Enterobacter sakazakii Targets DC-SIGN to Induce Immunosuppressive Responses in Dendritic Cells by Modulating MAPKs

Rahul Mittal, Silvia Bulgheresi, Claudia Emami and Nemani V. Prasadarao

*J Immunol* published online 21 October 2009
http://www.jimmunol.org/content/early/2009/10/21/jimmunol.0902029

Supplementary Material http://www.jimmunol.org/content/suppl/2009/10/21/jimmunol.0902029.DC1

**Why The JI?** Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Enterobacter sakazakii Targets DC-SIGN to Induce Immunosuppressive Responses in Dendritic Cells by Modulating MAPKs

Rahul Mittal,* Silvia Bulgheresi,§ Claudia Emami, † and Nemani V. Prasadarao2*‡

Enterobacter sakazakii (ES) is an emerging pathogen that causes meningitis and necrotizing enterocolitis in infants. Dendritic cells (DCs) are professional phagocytic cells that play an essential role in host defense against invading pathogens; however, the interaction of ES with DCs is not known. In this study, we demonstrate that ES targets DC-specific ICAM nonintegrin (DC-SIGN) to survive in myeloid DCs for which outer membrane protein A (OmpA) expression in ES is critical, although it is not required for uptake. In addition, DC-SIGN expression was sufficient to cause a significant inhibition in ES by HeLa cells and intestinal epithelial cells, which are normally not invaded by ES. OmpA+ ES prevented the maturation of DCs by triggering the production of high levels of IL-10 and TGF-β and by suppressing the activation of MAPKs. Pretreatment of DCs with Abs to IL-10 and TGF-β or of bacteria with anti-OmpA Abs significantly enhanced the maturation markers on DCs. Furthermore, DCs pretreated with various inhibitors of MAPKs prevented the increased production of proinflammatory cytokines stimulated by LPS or OmpA+ ES. LPS pretreatment followed by OmpA+ ES infection of DCs failed to induce maturation of DCs, indicating that OmpA+ ES renders the cells in immunosuppressive state to external stimuli. Similarly, OmpA+ ES-infected DCs failed to present Ag to T cells as indicated by the inability of T cells to proliferate in MLR. We conclude that ES interacts with DC-SIGN to subvert the host immune responses by disarming MAPK pathway in DCs. The Journal of Immunology, 2009, 183: 0000–0000.

Enterobacter sakazakii (ES) is a fairly ubiquitous organism, which can be found in milk powder, rice, vegetables, cheese, sausage meat, teas, and various spices (1–4). However, most of the attention of ES-related contamination of food products has focused on powdered infant formulae (5, 6). The U.S. Food and Drug Administration published a warning regarding the presence of ES in baby formula in 2002 and later several times (7). ES may exhibit long-term persistence in dried infant formula and has been described as the only organism isolated after a 2.5-year period of storage (8). Infants requiring formula feeding are at high risk for developing life-threatening ES infections, which are associated with significantly high morbidity and mortality rates ranging from 33 to 80% (9–11). More than half of the survivors suffer irreversible neurological sequelae, resulting in quadriplegia, developmental impairment, and impaired sight and hearing (12). Premature (<28 days old) or low-birth-weight (<2500 g) infants are more susceptible to ES infections (13, 14). The risk also appears to be particularly high in children with impaired immune defenses. Clinical presentations include meningitis (complicated by ventriculitis, brain abscess, cerebral infarction, and cyst formation), septicemia, and necrotizing enterocolitis (NEC) in infants (9). To date, a few very studies have been focused on the pathogenic mechanisms involved in the development of meningitis or NEC. Our recent studies have demonstrated that infection of newborn rats or mice with ES induces meningitis within 72 h of postinfection for which outer membrane protein A (OmpA) expression is critical (15). Similarly, newborn rats under hypoxia conditions also develop NEC by ES (16, 17). Nonetheless, the interaction of ES with professional phagocytes is not known. We speculate that ES might be interacting with resident macrophages and/or dendritic cells (DCs) initially in intestinal wall and therefore requires strategies to evade the phagocytic mechanisms of these cells for initiation of the disease.

