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## *In Vitro* and *ex Vivo* Activation of the TLR5 Signaling Pathway in Intestinal Epithelial Cells by a Commensal *Escherichia coli* Strain\*

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The capacity of non-pathogenic enteric bacteria to induce a pro-inflammatory response is under debate in terms of its effect on the symbiosis between the mammalian host and its commensal gut microflora. Activation of NF- $\kappa$ B and induction of interleukin-8 (IL-8) and CCL-20 by the commensal *Escherichia coli* strain MG1655 were first studied *in vitro* in the human intestinal epithelial cell (IECs) lines HT29-19A and Caco-2, transfected or not with plasmids encoding dominant negative Toll-like receptor (TLR) 5 and myeloid differentiation factor-88 (MyD88) adaptor protein. The response of enterocytes *in situ* was then assessed using murine ileal biopsies mounted in Ussing chambers. Commensal *E. coli* induced NF- $\kappa$ B DNA binding, NF- $\kappa$ B transcriptional activity, CCL-20 expression, and IL-8 secretion in the human IEC lines. *E. coli* MG1655 flagellin was necessary and sufficient to trigger this pro-inflammatory pathway via its interaction with TLR5 and the subsequent recruitment of the adaptor protein MyD88. Following epithelial cell polarization, signaling could be induced by live *E. coli* and flagellin on the apical side of HT29-19A. The *in vivo* relevance of our findings was confirmed, because immunohistochemical staining of murine ileum demonstrated expression of TLR5 in the apical part of enterocytes *in situ*. Furthermore, flagellin added on the mucosal side of murine ileal biopsies mounted in Ussing chambers induced a basolateral production of KC, a functional murine homolog of human IL-8. These findings provide strong evidence that flagellin released by flagellated commensal bacteria in the intestinal lumen can induce a pro-inflammatory response in enterocytes *in vivo*.

Intestinal epithelial cells (IECs)<sup>1</sup> serve as a barrier to bacteria colonizing the gut. Rather than a passive barrier, the

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<sup>1</sup> The abbreviations used are: IECs, intestinal epithelial cells; -CM, -conditioned medium; LPS, lipopolysaccharide; IL, interleukin; NF- $\kappa$ B, nuclear factor- $\kappa$ B; TLR, toll-like receptor; MyD88, myeloid differentiation factor 88; WT, wild-type; PBS, phosphate-buffered saline; PAMPs, pathogenic associated molecular patterns; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSA, bovine serum albumin; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent

intestinal epithelium is now considered to be an integral and essential component of the innate mucosal immune system of the host (1). IECs can respond to enteric pathogens (e.g. *Salmonella* species, *Yersinia enterocolitica*, and enteropathogenic *Escherichia coli*) either by the release of molecules directly endowed with bactericidal properties (2) or by the secretion of pro-inflammatory mediators (3–6) and the expression of adhesion molecules (7), which permit the recruitment of immune cells and the induction of a protective inflammatory response able to eradicate pathogens.

Many studies have demonstrated that the response of mammalian cells to pathogens is orchestrated through the activation of the nuclear transcription factor  $\kappa$ B (NF- $\kappa$ B) (8, 9) following recognition of specific prokaryote motifs named PAMPs (pathogenic associated molecular patterns) by cellular receptors. Toll-like receptors (TLRs), some of which are expressed by enterocytes, form the best characterized family of mammalian receptors to PAMPs (10). TLRs recognize an array of prokaryote motifs, including unmethylated CpG DNA motifs, lipopolysaccharide (LPS), lipoproteins, peptidoglycan, and flagellin (10–12), shared by both pathogenic and commensal bacteria, suggesting that either type of bacteria may have the potential to initiate innate immune host responses in IECs. Accordingly, Haller *et al.* (13) have recently shown that, following recognition of LPS by TLR4, the non-pathogenic commensal enteric bacteria *Bacteroides vulgatus* activates NF- $\kappa$ B nuclear translocation and thereafter pro-inflammatory gene expression in IEC lines as well as in primary enterocytes. In contrast, Neish *et al.* (14) showed that a non-pathogenic *Salmonella* strain not only failed to activate epithelial cells but could also prevent the pro-inflammatory response induced by a pathogenic strain of *Salmonella* (14). In addition, a recent study has shown that *Bacteroides thetaiotaomicron*, a prevalent anaerobe commensal of the human intestine, attenuates pro-inflammatory cytokine expression induced by a pathogenic *Salmonella* strain in IECs (15). Therefore, the capacity of commensal bacteria to activate a pro-inflammatory response in IECs remains controversial.

In this study, we have combined *in vitro* and *ex vivo* approaches to investigate the capacity of the *E. coli* MG1655 commensal strain (16) to induce a pro-inflammatory response in enterocytes. This bacterial strain was chosen as a paradigm of flagellated commensal enterobacteria. Furthermore, *E. coli* is usually the first colonizer of the human neonatal intestine and might therefore serve to prepare intestinal colonization by other commensals that are more prominent later on in life. *In vitro* studies allowed us to demonstrate the ability of this commensal strain to activate the NF- $\kappa$ B pathway in IEC lines

assay; ABC, avidin-biotin complex; EMSA, electrophoretic mobility shift assay; Dn, dominant negative.

and to ascribe this NF- $\kappa$ B induction to the sole interaction of bacterial flagellin with TLR5 in enterocytes. *Ex vivo* studies using Ussing chambers were used to show that this pro-inflammatory pathway can also be triggered *in vivo* in murine ileal enterocytes.

#### EXPERIMENTAL PROCEDURES

**Bacteria and Culture Conditions**—We chose *E. coli* K12 MG1655 strain as the representative of commensal non-pathogenic *E. coli*. It has been isolated from human stools and maintained in laboratories with minimal genetic manipulation, having only been cured of the temperate bacteriophage lambda and F plasmid, and its genome is entirely sequenced (16). Wild-type (WT) *E. coli* MG1655 and isogenic aflagellated  $\Delta$ *fliC* mutant were aerobically grown with shaking in Luria broth (LB) medium at 37 °C. Bacteria were harvested by centrifugation (2500  $\times$  g, 10 min) at stationary growth phase, washed two times with PBS (Invitrogen, Cergy-Pontoise, France), and subsequently diluted to obtain a final multiplicity of infection of 100 bacteria per cell.

**Production of Aflagellar Mutant**—The  $\Delta$ *fliC* *E. coli* mutant was constructed according to a method previously described (17). Briefly, PCR fragments of the Flp recognition target sequence-flanked chloramphenicol-resistant gene from the pKD3 plasmid were amplified with primers that contained a 40-bp 5' end extension, respectively, identical or complementary to nucleotides 371–410 or 1211–1250 of the *fliC* gene (relative to the +1 start codon) (5'-accgctatctgtcagaccagcttcaacggcgtgacgtgtgttaggctggagctgcttc-3' and 5'-gcacgtccagcgttccagcgatcgtgtttaccatcatatgaatctctcttag-3'). MG1655 *E. coli* strain, harboring plasmid pKD46 that encodes arabinose-inducible lambda phage Red recombinase and grown in medium containing 100 mg/liter ampicillin and 0.04% L-arabinose, was transformed by electroporation with the purified PCR product. Chloramphenicol-resistant clones, which had integrated the PCR product by homologous recombination by replacing the 410–1211 nucleotides in the *fliC* gene, were selected on LB agar plate supplemented with 30 mg/liter chloramphenicol, grown at 37 °C, and purified at 42 °C to cure the pKD46 plasmid of the strain. The insertion of the PCR product into the *fliC* gene was monitored by PCR using primers, respectively, identical or complementary to the nucleotides 21–43 or 1271–1294 of the *fliC* gene (5'-caacagcctctgctgatactc-3' and 5'-gcacgcaccgaggaagaacgg-3'). The chloramphenicol-resistant selected clone was then electroporated with the pCP20 plasmid, which carries a thermal-inducible FLP recombinase. By growing transformants at 42 °C, the pCP20 plasmid was cured of the strain, and the chloramphenicol resistance gene was removed by site-specific recombination between the Flp recognition target sites flanking the gene, resulting in an 800-nucleotide deletion in the *fliC* gene (from bp 410 to 1210). The deletion was confirmed by PCR. The motility of the bacterial populations was monitored in soft agar by plating dilutions of bacterial cultures into LB supplemented with 4.5 g/liter agar. Plates were dried 30 min at room temperature and incubated at 30 °C for 24 h. Mutant  $\Delta$ *fliC* *E. coli* non-motile colonies appeared as dots in contrast to WT colonies that spread out into large spots.

