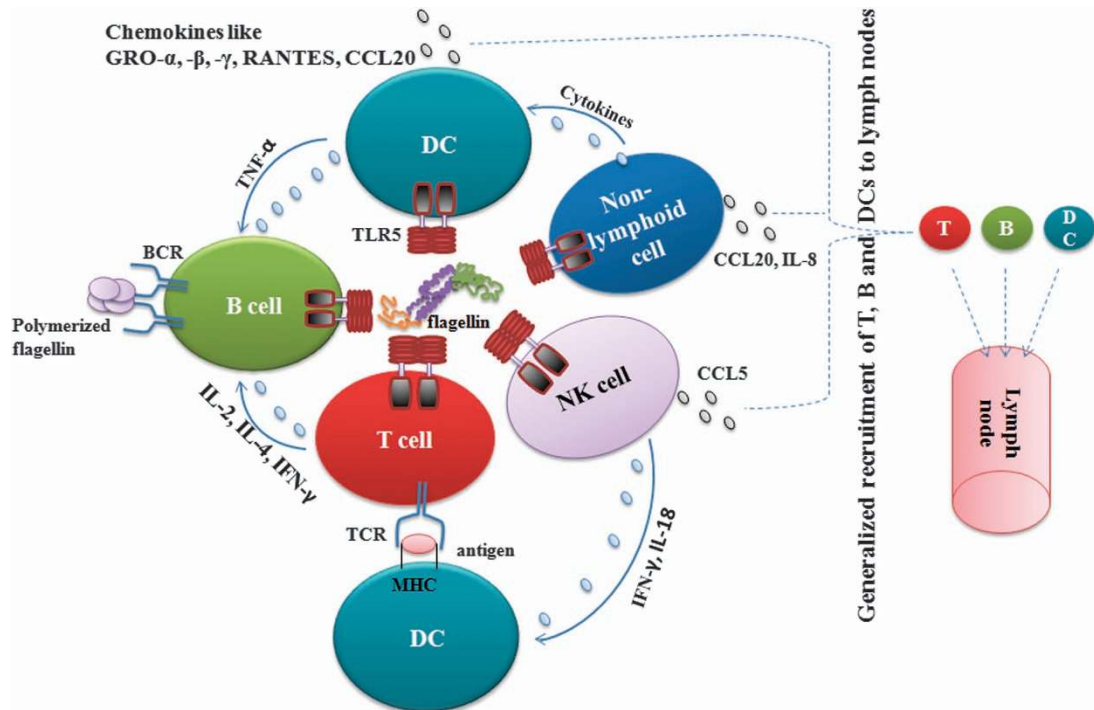


Figure 3 Flagellin interactions with different kinds of immune and non-immune cells. Flagellin directly activates a number of immune and non-immune cells, including T, B, DCs, NK and non-lymphoid cells (macrophages, epithelial, fibroblasts, stromal cells and neutrophils), through TLR5. The cumulative effect of this activation is the augmentation of immune responses through the generation of more potent antibodies and a Th1 response. Moreover, the interaction of flagellin with TLR5 culminates in the production of chemokines from a number of lymphoid and non-lymphoid cells, which results in the generalized recruitment of T and B cells to LNs, which maximizes the chances of an encounter with their cognate antigen and subsequent elicitation of potent immune responses. Instead of TLR5, polymerized flagellin directly stimulates B cells by cross linking BCRs, which might generate antibody responses of the IgM type.

that a common stop codon polymorphism in TLR5 in lung epithelial cells abolishes flagellin activity and increases the incidence of human lung infections by flagellated bacteria. This case signifies that TLR5 have an innate immune regulatory role in lung epithelial cells as well.

FLAGELLIN AS AN ANTI-TUMOR AGENT

TLR agonists have also been tested for anti-tumor activity in animal models and some of them have eventually made their way into human clinical trials (reviewed in refs 95–97). The immunobiology of TLRs in tumor progression or regression is very intricate and complex. Some studies show that activation of TLR4 or TLR9 on cancer cells promotes tumor progression and growth,^{98–100} whereas others show that TLR3 and TLR5 activation exhibits inhibitory effects.^{101,102} Among TLR agonists, the effect of flagellin is unique, as it does not involve the innate arm of immunity in combating tumor growth but rather is entirely dependent on the activation of adaptive immunity.¹⁰³ Sfondrini *et al.*¹⁰³ reported that administration of purified flagellin at the time of tumor transplantation enhanced tumor growth, whereas the same flagellin resulted in the regression of a palpable tumor. This finding can be explained by way of the immune bias induced by flagellin in response to weak or highly immunogenic tumors. The interaction of flagellin with highly immunogenic tumors induces a Th1 response and suppression of Tregs, resulting in the