DCs constitute a system of hemopoietic cells that are rare but ubiquitously distributed (18). Immature DCs are seeded throughout peripheral tissues to act as sentinels against invading pathogens (19). These APCs also play an important role in the modulation of specific immune responses. Upon pathogen capture, DCs are activated, process pathogen into antigenic peptides for presentation in association with either MHC class II or nonclassical MHC-like molecules such as CD1, and migrate to the secondary lymphoid organs where they activate naïve T cells to initiate adaptive immune response (20, 21). Activation of DCs is associated with the expression of costimulatory markers on their surface such as HLA-DR, CD40, and CD86. Phagocytosis of bacteria as well as contact with bacterial toxins or components of bacterial cell wall can activate resting DCs, resulting in the initiation of immune response and elimination of the pathogen (22–24). However, many pathogens have turned DCs into allies either by inactivating infected DCs and rendering them tolerogenic or by inducing the production of immunosuppressive factors such as IL-10 and TGF-β (25–27). To sense pathogens, DCs express pathogen recognition receptors.
like C-type lectins (28). DC-SIGN (DC-specific ICAM-grabbing nonintegrin, where ICAM is intercellular adhesion molecule) is a calcium-dependent C-type lectin expressed by DCs, containing a carbohydrate recognition domain at its extracellular COOH-terminal end that recognizes mannose-rich molecules (29). DC-SIGN was initially described as a receptor for ICAM-3 at the surface of T cells, triggering the formation of the immunological synapse between DCs and naive T lymphocytes (30). Interestingly, DC-SIGN binds to HIV and SIVs and is involved in the trans-infection of CD4 T lymphocytes by HIV- or SIV-infected DCs (31–34). DC-SIGN has also been implicated in the phagocytosis of Candida albicans, Mycobacterium tuberculosis, a lgB mutant of Neisseria gonorrhoeae, a nonpathogenic Escherichia coli strain, and Yersinia pestis (35–39). Although it is not known whether DCs are primary targets for ES during the infectious process, the knowledge about the steps involved in the pathogenesis of meningitis is essential to combat ES infections. In this study, we demonstrate for the first time that ES exploits DC-SIGN to enter DCs and interferes with the maturation of DCs by altering the MAPK pathway to render them tolerogenic.

Materials and Methods

Bacterial strains, cells, and reagents

ES (51329) and rat intestinal epithelial cells, IEC-6 (used between passages 20–26), were obtained from American Type Culture Collection. IEC-6 cells were maintained in DMEM supplemented with 10% FCS, 1 U \cdot ml^{-1} insulin, 100 U \cdot ml^{-1} penicillin G, and 100 U \cdot ml^{-1} streptomycin. All general chemicals, LPS isolated from E. coli K12, and mannan were purchased from Sigma-Aldrich. Mermaid is a DC-SIGN-like molecule expressed by the marine nematode Laxus oneistus. Recombinant histidine tagged mermaid (His-Mermaid) was expressed and purified as described previously (40). DC-SIGN cDNA cloned into pcDNA3 (41) and anti-DC-SIGN Abs (42) were obtained through National Institutes of Health AIDS Research and Reference Reagent program. Abs to phosphorylated forms of p38, ERK1/2, JNK, anti-CD64 Ab, and human rTNF-\alpha, IL-1\beta, and PGE_2 were obtained from BD Biosciences. MAPK inhibitors were purchased from Calbiochem and Sigma-Aldrich. For studies using chloramphenicol, optimization of time kill curves was performed in our bacterial culture system. At doses of 3–10 g \cdot ml^{-1}, bacterial concentrations (over 10^5–10^7 CFU \cdot ml^{-1}) remained static up to 24 h, as determined by serial dilution, plating on blood agar, and enumerating the bacteria after overnight incubation. Bacterial cultures from overnight incubation were centrifuged, washed with saline, and kept in water bath 30 min to obtain heat-killed ES.