**Preparation of Bacteria-conditioned Media**—After a 24-h culture in Dulbecco's modified Eagle's (DMEM, Invitrogen), bacteria were harvested by centrifugation (2500  $\times$  g, 10 min). Bacteria-CM (culture supernatant) were collected, filtrated through 0.22- $\mu$ m membranes, and frozen at -80 °C until used. In some experiments, bacteria-CM were treated with pepsin and trypsin to hydrolyze proteins. Briefly, pepsin (0.15 mg/ml, pH 1.8, Sigma) was added first for 4-h incubation at 37 °C. After adjusting the pH to 7.8, trypsin (0.15 mg/ml, Sigma) was added to the solution for a further 4-h incubation at 37 °C. After enzyme inactivation at 85 °C for 45 min, pH was adjusted to 7.4 and the hydrolyzed bacteria-CM were sterilized by filtration (0.22  $\mu$ m) and frozen until used. To further characterize the active bacterial product, bacteria-CM were ultrafiltrated through centrifugal filter devices Centrplus® YM-10 that retain molecules larger than 10,000 Da. Filtrated fractions were concentrated 50 times and stored at 4 °C until use. SDS-PAGE analysis of the concentrated bacteria-CM and Coomassie Blue staining revealed a single band of 55 kDa reacting with anti-flagellin antibody, which was absent in the concentrated  $\Delta$ *fliC* *E. coli*-CM (see "Results," Fig. 2, A and B). The concentration of flagellin in the bacteria-CM was therefore determined using the Bio-Rad Bradford assay, because this assay did not detect a significant amount of proteins in the concentrated  $\Delta$ *fliC* *E. coli*-CM. This concentrated flagellin was used for all tests.

**Cell Culture and Bacterial Stimulation of IECs**—The human IEC lines HT29-19A and Caco-2 were grown in DMEM supplemented with FCS (10 and 20%, respectively, Invitrogen) to 80–90% confluence in

6-well plates (Falcon®) at 37 °C in 5 and 10% CO<sub>2</sub>, respectively. After an overnight culture in medium without FCS, confluent cell monolayers were stimulated with either live WT *E. coli* or  $\Delta$ *fliC* mutant at a multiplicity of infection of 100 bacteria per cell, LPS (0.1–1  $\mu$ g/ml, from *E. coli* O111:B4, Sigma), concentrated flagellin (0.1–1000 ng/ml), or IL-1 $\beta$  (10 ng/ml, R&D Systems, Abingdon, UK), for 1–16 h. To prevent bacterial overgrowth in co-cultures of IECs with live *E. coli*, 100  $\mu$ g/ml gentamicin was added after 3 h of culture.

For polarized monolayers, HT29-19A and Caco-2 cells were seeded on microporous filters (Falcon™ and Costar®, respectively), at a concentration of 0.8  $\times$  10<sup>6</sup> cells/cm<sup>2</sup> in DMEM supplemented with FCS, for 3 weeks at 37 °C. Tightly polarized monolayers then displayed a trans-epithelial electrical resistance of ~150 ohm.cm<sup>-2</sup>. Polarized intestinal cell monolayers were treated as previously described, and electrical resistances were checked at the end of the experiments.

**Nuclear Extracts and Electrophoretic Migration Shift Assay**—HT29-19A and Caco-2 cells were stimulated for various times (1–6 h) as noted above, and nuclear extracts were prepared as previously described (18). Briefly, cells were washed twice in ice-cold PBS, scraped from the dishes, and centrifuged, and the cell pellets were resuspended in hypotonic buffer (20 mM HEPES, pH 7.8, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA) containing protease inhibitor mixture (1 tablet/10 ml, Roche Applied Science). After incubation on ice for 10 min, Nonidet P-40 (Sigma) was added at a final concentration of 1%, and samples were centrifuged for 5 min at 3,000  $\times$  g. Supernatants (cytoplasmic fractions) were discarded, and nuclear pellets were washed briefly with hypotonic buffer and resuspended in extraction buffer (10 mM HEPES, pH 7.8, 400 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, protease inhibitors, 25% glycerol). After incubation at 4 °C for 30 min with occasional vortex mixing, samples were centrifuged at 10,000  $\times$  g for 5 min. Supernatants (nuclear fractions) were recovered and frozen at -80 °C until use. Nuclear extracts (5  $\mu$ g) were placed in binding buffer (10 mM HEPES, pH 7.8, 100 mM NaCl, 1 mM EDTA, and 10% glycerol) containing 1  $\mu$ g of poly(dI-dC) and 0.5 ng of <sup>32</sup>P-labeled DNA probe corresponding to the  $\kappa$ B site (5'-AGTTGAGGGGACTTTC-CCAGG-3', Promega, Charbonnières, France) prepared according to the manufacturer's instructions. After incubation at room temperature for 30 min, samples were run on a 5% polyacrylamide gel in Tris-borate-EDTA buffer. The gel was dried, exposed to a PhosphorImager screen (Molecular Dynamics), and analyzed with the ImageQuant software (Molecular Dynamics). For NF- $\kappa$ B supershift assays, antibodies against p50 or p65 subunits (10 ng/ $\mu$ l, Santa Cruz, CA) were added after 15 min incubation of nuclear extracts with the <sup>32</sup>P-labeled DNA probe.

**Cytokine Assays**—Concentrations of IL-8 and KC were measured using enzyme-linked immunosorbent assays (Dusset kits, R&D Systems). Briefly, plates were coated overnight with anti-IL-8 or anti-KC capture antibodies (4  $\mu$ g/ml), and nonspecific binding sites were blocked by 1-h incubation with PBS-1% bovine serum albumin (BSA, Sigma). Culture supernatants, centrifuged for 10 min at 10,000  $\times$  g to eliminate residual bacteria, and standards were then added at appropriate dilutions, and the mixture was incubated for 2 h. Plates were subsequently added with biotinylated detection antibody (20 ng/ml) for 2 h, streptavidin-horseradish peroxidase (HRP) for 20 min, and tetramethylbenzidine, used as a substrate for HRP detection for 20 min. Absorbance values were read at 450 nm, and cytokine concentrations were determined by comparison with standard curves.

**Real-time Quantitative PCR for Analysis of mRNA Levels**—Two micrograms of total RNA extracted from cells with an RNeasy Mini kit (Qiagen) was transcribed into cDNA using murine-Moloney leukemia virus reverse transcriptase, random hexamers, and oligo(dT) (Promega) according to standard procedures. cDNA was then amplified with SYBR-Green PCR Master Mix (Applied Biosystems) and 300 nm of the corresponding primers with 40 cycles, including denaturation at 95 °C for 15 s, annealing, and extension at 60 °C for 1 min in an ABI Prism™ 7700 sequence detection system (Applied Biosystems, software version 1.6). Primers for CCL-20 (CCAAGAGTTTGCTCCTGGCT and TGCTTGCTGCTTCTGATTTCG) and glyceraldehyde-3-phosphate dehydrogenase (GAAGGTGAAGGTCGG-AGTC and GAGGGATCTCGCTCCTGGAAGA) yielded PCR products of 75 and 244 bp, respectively. Data were normalized referring to expression of glyceraldehyde-3-phosphate dehydrogenase.

