Appendix
Lysophospholipid sensing triggers secretion of flagellin from pathogenic salmonella

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Flagellin induces inflammatory and innate immune responses through activation of Toll-like receptor 5. Here we show that proinflammatory monomeric flagellin produced by salmonella during infection of intestinal epithelial cells was not derived from polymeric bacterial cell wall-associated flagellum but instead was synthesized and secreted de novo by the bacterium after direct sensing of host-produced lysophospholipids. Inhibition of lysophospholipid biosynthesis in intestinal epithelial cells reduced flagellin production and release from salmonella. Lysophospholipids induced a cAMP-dependent signaling pathway in salmonella that resulted in production and secretion of active flagellin. The induction of Toll-like receptor ligand synthesis and secretion by a host signal represents a previously unknown regulatory mechanism for inflammation and innate immunity during infection with a bacterial pathogen.

The innate immune system functions by recognizing conserved pathogen-associated molecular patterns through many pattern-recognition receptors such as Toll-like receptors (TLRs). In mice there are at least 11 members of the TLR family that recognize conserved components of pathogens such as bacterial lipopolysaccharide (LPS), peptidoglycan, bacterial flagellin, bacterial DNA, viral RNA and other types of ligands. Flagellin is the main protein component of bacterial flagella, which are motility structures known to be essential for the pathogenesis of many gastrointestinal, respiratory and renal tract bacteria. Both in vitro and in vivo studies have shown that flagella facilitate infection of host cells and bacterial colonization and provide important stimuli for eliciting host inflammatory responses.

The flagellin of many bacterial pathogens is a target of antibodies and T cells during infection. However, it is the recognition of flagellin by TLR5 expressed by many cell types, including intestinal epithelial cells (IECs), dendritic cells and macrophages, that is responsible for most host inflammatory responses produced by this molecule. A primary mechanism by which IECs generate inflammatory and innate immune responses after infection with pathogenic salmonella is detection of flagellin by TLR5 (ref. 2). The importance of this mechanism is emphasized by the inability of flagellin-deficient Salmonella typhimurium to generate inflammatory responses from IECs in vitro. A potential flagellin-TLR5-mediated induction of innate immune responses assumes an even greater importance in the gut because expression of the main LPS innate receptor TLR4 is downregulated at this mucosal site. The responses mediated through TLR5 are crucial in recruiting neutrophils, macrophages and dendritic cells to the site of salmonella infection, host cells that are vital to the systemic dissemination of this pathogen.

The capacity to generate inflammatory responses via TLR5 is associated exclusively with monomeric flagellin, as polymers of flagellin (flagella) do not bind to TLR5. Given that the main form of flagellin on the surface of an infectious bacterium is the polymeric flagellum, an important issue concerns the mechanism by which monomeric flagellin is made available to induce TLR5-mediated inflammation. We show here that production and release of biologically active monomeric flagellin occurred de novo by a regulated process activated in salmonella after sensing of host-produced lysophospholipids by the bacterium. Our results therefore characterize a previously unknown mode of regulation of inflammatory and innate immune responses.

RESULTS

Salmonella-IEC interaction promotes flagellin secretion

To understand how flagellin monomers are made available to the innate immune system, we began by analyzing the induction of inflammatory responses from IECs after infection with S. typhi, a common pathogenic salmonella strain. We used the model human IEC line Caco-2 for this study. The absence of a fully competent LPS receptor on this cell line, coupled with its competence for TLR5 signaling, made it a convenient system for analyzing host cell responses to flagellin. We infected Caco-2 cells with live or gentamycin-treated S. typhi and measured production of the chemokine interleukin 8 (IL-8) in the cell supernatants. Induction of IL-8 secretion after infection required metabolically active bacteria, as gentamycin-treated S. typhi did not trigger substantial IL-8 secretion even at a multiplicity of infection 10 times higher than that used with live S. typhi (Supplementary Fig. 1 online). Induction of IL-8 from Caco-2 was also efficiently reproduced by incubation of the cells with lysophospholipids...
Figure 1 Contact with IECs activates release of proinflammatory flagellin from pathogenic salmonella. (a) ELISA for IL-8 secreted by Caco-2 cells in response to activation with supernatants from S. typhi stimulated with Caco-2 cells or with Caco-2-CS. Data are represented as mean ± s.d. of triplicate samples and are representative of three experiments. (b) Immunoblot for flagellin in supernatants from S. typhi V\textsuperscript{i} or S. typhimurium stimulated with Caco-2 cells or Caco-2-CS. Results are representative of three independent experiments. (c) Immunoblot of flagellin released from S. typhimurium during interaction with the human IEC lines HT-29 and T-84. Data are representative of two experiments. RPMI, medium alone.