Generation of OmpA^+ ES and complementation with ompA gene

ES 51329 was grown in LB or tryptic soy broth medium without any antibiotics. ES was transformed with the plasmid pUC13 containing the gfp gene. Transformants were selected by ampicillin (100 \mu g/ml) and assessed for GFP expression by viewing under UV light. The ompA deletion mutant of ES was constructed by replacing ompA with a kanamycin cassette. Briefly, a spontaneous rifampicin-resistant mutant was isolated and named ESS1R. A 1.77-kb DNA containing ompA was amplified from ES with primers: 5’-GTGAGCTCCGGGCTAAAAATTCACTCAA-3’ (containing a SacI site) and 5’-CAGGTACCACGCGAGCTGAGTTGCA-3’ (containing a KpnI site). The DNA was cloned into pEP185.2 at the same sites, and

![FIGURE 1. Intracellular survival of ES in DCs. OmpA^+ and OmpA^- ES were incubated with DCs at a multiplicity of infection of 10 for indicated time points. The number of intracellular bacteria (A and B) was determined using gentamicin protection assays as described in Materials and Methods. Data represent mean log_{10} CFU per 5 \times 10^4 DCs \pm SD of triplicate samples from three independent experiments. DCs infected with the bacteria were processed from separate experiments for SEM (C) and TEM (D). Arrows indicate either intact or degraded bacteria.](http://www.jimmunol.org/DownloadedFrom)
FIGURE 2. Expression of maturation markers on the surface of DCs infected with ES. OmpA0, OmpA+, or OmpA−, or heat-killed ES (HK OmpA− ES) or LPS were incubated with DCs for 24 and 48 h. In some experiments, ES were pretreated with 20 μg·ml−1 chloramphenicol for 1 h at 37°C, washed, and adjusted the OD600 to 107 CFU·ml−1 and then added at a multiplicity of infection of 10 to DCs (ES+CM 0 h). In addition, chloramphenicol was added to bacteria-DC coculture 6 h postinfection (ES+CM 6 h). DCs were then washed, stained with Abs to CD40, HLA-DR, CD86, and CD25, fixed, and analyzed by flow cytometry. In separate experiments, DCs were first stimulated with LPS or MM for 24 h and then infected with OmpA+ ES for an additional 24 h. DCs were then washed and the expression of CD40 (D), HLA-DR (E), and CD86 (F) was analyzed by flow cytometry. The data represent geometric mean fluorescence intensity (MFI) of logarithmic data subtracted from isotype-matched controls. DCs infected with ES, LPS, or MM were added to naive T cells to examine the Ag presentation capacity of DCs as described in Materials and Methods (G). The error bars represent SDs from the means of triplicate samples. The results are representative of three independent experiments. The inhibition of cell surface marker expression or Ag presentation was significantly reduced in comparison to LPS-infected DCs. *, p < 0.001 by two-tailed t test.

