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Running Title: TLR5 neutralization by anti-FliC antibodies

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ABSTRACT

Toll-like receptors (TLRs) trigger immunity by detecting microbe-associated molecular patterns (MAMPs). Flagellin is a unique MAMP since it harbors (i) an antigenic hypervariable region and (ii) a conserved domain involved in TLR5-dependent systemic and mucosal pro-inflammatory and adjuvant activities. Here, the contribution of the flagellin domains in TLR5 activation was investigated. We showed that TLR5 signaling can be neutralized *in vivo* by flagellin-specific antibodies, which target the conserved domain. However, deletions of flagellin's hypervariable region abrogated the protein's intrinsic ability to trigger the production of neutralizing antibodies The fact that MAMP-specific antibodies block TLR-mediated responses shows that this type of neutralization is a novel mechanism for down-regulating innate immunity. The stimulation of mucosal innate immunity and adjuvancy to foreign antigen was not altered by the hypervariable domain deletions. In contrast, this domain is essential to trigger systemic innate immunity, suggesting that there are distinct mechanisms for TLR5 activation in systemic and mucosal compartments. In summary, specific MAMP determinants control the production of neutralizing antibodies and the compartmentalization of innate responses.

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INTRODUCTION

Toll-like receptors (TLRs) are instrumental in the coordinated induction of innate and adaptive immunity in mammals (1, 2). Since TLRs are expressed by a broad variety of cell types, they are able to trigger immunity throughout the body. Following infection by pathogenic microorganisms, TLRs recognize conserved motifs referred to as microbe-associated molecular patterns (MAMPs) (1). TLR engagement induces a gene expression program dedicated to both innate clearance of and acquired immunity to pathogenic microorganisms (2). For instance, TLRs induce the production of chemokines which, in turn, specifically attract the polymorphonuclear neutrophils (PMNs) directly involved in innate microbial clearance. Furthermore, TLRs promote the secretion of pleiotropic immune mediators (such as TNFα) and the functional maturation of dendritic cells (DCs) which specialize in antigen presentation to lymphocytes. Consequently, TLR agonists not only stimulate “broadly specific” pro-inflammatory immune responses but also enhance the adaptive immune response to defined antigens, and are thus considered to be adjuvants (3). Despite these potentially beneficial effects, the systemic toxicity of MAMPs has prompted efforts to develop derivatives that bias MAMP activity towards adjuvancy (4). Indeed, engineering molecules with unique properties is a major challenge in manipulating immune responses.

Bacterial flagellins (the major flagella components in many bacterial pathogens) are specific, unique agonists for TLR5 activation (5, 6). The FliC flagellin from *Salmonella enterica* Serovar Typhimurium (*S.* Typhimurium) is the paradigm for studies on flagellum structure-function, immunity and TLR5 signaling (6-9). It is a 494 amino-acid protein with two distinct domains. The amino- and carboxy-terminal conserved regions (comprising about 170 and 90 amino-acids, respectively) form a domain that

is essential for TLR5 activation. Furthermore, the motif 89-96 is absolutely required (8-10). The middle (outer) domain of flagellin FliC comprises amino acids from positions 170 to 400 and is not mandatory for TLR5 signaling (9, 10). It is designated as a hypervariable region, since the primary sequences greatly vary in composition and size from one bacterial species to another. In contrast, it is known that the hypervariable region is essential for flagellin antigenicity (11, 12). In fact, flagellins hypervariable region carries H-antigen specificity used for serotyping enteropthopathogenic bacteria, especially *Salmonella* strains. Deletion of the central domain decreases the flagellins' antigenicity. For example, the *S.* Typhimurium flagellin $FliC_{\Delta 204\text{-}292}$, which is truncated of 99 amino-acids positioned in middle part of FliC, is poorly recognized by antibodies directed against the whole flagellin (11). The question of whether or not the antigenic and TLR5-activating domains can be functionally dissociated had not been addressed until now.

TLR5 agonists are potent activators of systemic and mucosal innate responses. We and others have shown that intravenous (i.v.) injection of flagellins promotes a systemic response, characterized by the production of pro-inflammatory mediators (such as TNFα or IL-6) and DC activation (5, 6, 13-17). Furthermore, flagellins trigger mucosa-specific innate and adaptive defense mechanisms (18-20). For instance, epithelial cell lines and lung mucosa upregulate the production of chemokines like CXCL8 (IL-8) and CCL20 which, in turn, recruit mucosal PMNs and DCs, respectively (18, 19). Various authors have reported that flagellins are potent systemic and mucosal adjuvants that elicit (i) serum and/or secretory antibody responses and (ii) Th1 and Th2 cell responses to both the flagellins themselves and co-administered antigens (14, 17, 21, 22).

The goal of the present study was to determine whether or not MAMP domains could alter the outcome of innate and adaptive immune responses. Since MAMPs have adjuvant activity, they also promote intrinsic anti-MAMP responses which, in turn, can neutralize pro-inflammatory and adjuvant properties. We addressed this issue by using flagellin as a model. We found that (i) anti-flagellin antibodies neutralize TLR5 signaling, (ii) deletion of the hypervariable part of flagellin abrogates the latter's ability to induce neutralizing antibodies but does not significantly alter pro-inflammatory and adjuvant activities and (iii) systemic detection of flagellin does not involve the same molecular determinants as mucosal detection.

MATERIALS AND METHODS

Production of recombinant flagellins. The recombinant flagellins originated from the *Salmonella enterica* Serovar Typhimurium ATCC14028 flagellin FliC (accession number AAL20871). The flagellins FliC and $FliC_{\Delta 205-293}$ were either isolated from the *S.* Typhimurium strains SIN22 (*fljB*) and SJW46, as described previously (11, 15, 19), or purchased from Alexis Biochemicals (Switzerland). The constructs encoding $FliC_{\Delta 174-400}$ and $FliC_{\Delta 191-352}$ were generated by PCR on a pBR322-derived plasmid harboring the wild type *fliC* gene under the control of its own promoter and using the following primer pairs: *AGCACC*attcagcgtatccagacc / *GCTGGT*gctacaaccaccgaaaac, and *TCGAG*atatcctgtaacagttgcagcc / *ACTCGAG*gacggtacatccaaaactgcac (bases encoding a linker are in italics). Site-directed mutagenesis was also performed on the plasmid harboring $FliC_{\Delta 174-400}$ in order to replace the residues 89-96 (QRVRELAV) involved in TLR5 detection by the corresponding sequences from a non-signaling flagellin (DTVKVKAT); the resulting protein was thus $FliC_{\Delta 174-400/89-96*}$ (8). In $FliC_{\Delta 174-400}$, $FliC_{\Delta 191-352}$ and $FliC_{\Delta 174-400/89-96*}$, the asparagine located 6 residues from the end was changed into a serine. The truncated flagellins were purified from the supernatant of recombinant *S.* Typhimurium SIN41 *(fliC fljB*), as follows. *Salmonella* were grown in Luria-Bertani (LB) broth for 18 hours at 37°C with agitation. The supernatant was filtered and saturated with 60% ammonium sulfate (Sigma Aldrich, USA). The precipitated materials were recovered by centrifugation, solubilization in 20mM Tris/HCl pH7.5 and then dialysis. The proteins were further purified by successive rounds of hydroxyapatite and anion exchange chromatography (Bio-Rad Laboratories, USA). Lastly, the proteins were depleted of lipopolysaccharide (LPS) using a polymyxin B column (Pierce, USA). Using the *Limulus* assay (Associates of

Cape Cod Inc., USA), the residual LPS concentration was determined to be less than 30 pg LPS per µg recombinant flagellin. When specified, flagellins were treated for 1h at 37°C with 0.017% trypsin-EDTA (Invitrogen, USA) to totally hydrolyze the proteins, followed by heating at 70°C for 1h to inactivate the trypsin. Proteins were analyzed using standard SDS-PAGE and immunoblotting with FliC-specific polyclonal antibodies.

