

Blocking of the TLR5 Activation Domain Hampers Protective Potential of Flagellin DNA Vaccine¹

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Flagellin is a key component of the flagella of many pathogens, including *Pseudomonas aeruginosa*. Flagellin is an attractive vaccine candidate because it is readily produced and manipulated as a recombinant protein and has intrinsic adjuvant activity mediated through TLR5. Although DNA vaccines encoding native *Pseudomonas* B-type (FliC) or A-type (FlaA) flagellin are strongly immunogenic, the resultant Ab response interferes with the interaction of homologous flagellin with TLR5. This reduces the ability of the host to clear homologous, but not heterologous, flagellin-expressing *P. aeruginosa*. To circumvent this problem, a DNA vaccine encoding a mutant FliC R90A flagellin was developed. The mutant Ag encoded by this vaccine was highly immunogenic, but its ability to interact with TLR5 was reduced by >100-fold. Vaccination with this flagellin mutant DNA vaccine induced cross-reactive Abs against both FliC and FlaA, but few Abs capable of interfering with TLR5 activation. The flagellin mutant DNA vaccine provided excellent protection against both FliC- and FlaA-expressing *P. aeruginosa*. These findings suggest that vaccines against flagellated pathogens should avoid inducing Abs against TLR5 and raise the possibility that flagellated bacteria evade host elimination by facilitating the production of Abs that reduce the host's ability to mount an innate immune response. *The Journal of Immunology*, 2007, 179: 1147–1154.

Flagella are conserved organelles that play a critical role in providing motility to diverse bacterial species, including *Pseudomonas aeruginosa* (1). Reducing flagellar activity restricts motility, and thus limits an organism's ability to invade and effectively colonize a new host (2). Flagella are effectively targeted by the host immune system, and bacteria that delete or mutate their flagella can cause severe persistent infection (as in the case of *Legionella pneumonia* and *Salmonella typhimurium*) (3, 4).

Flagellin, a key protein component of flagella, can also trigger an innate immune response by interacting with a cell surface receptor TLR5 (5). The domains of flagellin involved in interacting with TLR5 have been investigated. The NH₂- and COOH-terminal regions of flagellin are well conserved among various Gram-neg-

ative bacteria (6). In studies of *S. typhimurium* FliC and *Salmonella muenchen* flagellin, deletion of either the NH₂- or COOH-terminal regions abolishes the interaction with TLR5. A single amino acid substitution in either region results in a loss of proinflammatory activity, suggesting that the structure at both ends of the molecule contribute to TLR5 interactions (7, 8). Characterization of flagellin chimeras containing domains from "active" (*Salmonella*) and "inactive" (*Helicobacter*) types demonstrated that type-specific NH₂- and COOH-terminal domains (particularly aa 89–96 at the NH₂ terminus) play a critical role in TLR5 activation. Similarly, aa 83–97 in the NH₂ terminus of *P. aeruginosa* A-type flagellin (FlaA)⁴ was essential for TLR5 activation (9). Taken together, these findings suggest that the tertiary structure generated by domains in both the NH₂ and COOH terminus regions are critical for TLR5 activation by flagellin.

Activating TLR5 expressed by epithelial cells, macrophages, or dendritic cells initiates a signaling cascade mediated through an adaptor molecule, MyD88, IL-1R-associated kinase, TNFR-associated factor 6, and I κ B kinase. This process culminates in the stimulation of innate immune responses characterized by the production of proinflammatory cytokines including TNF- α , IL-6, IL-10, and IL-12 and cell surface molecules including MHC, CD40, B7.1, and B7.2. Activation of this cascade has been shown to improve the induction of vaccine-specific adaptive immune responses (10–20).

Based on these findings, it was postulated that flagellin could be a very effective vaccine candidate. Flagellin Ags should trigger an adaptive immune response that targets multiple pathogens, while the NH₂- and COOH-terminal domains of the protein should act as intrinsic adjuvants, stimulating a TLR5-dependent innate immune response. Although there have been reports of full-length flagellin being used to induce the production of anti-flagellar Abs (21, 22),

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Received for publication March 26, 2007. Accepted for publication May 5, 2007.

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¹ This work was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and the Yokohama Medical Foundation (Yokohama City University Center of Excellence Program to N.J., 18590432 to F.T., and 18659126 to K.O.), the National Institute of Biomedical Innovation (to K.O.), Strategic Research Project of Yokohama City University (K18022 to F.T.), Advancement of Medical Sciences from Yokohama Medical Foundation (to F.T.), and Japan Science and Technology Agency (131-1 to F.T.).

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⁴ Abbreviations used in this paper: FlaA, A-type flagellin; WT, wild type; FliC, B-type flagellin; p.i., postinfection; MPO, myeloperoxidase.

there are no reports of full-length TLR5-activating flagellin protecting against subsequent bacterial infection. Indeed, vaccination studies indicate that flagellin-deficient mutant *Salmonella* induce superior protection when compared with wild-type (WT) parental bacteria (23, 24). Yet, when anti-flagellar Abs were raised by immunization with truncated forms of *P. aeruginosa* flagellin or a component vaccine targeting *Campylobacter jejuni* flagellin, protection against lethal doses of *P. aeruginosa* and *C. jejuni* was again observed (25, 26). These findings, and preliminary studies in our laboratory, suggested that full-length flagellin protein capable of interacting with TLR5 was limited in its ability to induce a protective immune response.