**Expression Plasmids and Transient Transfections of HT29-19A and Caco-2 Cells**—The NF- $\kappa$ B reporter Ig $\kappa$ -luciferase plasmid (19) and the MyD88 dominant negative expression plasmid were provided by Dr. D. J. Philpott (IHIS, Institut Pasteur, Paris). Co-transfection with 0.1  $\mu$ g of *Renilla* luciferase reporter vector (pRL-TK, Promega) was used to normalize the transfection efficiencies. Transfection was performed using Amaxa Nucleofector™ apparatus (Amaxa, Cologne, Germany) according to the manufacturer's instructions. HT-29-19A and Caco-2

cells were harvested when 50% confluent and resuspended in electroporation buffer at a final concentration of  $2.5 \times 10^7$  cells/ml. 0.1 ml of cell suspension was supplied with 1  $\mu$ g of the Ig $\kappa$ -luciferase plasmid, 0.1  $\mu$ g of the *Renilla* luciferase reporter vector, and 1  $\mu$ g of empty vector (control) or 1  $\mu$ g of plasmid encoding either dominant negative TLR5 (InvivoGen, San Diego, CA) or dominant negative MyD88, and was then transferred into a 2.0-mm electroporation cuvette, and electroporated. DNA quantity, cell concentration, and buffer volume were kept constant throughout all experiments. After electroporation, cells were immediately diluted with 2.5 ml of DMEM supplemented with 5% FCS and distributed into 24-well plates at a density of  $0.5 \times 10^6$  cells per well. After 24 h at 37 °C, cells were stimulated with bacteria, as described above, or treated with either IL-1 $\beta$  (10 ng/ml) or concentrated flagellin (100 ng/ml) for 8 h. Cells were then lysed in lysis buffer (Promega), and luciferase activity was quantified using the Dual-luciferase® reporter assay system (Promega).

**Western Blotting**—Proteins were run on 9% SDS-PAGE gels and electrotransferred onto nitrocellulose membranes (Amersham Biosciences) at 200 mA for 45 min. Membranes were blocked using 5% nonfat dry milk in TTBS (20 mM Tris at pH 7.5, 150 mM NaCl, and 0.05% Tween 20) and then successively incubated with *Salmonella* H antiserum poly a-z (Difco, BD Biosciences) at 1:1,000 dilution in TTBS containing 1% gelatin for 2 h and with 1:5,000 dilution of HRP-conjugated donkey anti-rabbit Ig (Jackson ImmunoResearch) for 1 h. HRP was revealed with ECL-Plus light detection reagents (Amersham Biosciences) and exposure to BioMax MR film (Eastman Kodak).

**Stimulation of Murine Ileal Biopsies Mounted in Ussing Chambers**—C3H/HeN mice, 8–12 weeks old, were raised at the breeding facility of Institut National de la Recherche Agronomique (INRA), in accordance with the regulations of the Animal Care Committee of INRA. Animals were killed by cervical dislocation, and their small intestine was removed. Ileal segments, 0.5 cm long, were taken from the middle part of the ileum, opened as a flat sheet, gently rinsed with cold Ringer solution, and laid on a Millipore filter (HAMK, 8  $\mu$ m). Filter and tissue were mounted within 5 min of sampling in adapted Ussing chambers exposing a surface area of 0.15 cm<sup>2</sup>. Tissue specimens were bathed on each side with 1 ml of Ringer's solution containing the following (in mM): 140 Na<sup>+</sup>, 5.2 K<sup>+</sup>, 120 Cl<sup>-</sup>, 25 HCO<sub>3</sub><sup>-</sup>, 1.2 Ca<sup>2+</sup>, 2.4 HPO<sub>4</sub><sup>2-</sup>, 1.2 Mg<sup>2+</sup>, and 4 glutamine. The solution was thermostatted, oxygenated, and maintained at pH 7.4 by a gas flow containing 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The mucosal and serosal bathing solutions were connected by agar bridges to calomel electrodes for measurement of the transmural potential difference and to Ag-AgCl electrodes for measurement of the short-circuit current (I<sub>sc</sub>). Biopsy specimens were pulsed at 5 mV regularly, and the increase in I<sub>sc</sub> was used to calculate the electrical resistance. Electrical parameters (potential difference, I<sub>sc</sub>, resistance) were recorded at 30-min intervals for 4 h. Concentrated flagellin (500  $\mu$ g/ml), LPS (100 ng/ml), or concentrated  $\Delta$ fliC-conditioned medium (10  $\mu$ l, corresponding to the volume of 500  $\mu$ g of concentrated flagellin) were added in the serosal compartment, and after a 4-h incubation period the mucosal and the serosal compartments were collected and assayed for KC by ELISA.

**Immunoperoxidase Staining of Paraffin Sections**—Segments of ileums from C3H/HeN mice were fixed in 4% formalin (Prolabo) and embedded in paraffin. Tissue sections, 4  $\mu$ m thick, were deparaffinized, rehydrated in PBS containing 0.1% BSA, and subsequently incubated with a 1:100 dilution of polyclonal goat anti-TLR5 antibody (TLR5 N-15, Tebu-bio, Le Perray en Yvelines, France) or control goat serum for 45 min, with a 1:500 dilution of biotinylated secondary rabbit anti-goat antibody (DakoCytomation, Denmark) for 30 min and then with avidin-biotin complex (ABC) linked to HRP (Vectastain Elite ABC kit, Vector Laboratories, AbCys, Paris, France) for 30 min. All reagents were diluted in PBS-0.1% BSA, and tissue sections were washed twice in this buffer between each incubation. HRP was revealed using the VIP peroxidase substrate kit (Vector Laboratories) according to the manufacturer's instructions. Sections were counterstained with methyl green (Vector Laboratories) and mounted with VectaMount (Vector Laboratories). Anti-TLR5 staining specificity was tested following overnight preincubation of goat anti-TLR5 antibodies with TLR5 blocking peptide (sc-8695P, Tebu-bio) at a ratio 1 V/10 V, at 4 °C.

**Measure of Endotoxin Contamination**—LPS concentration was determined using the Limulus amoebocyte lysate colorimetric assay QCL-1000 from BioWhittaker according to the manufacturer's instructions.

## RESULTS

**Commensal Non-pathogenic E. coli MG1655 Induces a Pro-inflammatory Signal in Human IECs**—To determine whether

a commensal *E. coli* can induce a pro-inflammatory response in IECs, NF- $\kappa$ B DNA-binding activity was assessed by electrophoretic mobility shift assay (EMSA) in the HT29-19A and Caco-2 human IEC lines that had been co-cultured with *E. coli* MG1655 at a multiplicity of infection of 100 bacteria per cell. As shown in Fig. 1A, stimulation by bacteria induced a strong increase in NF- $\kappa$ B DNA-binding activity in both cell lines after 1 h, which persisted over 6 h. For comparison, IL-1 $\beta$  used as a control induced only a transient increase in NF- $\kappa$ B DNA-binding activity, which was weaker in Caco-2 than in HT29-19A cells. Supershift assays with an antibody against the p50 subunit resulted in a partial shift of the NF- $\kappa$ B band, whereas this band was fully supershifted in the presence of the anti-p65 antibody (Fig. 1B), suggesting that the NF- $\kappa$ B binding complexes induced by commensal *E. coli* likely contained both p65 homodimers and p65/p50 heterodimers.