Figure 2 The host stimulus that induces flagellin release from salmonella is not proteinaceous. (a) Immunoblot for flagellin in supernatants from S. typhi incubated with heat-treated or proteinase K-treated Caco-2-CS. (b) ELISA for IL-8 secreted by Caco-2 cells after activation with supernatants from S. typhi stimulated with untreated Caco-2 cells (Live) or treated Caco-2 cells (Heat; Methanol). (c) Immunoprecipitation and immunoblot for flagellin in supernatants from S. typhi incubated for 1 h without (RPMI) or with (RPMI-10) 10% FCS. (d) ELISA for IL-8 produced by Caco-2 cells incubated with supernatants of S. typhi treated with FCS (concentration, horizontal axis). (e) ELISA for IL-8 produced by Caco-2 cells incubated with supernatants of S. typhi stimulated with 100 μg/ml of BSA or transferrin. Results are representative of three independent experiments. Error bars, mean ± s.d.
activate flagellin secretion from both salmonella strains was not restricted to LPA, as lysophosphatidylcholine (LPC) also stimulated release of flagellin, although LPA was a more potent inducer than LPC. In contrast, incubation of S. typhi with phosphatidic acid or phosphatidylcholine did not result in increased flagellin secretion above background, suggesting that the stimulatory activity was specific to lysophospholipids (Fig. 3c; response with phosphatidic acid similar to that with phosphatidylcholine). At the concentrations used, incubation with LPA or LPC did not affect bacterial growth or viability (data not shown).

To provide some indication that binding of LPA to salmonella occurred via a specific receptor, we did a limited competitive inhibition study in which membrane components from one strain of salmonella were used to compete for binding to exogenously added LPA. LPA-mediated release of flagellin from S. typhi was inhibited when the lipid was preincubated with protein extract derived from S. typhimurium membrane (Supplementary Fig. 2 online); however, there was no inhibition when the lipid was preincubated with S. typhimurium cytosol. These data support the hypothesis that salmonella strains express a membrane-associated receptor that can bind to lysophospholipids.

Blocking lysophospholipid production reduces flagellin secretion
After our demonstration that lysophospholipids could activate release of flagellin from two pathogenic salmonella strains, the next important issue was whether LPA, LPC or a related lysophospholipid was also the active component in Caco-2-mediated flagellin secretion. Lysophospholipids are generated in cells after hydrolysis of phospholipids by the phospholipase A₂ (PLA₂) class of enzymes, which include the calcium-dependent secretory PLA₂ (sPLA₂), calcium-dependent cytosolic PLA₂ (cPLA₂) and calcium-independent cell-associated PLA₂ (iPLA₂) enzymes. We therefore assessed the function of these enzymes in the capacity of Caco-2 cells to stimulate the release of flagellin from S. typhi. We pretreated cells with specific PLA₂ inhibitors (prostaglandin B₂ to inhibit sPLA₂ (ref. 29), arachidonoyl trifluoro-methylketone to inhibit cPLA₂ (ref. 30) and bromoeno-lactone to inhibit iPLA₂ (ref. 31), before infecting the cells with S. typhi and analyzing the supernatants for the presence of flagellin. At the same time, we also analyzed Caco-2-CS obtained from cells treated with the inhibitors for its capacity to stimulate secretion of flagellin from S. typhi. Inhibition of iPLA₂ abrogated the capacity of Caco-2 cells to stimulate release of flagellin, whereas inhibition of sPLA₂ suppressed the ability of Caco-2-CS to trigger secretion of flagellin (Fig. 4a).