DC culture and activation

DCs were generated from human PBMCs as described previously (44, 45). Briefly, monocytes were prepared from PBMCs by positive selection using Briefly, monocytes were prepared from PBMCs by positive selection using CD14 immunomagnetic beads (Miltenyi Biotec). CD14+ isolated cells were then cultured in RPMI 1640 supplemented with 10% FCS, 2.4 mM L-glutamine (Invitrogen), 50 ng·ml−1 human rGM-CSF and 20 ng·ml−1 human rIL-4 (PeproTech). DCs were used after 7 days of culture, and phenotype was determined by FACSCaliber flow cytometer (BD Biosciences). Immature DCs were CD3-negative, CD14-low, CD19-negative, CD83-negative, and CD25-negative and expressed low levels of HLA-DR, CD40, CD86, and CD1a. For stimulation experiments, DCs (5 × 105/ml) were cultured with live or killed ES at a multiplicity of infection of 10 (cell to bacteria ratio 1:10) for 24 and 48 h. DCs were also stimulated with LPS (Sigma-Aldrich) at a concentration of 10 μg·ml−1 as well as with a maturation mixture (MM) of TNF-α (10 ng·ml−1), IL-1β (10 ng·ml−1), and PGE2 (1 μg·ml−1) (46). In some experiments, DCs were pretreated for 20 min with anti-DC-SIGN Ab (5 μg·ml−1), mannan (500 μg·ml−1), or dextran (500 μg·ml−1), or ES was pretreated for 30 min with His-Mermaid (10 μg·ml−1). The concentrations used were based on our preliminary data and were selected based on the fact that at these concentrations, there was no influence on the survival of bacteria or DCs. Before Ab staining, an aliquot of DC culture was stained with trypan blue as well as with an Annexin V kit to assess the amount of cell death in coculture.

Transfection of HeLa and IEC-6 cells

HeLa- and IEC-6-DC-SIGN cells were generated by transfecting the respective cells with an expression plasmid containing human DC-SIGN gene, followed by selection for stable surface DC-SIGN expression as originally described (47). For invasion assays, ES (107 CFU/ml) were added to confluent monolayers of HeLa or IEC-6 cells separately at a cell ratio of 100:1 and incubated for 6 h. The monolayers were washed three times with RPMI 1640 medium followed by addition of gentamicin (100 μg·ml−1) and further incubated for 1 h at 37°C. The cells were then washed three times with RPMI 1640 and lysed with 0.5% of Triton X-100. The released bacteria were diluted with saline and enumerated by plating on blood agar. The total cell-associated bacteria were determined as described for the invasion, except that the gentamicin step was omitted.

Determination of ES uptake by DCs and intracellular survival

DCs (5 × 105 cells·ml−1) were washed three times in culture medium without antibiotics and then placed in 500 μl of culture medium in 12 × 75-mm polystyrene snap-cap tubes (BD Falcon). Varying concentrations of bacteria (10 μl) were added to the tubes. DCs and bacteria were then incubated for 1 h at 37°C. At different incubation periods, the cocultures were centrifuged at a low speed; aliquots from the supernatants were diluted and plated on blood agar. The number of bacteria present in the supernatants was subtracted from the bacteria added to cocultures to obtain the number of bacteria entered DCs. To assess intracellular bacteria at

the internal 876-bp Nru-I/BglII fragment was replaced with a 1.2-kb kana-mycin cassette from pUC-4K (Pharmacia). The recombinant plasmid was transferred from E. coli to ES51R by conjugation, and double-crossover mutants were selected. The deletion of ompA in ES51R was verified by PCR with the above primers. To restore the OmpA expression in OmpA mutants, the deletion of this gene, followed by selection for stable surface DC-SIGN expression as originally described (47). For invasion assays, ES (107 CFU/ml) were added to confluent monolayers of HeLa or IEC-6 cells separately at a cell ratio of 100:1 and incubated for 6 h. The monolayers were washed three times with RPMI 1640 medium followed by addition of gentamicin (100 μg·ml−1) and further incubated for 1 h at 37°C. The cells were then washed three times with RPMI 1640 and lysed with 0.5% of Triton X-100. The released bacteria were diluted with saline and enumerated by plating on blood agar. The total cell-associated bacteria were determined as described for the invasion, except that the gentamicin step was omitted.
different times postexposure, gentamicin was added to DC-bacteria coculture tubes at a final concentration of 100 \( \mu \text{g} \cdot \text{ml}^{-1} \) and incubated for an additional 60 min at 37°C. The cocultures were washed three times in RPMI 1640 containing no antibiotics and reconstituted with antibiotic-free culture medium. The cultures were then exposed to medium alone, treated with ES (anti-DC-SIGN Abs) or OmpA ES for 24 and 48 h. Culture supernatants were collected and the levels of TNF-\( \alpha \) (A), IL-1\( \beta \) (B), IL-6 (C), IL-10 (D), IL-12p70 (E), and TGF-\( \beta \) (F) were assessed by ELISA. The error bars represent SDs from the means of triplicate samples from four individual experiments. The suppression of cytokine production by OmpA\(^{\text{ES}}\) ES was significant in comparison to OmpA ES-, LPS-, or HK ES-induced levels.

