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REVIEW PAPER

Utilizing bacterial flagellins against infectious diseases and cancers

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Abstract The flagellum is the organelle providing motility to bacterial cells and its activity is coupled to the cellular chemotaxis machinery. The flagellar filament is the largest portion of the flagellum, which consists of repeating subunits of the protein flagellin. Receptors of the innate immune system including Toll like receptor 5, ICE protease activating factor, and neuronal apoptosis inhibitory protein 5 signal in response to bacterial flagellins. In addition to inducing innate immune responses, bacterial flagellins mediate the development of adaptive immune responses to both flagellins and coadministered antigens. Therefore, these proteins have intensively been investigated for the vaccine development and the immunotherapy. This review describes the utilization of bacterial flagellins for the construction of vaccines against infectious diseases and cancer immunotherapy. Furthermore, the key factors affecting the performance of these systems are highlighted.

Keywords Adjuvant · Carrier · Flagellin · Toll like receptor 5 · Vaccine

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Introduction

The flagellum is the organelle involved in the locomotion of bacterial cells. The flagellar activity is coupled to the chemotaxis machinery that senses environmental chemical and physical information and orchestrates the cellular migration for the bacterial growth and survival (Tindall et al. 2012). The bacterial flagellum consists of six components: a basal body, a motor, a switch, a hook, a filament, and an export apparatus (Macnab 2003). The flagellar filament is the largest portion of the flagellum, which is composed of repeating subunits of a protein known as flagellin. Amino acid sequence analyses of bacterial flagellins revealed conserved termini regions flanking a central region, which is hypervariable both in the amino acid composition and size. The N- and C-terminal chains of bacterial flagellins form packed α-helix structures including D0 and D1 domains, which are positioned in the filament core. On the other hand, the central portion of flagellin is exposed as a β-sheet folded structure including D2 and D3 domains on the outer surface of the filament (Winstanely and Morgan 1997; Beatson et al. 2006).

Starting in the late 1990s, flagellin was identified as the inducer of cytokine release from intestinal epithelial cells or human monocytes (Ciacci-Woolwine et al. 1998; Wyant et al. 1999a, b; Steiner et al. 2000). McDermott et al. (2000) reported that the cytokine inducing activity of flagellin was eliminated by trypsin treatment of monocytes, suggesting that this function

of flagellin results from its interaction with cell surface polypeptide receptors on monocytes. The molecular basis of the cytokine inducing activity of flagellin was revealed by Hayashi et al. (2001), who demonstrated that flagellin was the component of *Listeria* culture supernatant that activated the receptor of innate immune system, Toll like receptor 5 (TLR5). TLR5 expressed as a transmembrane protein by monocytes, macrophages, dendritic cells (DCs), and epithelial cells senses extracellular flagellin. The flagellin activated TLR5 signals through the adaptor protein, myeloid differentiation primary response protein 88 (MyD88) to trigger nuclear factor (NF)-kB mediated induction of proinflammatory cytokines, while also promoting the cell survival. In addition to sensing by TLR5, flagellin has recently been shown to be sensed by another class of innate immune receptors, Nod-like receptors (NLRs), which are expressed in the cytosol. Two NLR proteins, ICE protease activating factor (Ipaf) and neuronal apoptosis inhibitory protein 5 (Naip5), have been reported to signal in response to flagellin that attains an intracellular location. The primary consequence of this signaling pathway is activation of the inflammasome complex, which triggers the caspase-1 mediated cleavage of pro-IL-1ß and pro-IL-18 into their mature active forms (Akira et al. 2006; Carvalho et al. 2011; Franchi et al. 2006; Miao et al. 2006; Lightfield et al. 2008; Means et al. 2003). In addition to inducing innate immune responses, bacterial flagellins mediate the development of adaptive immunity to both flagellins and coadministered antigens (Ramos et al. 2004). Thus these proteins are attractive candidates for the development of vaccines and immunotherapy. In this mini-review, therefore, we intend to describe recent advances in the utilization of bacterial flagellins for the construction of vaccines against infectious diseases and cancer immunotherapy.

Applications of flagellins against infectious diseases

Flagellins as adjuvants

Bacterial flagellins have been investigated as adjuvants in vaccine formulations against several pathogenic organisms (Table 1). *Vibrio vulnificus* has a total of six flagellin structural genes, which are organized into two distinct genetic loci with three genes per locus. Among the six flagellins, *V. vulnificus* flagellin B (VVFB) appeared to be the most crucial building block of the flagellar filament. Mice immunized intranasally with tetanus toxoid (TT) plus VVFB were fully protected against systemic challenge with a superlethal dose of tetanus toxin. However, only 17 % of mice immunized with TT alone survived the challenge. Coadministration of VVFB with TT induced significantly enhanced TT-specific serum IgG and also TT-specific IgA in both serum and mucosal samples (saliva and vaginal washes) (Lee et al. 2006). In addition, flagellin of *Listeria monocytogenes* (LMF), phase 1 flagellin of *Salmonella* Typhimurium (STF), and VVFB of *V. vulnificus* showed similar efficiencies as tetanus toxoid mucosal adjuvants (US Patent No. 7914802, 2008).