Treatment with cPLA₂ inhibitor did not affect Caco-2-induced secretion of flagellin. As a control for specificity, these inhibitors did not inhibit release of LPS from S. typhi (data not shown). Therefore, lysophospholipids produced by iPLA₂ and or sPLA₂ in Caco-2 cells could stimulate S. typhi to secrete flagellin.

Consistent with those findings, we were also able to demonstrate the presence of LPC by thin-layer chromatography in culture supernatants derived from Caco-2 cells grown in serum-free conditions (Fig. 4b). In extraction conditions that normally lead to more recovery of LPA than LPC, we detected no LPA in Caco-2-CS supernatants. Instead, the supernatants contained LPC, indicating that LPC was the principal lysophospholipid contributing to the flagellin-inducing activity of Caco-2-CS. We confirmed the identity of LPC as the active molecule by staining the thin-layer chromatography plate with Dragendorf reagent, which specifically stains choline-containing lipids (data not shown). More notably, mass spectrometry of the LPC spot extracted from silica showed it was an oleoyl derivative of LPC (Supplementary Fig. 3 online). To rule out the possibility that LPC was released because of induction of apoptosis in Caco-2 cells when grown in serum-free conditions, we stained the cells with annexin V and propidium iodide, which showed they were not undergoing apoptosis (data not shown). The data were all consistent with the conclusion that the Caco-2 cells constitutively secrete LPC.

The iPLA₂-dependent secretion of flagellin in vivo
To establish that host signal-dependent secretion of flagellin from salmonella was relevant during infection of IECs in vivo, we analyzed the release of flagellin from S. typhimurium after interaction with intestinal cells ex vivo and in a ligated ileal loop model in vivo. Salmonella released flagellin during interaction with intestinal cells, which was inhibited when cells were preincubated with the iPLA₂ inhibitors.
secretion from Caco-2 cells infected with gentamycin-treated S. typhi (Supplementary Fig. 1). Finally, we confirmed induction of flagellin transcription in response to activation with lysophospholipids by analyzing expression of β-galactosidase by a strain of S. typhimurium carrying a promoterless Lac operon fused to the fliC promoter. Incubation of these bacteria with lysophospholipids, Caco-2 cells, Caco-2-CS or mouse intestinal explants led to a substantial increase in intracellular β-galactosidase, suggesting that the host stimuli could activate transcription from the fliC promoter (Fig. 6c). The increase in β-galactosidase after activation of bacteria with those stimuli was also associated with release of very small amounts of this enzyme into the supernatant (data not shown), indicating that fliC-driven expression of β-galactosidase might also result in the (inefficient) export of this enzyme through the flagellar export system.

Flagellin secretion occurs via cAMP-dependent signaling

To gain further insight into the mechanism of lysophospholipid-mediated induction of flagellin expression and secretion, we analyzed the involvement of cyclic AMP (cAMP) in this process. Both cAMP and cAMP receptor protein are known to regulate expression of flagellin and biosynthesis of flagella in salmonella. S. typhimurium strains lacking adenylyl cyclase or cAMP receptor protein are non-motile. To investigate the involvement of cAMP in LPC-induced flagellin secretion, we incubated bacteria with glucose before activating them with LPC. Glucose decreases cAMP in bacteria, a phenomenon commonly referred to as “catabolite repression.” Prior incubation with this sugar reduced the ability of S. typhi to secrete flagellin in response to activation with LPC, suggesting involvement of intracellular cAMP in LPC-mediated flagellin release. Consistent with this mechanism, the inhibitory effect of glucose on flagellin secretion was reversed when cAMP was provided during activation of S. typhi with LPC (Fig. 7a). However, there was no detectable flagellin secretion in the presence of cAMP alone. The involvement of cAMP in LPC-induced secretion of flagellin was also demonstrated by analysis of an adenylate cyclase-mutant strain of S. typhimurium (Δcya-27). Unexpectedly, this strain did secrete flagellin in response to LPC, but the amount secreted was very low. However, flagellin secretion was increased when cAMP was provided at the time of stimulation with the lipid (Fig. 7b). The electrophoretic mobility of flagellin released by this mutant strain of S. typhimurium was slightly different from that of

Figure 5 Inhibition of iPLA2 ex vivo or in vivo reduces the ability C57Bl/6 intestinal cells to trigger flagellin from S. typhimurium. (a) Immunoblot for flagellin in supernatants of C57Bl/6 intestinal cells treated with bromoethyl lactone for 15 h before incubation with S. typhimurium. (b) Immunoblot for flagellin in supernatants of the contents of ligated ileal loops of C57Bl/6 mice fed bromoethyl lactone or vehicle for 20 h and then infected with S. typhimurium SL1344. Bottom, the blot was stripped with a solution of low pH and was reprobed with antibody to LPS (012). Data are representative of two independent experiments.