To clarify the mechanisms underlying the protective potential of flagellin, a series of DNA vaccines encoding FlaA and B-type flagellins (FliC) from different serotypes of *P. aeruginosa* (PAK and PAO1) (27) were generated and characterized in a murine model of acute pneumonia. Results show that these flagellin vaccines efficiently protected mice against heterologous, but not homologous, bacterial challenge. Each flagellin vaccine raised Abs that cross-reacted with both types of flagellin, but also Abs that prevented TLR5 activation in a type-specific manner (thereby hampering innate bactericidal activity). A mutagenized form of flagellin (FliC R90A) that retained the immunogenicity of the parent molecule, but had significantly reduced ability to interact with TLR5, conferred optimal immune responses against infection by both strains of *P. aeruginosa*.

Materials and Methods

Bacterial strains and culture conditions

P. aeruginosa strains PAO1 and PAK were used for this study. PAO1 and PAK express different virulence factors, such as pili, flagella, and type III-secreted exoenzymes. All bacterial strains were cultured in Terrific Broth (Sigma-Aldrich) at 37°C for 16 h. For the intranasal challenge, the bacterial culture was centrifuged at $3000 \times g$ for 10 min, and the pellet was washed twice in PBS and resuspended in PBS at an OD of 1.0 at 550 nm. Thirty or 40 μ l of this suspension corresponded to ~ 2 LD₅₀ doses for both PAO1 and PAK.

Construction of mammalian expression plasmids for vaccination

Genomic DNA of the *P. aeruginosa* strain PAO1 and PAK were prepared as described previously (28). The *fliC* of PAO1 and *flaA* of PAK were PCR amplified and introduced into the mammalian expression plasmid pFLAG-CMV5 (Sigma-Aldrich) to obtain *fliC* and *flaA* genes fused to the FLAG signal sequence. The DNA fragments of *fliC*-FLAG and *flaA*-FLAG were then introduced into the *EcoRI* site of the pGACAG vector (29) to obtain pGACAG-FliC and pGACAG-FlaA. All of the plasmids were transformed into *Escherichia coli* DH5 α and purified using the Endo-free Maxiprep kit (Qiagen).

Generation of flagellin mutants

Several single alanine point mutations both at the NH₂ (L88A, R90A, and Q97A) and COOH (V404A, R415A, Q422A, N423A, and F425A) termini were introduced by a standard PCR-mutagenesis strategy (30). All mutations were verified by DNA sequencing. Recombinant proteins were expressed in *E. coli* DH5 α and purified as described elsewhere (31). Contaminated endotoxins were removed using Detoxi-Gel (Pierce). Finally, the amount of contaminated endotoxin was <0.003 ng/mg protein.

Immunization and challenge schedule

Eight-week-old female BALB/c, C57BL/6 TLR5^{+/-}, or TLR5^{-/-} mice (5) were used for this study. After being anesthetized with a ketamine and xylazine mixture, the mice (eight mice per group) were immunized with the plasmid solution (1 μ g/ μ l saline, 50 μ l/muscle) containing 50 μ g pGACAG (control empty plasmid), pGACAG-FliC, pGACAG-FliCR90A, or pGACAG-FlaA by an i.m. electroporation method as described previously (32). A booster immunization was given at 4 wk after the first immunization. Two weeks after the final immunization, the mice were anesthetized and challenged intranasally with 2 LD₅₀ of PAO1 or PAK. The mortality

of the challenged mice was monitored for the subsequent 10 days. All of the animal experiments were approved by the institutional animal care and welfare committee, and the mice were treated according to the institutional animal care guidelines.

Immunoblotting

Specificity of serum IgG was analyzed by using immunoblots of flagella prepared from PAO1 and PAK as described previously (29, 33). After separation by SDS-PAGE, samples were blotted on polyvinylidene difluoride membranes. The membranes were blocked with 10% skim milk and then reacted with mouse sera from various groups of vaccinated mice. After incubation with HRP-labeled anti-mouse IgG Ab, the blots were reacted with ECL plus substrate (Amersham Biosciences) and then analyzed by autoradiography.

Purification of flagella

Flagella was isolated and purified from the PAO1 and PAK strains of *P. aeruginosa* according to the method described previously (34), with some modifications. *P. aeruginosa* PAO1 or PAK was cultured at 37°C for 14 h and centrifuged at $10,000 \times g$ for 15 min. Over a period of 2 h, ammonium sulfate was added to the supernatant to reach 40% saturation. After centrifugation at $20,000 \times g$ for 40 min, the pellets were resuspended in 8 ml of 50 mM Tris-HCl (pH 9.5) containing 0.5 mM DTT and then dialyzed extensively against the same buffer. After centrifugation at $27,000 \times g$ for 40 min, the pellet (flagella) was resuspended in PBS, and stored at -80°C until use.

ELISA

Flagella-specific Ab was determined by ELISA as described previously (32). Flat-bottom 96-well plates (Nunc) were coated with flagella diluted to 2 μ g/ml in bicarbonate buffer (pH 9.6; FliC or FlaA) at 4°C for 18 h. Plates were then blocked with PBS containing 0.05% Tween 20 and 0.5% BSA for 2 h and incubated with appropriately diluted serum 37°C for 2 h. Plates were washed three times with PBST and incubated with HRP-labeled anti-mouse IgG at 37°C for 2 h. After washing, substrate buffer containing urea peroxidase and tetramethylbenzidine (Sigma-Aldrich) was added and incubated at 25°C for 15 min. The plates were read using a spectrophotometer (Benchmark Plus; Bio-Rad) at an OD of 405 nm, and the titer was generated using a high-standard antisera. TNF- α levels in the bronchoalveolar lavage fluid were measured according to the manufacturer's protocol (eBioscience).

