Expression, purification, and functional characterisation of Flagellin, a TLR5-ligand

Irshad Ahmed Hajam, Pervaiz Ahmed Dar, Shanmugam Chandra Sekar, Rajakishore Nanda, Subodh Kishore, Veerakyathappa Bhanuprakash & Kondabattula Ganesh

Foot and Mouth Disease Vaccine Quality Control and Assurance Laboratory, Indian Veterinary Research Institute, Bangalore, 560 024 Karnataka, India
ganesh_k60@yahoo.co.in

Keywords
Cytochines, Flagellin, Immunogenicity, *Salmonella* Typhimurium.

Summary
Flagellin, a Toll-like receptor 5 (TLR5)-ligand, is known for its activities like adjuvant, induction of pro-inflammatory cytokines and innate immunity. In this context, fliC gene of *Salmonella* Typhimurium was cloned into pET32a expression plasmid using in-house designed gene specific primers. The frame and orientation of the inserted fliC gene was confirmed upon colony PCR, restriction enzyme analysis and sequencing. Sequence analysis of fliC revealed proper orientation of the gene and had 1,485 nucleotides. Following transformation of pET-fliC plasmid into *Escherichia coli* BL21 (DE3) cells, the gene was expressed after inducing with IPTG (Isopropylβ-D-1-thiogalactopyranoside). The polyHis-tag-fliC was ~70kDa as confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The identity/authenticity of the recombinant-fliC was confirmed by its specific reactivity with commercial anti-fliC MAb of *S.* Typhimurium. Further, the antigenic and functional properties of recombinant-fliC were determined espousing its ability to induce antigen specific antibodies in G pigs and increased m-RNA expression of certain pro-inflammatory mediators like TNF-α and GM-CSF *in vitro*.

Espressione, purificazione e caratteristiche funzionali della Flagellina, un TLR5-legante

Parole chiave
Citochine, Flagellina, Immunogenicità, *Salmonella* Typhimurium.

Riassunto
La flagellina, legante il Toll-like receptor 5 (TLR5), è nota per le sue proprietà adiuvanti e per l'induzione di citochine pro-infiammatorie e immunità innata. L'articolo descrive i risultati della clonazione del gene fliC di *Salmonella* Typhimurium effettuata in un plasmide con espressione pET-32a, utilizzando primer specifici per il gene messi a punto nel Foot and Mouth Disease Vaccine Quality Control and Assurance Laboratory. La struttura e l'orientamento del gene fliC sono stati confermati tramite colony PCR e analisi con enzimi di restrizione e sequenziamento. L'analisi della sequenza di fliC ha evidenziato il corretto orientamento del gene e la presenza di 1.485 nucleotidi. Il plasmide pET-fliC è stato impiegato per la trasformazione genetica in cellule di *Escherichia coli* BL21 (DE3) e il gene è stato espresso tramite induzione con IPTG. La massa del poly His-tag-fliC pari a circa 70 kDa è stata confermata con elettroforesi su gel di poliacrilammide in presenza di sodio dodecil solfato (SDS-PAGE). L'identità/autenticità del fliC ricombinante è stata confermata dalla reattività specifica evidenziata con MAb anti-fliC di *S.* Typhimurium. Le proprietà antigeniche e funzionali del fliC ricombinante sono state determinate tramite l'induzione di anticorpi antigene-specifici nelle cavie e l'aumento *in vitro* dell'espressione dell'm-RNA di alcuni mediatori come TNF-α e GM-CSF.

Veterinaria Italiana 2013, **49** (2), 181-186. doi: 10.12834/VetIt.2013.492.187.192

Introduction

Bacterial flagellin (fliC) serves many functions like mobility, pathogenicity, adjuvanticity and toll like receptor (TLR)-ligand activity (1, 9, 19). TLRs, a group of conserved receptors play a crucial role in activating antigen presenting cells (APCs) and the adaptive response (2). The binding of fliC with TLR5 leads to a cascade of reactions that results in the production of pro-inflammatory cytokines like TNF-α, IL-6 and IL-12, thereby up-regulate the antigen presenting cell surface molecules (8). TLR5 is present on the surface of epithelial and immune system cells like dendritic cells (DC), monocytes, natural killer cells (NK), and T-lymphocytes (25). fliC activates the epithelial cells, well-known for their ability to encounter the pathogen and defend the body against it. Moreover, *in vivo* administration of fliC is much safer than the lipopolysaccharide (LPS) inducing shock (26). These properties make fliC an ideal and potent molecular adjuvant (6, 12, 17, 23). In addition, immunisation with fliC has been found to protect the host from a wide range of pathogens, chemicals and radiations (22, 26). Therefore, the present study was conceptualised to clone and express the fliC from *S.* Typhimurium in prokaryotic expression system, characterise the recombinant protein by Western blot and *in vitro* analysis of mRNA expression levels of various pro-inflammatory mediators.

