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Protection from Intestinal Inflammation by Bacterial Exopolysaccharides

Sara E. Jones,* Mallory L. Paynich,* Daniel B. Kearns,† and Katherine L. Knight*

Host inflammatory responses against pathogenic organisms can be abrogated by commensals; however, the molecular mechanisms by which pathogenesis is prevented are still poorly understood. Previous studies demonstrated that administration of a single dose of *Bacillus subtilis* prevented disease and inflammation by the enteric mouse pathogen *Citrobacter rodentium*, which causes disease similar to the human pathogen enteropathogenic *Escherichia coli*. No protection was observed when an exopolysaccharide (EPS)-deficient mutant of *B. subtilis* was used, suggesting that EPS are the protective factor. In this study, we isolated and characterized EPS and showed that they also prevent *C. rodentium*-associated intestinal disease after a single injection. Protection is TLR4 dependent because EPS-treated TLR4 knockout mice developed disease. Furthermore, protection could be conveyed to wild-type mice by adoptive transfer of macrophage-rich peritoneal cells from EPS-treated mice. We found that EPS specifically bind peritoneal macrophages, and because mice lacking MyD88 signaling in myeloid cells were not protected by EPS, we conclude that bacterial EPS prevent colitis in a TLR4-dependent manner that requires myeloid cells. These studies provide a simple means of preventing intestinal inflammation caused by enteric pathogens. The Journal of Immunology, 2014, 192: 000–000.

The gastrointestinal microbiota contributes to the development and maintenance of the host immune system. One benefit of a healthy microbiota is protection from colitis induced by enteric pathogens as well as by inflammatory agents such as dextran sulfate or 2,4,6-trinitrobenzene sulfonic acid (1–3). Although much work has been done to identify specific bacteria that prevent colitis, many questions remain about the mechanisms by which these bacteria elicit a protective response. We previously showed that a single oral dose of *Bacillus subtilis* protects mice from disease induced by the enteric pathogen *Citrobacter rodentium* (4), which shares many characteristics with the human pathogen enteropathogenic *Escherichia coli*. Symptoms of infection include diarrhea, systemic increases in proinflammatory cytokines, and altered colonic architecture, such as crypt hyperplasia, goblet cell depletion, and infiltration of immune cells, including neutrophils and T cells. However, mice administered *B. subtilis* in addition to *C. rodentium* display no evidence of diarrhea, have normal levels of proinflammatory cytokines, and normal colonic architecture (4).

During infection, *C. rodentium* disrupts the intestinal barrier (5), resulting in translocation of luminal contents and activation of the host pattern recognition receptors, which include TLRs. TLRs recognize conserved motifs of microbial proteins (e.g., flagella), lipids (e.g., LPS), and nucleic acids (e.g., CpG) as well as host danger-associated molecular patterns (6). Activation of TLRs results in translocation of NF-κB to the nucleus, production of chemokines and cytokines, and ultimately recruitment of immune cells to the site of infection (6). This inflammatory cascade is needed to clear the pathogen, but it also damages the host tissues (7–9). For example, MyD88 knockout (KO) mice do not develop colonic hyperplasia or recruit neutrophils but succumb to infection. In contrast, most immunocompetent strains of mice clear *C. rodentium* 3–4 wk postinfection.

*B. subtilis* is a Gram-positive spore-forming bacterium present in the gastrointestinal tract of both humans and mice (10, 11). Several groups report that select probiotic strains of *B. subtilis* relieve the symptoms associated with antibiotic-associated diarrhea and irritable bowel syndrome in human patients; however the mechanisms of protection have not been well established (10, 11). In a previous study, we showed that an exopolysaccharide (EPS) mutant failed to prevent *C. rodentium*-associated disease, suggesting that EPS are the bacterial components mediating protection (4). EPS are secreted heterogeneous structures composed primarily of carbohydrates that not only sometimes coat bacteria but are major components of the biofilm matrix (12). The role of EPS during pathogen infection is well appreciated. For example, pathogenic *Staphylococcus aureus* are coated with an EPS-containing capsule that prevents phagocytosis and allows adherence of the bacteria to host tissues and subsequent immune evasion (13). Less understood is the role of bacterial EPS during probiosis. EPS may be important for probiotic or commensal organisms to establish and maintain an intestinal niche that could prevent pathogen colonization. Alternatively, gut metabolism of EPS could contribute to short chain fatty acid synthesis, a process that regulates intestinal permeability (14). Interestingly, a few groups have demonstrated that EPS suppress disease by modulating the host inflammatory response via TLR2 signaling (1, 2). Collectively, these studies suggest that bacterial EPS, such as those produced by *B. subtilis*, could prevent intestinal disease using one or more of several different mechanisms, including alteration of pathogen colonization, reduction of gut permeability, and immunomodulation of the host response. We show in this study that *B. subtilis* treatment did not alter pathogen colonization nor prevent disruption of the...
epithelium, but instead, protection by *B. subtilis* EPS is a result of host immune modulation. After purifying EPS and showing that they mediate protection, we identified host immune cells that bind EPS and further showed that protection requires TLR4 and MyD88-signaling myeloid cells. Furthermore, cells from wild-type (wt) and TLR4 KO mice were adaptively transferred to naive wt mice to test whether these cells conveyed protection from enteric disease caused by *C. rodentium* and to identify which cells use TLR4. These studies identify bacterial polysaccharides, which after a single injection, have the capacity to prevent colitis in an infectious disease model in a TLR4-dependent manner.