inhibition of tumor growth. However, weakly immunogenic tumors polarize the response toward Th2, which prevents the apoptosis of Tregs and increases their suppressive effect on CD4⁺ Th1 cells,^{103,104} favoring tumor growth. This result also suggests that preactivated adaptive immunity is critical to the anti-tumor activity of flagellin. The combination of flagellin with CpG-ODN has been shown to abrogate the negative effect of flagellin on tumor growth and has produced a synergistic response in tumor inhibition.¹⁰³ This might be due to induction of IL-12 and IL-23 expression in DCs and consequent Th1 bias, as has been reported with the combination of flagellin and other TLR agonists.¹⁰⁵ Recently, *Salmonella typhimurium* has been used to express and secrete flagellin of *Vibrio vulnificus* as an anti-cancer agent against a variety of cancers in mouse models.¹⁰⁶ This study shows that flagellin-secreting *Salmonella* targeted to the tumor microenvironment exerted potent tumoricidal activity through two-step activation of the TLR4 and TLR5 signaling pathways. The tumor suppressive effects of flagellin-secreting *Salmonella* were far more potent than those mediated by *Salmonella* harboring an empty vector,¹⁰⁶ indicating synergistic effects of the TLR4 and TLR5 signaling pathways in combating tumor growth. In addition to the crosstalk between TLRs, Nod-like receptors (NLRs) inhibit tumor progression and act in concert with TLR5 to induce potent anti-tumor activity. A study by Garaude and Blander³⁶ shows that engineered flagellin-bearing tumor

cells elicit robust anti-tumor T-cell responses and tumor rejection in mice. This anti-tumor activity, mediated by flagellin, is dependent on the activation of both TLR5 and NAIP5, as abrogation of either NLR or TLR5 signaling restored the ability of flagellin-bearing tumor cells to form tumors *in vivo* and impaired immune responses, respectively. This explains why some TLR ligands tested as single adjuvants in phase II and III clinical trials failed at providing significant tumor regression.¹⁰⁷ The crosstalk of TLR5 and NOD1 is supported by another study, which shows that combined stimulation of both the receptors strongly potentiates NF- κ B activity, resulting in the elicitation of potent innate immune responses and protection against *Salmonella* infection.¹⁰⁸ These findings clearly indicate the great potential for dual targeting of TLRs and NLRs in the design of optimal cancer vaccines.

Cancer immunotherapy aims to mount effective anti-tumor T-cell responses to control tumor growth and metastasis. Although this approach holds great promise for cancer treatment, its therapeutic application in chemotherapy has been limited so far. Despite the innate ability of the immune system to recognize tumor cells, several potential mechanisms are exploited by cancer cells to create an immunosuppressive environment that enables them to escape immune destruction. Thus, novel strategies should be employed to overcome immunosuppressive mechanisms and the induction of effective anti-tumor immunity. A study by Yu *et al.*¹⁰⁹ shows that adenovirus expressing a fusion protein of flagellin and Grp170, the largest endoplasmic reticulum chaperone, induces a potent anti-tumor response against B16 melanoma and its distant lung metastasis compared to the co-administration of Grp170 and flagellin. The therapeutic potential of this treatment was also confirmed in mouse prostate cancer and colon carcinoma.¹⁰⁹ The flagellin-Grp170 fusion resulted in the superior presentation of tumor antigens by DCs to CD8⁺ T cells, which subsequently elicited potent cytotoxic CD8⁺ T-cell responses, both locally (that is, tumor site) and systemically.¹⁰⁹ The tumor microenvironment can also be ameliorated to elicit anti-tumor responses through TLR5 ligand-secreting T cells, as reported by Kaczanowska and Davila.¹¹⁰ These studies suggest that targeting the tumor microenvironment with such novel strategies has great potential to overcome the immunosuppressive mechanisms of tumors and to induce effective anti-tumor immunity. Flagellin exclusively acts as a potent T-cell adjuvant to mediate anti-tumor immunity. Leigh *et al.*¹¹¹ shows that a *Salmonella* flagellin-derived, pharmacologically optimized TLR5 agonist, CBLB502, stimulates robust anti-tumor activity through the direct activation of TLR5-expressing accessory immune cells, which subsequently stimulate cytotoxic CD8⁺ T cells. The anti-tumor potential of CBLB502 is further shown in another report. This report demonstrates that the strongest NF- κ B activation in response to CBLB502 occurs in the liver and GI tract, where liver hepatocytes and lamina propria DCs, respectively, are responsible for the primary CBLB502 response.¹¹² CBLB205 agonist suppresses the liver metastases of different kinds of cancer regardless of their TLR5 status, indicating the activation of TLR5-bearing cells present in the