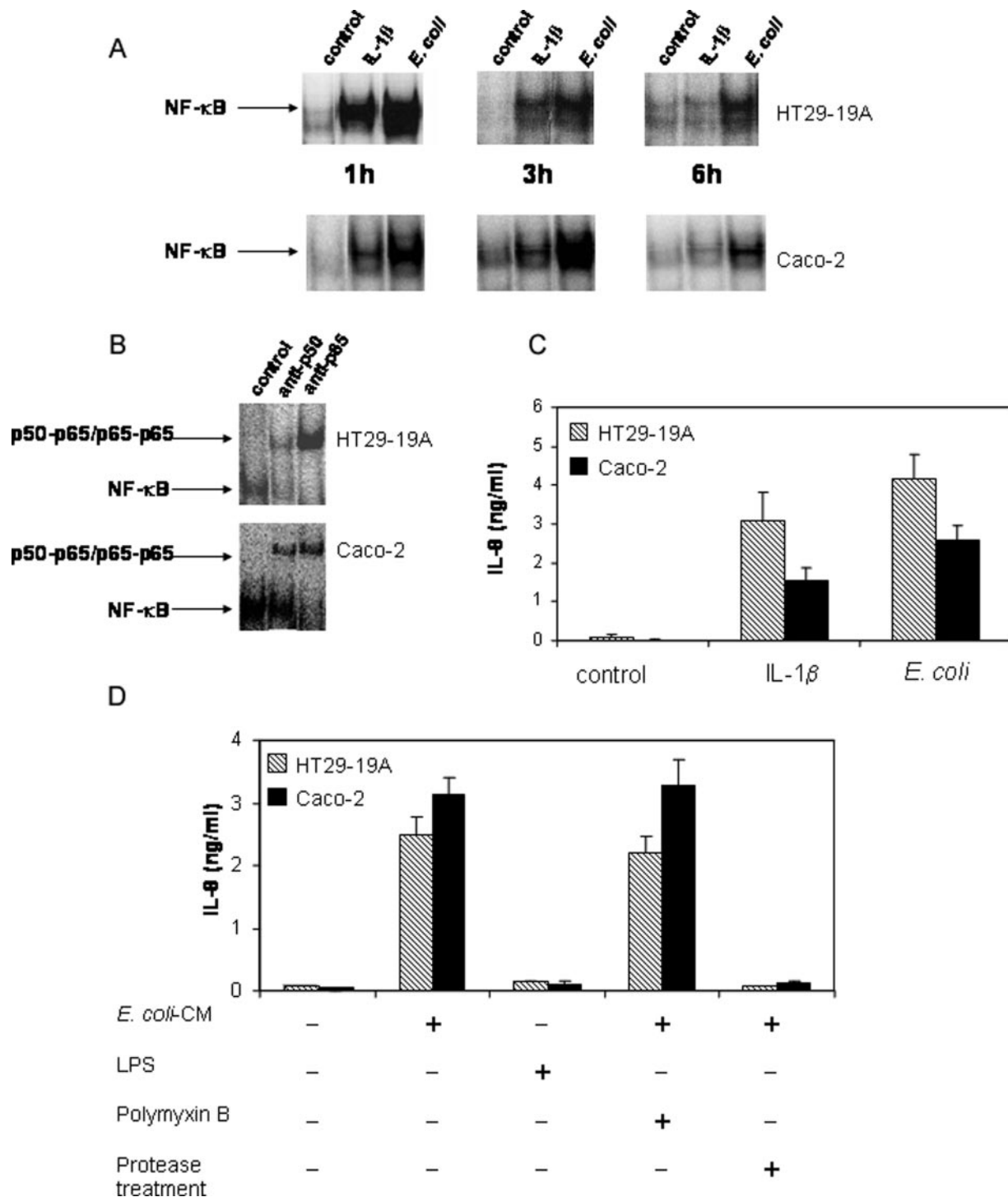
NF- $\kappa$ B is central to the activation of several pro-inflammatory genes in human IECs, including IL-8 (8, 20). IL-8 was therefore measured in culture supernatants by ELISA after 16 h of stimulation. As shown in Fig. 1C, *E. coli* stimulation, similar to IL-1 $\beta$  stimulation, induced the secretion of IL-8 in the two IEC lines tested, indicating that NF- $\kappa$ B DNA-binding activity resulted in the induction of cytokine secretion.

LPS of the commensal bacteria *B. vulgatus* was recently shown to activate the NF- $\kappa$ B signaling pathway in rat IECs (13). The pro-inflammatory effect of whole *E. coli* MG1655 was therefore compared with those of *E. coli*-conditioned medium (*E. coli*-CM) and *E. coli* LPS. *E. coli*-CM induced NF- $\kappa$ B activity (not shown) and IL-8 secretion in HT29-19A and Caco-2 cells as efficiently as whole bacteria, whereas LPS had no stimulatory effect even at a concentration of 1  $\mu$ g/ml (Fig. 1D). Furthermore, addition of polymyxin B (10  $\mu$ g/ml) to the *E. coli*-CM did not decrease its stimulatory effect, whereas its activity was abolished after treatment with proteases (Fig. 1D), pointing to the role of a proteinaceous factor.

**The Pro-inflammatory Signal Delivered by Commensal E. coli Is Mediated by Flagellin and Activation of the TLR5-MyD88 Signaling Pathway**—SDS-PAGE analysis of *E. coli*-CM and Coomassie Blue staining revealed a prominent protein band with an estimated molecular mass of 55 kDa, which was similar to that of *E. coli* flagellin (Fig. 2A). The identity of the 55-kDa band was confirmed by its immunoreactivity with an antiserum against *Salmonella* H7 flagellin and the absence of this protein in preparations from an isogenic *E. coli*  $\Delta$ fliC aflagellar mutant of the commensal *E. coli* MG1655 (Fig. 2B). When tested in soft agar, the  $\Delta$ fliC *E. coli* mutant colonies appeared as dots in contrast to WT colonies, which spread out into large spots (Fig. 2C). The non-motile phenotype confirmed that the mutant strain was aflagellated.

Flagellin from different bacterial strains was shown to induce IL-8 secretion and CCL-20 expression in several *in vitro* models of IECs (21–23). Flagellin, concentrated from *E. coli* MG1655-CM, induced the secretion of IL-8 and increased the levels of CCL-20-encoding mRNA in both HT29-19A and Caco-2 cells (Fig. 2, D and E, respectively). This effect was dose-dependent, and a concentration of 10 ng/ml was sufficient for a significant stimulation. Moreover, the efficiency of 100 ng/ml flagellin concentrated from *E. coli* was comparable to that observed with similar amounts of flagellin purified from *Salmonella typhimurium* (data not shown). As a control, the concentrated  $\Delta$ fliC *E. coli*-CM did not induce any stimulatory effect.

To determine whether the pro-inflammatory response induced by *E. coli* MG1655 in our cell lines could be exclusively ascribed to flagellin, we tested the pro-inflammatory properties of the live isogenic  $\Delta$ fliC aflagellar mutant of the commensal *E. coli* MG1655. As shown in Fig. 3A, the aflagellated *E. coli*