Flagellin release involves active signaling, not flagellar shearing

The results reported above showed that interaction with host-derived lysophospholipids could activate release of flagellin from salmonella. We needed to establish that this release was an active response to sensing of a host signal rather than a result of depolymerization or shearing of flagella into monomeric flagellin. To address this issue, we determined that incubation of S. typhimurium with LPC did not reduce flagellar density, as demonstrated by binding of monoclonal antibody to flagellin to intact bacteria; in fact, there was a modest increase in flagellin expression on bacteria treated with LPC (Supplementary Fig. 4 online). In addition, analysis of flagellar in the cytosol and supernatant of bacteria showed that LPC and Caco-2-CS induced expression of flagellin in the cytosol that was associated with its concomitant release in the supernatant (Fig. 6a), a finding consistent with the idea that translation of flagellin in salmonella is coupled to its secretion. LPC or Caco-2-CS did not modulate release of the TLR4 ligand LPS from salmonella, indicating that lysophospholipid sensing by this pathogen was specifically activating the expression of flagellin. Secretion of flagellin was considerably reduced in the presence of gentamycin, an inhibitor of bacterial protein synthesis, which indicated that the induction of flagellin release required new protein synthesis (Fig. 6b), a result consistent with the absence of IL-8

Figure 6 De novo expression of flagellin stimulated by host lysophospholipids. (a) Immunoblot for flagellin in S. typhi supernatants (top) and cytosolic lysates (bottom) at 10 and 60 min after stimulation with LPC (100 µM) or Caco-2-CS. Blots were stripped with a solution of low pH and reprobed with monoclonal antibody to S. typhi LPS. (b) Immunoblot for flagellin in supernatants of gentamycintreated or live S. typhi after activation with Caco-2-CS, LPC or LPA (200 µM). (c) Activity of β-galactosidase (β-gal) in an S. typhimurium strain containing the fliC promoter region fused to a promoterless lacZ, activated with various stimuli (horizontal axes). Error bars, mean ± s.d. (data in arbitrary units). Results are representative of three independent experiments.
the wild-type strain (for unknown reasons). Unlike the Δcyta-27 strain, however, a Δcyta-27-Δacr-27 strain of S. typhimurium (lacking both adenylate cyclase and the cAMP receptor protein) did not demonstrate any detectable flagellin secretion after incubation with LPC or cAMP (data not shown).

**DISCUSSION**

Early inflammatory and innate immune responses during microbial infection are initiated after recognition of conserved molecular patterns by TLRs. These responses constitute an essential defense mechanism against pathogenic microbes. TLR5 recognizes bacterial flagellin, the monomeric component of the flagellar filament. Flagellin is important in generating inflammatory and innate immune responses from IECs during infection with salmonella and many other bacterial pathogens. A unique feature of the flagellin-TLR5 interaction is that signaling through the receptor can be initiated only by monomeric flagellin because the receptor recognition domain is 'hidden' in the multimeric complex flagellum, the main form of flagellin in bacteria. Here we have identified a mechanism by which biologically active flagellin is produced from pathogenic salmonella through a host-dependent process. One consequence of this for the host is that the secreted flagellin can then be sensed by the innate pattern-recognition receptors to activate the innate immune system. The data provided here have shown that flagellin was not derived from shearing or depolymerization of flagella present on the surface of the bacterium but instead was newly synthesized and secreted after contact of the pathogen with host cells. We have identified the host molecules capable of bringing about release of flagellin from salmonella as lysophospholipids by three independent but complementary approaches. First, purified lysophospholipids such as LPA and LPC efficiently activated flagellin secretion from two strains of salmonella; second, LPC was the main lysophospholipid in Caco-2-CS and third, inhibition of iPLA₂ or SPLA₂ enzymes responsible for generating lysophospholipids, abrogated the ability of cells to stimulate flagellin secretion. The iPLA₂-dependent release of flagellin occurred not only with a model human IEC line but also with normal mouse intestinal cells, indicating the physiological importance of the phenomenon.