Quantification of *P. aeruginosa* in the lung

Two weeks after final immunization, the mice were anesthetized and inoculated intranasally with 5×10^5 CFU of PAO1 or PAK in 30 μ l of PBS. Twenty-four hours postinfection (p.i.), the mice were sacrificed under a CO₂-rich atmosphere, and then the lungs were removed and homogenized in 4 ml of PBS under sterile conditions. Serial 10-fold dilutions of lung homogenates were plated on Luria-Bertani broth agar plates (Sigma-Aldrich), incubated at 37°C for 24 h, and then the number of colonies was enumerated (29).

Myeloperoxidase (MPO) assay

The MPO assay was used to determine the levels of neutrophil infiltration into the lung as described previously (35). In brief, the lungs were homogenized in 50 mM HEPES buffer (4 μ l/mg lung) and centrifuged at $20,000 \times g$ at 4°C for 30 min. The pellet was suspended and homogenized in 0.5% cetyltrimethylammonium chloride (4 μ l/mg lung tissue). The pre-clear extract was collected and samples in duplicate (75 μ l) were mixed with equal volumes of the substrate buffer containing 3 mM 3,3',5,5'-tetramethylbenzidine dihydrochloride, 120 μ M resorcinol, and 2.2 mM H₂O₂ for 2 min. The reaction was stopped by adding 150 μ l of 2 M H₂SO₄ and the OD of 450 nm was measured.

Histopathology of lungs

After being intratracheally inoculated with 4% paraformaldehyde, the lungs were removed and fixed in 4% paraformaldehyde for 24 h. Frozen sections (thickness, 15 μ m) were subjected to H&E staining and then analyzed under microscopy.

Luciferase assay

Luciferase assay was performed according to a previously described method (36). In brief, HEK293 cells grown in 96-well plates were transiently transfected with 40 ng of mouse or human TLR5 expression plasmid in the presence of 5 ng of pNF- κ B-Luc (Stratagene) and pTK-RL

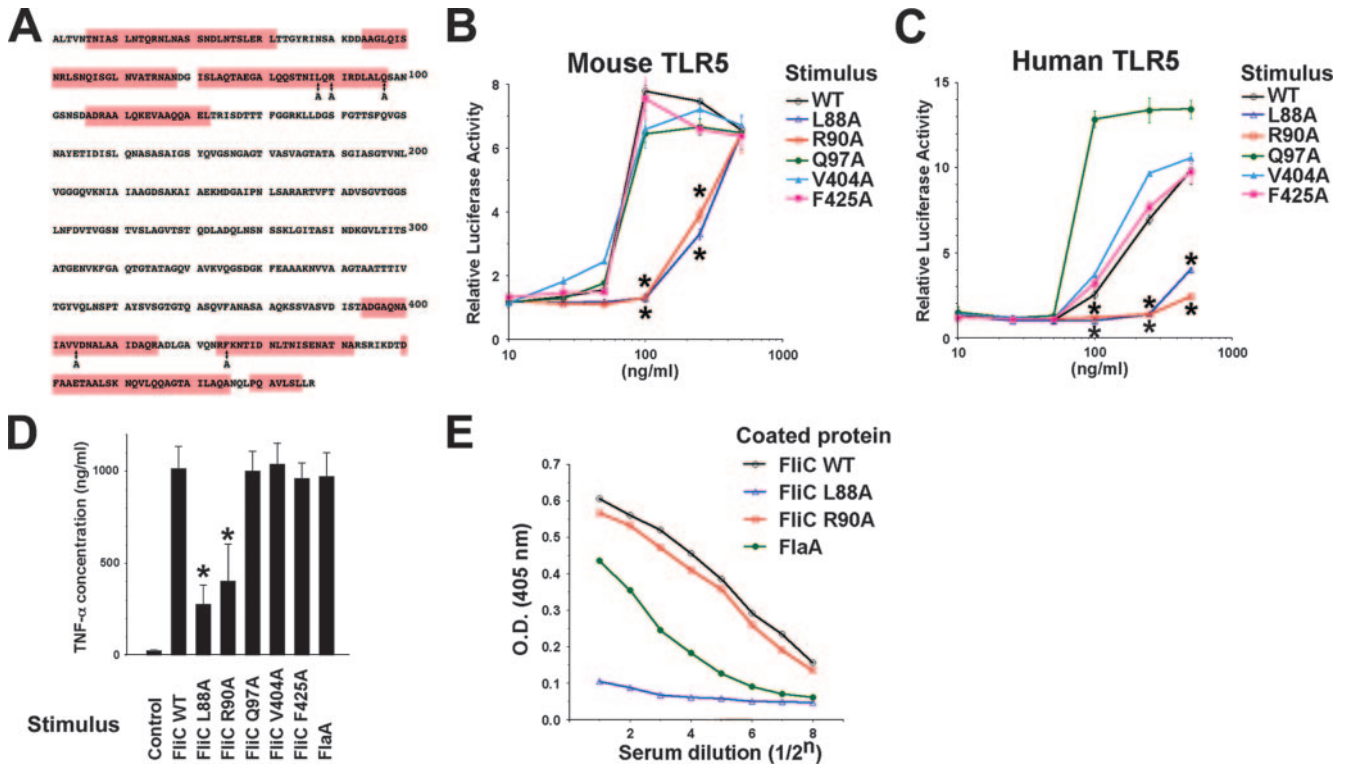


FIGURE 1. Characterization of flagellin mutants. *A*, Amino acid sequence of *P. aeruginosa* FliC. The sequences shaded with red indicate the regions from α helix whose intramolecular conformation corresponds to the TLR5 activation domain in *S. typhimurium* FliC. *B* and *C*, HEK293 cells were transiently transfected with either mouse or human TLR5 expression plasmid plus NF- κ B-dependent luciferase expression plasmid. Cells were then stimulated with FliC WT or mutants (L88A, R90A, Q97A, V404A, or F425A) at the concentration of 10–500 ng/ml, and luciferase activity was measured. The graph shows the mean \pm SD; *, $p < 0.01$ by Student's *t* test. *D*, Mice were intranasally inoculated with PBS alone (control) or 1 μ g of the recombinant protein of FliC WT or mutants (L88A, R90A, Q97A, V404A, or F425A). Four hours after inoculation, the bronchoalveolar lavage fluid was collected and TNF- α concentration was measured by ELISA. Data show the mean \pm SE, $n = 6$ mice/group. *, $p < 0.05$ by Student's *t* test. *E*, Reactivity of anti-FliC WT IgG raised in BALB/c mice with the recombinant protein of FliC WT, L88A, R90A, or FlaA was examined by ELISA. Two or three independent experiments gave similar results.

