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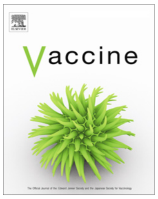

Recombinant flagellins with deletions in domains D1, D2, and D3: Characterization as novel immunoadjuvants

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ABSTRACT

Bacterial flagellin activates the innate immune system and ultimately the adaptive immune system through a Toll-like receptor 5 (TLR5)-dependent signaling mechanism. Given that TLR5 is widely distributed in epithelia, flagellin is currently being developed as a mucosal adjuvant. Flagellin FliC from *Salmonella enterica* has four domains: the conserved D0 and D1 domains and the hypervariable D2 and D3 domains. The deletion of D3 and partial deletion of D2 in the recombinant FliC$_{\Delta 174\text{-}400}$ strongly impairs flagellin's intrinsic antigenicity but does not affect the TLR5-dependent immunostimulation activity, i.e., the capacity to promote innate responses and adaptive responses to co-administered antigens. Here, we describe the development of novel recombinant flagellins with various deletions encompassing all of D2 and D3, and part of D1. Most of the recombinant molecules conserved an α-helical secondary structure that was as resistant to heat denaturation as the native protein. Whereas the recombinant flagellins' ability to trigger TLR5 varied markedly *in vitro*, most gave equivalent *in vivo* TLR5-dependent innate immune responses following intranasal administration of 2 µg of flagellin to mice. Concordantly, the recombinant flagellins were also valuable respiratory adjuvants for eliciting antibody responses to the foreign antigen ovalbumin, although their intrinsic antigenicity was decreased compared to the native flagellin and not increased compared to FliC$_{\Delta 174\text{-}400}$. Our results show that the additional deletions of D2 and the distal part of D1 of FliC$_{\Delta 174\text{-}400}$ does not impact on antigenicity and does not significantly modify the immunostimulatory adjuvant activity. Altogether, this study generated a novel set of recombinant flagellin that constitutes a portfolio of TLR5-dependent candidate adjuvants for vaccination.

1. Introduction

Flagellin, the structural protein of the flagellum, acts as a microbe–associated molecular pattern that strongly activates the innate and adaptive immune systems [1,2]. The three-dimensional structure of flagellin FliC from *Salmonella enterica* serovar Typhimurium has been solved. The 494 amino-acid protein is organized into four domains: D0 and D1 are essential for innate immune signaling and polymerization of the flagellar filament, whereas D2 and D3 harbor the main antigenic determinants of flagellin and are involved in phase variation [3–11] (Fig. 1A). Remarkably, D0 and D1 are highly conserved among the bacteria, whereas D2 and D3 are hypervariable. The D0, D1 and D2 domains are all constituted by primary sequences from both the amino- and carboxy-terminal regions of the polypeptide chain; for example, D0 is formed by the ND0 sequence (1–53) and the CD0 sequence (450–494). Importantly, D1 is rod-shaped and mainly formed by α-helices (57–99, 104–129, 406–447). Although D0 is organized into α-helices within the flagellum, it is totally disordered when the monomeric protein is free in solution. In contrast, the D2 and D3 domains of flagellin FliC are mostly made up of β-stands.

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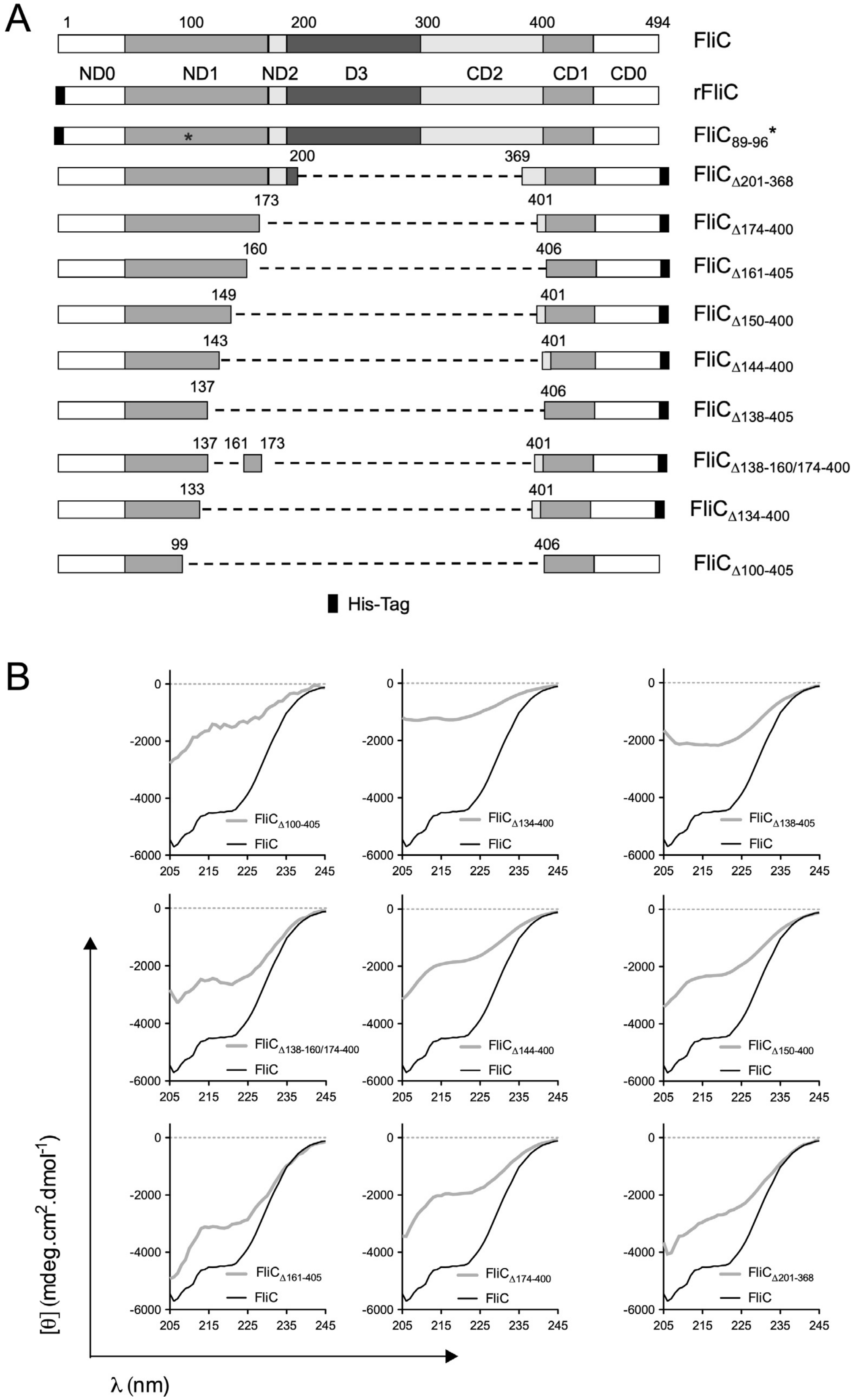

Fig. 1. Description and structural analysis of the recombinant flagellins. (A) Schematic representation of deletions on the primary sequence of flagellin FliC from *S.* Typhimurium (UniProt - P06179). Domains D0, D1, D2, and D3 are represented in different colors as indicated. The prefix N and C stands for amino- and carboxy-terminal region of the corresponding domain. The molecules were named according to the deletion performed into the primary sequence considering the cleavage of the initiator methionine. (B) Far UV circular dichroism spectra of each recombinant flagellin (gray line) is compared to that of native FliC (black line).

Various researchers have reported that the deletion of D2 and D3 strongly abrogates flagellin's intrinsic antigenicity but does not affect the protein's immunostimulatory activity [10–15].

