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Activation of Enteroendocrine Cells via TLRs Induces Hormone, Chemokine, and Defensin Secretion[1]

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Enteroendocrine cells are known primarily for their production of hormones that affect digestion, but they might also be implicated in sensing and neutralizing or expelling pathogens. We evaluate the expression of TLRs and the response to specific agonists in terms of cytokines, defensins, and hormones in enteroendocrine cells. The mouse enteroendocrine cell line STC-1 and C57BL/6 mice are used for in vitro and in vivo studies, respectively. The presence of TLR4, 5, and 9 is investigated by RT-PCR, Western blot, and immunofluorescence analyses. Activation of these receptors is studied evaluating keratinocyte-derived chemokine, defensins, and cholecystokinin production in response to their specific agonists. In this study, we show that the intestinal enteroendocrine cell line STC-1 expresses TLR4, 5, and 9 and releases cholecystokinin upon stimulation with the respective receptor agonists LPS, flagellin, and CpG-containing oligodeoxynucleotides. Release of keratinocyte-derived chemokine and β-defensin 2 was also observed after stimulation of STC-1 cells with the three TLR agonists, but not with fatty acids. Consistent with these in vitro data, mice showed increased serum cholecystokinin levels after oral challenge with LPS, flagellin, or CpG oligodeoxynucleotides. In addition to their response to food stimuli, enteroendocrine cells sense the presence of bacterial Ags through TLRs and are involved in neutralizing intestinal bacteria by releasing chemokines and defensins, and maybe in removing them by releasing hormones such as cholecystokinin, which induces contraction of the muscular tunica, favoring the emptying of the distal small intestine. *The Journal of Immunology,* **2007, 178: 4296–4303.**

The gastrointestinal tract is the site where the divergent needs of nutrient absorption and host defense meet. Because nutrient absorption requires a large surface area and a thin epithelium, features that potentially compromise host defense, it is not surprising that the gastrointestinal tract has evolved an elaborate network of surveillance systems comprised of both professional and nonprofessional immune cells (1).

Intestinal epithelium is composed of four adult types of cells: enterocytes, goblet cells, enteroendocrine cells (EECCs),[4] and Paneth cells. Enterocytes and Paneth cells were recently shown to participate in the innate immune response against pathogens by interacting with specific microbial molecular patterns through their cognate receptors (2–4). The best characterized of these receptors are the TLRs, which are mammalian homologs of the *Drosophila* protein Toll, involved in antifungal defense (5). Enterocytes and Paneth cells of the small intestine express different TLRs (6–8), and the interaction with TLR agonists results in the activation of NF-κB and the secretion of inflammatory cytokines (9–11) and antimicrobial peptides (12). The majority of these antimicrobial peptides are defensins, small cationic peptides containing sulfide bonds that exert their effect by damaging the bacterial cell membrane and by acting as chemokines (13–15). Defensins are classified as either α or β depending on the position of three intramolecular disulfide bonds. Enterocytes synthesize β-defensins (16) while Paneth cells secrete α-defensins (17).

EECCs are scattered as individual cells throughout the mucosa and, although they represent ~1% of the cells lining the intestinal lumen (18, 19), they are the largest population of hormone-producing cells in the body (20). In the gastrointestinal tract, these cells synthesize different types of gastrointestinal hormones and play an important role in the physiological functions of the alimentary tract (21). In vitro and in vivo studies indicate that EECCs share a common lineage with the three other principle cell lineages found in the intestine, all deriving from primitive intestinal stem cells located in the intestinal crypts (22, 23). This common lineage, along with a variety of data indicating the intimate link between the immune and neuroendocrine systems, led us to investigate whether EECCs of the intestinal tract might play a role in the innate immune response. In particular, we tested whether EECCs, in addition to their response to dietary luminal content, can directly recognize bacterial Ags and respond by producing immunostimulating and defensive factors and/or hormones that help remove pathogens by inducing contraction of the muscular tunica. Our data, obtained in vitro and in vivo in mice, indicate a role for EECCs in the control of intestinal microflora.

Materials and Methods

Reagents

Abs used were: goat anti-mouse TLR4 cross-reacting with human TLR4 (L-14; Santa Cruz Biotechnology), rabbit anti-mouse TLR5 cross-reacting with human TLR5 (H-127; Santa Cruz Biotechnology), biotinylated mouse

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[4] Abbreviations used in this paper: EECC, enteroendocrine cell; ODN, oligodeoxynucleotide; Fw, forward; Rw, reverse; TRITC, tetramethylrhodamine isothiocyanate; DAPI, 4′,6′-diamidino-2-phenylindole; KC, keratinocyte-derived chemokine; CCK, cholecystokinin; EIA, enzyme immunoassay; siRNA, small interfering RNA; PKC, protein kinase C.