(Promega) using FuGENE 6 reagent (Roche Diagnostic Systems). Eighteen hours after transfection, the cells were washed twice and subsequently stimulated with different concentrations of recombinant flagellin (0–1000 ng/ml) in the presence or absence of mouse IgG purified from vaccinated

mice for 6 h. Cells were then washed and lysed in Passive Lysis Buffer (Promega). Luciferase assays were performed using a Dual-Luciferase Reporter Assay System (Promega) and analyzed with a luminometer (Veritas; Promega).

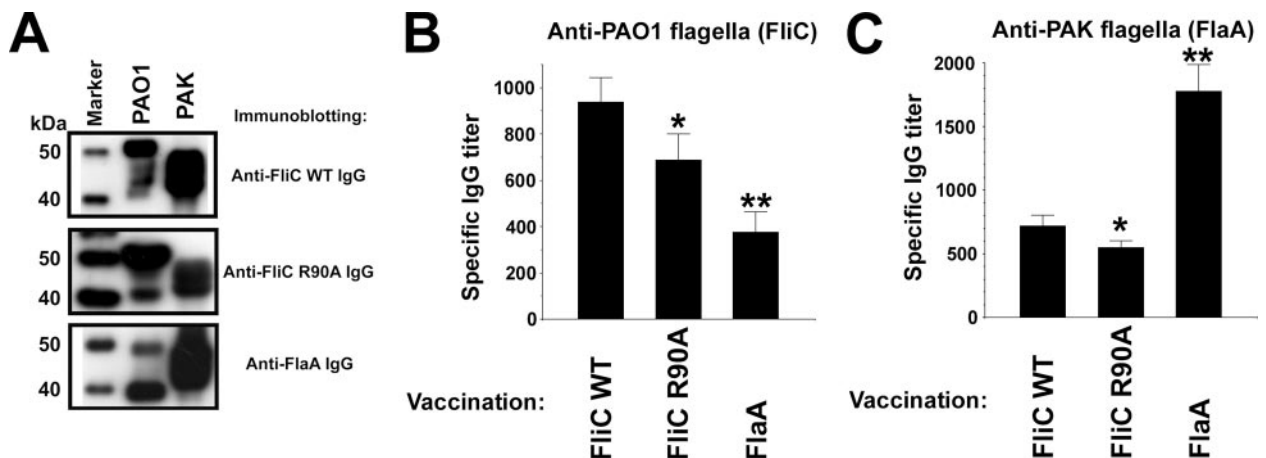


FIGURE 2. Immunogenicity of flagellin DNA vaccines. *A*, Cross-reactivity of IgG raised by FliC WT, R90A, or FlaA was examined by immunoblot analysis using flagella prepared from PAO1 or PAK endogenously expressing FliC or FlaA, respectively. For PAO1 blots, the proximal band corresponds to the size estimated from the amino acid sequence, whereas distal one was supposed to be a degraded product. *B–E*, BALB/c mice were immunized twice (0 and 3 wk) with either 50 μ g of pGACAG (vector), pGACAG-FliC WT, pGACAG-FliC R90A, or pGACAG-FlaA via i.m. electroporation. Blood was drawn 6 wk after the first immunization. Serum IgG titers against FlaA (*C*) or FliC (*B*) purified from PAK or PAO1, respectively, were determined by ELISA. The graph shows the mean \pm SD, $n = 8$ mice/group; *, $p < 0.05$ by Student's *t* test. Three independent experiments gave similar results.