Our results assign a new function to lysophospholipids and suggest that in addition to modulating adaptive immune responses through activation of specific G protein-coupled receptors on immune cells, these molecules might have a vital function in regulating inflammatory and innate immune responses by directly activating expression and secretion of a key bacterial TLR ligand. Increased PLA₂ has been reported in sera of typhoid patients; this could lead to increased production of lysophospholipids and consequently to propagation of inflammatory and or innate immune responses during infection with S. typhi. It is notable that these host-derived lipids engage a cAMP-dependent cascade of intracellular signaling in salmonella, a pathway known to be important in transcription of the flagellar regulon. The molecular details of the lipid sensor--transduced, intracellular signaling pathway will become clear once the lipid sensor is identified. The sensor could possibly be a two-component bacterial sensor or a G protein-coupled receptor; LPA and LPC are recognized by G protein-coupled receptors in mammalian cells.

In summary, our study has demonstrated a new mode of regulation of inflammatory and innate immune responses during infection with a bacterial pathogen. Activation of flagellin secretion from salmonella after interaction with IECs could, through induction of inflammatory mediators and consequently through recruitment of inflammatory cells to the site of infection, promote systemic dissemination of this pathogen. It may also, as suggested before, promote development of TH type 2 responses, a modulation that could favor establishment of infection with salmonella. In contrast to upregulation of flagellin during interaction with IECs, salmonella is believed to downregulate expression of flagellin when it establishes itself in macrophages; a response considered to be one of the immune-evading mechanisms used by this pathogen because flagellin is also a dominant target of T cells during infection of mice with S. typhimurium. Therefore, salmonella (and perhaps other bacterial pathogens) seems to have evolved sensory mechanisms by which, depending on the requirement, it can respond to the host environment in different ways at different stages of infection. Our results suggest that during infection of the gut with salmonella, sensing of host lysophospholipids serves as a key signal for activating release of flagellin from this pathogen. Given that flagellin is a potent mediator of innate and inflammatory responses in vitro and in vivo, and that it can modulate the suppressive function of human T regulatory cells in vitro, our findings have important implications for inflammation and immunity.

**METHODS**

Cell lines, bacterial strains and other reagents. The human IEC lines Caco-2, HT-29 and T-84 (American Type Culture Collection) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (RPMI-10) in a humidified atmosphere of 5% CO₂ at 37 °C. S. typhi (Vi-positive and Vi-negative isolates) and S. typhimurium were provided by G. Mehta (Lady Hardinge Medical College, New Delhi, India), S. typhimuriumSL1344 was provided by E. Charpentier (University of Vienna, Vienna, Austria). The S. typhimurium strain carrying a transcriptional fusion of βIC and lac operon was obtained from K. Kutsukake (University of Tokyo, Tokyo, Japan). The strains of S. typhimurium UK-1 (wild-type, adenylate cyclase mutant Δcyta-27) and adenylate cyclase--cAMP receptor protein double-mutant (Δcyta-27 Δacr-27) were made available by R. Curtiss (Arizona State University, Phoenix, Arizona). Bacteria were grown in Luria Bertani (LB) broth at 37 °C with shaking (200 r.p.m.). We obtained 18:1 LPA, 18:1 phosphatidic acid, 18:1 LPC and 18:1 phosphatidylcholine from Avanti Polar Lipids (18:1 indicates the 18-carbon unsaturated oleyl derivative of the lipid). Monoclonal antibodies to S. typhi flagellin and S. typhi LPS have been described. Rabbit antibody to S. typhimurium flagellin was purchased from BD Laboratories. Bromoelol lactone, arachidonoyl trifluoromethyl ketone and prostaglandin B₂ were purchased from Cayman Laboratories. Adenosine 5'-CAMP was obtained from Sigma-Aldrich.