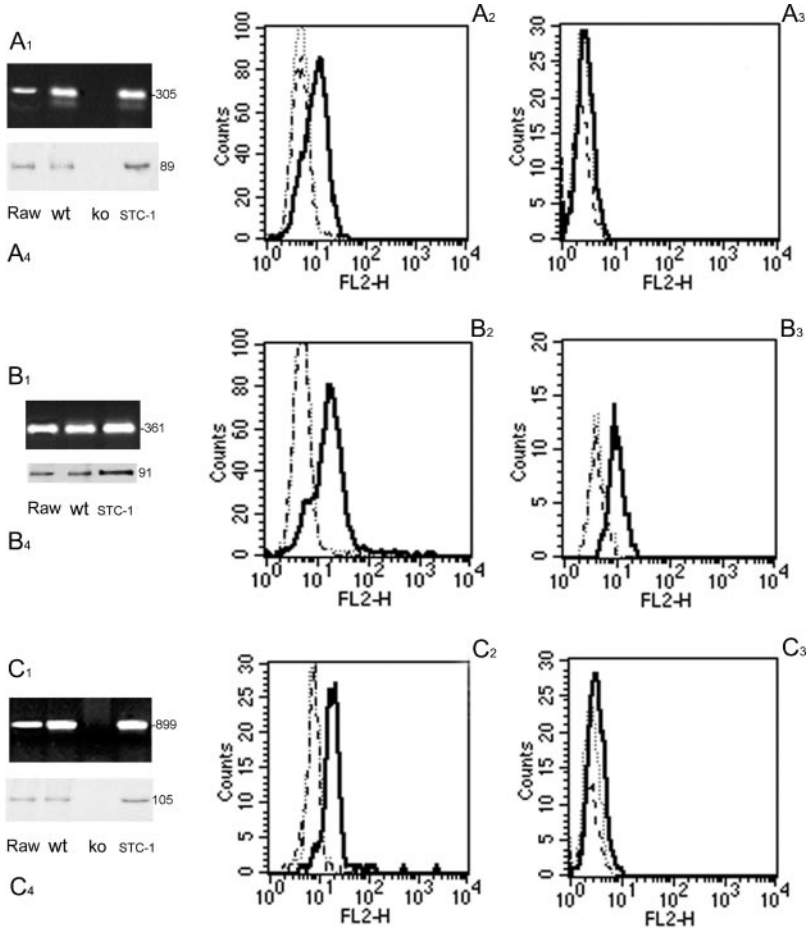

FIGURE 1. Expression of TLR4 (*A*), TLR5 (*B*) and TLR9 (*C*) in STC-1 cells. A_1–C_1, mRNA expression in RAW 264.7, peripheral blood cells of wild-type (wt) and knockout (ko) mice, and STC-1 cells. A_4–C_4, Western blot analysis of protein lysates from RAW 264.7, peripheral blood cells of wild- type and knockout mice, and STC-1 cells. Flow cytometric analysis of intracellular (A_2–C_2) and surface expression (A_3–C_3) of TLR4, TLR5, and TLR9 on STC-1 cells. Representative histograms: bold lines represent cells stained with TLR4, TLR5, or TLR9 Abs; bold discontinuous lines represent cells stained with secondary Abs; and dotted lines represent cells stained with unrelated Abs.

anti-human TLR9 cross-reacting with mouse TLR9 (HBT), and rabbit anti-mouse β-defensin 2 cross-reacting with human β-defensin 2 (Alpha Diagnostic International). Agonists used were: LPS (Sigma-Aldrich), CpG oligodeoxynucleotide (ODN) 1668 (M-Medical Genenco), and flagellin (InvivoGen).

Mice

The in vivo experiment was conducted using female wild-type C57BL/6 mice purchased from Charles River. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of Istituto Nazionale Tumori, Milano according to the guidelines of the United Kingdom Coordinating Committee on Cancer Research for animal welfare in experimental neoplasia (1998).

Cell culture

RAW 264.7 murine macrophages, NCTC 2544 human keratinocytes, and HEK293 (human embryonic kidney; purchased from American Type Culture Collection) were cultured in DMEM Glutamax (Invitrogen Life Technologies) supplemented with 10% FCS (Invitrogen Life Technologies), 10 ml/L penicillin and streptomycin, and 0.025% amphotericin B (all from Sigma-Aldrich). STC-1 mouse EECCs (a gift from Prof. G. Rindi, Department of Pathology and Laboratory Medicine, Università degli Studi di Parma, Parma, Italy) were maintained in DMEM (4500 mg/L glucose; Invitrogen Life Technologies) supplemented with 15% horse serum (Invitrogen Life Technologies), 2.5% FCS, 10 ml/L penicillin and streptomycin, and 2 mM glutamine (all from Sigma-Aldrich). All cultures were incubated at 37°C in a 5% CO_2 humidified environment; culture medium was changed three times per week until confluence.

Total RNA extraction, reverse transcription, and PCR

Expression of TLRs was investigated in STC-1, RAW 264.7, and peripheral blood cells of wild-type and knockout mice by RT-PCR. Total RNA was isolated from 1×10^6 washed and pelleted cultured cells using a Perfect RNA Eukaryotic Mini kit (Eppendorf). Total RNA was converted into cDNA as follows: 2 μg of total RNA, 1 μl of random primers (50 μg/ml; Promega) and 2 μl of dNTP mix (10 mM each; Eppendorf) were denatured at 65°C for 5 min; the reaction volume was brought to 20 μl by addition of 4 μl of RT_{plus}PCR buffer containing 25 mM Mg^{2+}, 1.5 U/μl cMaster RT Enzyme, 0.5 μl of Prime RNase inhibitor solution, and nuclease-free water (cMaster RTplusPCR System; Eppendorf); after incubation at 50°C for 60 min, the reaction was stopped by incubation at 85°C for 5 min. Using cDNA, PCR was performed with 400 nM primers, 200 μM of each dNTP, and 2 U of cMaster PCR Enzyme Mix (Eppendorf). Two cycles of amplification were performed for TLR4 and 5 using 0.5 μl of primary PCR product as the template for nested amplification. The primary PCR for TLR4 included denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, extension at 72°C for 50 s, and a final extension at 72°C for 5 min. The PCR profile for the TLR5 was similar except that annealing was done at 56°C. In the nested PCR for TLR4 and 5, annealing was done at 57°C for both genes and extension at 72°C for 30 s, 40 cycles for TLR4 and 30 cycles for TLR5. The PCR for TLR9 included denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The following primers (Primm) were used: TLR4 forward (Fw), 5′-ACACCAGGAAGCTTGAATCC-3′; TLR4 reverse (Rw), 5′-GCACTCATAATGATGGCACC-3′; TLR4 nested Fw, 5′-GACTTCATTCAAGACCAAGCC-3′; TLR4 nested Rw, 5′-ACACCTGCCAGAGACATTGC-3′; TLR5 Fw, 5′-ATGGATGGATGCTGAGTTCC-3′; TLR5 Rw, 5′-AGTTGAAGCTGAGCAGGAGC-3′; TLR5 nested Fw, 5′-TTGTCCGATCATCAGCTAAGC-3′; TLR5 nested Rw, 5′-GGCAGATTCTTCTTGTCTTGG-3′; TLR9 Fw, 5′-AACATGGTTCTCCGTCGAAGGA-3′; and TLR9 Rw, 5′-TATAGGACACCAGGAGGTACTC-3′.