\( \ast, p < 0.001 \) by two-tailed \( t \) test.

**Flow cytometry**

Expression of CD40, CD86, and HLA-DR, associated with DC maturation and activation, was detected by staining with appropriate monoclonal Abs (eBioscience). Cells were first preincubated for 20 min with IgG blocking buffer to prevent nonspecific binding. Cells were then fixed with BD Cytofix (BD Biosciences), washed, and incubated with 5 \( \mu \text{g} \cdot \text{ml}^{-1} \) anti-HLA-DR Ab (eBioscience), followed by Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). Cells were then allowed to adhere to poly-L-lysine-coated slides for 10 min (Paul Marienfeld) and mounted in an antifade Vectashield solution containing 4’,6-diamidino-2-phenylindole (Vector Laboratories). The cells were viewed with a Leica DMR microscope with Plan-apochromat oil immersion objectives. Images were acquired with a SkyVision-2/ VDS digital charge-coupled device camera (12-bit; 1280 \( \times \) 1024 pixels) in unbinned or 2 \( \times \) 2 binned models into the EasyFISH software, saved as 16-bit monochrome images, and merged as 24-bit RGB TIFF images (Applied Spectral Imaging). The TIFF images were assembled using Adobe Photoshop 7.0.

**Confocal laser microscopy**

DCs were infected with either OmpA\(^{\text{ES}}\) ES for varying periods, washed with PBS, and preincubated with IgG blocking buffer to prevent nonspecific binding. Cells were then fixed with BD Cytofix (BD Biosciences), washed, and incubated with 5 \( \mu \text{g} \cdot \text{ml}^{-1} \) anti-HLA-DR Ab (eBioscience), followed by Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). Cells were then allowed to adhere to poly-L-lysine-coated slides for 10 min (Paul Marienfeld) and mounted in an antifade Vectashield solution containing 4’,6-diamidino-2-phenylindole (Vector Laboratories). The cells were viewed with a Leica DMR microscope with Plan-apochromat oil immersion objectives. Images were acquired with a SkyVision-2/ VDS digital charge-coupled device camera (12-bit; 1280 \( \times \) 1024 pixels) in unprocessed or 2 \( \times \) 2 binned models into the EasyFISH software, saved as 16-bit monochrome images, and merged as 24-bit RGB TIFF images (Applied Spectral Imaging). The TIFF images were assembled using Adobe Photoshop 7.0.