Activation of salmonella with stimuli. S. typhi freshly grown in LB broth were washed with and resuspended in serum-free RPMI 1640 medium. Bacterial
numbers were determined by measurement of absorbance at 630 nm. Bacteria were incubated with Caco-2 cells at a pathogen/cell ratio of 100:1 or were stimulated with Caco-2-CS. Caco-2-CS was derived from Caco-2 cells grown 24 h for 10–15 h in serum-free conditions at a density of 1 x 10^6 to 2 x 10^6 cells/ml. Salmonella was also stimulated with varying concentrations of FCS, LPA, phosphatidic acid, LPC or phosphatidylycholine. LPA was found to precipitate in RPMI 1640 medium, possibly because of association with divalent cations such as Ca^2+ and Mg^2+. Therefore, in some experiments, bacterial stimulation with lipo- polysaccharide was done in PBS. Bacteria (2 x 10^6 suspended in 1 ml buffer) were incubated for 1 h at 37 °C with various bacterial suspensions were centrifuged at 13,000g for 5 min and the supernatants were filtered through a 0.22-μm membrane to remove bacteria and or any cellular debris. In some experiments, bacteria were treated for 1 h with gentamycine (100 μg/ml) before being incubated with various stimuli. For study of LPC-induced flagellin release, S. typhi was treated for 15 min with 100 mM (+)-glucosamine, followed by incubation for 10 min at 37 °C with LPC in the presence of 5 mM Ca^2+. In addition, wild-type S. typhimurium UK-1 and Agya-27 S. typhimurium UK-1 were incubated for 10 min at 37 °C with LPC in presence or absence of 5 mM Ca^2+ and the supernatants were analyzed for the presence of flagellin.

Preparation of bacterial membranes and cytosol. Salmonella were grown in LB medium and were washed and resuspended in PBS. The bacterial suspension was sonicated and then was spun at 13,000g to remove debris. The supernatant was centrifuged at 100,000g for 2 h at 4 °C to obtain bacterial membranes (pellet) and cytosol (supernatant).

Caco-2 treatment. Caco-2 cells were recovered from 75-cm^2 tissue culture flasks after treatment with trypsin-EDTA and were washed and resuspended in serum-free RPMI 1640 medium. Cells were seeded in a 24-well tissue culture plate at a density of 5 x 10^5 cells/well or in a 6-well plate at a density of 2 x 10^6 cells/well and were incubated at 37 °C. Cells were left untreated or were subjected to methanol fixation for 5 min or to heat inactivation at 80 °C for 1 h. Caco-2-CS was digested for 1 h at 37 °C with 1 μg/ml of proteinase K, followed by heat treatment at 80 °C for 45 min to inactivate proteinase K. In some experiments, cells were treated with 100 μM arachidonyl trifluoromethylketone, 20 μM prostataglandin B1, or bromoelone lactone (used at a concentration of 40 μM from a stock in ethanol or 10 μM from a stock in dimethyl sulfoxide, in which it was more readily soluble) for 10 h at 37 °C in an atmosphere of 5% CO_2. After inhibitor treatment, cells were washed thoroughly and were left in serum-free RPMI 1640 medium for another 3 h. S. typhi (2 x 10^6 bacteria/ml) were incubated for 1 h with inhibitor-treated cells as well as 3-hour culture supernatants from these cells and flagellin in the supernatants was analyzed by immunoblot.

Intestinal cells and ligated intestinal loop assays. Experiments with mice were done according to the guidelines provided by the Institutional Animal Ethical Committee of the National Institute of Immunology (New Delhi, India). Small intestines were removed from C57BL/6 mice and were washed with PBS and then the mucus was removed with 1 mM dithiothreitol. The intestine was washed repeatedly with PBS to remove all traces of dithiothreitol, was filled with RPMI medium, was tied at the ends and was incubated for 1 h at 25 °C in RPMI medium. Intestinal cells were flushed out with fresh medium and were seeded at a density of 2 x 10^6 cells/well on a six-well plate. Cells were treated with 5 μM bromoelone lactone for 15 min at 37 °C in an atmosphere of 5% CO_2. The inhibitor was washed off and cells were infected for 1 h with 2 x 10^6 S. typhimurium in 1 ml RPMI medium.