Western blot analysis

TLR expression. Proteins were extracted from STC-1, RAW 264.7, and peripheral blood cells from mice (wild-type and knockout) using lysis buffer (0.1 M NaCl, 0.01 M Tris-HCl (pH 7.6), and 0.001 M EDTA (Sigma-Aldrich), pH 8), 1% Triton X-100 (Sigma-Aldrich), 0.5 ng/ml leupeptin, 1 ng/ml pepstatin, 2 ng/ml aprotinin, and 100 μg/ml PMSF (Sigma-Aldrich)

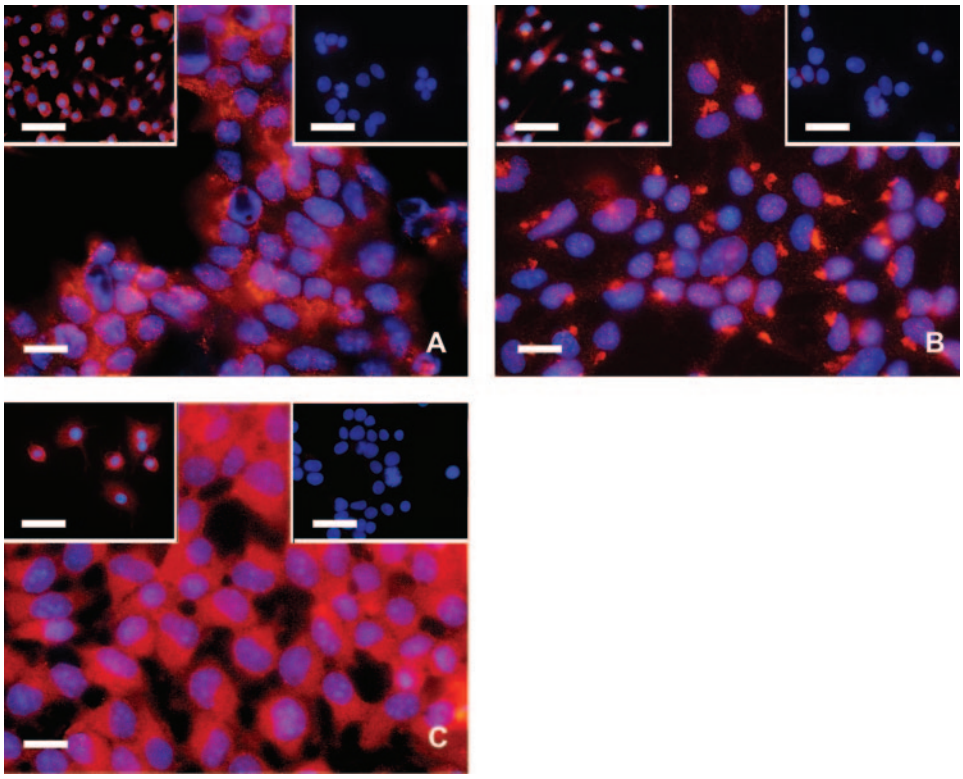

FIGURE 2. Immunofluorescence microscopy analysis of TLR4 (*A*), TLR5 (*B*), and TLR9 (*C*) in STC-1 cells. Double immunofluorescence image of STC-1 cells (bar, 10 μm), RAW 264.7 cells (*left inset*; bar, 30 μm), and HEK 293 (*right inset*; bar, 30 μm). Red, TLR staining; blue, DAPI nuclear staining.

on ice for 45 min. Proteins were estimated quantitatively using the BCA Protein Assay kit (Pierce). Protein samples (15 μg) were fractionated on an 8% acrylamide (Bio-Rad) slab gel containing 0.1% SDS (Sigma-Aldrich) and transferred onto a nitrocellulose filter (Amersham Biosciences) by electroblotting. After incubation for 1 h in TBS with 1% Tween 20 (Sigma-Aldrich) and 5% milk powder to block nonspecific binding sites, the filter was incubated with primary Abs directed to TLR4 (1/100 in TBS with 5% milk powder) for 1 h and to TLR5 (1/100 in TBS with 5% milk) (Sigma-Aldrich) for 45 min. After three washes for 20 min each in TBS, 1% Tween 20, and 5% milk powder, the filter was incubated with secondary Ab in TBS, 0.1% Tween 20, and 5% milk powder for 1 h at room temperature. Secondary Abs were: anti-goat peroxidase-conjugated Ab for TLR4 (1/1000) and anti-rabbit peroxidase-conjugated Ab for TLR5 (1/1000; Vector Laboratories). To detect the TLR9 signal, the filter was incubated for 1 h in TBS with 5% milk, washed in TBS for 5 min, incubated with biotinylated mAb to TLR9 (1/25 in TBS) for 1 h, washed three times in TBS-1% Tween 20, and examined using the ABC kit (Vector Laboratories). Bands were visualized using ECL Western Blotting Detection Reagents and autoradiography film (Amersham Biosciences).

DEFB2 expression. Total protein (10 μg) extracted from STC-1 cells treated with LPS (1 μg/ml), CpG-ODN (1 μM), or flagellin (100 ng/ml) or untreated was fractionated, along with DEFB2 control peptide (Alpha Diagnostic International) on a 10, 13, and 15% acrylamide gradient slab gel containing 0.2% SDS and electroblotted onto a nitrocellulose filter. After blocking in TBS with 0.5% glutaraldehyde (Merck) for 30 min (10 min of incubation and 20 min of shaking), the filter was incubated with primary Ab directed to mouse β-defensin (1/500 in PBS/0.25% BSA) for 18 h, washed three times for 20 min each in TBS-1% Tween 20, and incubated with secondary goat anti-rabbit peroxidase-conjugated Ab (1/1000 in TBS-1% Tween 20; Vector Laboratories) for 1 h at room temperature. The filter was washed three times in TBS-1% Tween 20 and once in TBS, and bands were visualized using ECL Western Blotting Detection Reagents and autoradiography film.

Flow cytometry

Flask-cultured STC-1 cells were trypsinized, washed in PBS, and fixed in 400 μl of 4% buffered formaldehyde. Immunofluorescence staining was performed by incubating STC-1 for 60 min at room temperature with anti-TLR4, TLR5, and TLR9 primary Abs diluted in PBS for cell surface staining or in saponin buffer (0.2% saponin and 0.5% BSA; Sigma-Aldrich) for intracellular staining. Matched specie/isotype Igs were used as control. The cells were then incubated with appropriate secondary Abs, washed with buffer, and analyzed by FACSCalibur flow cytometry.