tumor microenvironment, which in turn execute anti-tumor activity.¹¹² To elucidate the exact role of innate immune cells, especially NK and DCs, in mediating the anti-tumor potential of flagellin, Brackett *et al.*¹¹³ reported that the TLR5 agonist entolimod (formerly named CBLB502) mediates anti-tumor activity through the NK-dendritic-CD8⁺ T-cell axis. Entolimod activates NF- κ B-, AP-1- and STAT3-driven immunomodulatory signaling pathways specifically within the liver and rapidly induces chemokines, such as CXCL9 and -10, which recruit blood-borne CXCR3-expressing NK cells to the liver.¹¹³ NK cell-driven activation of DCs results in the elicitation of potent CD8⁺ T-cell responses, which exert both the anti-metastatic effect of entolimod and the induction of tumor-specific memory responses.¹¹³ These findings clearly indicate the potential of TLR5 agonists as potent organ-specific immunoadjuvants, enabling efficient anti-cancer immunization that does not rely on the recognition of tumor-specific antigens. In contrast to the TLR5-negative environment outside of the liver, the tumor must bear the TLR5 receptor to mediate the anti-tumor activity mediated by flagellin.

The expression of TLR5 has been used as a novel predictive marker for the recurrence and survival of various types of cancers.¹¹⁴ The expression of TLR5 has been shown to increase in various types of cancers, such as in gastric and colorectal carcinogenesis. Rhee *et al.*¹¹⁵ reported that gastric carcinoma expresses high levels of TLR5 and that treatment with flagellin elicits a potent anti-tumor activity. Flagellin induces the activation and proliferation of DCs and fibroblasts through the degradation of apoptosis-inducing protein¹¹⁶ p27, while the activation of TLR5 on carcinoma cells inhibits proliferation and anchorage-independent growth. The activation of separate signaling pathways by flagellin in different cell types clearly warrants further research. Nguyen *et al.*¹¹⁷ demonstrate that flagellin acts a potent adjuvant for E6/E7 proteins of human papillomavirus and induces a strong CD8⁺ T-cell response, which suppresses tumor growth and prolongs survival. This flagellin-based anti-tumor therapeutic vaccine resulted in the complete remission of lung metastasis through the activation of CD8⁺ T cells, involving the MyD88-dependent pathway. Thus, flagellin exclusively acts as an adjuvant for T cells and has the potential to be used for anti-cancer immunotherapy, even for TLR5 non-expressing tumor cells.

FLAGELLIN AS A RADIOPROTECTIVE AGENT

In addition to being a potent immunomodulatory and anti-cancer agent, flagellin also possesses a radioprotective property that protects the host against ionizing radiation. Radiation-induced tissue toxicity remains a major challenge in the delivery of tumoricidal doses of intestinal irradiation. Several significant studies have demonstrated the radioprotective potential of flagellin. Vijay-Kumar *et al.*²⁷ reported that systemic administration of flagellin protects mice against irradiation in a TLR5-MyD88 manner. This protection is mediated by the cells of the innate immune system, which release cytoprotective cytokines, such as G-CSF, a known radioprotectant.¹¹⁸ The exploitation of flagellin as a