Intranasal coadministration of either STF in a monomeric form or flagellin of *Salmonella* Enteritidis (SEF) in a polymeric form with an inactivated influenza virus vaccine resulted in the full protection of the vaccinated mice against a high dose intranasal lethal challenge by the homologous virus. In contrast, only 33 % of the mice immunized with the inactivated influenza virus vaccine alone survived the challenge. The adjuvanted vaccines induced enhanced systemic influenza specific IgA and IgG titers. In contrast, no significant increase in the secreted IgA level was observed in mucosal samples (saliva and lung washes). The presence of flagellins induced higher interleukin (IL)-4 production than the vaccine alone. Therefore, intranasal immunization with the adjuvanted vaccines is capable to elicit cellular immune responses as well as humoral responses. These results indicated that both monomeric and polymeric flagellins seem as promising adjuvants to improve protection against influenza epidemics (Skountzou et al. 2010).

Four tandem copies of the ectodomain of conserved influenza matrix protein M2 (M2e) linked genetically to phase 2 flagellin of *S.* Typhimurium (STF2) retained TLR5 stimulating activity. Mice immunized subcutaneously with the fusion protein (VAX102) in the absence of any exogenous adjuvants or other formulation additives exhibited higher levels of M2e-specific IgG than mice immunized with an equimolar dose of M2e peptide adsorbed to alum. The physical fusion of the antigen 4× M2e to the flagellin is required to achieve optimal immunogenicity of the M2e epitope. Immunization of mice with as little as

Table 1 Applications of bacterial flagellins against infectious diseases and cancers

Applied flagellin	Target agent	Immunization route	Immune response	Protection studies	Ref.
Flagellins as adjuvants					
VVFB, LMF, STF	Tetanus toxin	Nasal	Anti tetanus toxoid (TT) serum IgG, anti tetanus toxoid IgA in serum, saliva and vaginal washes	Protection against systemic tetanus toxin challenge	Lee et al. (2006); US Patent No. 7914802 (2008)
STF, polymeric SEF	Influenza A virus	Nasal	Influenza specific IgG and IgA in serum, cellular responses	Protection against lethal intranasal viral challenge	Skountzou et al. (2010)
STF2	Influenza A virus	Subcutaneous, nasal	Serum IgG against M2e	Protection against lethal intranasal viral challenge	Huleatt et al. (2008)
STF2	Influenza A virus	Subcutaneous	Serum IgG against the globular head domain of HA	Protection against lethal intranasal viral challenge	Song et al. (2008)
STF	*Plasmodium vivax*	Nasal, subcutaneous	Anti-MSP_{19} serum IgG, cellular immune responses	None performed	Bargieri et al. (2008)
KF	*Streptococcus mutans*	Nasal	AP specific serum IgG and saliva IgA	Protection against dental carries	Sun et al. (2012)
MAF, MAFR, STF2	None	Subcutaneous	Anti DUD serum IgG	None performed	Terron-Exposito et al. (2012)
STFΔ	None	Nasal	OVA specific IgG in serum and bronchoalveolar lavages, OVA specific IgA in bronchoalveolar lavages	None performed	Nempont et al. (2008)
SEF	*Yersinia pestis*	Nasal, intramuscular	Serum IgG against the F1 antigen of *Y. pestis*, serum IgG against the fusion of F1 and V antigens of *Y. pestis*	Protection against intranasal challenge with *Y. pestis*	Honko et al. (2006)
STF	Human immunodeficiency virus type-1 (HIV-1)	Oral	Induction of Gag specific IgA secreting cells	None performed	Kajikawa et al. (2012)
STF, STFΔ	HIV-1	Intramuscular, nasal	Env speccific serum IgG, Vaginal IgA and IgG against Env	Viral neutralizing antibodies elicited	Vassilieva et al. (2011)
STF2Δ	West nile virus (WNV)	Subcutaneous	Serum IgG against the E protein of WNV	Induction of viral neutralizing antibodies and protection against viral challenge	McDonald et al. (2007)
STF	*Streptococcus pneumoniae*	Nasal	Upregulated expression of genes coding for interleukin-6, tumor necrosis factor-α, chemokine CXC motif ligand 1, chemokine CXC motif ligand 2, and chemokine C–C motif ligand 20	Protection against nasal pneumococcal challenge	Munoz et al. (2010)