**Scanning and transmission electron microscopy (TEM)**

DCs were treated with ES and then fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.1). All samples were washed three times in 0.1 M cacodylate buffer for 15 min each. Then postfixed for 20 min in 1% osmium tetroxide at 4°C, followed by addition of EtOH (60%). Samples were dehydrated through 70, 80, 95, and 100% EtOH (two times, 15 min each) then into propylene oxide (two times, 15 min each) and into a 1:1, left overnight and capped, at room temperature. The 1:1 propylene oxide/Epone mixtures were decanted off and replaced with 100% Eponate mixtures. The samples were polymerized at 70°C for 48 h. Thin sections were then into propylene oxide (two times, 15 min each) and into a 1:1, left overnight and capped, at room temperature. The 1:1 propylene oxide/Epone mixtures were decanted off and replaced with 100% Eponate mixtures. The samples were polymerized at 70°C for 48 h. Thin sections were then dehydrated through 70, 80, 95, and 100% EtOH (two times, 15 min each) then into propylene oxide (two times, 15 min each) and into a 1:1, left overnight and capped, at room temperature. The 1:1 propylene oxide/Epone mixtures were decanted off and replaced with 100% Eponate mixtures. The samples were polymerized at 70°C for 48 h. Thin sections were then dehydrated through 70, 80, 95, and 100% EtOH (two times, 15 min each) then into propylene oxide (two times, 15 min each) and into a 1:1, left overnight and capped, at room temperature. The 1:1 propylene oxide/Epone mixtures were decanted off and replaced with 100% Eponate mixtures. The samples were polymerized at 70°C for 48 h. Thin sections were then dehydrated through 70, 80, 95, and 100% EtOH (two times, 15 min each) then into propylene oxide (two times, 15 min each) and into a 1:1, left overnight and capped, at room temperature. The 1:1 propylene oxide/Epone mixtures were decanted off and replaced with 100% Eponate mixtures. The samples were polymerized at 70°C for 48 h. Thin sections were then dehydrated through 70, 80, 95, and 100% EtOH (two times, 15 min each) then into propylene oxide (two times, 15 min each) and into a 1:1, left overnight and capped, at room temperature. The 1:1 propylene oxide/Epone mixtures were decanted off and replaced with 100% Eponate mixtures. The samples were polymerized at 70°C for 48 h.

**FIGURE 3.** Cytokine production by DCs infected with ES. DCs were exposed to medium alone, treated with various ES strains, LPS or heat-killed (HK) ES for 24 and 48 h. Culture supernatants were collected and the levels of TNF-\( \alpha \) (A), IL-1\( \beta \) (B), IL-6 (C), IL-10 (D), IL-12p70 (E), and TGF-\( \beta \) (F) were assessed by ELISA. The error bars represent SDs from the means of triplicate samples from four individual experiments. The suppression of cytokine production by OmpA\(^{\text{ES}}\) ES was significant in comparison to OmpA ES-, LPS-, or HK ES-induced levels.

\( \ast, p < 0.001 \) by two-tailed \( t \) test.
and/or TGF-β-neutralizing Abs on DC activation. DCs were pretreated with IL-10- or TGF-β-blocking Abs or isotype-matched Ab (Control Ab) and then infected with OmpA⁺ ES as described in Materials and Methods. The expression of CD40 (A), HLA-DR (B), and CD86 (C) was analyzed by flow cytometry. The error bars represent SDs from the means of triplicate samples. The results are representative of five independent experiments. The increase in the expression of surface markers in DCs treated with IL-10 and/or TGF-β is significantly greater compared with OmpA⁺ ES or control Ab-treated DCs at both time points. *, p < 0.001 by two-tailed t test.

Statistical analysis

Statistical significance was determined by paired, two-tailed Student’s t test. Values of p < 0.05 were considered to be statistically significant.