### Materials and Methods

#### Reagents and mice

Anti-F4/80 (clone BM8) and anti-CD11b (clone M1/70) were obtained from BioLegend (San Diego, CA); donkey anti-rabbit Ig was obtained from The Jackson Laboratory (Bar Harbor, ME). All other reagents were purchased from Sigma-Aldrich unless otherwise noted. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Usage Committee at Loyola University Medical Center (Maywood, IL). Specific pathogen–free C57BL/6, MyD88 KO, and TLR4 KO founders were purchased from The Jackson Laboratory. Mice lacking MyD88 in myeloid cells and epithelial cells were generated by crossing a Lyz2-Cre or Villin-Cre transgenic mouse, respectively, to a MyD88 floxed mouse as described previously (15). Mice used for these experiments (4–8 wk of age) were bred at Loyola University Chicago. Sterile standard chow and tap water were given to mice ad libitum.

#### Bacterial and spore preparation

*wt B. subtilis* 3610 spores were germinated via exhaustion as described previously (4). On the day of administration, *B. subtilis* spores were washed with ice-cold water, resuspended in 100 μl PBS, and administered to mice via oral gavage. For infection studies, *C. rodentium* ATCC 51549 was cultured for 16 h in Luria–Bertani medium and washed once in PBS, and an infectious dose was resuspended in 100 μl sterile PBS for administration to mice by oral gavage. MyD88 KO and epithelial MyD88-deficient mice received 10^15 CFUs; all other mouse strains received 5 × 10^14 CFUs pathogen.

#### In vivo imaging of *C. rodentium*

As previously described (5), *C. rodentium* ICC180 (*C. rodentium* lux+) was grown overnight at 37°C in Luria–Bertani medium and orally gavaged into C57BL/6 mice (5 × 10^10 CFU/mouse). Assessment of bioluminescence (photons s^-1 cm^-2 sr^-1) in living animals was measured using the IVIS100 system (Xenogen, Alameda, CA). A photograph (grayscale reference image) was taken under low illumination prior to quantification of photons emitted from *C. rodentium* ICC180 (medium binning, 5-min exposure) using the software program Living Image (Xenogen). A pseudocolor heat map image representing light intensity (blue [least intense] to red [most intense]) was generated using Living Image software and superimposed over the grayscale reference image.

#### *C. rodentium* colonization

*C. rodentium* colonization was assessed in fresh fecal samples homogenized in 500 μl sterile 20% glycerol in PBS. For mucosal studies, colonic fecal contents were removed and the tissue flushed with sterile PBS. The colon was homogenized in 2 ml sterile 20% glycerol in PBS. Serial dilutions were cultured on selective MacConkey plates for 16 h at 37°C; only colonies that displayed the characteristic pink center surrounded by a white rim (*C. rodentium*) were counted. Colonization was calculated and expressed as CFUs per gram feces.