Materials and methods

Bacterial strain

Stab-culture of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) of bovine origin available at FMD vaccine quality control unit, Indian Veterinary Research Institute (IVRI), Bangalore, was used as source for fliC gene.

Amplification of fliC gene

A colony of *S.* Typhimurium was grown in Luria-Bertani broth (LB broth) overnight at 37°C with constant agitation (220 rpm). Genomic DNA was extracted (21) and the fliC gene was amplified using in-house designed gene specific primers (fliC F; cgcggatccatggcacaagtcattaatacaaac and fliC R; gcgctcgagacgcagtaaagagaggacgtt) based on the known DNA sequence. For amplification of the fliC gene, the polymerase chain reaction (PCR) was standardised using 10 ng of genomic DNA, 20 pM of each gene specific primers, 10 mM of each dNTPs, 5 µl of 10× enzyme buffer and 0.5 U of Accu Taq LA DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA) in a 50 µl final reaction volume. The amplification was carried out with initial denaturation of DNA at 94°C (10 min) followed by 35 cycles of 94°C for 45 sec, annealing at 56°C for 90 sec, extension at 72°C (100 sec), and final extension at 72°C for 10 min. The amplified product was analysed by electrophoresis using 0.5% agarose gel and ethidium bromide as tracking dye.

Cloning and expression of flagellin

The amplified PCR product was cloned into prokaryotic expression vector pET-32a (Novagen, San Diego, California, USA) at *NcoI-XhoI* site. For ligation, the fliC gene PCR product and vector plasmid were used in the ratio of 2:1. The ligated product was initially propagated in Top 10 *Escherichia coli* competent cells (Invitrogen, Carlsbad, California, USA) (21). Transformed colonies were screened by colony PCR, restriction enzyme analysis and sequencing. The recombinant pET-fliC plasmid extracted from Top 10 *E. coli* cells was purified and transformed to *E. coli* BL21 (DE3) strain (Novagen, San Diego, California, USA) for expression of flagellin. The expression was induced by adding 1 mM Isopropylβ-D-1-thiogalactopyranoside (IPTG) to transformed BL21 (DE3) bacteria (OD_{600}=0.6) at 30°C. Zero-time aliquot (uninduced culture) was used as control. The samples collected after 4 h and 6 h were analysed for expressed protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (15). The recombinant protein was further confirmed by Western blot analysis using anti-fliC monoclonal antibody (MAb) of *S.* Typhimurium (Clone: X5A12, Invivogen, San Diego, California, USA).

Solubility and purification of the recombinant flagellin

The solubility of the expressed protein was determined by re-suspending the bacterial pellet (6 h post IPTG induction) in lysis buffer. After sonication, the lysate was centrifuged at 12,000 × *g* (15 min) and supernatant representing soluble fraction was collected. The pellet was again resuspended in the lysis buffer, which represented the suspension of the insoluble matter. Both extracts were analysed after running on 10% SDS-PAGE. Purification of the recombinant flagellin containing poly-His tag was done by using Ni-NTA Spin Columns (Qiagen, Hilden, Germany) under denaturation conditions as *per* the manufacturer's instructions. The positive elutes confirmed by SDS-PAGE were pooled and extensively dialysed (six washings) against phosphate buffered saline (pH 8.0) at 4°C. Purified protein was quantified by a Bradford assay (5), filtered, and stored in the same buffer at –20°C until use.

Antigenic and functional characterisation of recombinant flagellin

To determine the antigenic nature of recombinant-fliC three guinea pigs were immunised with the purified protein (6 µg/animal). Guinea pigs were maintained under standard conditions following the recommendations of the Institute Animal Ethics Committee (IAEC) under the guidelines set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Three animals were kept as controls. Blood samples were collected from all the animals directly from the heart on day 14 post immunisation and serum was separated, inactivated and stored at –20°C until use.