Results

OmpA expression is necessary for the survival of ES in DCs

Our previous studies have demonstrated that OmpA expressing ES induces meningitis in newborn mice whereas OmpA⁻ ES did not, suggesting that OmpA expression may be important for survival in animals (15). However, its interaction with immune cells has not been studied to date. Therefore, to examine whether ES survives in DCs in vitro, myeloid DCs were infected with OmpA⁺ and OmpA⁻ ES for varying periods. The results from gentamicin protection assays showed that OmpA⁺ ES survived inside DCs, whereas OmpA⁻ ES was killed within 2 h (Fig. 1A). To examine whether lack of OmpA⁻ ES in DCs is not due to lack of entry into the cells, intracellular bacteria from 15 to 90 min postinfection was determined. OmpA⁻ ES did enter the cells as early as 15 min and were killed by 75 min postinfection (Fig. 1B). To determine whether the observed survival of OmpA⁺ ES is due to the expression of OmpA, complementation of OmpA⁺ ES with a plasmid containing the entire ompA gene was performed. The wild-type ES and the complemented strain, pOmpA⁺ ES, expressed similar levels of OmpA as analyzed by Western blotting with anti-OmpA Abs (data not shown). Phagocytosis assays with pOmpA⁺ ES restored the ability of OmpA⁺ ES to persist in DCs, indicating that OmpA is involved in the survival of ES in DCs. Scanning electron microscopy of OmpA⁺ ES interaction with DCs revealed that ES was in the process of being engulfed by conventional phagocytosis by DCs at 15 min postinfection (Fig. 1C). The bacteria were completely engulfed by 60 min postinfection. DCs containing the bacteria showed rugged surface. OmpA⁻ ES were also engulfed by 60 min postinfection; however, DCs revealed no rough morphology as that of OmpA⁺ ES-infected cells. TEM images demonstrated
that OmpA<sup>+</sup> ES was either attached to the cell surface or enclosed within the cytoplasmic vesicles with the characteristics of phagosomes (Fig. 1D). The fully phagocytosed ES was completely surrounded by intact membranes, and sometimes two or more bacteria were seen in phagosomes, indicating that the bacteria might be multiplying inside DCs. In contrast, the OmpA<sup>−</sup> ES, although entered DCs, were killed by the cells as observed by the presence of several vacuole-like structures containing debris. These data suggest that both OmpA<sup>+</sup> and OmpA<sup>−</sup> ES enter DCs with a similar frequency; however, OmpA<sup>+</sup> ES survives and multiplies inside DCs, whereas OmpA<sup>−</sup> ES was killed within 2 h postinfection.

**OmpA<sup>+</sup> ES prevents the maturation and Ag presentation of DCs**

To test whether the survival of OmpA<sup>+</sup> ES could also lead to a difference in maturation of DCs, we investigated the cell surface expression of CD40, CD86, and HLA-DR after 24 and 48 h stimulation by LPS or proinflammatory stimuli, for which bacterial protein synthesis in ES to prevent the maturation of DCs was examined by incubating DCs with OmpA<sup>+</sup> ES treated with 10<sup>6</sup> CFU of bacteria) or isotype-matched control Ab for 1 h on ice before adding to DCs. The expression of CD40, HLA-DR, or CD86 was examined by flow cytometry (C–E). F. Confocal microscopy of DCs infected with ES. DCs were cocultured with GFP OmpA<sup>+</sup> or OmpA<sup>−</sup> ES at an multiplicity of infection of 10 for 15, 30, 60, and 120 min. The cells were washed, and DCs were allowed to adhere to poly-L-lysine slides and then stained with anti-DC-SIGN Ab, followed by Cy3-coupled secondary Ab. The slides were counterstained with 4′,6-diamidino-2-phenylindole and visualized by confocal laser microscopy. Only data with OmpA<sup>+</sup> ES at 60 min postinfection are shown. The error bars represent SDs from four individual experiments performed in triplicate. The decrease in the number of intracellular survival ES is shown. The error bars represent SDs from means of four individual experiments performed in triplicate.

ES interaction with DC-SIGN

**FIGURE 5.** Binding of ES to DC-SIGN. DCs were incubated with DC-SIGN-blocking Ab, mannan, His-Mermaid, dextran, anti-CD64 Ab, or control Ab for 1 h before incubating with ES. The number of intracellular survival ES was determined as described in Materials and Methods (A). In separate experiments, ES were treated with 10 μg of His-Mermaid and incubated for 1 h and washed, and the bound Mermaid was identified by probing with anti-His Ab followed by flow cytometry (B). Similarly, OmpA<sup>+</sup> ES were incubated with either anti-OmpA Abs (20 μl of antiserum with 10<sup>7</sup> CFU of bacteria) or isotype-matched control Ab for 1 h on ice before adding to DCs. The expression of CD40, HLA-DR, or CD86 was examined by flow cytometry (C–E). F. Confocal microscopy of DCs infected with ES. DCs were cocultured with GFP OmpA<sup>+</sup> or OmpA<sup>−</sup> ES at an multiplicity of infection of 10 for 15, 30, 60, and 120 min. The cells were washed, and DCs were allowed to adhere to poly-L-lysine slides and then stained with anti-DC-SIGN Ab, followed by Cy3-coupled secondary Ab. The slides were counterstained with 4′,6-diamidino-2-phenylindole and visualized by confocal laser microscopy. Only data with OmpA<sup>+</sup> ES at 60 min postinfection are shown. The error bars represent SDs from means of four individual experiments performed in triplicate. The decrease in the number of intracellular survival ES is shown. The error bars represent SDs from means of four individual experiments performed in triplicate.

