Reconstitution of a Functional Toll-like Receptor 5 Binding Site in *Campylobacter jejuni* Flagellin*

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Bacterial flagellin is important for intestinal immune homeostasis. Flagellins from most species activate Toll-like receptor 5 (TLR5). The principal bacterial food-borne pathogen *Campylobacter jejuni* escapes TLR5 recognition, probably due to an alternate flagellin subunit structure. We investigated the molecular basis of TLR5 evasion by aiming to reconstitute TLR5 stimulating activity in live *C. jejuni*. Both native glycosylated *C. jejuni* flagellins (FlaA and FlaB) and recombinant proteins purified from *Escherichia coli* failed to activate NF-κB in HEK293 cells expressing TLR5. Introduction of multiple defined regions from *Salmonella* flagellin into *C. jejuni* FlaA via a recombinatorial approach revealed three regions critical for the activation of human and mouse TLR5, including a β-hairpin structure not previously implicated in TLR5 recognition. Surprisingly, this domain was not required for the activation of chicken TLR5, indicating a selective requirement for the β-hairpin in the recognition of mammalian TLR5. Expression of the active chimeric protein in *C. jejuni* resulted in secreted glycosylated flagellin that induced a potent TLR5 response. Overall, our results reveal a novel structural requirement for TLR5 recognition of bacterial flagellin and exclude flagellin glycosylation as an additional mechanism of bacterial evasion of the TLR5 response.

Flagellin, the monomeric subunit of the bacterial motility apparatus, is the natural ligand of the innate immune sensor Toll-like receptor 5 (TLR5)[3] (1). Activation of TLR5 by flagellin initiates a powerful host response that provides crucial signals for maintaining intestinal immune homeostasis (2, 3). The immunostimulatory properties make flagellin an attractive vaccine carrier protein and potent vaccine adjuvant. Its intrinsic adjuvant activity is currently being employed in experimental recombinant vaccines against human influenza, West Nile fever, malaria, tuberculosis, and plague (4–9). In addition, flagellin-induced immune activation protects the intestine and other tissues against lethal irradiation due to potent TLR5-mediated anti-apoptotic effects (10, 11).

The immunological impact of flagellin stimulation has driven several bacterial pathogens to evolve mechanisms to escape the effective TLR5-mediated host defense. In *Salmonella enterica* serotype Typhi, this is achieved by repression of flagellin expression and secretion (12), whereas *Listeria* shuts off flagellin expression at the host temperature of 37 °C (13). The flagellins of the α- and ε-proteobacteria, which include the major food-borne pathogen *Campylobacter jejuni* and the gastric pathogen *Helicobacter pylori*, fail to activate TLR5 altogether (14–18). For these organisms the consequences of TLR5 evasion for infection are currently unknown. It has been demonstrated that purified *H. pylori* flagellin induces severely impaired adaptive immune responses in comparison to TLR5-activating flagellins (19).

The flagellin protein of *C. jejuni* clearly differs from bacterial flagellins that do activate TLR5. Electron microscopy shows that the *C. jejuni* flagellar filament comprises seven longitudinal helical arrays of stacked flagellin subunits (protofilaments) instead of the 11 present in *e.g. S. enterica* serotype *typhimurium*, suggesting differences in flagellin polymerization between these species (20). Consistent with this hypothesis, the amino acid regions of flagellin involved in filament assembly in *Salmonella* have diverged in *Campylobacter*. These changes may contribute to the TLR5 evasion in *C. jejuni* (18, 21). An additional difference between *C. jejuni* flagellin and most TLR5-activating flagellins is the presence of pseudaminic acid derivatives that cover the putative surface-exposed region of *C. jejuni* flagellin and that may comprise up to 10% of their total weight (22). The contribution of the post-translational modification of *Campylobacter* flagellin to evasion of the TLR5 response is currently unknown.

Considering the important role of TLR5 in intestinal biology and the potential of bacterial flagellin as a vaccine adjuvant, we sought to better define the molecular basis of the TLR5 evasion by *C. jejuni* flagellin by attempting to restore TLR5-stimulating activity. Mutagenesis or replacement of larger amino acid regions between flagellins has previously been instrumental in defining residues critical for TLR5 recognition (18, 21, 23–26). Using a series of recombinant chimeric flagellins, we succeeded in reconstituting a *Campylobacter* flagellin with TLR5 activating ability and in the engineering of *C. jejuni* that processes, glycosylates, and secretes flagellins that yield a potent TLR5 response. Activation of human TLR5 by *Campylobacter* was

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[3] The abbreviations used are: TLR5, Toll-like receptor 5; FlaA, *C. jejuni* flagellin A; FlaB, *C. jejuni* flagellin B; FliC, *S. enteritidis* or *S. typhimurium* Phase I flagellin; DMEM, Dulbecco's modified Eagle's medium; Naip5, Nod-like receptor (NLR) Ipaf and NLR apoptosis inhibitory protein 5; IL, interleukin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

ASBMB

TABLE 1

Primers used in this study

Product/primer	Primer sequence[a]	Template DNA
His$_6$-FlaA-F	Forward, 5′-<u>GGATCC</u>CACCACCACCACCACCACATGGGATTTCGT-3′	*C. jejuni* 81116
His$_6$-FlaA-R	Reverse, 5′-<u>ATCGAT</u>CTATTGTAATAATCTTAAAACATTTTGCTG-3′	
His$_6$-FlaB-F	Forward, 5′-<u>GGATCC</u>CACCACCACCACCACCACATGGGTTTTAGG-3′	*C. jejuni* 81116
His$_6$-FlaB-R	Reverse, 5′-<u>ATCGAT</u>TTATTGTAATAGTTTTAAAACATTTTGCTG-3′	
FlaA-(1–52)	Forward, 5′-CACCATGGGATTTCGTATTAACAC-3′	*C. jejuni* 81116
	Reverse, 5′-GATATTAGAAGTGAAGCGATCTGCTATCGCCATCCC-3′	
FliC(N)	Forward, 5′-GGGATGGCGATAGCAGATCGCTTCACTTCTAATATC-3′	*S. enteritidis* 706
	Reverse, 5′-GTATTAGCGATATTATCAAGTTCTTCCAGACGTTGCTGAA-3′	
FlaA-(123–576)	Forward, 5′-TTCAGCAACGTCTGGAAGAACTTGATAATATCGCTAATAC-3′	*C. jejuni* 81116
	Reverse, 5′-TTGTAATAATCTTAAAACATTTTGC-3′	
FlaA-(1–491)	Forward, 5′-CACCATGGGATTTCGTATTAACAC-3′	*C. jejuni* 81116
	Reverse, 3′-ACAATGCAGAATCAATTGAATCCATAACCGCCATTGC-3′	
FliC(C)	Forward, 5′-GCAATGGCGGTTATGGATTCAATTGATTCTGCATTGT-3′	*S. enteritidis* 706
	Reverse, 5′-ATTCTGCTGCTTTAACATTGGTTACCGTATTGCCAAG-3′	
FlaA-(527–576)	Forward, 5′-CTTGGCAATACGGTAACCAATGTTAAAGCAGCAGAAT-3′	*C. jejuni* 81116
	Reverse, 5′-TTGTAATAATCTTAAAACATTTTGC-3′	
FlaA-(1–122)	Forward, 5′-CACCATGGGATTTCGTATTAACAC-3′	*C. jejuni* 81116
	Reverse, 5′-TTAGAAACGCGATCGATATCTGCTATCGCCATCC-3′	
FliC(H)	Forward, 5′-GGATGGCGATAGCAGATATCGATCGCGTTTCTAA-3′	*S. enteritidis* 706
	Reverse, 5′-GTAAAACTTTGAGCACCAACATTGAACCCATCAA-3′	
FlaA-(177–576)	Forward, 5′-TTGATGGGTTCAATGTTGGTGCTCAAAGTTTTAC-3′	*C. jejuni* 81116
	Reverse, 5′-TTGTAATAATCTTAAAACATTTTGC-3′	
FliC(NVC)	Forward, 5′-GGGATGGCGATAGCAGATCGCTTCACTTCTAATATC-3′	*S. enteritidis* 706
	Reverse, 5′-ATTCTGCTGCTTTAACATTGGTTACCGTATTGCCAAG-3′	
FlaA3-topoF	5′-CACCATGGGATTTCGTATTAACAC-3′	
FlaA6-topoR	5′-TTGTAATAATCTTAAAACATTTTGC-3′	
FlaAB-mutant-R	5′-AAAGCTATTATTCCCTTACAGGATGAG-3′	*C. jejuni* 81116
FlaAFSphI	5′-<u>GCATGC</u>TAGTAAAATTGAAGATGAAAGAGAG-3′	*C. jejuni* 81116
FlaARNsiI	5′-<u>ATGCAT</u>TTTAAATCCTTTAAATAATTTC-3′	
Flagellin-pMA3-F	Forward, 5′-CC<u>GAGCTC</u>AAAAGGATTTAAAATGGGATTTCGTATTAACACAAATGT-3′	
Flagellin-pMA3-R	Reverse, 5′-CCC<u>CGCGG</u>CTATTGTAATAATCTTAAAACATTTTGCTG-3′	