#### Exopolysaccharide preparation

Exopolysaccharides were isolated from *B. subtilis* DS991 (sinRtasA mutant), a strain that produces and secretes large amounts of EPS; material from this strain is designated EPS+ (16). As a control, we used DS5187 (*sinRtasAspeekH* mutant), a strain that does not produce EPS (16) and material from this strain is referred to as EPS−. EPS were isolated as described previously (16). Briefly, stationary phase supernatants were mixed with an equal volume of 100% EtOH at 4°C for 90 min to precipitate the EPS. The precipitant was pelleted (15,000 × g, 4°C, 20 min), washed in PBS, and resuspended in 0.1 M Tris. Samples were digested with DNase (67 μg/ml) and RNase (330 μg/ml) at 37°C; after 1 h, proteinase K (40 μg/ml) was added, and samples were incubated at 55°C for 1 h. EPS was EtOH precipitated, resuspended in 0.1 M Tris (pH 8), and further purified by gel filtration on an S1000 column in 0.1 M Tris (pH 8) and then desalted by dialysis. EPS was quantified by a colorimetric phenol sulfuric acid assay using serial dilutions of fructose as standard (17). Sample purity was assessed by immunoelectrophoresis and Western blot analysis using anti-EPS antiserum.

#### Composition and linkage analysis of EPS

These analyses were performed at the Complex Carbohydrate Research Center (University of Georgia) (18). Gas chromatography/mass spectrometry analysis of per-O-trimethylsilyl derivatives of the monosaccharide methyl glycosides was performed on an Agilent 7890A GC interfaced to a 5975C MSD, using an Agilent DB-1 fused silica capillary column (30 m × 0.25 mm ID) and linkages were determined on an Agilent 7890A GC interfaced to a 5975C MSD (mass selective detector, electron impact ionization mode); separation was performed on a Supelco 2380 fused silica capillary column (30 m × 0.25 mm ID).

#### Generation of EPS-specific Abs

A New Zealand White rabbit was immunized by i.m. and s.c. injection of 100 μg EPS in TiterMax Gold adjuvant. Three weeks post primary immunization, the rabbit was boosted with 100 μg EPS in adjuvant. Eight days later, serum was collected. Ab to EPS was detected by Western blot analysis using donkey anti-rabbit (HiLJ-HRP (The Jackson Laboratory)) Abs as secondary Ab and an immunoelectrophoresis followed by staining with Coomassie brilliant blue to visualize Ag/Ab arcs of precipitation.

#### Study design

*B. subtilis* spores (10^10 in 100 μl PBS, orally administered) or 200 μl EPS (1 mM in 0.1 M Tris, i.p.) or hyaluronic acid (PBS, i.p.) were administered to mice 24 h prior to infection with *C. rodentium* by oral gavage. Age- and gender-matched mice were used for each experiment. To assess disease, all mice were euthanized 10 or 11 d postinfection (dpi) and tissues were collected, except for the MyD88 KO mice, which were euthanized 9 dpi. These days were chosen because at these times the pathogen is well established in each strain and colitis is evident (7–9). Before euthanization, blood was collected. Serum keratinocyte-derived cytokine (KC) levels were assessed by ELISA (R&D Systems, Minneapolis, MN). To assess diarrhea, feces were examined and scored 1–4 (19): 1, no diarrhea (hard, dry pellets); 2, slightly soft stool (mild diarrhea); 3, very soft stool (moderate diarrhea); and 4, unformed stool (severe diarrhea). Distal colons were collected and processed for histological analysis as follows: colons were fixed overnight in 10% formalin-buffered phosphate, dehydrated through an alcohol gradient, cleared with xylene, and infiltrated with paraffin. Tissues were sectioned longitudinally at 4 μm and stained with H&E. Epithelial hyperplasia in the distal colon was determined from images of each colon taken with a Leica DM IRB microscope equipped with MagnaFire charge-coupled device camera as described previously (20). Five well-oriented crypt heights/mouse were measured from two to three regions.

#### Assessment of EPS binding to cells

Peritoneal cells were obtained from mice (4–6 wk of age) injected i.p. with 5 ml DMEM (10% FBS). After lysing RBCs, cells were incubated with EPS, washed, and then incubated with anti-F4/80 (clone BM8), anti-CD11b (clone M1/70), or anti-EPS, followed by donkey anti-rabbit Ig as secondary Ab. Fluorescence intensity was assessed by flow cytometry.

#### Assessment of EPS-induced cytokine production

Peritoneal cells were obtained from euthanized mice (4–6 wk of age) injected i.p. with 5 ml DMEM (10% FBS). After lysing RBCs, cells were incubated with EPS (5, 15, or 30 μg/ml), LPS (100 ng/ml), or Pam3Cysk (100 ng/ml), and supernatant was collected at 2 and 6 h for measurement of KC and TNF-α, respectively, by ELISA. As a control, the same volume of material from the non–EPS-producing strain was used.