Immunofluorescence microscopy analysis

STC-1, RAW 264.7 (positive control), and HEK293 (negative control) cells were subcultured on coverslips in 24-well plates, washed with PBS for 5 min, and fixed for 5 min in 4% paraformaldehyde, 2% saccharose in PBS (TLR4 and TLR9), or 2% paraformaldehyde in PBS (TLR5). After washing once in PBS, cells were incubated for 60 min at room temperature with the primary Abs diluted 1/20 in saponin buffer (0.2% saponin and 0.5% BSA; Sigma-Aldrich) for TLR4 and TLR5 and 1/50 in the same buffer for TLR9. After washing with saponin buffer, cells were incubated for 1 h at room temperature with secondary Ab donkey anti-goat-tetramethylrhodamine isothiocyanate (TRITC; 1/:500 in saponin buffer; Jackson ImmunoResearch Laboratories) to detect TLR4, with goat anti-rabbit-TRITC (1/100 in saponin buffer; Jackson ImmunoResearch Laboratories) to detect TLR5, or with goat anti-mouse-TRITC (1/200 in saponin buffer; Molecular Probes) to detect TLR9. Cells were then washed with PBS, incubated with 4′,6′-diamidin-2-phenylindole (DAPI; 1/50,000 in PBS) for 5 min, washed again with PBS, and slides were mounted with Mowiol (Calbiochem).

Stimulation with TLR agonists and release of cholecystokinin (CCK) and keratinocyte-derived chemokine (KC)

The release of CCK and KC after TLR agonist treatment was quantified in both STC-1 cell supernatants and in mouse sera. For the in vitro studies, STC-1 cells were subcultured on coverslips in 24-well plates and treated with LPS (100 ng/ml, 1 and 10 μg/ml), CpG-ODN (100 nM, 1 and 10 μM), flagellin (10, 100 ng/ml and 1 μg/ml), fatty acid (1500 μM), or croton oil (20 μg/ml) (Sigma-Aldrich) added directly to the medium. After 1, 2, 4, 8, 12, and 18 h, supernatants were collected and kept at −80°C. For blocking studies, confluent cells were incubated for 30 min with anti-TLR4 and 5 Abs (20 μg/ml) before addition of LPS (1 μg/ml) and flagellin (100 ng/ml); after 4 h, supernatants were collected and kept at −80°C.

For in vivo studies, 40 mg/kg body weight of LPS, 1.6 mg/kg of CpG-ODN, 0.8 mg/kg of flagellin, and 3.2 mg/kg of fatty acids were administered orally with a stomach tube to groups of C57BL/6 and TLR4$^{-/-}$ or TLR9$^{-/-}$ mice (n = 5 for each group, previously treated or untreated with gentamicin for 1 wk). A control group (n = 5) was treated with water. After 3 and 18 h, blood was collected from all mice, incubated at 37°C for 20 min, and centrifuged (13,000 × g for 10 min) immediately thereafter to separate coagulated blood from sera. Serum samples were kept at −80°C.

Production of mouse CCK in supernatants and in plasma was quantified using the enzyme immunoassay (EIA) kit from Phoenix Pharmaceuticals according to the manufacturer's recommendations. Plasma samples were

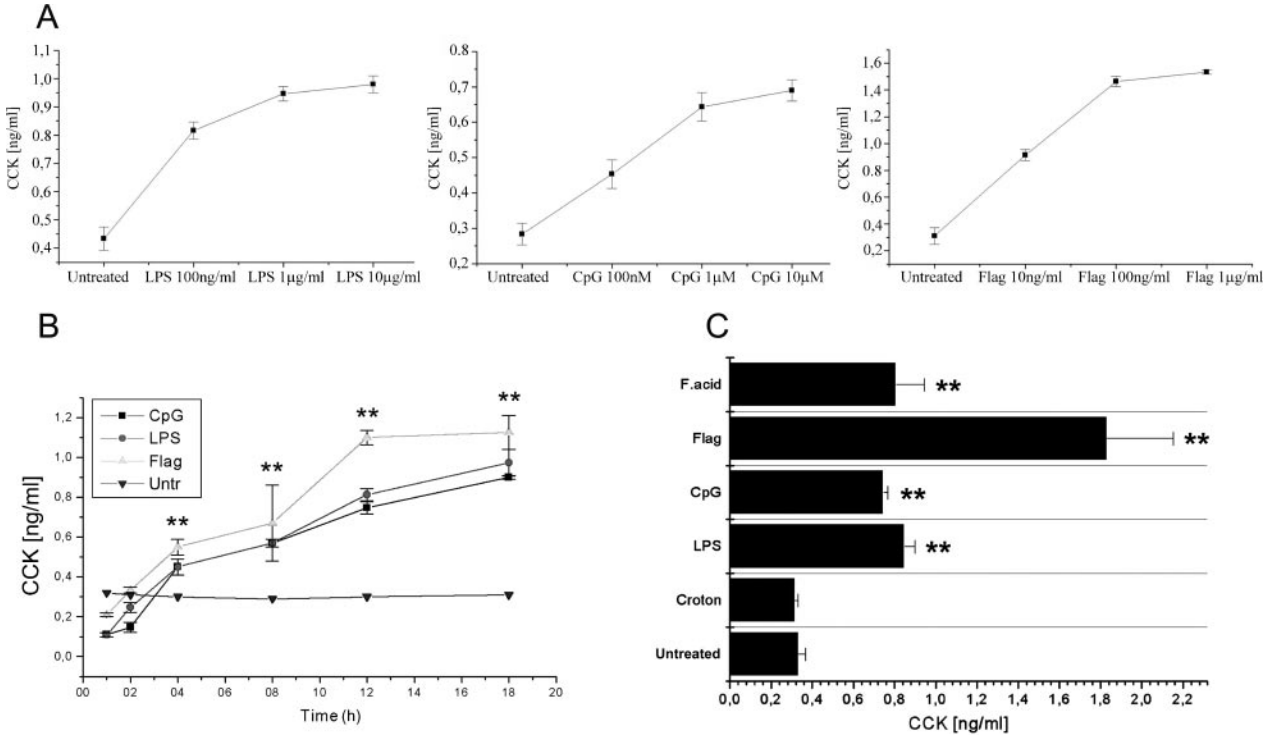

FIGURE 3. CCK release from STC-1 cells stimulated with TLR agonists. Analysis of supernatants by EIA: Dose (*A*)- and time (*B*)-response studies. *C*, Levels 18 h after stimulation with fatty acid (F. acid), flagellin (Flag), CpG-ODN (CpG), LPS, and croton oil (Croton). Data are mean ± SEM from three independent experiments (**, $p \leq 0.01$ vs untreated cells).

diluted 1/2 in PBS. Concentrations of mouse KC were evaluated using the ELISA kit from R&D Systems.