Animal experiments. Female NMRI mice (6-8 weeks old) were purchased from Charles River Laboratories (France) and maintained in a specific pathogen-free facility in an accredited establishment (#A59107; Institut Pasteur de Lille). All experiments complied with current national and institutional regulations and ethical guidelines. For hyper-immunization, animals were injected subcutaneously (s.c.) with the flagellin FliC (1µg per injection) emulsified in 200µl of complete Freund's adjuvant (CFA)/PBS on day 1 and incomplete Freund's adjuvant (IFA)/PBS on days 21, 35 and 49. On day 63, mice were given 200µl flagellin/PBS i.v. and were sacrificed 2h later by intraperitoneal (i.p.) injection of 5 mg sodium pentobarbital (CEVA Santé Animale, France) for serum and tissue sampling and analysis.

To characterize the mucosal innate response and adjuvant properties, 20µl of PBS ± proteins were administered intranasally (i.n.) to mice anesthetized i.p. with 1.5 mg ketamine (Merial, France) and 0.3 mg xylazine (Bayer, Germany) per 25g animal. To study pro-inflammatory responses, mice were sampled either at 2h (for RNA and gene expression assays) or 6h (to test cytokine production). For immunization assays, mice were administered i.n. with PBS ± LPS-depleted ovalbumin (OVA) (20µg, Sigma, grade VII, USA) ± flagellins (1µg) on days 1 and 21. Broncho-alveolar lavages (BALs) and serum were sampled on day 35.

To assess neutralization, immune and mock sera were heated for 30 min at 56°C to inactivate complement. Serial serum dilutions (in 200µl of PBS) were passively transferred to animals by the i.v. route 1h before systemic activation with flagellins. In some experiments, sera were mixed with flagellins diluted in PBS and administered i.n. to test mucosal neutralization.

BALs were collected after the intra-tracheal injection of 1ml PBS with Complete Protease Inhibitor Cocktail (Roche, Switzerland) and clarified by centrifugation. Blood samples were collected and clotted at room temperature, with the serum then being separated by centrifugation. Lung protein extracts were prepared by homogenizing tissue with 2 ml T-PER Tissue Protein Extraction Reagent (Pierce, USA) supplemented with protease inhibitors. All samples were stored at -80°C prior to analysis.

Analysis of antigen-specific antibody responses. Levels of OVA- or flagellin-specific antibodies in serum and BAL samples were assessed using ELISAs. Briefly, OVA (20µg per well in phosphate buffer 0.2M pH 6.5) and flagellin FliC (100 ng per well in PBS) were coated on MaxiSorp microplates (Nalge 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 1h at room temperature. Serial dilutions of samples were incubated for 1h at room temperature before development. Biotinylated anti-mouse IgG or IgA 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 450nm was determined. The IgG titer

was defined as the reciprocal of the highest sample dilution yielding an absorbance value of 0.15 OD for OVA and 0.5 OD for FliC and was systematically compared with a reference serum. Titers are given as geometrical means of titers from individual mice. Total IgA and OVA-specific IgA levels in BALs were measured and normalized using a calibration curve with commercial mouse IgA (Sigma). The specific IgA ratio (expressed in ng of OVA-specific IgA per µg total IgA) was determined for each mouse.

Cytokine-specific ELISA and gene expression. Mouse CXCL2 and CCL20 and human IL-8 (CXCL8) levels were measured in serum, BALs, total lung and/or cell culture supernatant using commercial ELISA kits (R&D Systems, USA).

Total RNA from mouse lungs was extracted with the Nucleospin RNA II kit (Macherey Nagel, Germany) and reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems, USA). The resulting cDNA was amplified using SYBR Green-based real-time PCR (Applied Biosystems). The specific primers are CGTCATCCATGGCGAACTG / GCTTCTTTGCAGCTCCTTCGT (*ACTB*, coding for β-actin), TTTTGGGATGGAATTGGACAC / TGCAGGTGAAGCCTTCAACC (*CCL20*), and CCCTCAACGGAAGAACCAAA / CACATCAGGTACGATCCAGGC (*CXCL2*). Relative mRNA levels ($2^{-\Delta\Delta Ct}$) were determined by comparing (a) the PCR cycle thresholds (Ct) for the gene of interest and *ACTB* (ΔCt) and (b) ΔCt values for treated and control groups (ΔΔCt), as described previously (19).

Cell-based assays. The Caco-2 human colon adenocarcinoma cell line was stably transfected with the plasmid harboring a luciferase gene under the control of the human *CCL20* promoter (23), giving rise to the Caco-Rumbo line. These intestinal

epithelial cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 10 mM HEPES, non-essential amino acids 1X, penicillin (100 U/ml) and streptomycin (100 U/ml) and (for transgene selection) 0.7 mg/mL G418 (Invitrogen). The human bronchial epithelial cell line BEAS-2B was cultured in Kaigh's F12 nutrient medium supplemented as for Caco-Rumbo medium plus 1 mM sodium pyruvate and insulin-transferrin-selenium mix (Invitrogen).

Cells were stimulated with recombinant flagellins for 6h for luciferase assays or for 16h before harvesting the supernatant for ELISA. Luciferase activity in cell extracts was measured using the Bright Glo Luciferase Assay (Promega, USA). Relative luminescence (RLU) was normalized as a percentage of the maximum activity with wild type flagellin for the activation test with the recombinant flagellins. For the *in vitro* neutralization test, the RLU was normalized as a percentage of the maximum activity for each protein: $[(RLU_{treated}/RLU_{untreated})/(RLU_{max}/RLU_{untreated})]\times 100$.

Statistical analysis. Statistical differences were analyzed using the Mann-Whitney test and were considered to be significant for p values <0.05. Unless otherwise specified, results are expressed as arithmetic means ± standard deviation.

RESULTS

Flagellin-specific antibodies neutralize TLR5-mediated signaling

Bacterial flagellins are known to elicit strong antibody responses, which are mainly directed against the hypervariable region (11, 12, 24). We hypothesized that anti-flagellin antibodies would neutralize the flagellins' TLR5-stimulating activity. Hence, mice were immunized s.c. with the flagellin FliC or a mock preparation (PBS alone or the irrelevant antigen ovalbumin (OVA) formulated in CFA), followed by boosts with IFA. ELISA analysis revealed that the anti-FliC sera exhibited specific IgG titers > 10^6, whereas mock sera titers were below the assay's detection threshold (10^2).