The functional characterisation of the protein was studied under *in vitro* conditions by determining the effect of recombinant-fliC on m-RNA expression levels of various inflammatory mediators and quantified semi-quantitatively by reverse transcriptase polymerase chain reaction (RT-PCR) using established primers (11) (Table 1). Peripheral blood mononuclear cells (PBMC) were isolated from the blood of normal healthy G pigs by density gradient (4) using Histopaque (M/s Sigma-Aldrich, Cat. No. 1083) and were cultured in RPMI-1640 medium (M/s Sigma-Aldrich, Cat. No. R1383) supplemented with 10% fetal calf serum and penicillin and streptomycin at 50 µg and 100 IU/ml, respectively. Harvested cells were counted and distributed (2x10^6/well) in 24-well culture plate and incubated with or without purified recombinant-fliC (5µg/ml) at 37˚C, 5% CO_2 for 8 h. RNA was extracted by RNeasy Mini kit (Qiagen, Hilden, Germany) and cDNA was prepared using SuperScript™ III Reverse Transcriptase kit (Invitrogen, San Diego, California, USA) as *per* the manufacturer's instructions. Amplification of the cytokine genes was carried out as described previously (11). The density of electrophoretic bands of amplified PCR products was done using band leader 3.0.

Table I. *Sequence of primers used for the detection and quantification of the inflammatory mediators by reverse transcriptase-polymerase chain reaction.*

Primer name	5´-3´ sequence	Product length
GAPDH (F-RT)	ACC ACA GTC CAT GCC ATC AC	343 bp
GAPDH (R-RT)	TGT CGC TGT TGA AGT CA	
GM-CSF (F-RT)	CTG TGG TTT GCA GCA TCT GT	171 bp
GM-CSF (R-RT)	GGG GCT CAA ACT GGT CAT AG	
TNF-α (F-RT)	ATC TAC CTG GGA GGC GTC TT	184 bp
TNF-α (R-RT)	GAG TGG CAC AAG GAA CTG GT	
iNOS (F-RT)	GCA CAC GTT GGC TTC CCT CT	456 bp
iNOS (R-RT)	TGG GCC AGT GCT TCT GAT TTT CC	

Results

Construction of the expression vector pET32a-fliC

The fliC gene (~1.5 kb) sequence of *S.* Typhimurium was successfully cloned into expression vector, pET-32a using *NcoI* and *XhoI* restriction enzymes. The colony PCR product of recombinant clones has shown amplification of ~2 kb product using vector specific T7 forward and gene specific reverse primers and thus indicated that the gene was in proper orientation. The release of ~1.5 kb insert upon restriction enzyme digestion and gene sequence analysis of 1,986 bases of recombinant plasmid including 501 bp of vector and 1,485 bp of fliC gene, further confirmed the proper orientation of the insert (Figure 1). The sequence was published in GenBank with accession number HM 920247.1.