Flagellins are detected by Toll-like receptor 5 (TLR5)-dependent signaling mechanism on the cell surface [16]. In the mouse model, flagellin also triggers the inflammasome via the cytosolic machinery, including nucleotide-binding and oligomerization domain-like receptor (NLR) apoptosis-inhibitory proteins 5 and 6 (NAIP5 and NAIP6), and NLR family caspase recruitment domain-containing 4 (NLRC4) [2]. Remarkably, flagellin monomers induce TLR5 signaling but filamentous flagella do not [17]. In the D1 domain in *S.* Typhimurium FliC, the ND1 motifs 82–101 and 110–118 and the CD1 motif 412–438 are especially important for flagellin's interaction with TLR5 and thus for signal transduction [5–7,18]. The replacement of motif 89–96 in *S.* Typhimurium FliC by the corresponding motif from *Helicobacter pylori* abrogates the interaction with TLR5, as demonstrated by cell signaling assays and a structure-based analysis [19,20]. The FliC CD0 region's contribution to NAIP/NLRC4 and TLR5 signaling has been highlighted by several studies [6,7,21–24]. However, it is still not completely understood how D0 interacts with TLR5 or NAIP.

In view of the flagellins' ability to modulate innate immune signaling and their various potential clinical applications, these proteins have been the focus of major research and development efforts [1,25]. In particular, flagellins are being developed as a vaccine adjuvant for systemic as well as mucosal routes of immunization [25,26]. Flagellin stimulates various compartmentalized immune responses as a function of its administration route. When flagellin is administrated via the mucosal route, the epithelium is the main signaling site. The prevalent expression of TLR5 on the mucosal epithelium has been linked to flagellin's ability to act as adjuvant for vaccine antigens and antibiotics. When flagellin is delivered intranasal (i.e. to the respiratory tract), the epithelial TLR5 signaling but not the NLRC4 signaling activates and/or recruits immune cells, including polymorphonuclear neutrophils, inflammatory monocytes, and conventional dendritic cells (DCs). Previous work in our laboratory has shown that maturation and enhanced antigen presentation by mucosal conventional DCs in the epithelium are essential for adjuvant activity in the adaptive immune system [27,28]. Thus, the mucosal co-administration of native enterobacterial flagellin and various antigens stimulates specific cellular responses and systemic and mucosal antibody responses, including IgA production in luminal compartment of mucosa. Intramuscular, subcutaneous and intraperitoneal flagellin-based vaccines are very potent inducers of cellular responses and systemic antibody responses [29–32]. The systemic administration of flagellin targets several TLR5-responsive sentinel cells, including lymphoid tissue-resident and mucosal DCs. Both systemic and mucosal route of administration are related to T helper responses that are biased towards Th2 [28,31,32]. The flagellins' ability to accommodate protein domains in the amino- and carboxy-termini or in the D3 domain has been exploited to cargo antigens in mucosal and systemic vaccines. The genetic or chemical fusion of flagellin with an antigen of interest also enhances the adaptive immune response [33,34]. Lastly, flagellin is a potent adjuvant for use in older adults; in contrast to other TLRs, the activity of TLR5 signaling does not decline with age [35,36].

The flagellins' intrinsic antigenicity may be an obstacle for clinical development and application, since the generation of flagellin-specific antibodies can preclude the presence of adjuvant activity in consecutive applications [14]. Consequently, further investigation of the molecular determinants of adjuvanticity and antigenicity is of value in these applications. Adjuvant activity has been consistently linked to the ability to trigger TLR5 signaling. Genetic approaches have been used to show that the TLR5 target sequence is located in a conserved site in D1 required for protofilament formation and bacterial motility [17]. Recently, the resolution of the crystal structure of the zebrafish TLR5-*Salmonella* flagellin D1 complex confirmed these findings and showed that the contact area is extensive and involves several critical sites [6]. Functional studies have also shown that D0 is also involved in the interaction with TLR5 [7,23].

Flagellin's immunodominant regions have been mapped to D3 [11,37]. Earlier studies have shown that deletion of this hypervariable region lowers flagellin's antigenicity, although differing effects on the region's ability to activate TLR5 have been reported [9,13,38]. In particular, our laboratory demonstrated that *S.* Typhimurium FliC deleted for aminoacids 174–400 ($\text{FliC}_{\Delta174\text{-}400}$, lacking ND2, D3, and a significant part of CD2) stimulated murine and human TLR5 but displayed very low intrinsic antigenicity [14]. In order to improve our understanding on the influence of the ND1 and CD2 subdomains in flagellin's antigenicity and ability to activate TLR5, we generated a set of flagellin variants bearing extensive deletions in these regions and then characterized the proteins' structural characteristics and *in vivo* and *in vitro* immunological properties. Our results indicate that the deletion of CD2 and ND1 significantly does not increase protein's antigenicity compared to the parental recombinant $\text{FliC}_{\Delta174\text{-}400}$ and does not impact on the TLR5-mediated immunostimulation activity when delivered as intranasal vaccine adjuvant.

2. Material and methods

2.1. Production of recombinant flagellins

A schematic diagram of flagellins evaluated in this study is presented in Fig. 1A and aminoacid sequences are given in Table S1. The recombinant proteins originated from the *Salmonella enterica* serovar Typhimurium ATCC14028 flagellin FliC (accession number P06179). Native flagellin FliC, rFliC, $\text{FliC}_{89\text{-}96^*}$, and the novel recombinant truncated flagellins (most harboring an amino-terminal histidine Tag) were produced as described previously [39,40]. The rFliC is equivalent to the native protein and $\text{FliC}_{89\text{-}96^*}$ contains TLR5-non-signaling residues at positions 89–96. The poorly antigenic TLR5-stimulating $\text{FliC}_{\Delta174\text{-}400}$ harboring a carboxy-terminal histidine Tag were generated by PCR-mediated In-Fusion® HD Cloning Kit (Clontech, Mountain View, CA, USA) using the construct described by Nempont et al. [14] and cloned into the expression vector pET22b+. This DNA template was used for PCR-mediated mutagenesis to create extensive deletions. Finally, $\text{FliC}_{\Delta201\text{-}368}$ was previously designed by gene synthesis and cloning into pET11a [41]. The recombinant flagellins were produced in *E. coli* BL21(DE3), purified using fast protein liquid chromatography (GE Healthcare, Pittsburgh, PA, USA) and depleted of lipopolysaccharide (LPS) using Triton X-114 extraction and polymyxin B columns (ThermoFisher Scientific, Waltham, MA, USA). In a *Limulus* assay (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit), the residual LPS concentration was determined to be less than 30 pg per μg of flagellin. To ensure that the flagellins were mostly monomers, the corresponding samples were heated for 10 min at 65 °C before use.

2.2. Circular dichroism (CD)

The CD spectra of recombinant flagellins were recorded between 195 and 320 nm on a spectropolarimeter (Jasco J-815) with Peltier temperature control system, using a 0.1 cm path length quartz cell. A scanning speed of 100 nm/min, a response time of 2 s, a data pitch of 0.1 nm, and a bandwidth of 2 nm were set. For each sample, three spectra were recorded and the result presented as the average spectrum to reduce background noise.

Data were collected at 25 °C in PBS buffer. Protein concentration was 15 μM. The samples were incubated for 15 min at 25 °C before taking CD measurements. Molar ellipticity was calculated by the following formula:

$$[\theta](\mathrm{deg} \times \mathrm{cm}^2 \times \mathrm{dmol}^{-1}) = \theta(\mathrm{mdeg})/10 \times \mathrm{L}(\mathrm{cm}) \times [\mathrm{M}](\mathrm{M})$$

where θ is the raw signal, L the path and [M] the protein concentration.

The heat-induced denaturation of flagellin variants in PBS buffer was followed by measuring the CD signal at 222 nm. The samples were treated by increasing the temperature ranging 20–60 °C at a speed of 4 °C/min. The molar ellipticity at 222 nm was measured every 0.1 °C. The data was fitted to sigmoidal functions and inflection points were calculated using GraphPad Prism software.

2.3. In vitro measurement of TLR5-stimulating activity

The human transgenic cell line Caco-*CCL20-LUC* was grown in DMEM supplemented with 10% FCS, 10 mM HEPES, 1 μM nonessential amino acids, 100 U/ml penicillin, and 100 U/ml streptomycin as described [14]. Cells were mock-stimulated or stimulated with native FliC or recombinant flagellins with 5-fold dilution series, starting at 1 μg/ml. After 6 h of stimulation, firefly luciferase activity in cell extracts was measured using the Luciferase Assay (Promega). Fold increase was calculated as the ratio of the luminescence induced by each recombinant flagellin and the luminescence of unstimulated cells.