Table 1 continued

Applied flagellin	Target agent	Immunization route	Immune response	Protection studies	Ref.
STF	*Clostridium difficile*	Intraperitoneal	None described	Protection against oral lethal challenge with *C. difficile*	Jarchum et al. (2011)
STF	Vancomycin resistant *Enterococcus* (VRE)	Intraperitoneal	None described	Protection against oral challenge with VRE	Kinnebrew et al. (2010)
Flagellins as antigens					
SEF	*Salmonella* Enteritidis	Oral	Cellular responses	Protection against oral challenge with *S.* Enteritidis	Kajikawa et al. (2007)
SPF	*Salmonella* Paratyphi A	Nasal	Serum anti SPF IgG	Protection against lethal intraperitoneal challenge with *S.* Paratyphi A	Gat et al. (2011)
PAF	*Pseudomonas aeruginosa*	Subcutaneous, intraperitoneal	Serum anti PAF IgG	Protection against *P. aeruginosa* infection in burned mice	Faezi et al. (2012)
Truncated CCF	*Campylobacter jejuni*	Nasal	Antigen specific serum IgG, antigen specific intestinal secretory IgA	Protection against oral challenge with *C. jejuni*	Lee et al. (1999)
HPF, cHPF	*Helicobacter pylori*	Nasal, subcutaneous	Antigen specific serum IgG and IgA	Protection against *H. pylori* challenge	Mori et al. (2012)
Flagellins as both antigens and adjuvants/carriers					
PAF	*P. aeruginosa*	Intramuscular	OprI, PAF, and OprF specific serum IgGs	Protection against intranasal challenge with *P. aeruginosa*	Weimer et al. (2009)
PAF	*P. aeruginosa*	Subcataneous, intravaneous	PMA and PAF specific serum antibodies	Protection against intranasal challenge with *P. aeruginosa*	Campodonico et al. (2011)
SEF	*S.* Enteritidis	Intramuscular	LPS and SEF specific serum IgGs	Protection against lethal intraperitoneal challenge with *S.* Enteritidis	Simon et al. (2011)
BPF	*Burkholderia pseudomallei*	Intramuscular, intravaneous	LPS and BPF specific serum IgGs	Protection against intraperitoneal challenge with *B. pseudomallei*	Brett and Woods (1996)
Flagellins against cancer					
PFlgs	B16-OVA melanoma cells	Intravaneous	Cellular immune responses, OVA specific serum IgG	Protection against tumor growth following injection of B16-OVA cells	Faham and Altin (2010)

Table 1 continued

Applied flagellin	Target agent	Immunization route	Immune response	Protection studies	Ref.
9Flg	B16-OVA melanoma cells	Intravaneous	Cellular immune responses	Protection against tumor growth following injection of B16-OVA cells	Faham et al. (2011)
STF	Colonic cancer cells	Peritumoral injection	Neutrophil infiltration	Regression of tumor growth in mouse xenograft models of colon cancer	Rhee et al. (2008)
STF	Breast cancer cells	Peritumoral injection, intravenous	Neutrophil infiltration	Inhibition of tumor growth in xenograft mouse breast tumor models	Cai et al. (2011)
CBLB502	Radiation induced injuries	Subcutaneous	None described	Protection of healthy tissues against radiation	Burdelya et al. (2008)
CBLB502	Lung cancer cells	Subcutaneous	Neutrophil infiltration	Inhibition of tumor growth in mouse xenografts of lung cancer	Zhou et al. (2012)

VVFB, *Vibrio vulnificus* flagellin B; LMF, *Listeria monocytogenes* flagellin; STF, phase 1 flagellin of *S*. Typhimurium; SEF, *S*. Enteritidis flagellin; STF2, phase 2 flagellin of *S*. Typhimurium; M2e, the ectodomain of conserved influenza matrix protein M2; HA, influenza hemagglutinin antigen; KF, *Escherichia coli* flagellin; MAF, MAFR *Marinobacter algicola* flagellins; DUD, dynein union domain in the p54 protein of the African swine fever virus; STFΔ, a mutant of STF with a deleted hypervariable region; STF2Δ, a mutant of STF2 with a deleted hypervariable region; SPF, *S.* Paratyphi A flagellin; PAF, *P. aeruginosa* flagellin; CCF, *Campylobacter coli* flagellin; HPF, *H. pylori* flagellin; cHPF, chimeric HPF, OprI, OprF outermembrane proteins of *P. aeruginosa*; PMA, polymannuronic acid; LPS, lipopolysaccharide; BPF, *B. pseudomallei* flagellin; PFlgs, peptides derived from TLR5 binding region of STF; 9Flg, a peptide spanning amino acids 85-111 of STF; CBLB502, a polypeptide derived from flagellin of *Salmonella* Dublin

0.3 μg of VAX102 provided 90–100 % protection against a lethal intranasal challenge with influenza A virus, while mice immunized with 4× M2e were not protected. The efficacy of VAX102 delivered intranasally is comparable to the efficacy when delivered subcutaneously. This is an important finding because needle free delivery of a universal influenza vaccine could make the vaccine available to a larger population (Huleatt et al. 2008). Results of a complete phase I clinical trial of the influenza vaccine candidate VAX102 demonstrated the safety and immunogenicity of the fusion protein in healthy adults (Turley et al. 2011).

STF2 fused to the globular head domain of the influenza hemagglutinin (HA) antigen (VAX125) was highly immunogenic and efficacious against a lethal intranasal challenge in mouse models of influenza A infection (Song et al. 2008). The influenza vaccine candidate VAX125 underwent a complete phase I clinical study. The results from this trial showed that the recombinant flagellin was generally well tolerated by vaccinated individuals. Importantly, 91 % of individuals who received any dose of the recombinant protein developed titers of functional antibodies compatible with the protective status against the influenza infection (Treanor et al. 2010).

The adjuvant properties of STF were investigated in malaria vaccine formulations based on the recombinant 19 kDa C-terminal fragment of *Plasmodium vivax* Merozoite Surface Protein-1 ($MSP1_{19}$). STF can act as a potent adjuvant to elicit strong $MSP1_{19}$ specific IgG responses either admixed or genetically fused to the malarial antigen. However, mice vaccinated via the intranasal route mounted lower anti $MSP1_{19}$ specific antibody titers when compared to animals immunized via the subcutaneous route. MSP_{19} specific interferon (IFN)-γ secretion by immune spleen cells of mice vaccinated with the malarial antigen linked genetically to STF was higher than that of mice vaccinated with $MSP1_{19}$ admixed to STF. Therefore, even though the antibody responses were similar, the linkage of the antigen to flagellin may be an important strategy to improve the adjuvant activity for cell mediated immune responses (Bargieri et al. 2008).