Inhibition of TLR-induced CCK secretion in STC-1 cells

MyD88, an adapter molecule involved in the earliest events of TLR signaling cascade, was silenced by small interfering RNA (siRNA) transfection. Briefly, STC-1 cells (3×10^5/well) seeded in 6-well plates at 60–80% of confluence were washed in Optimem (Invitrogen Life Technologies) medium and then transfected with a pool of siRNA oligonucleotides targeting mouse MyD88 or a scrambled RNA duplex (Dharmacon), at a final concentration of 100 nM. Six μg/μl Lipofectamine 2000 (Invitrogen) were used as transfection reagent. After 24 h, the transfection mixture was aspirated from the cells and replaced with culture medium and treated for 18 h with TLR agonists and fatty acids as reported above.

Protein kinase C (PKC), one of the proteins involved in the TLR signaling pathway, was inhibited with a 5 μM mixture of rottlerin and bis (Sigma-Aldrich). PKC-inhibited STC-1 cells were then treated with the TLR agonists and fatty acids as reported above.

Supernatants obtained from siRNA and PKC-inhibition experiments were evaluated by EIA as described above.

Stimulation with TLR agonists and release of β-defensin

STC-1 cells, subcultured on coverslips in 24-well plates, were treated with LPS (1 μg/ml), CpG-ODN (1 μM), or flagellin (100 ng/ml). NCTC 2544 cells, subcultured on coverglass in 24-well plates, were treated with LPS (1 μg/ml). After 18 h, cells were incubated for 5 min in methanol at −20°C, washed in PBS, permeabilized for 5 min with 0.5% Triton X-100 in PBS, and washed with PBS. Glycine (1%; Sigma-Aldrich) in Tris-HCl was added for 5 min to eliminate autofluorescence, and, after washing in PBS, by addition of normal swine serum (10% in PBS; DakoCytomation) for 30 min, cells were then incubated with primary Ab rabbit anti-mouse DEFB2 (1/100 in PBS) for 60 min at 37°C, washed with PBS, and incubated with secondary Ab goat anti-rabbit-FITC (1/200 in PBS; Molecular Probes) for 60 min at room temperature. After washing with PBS, cells were incubated with DAPI (1/50000) in PBS for 5 min, washed with PBS, and slides were mounted with Mowiol.

Statistical analysis

Student's *t test* (paired two-tailed) and GraphPad Prism software (GraphPad) were used for comparisons between groups. Values of $p < 0.05$ were considered to be significant.

Results

Expression of TLR4, TLR5, and TLR9 in EECCs

The expression of TLR4, TLR5, and TLR9 was investigated by RT-PCR in total RNA extracted from the mouse enteroendocrine cell line STC-1. Total RNA from the murine macrophage line RAW 264.7 and peripheral blood cells of wild-type C57BL/6 mice (positive controls), and from TLR4$^{-/-}$ or TLR9$^{-/-}$ C57BL/6 mice (negative controls) was also examined. PCR products encoding TLR4, 5, and 9 mRNA of the expected size were generated from RNA of STC-1, RAW 264.7 and C57BL/6 peripheral blood cells, whereas no TLR4 or TLR9 mRNA was detected in peripheral blood cells from TLR4$^{-/-}$ or TLR9$^{-/-}$ mice (Fig. 1, A_1–C_1). Expression of TLR4, TLR5, and TLR9 was then evaluated in STC-1 by flow cytometry analysis. All three receptors were found to be expressed at cytoplasm level (Fig. 1, A_2–C_2), while staining at the cellular membrane was observed only for the anti-TLR5 Ab (Fig. 1, A_3–C_3). Western immunoblotting detected proteins of the appropriate molecular mass in STC-1, RAW 264.7, and wild-type blood cells, confirming the specificity of the commercially available anti-TLR4, 5, and 9 Abs, while blood cells from TLR4$^{-/-}$ or TLR9$^{-/-}$ mice were negative for the relevant protein expression (Fig. 1, A_4–C_4).

Microscopy immunofluorescence analysis revealed in both STC-1 and RAW 264.7 cells expression of TLR4, 5, and 9 proteins localized primarily in the cytoplasm, while HEK293 were negative (Fig. 2, *A–C* and *insets*).

STC-1 cells activated by TLR4 agonist LPS, TLR5 agonist flagellin, or TLR9 agonist CpG-ODN produce CCK

The physiological response of EECCs to dietary luminal content is the secretion of enteroendocrine hormones such as CCK. Accordingly, STC-1 cells stimulated by fatty acids exhibit an increase in CCK production (24). Analysis of CCK production by STC-1 cells

Table I. *CCK levels in culture media of treated or untreated STC-1 after siRNA-mediated silencing of MyD88*[a]

	Naïve MyD88 Cells (ng/ml)	Scrambled-Treated Cells (ng/ml)	MyD88 siRNA-Treated Cells (ng/ml)
Culture medium	0.34 ± 0.04	0.32 ± 0.02	0.33 ± 0.04
LPS *Escherichia coli*	0.95 ± 0.05	0.97 ± 0.04	0.45 ± 0.06[b]
CpG-ODN	0.75 ± 0.02	0.77 ± 0.03	0.39 ± 0.05[b]
Flagellin	1.85 ± 0.31	1.79 ± 0.26	0.40 ± 0.08[b]
Fatty acids	0.78 ± 0.09	0.81 ± 0.08	0.80 ± 0.07

[a] As negative control, cells were transfected with an unrelated oligonucleotide (scrambled-treated cells).
[b] Student's *t* test; $p \leq 0.01$ vs scrambled treated cells.

Table II. *CCK levels in culture media of treated or untreated STC-1 after inhibition of PKC with a mixture of rottlerin and bis*

	Naïve PKC Cells (ng/ml)	PKC-Inhibited Cells (ng/ml)
Culture medium	0.331 ± 0.035	0.329 ± 0.010
LPS *Escherichia coli*	0.846 ± 0.054	0.337 ± 0.027[a]
CpG-ODN	0.757 ± 0.025	0.326 ± 0.020[a]
Flagellin	1.830 ± 0.030	0.402 ± 0.004[a]
Fatty acids	0.797 ± 0.015	0.335 ± 0.023[a]

[a] Student's *t* test; $p \leq 0.01$ vs naïve PKC cells.

stimulated in vitro with the TLR agonists LPS, flagellin, and CpG-ODN revealed a progressive increase in CCK levels with dose-dependent responses for each agonist, going to plateau after 18 h of treatment (Fig. 3, *A* and *B*). At 18 h of treatment, a significant increase in CCK levels, which was at least 2-fold higher than in untreated samples, was observed for each of the three agonists. Fatty acids were used as positive control and croton oil, an inflammatory agent, was used as a nonspecific stimulus. Interestingly, flagellin appeared to produce a more potent effect than fatty acids. Croton oil did not increase CCK production in STC-1 cells (Fig. 3*C*).