We and others have previously used human intestinal epithelial cell lines as unique reporters of flagellin/TLR5-stimulatory activity, based on expression of the chemokine CCL20 (also known as "liver-activated and -regulated chemokine", LARC) (19, 23, 25). Using Caco-Rumbo cells harboring the luciferase gene under the control of the *CCL20* promoter (23), we demonstrated that an anti-FliC serum is able to fully neutralize FliC's TLR5 agonist activity (Fig. 1A). The neutralizing effect of FliC-specific antibodies on TLR5 signaling was then directly assessed in immunized animals. To this end, systemic pro-inflammatory responses in mice (production of CCL20 and CXCL2 chemokines) were studied after i.v. injection of FliC (Fig. 1B-C). In mock-immunized animals, a FliC challenge triggered a significant increase in serum levels of CCL20 and CXCL2, compared with a PBS challenge. In contrast, chemokine production in FliC-immunized animals was not enhanced by any of the challenges. Using passive serum transfer in naive animals, a close correlation was found between the amount of antibody injected and the systemic innate response, as

shown in Fig. 1D. In conclusion, pre-existing immunity to flagellin can neutralize the latter's TLR5-stimulating activity, both *in vitro* and *in vivo.*

Deletion of flagellin's hypervariable region impairs antigenicity but does not modify TLR5-stimulating activity

Since flagellin's antigenic domain (i.e. the hypervariable central region of the molecule) is not mandatory for TLR5 signaling, we sought to engineer flagellin variants which could not be neutralized by antibodies (11, 24). These recombinant molecules were designed on the basis of flagellin's three-dimensional structure and reported immunological properties (7-9). Two novel flagellin molecules ($FliC_{\Delta 191-352}$ and $FliC_{\Delta 174-400}$, composed of 336 and 271 amino-acids, respectively) were constructed by internal deletion (Fig. 2A). As a control, we used the previously characterized variant $FliC_{\Delta 204-292}$, which has a partial deletion in the antigenic domain (11) (Fig. 2A). As a negative control for *in vitro* and *in vivo* experiments, mutations that impair TLR5 signaling were introduced into $FliC_{\Delta 174-400}$, yielding the recombinant protein $FliC_{\Delta 174-400/89-96*}$. The predicted structures of the respective flagellins indicated that the motif 89-96 and the overall structure of the conserved regions were unchanged (Fig. 2A). With the exception of $FliC_{\Delta 204-292}$, the variants were unable to complement the motility of flagellin-deficient bacteria and were secreted into the culture supernatant.

Next, we assessed the intrinsic antigenicity of the recombinant flagellins. To this end, saturating concentrations of flagellins were coated onto microplates and probed by ELISA, using a hyperimmune serum specific for FliC or $FliC_{\Delta 174-400}$. As illustrated in Fig. 2B, we observed 3- to 10-fold lower antibody titers when anti-FliC serum was titrated against FliC variants than against wild type FliC. In contrast, the

reactivity of hyperimmune serum specific for $FliC_{\Delta 174-400}$ was similar, whatever the target flagellin. These results suggest that the central hypervariable region is the major target for anti-flagellin antibodies.

Lastly, we sought to establish whether or not the recombinant molecules retained any TLR5-stimulating activity. A dose-response analysis was performed using Caco-Rumbo reporter cells and the lung epithelial cell line BEAS-2B. Activation was assessed by measuring luciferase activity in Caco-Rumbo cells and IL-8 secretion by BEAS-2B cells. As shown in Fig. 3A-B, $FliC_{\Delta 204-292}$, $FliC_{\Delta 191-352}$ and $FliC_{\Delta 174-400}$ were all potent cell activators. The flagellins' respective EC_{50} values varied slightly with the cell type but fell within the previously described ng/mL range (9). The recombinant flagellins' activity was found to be fully dependent on TLR5, since $FliC_{\Delta 174-400/89-96*}$ was unable to activate epithelial cells. The requirement for TLR5 signaling was further confirmed by using bone marrow macrophages derived from TLR5-deficient mice. The cells did not synthesize any detectable IL-12 p40 (< 18 pg/ml) upon stimulation with 0.5 µg/mL recombinant flagellins in contrast to cells derived from wild type C57BL/6 animals (ranging from 500.7 to 709.5 pg/ml IL-12 p40).

Deleted flagellins stimulate TLR5-dependent mucosal innate responses

TLR5 stimulation by recombinant flagellins was then studied *in vivo* by the mucosal route. To this end, *CCL20* and *CXCL2* expression in the lungs of mice treated i.n. with flagellins was quantified using qRT-PCR (Fig. 3C). Within 2 hours, *CCL20* mRNA pulmonary levels were about 30-fold higher in animals treated with wild type or recombinant flagellins than in mock-treated animals. Furthermore, CCL20 chemokine production was detected at 6h post-instillation, both in lung

homogenates and BALs (Fig. 3D). In control experiments, $FliC_{\Delta 174\text{-}400/89\text{-}96^*}$ and trypsin-digested flagellins did not induce this type of effect. Similar findings were observed for CXCL2 (data not shown). These results confirmed that the *in vivo* pro-inflammatory response was exclusively due to the recombinant flagellins. Overall, flagellins with deletions in the hypervariable region displayed mucosal pro-inflammatory properties equivalent to those of the wild type FliC counterpart.

Recombinant flagellins exhibit mucosal adjuvant activity

Intranasal administration of flagellins is known to promote mucosal adaptive immunity (14, 22, 26). In order to characterize the adjuvant properties of our recombinant molecules, antibody responses in serum and secretions were studied after i.n. immunizations. Ovalbumin (OVA) was used as a model antigen, formulated with or without the various flagellins or with cholera toxin (CT) as a gold standard mucosal adjuvant. The co-administration of FliC with OVA significantly increased the OVA-specific IgG response (both in serum and the BAL, about 300- and 100-fold, respectively), compared with animals immunized with OVA alone (Fig. 4A-B). Moreover, the OVA-specific IgA response was enhanced in BAL, thereby suggesting that FliC promotes the archetypal secretory antibody response of a mucosal adjuvant (Fig. 4C). Interestingly, FliC's effect was similar to that of CT. Like FliC, the recombinant flagellins $FliC_{\Delta 204\text{-}292}$, $FliC_{\Delta 191\text{-}352}$ and $FliC_{\Delta 174\text{-}400}$ were thus able to potentate systemic and mucosal responses. In contrast, $FliC_{\Delta 174\text{-}400/89\text{-}96^*}$ and trypsin-treated flagellins lacked potency (Fig. 4 and Table 1). Hence, the deletion of flagellin's hypervariable region did not significantly influence the TLR5-mediated mucosal adjuvant properties. Our data also showed that the recombinant molecules' respective effects on innate and adaptive immunity are correlated.

Deletion of the hypervariable region impairs the ability to elicit anti-flagellin antibodies.