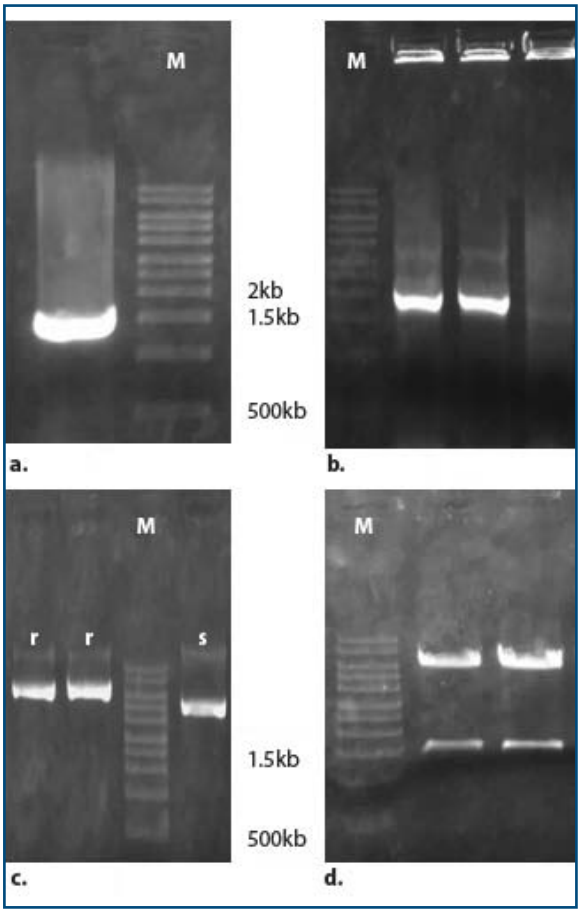

Figure 1. *Amplification of fliC gene and confirmation of recombinant clone.*
(a) PCR amplification of fliC gene.
(b) Confirmation of recombinant clones by colony PCR using vector specific T7 primer and gene specific reverse primer.
(c) Recombinant plasmid (r) showing less mobility than neat pET32a vector plasmid (s) in agarose gel.
(d) Insert release (1,485 bp) by using *NcoI* and *XhoI* restriction enzymes.

Expression and characterisation of flagellin protein

The confirmed pET-fliC plasmid was transformed into *E. coli* BL21 (DE3) host cells for expression of flagellin. As shown in Figure 2c, the Coomassive brilliant blue stained gel indicated the polyHis$_6$-tagged flagellin protein to be ~70kDa. For characterisation of the recombinant-fliC, the IPTG-induced cell lysate was fractionated on SDS-gel, transferred to a nitrocellulose membrane and probed with specific anti-fliC MAb. The protein reacted specifically giving a characteristic single band (Figure 2d), thus confirming the authenticity of the protein.

Solubility and purification of the recombinant flagellin

The expressed recombinant-fliC was roughly about two third as soluble and one-third as insoluble as determined by the band intensity of the coomassive stained SDS-PAGE gels by loading equal volumes of supernatant and re-suspended pellet samples of lysed bacteria (Figure 2b). The protein was produced in bulk and purified under denatured conditions using Ni-NTA spin columns. The protein was re-natured by dialysis against PBS (pH 7.4) and the purity of the Ni-NTA purified recombinant protein was >95% as analysed by SDS-PAGE (Figure 2c). The amount of the recombinant-fliC was 2 mg/100 ml of bacterial culture.

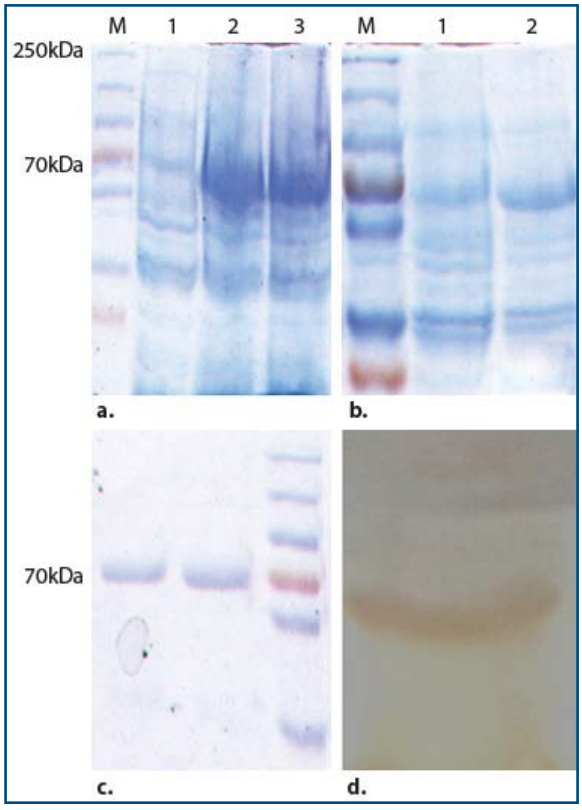

Figure 2. *Expression of poly His6-tagged flagellin protein in* Escherichia coli *BL21(DE3) cells using pET32a vector.*
(a) Protein expression at 4 h (lane 2), 6 h (lane 3) post IPTG induction, and uninduced control (lane 1).
(b) Expressed protein as soluble (lane 2) and insoluble (lane 1).
(c) Purified poly His tagged protein isolated using Ni-NTA spin columns.
(d) Immunoblot of the expressed protein using anti-fliC monoclonal antibody of *Salmonella* Typhimurium.

Antigenic and functional characterisation of recombinant flagellin

The recombinant-fliC was found to be immunogenic as the sera collected from all the three animals that received expressed protein showed specific immunoreactivity on Western blotting analysis. Furthermore, *in vitro* studies of flagellin indicated that it was functional and increased the m-RNA expression levels in the stimulated PBMC. The results indicated that recombinant-fliC induced higher levels of pro-inflammatory mediators TNF-α and GM-CSF when compared to the un-stimulated control wells (Figures 3 and 4).