[a] Underlines indicate restriction sites used for cloning.

not influenced by flagellin glycosylation but required introduction of three defined domains of *Salmonella* flagellin. Differential activation of mammalian and chicken TLR5 by recombinant flagellins led to the discovery of a β-hairpin structure not previously implicated in mammalian TLR5 recognition.

EXPERIMENTAL PROCEDURES

Cell Lines and Bacterial Strains—HeLa 57A cells stably transfected with a NF-κB luciferase reporter construct (27), HEK293 cells, and HT-29 intestinal epithelial cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5% fetal calf serum at 37 °C under 5% CO_2. NHEK human primary keratinocytes were propagated under the same conditions in KGM-2 Keratinocyte Growth Medium-2 (Lonza).

C. jejuni strains 81116 (NCTC11828) (28), NCTC 11168H1 (29), and their derivatives were grown at 37 °C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) on saponin agar medium containing 4% lysed horse blood with the appropriate antibiotics. *S. enterica* ssp. *enteritidis* 90-13-706 and *S. enteritidis* 90-13-706Δ*fliC* (30) and *Escherichia coli* BL21(DE3) Star (Invitrogen), used to express recombinant flagellins, were grown in Luria Bertani broth at 37 °C.

Purification of Native Flagellin—Native flagellin of *S. enteritidis* was purified overnight cultures as described (31), with minor modifications. Briefly, bacteria were resuspended in 10 mM Tris·HCl, 145 mM NaCl, pH 7.4, homogenized (2 min), and centrifuged twice (10,000 × g, 20 min, 4 °C), discarding the pellet after each centrifugation. Flagella were collected from the supernatant by centrifugation (100,000 × g, 60 min, 4 °C) and depolymerized in 0.2 M glycine, pH 2 (30 min, 20 °C, with stirring). After centrifugation (100,000 × g, 60 min, 4 °C), the

supernatant containing monomeric flagellin was adjusted to pH 7.2 with 1 M NaOH, and ammonium sulfate was added to a final concentration of 2.67 M. After overnight incubation (20 °C), repolymerized flagellins were collected by centrifugation (14,000 × g, 15 min, 4 °C), dissolved in H_2O, and dialyzed against H_2O (24 h, 4 °C). Native flagellin of *C. jejuni* strain 81116 was purified as described (32) and stored at −20 °C.

Construction and Purification of Chimeric Flagellins—The construction of recombinant *Salmonella* FliC has been described (33). Recombinant FlaA and FlaB of *C. jejuni* strain 81116 were obtained after amplification of the corresponding genes with *pfu* polymerase (Promega) using primer pairs His$_6$-FlaA-F and His$_6$-FlaA-R and pairs His$_6$-FlaB-F and His$_6$-FlaB-R, respectively (Table 1). Products were ligated into expression vector pT7-7 (34) using restriction enzymes BamHI and ClaI. Chimeric flagellins were constructed by overlap extension PCR using primers and a template as depicted in Table 1, unless stated otherwise. For construction of chimera FlaA^N, the template was a mixture of FlaA-(1–52), FliC(N), and FlaA-(123–576); for chimera FlaA^C, the template was a mixture of FlaA-(1–491), FliC(C), and FlaA-(527–576); for FlaA^{NC}, the template was FlaA-(1–491) (prepared with template *flaA^N*), FliC(C), and FlaA(527–576); for FlaA^{NVC}, the template was a mixture of FlaA-(1–52), FliC(NVC), and FlaA-(527–576); for FlaA^H, the template was a mixture of FlaA-(1–122), FliC(H), and FlaA-(177–576); for FlaA^{NH}, the template was FlaA-(1–122) (prepared with template *flaA^N*), FliC(H), and FlaA-(177–576); for FlaA^{HC}, the template was a mixture of FlaA-(1–122), FliC(H), and FlaA(177–576) (prepared with template *flaA^C*); for FlaA^{NHC}, the template was FlaA(1–122) (prepared with template *flaA^N*), FliC(H), and FlaA-(177–576) (prepared with

template *flaA*^{C}). The obtained chimeric flagellin genes were amplified using primers FlaA-topoF and FlaA-topoR and ligated in pET101/D-TOPO (Invitrogen). All plasmid constructs were propagated in *E. coli* DH5α and transformed into *E. coli* BL21(DE3) Star for protein expression.

Recombinant His$_6$-tagged proteins were obtained by incubating pellets of isopropyl-1-thio-β-D-galactopyranoside-induced (1 mM, 5 h, 37 °C) bacterial cultures in 8 M urea for 17 h (20 °C). After sonication and centrifugation (5000 × *g*, 15 min, 20 °C) to remove debris, His$_6$-tagged flagellin was purified with nickel-nitrilotriacetic acid-agarose (Qiagen). After washing with 8 M urea, pH 6.4, flagellin was eluted in steps with 8 M urea, pH 5.3, and 8 M urea, pH 4.5. For recombinant FliC, FlaA, and FlaB, fractions containing flagellin were pooled, dialyzed (24 h, 4 °C) against 10 mM of Tris·HCl (pH 9.0), and centrifuged (100,000 × *g*, 60 min) to remove protein aggregates. Chimeric flagellins and control recombinant FlaA and FliC were kept at −20 °C in 8 M urea, pH 4.5, at a concentration of 500 μg ml^{-1}. Proteins were analyzed on SDS-PAGE, and concentrations were determined by using the BCA protein assay kit (Thermo Scientific Pierce).

Construction of C. jejuni Mutants—The complete *flaA-flaB* region of *C. jejuni* 81116 was amplified by PCR with primers FlaA3-topoF and FlaAB-mutant-R (Table 1) and ligated into pGEM-T easy (Promega). EcoRV was used to remove the last 714 nucleotides of *flaA* and the N-terminal 1017 nucleotides of *flaB*. This fragment was replaced with a chloramphenicol resistance cassette obtained by digestion of pAV35 (35) with PvuII (resulting in plasmid pMR108). *C. jejuni* mutant strain 11168H1Δ*flaAB* was constructed by homologue recombination through electroporation using the pMR108 deletion plasmid and strain *C. jejuni* 11168H1, as described (36). The $\sigma28$ *flaA* promoter region was amplified from *C. jejuni* 81116 with primers FlaAFSphI and FlaARNsiI and cloned into pMA1 (37) using SphI and NsiI, resulting in pMA3. For the expression of flagellin proteins in *C. jejuni*, *flaA*, *flaA*^{NC}, and *flaA*^{NHC} were PCR-amplified with primers flagellin-pMA3-F and flagellin-pMA3-R using the His-tagged expression constructs as template, digested with SacI and SacII, and cloned into the multiple cloning site of pMA3. Conjugation to *C. jejuni* was performed as described (37).