Deletion of the antigenic domain is expected to decrease the flagellin-specific immune response and thereby any neutralization of TLR5-mediated immunity, especially with repeated administration. We therefore decided to assess the efficacy of i.n. immunization with respect to the induction of FliC-specific antibodies. As expected, FliC elicited a strong IgG response in serum and BALs (Fig. 5). In contrast, $FliC_{\Delta 204-292}$ triggered 10-fold lower antibody levels in both fluids than did FliC and a more pronounced effect was observed after immunization with $FliC_{\Delta 191-352}$ and $FliC_{\Delta 174-400}$ to reach non detectable levels as for flagellin treated with protease (Table 1). In conclusion, the flagellins' antigenic and immunostimulatory domains are functionally uncoupled. Therefore, $FliC_{\Delta 191-352}$ and $FliC_{\Delta 174-400}$ are molecules of interest for preventing or attenuating the generation of flagellin-specific antibodies with neutralizing activity.

Removal of the hypervariable region does not impair flagellin's susceptibility to antibody neutralization.

To determine whether or not $FliC_{\Delta 174-400}$ can escape antibody-mediated neutralization, we performed experiments *in vitro* and *in vivo*. First, we found that an anti-FliC hyperimmune serum is able to neutralize to similar extent the stimulation of epithelial cells by the TLR5 agonists FliC and $FliC_{\Delta 174-400}$ (Fig. 6A-B), indicating that deletion of hypervariable domain does not impair neutralization. We further investigated the capaicity of neutralization *in vivo*. The effective doses needed to

initiate TLR5-mediated innate responses by the i.n. route was determined. FliC and $FliC_{\Delta 174-400}$ displayed similar dose-response profiles and the 0.1µg dose was selected for subsequent neutralization assays (Fig. 6C-D). To this end, animals were hyper-immunized i.n. with FliC to elicit strong, FliC-specific mucosal IgG responses (mean titer ~ 45,000) and then challenged i.n. with 0.1µg FliC or $FliC_{\Delta 174-400}$ flagellins. Pro-inflammatory chemokine production in BALs was monitored. Challenge with FliC or $FliC_{\Delta 174-400}$ led to CCL20 production (4.28± 1.98 vs 1.08± 0.54 ng/ml and 2.48± 1.22 vs 0.93± 0.48 ng/ml in mock- and FliC-immunized mice, respectively) as observed in naïve animals (Fig. 6C-D). Assuming that anti-FliC ELISA titers correlate to neutralizing titers (Fig. 1D), we predicted that the neutralizing activity in BALs was too low to promote blockade of TLR5 signaling in the lungs, whatever the type of flagellin. To definitely define whether neutralization can operate in the lungs, flagellins were incubated with anti-FliC sera prior to administration (Fig. 7). Both TLR5 agonistic activity of FliC and $FliC_{\Delta 174-400}$ were blocked in these conditions. In conclusion, deletion of the flagellin hypervariable region did not enable the resulting molecules to escape from antibody neutralization of TLR5 immune responses. These data also indicated that the FliC neutralization epitopes are located within the conserved sequences 1-173 and 401-494.

Mucosal and systemic TLR5-dependent responses depend to different extents on the hypervariable flagellin region

We also wanted to study the neutralization by flagellin-specific antibodies of TLR5-dependent responses induced after i.v. injection of the recombinant flagellins. To analyze the systemic activation of innate immunity, the production in circulating pro-inflammatory chemokines CCL20 and CXCL2 was measured by ELISA in serum

(Fig. 7). Unexpectedly, we observed that $FliC_{\Delta 174-400}$ was about 100-fold impaired in its ability to trigger systemic pro-inflammatory effects, compared with the wild type FliC. Whereas 10µg $FliC_{\Delta 174-400}$ stimulated a slight chemokine production, the variant mutated within the TLR5 motif $FliC_{\Delta 174-400/89-96*}$ was devoid of activity (0.85 ± 0.27 vs 0.02 ± 0.00 ng/ml for CCL20). This contrasted with $FliC_{\Delta 204-292}$ and $FliC_{\Delta 191-352}$, which were both potent activators like FliC (data not shown). Hence, certain molecular determinants on the hypervariable region (or dependent on the latter) are required for systemic TLR5 stimulation but not mucosal TLR5 stimulation. Taken as a whole, our results indicate that TLR5 activation within the mucosal and the systemic compartments is controlled by distinct mechanisms.

DISCUSSION

Over recent years, flagellins have been the focus of many studies on the role of TLR5 in systemic and mucosal immunity (6). In addition to TLR5-dependent stimulatory activity, flagellins display strong antigenic potency. Thus, flagellins are immunodominant antigens in the body's responses to pathogenic bacteria and in chronic inflammatory disorders, since they elicit prominent T-cell and antibody responses (6, 27-29). This dual nature as an innate immunity activator and an antigen means that flagellins are attractive immunological models. It is known that flagellins are constituted by a conserved domain (with a TLR5-activating motif) and a hypervariable region (assigned to antigenicity, especially antibody response) (9, 11, 12). Here, we sought to establish whether TLR5 signaling can be regulated by flagellin-specific antibodies and whether TLR5-stimulating and antigenic activities are linked and affect each other. Using truncated forms of the *S.* Typhimurium flagellin FliC and anti-FliC hyperimmune serum, we showed for the first time that pre-existing flagellin-specific antibodies are capable of neutralizing TLR5 signaling effects *in vivo*. Additionally, we demonstrated that deletion of flagellin's hypervariable region promotes escape from neutralization by decreasing the protein's potency for generating antagonistic antibodies. These data support that the flagellin TLR5-stimulating and antigenic domains can be dissociated but that their respective activities can affect the final outcome of immune responses. Lastly, we found that TLR5 signaling is compartmentalized, since the $FliC_{\Delta 174\text{-}400}$ flagellin (i.e. lacking the hypervariable region) stimulated immunity in the mucosa but was devoid of any systemic activity.

Whereas innate TLR signaling clearly orchestrates adaptive immunity, the reverse process has been little explored. Although recent evidence supports an

antigen-independent role for T lymphocytes in the regulation of innate immunity (30), the question of how B cells and, in particular, TLR agonist-specific antibodies influence innate responses has not been resolved. Our study indicates that animals can develop flagellin-specific antibodies that efficiently neutralize the onset of TLR5-mediated responses *in vitro* and *in vivo* (Fig. 1 and 7). Therefore, *in vivo* blockade of TLR signaling by MAMP-specific neutralization antibodies is a novel mechanism for down-regulating innate immunity. Our results are consistent with the report by Saha *et al.*, which suggested that ex vivo antibody blockade of the TLR5 activation motif of *Pseudomonas aeruginosa* flagellin reduces its efficacy to induce lung innate responses (31). Interestingly, we found that neutralizing epitopes in *S.* Typhimurium FliC are embedded within the conserved region (1-173 and 401-494) that also carries the activation motif 89-96 (8, 9). We did not perform any cross-neutralization experiments using flagellins isolated from other bacteria: however, if neutralizing antibodies indeed target the conserved TLR5 signaling motif with high affinity, one can expect blockade of the innate responses to any flagellins. We further demonstrated that the mechanisms of action of flagellin-specific antibodies rely on immediate neutralization of TLR5 signaling, since NF-κB-dependent chemokine gene transcription was not turned on early after challenge (Fig. 3). It is known that LPS can be sequestered by secretory IgA within endosomes in intestinal epithelial cells, thereby blocking TLR4-mediated NF-κB activation (32). Hence, one can assume that high-affinity flagellin-specific neutralizing antibodies bind to the flagellin signaling motif and thereby prevent any interaction between flagellin and its cognate detector TLR5. Further investigations with flagellin-specific mAbs are needed to dissect the mode of action and the antibodies' targets.