#### Transfer studies

Peritoneal cells were isolated from mice (4–6 wk of age) injected i.p. with 5 ml DMEM (10% FBS) 2 to 3 d posttreatment with EPS (i.p.). Cells (6 × 10^6) were injected (300 μl, i.p.) into naive mice (4–6 wk of age) at i +, i−, and i− with *C. rodentium*.

#### Statistical analysis

All experiments were performed a minimum of three times and analyzed using the Student *t* test. Error bars denote SEM. Differences were considered statistically significant if *p* < 0.05.
Results

Effect of B. subtilis on C. rodentium colonization and pathogen-induced gut leakiness

*B. subtilis* could prevent disease by altering pathogen adherence and/or colonization, by maintaining epithelial barrier integrity, or by changing the host inflammatory response. To test whether pathogen colonization was altered in the presence of *B. subtilis*, we performed in vivo imaging using lux⁺ *C. rodentium* as well as traditional plating techniques. Mice were orally administered *B. subtilis* (10⁸ CFU), followed 24 h later by *C. rodentium* (5 × 10⁸ CFU), and we detected the lux⁺ *C. rodentium* during the course of disease using an in vivo imaging system. We found that administration of *B. subtilis* did not change the localization or quantity of luminescence of *C. rodentium* (Supplemental Fig. 1). We also assessed the quantity of adherent and lumenal *C. rodentium* by plating colonic (adherent) and fecal (lumenal) samples and did not detect little to no FITC–dextran in serum. However, we found that *B. subtilis* does not protect mice by altering the localization, adherence, or density of the pathogen.

To test whether *B. subtilis* prevents disease by maintaining epithelial barrier integrity, we orally administered FITC–dextran to mice and then assessed the serum for fluorescence. If *B. subtilis* functions by preventing epithelial damage, then we expected to detect little to no FITC–dextran in serum. However, we found that mice infected with *C. rodentium* as well as those that received *B. subtilis* prior to pathogen infection had increased quantities of serum FITC–dextran (6.3 and 5.2 µg/ml, respectively) when compared with PBS-treated control mice (3.3 µg/ml) (Fig. 1C). These data suggest that *B. subtilis* does not protect from *C. rodentium*–induced colitis by preventing pathogen-induced disruption of the epithelium.

Analysis of EPS composition and structure

Because an *epsH* mutant, which does not produce EPS, failed to protect mice from *C. rodentium*–induced disease (7), we hypothesized that EPS may have immunomodulatory activity. To begin to test this idea, we first isolated EPS and analyzed its structure. EPS were purified from the *sinRtasA* mutant (DS991), which overproduces and secretes EPS into the supernatant (EPS⁺); as a control, supernatant of the *sinRtasAepsH* mutant (DS5187), which is unable to synthesize EPS (8), was subjected to the same purification process (EPS⁻). The purity of EPS were assessed by immunoelectrophoresis and Western blot analysis using rabbit anti-EPS antiserum. By immunoelectrophoresis, we observed only a single precipitation arc (Fig. 2A); no bands were observed with preimmune serum or with the EPS material (data not shown). By Western blot analysis, we observed only a single band of the expected size (~300 kDa) produced by the EPS⁺ strain (Supplemental Fig. 2). The OD₂₈₀ and OD₂₉₀ of purified EPS at a concentration of 1 mg/ml was 0.091 and 0.013, respectively, indicating that EPS were contaminated by little to no protein or nucleic acid.

The structure of purified EPS was analyzed by gas chromatography/mass spectrometry at the Complex Carbohydrate Research Center (University of Georgia), and the carbohydrate portion was found to be primarily mannose (88%) and glucose (11.9%) (Table I). Further structural analysis to determine the carbohydrate linkages revealed that the primary linkages are 2,6-mannose (31.8%), terminal mannose (29.9%), 3-mannose (15%), 2-mannose (4.7%), 6-mannose (4.7%), 6-glucose (3.7%), and terminal glucose (3.5%) (Table II); these data are consistent with the compositional analysis that indicates that mannose is the primary component of EPS.