Alternatively, transient transfection experiments using hTLR5 and mTLR5 were performed on HEK293 cells as previously described [23]. Briefly, cells were seeded into 96-well Costar plates (Corning) at 3×10^4 cells/well and incubated overnight. The next day, the cells were transfected by using JetPEI (Polyplus Transfection) with plasmids expressing TLR5 [pUNO-hTLR5 or pUNO-mTLR5-HA (InvivoGen) coding for hTLR5 and mTLR5, respectively, or pcDNA3 (Invitrogen) as control], pELAM-1 expressing NF-κB-dependent firefly luciferase as a reporter of pro-inflammatory signaling (C. Kirschning, University of Duisburg-Essen, Germany), and phRL-TK constitutively expressing *Renilla* luciferase (Promega) for normalization of transfection. After 24 h, the cells were stimulated with native FliC or recombinant flagellins for 16 h. Thereafter cells were lysed in a passive lysis buffer (Promega) and analyzed using a dual-luciferase reporter assay on a Mithras LB940 luminometer. Mock-transfected cells (i.e., transfected with the reporter plasmids pELAM-1, and phRL-TK) were stimulated with 1 μg/ml of recombinant flagellin and the values obtained were subtracted from the values obtained in transfected cells stimulated with the same recombinant flagellin. The relative luminescence was then calculated by normalizing firefly luciferase to *Renilla* luciferase activity in each sample and fold increase was calculated as the ratio of the relative luminescence induced by each recombinant flagellin and the relative luminescence of unstimulated transfected cells.

The human reporter cell line derived from respiratory alveolar epithelium A549-Dual™ cells (that harbors NF-κB- & IRF-specific reporter enzymes) was grown and activity in response to recombinant flagellin was measured as recommended by the manufacturer (Invivogen).

The normal human bronchial epithelial cell BEAS-2B were grown in complete epithelial cell culture medium (Promocell GmbH) as described previously [28]. Cells were stimulated with recombinant flagellin for 16 h before harvesting the supernatant for IL-8 production analysis using a commercial ELISA kit from R&D Systems.

2.4. Non-covalent interactions estimation

As most of the deletions affect D2 and D3 flagellin domains, we used PIC server [42] to estimate the number of non-covalent interactions in D0 and D1 domains of each recombinant flagellin as a measure of its corresponding stability. The total number of non-covalent interactions in D0 and D1 domains was estimated using the structure of *S.* Typhimurium native flagellin (PDB 1UCU), and for each recombinant flagellin the assessment was performed using as template the native flagellin deleting the corresponding residues.

2.5. Animal experiments

Six-to-ten-week old C57BL/6J or out bred NMRI mice were purchased from Janvier Laboratories (Saint Berthevin, France). *Tlr5*$^{-/-}$ backcrossed on C57BL/6J genetic were bred in house. Animals were maintained in a specific pathogen-free facility (#A59-350009, Institute Pasteur de Lille) and experiments complied with national regulations and ethical guidelines (B59-350009 – Institut Pasteur de Lille – protocol CEEA060211).

Intranasal (i.n.) administration of flagellin, LPS, or vaccines was performed as follows. Mice were anesthetized by intraperitoneal injection of ketamine-xylazine (1.5 mg and 0.3 mg respectively per 25 g animal body weight) or gaseous isoflurane (Belmont, France) and 30 μl of PBS ± *Escherichia coli* O111:B4 LPS ± recombinant flagellin ± endograde ovalbumin (OVA, Hyglos, Germany) were introduced into nostrils using a micropipette. For evaluation of activation of innate responses, lung tissues were sampled after i.n. administration of each recombinant flagellin or PBS. Immunization regimens consisted in a single i.n. administration of OVA ± recombinant flagellin or at day 1 followed by sampling at day 35 for OVA-specific antibodies. For sampling of tissues and sera, mice were sacrificed at selected times by intraperitoneal injection of 5.47 mg of sodium pentobarbital in 100 μl PBS.

2.6. Gene expression

Total RNA from lung tissues were extracted with the Nucleospin RNA kit (Macherey Nagel, Germany). RNA was reverse-transcribed with the High-Capacity cDNA Archive Kit (Life Technologies, USA). The resulting cDNA was amplified using SYBR Green-based real-time PCR and the 7300 Real Time PCR system (Applied Biosystems, USA) following manufacturers protocol. Relative quantification was performed as previously described using the genes coding β-actin (*Actb*) and β2-microglobulin (*B2m*) as endogenous controls [14]. The specific primers are CGTCATCCATGGCGAACTG/GCTTCTTTGCAGCTCCTTCGT (*Actb*), TGGTCTTTCTGGTGCTTGTC/GGGTGGCGTGAGTATACTTGAA (*B2m*), AAGAGGGATGCTGCCCTTAC/CCATTTTGTCTACGGGACGA (*Gadph*), TTTTGGGATGGAATTGGACAC/TGCAGGTGAAGCCTTCAACC (*Ccl20*), CCCTCAACGGAAGAACCAAA/CACATCAGGTACGATCCAGGC (*Cxcl2*), AATCTATACCTGTCCTGTGTAATGAAAGAC/TGGGTATTGCTTGGGATCCA (*Il1b*), and GTTCTCTGGGAAATCGTGGAAA/GTTCTCTGGGAAATCGTGGAAA (*Il6*). Relative mRNA levels ($2^{-\Delta\Delta Ct}$) were determined by comparing (a) the PCR cycle thresholds (Ct) for the gene of interest and the three housekeeping genes *Actb, B2m, and Gadph* (ΔCt) and (b) ΔCt values for treated and control groups (ΔΔCt).

2.7. Antigen- and adjuvant-specific antibody responses

Levels of native FliC- and OVA-specific IgG antibodies in serum samples were assessed by ELISA as described previously using biotinylated anti-mouse IgG (Southern Biotechnology Associates, USA) [28]. The concentration of immunoglobulin was defined using a standard curve with purified mouse IgG (Sigma). For isotype determination, ELISA were performed using serum that were diluted 200-fold, and biotinylated anti-mouse IgG1, IgG2a, IgG2b, and IgG2c (Southern Biotechnology Associates, USA).

To define the antigenicity, native or recombinant flagellins (100 ng per well in PBS) were coated on MaxiSorp microplates (Nalgen Nunc Int., USA) overnight at 4 °C. All microplates were washed with PBS/Tween20 0.05% and then blocked with PBS/Dry Milk 1% for 1 h at room temperature. Flagellin-specific "hyperimmune" serum was obtained on day 63 after subcutaneous injection of native flagellin FliC (1 µg per injection) emulsified in 200 µl of complete Freund's adjuvant /PBS on day 1 and incomplete Freund's adjuvant/PBS on days 21, 35 and 49. Serial dilutions of a FliC-specific "hyperimmune" serum were incubated for 1 h at room temperature before development as described [28].

Biotinylated anti-mouse antibodies (Southern Biotechnology Associates, USA), HRP-conjugated streptavidin (GE Healthcare, USA) and 3,3′,5,5′tetramethylbenzidine (Becton Dickinson Bioscience, USA) were used as development reagents. The reaction was stopped by addition of H_2SO_4 and the OD at 450 nm was determined.

2.8. Statistical analysis

The Mann Whitney test was used for comparison between two groups. For multiple group comparisons, One-way analysis of variance (ANOVA) test was performed. The differences were considered statistically significant for $P < 0.05$. All analyses were performed using the program GraphPad Prism version 5.0.

3. Results

3.1. Structural features of novel recombinant flagellins

The primary objective of the present work was to identify flagellin variants with low antigenicity but conserved ability to activate TLR5, i.e., immunostimulatory molecules. The native *S.* Typhimurium FliC was used as the template for a set of novel recombinant flagellins (Fig. 1A). As described elsewhere, rFliC is a tagged version of the native protein, whereas FliC$_{89\text{-}96^*}$ is impaired in TLR5 recognition [14,39,40]. We used FliC$_{\Delta 201\text{-}368}$ and FliC$_{\Delta 174\text{-}400}$ as reference flagellins, with low intrinsic antigenicity and potent ability to stimulate TLR5 [14,41]. Starting from FliC$_{\Delta 174\text{-}400}$, a novel panel of recombinant flagellins was engineered by further deleting the CD2/ND2 domains and part of the ND1 region (Fig. 1A and Table S1). The molecules FliC$_{\Delta 161\text{-}405}$, FliC$_{\Delta 150\text{-}400}$, FliC$_{\Delta 144\text{-}400}$, FliC$_{\Delta 138\text{-}405}$, FliC$_{\Delta 134\text{-}400}$, FliC$_{\Delta 138\text{-}160/174\text{-}400}$, and FliC$_{\Delta 100\text{-}405}$ were purified from *E. coli* by ion exchange chromatography or affinity chromatography, followed by endotoxin elimination on a polymyxin column. Endotoxin activity was determined in a Limulus assay, and was consistently below 0.3 endotoxin units/µg protein (Table S1).