Transient Transfection—HEK293 and HeLa 57A cells (~70% confluent) kept in 48-well plates were transiently transfected with 50 μl of a mixture of plasmid DNA and FuGENE 6 (Roche Diagnostics) in DMEM at a lipid to DNA ratio of 3 to 1. HEK293 cells were transfected with 50 ng of NF-κB-luc plasmid and 70 ng of pTK-LacZ, which was used for normalization of transfection efficiency. HeLa 57A cells were transfected with 125 ng of pFLAG-human-TLR5, pFLAG-mouse-TLR5, pFlAG-chicken-TLR5 (33), or pFLAG-CMV1 empty vector (Sigma) together with 125 ng of pTK-LacZ. Cells were used in TLR5 stimulation assays at 48 h after transfection.

Toll-like Receptor 5 Stimulation Assays—Transfected cells were placed in 0.5 ml of fresh DMEM with 5% fetal calf serum before stimulation with bacteria or purified flagellin. For cell stimulation, *S. enteritidis* and *C. jejuni* were grown (17 h) in Luria Bertani broth and heart infusion broth, respectively, collected by centrifugation (5000 × *g*, 10 min, 22 °C), and resus-

pended in Dulbecco's phosphate-buffered saline. Bacteria were added to the transfected cells at an m.o.i. of 1:100. After 3.5 h of stimulation, cells were rinsed 3 times with DMEM-5% fetal calf serum to prevent bacterial overgrowth and further incubated in fresh DMEM-5% fetal calf serum. Bacterial culture supernatants were collected (5000 × *g*, 10 min, 22 °C) after 16 h of growth, filtered (0.22 μm, Millipore), and added to transfected cells (10 μl per well). Native flagellin was depolymerized at 70 °C for 20 min before stimulation and added to the cells at the indicated concentrations (ng ml^{-1}). Recombinant FlaA, FliC, and chimeric flagellins, stored in 8 M urea, pH 4.5, at a concentration of 500 μg ml^{-1} (see above) were instantly diluted 500-fold by adding 1 μl of protein solution per well. All flagellin stimulations were stopped after 5 h by rinsing the cells twice with Dulbecco's phosphate-buffered saline, lysis in 0.1 ml of reporter lysis buffer (Promega), and freezing at −80 °C. Luciferase activity was measured in a luminometer (TD-20/20, Turner Designs) after mixing 20 μl of thawed cell lysate with 0.1 ml of luciferase reagent (Promega). For normalization of transfection efficiency, luciferase values were adjusted to β-galactosidase values determined with the β-galactosidase assay (Promega). Results were expressed in relative light units and represent the means of duplicate values of three independent experiments.

Reverse Transcription-PCR—RNA from HT-29 and NHEK cells stimulated for 2 h with 1 μg ml^{-1} of recombinant flagellins was isolated using RNA-Bee (Bio-Connect). Subsequent DNase I treatment and reverse transcription-PCR analysis for actin and IL-8 mRNA was performed as described previously (38).

Detection of Flagellins Produced by C. jejuni—Whole bacterial lysates and culture supernatant were prepared from 17 h of *C. jejuni* cultures. After centrifugation (5000 × *g*, 10 min, 22 °C), the supernatant was collected, and the pellet was resuspended in an equal amount of Dulbecco's phosphate-buffered saline for SDS-PAGE analysis. Secreted and intracellular flagellins were detected by Western blot analysis using anti-FlaA antibody CF1 (1:500 dilution) (39) and horseradish peroxidase-conjugated goat-anti mouse IgG (Sigma). Reactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermal Scientific Pierce). CF1 recognizes an epitope in a 193-amino acid stretch in the variable domain of *C. jejuni* 81116 FlaA. In chimera FlaA^{NHC}, the first 30 amino acids of this stretch has been replaced by the corresponding *S. enteritidis* sequence, which did not influence CF1 recognition. For two-dimensional electrophoresis, culture supernatant was concentrated 20 times using Centricon YM-30 filters (Millipore) and mixed with rehydration solution (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% IPG buffer, pH 4–7 (Amersham Biosciences), and 0.3% (w/v) dithiothreitol). First-dimension isoelectric focusing was performed on an IPGphor (Amersham Biosciences) with immobilized nonlinear pH (3–10) gradient strips (Amersham Biosciences) using the following isoelectric focusing parameters: 12 h at 30 V, 30 min at 500 V, 30 min at 100 V, 1 h at 40 min 6000 V, and 2 h at 500 V. Isoelectric focusing strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 10 mg ml^{-1} dithiothreitol followed by 15 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 25 mg ml^{-1} iodoacetamide.
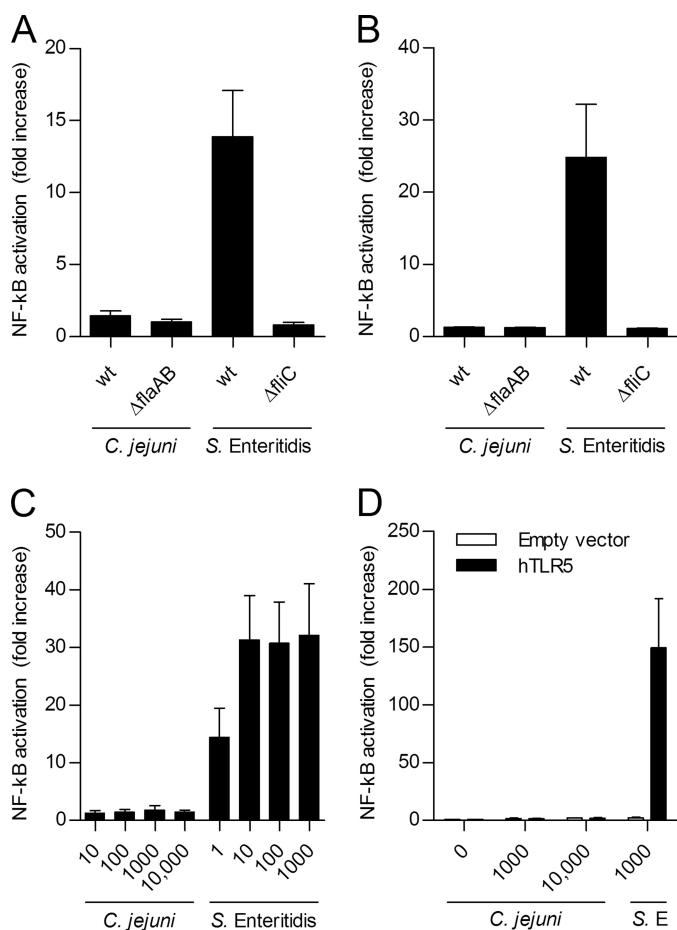
FIGURE 1. ***C. jejuni* fails to activate TLR5.** *A* and *B*, NF-κB activation was measured for TLR5-expressing HEK293 cells stimulated with live bacteria (*A*) or culture supernatant (*B*) of wild type (*wt*) *C. jejuni*, *C. jejuni* Δ*flaAB*, wt *S. enteritidis*, or *S. enteritidis* Δ*fliC*. *C*, NF-κB activation was measured for TLR5-expressing HEK293 cells stimulated for 5 h with purified native *C. jejuni* flagellin or *S. enteritidis* flagellin at the indicated concentrations (ng ml^{-1}). *D*, NF-κB activation was measured for human TLR5 (*hTLR5*)-transfected or control HeLa 57A cells stimulated for 5 h with purified native *C. jejuni* and *S. enteritidis* (*S. E*) flagellin at the indicated concentrations (ng ml^{-1}). Values represent the increase of NF-κB-induced luciferase activity in stimulated cells compared with non-stimulated cells and are the mean ± S.E. of three independent experiments.