MyD88 silencing and PKC inhibition decrease TLR-induced CCK release

STC-1 cells treated with specific siRNA to down-regulate expression of the early adaptor molecule MyD88 (25) showed reduced CCK production upon stimulation with TLR agonists but not after fatty acid stimulation (Table I). Moreover, specific inhibition of PKC, a molecule known to be involved in the TLR signaling pathway (26, 27), led to a nearly complete block in CCK production by STC-1 cells subsequently stimulated with TLR agonists or fatty acids (Table II).

Oral treatment with LPS, flagellin, or CpG-ODN enhances CCK serum levels

EECCs are the only CCK-producing cell type in direct contact with the luminal content; enhancement of serum levels of this hormone after oral treatments with substances that do not cross intact epithelial barriers is expected to be related to activation of EECCs. To determine whether intestinal EECCs express TLRs and whether activation of these receptors by specific agonists induce CCK production, as observed in STC-1 cells, we measured serum CCK levels in mice depleted or not of intestinal commensal microflora by antibiotic treatment and subsequently treated orally with LPS, flagellin, CpG-ODN, or fatty acids (positive control). After 3 h, serum CCK levels were increased mainly in mice treated with LPS (data not shown). After 18 h. the levels were significantly increased in mice treated with fatty acids and the TLR4, TLR5, and

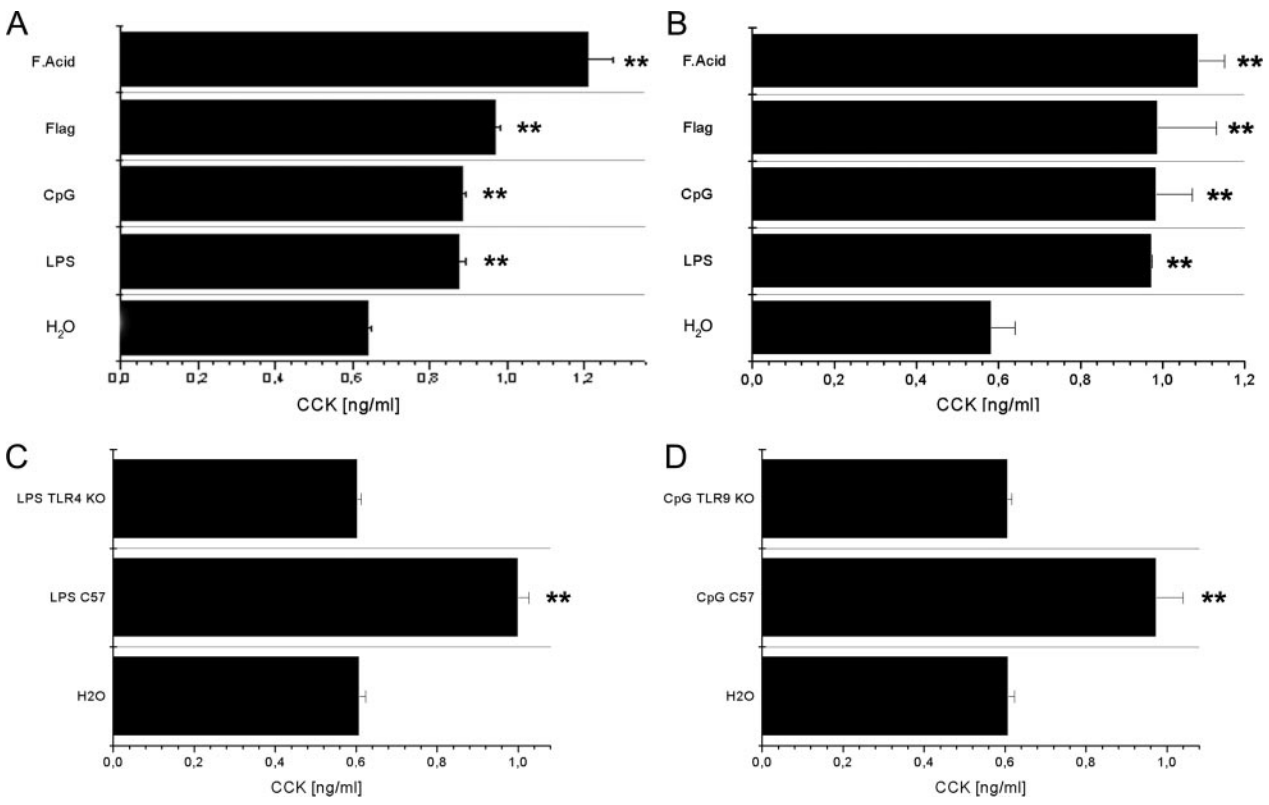

FIGURE 4. CCK serum levels in mice treated with TLR agonists. C57BL/6 mice untreated (*A*) or antibiotic-treated (*B*; 5 five per group), (*C*) TLR4$^{-/-}$ (*C*) and TLR9$^{-/-}$mice (*D*) were orally treated with fatty acid (F Acid), flagellin (Flag), CpG-ODN (CpG), and LPS. CCK serum levels were measured 18 h later by EIA. Data are mean ± SD (**, $p \leq 0.01$ vs untreated mice).

TLR9 agonists as compared with levels in control mice (Fig. 4*A*). The reduction of intestinal flora, induced by antibiotic gentamicin treatment, was found to only weakly increase the induction of CCK release following oral treatment with TLR ligands. Commensal bacteria seem to not affect the basal levels of CCK because no significant differences were observed in mice depleted or not of intestinal commensal microflora by antibiotic treatment (Fig. 4*B*). TLR4$^{-/-}$ and TLR9$^{-/-}$ mice serum levels of CCK were not increased by stimulation with LPS and CpG-ODN, respectively (Fig. 4, *C* and *D*). Since among the three TLR ligands tested LPS is certainly the more active biologically, we evaluated whether the observed increase of CCK levels was still present at lower doses of LPS; using amounts of LPS 10–100 times lower, the CCK levels in the serum were comparable to the one induced by the previously tested doses of LPS (1.02 ± 0.01 ng/ml with LPS 4 mg/kg; 0.99 ± 0.02 ng/ml with LPS 0.4 mg/Kg).