Discussion

Pathogen associated molecular patterns (PAMPS) like lipopolysaccharides (LPS), flagellin, capsule and other molecules are conserved evolutionarily. They

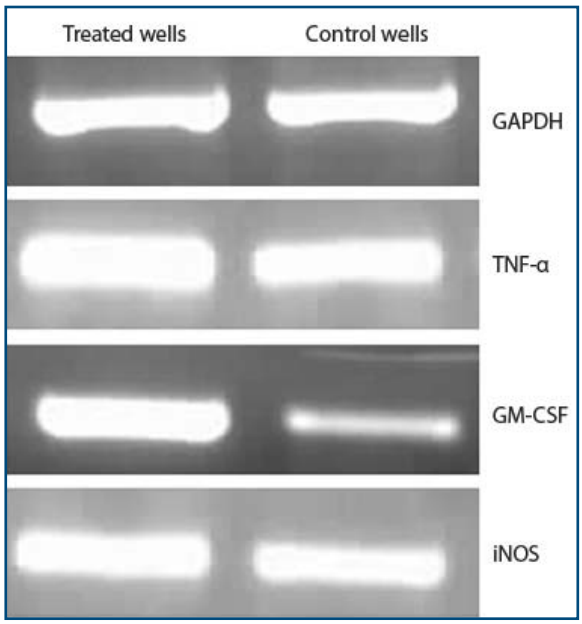

Figure 3. *Semi-quantitative RT-PCR of cytokine genes. PBMCs from healthy guinea pigs were cultured with or without flagellin.* Total RNA was isolated and RT-PCR of various cytokines was done. The bands obtained in flagellin stimulated and control cells were used for determining the relative mRNA levels of TNF-α, GM-CSF and iNOS semi-quantitatively as shown in Figure 4

Veterinaria Italiana 2013, **49** (2), 181-186. doi: 10.12834/VetIt.2013.492.187.192

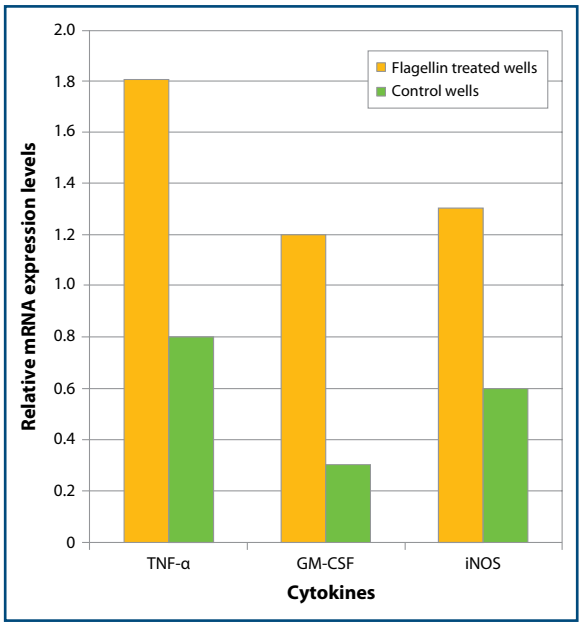

Figure 4. *Relative gene expression analysis of TNF-α and GM-CSF based on the density of various electrophoretic bands for Figure 3 by using band leader 3.0.* The expression levels were evaluated by taking the data of GAPDH bands as the background. TNF-α showed highest mRNA levels.'

are structurally or functionally crucial for survival, growth and/or development of the microorganisms (13, 16). In general, flagellin is mostly associated with Gram-negative bacteria and is required for motility, attachment to host cells, pathogenesis and virulence of bacteria. In particular, the flagellin of *Salmonella* is associated with virulence and immune responses. Flagellin, either in its native or truncated recombinant forms has been successfully used for the diagnosis of Salmonellosis in human beings and also production of monoclonal antibodies (20, 24). Besides, flagellin is a potent and safe adjuvant in inducing early, robust and broad-spectrum immune responses. Owing to its immune augment activity, the present investigation was conceptualised to produce purified form of recombinant whole flagellin of *S.* Typhimurium in prokaryotic system and characterise for its functionality. This will be useful in exploring its diagnostic, ligand and adjuvant activities in the near future in our laboratory.