**Effect of B. subtilis EPS on C. rodentium–associated disease**

To test whether EPS are sufficient to prevent disease, we administered purified EPS i.p. to wt mice and 24 h later infected them with *C. rodentium*. Disease was assessed 10 dpi by examining the colon, serum, and feces. Mice that received EPS displayed no evidence of disease (Fig. 2B–E), whereas mice that received material from the non–EPS-producing strain (EPS⁻), or no treatment other than PBS, displayed levels of proinflammatory KC (Fig. 2C), and diarrhea (Fig. 2D). These data indicate that EPS from *B. subtilis* are sufficient to protect wt mice from inflammation postinfection with *C. rodentium*.

Role of MyD88 and TLR4 in B. subtilis–mediated protection

Bacterial carbohydrates are ligands for many host pattern recognition receptors, including C-type lectins and TLRs, which are MyD88 dependent. *C. rodentium*–induced crypt hyperplasia is dependent on MyD88 signaling (9), and because we observed that *B. subtilis* and EPS suppressed crypt hyperplasia, we hypothesized that *B. subtilis* could mediate protection via this signaling pathway. Because MyD88 KO mice are highly susceptible to *C. rodentium* and succumb to disease 3–6 dpi (9, 21), we titrated the *C. rodentium* inoculum and found a minimal dose (10⁷ CFU) for which all mice developed disease (soft stool) at 5–7 dpi, similar to that observed with wt mice. Postinfection of MyD88 KO mice with *C. rodentium* (10⁷ CFU), mice lost weight (8–9 dpi), failed to clear the pathogen, and succumbed to disease by 11 dpi; administration
of B. subtilis did not protect mice (data not shown). We conclude that MyD88 signaling plays a role in B. subtilis–mediated protection of C. rodentium–induced colitis.

To identify the relevant MyD88-dependent TLR needed for protection in our model, we began to test individual TLR KO mice for susceptibility to C. rodentium after EPS treatment and started with TLR4 KO. EPS-treated TLR4 KO mice infected with C. rodentium showed evidence of disease including crypt hyperplasia, elevated serum KC, and diarrhea comparable to infected animals without EPS (Fig. 3A–C). As expected, neither material from the (EPS2) strain nor B. subtilis spores protected TLR4 KO mice from disease induced by the enteric pathogen (data not shown). These data suggest that EPS mediate protection via TLR4.

Because TLR4 is required for EPS-mediated protection, we tested whether a TLR4 agonist, hyaluronic acid was sufficient to prevent C. rodentium–associated disease. Mice were injected with hyaluronic acid (i.p.) prior to infection with C. rodentium, and disease was assessed 10 dpi. Hyaluronic acid did not protect mice at any of the concentrations tested (Fig. 3D–F), indicating that a TLR4 agonist is not capable of, or sufficient for, preventing disease. These data suggest that EPS does not act as a TLR4 agonist but instead may prevent disease by antagonizing TLR4.

Identification of EPS-binding cells

Because i.p. administration of EPS prevents C. rodentium–induced colitis, we searched by flow cytometry for peritoneal cells that

<table>
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<th>Glycosyl Residue</th>
<th>Mass (μg)</th>
<th>Molecular Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribose</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>Arabinose</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>Fucose</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>Xylose</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>Mannose</td>
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<tr>
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<tr>
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<td>11.9</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
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<td>0.1</td>
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<td>—</td>
</tr>
<tr>
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<td>100</td>
</tr>
</tbody>
</table>

*Values are expressed as mole percent of total carbohydrate. The total percentage may not add up to exactly 100% because of rounding.

Table II. Linkage analyses of EPS by gas chromatograph and mass spectroscopy

<table>
<thead>
<tr>
<th>Glycosyl Linkage Residue</th>
<th>EPS % Present</th>
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<tr>
<td>2-Rhamnopyranosyl residue (2-Rha)</td>
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<tr>
<td>Terminal Mannopyranosyl residue (t-Man)</td>
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</tr>
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</tr>
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<td>2,3,6 linked Mannopyranosyl residue (2,3,6-Man)</td>
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</tr>
<tr>
<td>2,4,6 linked Mannopyranosyl residue (2,4,6-Man)</td>
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<tr>
<td>2,3,4,6 linked Mannopyranosyl residue (2,3,4,6-Man)</td>
<td>0.5</td>
</tr>
<tr>
<td>4 linked N-acetyl Glucosamine (4-GlcNAc)</td>
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</tr>
</tbody>
</table>
bind EPS. We found that EPS bind cells in the granulocyte gate, with little to no binding to cells in the lymphocyte gate (Fig. 4A, 4B). More than 70% of cells in the granulocyte gate are F4/80+CD11b+ macrophages, and we found that EPS bind nearly all of these peritoneal macrophages (Fig. 4C, 4D). Although macrophages are "sticky" and readily bind polysaccharides, we think EPS binding is specific because EPS did not bind splenic macrophages (Fig. 4C, 4D). Fluorescence intensity represents EPS binding. Data shown are from one of three independent experiments.