radioprotective agent seems to be dependent on the time of the administration of flagellin and the dose of radiation. The administration of flagellin is beneficial up to 4 h following irradiation, whereas there is no beneficial effect following 24 h post irradiation.²⁷ This indicates that the early activation of innate immune cells plays an important role in combating the adverse toxicity of radiations. Other studies have also reported the radioprotective potential of flagellin. Burdelya *et al.*¹¹⁹ reported the radioprotective activity of a TLR5 agonist, CBLB502, and demonstrated that a single injection of this drug before lethal total-body irradiation protected mice and rhesus monkeys from both gastrointestinal and hematopoietic acute radiation syndromes and prolonged survival. Improved survival is also observed after irradiation, but at lower radiation doses. This radioprotective property is dependent on the activation of NF- κ B signaling pathway, which subsequently culminates in the production of anti-apoptotic proteins, scavengers of ROS and growth factors. These factors play an important role in CBLB205's protection against gastrointestinal and hematopoietic acute radiation syndromes.^{112,119} CBLB502 also induces additional prosurvival signals via the STAT3 and AP-1 signaling pathways, which are responsible for liver resistance to Fas-mediated apoptosis.¹¹² Furthermore, CBLB502-treated mice show higher concentrations of G-CSF and IL-6 in blood, which are known stimulators of hematopoietic stem cells (G-CSF) and the thrombopoietic lineage of hematopoiesis (IL-6).¹¹² The radioprotective potential of flagellin is also attributed to its ability to modify the recruitment and phenotype of macrophages. A study by Lacavé-Lapalun *et al.*¹²⁰ shows that the administration of flagellin 3 days after irradiation is associated with the transition of macrophages from a primarily proinflammatory M1 to a more anti-inflammatory M2 phenotype. This transition of phenotype resulted in a notable decrease of the acute damaging effects of radiation by reducing the inflammatory response and enhancing epithelial repair. All these findings clearly suggest that flagellin and a flagellin-derived agonist could potentially improve/expand the therapeutic window of tumoricidal doses of irradiations and thus serve as a biological protectant in radiation emergencies.

BENEFITS OF FLAGELLIN-BASED VACCINES

Flagellin-based vaccines have the potential to provide protection against various insults, including radiation, chemicals and pathogens. Previously, LPS has been used as a protective agent against such challenges and as vaccine adjuvant, but only to a limited extent, as it induces sepsis and pulmonary lung inflammation, even at low doses.^{121,122} Furthermore, LPS is a poor activator of epithelial cells,¹²³ which are often the first cells to encounter pathogens and chemicals, and hence it may not provide optimum protection. The expression of TLRs varies in various organs and thus its protection varies against various kinds of challenges. A study by Burdelya *et al.*¹¹² demonstrates that treatment with a TLR5 agonist, CBLB205, but not with LPS, improves survival of mice infected with a lethal dose of *S. typhimurium*. This study further reports that in

response to CBLB502 treatment, liver hepatocytes show the highest NF- κ B expression, while LPS-activated NF- κ B expression specifically occurs in the lungs, spleen and kidney; thus, these results make biological sense, as the liver is the primary site of *Salmonella* residence during infections. This indicates that the tissue specificity of TLR5 and TLR4 expression plays an important role in providing protection against different kinds of challenges and that LPS and flagellin may act as organ-specific immunomodulators. The use of flagellin over LPS is advantageous. Flagellin and a flagellin-based TLR5 agonist are significantly less toxic than agonists of many other TLRs.^{27,112} Flagellin induces the non-pathologic profile of cytokines and mediates less of the adverse effects associated with LPS.^{112,124} Flagellin is a potent activator of epithelial cells and a generally poor activator of hemopoietic cells, such as macrophages and DCs, which mediate sepsis.²¹ Flagellin-induced TLR5 signaling in epithelial cells results in the upregulation of cytokines, cytoprotective genes and other host-defense genes, which provides nonspecific protection against a variety of challenges, including orally ingested chemicals and pathogens, such as *Salmonella* and rotavirus infections.²⁷ There is also a report that flagellin treatment curtails the symptoms of allergy that are usually associated with an allergen.¹²⁵ Flagellin also mediates protection against ionizing radiations, which cause damage to various organs, particularly rapidly dividing mucous membranes. A 2-h prior exposure of flagellin has been found to provide protection against radiation and thus can be used prophylactically at the time of nuclear disaster.²⁷ Flagellin has also been shown to induce antibacterial gene expression and heat shock proteins, which equips epithelial cells to better address pathogens and radiation.¹²⁶ A flagellin-based TLR5 agonist, entolimod (CBLB502), has been used to reduce the systemic toxicity of 5-fluorouracil, an anti-tumor chemotherapeutic drug, and has improved the integrity of intestinal tissue and has stimulated hematopoiesis following treatment.¹²⁷ This indicates that flagellin or a flagellin-based agonist has the clinical potential to broaden the therapeutic window of genotoxic anti-tumor drugs. Therefore, approaches should include flagellin in therapies or prophylaxis to provide broad-spectrum protection against various kinds of challenges.