Second-dimension SDS-PAGE was performed using 10% polyacrylamide gels. Flagellins were detected as described above.

RESULTS

TLR5-activating Properties of Campylobacter Flagellin—The inability of *C. jejuni* flagellin to activate TLR5 has been demonstrated for native and recombinant flagellin from strain 81–176 (14, 15, 18). As *C. jejuni* flagellins show considerable sequence variation between different strains, are present in two differentially regulated isoforms (FlaA and FlaB) (40), and show variable glycosylation (41–43), we first examined the efficacy of *C. jejuni* strain 81116 to signal via TLR5. Flagellin activity was measured in HEK293 cells expressing TLR5 with an NF-κB-luciferase reporter as a read-out system. In this system, both *S. enteritidis* and its culture supernatant (containing secreted flagellin), but not the flagellin deficient (Δ*fliC*) strain, induced a robust TLR5 response (Fig. 1, *A* and *B*). In contrast, neither *C. jejuni* strain 81116 nor its culture supernatant activated

NF-κB in the TLR5-expressing cells (Fig. 1, *A* and *B*). Analysis of 10 additional clinical *C. jejuni* isolates confirmed the evolutionary conservation of this trait (data not shown). To exclude limited monomeric flagellin release as a cause of the inability to activate TLR5, native *C. jejuni* flagellin was purified. Isolated *C. jejuni* 81116 flagellin was also unable to activate TLR5, even at concentrations 10,000-fold higher than native flagellin of *S. enteritidis* (Fig. 1*C*). Experiments with HeLa 57A cells transfected with TLR5 instead of HEK293 cells yielded similar results (Fig. 1*D*). Competition assays showed that an excess of native *C. jejuni* 81116 flagellin did not antagonize HEK293 activation by flagellin of *S. enteritidis* (data not shown).

TLR5-stimulating Activity of Recombinant C. jejuni FlaA and FlaB—TLR5 activation by *Salmonella* flagellin requires the amino acids 89–96 at the bridge of the α-helices ND1a and ND1b in the N-terminal conserved domain (Fig. 2, *black box*) (18, 21). An additional region, located in the center of the conserved C-terminal CD1 α-helix (Fig. 2, *gray box*), appears critical for stability of the N-terminal TLR5 binding domain (18, 21, 24). Sequence analysis of *C. jejuni* FlaA shows considerable deviation of both the relevant N- and C-terminal regions from the corresponding regions of *Salmonella* FliC (Ref. 18 and Fig. 2). The independently expressed *C. jejuni* FlaB subunit is identical to FlaA in its N-terminal TLR5 binding site but differs at several amino acids in the center of the CD1 α-helix (Fig. 2, indicated by *asterisks*). To examine the potential relevance of these changes in amino acid composition for TLR5 activation, we expressed both FlaA and FlaB of *C. jejuni* strain 81116 as polyhistidine-tagged proteins in *E. coli* and purified them by Ni^{2+}-affinity chromatography. SDS-PAGE analysis of the native and recombinant *C. jejuni* flagellins demonstrated a markedly lower apparent molecular mass for the recombinant proteins compared with native *C. jejuni* flagellin, consistent with the absence of attached glycan moieties (Fig. 3*A*). The difference in electrophoretic mobility was not observed for recombinant and native *S. enteritidis* flagellin (FliC), in agreement with the lack of flagellin glycosylation in this species. Functional assays using TLR5-expressing HEK293 cells showed that both recombinant FlaA and FlaB failed to activate NF-κB (Fig. 3*B*), whereas purified recombinant *Salmonella* FliC induced a potent response. These results demonstrate that the non-glycosylated forms of both *C. jejuni* FlaA and FlaB lack TLR5-stimulating activity. As glycosylation of FlaA and FlaB is needed for flagella assembly (44) and, thus, possibly for appropriate folding of the protein, we also tested native flagellins isolated from *Campylobacter* 81116 FlaA and FlaB mutant strains. These proteins also failed to activate TLR5 (data not shown). Together, the data indicate that neither of the *C. jejuni* flagellins is able to activate TLR5 irrespective of their state of glycosylation.

Construction and Function of Chimeric Flagellins—In an attempt to restore the ability of *C. jejuni* flagellin to bind and activate TLR5, we replaced a part of its ND1 α-helix region with the corresponding region of *S. enteritidis* FliC that contains the putative TLR5 binding site (chimera FlaA^N, Fig. 4). Comparative modeling of FlaA on the structure of *S. enteritidis* flagellin was used to select amino acids regions that were predicted to yield minimal changes in the overall protein configuration. A
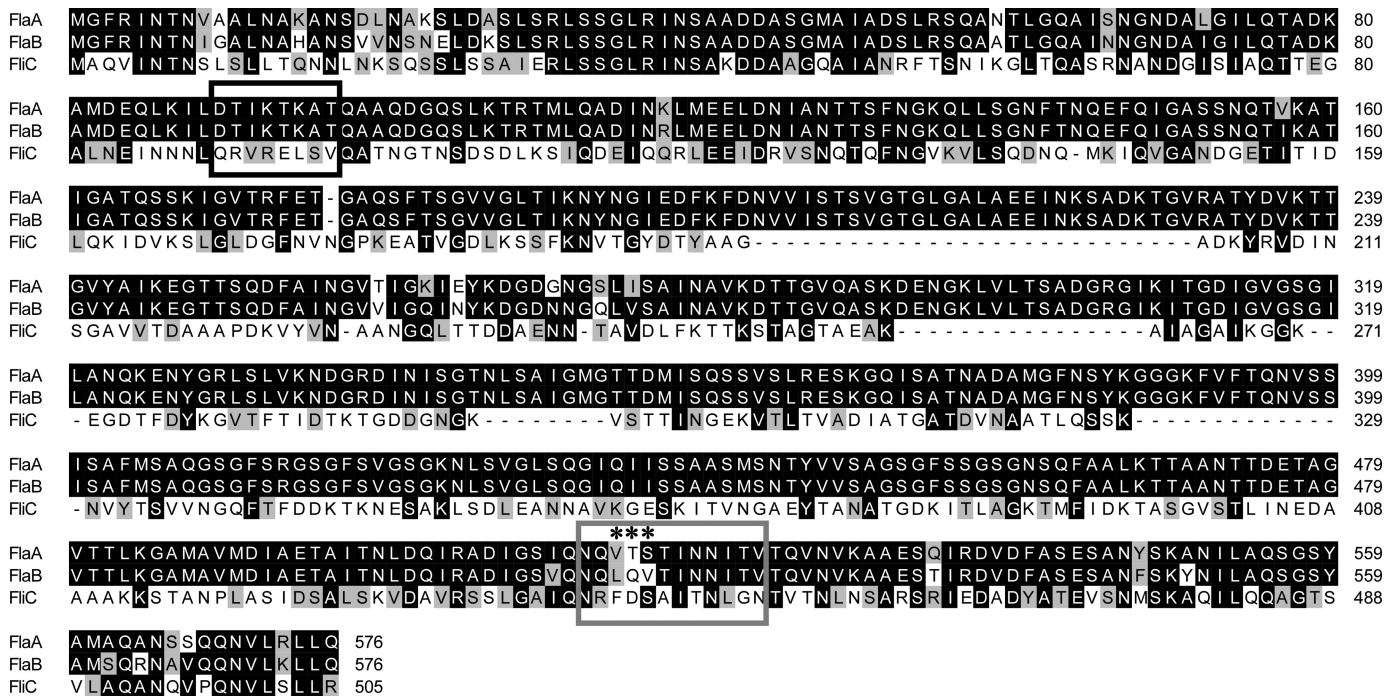
FIGURE 2. **ClustalW alignment of *C. jejuni* 81116 FlaA and FlaB and *S. enteritidis* 706 FliC.** The stretch of amino acids proposed to bind TLR5 in the N-terminal conserved domain are *boxed in black*, and the crucial residues for TLR5 activation in the C-terminal conserved domain are *boxed in gray*. Asterisks indicate differences in amino acid sequence between FlaA and FlaB in the C-terminal domain.