Production of keratinocyte-derived chemokine (KC) and β-defensin 2 by STC-1 cells activated by TLR4 agonist LPS and TLR5 agonist flagellin

Activation of professional and nonprofessional immune cells via TLR4, 5, or 9 agonists has been reported to induce the release of IL-8 in humans and of its functional counterpart, KC, in mice (28). Using the murine chemokine KC as a marker, we tested whether activation of STC-1 cells via TLR induces production of molecules implicated in activation of the innate immune system. Supernatants of STC-1 cells, collected 18 h after stimulation with all three TLR agonists, but not after fatty acids stimulation, were found to contain a significant amount of KC; the KC release was particularly high after LPS and flagellin treatments (Fig. 5*A*). Inhibition experiments confirmed that the release of KC induced by LPS and flagellin was via TLR4 and TLR5. Indeed, using Abs against TLR4 and 5, after 4 h of stimulation with LPS and flagellin, respectively, KC release was significantly inhibited (Fig. 5*B*); no significant inhibition was found after 18 h of stimulation (data not shown).

As noted above, different types of cells in the gut combat bacterial invasion not only by producing cytokines/chemokines to activate and recruit cells of the innate immune system, but also by producing molecules with bactericidal properties, such as defensins. Immunofluorescence analysis with anti-DEFB2 Ab to determine whether stimulation with TLR agonists influences the production of the epithelial defensin molecule by STC-1 cells revealed a basal production of DEFB2 in untreated STC-1 cells (Fig. 6*A*) that was strongly increased after LPS and flagellin treatments (Fig. 6, *B* and *C*), but not after CpG-ODN treatment (Fig. 6*D*). The primary Ab was omitted in the negative control experiment (Fig. 6*E*), while LPS-stimulated keratinocytes NCTC 2544 keratinocytes (29) served as the positive control (Fig. 6, *F* and *G*). Western blot analysis of extracts from STC-1 cells stimulated with LPS and flagellin revealed DEFB2 peptide of the appropriate molecular mass, while no bands were observed in extracted cells treated with CpG-ODN or left untreated (Fig. 6*H*).

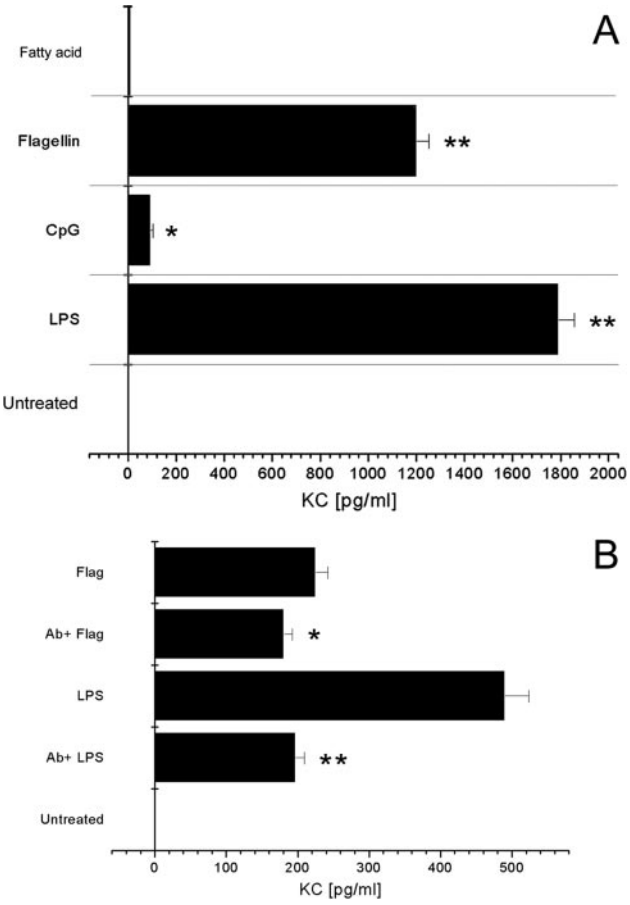

FIGURE 5. *A*, KC release from STC-1 cells stimulated with TLR agonists. Eighteen hours after stimulation with fatty acid, flagellin (Flag), CpG-ODN (CpG), and LPS, supernatants were collected and tested by ELISA. Data are mean ± SEM from three independent experiments (**, $p \leq 0.01$; *, $p \leq 0.05$ vs untreated cells). *B*, Block in KC production from STC-1 cells stimulated with flagellin and LPS by anti-TLR4 and 5 Abs. Abs were added to the cells 30 min before LPS and flagellin stimulation; 4 h after stimulation, supernatants were collected and tested by ELISA (**, $p = 0.0078$ LPS- vs Ab + LPS-treated cells, *, $p = 0.0478$ flagellin vs flagellin-treated cells).

Discussion

Our results clearly indicate that enteroendocrine STC-1 cells express TLR4, TLR5, and TLR9 receptors, which were detectable at both the RNA and protein levels. Immunofluorescence experiments revealed the expression of all three receptors in the cytoplasm, while cellular membrane staining was observed only for TLR5. The intracellular localization, which is normally observed for TLR9 and has been previously described in intestinal cells for TLR4 (30–33), might reflect a restriction of recognition via TLRs to internalized agonists in EECCs. We aimed to colocalize TLRs and EECCs in the murine intestine; however, our immunodetection experiments were not conclusive, since with the available Abs it was not possible to perform immunofluorescence staining on tissue and double immunofluorescence staining with Abs for EECCs detection.

The demonstration of TLR expression by EECCs represents further evidence that endocrine cells can express these receptors. Previous studies have shown that TLR2 is expressed in both mouse and human adrenal glands under basal conditions, and that TLR2-deficient mice are impaired in their ability to release adrenal corticosterone after inflammatory stress induced by bacterial cell wall components (34). TLR4 mRNA expression has been detected in normal and transformed endocrine epithelial pituitary cell types, and LPS appeared to stimulate production of IL-6 by pituitary tumor cells via TLR4 (35).