For experiments with ligated intestinal loops, mice were starved for 8 h before they were fed 10 μM bromoelone lactone in water for 20 h. Mice were anesthetized by intraperitoneal injection of a ketamine-xylazine mixture and were kept anesthetized for the duration of the experiment. A ileal loop 3–3.5 cm in length from control mice or mice fed bromoelone lactone was injected with 2 x 10^6 S. typhimurium SL1344 in 300 μl PBS. At 30 min after injection, the contents of the loop were collected and were centrifuged at 13,000g to pellet bacteria and cellular debris. Flagellin in supernatants was analyzed by immunoblot.

Immunoprecipitation and immunoblot analysis. The presence of flagellin in supernatants and cytosol derived from S. typhi activated with various stimuli was assessed by immunoblot. Supernatants from bacteria were first immunoprecipitated with monoclonal antibody and then were analyzed by immunoblot. Supernatants were treated with protein G-Sepharose beads preloaded with antibody to washed with PBS containing 0.5% Nonidet-P40 and were blocked with Laemmli sample buffer (nonreducing). Samples were separated on PAGE, were transferred to nitrocellulose and were blotted with anti-flagellin. Reactive bands were visualized with Enhanced Chemiluminescence Reagent (Amersham Pharmacia Biotech). For detection of LPC, blots were stripped with a solution of low pH (0.1 M NaOH containing acetic acid, and were reprobed with monoclonal antibody to S. typhi LPC.

IL-8 enzyme-linked immunosorbent assay (ELISA). Filter-sterilized supernatants obtained from bacteria activated with various stimuli were added in triplicate to Caco-2 monolayers in a 96-well plate (100 μg/ml supernatant plus 100 μM LPC-10 per well) and were incubated for 5 h at 37 °C in an atmosphere of 5% CO_2. Supernatants were collected and assayed for IL-8 by a commercially available ELISA kit (Opt EIA Human IL-8 Set; BD Pharmingen).

Flagellar density on Salmonella. S. typhi incubated with various concentrations of LPC for 1 h were pelleted at 13,000g for 5 min and were incubated with monoclonal antibody to S. typhi flagellin. Bacteria were washed with PBS containing 1% BSA and were incubated with horseradish peroxidase-conjugated antibodies to mouse immunoglobulin. Enzyme activity was assessed with a freshly prepared substrate solution containing H_2O_2 and O-phenylenediamine, and absorbance at 490 nm was measured after reactions were stopped with 2 N H_2SO_4.

β-galactosidase assay. The S. typhimurium strain containing the lacZ (4 x 10^6 bacteria/ml) was activated for 10 min at 37 °C with various stimuli (LPA, LPC, Caco-2 cells, Caco-2-CS and 1-cm intestinal fragments from C57BL/6 mice). Bacterial suspensions were centrifuged at 13,000g for 5 min. Pellets were resuspended in 1 ml PBS and sonicated and the debris were pelleted at 13,000g. The β-galactosidase activity in the supernatants was assayed by a colorimetric assay with chlorphenol red β-D-galactoside as the substrate, and absorbance at 570 nm was measured.

Caco-2-CS lipid analysis. Caco-2-CS was filtered to remove any cellular debris and was dried by lyophilization. Lyosphospholipids were extracted as described. The lyophilized cell supernatant was reconstituted in buffer containing 30 mM citric acid and 40 mM Na_2HPO_4, pH 4. For extraction of lipids, 4 ml of 1-butanol and 2 ml of water-saturated 1-butanol were added to 1.5 ml reconstituted supernatant and the mixture was vortexed vigorously. After phase separation, the organic extracts were pooled and were dried by lyophilization. Lipids were dissolved in chloroform and were separated by thin-layer chromatography, alongside LPA and LPC standards, with chloroform/methanol/distilled water/25% ammonia (32:5:15:2, volume/volume) as the mobile phase. Spots were detected after exposure to iodine vapor or were stained with Dragendorf reagent as described. The spot corresponding to LPC was scraped off, was extracted by sonication from the silica gel in chloroform/methanol/water (4:4:1, volume/volume) and was analyzed by electrospray-ionization mass spectrometry.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.