**FIGURE 3.** Assessment of *C. rodentium*–associated disease in EPS-treated TLR4 KO mice or TLR4 agonist–treated wt mice. Quantification by ELISA of proinflammatory KC in serum of TLR4 KO mice infected with *C. rodentium* (Cr) with or without EPS (EPS+); PBS and EPS+ are negative controls (A). Summary of colonic crypt heights from each treatment group (B). Diarrhea (C) also served as a disease marker. Results are averages from at least three independent experiments; a total of 5–10 mice were assessed for each group. (D–F) wt mice were treated with 50, 100, or 150 μg of the TLR4 agonist hyaluronic acid (HA) prior to infection and then assessed for disease 10 dpi. Serum KC was measured by ELISA (D), colonic crypt heights from each treatment group were measured (E), and diarrhea (F) also served as a disease marker. Results are averages from at least two independent experiments; a total of four to five mice were assessed for each group.

not TLR4 KO, peritoneal cells produced KC and TNF-α when incubated with the TLR4 agonist (LPS), and all cells produced proinflammatory cytokines in response to a TLR2 agonist (Pam3Cys4). We also used ELISA to test for production of IL-10 by peritoneal cells from EPS-treated mice but found no evidence that EPS induced production of IL-10 (data not shown). These data indicate that EPS does not induce a proinflammatory response by peritoneal cells. Similarly, we have no evidence that an IL-10–mediated anti-inflammatory response is stimulated.

**Effect of adoptively transferred EPS-treated peritoneal cells on development of *C. rodentium*–induced disease**

Because we observed that i.p. administration of EPS prevented disease and because EPS bound peritoneal macrophages, we hypothesized that peritoneal cells from an EPS-treated mouse could convey protection to naive mice infected with *C. rodentium*. Peritoneal cells were collected by lavage 2–3 d after i.p. injection with EPS+ or EPS−, and 6 × 10⁶ cells were injected i.p. into...
recipient mice on −1, 1, and 3 dpi. Disease was assessed 10 dpi, and we found no evidence of disease in mice that received peritoneal cells from wt and TLR4 KO mice. Peritoneal cells were incubated with EPS (EPS+) (30 μg/ml), material from the non–EPS-producing strain (EPS−), LPS (100 ng/ml), Pam3Cys (100 ng/ml), or without addition (sham). Results are averages from three independent experiments. ND, not detectable.

Because TLR4 signaling is necessary for protection by EPS, we tested whether peritoneal cells require TLR4 signaling. TLR4 KO and wt mice were treated with EPS, and donor peritoneal cells were transferred into wt or TLR4 KO recipients with the expectation that if TLR4 signaling is required by peritoneal cells to mediate protection, then EPS-treated peritoneal cells from mice lacking TLR4 will not protect wt mice from pathogen-associated disease. As predicted, we found that EPS-treated TLR4 KO peritoneal cells did not protect wt mice from disease as evidenced by elevated serum KC, crypt hyperplasia, and diarrhea (Fig. 7D–F and PBS controls in Fig. 2B–D). In contrast, TLR4 KO recipient mice were protected by injection of EPS-treated peritoneal cells from wt mice. These data confirm the requirement of TLR4 in our model and suggest that peritoneal cells use TLR4 to mediate protection.

Discussion
The peritoneal cavity contains a variety of host immune cells, the most numerous of which are macrophages (~30–50%) and B cells (~40%) (22). To identify the cells that contribute to protection in our model, we searched for cells that bind EPS and found that they bind peritoneal F4/80+CD11b+ macrophages, suggesting a role for macrophages in EPS-mediated protection. Transfer of total peritoneal cells from an EPS-treated mouse was sufficient to protect naive mice from C. rodentium–induced enteric inflammation. In contrast, cells from a mouse treated with the EPS− material or TLR4 KO peritoneal cells from EPS-treated mice did not protect mice from disease, demonstrating that EPS and TLR4 signaling are required for protection. Because EPS do not protect mice that lack MyD88 in myeloid cells, the TLR4-dependent immunosuppressive cells in the peritoneal cavity are likely macrophages.