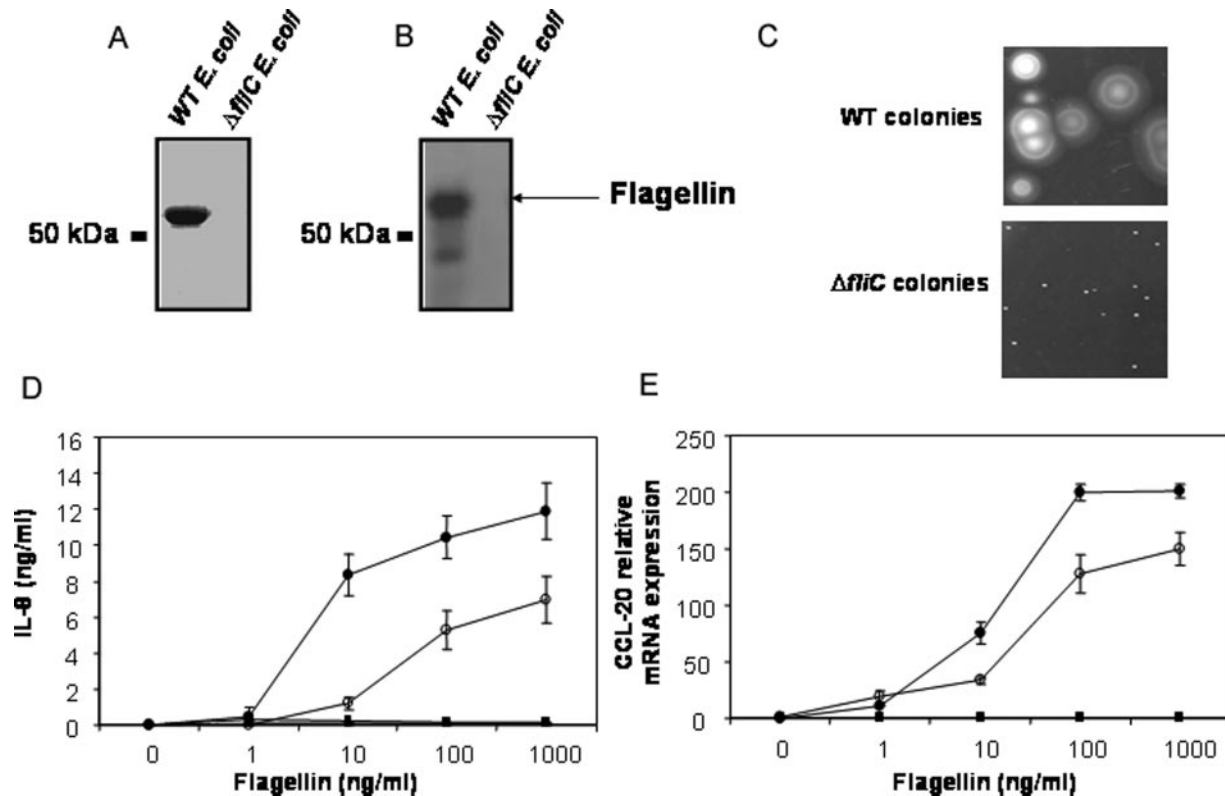


**FIG. 1. *E. coli* induces NF-κB DNA-binding activity and IL-8 secretion by IECs via a soluble protein.** *A*, time course of NF-κB DNA-binding activity was assessed by EMSA in HT29-19A and Caco-2 cell lines stimulated either with the commensal *E. coli* MG1655 strain (100 bacteria/cell), with IL-1β (10 ng/ml) or with medium alone (control), for 1–6 h. *B*, for supershift assays, nuclear extracts from HT29-19A and Caco-2 cell were stimulated with the commensal *E. coli* MG1655 strain for 3 h and NF-κB DNA-binding activity was tested by EMSA before (control) or after preincubation with antibodies against p50 or p65 NF-κB subunits. *C*, IL-8 protein secretion was measured by ELISA in supernatants of HT29-19A (hatched bars) or Caco-2 (black bars) cells stimulated for 16 h. *D*, HT29-19A (hatched bars) and Caco-2 (black bars) cells were cultured with either *E. coli*-conditioned medium (-CM) (1:6 dilution) with or without polymyxin B (10 μg/ml), LPS (1 μg/ml), or *E. coli*-CM after protease treatment. After 16 h of stimulation, IL-8 protein secretion was measured by ELISA in supernatants. Data are mean ± S.E. for a triplicate experiment representative of at least three (*D*) or five (*C*) experiments.

mutant was unable to activate NF-κB DNA binding and failed to induce IL-8 secretion (Fig. 3B) and CCL-20 mRNA expression (Fig. 3C) in either IEC line.

The prominent role of flagellin was further demonstrated by examining the signaling pathway elicited by *E. coli* MG1655 in IECs. Activation of NF-κB by flagellin from enteropathogenic

bacteria such as *S. typhimurium* and enterohemorrhagic *E. coli* results from its binding to TLR5, and from the subsequent recruitment of the MyD88 adaptor protein (24). In addition, binding to TLR5 involves a target site on flagellin that is highly conserved between bacterial species (25, 26). Reverse transcription-PCR analysis confirmed the presence of TLR5 in both

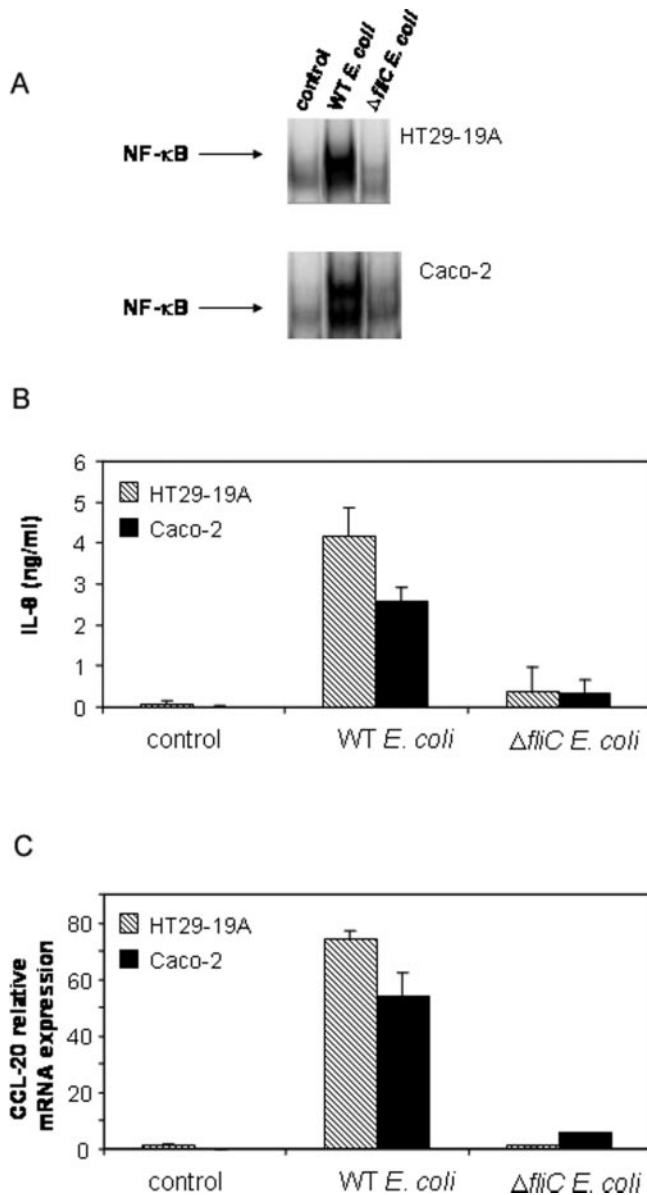


**FIG. 2. Dose-dependent induction of IL-8 secretion and CCL-20 expression by concentrated *E. coli* flagellin.** Concentrated WT and  $\Delta$ *fliC* *E. coli*-CM were analyzed after SDS/PAGE and Coomassie Blue staining (A) and by immunoblotting with an antiserum against *Salmonella* H7 flagellin (B). C, growth at 24 h on soft agar plates of WT and  $\Delta$ *fliC* *E. coli* colonies. D and E, HT29-19A (filled symbols) and Caco-2 (open symbols) IECs were stimulated either with flagellin concentrated from WT *E. coli*-CM (circles) at the indicated concentrations (1–1000 ng/ml) or with equivalent volumes of concentrated  $\Delta$ *fliC* *E. coli*-CM (squares). D, IL-8 secretion was assessed by ELISA after a 16-h stimulation. E, CCL-20 mRNA expression was analyzed by real-time quantitative PCR after a 6-h stimulation. Data are mean  $\pm$  S.E. for a triplicate experiment representative of at least three experiments (D and E).