The two highly conserved regions (the AP fragment) of the 190-kDa surface protein antigen (PAc) of *Streptococcus mutans* were inserted at the C-terminus of flagellin derived from *Escherichia coli* (KF) to produce a single recombinant protein (KF-AP). It was shown that KF-AP promoted higher AP-specific antibodies in the sera of intranasally immunized rats as well as in their saliva compared with AP alone or a mixture of AP and KF. In addition, a significant reduction of dental carries (64 %) was observed in rats vaccinated with the fusion protein (Sun et al. 2012).

Flagellins are highly immunogenic and their repeated use may lead to neutralization of their adjuvant capacities by systemic antibodies. This issue can be addressed by using flagellin proteins from different bacteria when antibodies raised against one of them do not neutralize the others. In a study by Terron-Exposito et al. (2012), it was shown that *Marinobacter algicola* derived flagellins (MAF and MAFR) induced the expression of IL-8 and chemokine C–C motif ligand 2 (CCL2) in Caco-2 cells with similar strength as STF2. These cytokines are elicited following the TLR5 stimulation. Therefore, these findings suggest that MAF and MAFR, and STF2 have similar TLR5 stimulating activity. The three bacterial flagellins also induced similar levels of IgG antibodies against a model antigen. On the other hand, MAF and MAFR were fully functional, in vitro or in vivo, in the presence of a high concentration of neutralizing anti-STF2 antibodies, and STF2 was not inhibited by neutralizing antibodies against *M. algicola* flagellins. These results demonstrate that the use of *M. algicola* flagellins (MAFR or MAF) and STF2 as adjuvants independently or sequentially (prime-boost) could be useful for the rational design of flagellin containing vaccines, thereby circumventing the systemic neutralization of adjuvants. Nempont et al. (2008) showed that the generation of flagellin specific antibodies in mice immunized with flagellin mutants deleted in the hypervariable region was remarkably attenuated compared with that in the animals vaccinated with full length flagellin. Therefore, the adjuvant neutralization in the repeated use of the mutant flagellins can be less pronounced than that of the full length flagellin. However, this matter was not elucidated experimentally in their study. Moreover, the reactivity of antibodies elicited against full length flagellin in mice with the deleted flagellin mutants was much lower than that with full length flagellin. Therefore, the sequential use of flagellin and the deleted flagellin mutants may be beneficial to avoid the adjuvant neutralization. The stimulation of mucosal innate immunity and mucosal adjuvancy to a foreign antigen

ovalbumin (OVA) was not altered by the hypervariable domain deletions. In contrast, this domain is essential to trigger the systemic innate immunity.

Honko et al. (2006) evaluated the efficiency of SEF as an adjuvant in an acellular plague vaccine. Mice immunized intranasally with a mixture of the F1 antigen of *Yersinia pestis* and SEF exhibited significant increases in anti F1 plasma IgG titers. In contrast, control mice immunized with the F1 antigen had low or undetectable antibody responses. Mice vaccinated with F1 and SEF were protected against an intranasal challenge with *Y. pestis* with a 93–100 % survival rate, versus only 7–10 % in the control group. Moreover, the evaluation of effectiveness of immunization with F1 and SEF in the presence of a high titer of serum anti flagellin IgG showed that preexisting anti flagellin antibodies had no significant effects on the adjuvant activity of SEF. This result indicated that the flagellin administration through the intranasal route circumvented the neutralization of flagellin's adjuvant capacity by serum antibodies. Intranasal or intramuscular vaccination of cynomolgus monkeys with SEF and a fusion of the F1 and V antigens of *Y. pestis* induced anti F1/V plasma IgG responses. These results suggest that SEF is an effective adjuvant for the development of an antibody response in nonhuman primates.

Two distinct recombinant *Lactobacillus acidophilus* strains displaying Gag of human immunodeficiency virus type 1 (HIV-1) on the bacterial cell surface were established by genetic fusion of Gag with the signal peptide and anchor motif of a mucus binding protein (Mub) from *L. acidophilus* with or without coexpression of STF fused to the signal peptide and anchor motif of another Mub from *L. acidophilus*. The surface exposed flagellin retained its TLR5 stimulating activity. Gag specific IgA producing cells were found in the large intestine and female reproductive tract of mice immunized orally with the recombinant *Lactobacillus* strain displaying Gag and STF but not those immunized with the recombinant bacterium displaying Gag alone. This result suggested that coexistence of STF conferred an adjuvant effect on local IgA secretion. In contrast, the *L. acidophilus* strain expressing Gag alone induced specific IFN-γ producing cells most efficiently at the local mucosa. This result implies that this strain promotes cellular immunity rather than humoral immunity. Thus the two different Gag displaying *L. acidophilus* strains have dissimilar immunogenicity depending on the coexpression of STF (Kajikawa et al. 2012).