The recombinant flagellins' secondary structures and folding properties were analyzed by circular dichroism (CD) (Fig. 1B). As expected, the presence of α-helices in the D1 domain of native FliC in solution was revealed by high absorbance at low wavelengths (i.e. at 206 nm and at 215–219 nm). All the deletions impacted the CD spectra but the recombinant flagellins with the largest deletions (e.g. FliC$_{\Delta 138\text{-}405}$, FliC$_{\Delta 134\text{-}400}$, and FliC$_{\Delta 100\text{-}405}$) showed the greatest changes in the CD spectrum with regard to the native molecule - implying a significant loss of α-helix structure (Fig. 1B). The thermal stability of the recombinant flagellins was studied by measuring the molar ellipticity at 222 nm. The flagellins' melting temperatures ranged from 32 °C to 42 °C. All the flagellins recovered their initial secondary structure following thermal denaturation at 100 °C, indicating that the molecules are able to spontaneously and reversibly refold into the native state (data not shown).

Lastly, we looked at whether the deletions induce structural constraints in the flagellins. This analysis was performed *in silico* by measuring the mean distance between the deletion borders (Table 1). We assumed that shorter distances were associated with less distortion of the original contacts in the structure and perhaps with less extensive structural perturbations. The distances between deletion borders ranged from 9 Å to more than 50 Å. Interestingly, the greatest distance was found in FliC$_{\Delta 150\text{-}400}$; however, the long, flexible linker "GGGGSGGGGS" was introduced between deletion borders, thereby decreasing constraints. Similarly, a GAAG linker was present in FliC$_{\Delta 161\text{-}405}$ and FliC$_{\Delta 138\text{-}405}$, whose deletion borders were 25 and 30 Å apart, respectively. FliC$_{\Delta 134\text{-}400}$ and FliC$_{\Delta 144\text{-}400}$ (lacking a linker) had distances of 32 Å and 40 Å, respectively, and so were considered to have strong structural constraints. Lastly, FliC$_{\Delta 100\text{-}405}$, FliC$_{\Delta 174\text{-}400}$ (both of which harbor the GAAG linker) and the linker-free FliC$_{\Delta 201\text{-}368}$ displayed distances of approximately 10 Å - suggesting minimal constraints. Furthermore, the number of weak interactions among the recombinant flagellins' aminoacids (considered as an index of folding stability) was estimated using the Protein Interactions Calculator [42] (Fig. S1). As expected, FliC$_{\Delta 201\text{-}368}$ (with intact D0-D1 domains) conserved all the interactions among the D0-D1 residues. FliC$_{\Delta 174\text{-}400}$ conserved much the same interactions as native FliC and FliC$_{\Delta 201\text{-}368}$, indicating that the partial deletion of CD1 has little impact on the D0-D1 region's folding stability. In contrast, FliC$_{\Delta 100\text{-}405}$ had only 60% of the native FliC's interactions. The other recombinant flagellins conserved about 80% of the non-covalent interactions in the D0-D1 region.

Table 1
Structural feature and biological activity of the recombinant flagellins.

Recombinant molecule	Distance from deletion ends (Å)[*]	Linker	EC_{50} on Caco-*CCL20-LUC* in ng/ml (nM) of flagellin[**]	EC50 on A549-Dual™ in ng/ml (nM) of flagellin[**]	EC50 on BEAS-2B in ng/ml (nM) of flagellin[***]
FliC		Not applicable	15 ± 2 (0.29 ± 0.04)	2.5 (0.05)	2.83 (0.054)
FliC$_{\Delta 201\text{-}368}$	8.90	None	8 ± 3 (0.23 ± 0.09)	34 (0.98)	Not done
FliC$_{\Delta 174\text{-}400}$	11.05	GAAG	9 ± 1 (0.30 ± 0.03)	38 (1.27)	1.30 (0.044)
FliC$_{\Delta 161\text{-}405}$	25.00	GAAG	135 ± 15 (4.82 ± 0.54)	31 (1.11)	32.48 (1.16)
FliC$_{\Delta 150\text{-}400}$	53.00	GGGGSGGGGS	230 ± 15 (8.31 ± 0.54)	178 (6.43)	106.30 (3.84)
FliC$_{\Delta 144\text{-}400}$	40.5	None	260 ± 20 (9.82 ± 0.76)	>5000 (>190)	51.60 (1.95)
FliC$_{\Delta 138/160\text{-}174/400}$	Not applicable	GAAG	130 ± 10 (5.08 ± 0.39)	29 (1.06)	Not done
FliC$_{\Delta 138\text{-}405}$	30.15	None	1500 ± 200 (54.58 ± 7.28)	>5000 (>190)	Not done
FliC$_{\Delta 134\text{-}400}$	32.20	None	7800 ± 250 (307.20 ± 9.85)	>5000 (>190)	79.85 (3.14)
FliC$_{\Delta 100\text{-}405}$	9.85	GAAG	7500 ± 300 (368.91 ± 14.76)	>5000 (>190)	Not done

The EC50 data are expressed as mean ± SEM of three experiments.

[*] Distance between the deleted aminoacids into the native flgellin molecule were calculated using Pymol.

[**] Half maximal effective concentration (EC50) was defined from a flagelin dose response fitted curve based on luciferase activity.

[***] EC50 was defined from a flagelin dose response fitted curve based on secretion of IL-8 in culture supernatant of BEAS-2B cells that were stimulated for 16 h.

3.2. The immunostimulatory activity of recombinant flagellins

In order to analyze the recombinant flagellins' TLR5-stimulating activity, dose-response assays were performed with the human intestinal epithelial reporter cell line Caco-*CCL20-LUC* (Fig. 2A). Cells were treated with serial dilutions of the various recombinant flagellins, including FliC$_{89\text{-}96*}$ defective in TLR5 signaling. As expected, native flagellin and rFliC were both strong stimulators, whereas FliC$_{89\text{-}96*}$'s immunostimulatory activity was abrogated (Fig. 2 and data not shown). Interestingly, all the novel recombinant flagellins were able to stimulate TLR5. We determined that the EC$_{50}$ values with the Caco-*CCL20-LUC* reporter were spread over several orders of magnitude (Fig. 2A and Table 1). The flagellins FliC$_{\Delta174\text{-}400}$ and FliC$_{\Delta201\text{-}368}$ displayed much the same level