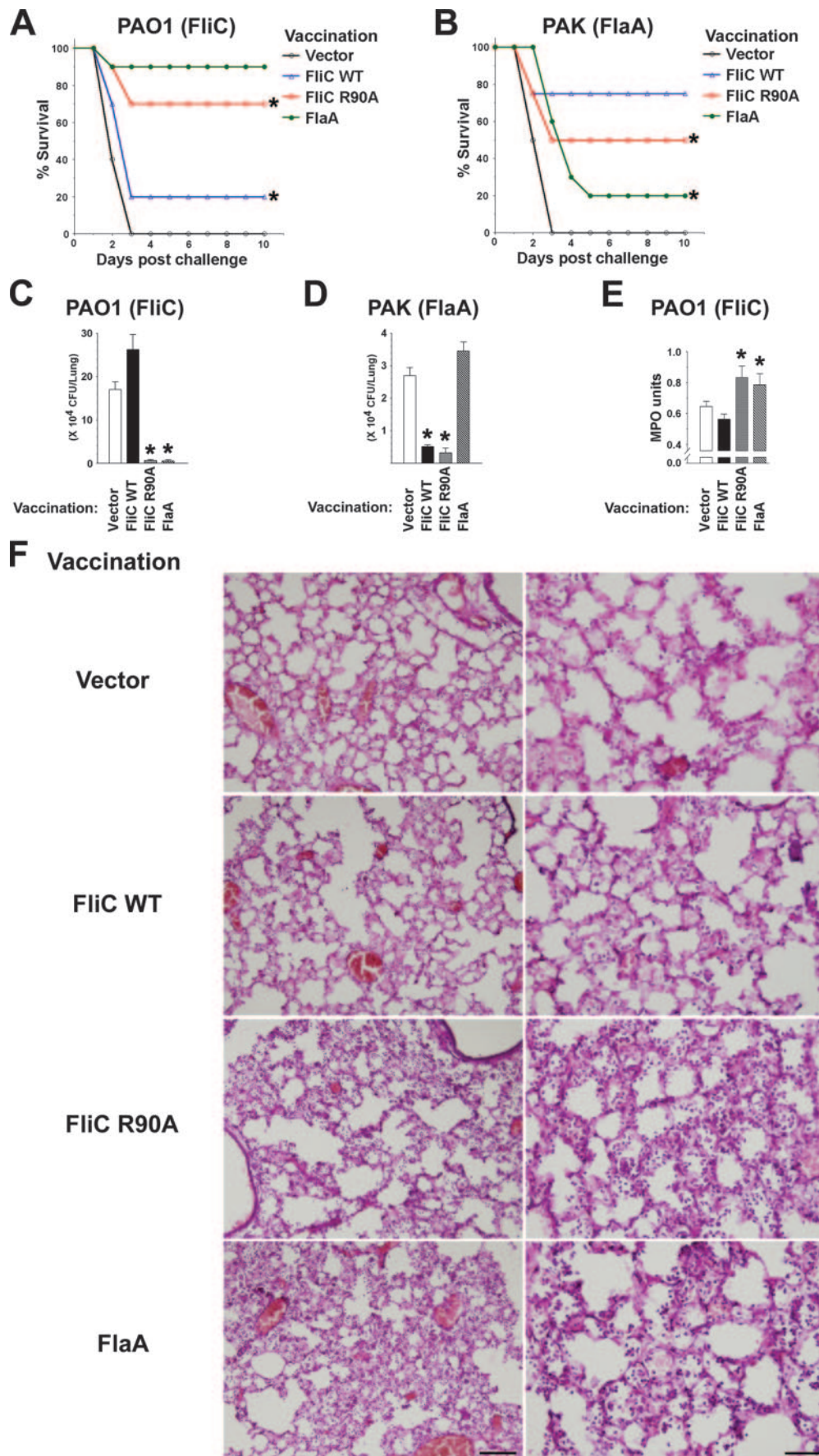
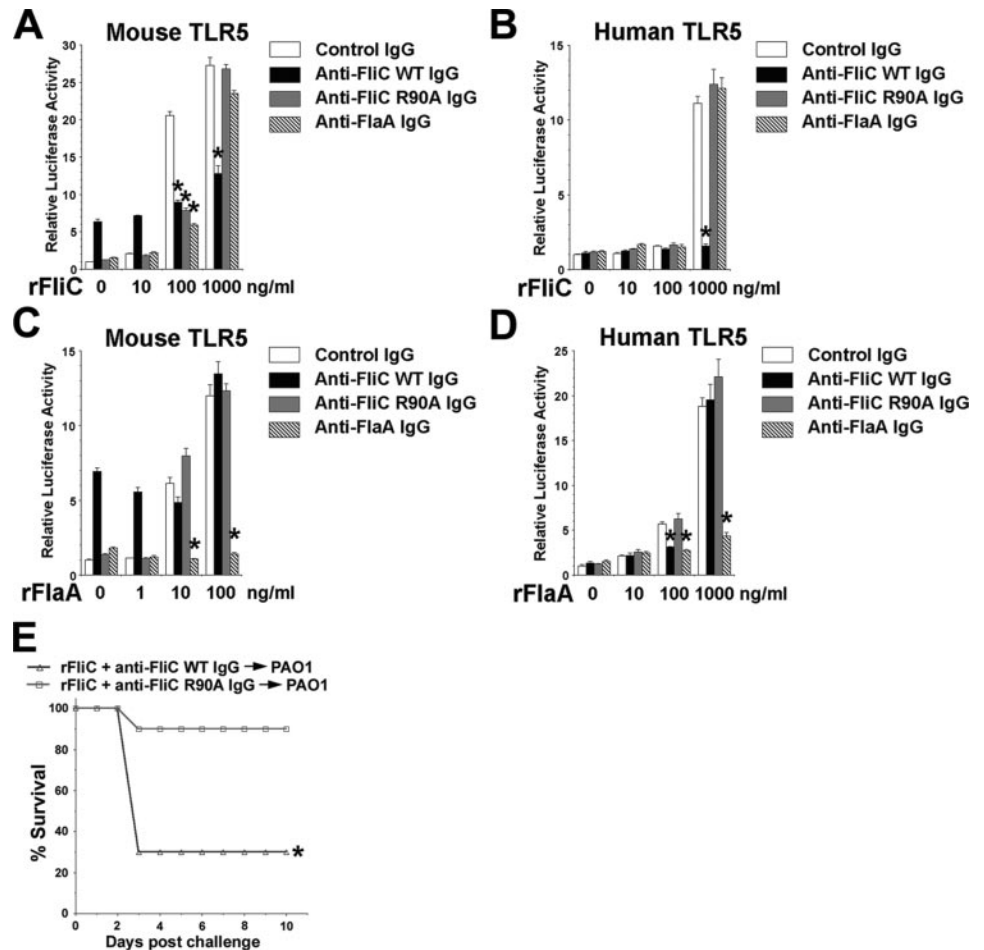


FIGURE 3. Protective potential of flagellin DNA vaccines. Mice were immunized as described in Fig. 2. *A* and *B*, Survival rate was monitored following intranasal challenge with 2 LD₅₀ doses of PAO1 or PAK. Three independent experiments gave similar results; *, $p < 0.05$ by Mantel-Cox log rank test. *C* and *D*, Two weeks after final immunization, mice were challenged intranasally with 5×10^5 CFU of PAO1 or PAK. Twenty-four hours p.i., mice were