A modified form of the gp120-gp41 Env protein of HIV-1 with heterologous transmembrane/cytoplasmic domains derived from the mouse mammary tumor virus glycoprotein was incorporated into Gag derived HIV-1 virus like particles (VLPs) at 10–15 fold higher levels than native Env. To further improve the immunogenicity of such VLPs (E/G VLPs), membrane anchored forms of STF or a STF with a truncated variable region (STFΔ) were constructed to be incorporated into the VLPs as adjuvants using a recombinant baculovirus expression system. HIV-1 specific immune responses induced by VLPs were determined in a guinea pig model. Incorporation of the flagellin proteins into E/G VLPs induced enhanced systemic antibody responses by either intramuscular or intranasal vaccination, as well as increased vaginal mucosal immune responses by intranasal vaccination. Chimeric VLPs containing full length STF were more effective in inducing systemic immune responses, whereas those VLPs containing STFΔ were more effective in eliciting mucosal IgA responses. The quality of antibody responses was improved by incorporation of flagellins into E/G VLPs as shown by enhanced virus neutralization capacities. STF did not show an adjuvant effect by intramuscular immunization and had a low adjuvant effect by intranasal immunization when mixed with the VLP antigens. Thus the association pattern of flagellin with antigens could contribute to its ability to function as an adjuvant (Vassilieva et al. 2011).

A modified version of STF2 was generated by replacing the hypervariable region that spans amino acid residues 170–415 with a short flexible linker (STF2Δ). The linker was used to facilitate the interaction of N- and C-terminal regions of the protein because this interaction is necessary for TLR5 signaling. STF2 and STF2Δ showed similar in vitro TLR5 stimulating activity, which confirms that removal of the hypervariable region from the flagellin protein does not affect TLR5 signaling. STF2Δ fused genetically to the EIII domain of the west nile virus (WNV) E protein showed in vitro TLR5 stimulating activity, which was similar to that of STF2Δ. In mice immunized subcutaneously with the fusion protein, a strong E specific serum IgG response was elicited that neutralized the viral infectivity and conferred full protection against a lethal WNV challenge.

Immunization of mice with EIII alone did not elicit E specific antibodies and only 10 % of the animals survived the challenge. The physical linkage of STF2Δ to the EIII domain, rather than a simple mixture of the two components, showed to be critical for the induction of a potent immune response (McDonald et al. 2007).

STF could exert protective responses against *Streptococcus pneumoniae* infection when administered before the infection. All BALB/c mice receiving STF intranasally 12–24 h before nasal pneumococcal challenge survived, while all untreated mice died of the infection. Coadministration of STF with *S. pneumoniae* resulted in the survival of 75 % of BALB/c mice after the challenge. The capacity of flagellin to induce protection against *S. pneumoniae* was also assessed in C57BL/6 mice and in mice of the outbred strain NMRI. Administration of STF 12 h before the challenge induced 80 % protection in C57BL/6 mice. One hundred percent protection was achieved in NMRI mice when the flagellin protein was administered 6–32 h before the challenge. Flagellin was also protective when coadministered with *S. pneumoniae* to mice of C57BL/6 and NMRI strains, albeit to a lower extent (40 %). Although *S. pneumoniae* does not have flagella, these results indicated the protective effect of flagellin in different mice strains, which is exerted through activation of innate immunity. When a flagellin mutant unable to signal through TLR5 was used, all of the treated mice died after the challenge. Therefore, TLR5 signaling is required for the protection induced by flagellin (Munoz et al. 2010).

Intraperitoneal STF administration protected antibiotic treated mice substantially from *Clostridium difficile* induced death by delaying *C. difficile* growth and toxin production in the colon and cecum. In addition, the flagellin protein protects the integrity of intestinal epithelial barrier during *C. difficile* infection. The observed protective effects of flagellin against *C. difficile* were speculated to result from restoring the intestinal innate immunity by TLR5 stimulation in antibiotic treated mice (Jarchum et al. 2011). Furthermore, intraperitoneal administration of STF protected antibiotic treated mice against intestinal colonization of vancomycin-resistant *Enterococcus* (VRE). RegIIIγ is a secreted C-type lectin that kills gram positive bacteria including VRE. Systemic administration of STF induces the expression of RegIIIγ in intestinal epithelial cells and Paneth cells along the entire length of small intestine. Flagellin induced TLR5 signaling results in the production of IL-22, which is required for the expression of RegIIIγ (Kinnebrew et al. 2010; Cash et al. 2006; Van Maele et al. 2010).

Flagellins as Antigens

Recombinant *Lactobacillus casei* cells expressing SEF on their cell surface (LCF) were constructed. Intragastric immunization of mice with the recombinant lactobacilli resulted in a significant level of protective immunity against an oral challenge with *S.* Enteritidis. There was no significant difference in the level of protection after immunization with the recombinant lactobacilli compared with the free SEF isolated from *S.* Enteritidis, although the amount of SEF carried by LCF was less than that of the free SEF. The immunization of mice with the recombinant lactobacilli did not result in antigen-specific antibody responses in either feces or sera but did induce the release of IFN-γ on restimulation of primed lymphocytes ex vivo. These results suggested that the protective efficacy provided by SEF expressing *L. casei* was mainly attributable to cell-mediated immune responses. When the levels of IFN-γ produced by primed and SEF-restimulated lymphocytes were compared between the recombinant *L. casei* cells expressing SEF on their cell surface, and a mixture of the purified SEF and normal *L. casei*, the results indicated that the *Lactobacillus* strain functions as an adjuvant only when SEF is expressed on the cell surface (Kajikawa et al. 2007).