TLR signaling neutralization is a major strategy for managing uncontrolled inflammation in sepsis or chronic disease (33). Different targets can promote a TLR signaling blockade, including TLRs themselves and cognate MAMPs. Most efforts seek to block the TLRs and a recent study showed that an anti-TLR4 mAb efficiently inhibited LPS-induced immune responses in acute polymicrobial infections (34). As shown in the present work, MAMP targeting represents an effective and attractive neutralization strategy. Chronic TLR stimulation may contribute to the physiopathology of some diseases and, when conjugated with the intrinsic adjuvant and antigenic activities of MAMPs, it may elicit anti-MAMP neutralizing antibodies. In turn, the neutralization of TLR-specific responses could fully suppress innate responses. Flagellin's hypervariable region is not essential for signaling (Fig. 3 and 4) - a finding which is consistent with the known TLR5 stimulatory activity of *Listeria monocytogenes* flagellin, which almost completely lacks a variable region (5). Thus, flagellated bacteria could evade host defenses by facilitating the production of antibodies that reduce the host's ability to mount an innate immune response. The high antigenicity of the flagellin variable domain may be critical in the potentiation of this type of antibody production. Remarkably, the study by Honko *et al.* showed that i.n.-administered anti-flagellin antibodies were unable to interfere with TLR5 (22). Accordingly, we were unable to detect neutralization in similar conditions.

In contrast to profilin (that depends on TLR11 for effective antigenicity (35)), flagellin's reduced immunogenicity following hypervariable region deletion does not rely on a TLR5 signaling failure. The deletion of major Th or B epitopes may explain the decreased ability of $FliC_{\Delta 174\text{-}400}$ to elicit antibodies. Previous studies identified a dominant Th epitope in flagellin's conserved domain and highlighted a major role for CD4 T cells in antibody responses (29, 36). Deletion of flagellin's hypervariable

region is therefore not absolutely required for effective help for B cell responses. Likewise, i.n. co-administration of $FliC_{\Delta 174\text{-}400}$ with OVA (which provides external help) did not enhance anti-FliC antibody titers, compared with instillation of $FliC_{\Delta 174\text{-}400}$ alone. It seems that deletion of a dominant B cell epitope on the hypervariable region is essential for presentation of a subdominant, neutralizing epitope located within the conserved region.

We previously suggested that early epithelial CCL20 production correlates with mucosal adjuvant properties - probably through DC recruitment within the mucosa (19). Our study supports this paradigm, since the mucosal adjuvancy of flagellin molecules correlates with early CCL20 production in lung tissue and BALs (Fig. 3, 6 and 7). TLR5 signaling is absolutely required for flagellin-induced enhancement of immune responses to co-instilled antigens. The deleted flagellin $FliC_{\Delta 174\text{-}400}$ also retains adjuvant properties when administered s.c, suggesting that mucosal and dermal responses behave similarly and thus differ from the systemic response (data not shown). Studies with the *S.* Typhimurium flagellin FljB (which harbors a similar deletion in the hypervariable region) have been recently performed with a view to using this type of molecule as an antigen carrier (14, 21). Similar findings were obtained using the deleted flagellin FljB as a carrier for foreign antigens in s.c. immunization (21).

The use of wild type flagellin as an adjuvant could lead to harmful effects because production of neutralizing antibodies may attenuate both the booster effect of the adjuvant and the innate responses to pathogenic flagellated bacteria. We established that $FliC_{\Delta 174\text{-}400}$ has more prominent beneficial properties, due to its poor capacity to generate neutralizing antibodies. In addition, we found that $FliC_{\Delta 174\text{-}400}$ is strongly attenuated for systemic signaling compared with wild type flagellin, whereas

mucosal activity was unaffected. This type of effect has been already observed for the TLR4 agonist LPS (4). Recent observations indicated that LPS's molecular features are essential for its biological activity (4, 37, 38). The LPS's O chain composition, the number and the length of the acyl chains and the type of substitutions all affect the outcome of TLR4 signaling. Discrimination relies on a specific combination of co-receptors and adaptor molecules. Indeed the monophosphoryl lipid A is a portion of LPS that preferentially signals through the adaptor TRIF but not MyD88, thereby rendering the molecule less toxic but nevertheless adjuvant (4). Moreover, signaling in the mucosa only has indeed been observed for LPS. For instance, bladder epithelial cells are devoid of the LPS co-receptor CD14 but can detect uropathogenic *Escherichia coli* LPS by using alternative mechanisms involving fimbriae (38). Interestingly, asialo-GM1 has been proposed as a flagellin co-receptor in the lung (39). Lastly, it has been shown that intracellular detectors like IPAF and NAIP5 participate in flagellin detection (40). Whether flagellin-mediated activation operates according to the same mechanisms in both mucosal and systemic compartments remains to be determined.

Our findings open up new prospects for the development of antagonistic strategies for manipulating host innate responses and specific inflammatory disorders. Further studies will have to establish whether or not the neutralization of various MAMPs protects or exacerbates bacterial infections or inflammatory diseases.

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FIGURE LEGENDS

Figure 1. Neutralization of TLR5 signaling by flagellin-specific antibodies. NMRI mice were immunized s.c. at week 1 with 1µg flagellin FliC and CFA, followed by boosts at weeks 3, 5, 7 with FliC and IFA. In mock conditions, animals were similarly treated with ovalbumin and adjuvants or adjuvants alone. Experiments were carried out at week 9. (A) *In vitro* TLR5-neutralizing activity of flagellin-specific immune serum. Caco-Rumbo epithelial cells harboring the reporter construct *CCL20-luc* were activated for 6h with the flagellin FliC incubated with 50% v/v FliC hyper-immune (open circles) or mock (black circles) sera. Luciferase activity was determined and normalized to the activity obtained with 100 ng/ml FliC. Results are representative of 1 of 3 independent experiments. (B, C) *In vivo* TLR5-neutralizing activity of flagellin-specific immune serum. Immunized animals (n=3) were injected i.v. with PBS (black bars) or 0.1 µg (grey bars) or 1 µg of flagellin FliC (open bars). Sera were collected 2h later and the concentrations of CCL20 (B) and CXCL2 (C) were determined by ELISA. (D) The neutralizing activity of immune serum. Animals (n=3 per dose) were passively transferred i.v. with various amounts of flagellin-specific or mock serum, and treated 1h later i.v. with recombinant flagellins, as indicated. Chemokine production in serum 2h post-challenge was measured by ELISA. Statistical significance (*p>0.05)* was determined using a Mann-Whitney test.