second chimeric flagellin was constructed in which the CD1 α-helix region was replaced (chimera FlaA^C), and a third was constructed by exchanging both the α-helices ND1 and CD1 (chimera FlaA^{NC}). Finally, a control chimera was constructed that contained both *S. enteritidis* α-helices ND1 and CD1 together with the entire central variable region (chimera FlaA^{NVC}, Fig. 4). All recombinant proteins were expressed in *E. coli*, purified, and tested for their ability to activated TLR5 in HEK293 cells. The control FlaA^{NVC} chimera was fully able to induce NF-κB translocation (Fig. 5*A*), confirming data that the structurally disordered extreme N- and C-terminal regions of flagellin are not involved in TLR5 engagement (23) and excluding the possibility these regions inhibit receptor activation. Functional analysis of the other three chimeric proteins unexpectedly showed that none of the chimeric flagellins had regained the ability to activate NF-κB (Fig. 5*A*) regardless the presence of both conserved regions critical for *Salmonella* flagellin to activate TLR5.

Reconstitution of Human TLR5 Recognition in C. jejuni FlaA Requires a Variable β-Hairpin Region of S. enteritidis FliC—In the search for additional regions required for restoration of TLR5 activation in a *Campylobacter* flagellin backbone, we focused on the β-hairpin region after the ND1b helix in *Salmonella* flagellin. This hairpin structure is involved in multimerization of flagellin subunits and may further stabilize the intramolecular structure formed by the highly conserved α-helices (45). Due to low sequence homology between bacterial species, the β-hairpin region has thus far been ignored as part of a direct TLR5 binding site. To assess the role of the 56-amino acid β-hairpin, we constructed a second series of chimeric flagellins (Fig. 4). Replacement of the β-hairpin region of *C. jejuni* FlaA with the β-hairpin from *S. enteritidis* FliC (FlaA^H)

was not sufficient to induce TLR5 activation (Fig. 5*B*). Similarly, chimeras consisting of *C. jejuni* flagellin with two of three *Salmonella* regions (chimera FlaA^{NH} and chimera FlaA^{CH}) were inactive (Fig. 5*B*). However, a chimeric flagellin containing the conserved ND1 and CD1 regions together with the variable β-hairpin region (chimera FlaA^{NHC}) strongly activated TLR5 in both HEK293 cells (Fig. 5, *B* and *C*) and HeLa 57A carrying human TLR5 but not empty vector (Fig. 5*D*). To further verify that the β-hairpin plays a role in TLR5 stimulation, we tested the activity of the chimeric flagellins FlaA^{NC} and FlaA^{NHC} in the non-transfected human intestinal epithelial cell-line HT-29 and in non-transformed primary human epithelial cells, which both endogenously express TLR5. FlaA^{NHC}, but not FlaA or FlaA^{NC}, enhanced IL-8 transcript levels in both cell types (Fig. 6). Together, these results indicate that at least three distinct sections of *Salmonella* flagellin are required to reconstitute human TLR5-stimulating activity in *Campylobacter* flagellin.

The β-Hairpin Region of Flagellin Determines TLR5 Species Specificity—To further explore the importance of the β-hairpin region in TLR5 recognition, we tested the abilities of constructed chimeric flagellins to activate TLR5 from different species. This approach has previously been instrumental in dissecting ligand properties required for TLR activations (33, 46, 47). All constructed chimeras that failed to activate human TLR5 were unable to activate mouse TLR5, except for FlaA^{NHC} (Fig. 7, *A* and *B*). FlaA^{NHC} induced lower levels of NF-κB activation in mouse TLR5 than in human TLR5-transfected cells. This effect was also observed for *Salmonella* FliC (data not shown) and is likely caused by intrinsic differences in TLR5, different expression levels, and/or the expression of mTLR5 in a heterologous (human) background. Chimeric flagellin FlaA^{NHC} was also able to activate chicken TLR5 (Fig. 7*C*). Unexpectedly,
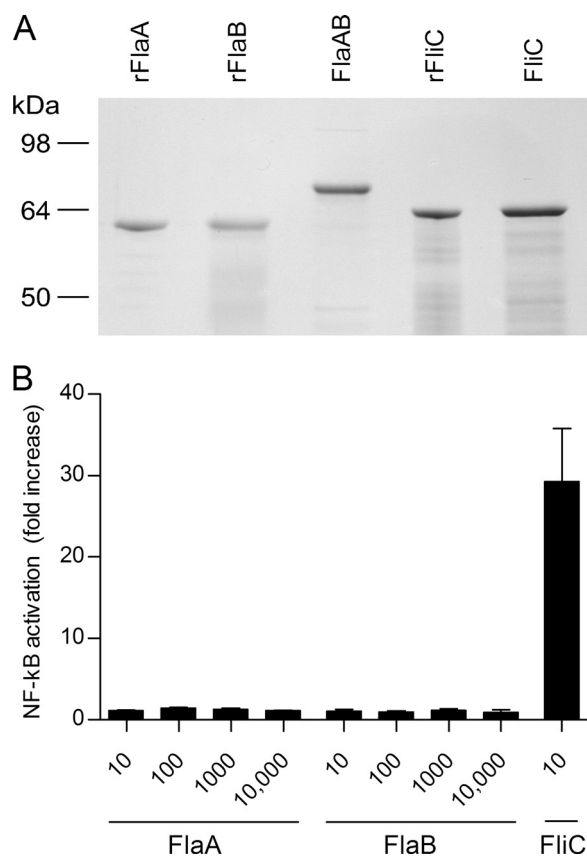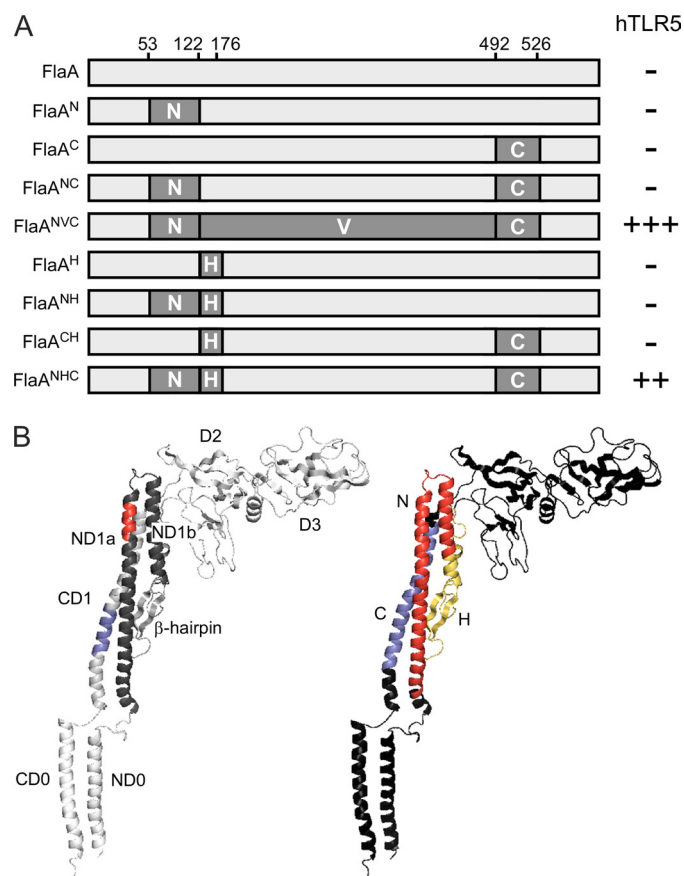
FIGURE 3. **Recombinant, non-glycosylated *C. jejuni* FlaA and FlaB fail to activate TLR5.** *A*, SDS-PAGE was performed to examine differences in electrophoretic mobility between recombinant non-glycosylated *C. jejuni* flagellins (*rFlaA* and *rFlaB*) and native glycosylated *C. jejuni* flagellin (*FlaAB*). As a control, recombinant and native *S. enteritidis* flagellin (*rFliC* and *FliC*, respectively) were analyzed. *B*, NF-κB translocation was measured in TLR5-expressing HEK293 cells after stimulation (5 h) with recombinant non-glycosylated *C. jejuni* FlaA and FlaB at the indicated concentrations (ng ml^{-1}). *S. enteritidis* FliC (10 ng ml^{-1}) was used as a positive control. Values represent the increase of NF-κB-induced luciferase activity in stimulated cells compared with non-stimulated cells and are the mean $\pm$ S.E. of three independent experiments.