In the present study, full length fliC gene was expressed in *E. coli*. Expression of such exogenous proteins in *E. coli* have aplenty of deleterious effects on the host cell due to over expressed recombinant protein or its inclusion bodies. This in turn, inhibits the growth of bacteria (7). In order to overcome these problems in this study, the host cell, *E. coli* were grown at 30°C. Upon studying the expression kinetics, it was found that the maximum fliC was expressed 6 h post IPTG induction. Furthermore, most of the expressed protein was in the soluble form even after purification, a requirement in eliciting immune response. The authenticity of the recombinant-fliC protein was confirmed by immune-blotting with anti-fliC MAb of *S.* Typhimurium and anti-sera raised against it.

Flagellin is known for its innate immune agonist activity and it binds to TLR5 receptor (3, 10, 16). Flagellin stimulates the secretion of inflammatory cytokines like TNF-α and GM-CSF. The other additional advantage of fliC is that, contrary to LPS, it does not induce shock (26). This unique feature makes fliC a better adjuvant for vaccines than other adjuvants. Because of this property, fliC has been used with many vaccine preparations to augment the immune responses delivered through different routes (6, 12, 17, 23). Further, the recombinant-fliC was tested for its increased pro-inflammatory activity in terms of expression of TNF-α and GM-CSF genes in PBMCs *in vitro*. The relative expression levels of these mediators were quantified semi-quantitatively by RT-PCR, which revealed that the most prominent change was on TNF-α. GM-CSF and that TNF-α have pleiotropic effects and induce general immune stimulation *in vivo*. TNF-α is a key molecule in coordinating the inflammatory response and activation of cytokine cascade while GM-CSF is responsible for proliferation and maturation of neutrophils, macrophages and dendritic cells (14, 18). In conclusion, the recombinant-fliC is immunogenic in guinea pig and known to induce pro-inflammatory cytokines. In the near future the expressed fliC will be explored for its various adjuvant and TLR ligand activities, particularly with FMD vaccine preparations, at the authors' laboratory.

Acknowledgments

The authors are very thankful to the Joint Director, IVRI in Bangalore, for providing necessary facilities and also Dr. V. Balamurugan, Senior Scientist, Project Directorate on Animal Disease Monitoring and Surveillance, H A Farm, Hebbal, Bangalore for providing *E. coli* BL21 cells.