Figure 2. Characteristics and cross-reactivity of hypervariable region-deleted flagellins. (A) A schematic 3D view of the recombinant flagellins. The structure of wild-type flagellin FliC is presented in the left-hand panel using Pymol (http://www.pymol.org). In the monomer, terminal regions (1-170 and 400-494) are

tightly folded in α-helixes and form a structural domain involved in flagellum function. The motif 89-96 (black) is essential for TLR5 signaling. The FliC "hypervariable" domain is mainly constituted of β structures and β turns. Using Swiss-Model (http://www.expasy.org/spdbv/), an overall structure was predicted for $FliC_{\Delta 204-292}$ and $FliC_{\Delta 174-400}$, showing partial and total deletion of the hypervariable region, respectively. For $FliC_{\Delta 191-352}$, the positions of amino acids delineating the deletion are shown on the left-hand panel. $FliC_{\Delta 174-400}$ and $FliC_{\Delta 191-352}$ contain GAAG and LELE linkers at the deletion junction, respectively. (B, C) Cross-reactivity of FliC-specific sera. Hyperimmune sera were obtained after s.c. administration of flagellin formulated with CFA for priming, followed by IFA boosts. Serum was titrated in ELISAs for FliC, $FliC_{\Delta 204-292}$, $FliC_{\Delta 191-352}$, and $FliC_{\Delta 174-400}$. The results are representative of 2 experiments. (B) Cross-reactivity of anti-FliC serum. (C) Cross-reactivity of anti- $FliC_{\Delta 174-400}$ serum. Statistical significance (*p>0.05* in a Mann-Whitney test) is indicated by an asterisk.

Figure 3. Epithelial and mucosal pro-inflammatory activity of hypervariable region-deleted flagellins. (A, B) Activation of epithelial cells by recombinant flagellins. Human epithelial cells were activated with flagellins FliC, $FliC_{\Delta 204-292}$, $FliC_{\Delta 191-352}$, $FliC_{\Delta 174-400}$ or $FliC_{\Delta 174-400/89-96*}$ at the indicated concentrations. Caco-Rumbo cells harboring the reporter fusion *CCL20-luc* were activated for 6h and luciferase activity was normalized to the maximal activity measured with saturating FliC levels (A). BEAS-2B bronchial epithelial cells were stimulated for 16h before measuring IL-8 levels in the supernatant. Results are representative of 1 of 2 independent experiments. (C-D) Stimulation of the mucosal innate response by deleted flagellins. Recombinant flagellins or trypsin-treated preparations (1µg

equivalent) were administrated i.n. to anesthetized mice (n=3-5). *CCL20*-specific mRNA levels in the whole lungs were determined 2h later using real time qRT-PCR (C). Six hours after instillation, BALs (black bars) and lungs (open bars) were sampled to measure the CCL20 concentration (D). Statistical significance (*p>0.05*) was determined in a Mann-Whitney test.

Figure 4. Adjuvant effect of flagellins with hypervariable region deletion. Mice (n=8) were immunized i.n. with ovalbumin (OVA) ± flagellins or cholera toxin (CT) on days 1 and 21. On day 35, OVA-specific IgG titers were measured in the serum (A) and BALs (B). The concentration of OVA-specific IgA in BALs was determined (C). Results are representative of 1 of 2 independent experiments. Statistical significance (*p>0.05*) was determined in a Mann-Whitney test.

Figure 5. Intrinsic antigenic properties of flagellins lacking a hypervariable region. Mice (n=8) were immunized i.n. with ovalbumin (OVA) ± flagellins or cholera toxin (CT) or LPS on days 1 and 21. On day 35, FliC-specific IgG titers were measured in the serum (A) and BALs (B). Results are representative of 1 of 2 independent experiments. Statistical significance (*p>0.05*) was determined in a Mann-Whitney test.

Figure 6. Neutralization of TLR5 signaling induced by hypervariable region-deleted flagellin FliC$_{\Delta 174\text{-}400}$. (A, B) Intranasal dose response activity of flagellins. Various amounts of flagellin FliC (black squares) or FliC$_{\Delta 174\text{-}400}$ (open squares) were administrated i.n. The concentrations of CCL20 (A) and CXCL2 (B) were determined 6h later in BALs using an ELISA. Statistical significance (p*>0.05)* was determined in a Mann-Whitney test. (C, D) Epithelial neutralization of TLR5 signaling by flagellin-

specific immune serum. Caco-Rumbo epithelial cells harboring the reporter construct *CCL20-luc* were activated for 6h with 10 ng/ml FliC (A) or $FliC_{\Delta 174-400}$ (B) incubated with various dilutions of FliC hyper-immune (open circles) or mock (black circles) sera. Luciferase activity was determined and normalized to the activity obtained with 10 ng/ml FliC or $FliC_{\Delta 174-400}$ in absence of serum. Results are representative of 1 of 2 independent experiments. (C, D) *In vivo* neutralization of TLR5 activity stimulated by $FliC_{\Delta 174-400}$. Animals (n=3 per dose) were instilled i.n. with 50 ng FliC (C) or $FliC_{\Delta 174-400}$ (D) supplemented with various quantities of anti-FliC or mock sera, as indicated. Chemokine production in BAL was assessed by ELISA 6h post-challenge. Statistical significance ($p>0.05$) was determined in a Mann-Whitney test.

Figure 7. Alteration of the systemic activation ability of hypervariable region-deleted flagellin $FliC_{\Delta 174-400}$. Various amounts of flagellin FliC (black squares) or $FliC_{\Delta 174-400}$ (open squares) were administrated i.v. The concentrations of CCL20 (A) and CXCL2 (B) were determined 2h later in the serum using an ELISA. Statistical significance ($p>0.05$) was determined in a Mann-Whitney test.

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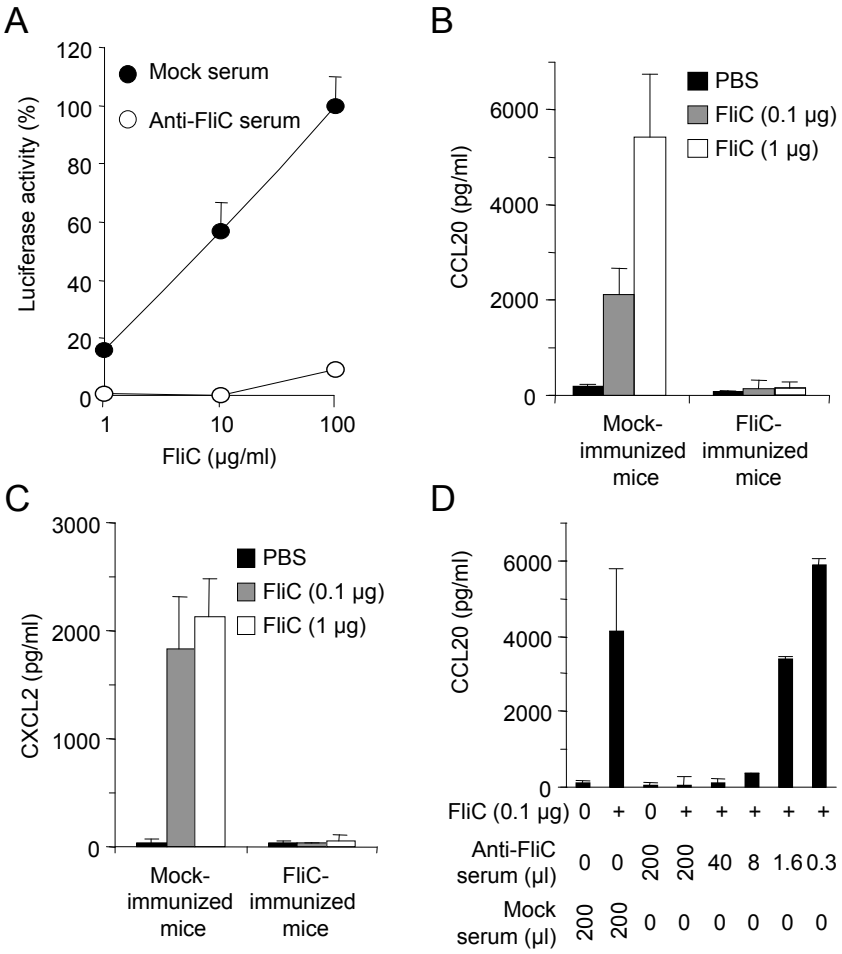