FIGURE 4. **Characteristics of chimeric flagellin proteins.** *A*, schematic overview of the constructed chimeric flagellins. Numbers refer to the start and end amino acid positions of the exchanged FlaA domains N, H, V, and C. *hTLR5*, human TLR5. *B*, the structure of *S. typhimurium* flagellin (PDB code 1UCU) shows the different flagellin domains as well as the N- and C-terminal regions proposed to be involved in TLR5 activation (in *red* and *blue*, respectively) (*left*), and the location of the exchanged N (*red*), H (*yellow*), and C (*blue*) domains (*right*). The potential of the recombinant flagellins to activate human TLR5 is indicated on the right as $-$, $+$, $++$, or $+++$.

however, chicken TLR5 responded also to chimeric flagellin FlaA^{NC}, in clear contrast to human and mouse TLR5. As the only difference between flagellin FlaA^{NC} and FlaA^{NHC} is the presence of the *S. enteritidis* β-hairpin, these results indicate that this hairpin structure is not merely needed for proper folding of the flagellin but, rather, is essential for activation of mammalian TLR5 but not chicken TLR5.
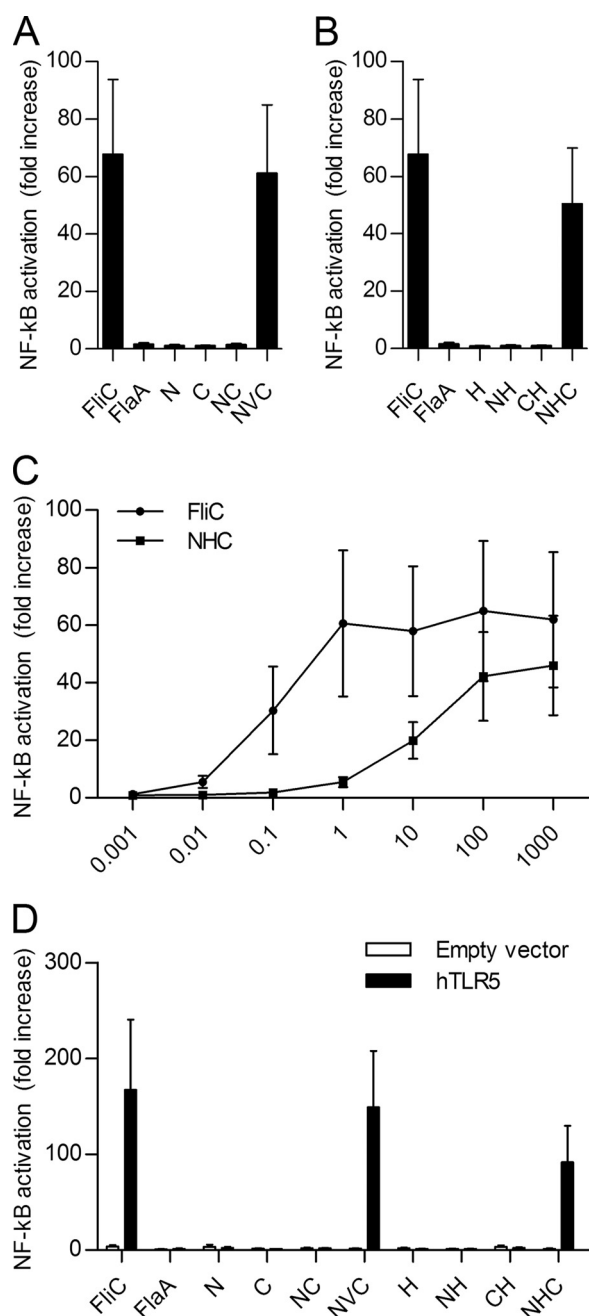
Glycosylation and Secretion of Biologically Active FlaA^{NHC} by C. jejuni—The TLR5-activating *Campylobacter* flagellins used above were overexpressed in *E. coli*, purified under denaturing conditions, and refolded *in vitro*. In *Campylobacter*, flagellins are only successfully secreted after post-translational modification. To engineer *C. jejuni* that express TLR5-activating flagellins, we expressed the genes encoding wild type FlaA, the chimeric flagellin FlaA^{NC}, and TLR5-activating chimeric flagellin FlaA^{NHC} in *C. jejuni* strain 81116. The genes were cloned into plasmid pMA3, a shuttle vector suitable for protein expression in *C. jejuni* under the control of the endogenous *flaA* σ^{28} promoter, and transformed into a flagella-deficient and non-motile *C. jejuni* 11168H1Δ*flaAB* mutant. Introduction of the plasmid encoding FlaA but not FlaA^{NC} or FlaA^{NHC} flagellin restored

flagella formation and bacterial motility in a Δ*flaAB* background (data not shown). Western blot analysis of whole cell lysates using anti-FlaA antibody CF1 as a probe yielded reactive proteins for both FlaA^{NC} and FlaA^{NHC} (Fig. 8*A*). Analysis of the bacterial culture supernatants also yielded reactive flagellin bands for both strains that were larger in size than the non-secreted intracellular proteins, consistent with the attachment of glycan moieties during protein export (Fig. 8*A*). Two-dimensional gel electrophoresis followed by immunoblotting with anti-FlaA antibodies demonstrated that both wild type flagellin produced by 11168H1Δ*flaAB*+FlaA and secreted FlaA^{NHC} appeared as an array of similarly sized proteins of different isoelectric points, a pattern shown by mass spectrometry to be typical for variable glycosylation of the protein (42, 48) (Fig. 8, *B* and *C*). Overall, these results indicate that the chimeric flagellins were expressed, processed, and secreted in the *C. jejuni* native background.

Infection of TLR5-expressing HEK293 cells with live *C. jejuni* that secrete chimeric glycosylated FlaA^{NHC} flagellin yielded a potent NF-κB response, whereas no activation was observed for *C. jejuni* producing the chimera FlaA^{NC} and wild type FlaA (Fig. 9, *A* and *B*). Similar results were obtained with

FIGURE 6. **IL-8 mRNA induction by FlaA^{NHC} in human non-transfected intestinal and primary epithelial cells.** HT-29 cells (*A*) and non-transformed primary human cells (*B*) were stimulated (2 h) with 1 μg ml^{-1} recombinant FlaA, FlaA^{NC}, or FlaA^{NHC}. IL-8 transcripts were analyzed by reverse transcription-PCR and are presented as -fold increase in mRNA levels in stimulated *versus* non-stimulated cells. Values are the mean $\pm$ S.E. of three independent experiments.

exploit bacterial components for targeted modulation of the immune system. In the present study we took advantage of the inability of *C. jejuni* flagellin to activate TLR5 to better define the molecular requirements for TLR5 recognition. Using a reverse-engineering approach, we reconstituted TLR5-stimulating activity in *C. jejuni* and discovered that besides the conserved N-terminal ND1a and ND1b α-helices and the C-terminal CD1 α-helix in flagellin, an adjacent β-hairpin structure is required for activation of mammalian TLR5. This β-hairpin was not required for activation of chicken TLR5 (Fig. 7*C*), indicating species-specific interaction of the flagellin with TLR5. *C. jejuni* O-linked glycosylation of flagellin did not interfere with TLR5 activation, which may hold promise for modification of flagellin to alter its physical properties when used *e.g.* as a vaccine adjuvant.