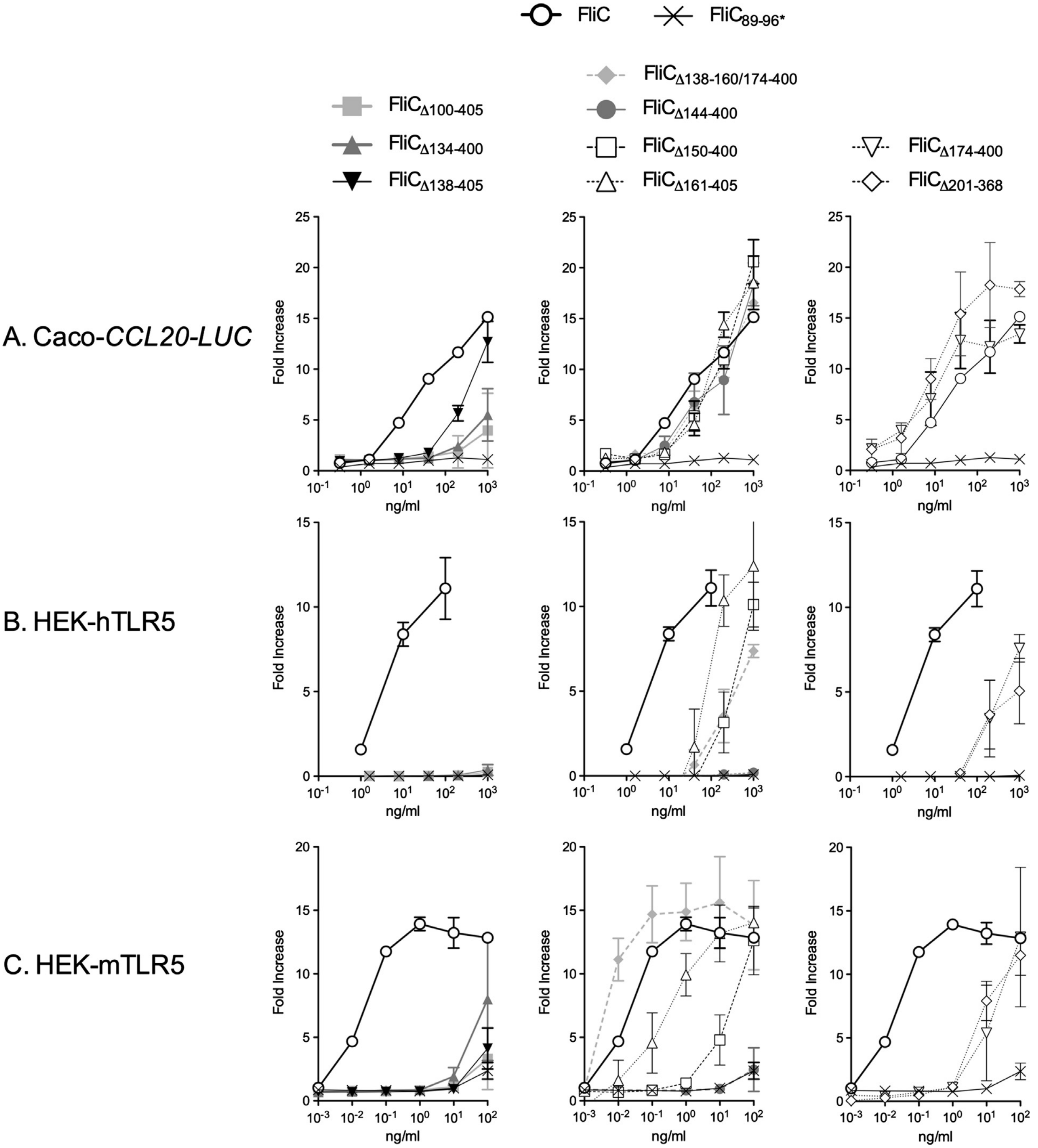

Fig. 2. *In vitro* proinflammatory activity of the recombinant flagellins. Reporter cells were activated for 6 h (A) or 16 h (B) with serial dilutions of recombinant flagellins. The native FliC and FliC$_{89\text{-}96*}$ were used as positive control and negative control of TLR5-specific activation, respectively. After incubation, cell extracts were prepared and assessed in luciferase assays. (A) Activation of Caco-*CCL20-LUC*. These reporter cells harbor the transcriptional fusion *CCL20-LUC* that is highly specific of flagellin-TLR5 signaling. The activation is expressed as fold increase; i.e. the ratio of firefly luciferase activity in flagellin-treated cells normalized to the activity measured with mock treatment. Results are representative of 4 experiments. (B-C) Activation of transgenic HEK293 cells. To this aim, cells were transfected with plasmids coding constitutive *Renilla* luciferase, NF-κB-dependent firefly luciferase, and constitutive expression of human (B) or mouse TLR5 (C) or mock (no plasmid). Assays measuring Renilla and Firefly luciferases were used to measure the relative luminescence. The RLU values of mock transfected cells was next subtracted from RLU values of TLR5-transfected cells. The activation is expressed as fold increase of flagellin-stimulated compared to unstimulated transfected cells. Results are representative of 2 experiments.

of activity as native flagellin (EC$_{50}$ ~ 10 ng/ml). The molecules FliC$_{\Delta 144\text{-}400}$, FliC$_{\Delta 150\text{-}400}$, FliC$_{\Delta 138\text{-}160/174\text{-}400}$ and FliC$_{\Delta 161\text{-}405}$ exhibited EC$_{50}$ values ranging from 100 to 250 ng/ml. Lastly, the recombinant flagellins with large deletions including FliC$_{\Delta 138\text{-}405}$, FliC$_{\Delta 134\text{-}400}$ and FliC$_{\Delta 100\text{-}405}$ had EC$_{50}$ > 1500 ng/ml. It is noteworthy that the altered biological activity flagellin correlated strongly with the loss of α-helical structure and the loss of interactions stabilizing D0-D1 folding.

The recombinant flagellins' stimulatory activity was also assessed with HEK293 cells that had been transfected with plasmids coding for human TLR5 (hTLR5) or mouse TLR5 (mTLR5) and reporter plasmids (Fig. 2B-C). Native flagellin's activity in the HEK293-hTLR5 reporter system was similar to that observed with Caco-2 reporter cells. Flagellins FliC$_{\Delta 174\text{-}400}$ and FliC$_{\Delta 201\text{-}368}$ elicited a weaker response in HEK293-hTLR5 reporter system than in the Caco-*CCL20-LUC* reporter system. In contrast, FliC$_{\Delta 144\text{-}400}$, FliC$_{-\Delta}$150-400, FliC$_{\Delta 138\text{-}160/174\text{-}400}$ and FliC$_{\Delta 161\text{-}405}$ showed less stimulatory activity than native FliC did, and FliC$_{\Delta 138\text{-}405}$, FliC$_{\Delta 134\text{-}400}$ and FliC$_{\Delta 100\text{-}405}$ were strongly impaired in their stimulatory activity. HEK293-mTLR5 reporter cells were activated at generally low flagellin concentrations, indicating the presence of differences between the species dose/response activity. Interestingly, some molecules (FliC$_{\Delta 161\text{-}405}$, and FliC$_{\Delta 150\text{-}400}$) activated TLR5 just as strongly the native flagellin FliC, suggesting a differential impact of the deletions on the interaction with mouse or human TLR5. The recombinant flagellins FliC$_{\Delta 138\text{-}405}$, FliC$_{\Delta 134\text{-}400}$ and FliC$_{\Delta 100\text{-}405}$ showed the lowest activity but were nevertheless able to activate TLR5 at concentrations $\geq$1000 ng/ml. Finally, we examined the dose-response activity of recombinant flagellins on the human bronchial epithelial cells BEAS-2B and the human alveolar epithelial cell reporter line A549-Dual™. In both cells (Table 1), results were overall coincident with results obtained on Caco-2 cells, being FliC$_{\Delta 174\text{-}400}$, FliC$_{\Delta 201\text{-}368}$ and FliC$_{\Delta 161\text{-}405}$ the most active. In BEAS-2B cells, immunostimulation activity was monitored using endogenous IL-8 cytokine secretion, i.e. a physiological process, rather than reporter systems such transcriptional fusion with luciferase. Recombinant flagellins were all able to induce IL-8 secretion at lower EC$_{50}$ values than in reporter cells. Moreover, the range of EC$_{50}$ among the various recombinant flagellins was less pronounced with a global variation over 100-fold (Table 1). In conclusion, the recombinant molecules differed in their ability to stimulate TLR5 reporter systems - suggesting that activation varies as a function of the physiological context.