Figure 1 Nempont *et al.*

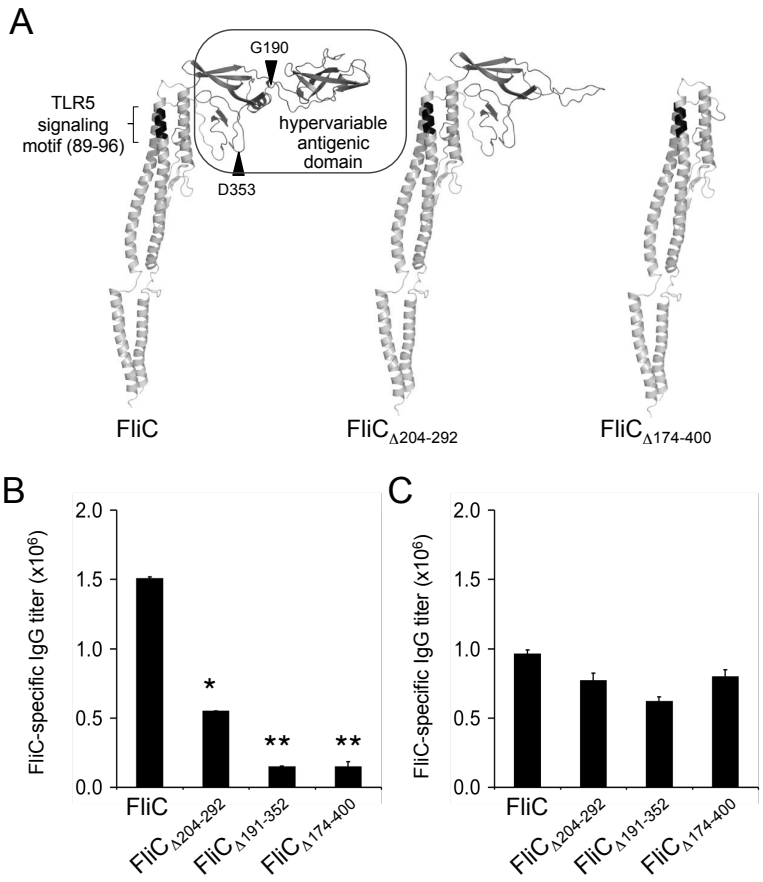


Figure 2 Nempont *et al.*

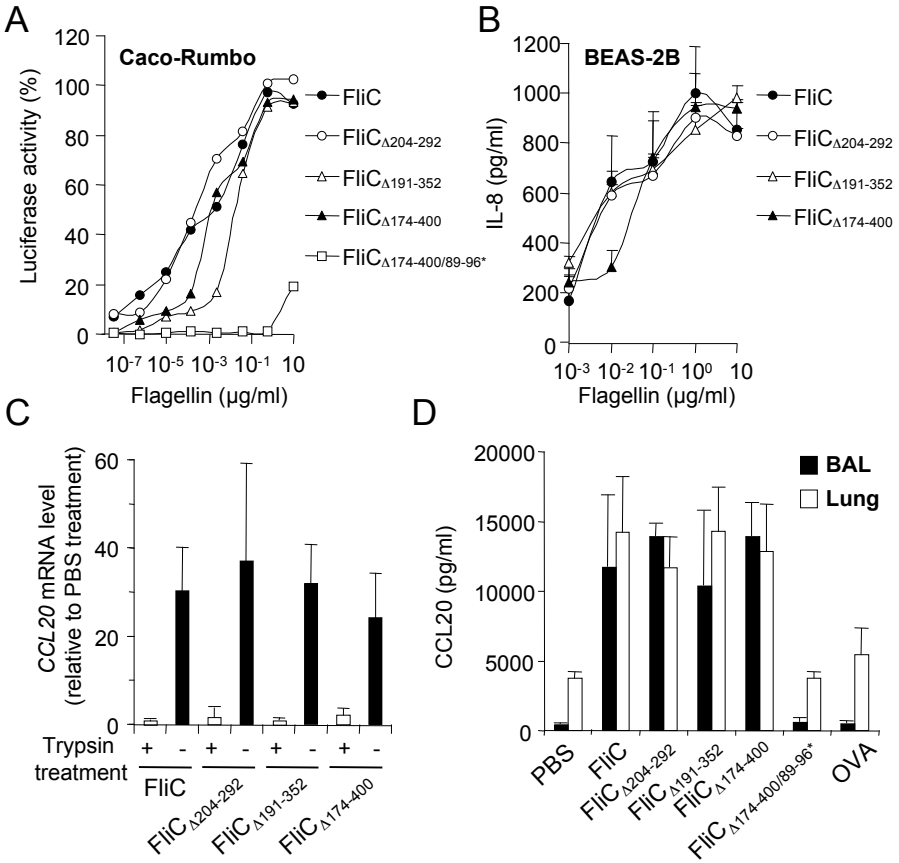
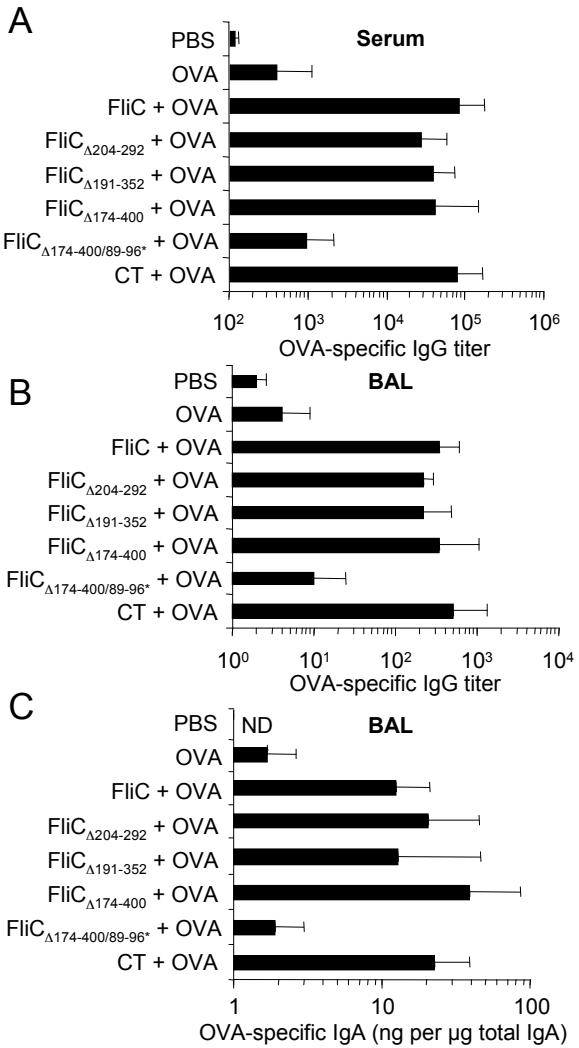