Previous studies on the ability of *Campylobacter* to avoid TLR5 recognition were performed with *C. jejuni* strain 81–176 (14, 15, 18). However, *C. jejuni* flagellins are known to show high sequence variability between strains and are present in two differentially regulated isoforms, FlaA and FlaB, that consistently differ at 12 amino acid positions in their conserved N- and C-terminal regions (49). Our results demonstrate that the evasion of the TLR5 response is a conserved trait among the *C. jejuni* species and holds for both FlaA and FlaB (Fig. 3*B* and data not shown) irrespective of the composition of the flagella (FlaA/FlaB subunit ratio) or the variable glycan modification. These results lend support to the notion that this species and other α- and ϵ-Proteobacteria have evolved an alternate class of flagellins that may provide a selective advantage in the host by evading the TLR5 innate immune response (18). The different flagellin structure may explain why the *Campylobacter* flagella fiber is formed by 7 instead of 11 subunit helices (28). Although its contribution to pathogenesis is unknown, the widespread evolutionary conservation of TLR5 evasion suggests that this trait adds a valuable selective advantage during colonization or infection.

The evasion of the mammalian TLR5 sensing machinery by *C. jejuni* has thus far been mostly attributed to deviations in the
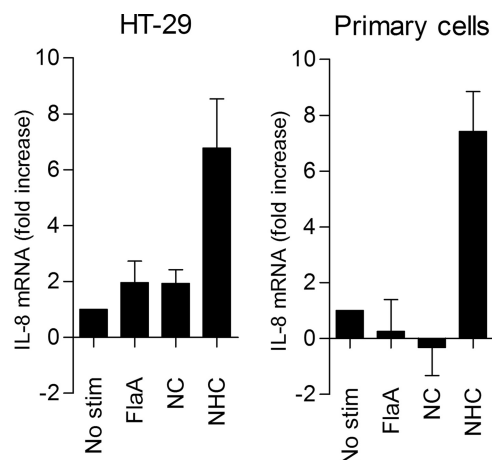

FIGURE 5. **The TLR5 stimulatory activity of the recombinant chimeric flagellins.** *A* and *B*, NF-κB activation was measured for TLR5-expressing HEK293 cells after 5 h of stimulation with the indicated flagellins (1 μg ml^{-1}). *C*, TLR5-expressing HEK293 cells stimulated with increasing concentrations (ng ml^{-1}) of recombinant FlaA^{NHC} or FliC show the dose-response relationship. *D*, NF-κB activation in HeLa 57A cells transfected with either human TLR5 (*hTLR5*) or empty vector after stimulation (5 h) with 1 μg ml^{-1} of the indicated recombinant flagellins. Values represent the increase of NF-κB-induced luciferase activity in stimulated cells compared with non-stimulated cells and are the mean $\pm$ S.E. of three independent experiments.

sterile culture supernatants of *C. jejuni* secreting FlaA^{NHC}. These results indicate that viable *C. jejuni* strains can be engineered that activate TLR5 and that glycosylation of flagellin does not interfere with TLR5 receptor recognition.

DISCUSSION

Knowledge of the molecular basis of TLR recognition is important to understand bacteria-host interactions and to
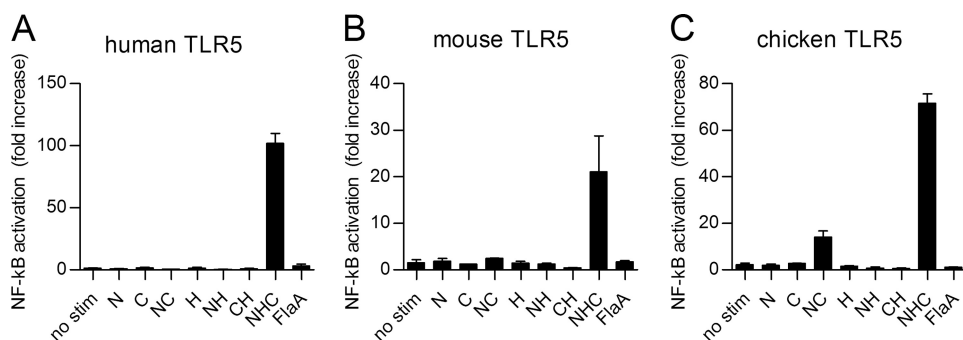
FIGURE 7. **Species-specific activation of TLR5 by FlaA^{NHC}.** *A*, *B*, and *C*, HeLa 57A cells were transfected with either human (*A*), mouse (*B*), or chicken (*C*) TLR5. NF-κB translocation was measured after stimulation with 1 μg ml^{-1} of the indicated recombinant flagellins. Values represent the increase of NF-κB-induced luciferase activity in stimulated cells compared with non-stimulated cells and are the mean $\pm$ S.E. of three independent experiments.
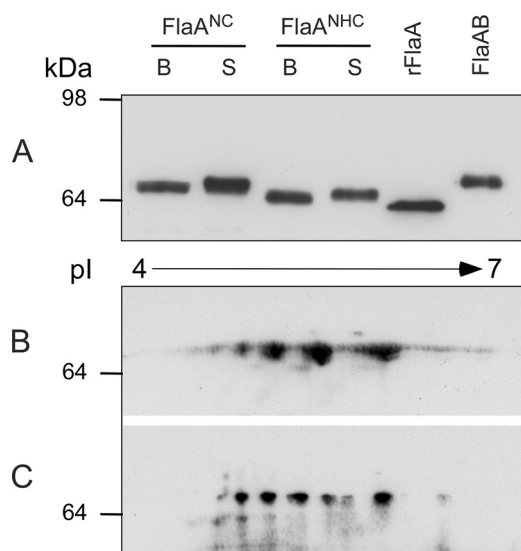
FIGURE 8. **C. jejuni expresses, glycosylates, and secretes chimeric flagellins FlaA^{NC} and FlaA^{NHC}.** *A*, Western blotting was performed to examine the electrophoretic mobility of bacteria-associated (*B*) and secreted (*S*) chimeric flagellins FlaA^{NC} and FlaA^{NHC} produced by *C. jejuni*. Blots were probed with the flagellin-specific antibody CF1. As controls, recombinant FlaA (*rFlaA*) and native *C. jejuni* flagellin (*FlaAB*) were used. *B* and *C*, two-dimensional electrophoresis followed by Western blotting using antibody CF1 was performed to visualize the flagellin glycosylation pattern on *C. jejuni* produced and secreted (*B*) FlaA and (*C*) FlaA^{NHC}.

proposed TLR5 binding region, a stretch of eight amino acids located in the N-terminal conserved domain flagellin and crucial for flagella formation in *Salmonella* (18). The successful engineering of a recombinant *Campylobacter* flagellin that activates human TLR5 required, besides the known N- and C-terminal regions, the presence of the β-hairpin domain from *Salmonella* FliC (Fig. 5). This domain has previously been discarded as a potential binding region for TLR5 due to low sequence homology among bacteria, although disruption of the β-hairpin domain by transposon insertion of a 31-amino acid polypeptide resulted in a significant decrease in TLR5 activation (21). As the construction of chimeric proteins brings the possibility of incorrect protein folding, it could be argued that proper flagellin folding and subsequent TLR5 activation in humans is only achieved with the presence of three distinct flagellin domains from the same origin. Indeed, in *Salmonella* flagellin, the ND1 and CD1 helices form multiple intramolecu-

lar domain-domain interactions that provide structural strength in the flagellin protein. The absence of the interactions in FlaA^N and FlaA^C, which contain one helix of *Salmonella* and one of *Campylobacter*, may explain the biological inactivity of these chimeras. Evidence that the β-hairpin structure likely confers more than protein folding and stability is that the *Salmonella* β-hairpin proved necessary for activation of human and mouse TLR5 but not chicken TLR5. This receptor was activated by both the chimeras FlaA^{NC} and FlaA^{NHC} (Fig. 7*C*). The activation of chicken TLR5 by Fla^{NC} (but not Fla^N or Fla^C) indicates that the protein is folded into a TLR5 activating state.