We next investigated the recombinant flagellins' ability to stimulate TLR5 *in vivo*. First, we studied the dose-dependent effects of the intravenous injection of flagellins from FliC$_{\Delta 134\text{-}400}$, FliC$_{\Delta 150\text{-}400}$ and FliC$_{\Delta 174\text{-}400}$ on the proinflammatory systemic response (Fig. S2). To this end, sera were sampled 2 h post-injection and assayed for the inflammatory mediators CCL20 and IL-6. Interestingly, FliC$_{\Delta 174\text{-}400}$ was approximately 10-fold more potent (like native flagellin) than FliC$_{\Delta 150\text{-}400}$ or FliC$_{\Delta 134\text{-}400}$. This finding suggests that *in vitro* biological activity was correlated with systemic *in vivo* activity but the variation *in vivo* was attenuated compared to *in vitro*. We next evaluated the recombinant flagellins' activity when administered intranasally - an administration route of major interest for mucosal vaccine adjuvants. To this aim, we take advantage of some surrogate lung markers that were identified after administration of native flagellin in the intranasal delivery of flagellin-adjuvanted vaccine [27,28]. The level of transcripts coding for the chemokines CCL20, CXCL1, and CXCL2 and the interleukins IL-1β and IL-6 in the lungs of *Tlr5*$^{-/-}$ and congenic wild type C57BL/6J mice was assayed after the intranasal administration of 2 μg of recombinant flagellin (Fig. 3). As expected, our data demonstrated that *Tlr5*$^{-/-}$ and wild type mice were both responsive to the TLR4 agonist lipopolysaccharide (LPS), as illustrated by the upregulation of gene expression in the lung. In agreement with previous observations [28,39,40], FliC$_{89\text{-}96^*}$ was unable to activate lung proinflammatory responses in wild type mice. Remarkably, recombinant flagellins FliC$_{\Delta 134\text{-}400}$, FliC$_{\Delta 138\text{-}160/174\text{-}400}$, FliC$_{\Delta 150\text{-}400}$, FliC$_{\Delta 161\text{-}405}$, FliC$_{\Delta 174\text{-}400}$, and FliC$_{\Delta 201\text{-}368}$ were all able to upregulate expression of *Ccl20, Cxcl1, Cxcl2, Il1b, and Il6* in C57BL/6J mice. Overall, a 20- to 200-fold increase in gene expression was observed for all recombinant flagellins, relative to control (PBS-treated) animals. In contrast, the activation of gene expression was totally abrogated in *Tlr5*$^{-/-}$ mice treated with the recombinant flagellins compared to PBS-treated *Tlr5*$^{-/-}$ animals. These results showed that in our experimental conditions, the increases in expression of lung pro-inflammatory mediators required TLR5 signaling and were independent of the recombinant flagellins' *in vitro* biological activity.

3.3. The activity of recombinant flagellins as mucosal adjuvants

To further characterize the respiratory response, the mucosal adjuvant activity of recombinant flagellins was evaluated in mice by using ovalbumin (OVA) as a model antigen (Fig. 4). Mice were thus immunized intranasally with OVA, OVA + FliC or OVA + recombinant flagellin; the OVA-specific serum antibody response was then assessed (Fig. 4A-B). The amount of flagellin used for these experiments was fixed to 2 μg, usual dose in vaccination studies [1,26,28]. The various recombinant flagellins, and native FliC were all similarly potent with regard to increasing levels of OVA-specific IgG (by approximately 80-to100-fold, relative to OVA alone). We also screened the sera of animals for the IgG isotypes: IgG1, IgG2a, IgG2b, and IgG2c triggered by the intranasal immunization (Fig. S3). As previously described [27,28], intranasal administration of the various flagellins as adjuvant elicited mainly IgG1 isotype, a feature associated to T helper 2-biased adaptive responses. Although the deleted flagellins varied in their *in vitro* activity, we demonstrated that their adjuvant activity was conserved *in vivo*, using standard experimental conditions.

Lastly, the recombinant flagellin's intrinsic antigenicity was evaluated by using two approaches: an analysis of flagellin-specific antibody responses elicited *in vivo* by mucosal vaccination, and an *in vitro* ELISA of antigenicity relative to FliC-specific "hyperimmune" serum. Firstly, only native flagellin FliC was able to elicit native flagellin FliC-specific antibodies in serum, whereas the antigenicity of recombinant flagellins was abrogated in our experimental conditions of immunization (Fig. 4C-D). We also assessed the serum antibody response generated upon the intranasal immunizations against the truncated recombinant flagellins FliC$_{\Delta 134\text{-}400}$, FliC$_{\Delta 174\text{-}400}$, and FliC$_{\Delta 201\text{-}368}$ (Fig. S4). As previously described [14,27], only animals immunized intranasally with native flagellin were able to mount a significant response against the recombinant flagellins. This suggest that the deletion of the D2 and D3 domains strongly impact on the overall antigenicity of recombinant flagellins and the capacity to elicit any antibody response specific for flagellin. The second set of experiments aims at defining whether the recombinant flagellins are still able to expose some antigenic determinants (B epitopes). All the recombinant flagellins were found to be cross-reactive with a FliC-specific "hyperimmune" serum (Fig. 5). However, the recombinant flagellins were all roughly 10-fold less cross-reactive than the native FliC. These data show that the recombinant flagellins have antigenic determinants in the distal domains ND1, CD1, ND2 and CD2 that can be recognized by highly potent anti-FliC hyperimmune serum but at the doses that we used in intranasal immunization, such antibody responses are not elicited. Our data also indicated that the additional deletions of CD2 and ND1 that is associated with structural changes (Fig. 1) do not unmask novel antigenic determinants specific of the antibody response compared to FliC$_{\Delta 174\text{-}400}$ and do globally affect the capacity to trigger response against the remaining antigenic