FIGURE 4. Type-specific anti-flagellin Ab inhibits the TLR5 activation by homologous, but not heterologous, type of flagellin. *A–D*, HEK293 cells were transiently transfected as described in Fig. 1. The cells were stimulated with different concentrations of either recombinant FliC or FlaA in the presence of either 10 $\mu\text{g}/\text{ml}$ control, anti-FliC WT, FliC R90A, or FlaA IgG, and luciferase assay was performed. Three independent experiments gave similar results; *, $p < 0.01$ by Student's *t* test. *E*, Two micrograms of rFliC was preincubated with 10 μg of anti-FliC WT or R90A IgG at 37°C for 30 min. BALB/c mice were inoculated intranasally with this mixture. Four hours after inoculation, mice were challenged with 2 LD₅₀ doses of PAO1, and the survival rate was monitored for the next 10 days, $n = 10$ mice/group; *, $p < 0.01$ by Mantel-Cox log rank test. Similar results were obtained in three independent experiments.



Cytoprotective assay

The cytoprotective effect of flagellin was performed according to the method described previously, with a little modification (4). Six-week-old female BALB/c mice were used. Purified flagellin (2 μg) was preincubated with 10 μg of each purified IgG at 37°C for 30 min. The mice (eight mice per group) were then inoculated intranasally with this mixture. Four hours after inoculation, the mice were challenged with 2 LD₅₀ doses of *P. aeruginosa* PAO1, and mortality was monitored for the subsequent 10 days.

Statistical analysis

ANOVA Bonferroni multiple comparison test and Mantel-Cox log rank test were used for the analysis of data. A value of $p < 0.05$ was considered as statistically significant.

Results

Characterization of flagellin FliC mutants for their TLR5 engagement

To examine the contribution of TLR5 activation by flagellin to its immunogenicity and protective potential against *P. aeruginosa* infection, TLR5 activation-null mutants (inactive flagellin) were designed. Previous studies showed that TLR5 activation by the FliC of *S. typhimurium* or FlaA of *P. aeruginosa* is mediated by a domain composed of the NH₂- and COOH-terminal regions forming several α helices (7, 9). Based on this information, five *Pseudo-*

monas FliC mutants were generated by replacing a single amino acid in each α helical region with an alanine (Fig. 1A). Each recombinant protein was purified under endotoxin-free conditions and characterized for its ability to trigger through TLR5. HEK293 cells were transfected with a mouse or human TLR5 expression plasmid plus an NF- κ B-dependent luciferase reporter plasmid. These cells responded to stimulation with full-length flagellin, but not with LPS, peptidoglycan, or recombinant GST (Fig. 1, B and C, and data not shown). Of interest, the mutagenized L88A and R90A mutants triggered TLR5-dependent NF- κ B activation >100-fold less efficiently than WT FliC ($p < 0.01$).

The biological activity of these flagellar proteins was then examined. Intranasal inoculation of recombinant FliC WT, FliC Q97A, FliC V404A, FliC F425A, or FlaA WT strongly up-regulated the pulmonary production of TNF- α (Fig. 1D). By comparison, the level of TNF- α in bronchoalveolar lavage fluids from mice treated with FliC L88A or FliC R90A was significantly reduced, suggesting that these mutants are inactive flagellin for TLR5-mediated proinflammatory responses ($p < 0.05$; Fig. 1D).

To determine whether these mutants retained their antigenic epitopes, the ability to bind IgG Abs raised against FliC was examined by ELISA. Results indicate that anti-flagellin Abs bound equally well to FliC WT and R90A, but not to L88A. Anti-FliC

sacrificed and the lung was removed. *C* and *D*, The number of live bacteria in the lung homogenate was enumerated. The graph shows the mean \pm SD, $n = 5$ mice/group. Similar results were obtained in three independent experiments; *, $p < 0.0001$ by Student's *t* test. *E*, MPO activity in the lung homogenates was determined at 24 h following intranasal challenge with PAO1. *F*, Lung tissue inflammation was analyzed at 24 h following intranasal challenge with PAO1. The lung sections were stained with H&E. Bar, 100 μm (left) and 50 μm (right), respectively. The graph shows the mean \pm SD values, $n = 5$ mice/group; *, $p < 0.05$ by Student's *t* test. Similar results were obtained in three independent experiments.

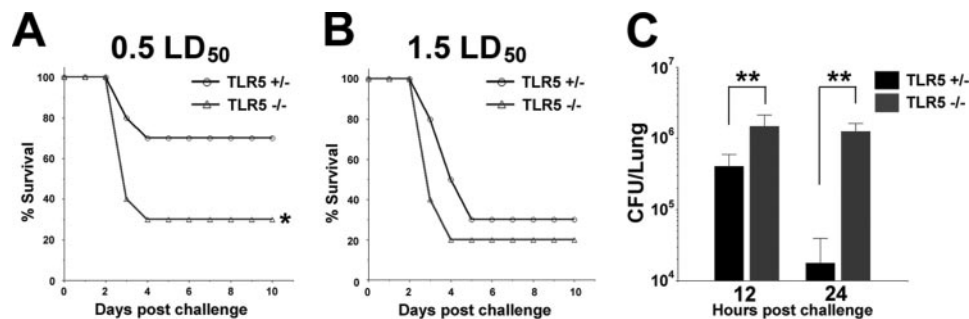


FIGURE 5. TLR5 plays crucial roles in the host's bactericidal activity against *P. aeruginosa*. TLR5-deficient (TLR5^{-/-}, blue) or control (TLR5^{+/-}, black) mice were challenged with 0.5 LD₅₀ (A and C) or 1.5 LD₅₀ (B) doses of the *P. aeruginosa* strain PAO1. A and B, Survival rate was monitored for 10 days following intranasal challenge. C, Twelve or 24 h p.i., mice were sacrificed and the lungs were removed, *n* = 10 mice/group. The number of live bacteria in the lung homogenate was enumerated. The graph shows the mean ± SD; *, *p* < 0.05; **, *p* < 0.01 by Student's *t* test. Two independent experiments gave similar results.

serum also cross-reacted with recombinant FlaA, but with lower affinity (Fig. 1E). These findings suggest that FliC R90A, but not L88A, retained the antigenic conformation of flagellin. Taken together, these findings indicate that FliC R90A is antigenic but triggers via TLR5 less well than WT flagellar proteins (inactive flagellin).