TLR signaling during C. rodentium infection is complex; some TLRs contribute to host defense and others promote host damage
toneal cells from wt or recipient wt (for all mice 10 dpi; serum KC (7–9, 21, 23). Previous studies using MyD88 KO mice infected with C. rodentium (21, 28, 29). In contrast, B. subtilis EPS are composed of three sugars (mannose [88%], glucose [11.9%], and N-acetylglucosamine [< 0.1%]), bind macrophages, and are larger than PSA (>250 kDa). On the basis of the carbohydrate analysis, the structure of B. subtilis EPS is significantly different from that of B. fragilis PSA, and they likely modulate the host immune response differently.

The probiotic C. butyricum promotes development of anti-inflammatory IL-10–producing F4/80+CD11b+CD11cint macrophages, which are critical for preventing dextran sulfate–induced colitis (1). In this case, the active bacterial molecules have not been identified. Although protection by B. subtilis EPS may be mediated by macrophages, the mechanism is likely different from B. fragilis or C. butyricum because they require TLR2 signaling whereas B. subtilis EPS requires TLR4 signaling. Collectively, our results and previous studies highlight the importance of selective modulation of TLR by commensal and probiotic bacteria to maintain intestinal homeostasis of CD4+ regulatory T cells and macrophages.

We do not know whether EPS causes an anti-inflammatory response, for example, production of IL-10 or other anti-inflammatory cytokines as is the case with B. fragilis and C. butyricum, or whether it inhibits induction of the inflammatory response initiated by C. rodentium infection. Because in preliminary studies we do not find evidence of increased anti-inflammatory cytokines after administration of EPS or B. subtilis, we hypothesize that EPS functions by altering macrophages in a TLR4-dependent manner to generate suppressor M2-like macrophages, which upon injection into wt recipient mice, prevent the inflammatory response caused by C. rodentium. Future experiments are needed to elucidate the mechanisms by which EPS and peritoneal macrophages prevent C. rodentium–induced colitis.

How can i.p. injection of macrophages suppress inflammation at a distant mucosal site? We hypothesize that peritoneal macrophages convey protection by one or both of two mechanisms. First, they could secrete a soluble immunosuppressive factor that modulates other immune cells. Alternatively, select peritoneal macrophages may migrate to the colon and suppress pathogen-induced colonic inflammation similar to that observed by Fraga-Silva et al. (30) who demonstrated that peritoneal macrophages migrate to areas of fungal infections.

Oral administration of B. subtilis provides protection, but administration of EPS by oral gavage does not protect against C. rodentium–induced colitis (data not shown). We showed previously that protection by B. subtilis requires it to be motile (4), and it may be that motile B. subtilis localizes to a particular niche in the gut and secretes a concentrated quantity of EPS, whereas administered by oral gavage, EPS do not reach this niche. Alternatively, EPS delivered by oral gavage may be degraded in the stomach before they can suppress inflammation. We know that B. subtilis can prevent C. rodentium–associated disease when ad-
These studies and ours indicate that commensals produce material from an EPS strain, prevent inflammatory disease induced by the enteric pathogen, C. rodentium. EPS-mediated protection requires TLR4 signaling, and although TLR signaling is known to regulate pathogen colonization and intestinal permeability, the protective effects of EPS seem to be a result of immunomodulation. Adoptive transfer studies demonstrate that TLR4 signaling on macrophage-rich peritoneal cells is required for EPS-mediated protection. Consistent with the idea that macrophages mediate protection in our model, we show that EPS bind peritoneal macrophages and that mice with MyD88-deficient myeloid cells are not protected by EPS. This study highlights how a single dose of purified bacterial molecules, such as EPS, can impact the host immune responses during infection with an enteric pathogen.

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Disclosures

The authors have no conflicts of interest.

References

S1. Effect of *B. subtilis* on *C. rodentium* colonization. Lux+ *C. rodentium* detected by IVIS 3 dpi (A-B) and 6 dpi (D-E) in the presence (B and E) and absence of *B. subtilis* (A and D). No luciferase was detected in uninfected (PBS) control mice (C). These results are representative images from 3 independent experiments with 2-3 mice/experiment.
S2. Analysis of EPS purity.
Western blot analysis of EPS using rabbit anti-EPS and donkey anti-rabbit Ig.