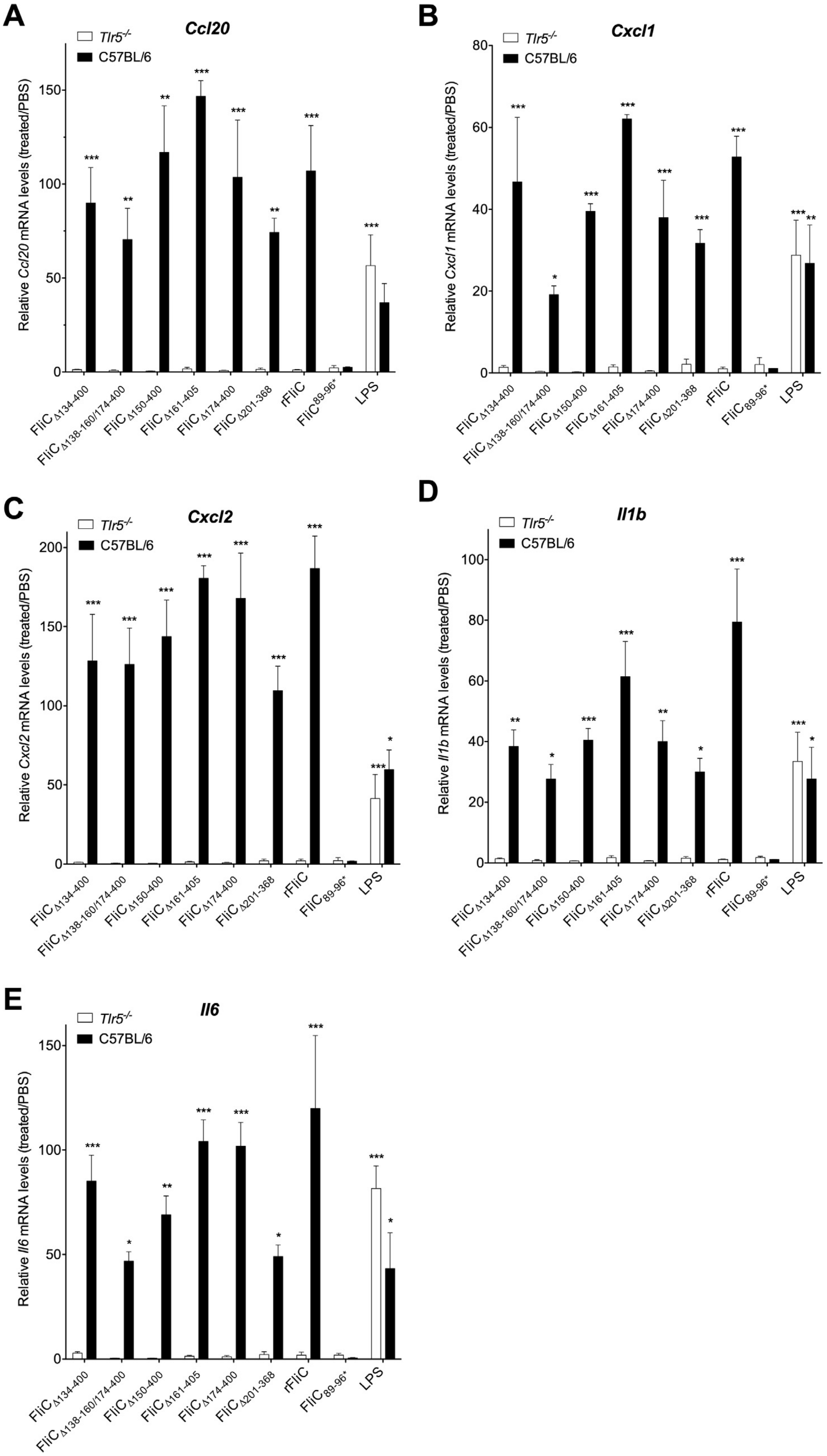

Fig. 3. Transcriptional activation of the mucosal innate response by the recombinant flagellins. C57BL/6J or *Tlr5*$^{-/-}$ mice (n = 3–4 per group) were treated i.n. with the recombinant flagellins (2 μg per animal) or LPS (1 μg per animal) or PBS as indicated. Two hours after administration, lungs were collected for analysis of mRNA levels by quantitative RT-PCR. Messenger RNA levels of *Ccl20* (A), *Cxcl1* (B), *Cxcl2* (C), *Il1b* (D), and *Il6* (E) for individual mice are expressed relative to the PBS-treated group of the corresponding genotype (arbitrarily set to a value of 1) and are shown as the mean ± SEM. Statistical significance between flagellin- and PBS-treated groups for each mouse genotype was assessed by One-way ANOVA test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

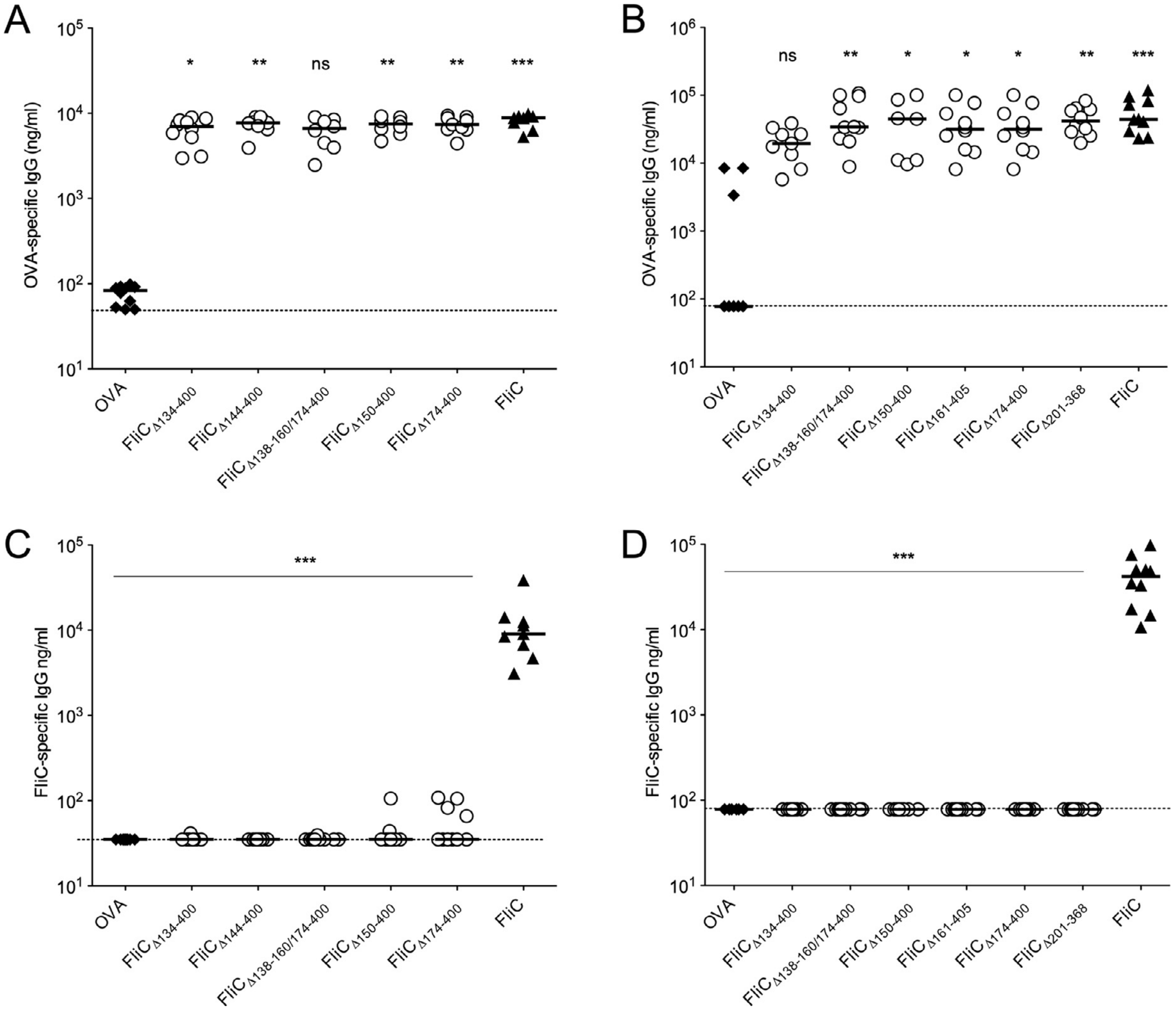

Fig. 4. Mucosal adjuvant activity of the recombinant flagellins. NMRI outbred mice were immunized i.n. on day 1with ovalbumin (OVA), OVA + FliC or OVA + recombinant flagellin as indicated. On day 35, OVA-specific IgG (A, B) FliC-specific IgG (C, D) were measured in serum by ELISA. (A, C) Immunization experiment 1 with 5 μg OVA. (B, D) Immunization experiment 1 with 10 μg OVA. Concentrations of antigen-specific IgG are given for individual animal and as median values. The dotted lines represent the limit of detection of the assays. Statistical significance was assessed by the ANOVA Kruskal-Wallis test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, *not significant*).

determinants. Taken as a whole, these data indicate that recombinant flagellins are much less antigenic than the native flagellin - irrespective of the extent of deletion beyond the D3/ND2 domains.

4. Discussion

In the present study, we investigated the structural organization and immunological activity of deletion variants of flagellin from *S.* Typhimurium. Previous research had demonstrated that flagellin's D2 and D3 domains are not essential for TLR5 signaling activity but contribute to antigenicity. The study's main objective was to gain information on the contribution of the ND1 and CD2 regions to flagellin's immunostimulatory, adjuvant and intrinsic antigenic properties. Our results demonstrated that recombinant flagellins generated by extensive deletions within ND1 and CD2 can be classified into strong and low potency TLR5 agonists. In an *in vitro* TLR5 stimulation assay with human and murine reporter cell lines, the agonist activity ranged over at least three orders of magnitude. Furthermore, the low-potency group displayed profound alterations in its α-helix content – the main secondary structure in the D1 domain. In a physiological cell system such the bronchial epithelial cell BEAS-2B, the range is attenuated suggesting that the reporter systems exacerbate and reveals additional differences among the recombinant flagellins unrelated to physiological immunostimulation activity. Despite this variability, our *in vivo* assay results strongly suggest that (i) TLR5 stimulation after intranasal administration is less tightly related to the structure-activity relationships observed *in vitro*, and (ii) the potency of recombinant molecules in clinical applications will be difficult to predict based only on *in vitro* assays, particularly transgenic reporter systems.