HT29-19A and Caco-2 cells (data not shown). To further examine the contribution of TLR5 to NF- $\kappa$ B activation by *E. coli* MG1655, HT29-19A and Caco-2 cells were transiently transfected with vector constructs encoding dominant negative forms of either TLR5 (TLR5Dn) or MyD88 adaptor protein (MyD88 Dn), together with an NF- $\kappa$ B Ig $\kappa$  luciferase reporter. As shown in Fig. 4, both flagellin concentrated from WT *E. coli*-CM and live WT *E. coli*, but not the aflagellated mutant  $\Delta$ *fliC*, induced the activation of NF- $\kappa$ B, expressed in relative luciferase activity compared with non-stimulated HT29-19A cells (confirming our results by EMSA). In contrast, HT29-19A cells transfected with TLR5 Dn showed a  $77 \pm 23\%$  and  $74 \pm 13\%$  decrease in relative luciferase activity compared with cells transfected with vector alone upon stimulation with concentrated flagellin or live WT *E. coli*, respectively (Fig. 4A). Similar results were observed in HT29-19A cells transfected with MyD88 Dn (Fig. 4B), with  $66 \pm 15\%$  and  $55 \pm 14\%$  decrease in relative luciferase activity after stimulation with concentrated flagellin and live WT *E. coli*, respectively. As expected, IL-1 $\beta$ -induced NF- $\kappa$ B activity was significantly decreased in the presence of MyD88 Dn ( $71 \pm 6\%$ ; Fig. 4B), but was not affected in the presence of TLR5 Dn (Fig. 4A), validating the specificity of the transfection assays. Similar results were observed with the Caco-2 IECs line (data not shown). Altogether, these data indicated that flagellin concentrated from WT *E. coli*-CM was the only effector of the pro-inflammatory activity of *E. coli* MG1655 in the model IEC lines examined.

**Polarized IECs Produce IL-8 in Response to Apical Commensal *E. coli* MG1655 or Flagellin Concentrated from *E. coli* MG1655-CM**—Commensal bacteria remain mainly within the intestinal lumen, and should therefore only interact with IECs via their apical membranes. Accordingly, it has been argued

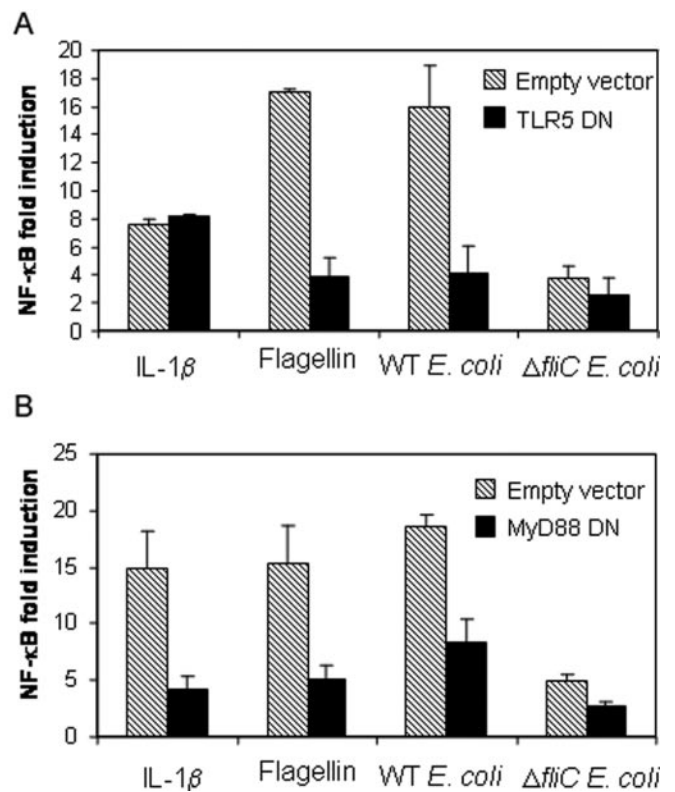
that the localization of TLR5 at the basolateral pole of IECs may be responsible for the discrimination between commensal and pathogenic flagellated bacterial strains, with only flagellated pathogens being able to invade the cells and to induce a pro-inflammatory response at the basolateral side (22, 27, 28). To assess the possible *in vivo* pro-inflammatory effects of *E. coli* or of its flagellin, concentrated from *E. coli*-CM, we tested the IL-8-inducing activity of these two stimuli on polarized HT29-19A and Caco-2 cells, which had been grown for 3 weeks on filters in Transwells®. Polarization was controlled by checking that the resistance of IECs monolayers mounted in Ussing chambers was  $\sim 150 \text{ ohm.cm}^{-2}$ . IL-8 secretion in response to basolaterally added concentrated flagellin or IL-1 $\beta$  was, respectively, 5- and 30-fold less in polarized than in non-polarized Caco-2 cells (data not shown), precluding the use of this cell line to investigate apical *versus* basolateral stimulation after polarization. In contrast, polarization did not affect significantly IL-8 secretion in the HT29-19A cell line, which was therefore solely used for this part of the study. As shown in Fig. 5A, when added onto the apical side of HT29-19A-polarized cells, WT but not the *E. coli*  $\Delta$ *fliC* mutant strain induced a robust basolateral secretion of IL-8. Apical stimulation of HT29-19A cells by concentrated flagellin, although less efficient than basolateral stimulation, also resulted in a strong secretion of IL-8 comparable to that observed with WT *E. coli* (Fig. 5B). Noticeably, transepithelial resistance of the monolayers was unchanged at the end of the stimulation period, thereby excluding the possibility that some apically added flagellin (soluble or released by live bacteria) had gained access to the basolateral chamber through leaky tight junctions between epithelial cells.



**FIG. 3. Flagellin is the only factor responsible for the pro-inflammatory response of IECs to *E. coli* MG1655.** IEC lines were stimulated with either WT *E. coli*, mutant aflagellated  $\Delta fliC$  *E. coli*, or medium alone (control) as described in Fig. 1. A, NF- $\kappa$ B DNA-binding activity was assessed by EMSA after 3 h. B, IL-8 protein secretion was measured by ELISA in supernatants of HT29-19A (hatched bars) or Caco-2 (black bars) cells stimulated for 16 h. C, CCL-20 mRNA expression was measured by real-time quantitative PCR on RNA extracted from IECs after a 6-h stimulation. Data are mean  $\pm$  S.E. for a triplicate experiment representative of at least five experiments (B and C).

*Ileal Biopsies from C3H/HeN Mice Produce KC in Response to Apical Stimulation by Flagellin Concentrated from E. coli MG1655-CM*—To define whether flagellin released by intraluminal commensal bacteria is susceptible to activate epithelial cells *in vivo*, TLR5 expression was studied by immunohistochemistry in mouse ileal tissue sections using a polyclonal antibody reacting with murine TLR5. TLR5 staining was detected in the upper two-thirds part of the villi and in the apical part of enterocytes (Fig. 6A, upper panel). Antibody staining was specific, because the labeling was markedly reduced following preincubation of the antiserum with a TLR5 blocking peptide (Fig. 6A, lower panel).

To further define whether apically expressed TLR5 is accessi-



**FIG. 4. Commensal *E. coli*-induced NF- $\kappa$ B activity in IECs depends on the TLR5- and MyD88-signaling pathway.** HT29-19A cells were transfected with NF- $\kappa$ B Ig $\kappa$  luciferase reporter alone (A and B, hatched bars) or in combination with either dominant negative TLR5 (A, black bars) or dominant negative MyD88 (B, black bars). Cells were then stimulated with either WT *E. coli*, mutant aflagellated  $\Delta fliC$  *E. coli*, IL-1 $\beta$  as in Fig. 1, or flagellin concentrated from WT *E. coli*-CM (100  $\mu$ g/ml) for 8 h. Luciferase activities were determined and normalized based on *Renilla* luciferase activity. Data are mean  $\pm$  S.E. of triplicates in one experiment representative of at least three distinct experiments.

ble to intraluminal stimulation, the effect of concentrated flagellin was assessed in murine ileal biopsies mounted in Ussing chambers, because this device allowed us to stimulate exclusively the luminal side of the epithelium. Electrical resistance of the ileal biopsies was monitored throughout the assay to control epithelial barrier integrity. An electrical resistance threshold of 12 ohms.cm<sup>-2</sup> was fixed as the limit for tissue integrity, because previous studies have demonstrated that there is no paracellular leakage of peptides for electrical resistance superior to 6 ohms.cm<sup>-2</sup> (29). Biopsies were therefore stimulated for 4 h by flagellin concentrated from *E. coli* MG1655-CM added to the apical compartment. Release of KC, a murine cytokine that shares functional properties with human IL-8 (30), was evaluated in the media bathing the mucosal and serosal sides of the biopsies. As shown in Fig. 6B, within this short 4-h stimulation period, flagellin concentrated from *E. coli* (500 ng/ml) induced a 2.5-fold increase in KC production in the basolateral compartment, compared with unstimulated control biopsies. In contrast, purified LPS from *E. coli*, at a concentration comparable to that detected in the preparations of concentrated flagellin by the limulus test (100 ng/ml), did not increase KC secretion over the background of 200 pg/ml. Finally, concentrated aflagellated  $\Delta fliC$  *E. coli*-CM did not induce any KC secretion, confirming that flagellin was the only soluble bacterial product responsible for the pro-inflammatory response.