Amino acid sequence analysis suggests that *C. jejuni* flagellin contains a β-hairpin structure at grossly the same position as in *Salmonella* flagellin. Although several amino acids are conserved between the β-hairpin of *Campylobacter* and *Salmonella*, the inactivity of chimera FlaA^{NC} toward human and mouse TLR5 shows that the *C. jejuni* β-hairpin domain cannot substitute for the *Salmonella* β-hairpin structure in the receptor interaction. Chicken TLR5 is activated by flagellins that contain either the *Salmonella* or *Campylobacter* β-hairpin. This may indicate that chicken TLR5 has a more relaxed ligand specificity than mammalian TLR5 with respect to the β-hairpin. Indeed, we previously demonstrated that chicken TLR5 has different flagellin sensing qualities compared with human TLR5 (33). In addition, Smith *et al.* (21) showed that the disruption of the β-hairpin in flagellin in *Salmonella* significantly decreased biological activity of flagellin for human TLR5 but not mouse TLR5. Together, these data suggest the hairpin stretch contributes to the species specificity of flagellin recognition by TLR5.

Campylobacter flagellins are heavily decorated with an array of variably modified pseudaminic acid residues, which is needed for flagella formation (44). In *Pseudomonas aeruginosa*, flagellin glycosylation promotes TLR5 stimulation (50). The glycosylation moieties present on the flagellin of *C. jejuni* are located on the predicted surface exposed variable domain, mostly in a 200-amino acid hydrophobic patch. Structural modeling of *C. jejuni* FlaA on the crystal structure of *Salmonella* flagellin reveals that the sugar moieties are not in close proximity to the predicted TLR5 binding site. Expression of chimeric FlaA^{NHC} by live *Campylobacter*, which resulted in glycosylated and secreted proteins, presented us with the opportunity to, for the first time, directly assess the role of the flagellin-glycosylation on TLR5 activation. Culture supernatants containing glycosylated FlaA^{NHC} as well as live *C. jejuni* secreting glycosylated flagellins showed the ability to strongly activate TLR5, suggesting that the modification of *C. jejuni* flagellin does not serve as an additional mechanism to prevent or promote activation of TLR5.

The successful engineering of *Campylobacter* strains, which secrete flagellins that variably activate TLR5, indicates that the
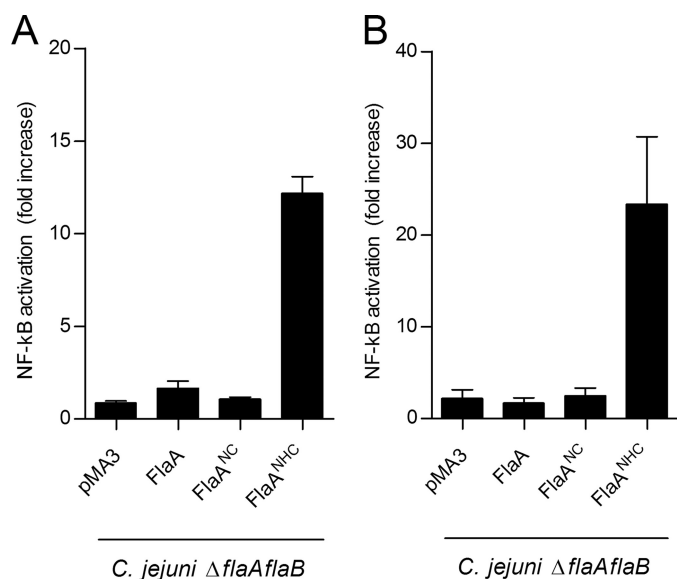
FIGURE 9. **C. jejuni expressing FlaA**[NHC] **induces NF-κB activation in HEK293 cells.** NF-κB activation in TLR5-expressing HEK293 cells was measured after stimulation (5 h) with live bacteria (A) or culture supernatant of C. jejuni ΔflaAflaB expressing FlaA, FlaA[NC] or FlaA[NHC] (B). As a control, C. jejuni ΔflaAflaB carrying the empty expression vector pMA3 was used. Values represent the increase of NF-κB-induced luciferase activity in stimulated cells compared with non-stimulated cells and are the mean ± S.E. of three independent experiments.

modified regions are not critical for transport through the *C. jejuni* flagellar secretion apparatus. Successful secretion of flagellin through the flagellar basal body requires the ND0 domain, which contains a secretion signal (51), and the CD0 region, which binds chaperone FliS to inhibit cytosolic flagellin polymerization (52, 53). None of the chimeric flagellins constructed in this study have alterations in either the putative secretion signal in domain ND0 or in domain CD0, and the chimeras are, thus, predicted to bind *C. jejuni* FliS in a similar fashion as the wild type flagellin. Furthermore, as in *Campylobacter* flagellin glycosylation is essential for secretion (44), all putative glycosylation sites in the constructed chimeric flagellins were left intact. The conservation of the basic *Campylobacter* flagellin architecture may explain the successful secretion of flagellins with the incorporated foreign domains needed for TLR5 activation. However, despite secretion, none of the chimeric flagellins in *C. jejuni* assembled into a filament. This may indicate a dysregulation of the flagellar components needed for fiber assembly and/or incompatibility of the chimeric structure with *e.g.* the *Campylobacter* filament capping protein FliD, a defective multimerization, or altered axial interactions between the flagellin subunits. Elucidation of the crystal structure of *C. jejuni* flagellin may resolve this issue.

Recent studies have identified two additional cellular receptors for flagellin, the intracellular Nod-like receptor (NLR) Ipaf and NLR apoptosis inhibitory protein 5 (Naip5) (54, 55). Localized intracellularly, these receptors are involved in sensing flagellin that is injected into the host cell, for instance through the type III secretion system (T3SS) of *S. typhimurium*, or the type IV secretion system (T4SS) of *Legionella pneumophila*. Activation of Ipaf and/or Naip5 results in caspase-1-dependent IL-1β and IL-18 secretion. So far, a functional injection

machinery like T3SS or T4SS has not been found in *Campylobacter*. In the case that *Campylobacter* flagellin gains access to the cytosol, it may activate Ipaf and Naip5, as the C-terminal 35-amino acid flagellin domain that is sensed by these receptors is highly conserved when compared with *Legionella* FlaA (56). As this region is not altered in FlaA[NHC], this chimeric flagellin is predicted to activate both TLR5 and Ipaf/Naip5.

In conclusion, we constructed a live *C. jejuni* secreting glycosylated flagellins with reconstituted TLR5 activity by the introduction of multiple domains from *Salmonella* flagellin. Through the construction of a series of chimeric flagellins, we identified a previously unknown role for the flagellin β-hairpin domain in the activation of TLR5 and showed that this structure determines TLR5 species specificity in flagellin response. These results provide more insight in the flagellin-TLR5 interaction and contribute to the current knowledge on the application of flagellin for vaccination purposes.

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