In accordance with the literature data, deletions of the D3-D2 domains induced small changes in flagellin's CD spectrum [9]. The CD analyses indicated that six of the nine recombinant flagellins conserved the D1 domain's archetypal α-helical structure (Fig. 1B). This feature was strongly related to the flagellins' ability to stimulate an innate immune pathway *in vitro*, since the three flagellins with lower capacity to trigger TLR5 activation in reporter systems FliC$_{\Delta138\text{-}405}$, FliC$_{\Delta134\text{-}400}$ and FliC$_{\Delta100\text{-}405}$ displayed the greatest changes in secondary structure (Fig. 2). The recombinant flagellins varied in their ability to activate TLR5 *in vitro*, despite that in most of the cases they contain D1 residues that are critical for the TLR5-flagellin interaction [6,7,23,24]. These variations might be related to the protein's overall conformation, and the mode and robustness of D1's folding. The flagellins FliC$_{\Delta201\text{-}368}$ and FliC$_{\Delta174\text{-}400}$ (exhibiting the highest *in vitro* activity) were characterized by short distances between the deletion borders (i.e. low structural constraints), the maintenance of α-helices in D1, and

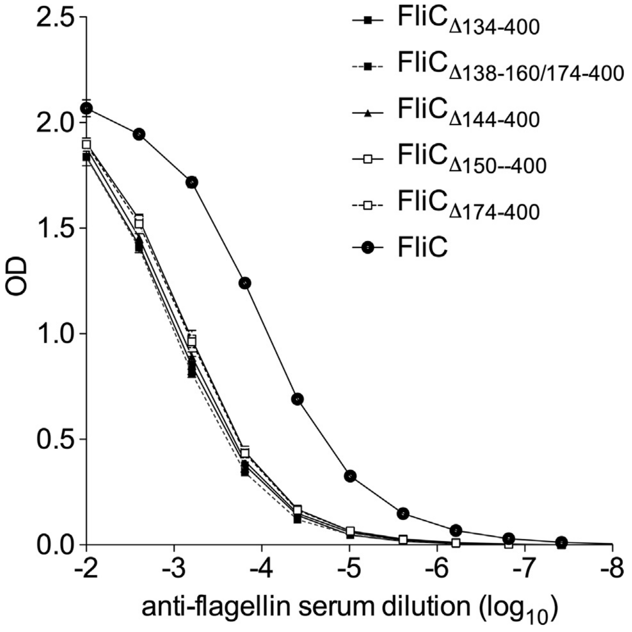

Fig. 5. Cross-reactivity of the recombinant flagellins. A hyperimmune serum specific for native flagellin FliC was titrated in ELISAs for FliC and the recombinant flagellins. The results are representative of two experiments and values represent the absorbance versus dilutions of the hyperimmune serum.

small alterations in the non-covalent interactions between D0-D1 aminoacids (Fig. 2 and Fig. S1). In contrast, FliC$_{\Delta134\text{-}400}$ and FliC$_{\Delta100\text{-}405}$ have deletions in loops and β-strands extending into D1 domain and that might contribute to the stability of the D1 domain. Furthermore, it has been recently described that ND1 region 125–135 contribute to flagellin-TLR5 interaction [43]. In conclusion, proper folding of the D1 domain is essential for optimal signaling.

Reducing the ability of a TLR agonist to trigger an innate response may ease safety concerns. By way of an example, monophosphoryl lipid A (MPLA) has been derived from LPS because of its weak ability to stimulate TLR4 signaling [44]. The EC_{50} of MPLA is >10,000-fold higher than that of LPS. Thus, MPLA shows attenuated inflammatory side effects, has a good safety profile, and has been licensed as a vaccine adjuvant. In *in vitro* assays, the recombinant flagellins FliC$_{\Delta138\text{-}405}$, FliC$_{\Delta134\text{-}400}$, FliC$_{\Delta100\text{-}405}$, FliC$_{\Delta144\text{-}400}$, FliC$_{\Delta150\text{-}400}$, FliC$_{\Delta138\text{-}160/174\text{-}400}$ and FliC$_{\Delta161\text{-}405}$ displayed significant changes in EC_{50} (from 10-fold to 1000-fold higher than native flagellin, depending on flagellin deletion and cell line considered, indicating reduced ability to stimulate TLR5 signaling. Using *in vivo* dose-response assays, FliC$_{\Delta174\text{-}400}$ was found to have lower pro-inflammatory activity than native flagellin following mucosal and systemic administration in mice [14]. The results of the dose-response confirmed that systemically administered FliC$_{\Delta174\text{-}400}$ is attenuated in its ability to trigger IL-6 production but not CCL20 production (Fig. S2). The ability of FliC$_{\Delta134\text{-}400}$ and FliC$_{\Delta150\text{-}400}$ to stimulate serum CCL20 and IL-6 production after systemic injection was more than 10-fold and 100-fold lower, respectively, than for native flagellin. These observations corroborate the attenuated activity found in *in vitro* cell assays but the impact of deletions on flagellin *in vivo* immunostimulation activity is less marked. It remains to be established how our recombinant flagellins alters TLR5 signaling, i.e. through changes in the primary interaction, receptor dimerization, adaptor recruitment or other molecular event. It is noteworthy that intranasal administration consisted of a single dose of 2 μg in 30 μl PBS (i.e. 67 μg/ml), which would result in concentrations above the EC_{50} of any of the recombinant flagellins and thus above the threshold for the saturation of immune signals. This may explain that, irrespective of their *in vitro* potency, the recombinant flagellins were able to stimulate the innate and adaptive immune systems in our animal experiments.

The recombinant flagellins stimulated mouse and human TLR5 transfected reporter systems to different extents. The flagellin concentrations required to activate mouse TLR5 were generally lower than those required for human TLR5 [45,46]. Consequently, mTLR5 was assumed to detect a broader set of flagellin variants, whereas hTLR5 was more sensitive to variations in flagellin structure [43,46]. In physiologically non-transgenic relevant models, i.e., BEASB-2B human bronchial epithelial cells, the recombinant flagellins were found highly active compared to reporter models and the overall differences are attenuated. These observations suggest therefore that the recombinant flagellins are effective in human and could be developed as adjuvant for vaccination by conventional route of immunization as well as respiratory route.

The production of flagellin-specific antibodies is undesirable when flagellin is to be used as an adjuvant. Indeed, specific antibodies can neutralize the flagellin's ability to trigger innate immunity, and thus affect its adjuvant activity [14,28,47,48]. Previous research has shown that the hypervariable D2 and D3 domains of enterobacterial flagellins harbors the major determinants of the antibody response, and thus are useful for serotyping bacteria [10,12,37]. Recently, vaccination studies showed that partial deletion of D3 (flanking aminoacids 190–320 in *S. typhi*, and 204–292 in *S.* Typhimurium) does not abrogate antigenicity [13,14]. However, flagellins with deletions encompassing the whole D3 domain and most of the D2 domain (aminoacids 180–400 in *S. typhi* and 174–400 in *S.* Typhimurium) do not induce flagellin-specific antibody responses in mice [13,14]. Here, the novel recombinant flagellins with deletions beyond the D3/D2 region (including CD2 and ND1) did not trigger a flagellin-specific response after one dose of intranasal vaccination, and so were impaired to much the same extent as FliC$_{\Delta174\text{-}400}$ (Figs. 4, 5 and S4). Consequently, extension of the deletion beyond flagellin's region 174–400 (to ND1 and D2) does not modify the recombinant protein's intrinsic antigenicity. Interestingly, entolimod (a TLR5 agonist in late-stage clinical development) harbors deletions in D3 and part of D2. It is derived from the 504- amino-acid *Salmonella enterica* serovar Dublin flagellin (deleted from position 176 to position 402 but with a 16 amino-acid linker), and is a potent radioprotective drug [49]. Recent advances in TLR5-flagellin interaction have improved our knowledge at the structural biology level. To better understand the adjuvant and *in vivo* proinflammatory effects, our results confirm that several important aspects have to be considered: the biochemical aspects of the recombinant flagellins' binding to TLR5, the molecules recruited for signaling, the general outcome of activation, and the tissue compartment to which the molecules are administered. Lastly, understanding flagellin's pharmacokinetic *in vivo* will help to optimize the design of TLR5-dependent adjuvants.

In conclusion, our results constitute a framework for generating structures with low intrinsic antigenicity but a conserved ability to act as immunostimulatory adjuvants. The different capacity of the recombinant flagellins to stimulate TLR5 may be an important feature to prepare adjuvant with a variable attenuation in pro-inflammatory signaling to target appropriate populations and various routes of immunization regarding the responsiveness to adjuvant, the safety and the tolerable side effects. Although it has been shown that flagellin-TLR5 interaction involves several regions of D1 and D0 domains, we have shown that deletion of ND2 and CD1 reduces flagellin's antigenicity but does not abrogate the global potency of immunostimulation, and thereby D1 and D0 domains interactions with TLR5.

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Conflict of interest

The authors declared that there is no conflict of interest. JC Sirard is the inventor of the patent WO2009156405 that describes several recombinant flagellins of this study.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.12.009.

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