Intranasal immunization of mice with an attenuated mutant of *Salmonella* Paratyphi A expressing cell associated flagella conferred superior protection (90 %) against a lethal intraperitoneal challenge with *S.* Paratyphi A, compared with the flagellin monomer (SPF) exporting mutants of the attenuated bacterium (30–47 % protection). Serum IgG antibodies against the flagellin protein reached similar levels for all of the studied mutants. However, the superior protection induced by the flagella attached mutant was associated with an increased IgG2a:IgG1 ratio of flagellin specific antibodies with enhanced opsonophagocytic capacity (Gat et al. 2011).

Flagellin of *Pseudomonas aeruginosa* (PAF) was highly immunogenic and protective against *P. aeruginosa* infection in mouse models of burn wounds. Furthermore, passive immunization of mice with anti-PAF IgG

substantially improved the survival of *P. aeruginosa* infected burned mice (Faezi et al. 2012).

Flagellin of *Campylobacter coli* (CCF) is highly homologous to flagellin of a heterologous strain of campylobacter, *Campylobacter jejuni* (98.1 %) in the region comprising amino acids 5–337. The protective efficacy of this region produced as a recombinant protein in *E. coli* was studied against challenge by *C. jejuni*. Mice were vaccinated intranasally with the recombinant truncated CCF with or without using *E. coli* heat-labile enterotoxin (LT_{R192G}) as a mucosal adjuvant. The animals were then challenged orally with *C. jejuni.* Control animals immunized with either buffer or LT_{R192G} were colonized by the bacterium throughout the experiment course. 100 % of mice immunized with the truncated CCF alone were colonized by the *C. jejuni* on day 7 postchallenge. However, only 20 % of animals vaccinated with the truncated CCF plus LT_{R192G} were colonized at the same time (Lee et al. 1999).

Recently it has been shown that many α and ε-proteobacteria including *Campylobacter*, *Helicobacter* and *Bartonella* have flagellin proteins that evade TLR5 recognition because they carry some changes in the primary amino acid sequence at the D1 domain that make them unable to bind to TLR5 (Andersen-Nissen et al. 2005). The ability of flagellin of *H. pylori* (HPF) for the recognition by TLR5 was restored through engineering a chimeric flagellin (cHPF), in which both terminal segments of HPF were replaced by the corresponding segments from TLR5 activating *E. coli* flagellin. Variable domains of HPF represent the immunodominant epitopes. Increased serum IgG and IgA antibody responses were measured in mice vaccinated with the cHPF in comparison to mice vaccinated with HPF. The elicited antibodies were able to bind the native flagellin from *H. pylori* lysates. Vaccination with cHPF provided mice with significant protection against *H. pylori* (Mori et al. 2012).

Flagellins as both antigens and adjuvants/carriers

PAF is a potent adjuvant as well as a protective antigen (McDermott et al. 2000; Faezi et al. 2012). Weimer et al. (2009) investigated the protective potential of multivalent vaccines containing flagellin and outer membrane proteins (OprF and OprI) of *P. aeruginosa* against nonmucoid *P. aeruginosa*. Mice immunized intramuscularly with either OprI–PAF or OprF–OprI–PAF fusion proteins exhibited a robust OprI-specific IgG response in the sera. In contrast, there was no significant OprI-specific IgG response in mice given only OprI or a mixture of OprI and the PAF protein. In all cases, high flagellin specific IgG responses were observed. Mice immunized with OprF–OprI–PAF also exhibited a high level of OprF-specific IgG. Mice immunized with the OprF–OprI–PAF fusion protein displayed a markedly lower bacterial burden in lungs 3 days after respiratory challenge and cleared the infection faster than control mice immunized with the OprF–OprI fusion protein. Moreover, mice immunized with OprF–OprI–PAF had less inflammation and lung damage throughout the infection than OprF–OprI-immunized mice. Although the adjuvants Pam3Cys and Pam2Ser as well as alum were found to enhance the immunological response to *P. aeruginoa* antigens, it appears that flagellin is far more effective, as evidenced by the great differences in the amount of antigens required and the resultant titers of antigen specific IgG.

Epidemiological studies have linked opsonic antibodies specific to alginate in the sera of cystic fibrosis (CF) patients with a lack of chronic mucoid *P. aeruginosa* colonization and better overall lung function. Purified *P. aeruginosa* alginate is safe when administered to humans but alginate is poorly immunogenic in most humans, failing to elicit high titers of protective antibodies. In order to increase the immunogenicity of alginate, it has been conjugated to carrier proteins. However, a major challenge to this approach is that alginates from different mucoid strains have variable ratios of mannuronic to guluronic acids, making it difficult to find an alginate preparation that gives rise to antibodies reactive with multiple strains of mucoid *P. aeruginosa*. Nonetheless, blocks of O-acetylated polymannuronic acid (PMA) are present within most *P. aeruginosa* alginates, suggesting that a preparation of alginate rich in PMA residues can induce broadly reactive antibodies. PMA conjugated to PAF was able to elicit high titers of specific antibodies to PMA and PAF in mice and rabbits. Rabbit antibodies to the conjugate showed evidence of protective efficacy against both mucoid and nonmucoid types of *P. aeruginosa* in a mouse model of lung infection. Conjugation of PMA to PAF enhanced the immunogenicity of PMA without eliciting antibodies that inhibit TLR5, indicating that desirable protective antibodies were elicited but not at the price of possibly

inhibiting an important and conserved mechanism of innate immune resistance to pathogens (Campodonico et al. 2011).