Immunogenicity of DNA vaccines encoding active or inactive flagellin (FliC WT, R90A, or FlaA)

Mice were immunized and boosted with DNA vaccines encoding active or inactive flagellin (FliC WT, FliC R90A, or FlaA). Sera from these animals were studied for reactivity with flagellar proteins from the PAO1 and PAK strains of *P. aeruginosa* (expressing FliC and FlaA, respectively). Sera from unvaccinated mice did not react with either protein (data not shown). Sera from all of the immunized mice reacted with both types of flagella, suggesting that each vaccine induced Abs capable of cross-reacting with both types of flagellin (Fig. 2A). Of interest, serum raised against FlaA reacted better to the degraded or an alternative form (Fig. 2A, lower band) than full-length FliC (Fig. 2A, upper band). This may reflect recognition of some cryptic epitopes of FliC by anti-FlaA Abs.

The magnitude of each serum IgG response was quantitated by ELISA (Fig. 2, B and C). After boosting, significant IgG anti-PAO1 (FliC) and anti-PAK flagella (FlaA) responses were present in all vaccination groups. The FliC R90A DNA vaccine induced the weaker IgG responses against both types of flagella compared with those by FliC WT vaccine (*p* < 0.05; Fig. 2, B and C), suggesting either that the mutation introduced into R90A affected its immunogenicity or that TLR5-mediated activation of the innate immune system contributed to maximizing the anti-flagellar response elicited by vaccines encoding WT flagellin.

Protective activity of DNA vaccines targeting flagellin

The capacity of each DNA vaccine to protect against *P. aeruginosa* infection was assessed by challenging mice intranasally with the PAO1 or PAK strain of bacteria (Fig. 3, A and B). Unexpectedly, mice vaccinated with FlaA were better protected against PAO1 (which expresses FliC) than PAK (which expresses FlaA), whereas mice vaccinated with the FliC DNA vaccine were better protected against PAK than PAO1. These findings suggest that each flagellar vaccine elicited stronger protection against bacteria expressing the heterologous type of flagellin. In contrast, the DNA vaccine targeting the inactive flagellin FliC R90A induced strong protection against both strains of *P. aeruginosa* (Fig. 3, A and B).

The effect of each DNA vaccine on host susceptibility to bacterial infection was further evaluated by examining bacterial persistence and neutrophil recruitment in the lung following intranasal infection with a sublethal dose of *P. aeruginosa*. Consistent with data from the protection studies, mice immunized with DNA vaccines encoding FlaA or FliC R90A efficiently eliminated PAO1, whereas mice immunized with the FliC WT or R90A vaccines efficiently eliminated PAK (Fig. 3, C and D). When neutrophil recruitment was examined by monitoring MPO activity, both FliC R90A and FlaA vaccines elicited stronger MPO activity than FliC WT following challenge with PAO1 (Fig. 3E). The lung inflammation associated with *P. aeruginosa* infection was also assessed histopathologically. When mice were challenged with PAO1, the lung inflammation was characterized by massive neutrophil infiltration among mice vaccinated with FliC R90A or FlaA relative to those vaccinated with vector or FliC WT (Fig. 3F). Taken together, these findings raise the interesting possibility that the presence of a homologous TLR5 activation domain in a flagellin-based vaccine hampers the induction of protective inflammatory responses against homologous, but not heterologous, strains of bacteria.

Anti-flagellin Ab blocks TLR5 activation in a type-specific manner

To clarify the mechanism underlying the lower protection against bacteria-expressing flagellin homologous to that present in the vaccine, sera from immunized mice were analyzed for their ability to block TLR5 activation. Recombinant flagellin dose-dependently stimulated both mouse and human TLR5 in the presence of control IgG (Fig. 4, A–D). Anti-FliC WT, but neither FliC R90A nor FlaA IgG, significantly suppressed the activation by a higher concentration of rFliC (1000 ng/ml, *p* < 0.001; Fig. 4A). A similar result was observed when human TLR5 activation was examined (Fig. 4B). Anti-FlaA IgG specifically suppressed both mouse and human TLR5 activation by rFlaA (*p* < 0.01; Fig. 4, C and D). Combined with data from the protection studies, these results suggest that anti-flagellin Abs induced against the domain responsible for TLR5 activation hampered the host's ability to generate an innate immune response against *P. aeruginosa*, thereby increasing susceptibility to infection. It was also noted that serum from mice immunized with the FliC DNA vaccine weakly but significantly stimulated mouse TLR5. This may reflect epitope mimicry between FliC and the Id of the anti-FliC Ab, as reported in previous studies (37).