We observed that STC-1 cells release CCK not only when stimulated with fatty acids, but also when treated with TLR 4, 5, and 9 agonists, while no CCK increase release was associated with the nonspecific inflammatory agent croton oil. MyD88 silencing experiment confirmed the involvement of TLR signaling in TLR agonist-induced CCK production. Moreover, the PKC blocking experiment demonstrated that the PKC is involved in TLR agonist-induced CCK production. Interestingly, production of the proinflammatory cytokine KC was stimulated in EECCs by

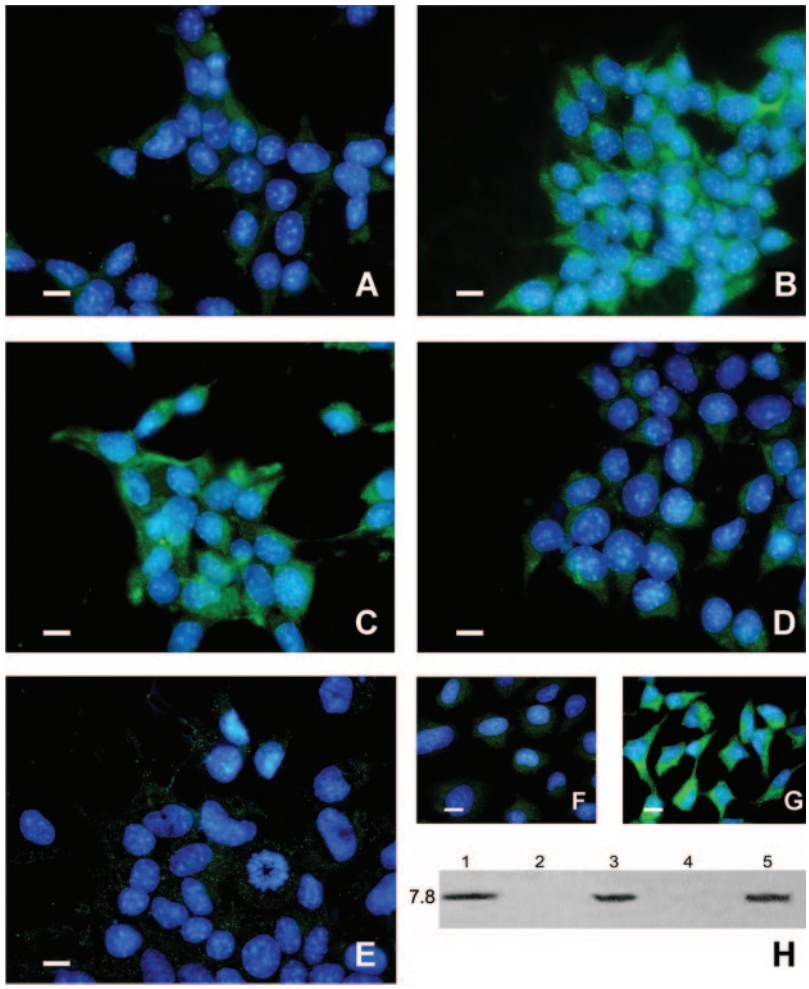

FIGURE 6. β-Defensin 2 expression in STC-1 cells at 18 h after TLR agonist treatments. Double immunofluorescence images show cytoplasmic localization of DEFB2 (green) and DAPI nuclear staining (blue) in STC-1 (*A–E*) and NCTC 2544 cells used as positive control (*F* and *G*). *A*, Untreated; *B*, LPS-treated; *C*, flagellin-treated; *D*, CpG-ODN-treated STC-1 cells (bar, 10 μm) or *E*, stained without primary Ab (bar, 15 μm); *F*, untreated cells; *G*, LPS-treated NCTC cells (bar, 15 μm). (*H*) *Western blot of β-defensin 2 expression*. *Lane 1*, DEFB2 peptide; *lanes 2–5*, extracts from STC-1 cells either untreated or treated with LPS, CpG-ODN, and flagellin, respectively.

the TLR4, 5, and 9 agonists, but not by fatty acids. CpG-ODN was less potent than LPS or flagellin in inducing KC release, consistent with previous studies reporting a weaker inflammatory response after CpG-ODN than after other TLR agonist stimulation (36–39). KC production might activate innate and adaptive immune responses by recruiting neutrophils and APCs (macrophages, dendritic cells), thus contributing to the defense of the gut (40). Moreover, preliminary results indicate that TLR agonist-activated STC-1 cells, releasing soluble molecules and/or by direct cell contact, might induce the expression of activation markers, such as CD80 and CD86, on dendritic cells, suggesting that EECCs cross-talk with dendritic cells, as previously reported for intestinal epithelial cells (41–43).

Immunofluorescence and Western blot analyses indicated that the activation of EECCs via TLR4, 5, and 9 agonists induced the release of the defensin molecule DEFB2, suggesting that EECCs control microbial attack not only indirectly by producing chemokines, but also directly by reducing the presence of bacteria in the gut lumen via up-regulation of antibiotic peptide production. Together, these data indicate that the response of EECCs to pathogen-associated molecular patterns is similar to that of Paneth cells and enterocytes of the same epithelial origin.

The increased CCK levels observed in sera of mice treated orally with TLR4-, TLR5-, and TLR9-specific agonists clearly demonstrated the ability of EECCs in vivo to respond to TLR agonists, since only EECCs are able to release CCK after an oral stimulation. Low CCK levels in TLR4$^{-/-}$ or TLR9$^{-/-}$ mice serum demonstrated the specificity of agonists stimulation. CCK acts to favor the emptying of the small intestine, leading to contraction of the muscular tunica of the distal small intestine and to relaxation of the proximal part of the large intestine (44–49). Thus, it would be hypothesized that the detection of pathogenic bacteria by EECCs in the small intestine, followed by the release of CCK, constitutes a mechanism by which these cells remove the bacteria from the intestine. CCK also stimulates extrinsic vagal afferent neurons, which play a role in the communication between the peripheral immune system and CNS. In particular, CCK, acting via CCK receptors, mediates part of excitatory action of IL-1β on the vagal system, participating in the behavioral responses to infection and inflammation (1, 50).

Together, these data suggest that EECCs are stimulated by bacteria and not only by dietary stimuli. The ability of EECCs to release KC and defensins establishes a functional commonality with other intestinal cells, i.e., Paneth cells and enterocytes. Although the small number of EECCs as compared with enterocytes might render their local release of defensins and proinflammatory molecules only marginally important in the control of pathogens, EECCs, unlike Paneth cells or enterocytes, release hormones such as CCK in response to pathogens. Because the systemic action of these hormones induces the emptying of the distal small intestine by the contraction of the muscular tunica, this releasing might play a crucial role in the control of pathogens in the small intestine.

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Disclosures

The authors have no financial conflict of interest.

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