Outer membrane lipopolysaccharide (LPS) of *Salmonella* is structurally characterized by a terminal lipid A group at the 3-deoxy-D-manno-octulosonic acid (KDO) terminus of the conserved core polysaccharide (Heinrichs et al. 1998). The serovar specific O-polysaccharide (OPS) region extends as a repeating polymer from the distal end of the core polysaccharide (Palva and Makela 1980; Samuel and Reeves 2003). *Salmonella* OPS and flagellins are virulence factors and protective antigens (Kajikawa et al. 2007; Jimenez-Lucho et al. 1987; Liang-Takasaki et al. 1983; Bergman et al. 2005; Strindelius et al. 2002, 2004). However, the surface polysaccharides of *Salmonella* are poorly immunogenic and do not confer immunologic memory. These limitations can be overcome by covalently attaching the polysaccharides to carrier proteins (Gonzalez-Fernandez et al. 2008; Pollard et al. 2009). Core polysaccharide-OPS (COPS) of *S*. Enteritidis LPS was therefore conjugated to SEF. Mice immunized with COPS-SEF conjugates mounted higher anti-LPS IgG levels than mice receiving unconjugated COPS and exhibited high anti SEF IgG; anti LPS and anti SEF IgG levels increased following booster doses. Mice immunized with COPS-SEF conjugates were significantly protected from lethal intraperitoneal challenge with *S*. Enteritidis (80–100 % protection). In contrast, 100 % mortality was observed in phosphate buffer saline (PBS) immunized group of mice. Since the core polysaccharide is virtually identical across most *Salmonella* serovars, using COPS-SEF may also provide some degree of cross protection across serovars. However, it remains to be elucidated (Simon et al. 2011).

Polyclonal and monoclonal antisera elicited against *Burkholderia pseudomallei* flagellin protein (BPF) and LPS provided passive protection against *B. pseudomallei* in animal models. The O-polysaccharide moiety of *B. pseudomallei* LPS conjugated to BPF induced high titers of IgG to both the protein and carbohydrate components of the construct in rabbits following intraperitoneal immunisation. Diabetic rats passively immunized with the rabbit IgG by intravenous injection were protected against intraperitoneal challenge with *B. pseudomallei*. However, the level of protection was less than those previously reported for an anti LPS monoclonal antibody and flagellin specific polyclonal antisera. The absence of conformational epitopes in the conjugate preparation may explain the loss in the protective capacity of conjugate antisera (Brett and Woods 1996).

Applications of flagellins against cancers

Activation of DCs by microbial products via TLRs is instrumental in the induction of immunity. Immature DCs are resident sentinel cells in tissues that are specialized in antigen capture. Upon activation, DCs migrate to draining lymphoid tissues, where they present antigens to naïve T cells and provide signals for the development of appropriate $CD4^+$ T helper (Th) responses. Flagellin induces maturation of TLR5 expressing DCs and the upregulation of co stimulatory molecules and antigen presenting capacity in a MyD88 dependent manner. Activation of the TLR5 signaling cascade by flagellin stimulates DCs to shape Th2 biased immunity via production of Th2 promoting or Th1 suppressing factors. The $CD4^+$ T cells committed to Th2 phenotype produce IL-4 and promote strong antibody responses (Didierlaurent et al. 2004). Bates et al. (2011) found that the ability of flagellin to promote an antigen specific $CD8^+$ T cell response is independent of its ability to signal via TLR5 and operates through delivery of an antigen in a form that facilitates antigen processing e.g. the flagellin-antigen fusion form.

The region spanning amino acids 79–117 in the conserved N-terminus of STF contains epitopes that can interact with TLR5 (Smith et al. 2003). Therefore, peptides derived from this region (PFlgs) can be used for targeted delivery of liposomal antigens to antigen presenting cells (APCs) in vitro and in vivo. When engrafted onto liposomes, two PFlgs denoted as 9Flg (spanning amino acids 85–111) and 42Flg (spanning amino acids 98–117), promoted strong liposome binding to murine bone marrow derived DCs (BMDCs) and splenic DCs (SpDCs); the cell binding correlated with the expression of TLR5, with little or no binding to cells lacking the expression of TLR5. Importantly, the cell bound liposomes were efficiently internalized by cultured DCs, permitting intracellular antigen processing and presentation. In addition, engrafting PFlgs onto liposomes induced a significant increase in maturation of DCs relative to engrafting a histidine tag (12 His) onto liposomes both in vitro and