One of the major concerns with use of protein-based adjuvants, such as flagellin, is the induction of antibody responses that might neutralize their adjuvant potential and diminish or suppress the immune response against the antigen on subsequent immunizations. Prior immunity to flagellin does not hamper antibody production against the antigen.¹⁷ Furthermore, Eaves-Pyles *et al.*¹⁴ reported that conserved regions of flagellin are sufficient to induce a proinflammatory response in immune cells. Thus, the anti-flagellin immune response could be minimized by using the conserved regions only. Moreover, the plasticity of flagellin allows the creation of fusion proteins without compromising the conformational epitopes.²⁰ The foreign antigens can be physically linked to the N- or C-terminal or the hypervariable region can be replaced. Song *et al.*¹²⁸ fused the HA globular head of H1N1 influenza virus to the C-terminal of flagellin, which protected

mice against lethal challenge and was immunogenic and well tolerated in humans. In a subsequent study, they replaced the hypervariable region of flagellin with the HA globular head of H5N1 and found that it was more efficacious and immunogenic than the previous design.¹²⁹ These influenza vaccines have elicited robust, long-lasting (>8 month) neutralizing antibody responses, have protected mice against lethal challenges and are currently under clinical trials.^{129,130} Immunogenic antigens of other pathogens have also been fused to flagellin; for instance, fusion of the C-terminal end of merozoite surface protein-1 (MSP-119) with the C-terminal end of flagellin developed strong, specific and long-lasting antibody-mediated responses.⁷⁹ The booster immunization showed that the immune response was similar to emulsified complete or incomplete Freund's adjuvanted vaccine.⁷⁹

Flagellin has shown adjuvant potential both as a fusion protein or in its co-administration with an antigen without any physical linking.^{19,42,43} However, there is a minor discrepancy in the literature regarding the use of flagellin in the context of a fusion partner or in its co-administration with an antigen. Some studies have shown that the co-administration of flagellin with an antigen does not augment the immune response and therefore, physical linking of flagellin with an antigen is required for its adjuvant activity.¹³¹ We and others have shown that there is no requirement for the physical linking of flagellin to the antigen and that co-administration generates potent immune responses.^{42,43,132} All these studies show that flagellin augments immune response manifolds and protects animals against a lethal challenge. Linking the antigen to flagellin might be a superior strategy due to the better activation of DC through TLR5 signaling, which provides a means for efficient antigen internalization and access to the antigen processing pathway. In addition, flagellin has the potential to act as an adjuvant of its own against one or more epitopes present in the hypervariable region.¹³³

Most adjuvants are effective in young or middle-aged populations and are less efficient in older people, who are in dire need of good quality adjuvants or vaccines. Flagellin has shown effective adjuvant activity in aged mice and has enhanced antigen-specific systemic and mucosal immune responses, but this response occurred at a less robust level than that in young mice.^{134,135} Flagellin induced an efficient immune response in elderly subjects immunized with a recombinant hemagglutinin influenza–flagellin fusion vaccine (VAX125).¹³⁶ In addition, flagellin has been shown to enhance cross-protective responses of related antigens; for instance, intranasal immunization of mice with recombinant PspA fused to flagellin enhances cross-protective immunity against *Streptococcus pneumoniae* infection.⁷⁷ All these findings suggest that the benefits and potential of flagellin are unlimited.

CONCLUSION

Related to the use of flagellin as an adjuvant and anti-tumor agent, there are many areas worthy of continued investigation. What are the long-term consequences of the use of flagellin in the context of dosage and the route of administration? Are

other receptors or pathways, besides TLR5 and NLRC4, involved in flagellin perception and/or mediation of immunity in mammals? Do distinctive pathways exist for flagellin in different kinds of cells, for instance, in normal and cancerous cells? To what extent does inflammasome-mediated recognition of flagellin contribute to the overall innate immune response and the adaptive immune response? The answers to these questions should provide a solid basis for the use of flagellin as an effective adjuvant and anti-tumor agent in the future.

In addition, there is accumulating evidence that implicates flagellin for inflammatory diseases, such as acute lung inflammation, cardiovascular collapse, inflammatory bowel disease and lung injury.^{90,137} These effects from the use of flagellin could be minimized or avoided by using very small doses of flagellin or flagellin-based fusion proteins. For instance, 'Vax-Innate's Universal Flu Vaccine' has shown in phase 1 clinical study that 0.3 and 1.0 µg doses are safe and immunogenic, while 3 and 10 µg doses were associated with flu-like symptoms. A study by Treanor *et al.*¹³⁶ in humans has also demonstrated that doses above 3 µg have produced moderately severe systemic symptoms accompanied by substantial increases in serum C-reactive protein. Another alternative to minimize toxicity concerns might be the use of a combination of flagellin with another TLR agonist, such as TLR3 or TLR9 ligand, or the use of flagellin in combination with NLRs. These strategies will result in more pronounced effects than individual agonists, especially against tumors^{36,103} and will help reduce the dose of the agonists and toxicity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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