Figure 3 Nempont *et al.*



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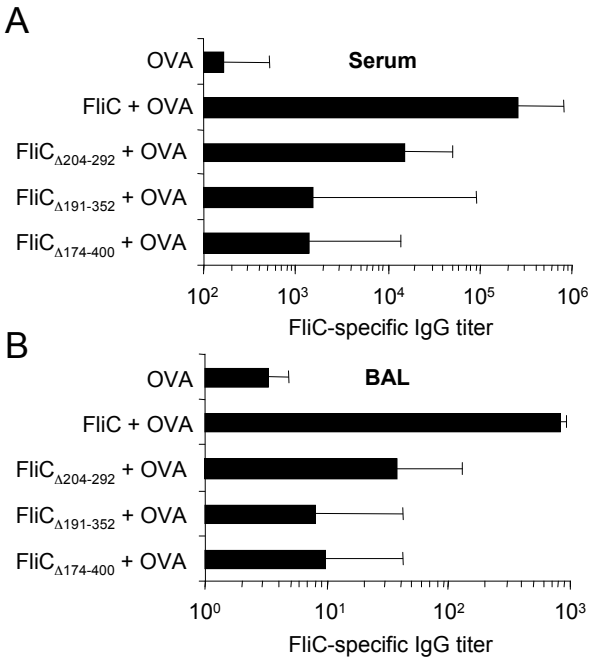

Figure 5 Nempont *et al.*

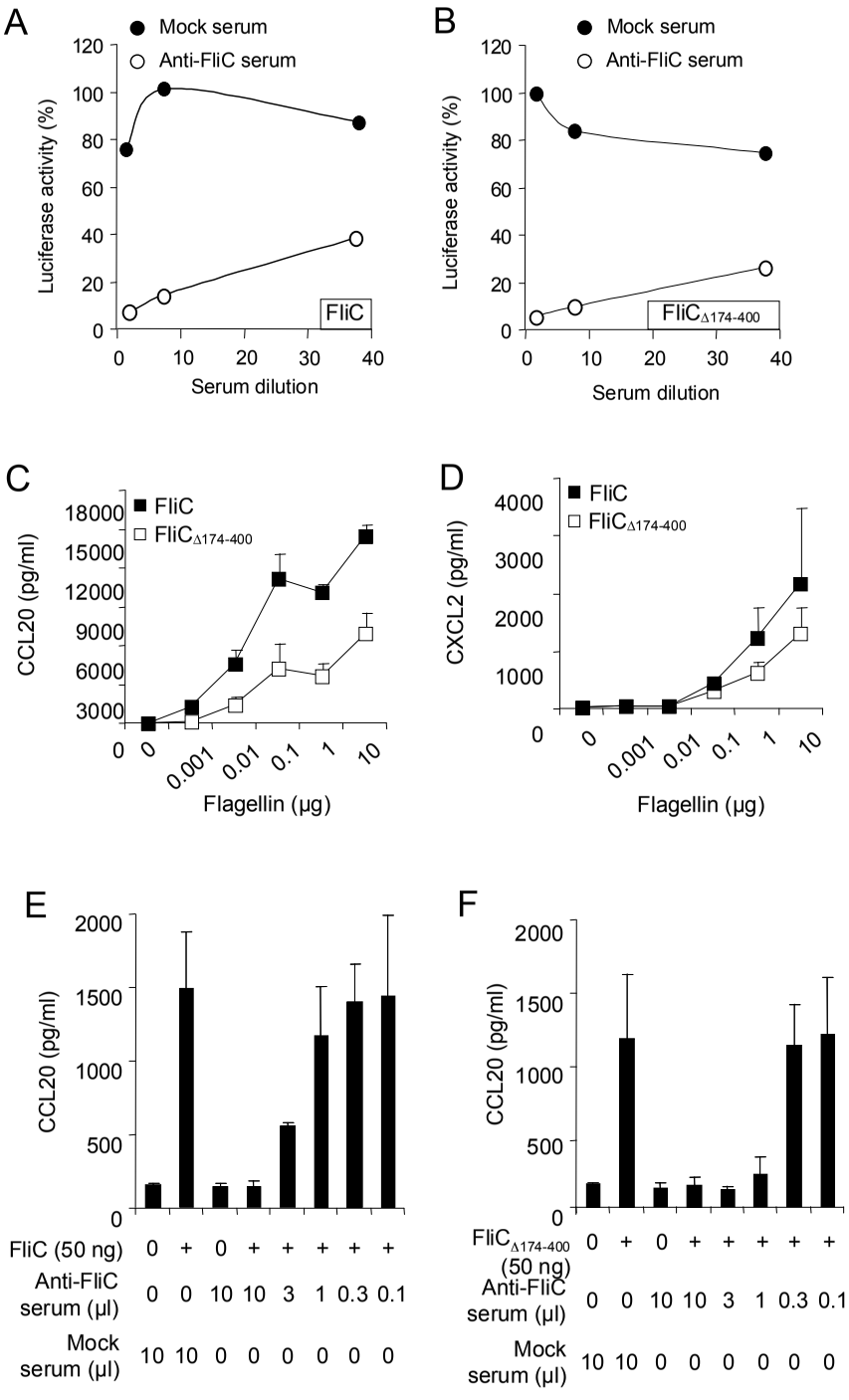

Figure 6 Nempont *et al.*

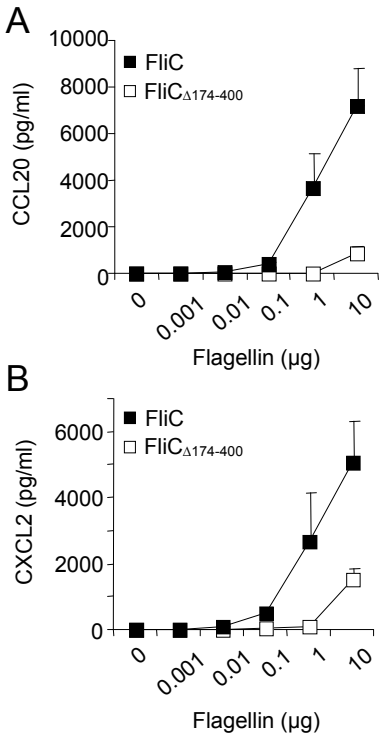

Figure 7 Nempont *et al.*