in vivo. Vaccination of mice with PFlg engrafted liposomes containing the model antigen OVA (PFlg-OVA liposomes) induced OVA specific T cell priming, increased number of antigen responsive IFN-γ-producing $CD8^+$ T cells, and increased antigen specific serum IgG. However, such responses were not observed with 12 His engrafted liposomes containing OVA. These results demonstrate that vaccination with PFlg-OVA liposomes is effective at inducing humoral and cell mediated immune responses to the liposome associated antigen. Moreover, immunization with PFlg-OVA liposomes and B16-OVA-derived plasma membrane vesicles engrafted with 9Flg and 42Flg inhibited tumor growth in mice challenged with B16-OVA melanoma cells in lung tumor models (Faham and Altin 2010). Liposomes engrafted with 9Flg can be used to target antigen encoding plasmid DNAs (pDNAs) to APCs. High cellular immune responses were elicited in mice vaccinated intravenously with 9Flg engrafted liposomes containing pDNAs that encode the epitope $OVA_{257–264}$. DNA vaccines have conventionally been administered via either intramuscular or subcutaneous route. Intravanous administration of $OVA_{257–264}$ encoding pDNAs loaded onto 9Flg engrafted liposomes elicited immune responses that are substantially more potent than those induced by intramuscular vaccination with $OVA_{257–264}$ encoding pDNAs in naked form. The intravanous route can potentially allow a more efficient delivery of the 9Flg engrafted liposomes to APCs in the blood circulation as well as those in more immunologically relevant sites such as spleen, liver, and lung. Compared to intramuscular injection of the plasmids in naked form, the intravaneous administration of 9Flg engrafted liposomes containing $OVA_{257–264}$ encoding pDNAs to mice induced more potent antitumour response in the B16-OVA melanoma tumor models (Faham et al. 2011).

Recent evidence has shown that activation of TLR5 by peritumoral flagellin treatment substantially inhibits colon tumor growth in a mouse xenograft model of human colon cancer. However, blocking of TLR5 dependent signaling in this mouse model suppresses innate immune responses represented by reduced neutrophil infiltration and tumor necrosis inside the tumors, which is associated with enhanced tumor growth. The inhibitory role by flagellin/TLR5 engagement in tumor growth is effective in the initial step of tumor development but not sufficient to modulate established tumors (Rhee et al. 2008).

TLR5 was highly expressed in human breast carcinomas and TLR5 signaling was overly functional in human breast cancer cells. Triggering of TLR5 signaling pathway by flagellin in breast cancer cells inhibits cell proliferation in vitro. In addition, peritumoral flagellin treatment resulted in increased neutrophil infiltration and retarded tumor growth in mouse xenografts of human breast cancer cells. Intravenous administration of flagellin also inhibited the tumor growth (Cai et al. 2011).

A polypeptide derived from flagellin of *Salmonella* Dublin, designated CBLB502 includes the complete N- and C-terminal domains of flagellin separated by a flexible linker. CBLB502 produced in *E. coli* as a recombinant protein retains entirely the NF-kB inducing activity and exceptional stability of flagellin, yet is substantially less immunogenic. This polypeptide protects healthy tissues against radiation toxicity without diminishing the therapeutic effect of radiation on tumors and without promoting radiation induced carcinogenicity. The differential radioprotective effect of CBLB502 in tumor versus normal tissues is likely due to the constitutive activation of NF-kB in most tumor cells and/or inhibition of downstream TLR5 signaling by the activated phosphatidylinositol-3 kinase present in many tumor cells. There was no evidence of desensitization with multiple injections of CBLB502. Thus the therapeutic index of cancer radiotherapy can be improved by TLR5 agonists (Burdelya et al. 2008).

In a study by Zhou et al., the effects of CBLB502 on the growth and radiosensitivity of A549 lung cancer cells were investigated. Knocking down the expression of MyD88 or TLR5 in A549 cells enhanced tumor growth in mouse xenografts of A549 lung cancer cells. CBLB502 inhibited the tumor growth in the xenograft mice. However, the polypeptide did not affect the radiosensitivity of tumors in vivo (Zhou et al. 2012).

Concluding remarks

Flagellins are potent immune activators, stimulating diverse biologic effects that mediate innate immune responses as well as the development of adaptive immunity. Therefore, flagellins can be used for the construction of vaccines against infectious diseases

and are applicable in cancer immunotherapy. Results of several studies have established that flagellins are potent systemic and mucosal adjuvants that elicit adaptive immunity responses to both the flagellins and coadministered antigens. The genetic fusion of antigens to flagellins, the genetic modification of flagellins in the hypervariable region, and the vaccine administration route can remarkably influence the efficiency of flagellin adjuvanted vaccines. When these vaccines should be used in prime-boost immunization or independently, the effect of preexisting antibodies against flagellins should be considered because anti flagellin antibodies can neutralize adjuvant properties of flagellins. In addition, these antibodies may attenuate the innate immune responses to flagellated bacteria encountered the host. Applications of flagellins from distinct bacteria, the deletion of hypervariable region of flagellins to decrease their immunogenicities, and intranasal administration of flagellins represent useful approaches to prevent or attenuate the undesired effects of preexisting anti flagellin antibodies. Other developments involve the improvement of genetic tools for the production of flagellin containing vaccines. Studies about expression hosts and signals can ensure the production efficiency of these vaccines. Furthermore, the use of appropriate vaccine delivery vehicles can improve the efficiency of flagellin containing vaccines. Comparisons of immunological properties of flagellins from distinct bacteria especially non pathogenic bacteria represent another attractive subject for future studies.

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