Anti-flagellin Ab inhibits host protection against *P. aeruginosa* infection

To evaluate the contribution of anti-FliC Ab response to host protection against *P. aeruginosa*, mice were inoculated intranasally with rFliC in the presence of anti-FliC WT or FliC R90A IgG and then challenged with the PAO1 strain of bacteria (Fig. 4E). Pretreatment with 2 μ g of rFliC for 4 h conferred protection against 2 LD₅₀ doses of intranasal challenge with bacteria (data not shown). This protection was blocked when rFliC was preincubated with anti-FliC WT IgG. However, anti-FliC R90A IgG had weaker potential for the blocking of the rFliC-mediated protective effect ($p < 0.01$; Fig. 4E). This observation suggests that inactive flagellin FliC R90A vaccine raises a weaker level of blocking Ab production, which tolerates a rFliC-mediated anti-*Pseudomonas* innate immune response required for the elimination of bacteria.

TLR5 activation contributes to protection against *P. aeruginosa* infection

The ability of flagellin to elicit a protective innate immune response through its interaction with TLR5 was further investigated (Fig. 5). TLR5-deficient (TLR5^{-/-}) and control (TLR5^{+/-}) mice were challenged intranasally with 0.5 or 1.5 LD₅₀ doses of *Pseudomonas*. The TLR5-deficient mice were significantly more susceptible to challenge with 0.5, but not with 1.5, LD₅₀ doses than were the controls ($p < 0.05$; Fig. 5, A and B). When challenged with sublethal doses of *Pseudomonas*, the number of infectious bacteria persisting in the lungs was higher in TLR5-deficient than control mice, suggesting that bactericidal activity against *P. aeruginosa* is impaired in TLR5-deficient mice ($p < 0.01$; Fig. 5C). These findings are consistent with flagellin-mediated TLR5 activation improving host resistance to infection by flagellated bacteria (20).

Discussion

The activation of TLR5 by flagellin has an adjuvant-like effect that enhances the immunogenicity of epitopes expressed on the flagellin molecule (13, 20). However, Abs generated against the TLR5 activation domains expressed by WT flagellin hamper the host's ability to mount a protective innate immune response against flagellated pathogens. Indeed, although vaccination with WT flagellin induces a strong Ag-specific Ab response, it does not reduce host susceptibility to infection by bacteria expressing homologous TLR5 activation domains. This contrasts to the effect of the mutant flagellin FliC R90A, which interacts poorly with TLR5 and, thus, induces only minimal levels of anti-TLR5-blocking Abs. Vaccination with FliC R90A provided protection against bacteria expressing either homologous or heterologous types of flagellin. Studies have been initiated to examine the breadth of the protection conferred by flagellin FliC R90A against a wide variety of *P. aeruginosa* strains.

The magnitude of the Ab response induced by flagellin is significantly improved by activation of the innate immune system. Sanders et al. (38) demonstrated that Ab production following *Salmonella* rFliC immunization was severely impaired in MyD88 knockout mice. *Helicobacter pylori* FlaA, which interacts poorly with TLR5 and, thus, induces only weak anti-FlaA Ab, becomes highly immunogenic when coadministered with CFA (38). Our results are consistent with these observations. The inactive *Pseudomonas* flagellin FliC R90A induced significantly lower levels of Ab production when compared with active *Pseudomonas* flagellin FliC or FlaA (Fig. 2, B and C). Thus, TLR5- and MyD88-mediated innate immune activation influences the magnitude of the anti-flagellin Ab response. However, this response includes both

protective anti-flagellin Abs and Abs that neutralizing TLR5 activity.

P. aeruginosa possesses another major pathogen-associated molecular pattern, LPS, which is the potent activator of the host's defensive innate immune responses (39). However, previous studies have demonstrated that lung epithelial cells poorly recognize LPS (40, 41). Results from the present study suggest that flagellin may be the dominant inflammatory trigger of airway epithelia (Fig. 5). Yet, it should be noted that we observed no significant difference between TLR5-deficient and its counterpart control mice in their resistance to relatively high challenge doses of *P. aeruginosa* (42).

Several classes of flagellin-mediated protection mechanisms have been implicated in the host's mucosal immunity. Molofsky et al. (3) demonstrated that aflagellated *Legionella* persists longer than WT bacteria following pulmonary infection, in part due to the apoptosis of lung epithelial cells. Aflagellated *Salmonella* elicited severe mucosal damage by a mechanism that also appears to involve the induction of apoptosis in gut epithelia via inhibition of NF- κ B activation (4). Thus, certain bacterial flagellin may modulate host's mucosal inflammation by triggering NF- κ B and altering apoptosis signaling in epithelial cells. Blocking the TLR5 activation domain of flagellin may therefore permit bacteria to evade the host's innate immune responses and alter the level of tissue damage associated with late-stage bacterial infection.

Many studies have demonstrated that pathogenic bacteria possess versatile systems to evade from the host's immune surveillance. For instance, *Yersinia pestis* produces tetra-acylated LPS, which acts as an antagonist to TLR4, preventing the induction of TLR4-mediated inflammatory responses upon infection. After colonization, *P. aeruginosa* down-regulates the expression levels of flagella to evade from the TLR5-mediated host's innate immune activation (43). *H. pylori* may have evolved to express inactive flagellin to prevent activation of the TLR5-mediated host's mucosal immunity, enabling persistent infection in the stomach epithelia (6). Current data provide another mechanism by which bacteria can subvert host induction of an innate immune response.

In summary, these studies are the first to demonstrate that Ab responses directed against the TLR5 activation domain of flagellin actually hamper the induction of protective immunity. By modifying flagellin to prevent this Ab response, the host's ability to mount protective innate immune responses against flagellated pathogens is significantly improved. These observations have important implications for vaccine development against flagellated pathogens.

Acknowledgments

We thank Dr. K. Ogata for discussion, A. de la Fuente for secretarial assistance, and M. Tozuka and K. Takase for technical assistance.

Disclosures

The authors have no financial conflict of interest.

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