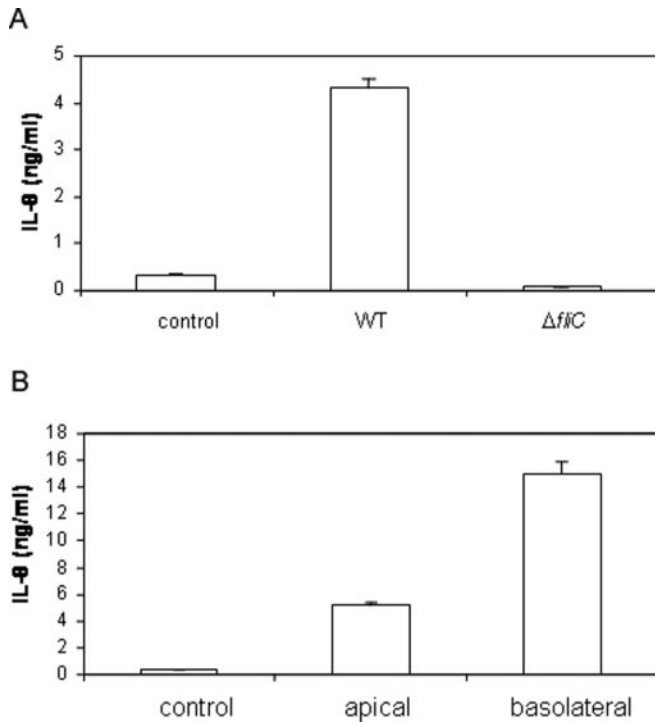


FIG. 5. Apical stimulation of polarized HT29-19A cells by live *E. coli* or flagellin induces basolateral secretion of IL-8. A, basolateral secretion of IL-8 in polarized HT29-19A cells stimulated on their apical side with either the WT *E. coli*, the  $\Delta$ *fliC* mutant (100 bacteria/cell), or medium alone (*control*) for 16 h. B, polarized HT29-19A cells were not stimulated (*control*) or stimulated with flagellin concentrated from WT *E. coli*-CM (100 ng/ml) added either to the apical or to the basolateral compartment. IL-8 secretion was assessed by ELISA in the basolateral supernatants. Data are mean  $\pm$  S.E. of triplicates in one experiment representative of at least three distinct experiments.

#### DISCUSSION

It is generally assumed that induction of an intestinal pro-inflammatory response is a property of pathogenic intestinal bacteria, whereas commensal bacteria should not induce this response as this might hamper establishment of mutualistic relationships between the bacteria and their mammalian host. Epithelial cells lining the gut have recently appeared to be a key player in the regulation of the initial steps of host pro-inflammatory responses to intraluminal bacteria via their controlled expression of receptors for PAMPs. One attractive hypothesis is that intestinal epithelial cells are hyporesponsive to commensal intraluminal bacteria due to the low expression of PAMP receptors at their apical surface (12), whereas invasive bacteria have access to, and can activate PAMP receptors, preferentially expressed intracellularly, or on the basolateral membrane. Challenging this hypothesis, our study shows that a commensal enteric *E. coli* strain can activate an NF- $\kappa$ B-dependent pro-inflammatory response not only in some polarized IEC lines, but also in native murine ileal epithelial cells in biopsies mounted in Ussing chambers. Induction of the NF- $\kappa$ B pathway relies on the interaction of flagellin common to all flagellated bacteria with TLR5 expressed *in situ* in the ileum, both at the basal and apical compartments of enterocytes.

*In vitro* studies using HT29-19A and Caco-2 IEC lines allowed us to demonstrate that the commensal *E. coli* MG1655 strain can, in common with pathogenic Gram-negative bacteria such as invasive *Salmonella* species (8), invasive *Shigella* species (9), and enteroadherent *E. coli* (20), activate the NF- $\kappa$ B pathway and thereby induce expression of chemokines endowed with bactericidal properties and/or able to recruit cells of the innate immune system. These *in vitro* studies also demon-

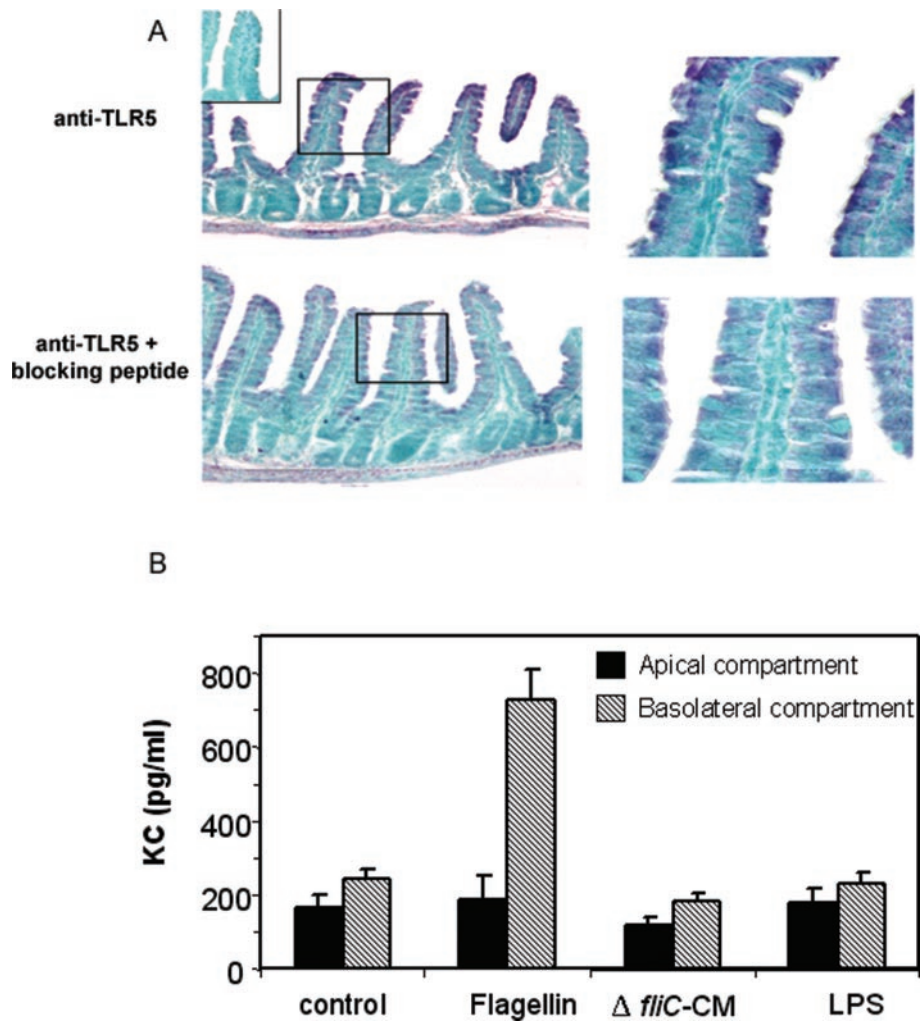
strated the unique contribution of the interaction between bacterial flagellin and TLR5. Thus, the NF- $\kappa$ B-mediated pro-inflammatory response induced by *E. coli* was resumed by flagellin concentrated from *E. coli* MG1655-CM and, on the contrary, was abolished in the presence of whole bacteria or conditioned medium derived from the same *E. coli* strain in which the flagellin encoding gene had been interrupted ( $\Delta$ *fliC* strain). This result is in keeping with previous studies indicating that flagellin produced by pathogens such as *S. typhimurium* (21, 22), but also from non-invasive *E. coli* pathogens (23, 31), can induce an NF- $\kappa$ B response. Sustaining the prominent role of flagellin, which admits TLR5 as a unique ligand at the surface of mammalian cells, transfection of IEC lines with a plasmid encoding dominant negative TLR5 abolished the induction of NF- $\kappa$ B in response to both *E. coli* derived flagellin and whole live *E. coli*. Consistent with the role of TLR5 in flagellin recognition, induction of NF- $\kappa$ B activity by live *E. coli* or flagellin was also significantly diminished in IECs transfected with dominant negative MyD88, the proximal relay of TLR5 in the NF- $\kappa$ B pathway (24). The highly conserved structure of flagellin between flagellated bacteria (25, 26) thus endows non-pathogenic as well as pathogenic bacteria with the capacity to activate the NF- $\kappa$ B signaling cascade in IECs.