References

1. Allen-Vercoe E. & Woodward M.J. 1999. Adherence of *Salmonella enterica* serovar Enteritidis to chick gut explant: the role of flagella but not fimbriae. *J Med Microbiol*, **48**, 771-780.
2. Banchereau J., Briere F., Caux C., Davoust J., Lebecque S., Liu Y., Pulendran B. & Palucka K. 2000. Immunobiology of dendritic cells. *Ann Rev Immunol*, **18**, 767-811.
3. Barton G.M. & Medzhitov R. 2002. Control of adaptive immune responses by Toll-like receptors. *Curr Opin Immunol*, **14**, 380-383.
4. Boyum A. 1968. Separation of leukocytes from blood and bone marrow with special reference to factors which influence and modify sedimentation properties of haematopoietic cells. *Scand J Clin Lab Invest Suppl*, **97**, 77-89.
5. Bradford M.M. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248-254.
6. Cuadros C., Lopez-Hernandez F.J., Dominguez A.L., McClelland M. & Lustgarten J. 2004. Flagellin fusion proteins as adjuvants or vaccines induce specific immune responses. *Infect Immun*, **72**, 2810-2816.
7. Dabrowski S. & Kur J. 1998. Cloning and expression in *Escherichia coli* of the recombinant His-tagged dna polymerases from *Pyrococcus furiosus* and *Pyrococcus woesei*. *Protein Expr Purif*, **4**, 131-138.
8. Didierlaurent A., Ferrero I., Otten L.A., Dubois B., Reinhardt M., Carlsen H., Blomhoff R., Akira S., Kraehenbuhl J.P. & Sirard J.C. 2004. Flagellin promotes myeloid differentiation factor 88-dependent development of Th2-type response. *J Immunol*, **172**, 6922-6930.
9. Eaves-Pyles T.D., Wong H.R., Odoms K. & Pyles R.B. 2001. *Salmonella* flagellin dependent proinflammatory responses are localized to the conserved amino and carboxyl regions of the protein. *J Immunol*, **167**, 7009-7016.
10. Hayashi F., Smith K.D., Ozinsky A., Hawn T.R., Yi E.C., Goodlett D.R., Eng J.K., Akira S., Underhill D.M. & Aderem A. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*, **410**, 1099-1103.
11. Hiroyuki Y., Tadashi U., Satoru M., Kumiko H. & Isamu S. 2005. Newly designed primer sets available for evaluating various cytokines and iNOS Mrna expression in guinea pig lung tissues by RT-PCR. *Exp Anim*, **54**, 163-172.
12. Hong S.H., Byun Y., Nguyen C.T., Kim S.Y., Seong B.L., Park S., Woo G., Yoon Y., Koh J.T., Fujihashi K. & Rhee J.H. 2011. Intranasal administration of a flagellin-adjuvanted inactivated influenza vaccine enhances mucosal immune responses to protect mice against lethal infection. *Vaccine*, **30**, 466-474.
13. Janeway C.A. Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symp Quant Biol*, **54**, 1-13.
14. Kielian T., Nagai E., Ikubo A., Rasmussen C.A. & Suzuki T. 1999. Granulocyte/macrophage-colony-stimulating factor released by adenovirally transduced CT26 cells leads to the local expression of macrophage inflammatory protein 1alpha and accumulation of dendritic cells at vaccination sites *in vivo*. *Cancer Immunol Immunother*, **48**, 123-131.
15. Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophages T4. *Nature*, **227**, 680-685.
16. Medzhitov R. & Janeway C.A. Jr. 2000. Innate immune recognition: mechanisms and pathways. *Immunol Rev*, **173**, 89-97.
17. Moigne V.L., Robreau G. & Mahana W. 2008. Flagellin as a good carrier and potent adjuvant for Th1 reponse: Study of mice immune response to the p27 (Rv2108) *Mycobacterium tuberculosis* antigen. *Mol Immunol*, **45**, 2499-2507.
18. Pulendran B., Smith J.L. & Caspary G. 1999. Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. *Proc Natl Acad Sci USA*, **96**, 1036-1041.
19. Reichhart J.M. 2003. TLR5 takes aim at bacterial propeller. *Nat Immunol*, **4**, 1159-1160.
20. Sadallah F., Brighouse G., Del-Giudice G., Drager-Dayal R. & Hodne M. 1990. Production of specific monoclonal antibodies to *Salmonella typhi* flagellin and possible application to immunodiagnosis of typhoid fever. *J Infect Dis*, **161**, 59-64.
21. Sambrook J. & Russell D.W. 2001. Molecular cloning: a laboratory manual, Third ed. Cold Spring Harbor laboratory Press, Cold Spring Harbor, New York, pp. 116-118.
22. Sanders C.J., Yu Y., Moore D.A., Williams III I.R. & Gewirtz A.T. 2006. Humoral immune response to flagellin requires T cells and activation of innate immunity. *J Immunol*, **177**, 2810-2818.
23. Skountzou I., Mdel P., Martin B., Wang L., Koutsonanos Y.D., Weldon W., Jacob J. & Compans R.W. 2010. *Salmonella* flagellins are potent adjuvants for intranasally administered whole inactivated influenza vaccine. *Vaccine*, **28**, 4103-4112.
24. Sukosol T., Sarasombath S., Songsivilai S., Ekpo P., Rungpitarangsi B. & Pang T. 1994. Fusion protein of *Salmonella typhi* flagellin as antigen for diagnosis of typhoid fever. *Asian Pacific J Allergy Immunol*, **12**, 21-25.
25. Tallant T., Deb A., Kar N., Lupica J., Veer M.J.D. & DiDonato J.A. 2004. Flagellin acting via TLR5 is the major activator of key signaling pathways leading to NF-κB and proinflammatory gene program activation in intestinal epithelial cells. *BMC Microbiol*, **4**, 33. doi:10.1186/1471-2180-4-33.
26. Vijay-Kumar M., Aitken J.D., Sanders C.J., Frias A., Sloane V.M., Xu J., Neish A.S., Rojas M. & Gewirtz A.T. 2008. Flagellin treatment protects against chemicals, bacteria, viruses, and radiation. *J Immunol*, **180**, 8280-8285.