One recent study also suggested that a commensal bacteria, *B. vulgatus*, can activate NF- $\kappa$ B in rat IECs (13). The latter bacteria are aflagellated, and the pro-inflammatory factor was identified as LPS, another motif common to commensal and pathogenic Gram-negative bacteria, which activated the NF- $\kappa$ B cascade upon binding to TLR4 (13). In that study, the inducing effect of LPS was also observed in primary rat epithelial cells, but the ability of LPS to induce this pathway *in vivo* was not unambiguously demonstrated, because experiments were carried on non-polarized cells isolated from the rat intestine, preventing to ascertain that TLR4 was accessible *in situ* to LPS released by intraluminal bacteria.

The same criticism might apply to TLR5-flagellin interactions, because it has been argued, based on the study of the T84 epithelial cell line, that its localization at the basolateral pole of IECs allows enterocytes to discriminate between commensal strains remaining in the gut lumen and pathogenic strains able to reach the basolateral membrane upon invasion (22, 27, 28). Induction of IL-8 by apical stimulation has, however, been observed in polarized Caco-2 and HCA-7 epithelial cell lines in response to flagellin from *S. typhimurium* and enterohemorrhagic *E. coli*, respectively (21, 31). Our results using the HT29-19A cell line provided consistent results with the latter studies. NF- $\kappa$ B signaling could be triggered in polarized HT29-19A by apical stimulation with live commensal *E. coli* or its soluble flagellin, resulting in a robust polarized basolateral secretion of IL-8. IL-8 production was, however, 2- to 3-fold higher when flagellin was added to the basolateral membrane, suggesting some differential distribution of the signaling machinery in polarized HT29-19A cells. Discrepancies in the results obtained with various cell lines suggest that IEC response and/or TLR5 distribution might vary depending on cell lines but also on cellular culture conditions within individual laboratories, casting doubt on the possible transfer of these *in vitro* observations to the *in vivo* situation.

We therefore determined whether flagellin could activate epithelial cells *in vivo* when released in the intestinal lumen by commensal bacteria. Analysis of TLR5 expression in murine ileal biopsies by immunohistochemistry indicated that this receptor was not restricted to the basolateral side of the epithelium, but in contrast was also strongly expressed at the apical part of the villous enterocytes, a result in keeping with recent observations in humans (32). To further assess the *in vivo*





**FIG. 6. Apical commensal *E. coli* flagellin induces basolateral secretion of KC by murine ileal biopsies.** *A*, staining of enterocytes by anti-TLR5 antibody. *Upper panel*: ileal sections from C3H/HeN mice were stained with an anti-TLR5 goat antibody (*upper left panel*,  $\times 100$ ; *upper right panel*,  $\times 400$ ). The *inset* in the *upper left panel* shows staining with a control polyclonal goat antibody. *Lower panel*: anti-TLR5 antibody was preincubated overnight with TLR5 blocking peptide prior to immunostaining (*lower left panel*,  $\times 100$ ; *lower right panel*,  $\times 400$ ). *B*, KC secretion by intestinal biopsies. Murine ileal biopsies were mounted in Ussing chambers and stimulated apically with either flagellin concentrated from WT *E. coli*-CM (500  $\mu\text{g/ml}$ ), concentrated  $\Delta fliC$  *E. coli*-CM (equivalent volume of concentrated WT *E. coli*-CM), LPS (100  $\text{ng/ml}$ ), or medium alone (*control*). After a 4-h stimulation, KC secretion was measured by ELISA in both apical (*black bars*) and basolateral (*hatched bars*) compartments. Data are mean  $\pm$  S.E. of triplicates in one experiment representative of at least five distinct experiments.

capacity of enterocytes to respond to intraluminal flagellin, murine ileal mucosa was mounted in Ussing chambers, and flagellin was added on the luminal side. This apical *ex vivo* stimulation induced a significant basolateral secretion of KC, a murine cytokine that shares functional properties with human IL-8 (30). In common with IL-8, murine KC is under NF- $\kappa$ B transcriptional control and can be stimulated by several pro-inflammatory substances, including LPS, in various cell types including epithelial cells (33–35). The human IEC lines tested in the present study failed to respond to LPS, as previously observed by Abreu *et al.* (36). In contrast, a murine IEC line was recently found to respond to LPS, following internalization and interaction of LPS and TLR4 in the Golgi apparatus (37, 38). Furthermore, results by Haller *et al.* (13) suggest that LPS can stimulate primary rat IECs. The contribution to KC induction of LPS contaminating the preparation of flagellin from *E. coli* is however unlikely. Indeed, supernatants derived from  $\Delta fliC$  bacteria had no effect, confirming the prominent, if not exclusive, role of flagellin in KC induction in murine ileal biopsies.

Altogether, these data raise the question of the strategies developed by the host and commensal bacteria to maintain gut homeostasis and avoid inappropriate inflammation. As suggested by Haller *et al.* (13, 39), rapid intestinal production of immunoregulatory cytokines might be able to counteract the pro-inflammatory effect of commensal bacteria. Thus, enterocyte-derived transforming growth factor- $\beta$ 1 could inhibit *B. vulgatus*-induced NF- $\kappa$ B recruitment to the IL-6 promoter

by modulating histone acetylation (39). Alternatively, some components of the resident gut microflora might themselves dampen the IEC pro-inflammatory response. Neish *et al.* (14) observed that an avirulent *Salmonella* strain abrogated production of inflammatory cytokines in cultured human IECs in response to diverse pro-inflammatory stimuli. More recently, the commensal bacteria *B. thetaiotaomicron*, a prominent anaerobe of the human intestine, was found to inhibit *in vitro* and *in vivo* inflammatory responses induced by a pathogenic *Salmonella* strain (15). In both cases NF- $\kappa$ B was entrapped in the cytosol of epithelial cells and failed to translocate into the nucleus, due either to blockade of I $\kappa$ B ubiquitination and degradation (14) or to cytosolic redistribution of peroxisome proliferator-activated receptor- $\gamma$ , which complexed NF- $\kappa$ B (15). It is noticeable that *E. coli* is only prominent in the human intestine early on in life. Does it trigger an inflammatory response that contributes to its elimination and replacement by less pro-inflammatory strains? Alternatively, the rapid induction of an IgA response upon colonization may limit contact between commensal bacteria and the epithelial barrier, thereby preventing protracted inflammation (40). It is interesting, however, that  $\sim 15\%$  of normal humans possess a mutated form of TLR5 unable to transmit pro-inflammatory signals (41). It is tempting to hypothesize that such a mutation has provided a selective advantage by improving mutual relationships between *E. coli* and its human host. Further *in vivo* studies should help to delineate how commensal *E. coli* colonize

the intestine without inducing an intestinal inflammatory response deleterious for the host.

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***In Vitro* and *ex Vivo* Activation of the TLR5 Signaling Pathway in Intestinal Epithelial Cells by a Commensal *Escherichia coli* Strain**

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