To test whether the survival of OmpA<sup>+</sup> ES led to a significant increase in the expression of costimulatory markers. In addition, the requirement of bacterial protein synthesis in ES to prevent the maturation of DCs was examined by incubating DCs with OmpA<sup>+</sup> ES treated with bacteriostatic doses of chloramphenicol (ES+CM 0 h; Fig. 2, A–C). Similar to that observed by coculturing with killed ES, the expression of costimulatory markers increased. However, a 6-h delay in addition of chloramphenicol (ES+CM 6 h; Fig. 2C) allowed sufficient protein synthesis to down-regulate costimulatory surface markers of DCs (Fig. 2, A–C). The interference with DC maturation by ES raised the question, whether the bacteria would inhibit maturation induced by the LPS or MM of TNF-α, IL-1β, and PGE<sub>2</sub>, which are shown to be present at the site of infection in several other studies (52). Despite pretreatment with these inducers, DCs subsequently infected with OmpA<sup>+</sup> ES showed down-regulation of maturation markers CD40, CD86, and HLA-DR (Fig. 2, D–F). Taken together, these data demonstrate that OmpA<sup>+</sup> ES inhibited phenotypic maturation of DCs induced by LPS or proinflammatory stimuli, for which bacterial protein synthesis is required. In addition, infection of DCs with OmpA<sup>+</sup> ES renders them into an immunosuppressive state.

To further determine whether suppression of phenotypic maturation by OmpA<sup>+</sup> ES correlates with prevention of functional maturation, DCs were tested for their ability to stimulate allogeneic...
purified naive CD4+ T cells. Mature DCs normally induce significant allogeneic lymphoproliferation at DC and naive T cell ratios of 1:300 (50). OmpA+ ES-activated DCs were effective in stimulating allogeneic CD4+ T cells, whereas OmpA+ ES-treated DCs induce very weak or no T cell proliferation (Fig. 2G). As observed with maturation markers, LPS treatment also induced the stimulation of CD4+ T cells, which was significantly decreased by OmpA+ ES infection. Similarly, MM-induced activation of the cells was also prevented by infection of DCs with OmpA+ ES. Activation of DCs following exposure to bacteria is associated with secretion of chemokines and cytokines, which play a crucial role in deciding the ultimate outcome of an infection by switching on Th1 or Th2 immune response (53, 54). Therefore, we speculated that ES might be exploiting at similar strategy. To test whether ES interacts with DC-SIGN, DCs were pretreated with anti-DC-SIGN Abs was due to absence of bacteria or DCs (data not shown). Lack of survival of OmpA+ ES failed to show activation of DCs. These results indicate that production of both IL-10 and TGF-ß by DCs infected with OmpA+ ES plays an important role in preventing the maturation of these cells.

**DC-SIGN is involved in internalization of ES by DCs**

Several microorganisms such as HIV and *Mycobacterium tuberculosis* use the immunoreceptor DC-SIGN to escape immune surveillance by interfering with DC maturation (31–35). Therefore, we speculated that ES might be exploiting at similar strategy. To test whether ES interacts with DC-SIGN, DCs were pretreated with DC-SIGN blocking Ab for 20 min, followed by addition of bacteria. As shown in Fig. 5A, OmpA+ ES failed to survive in DCs pretreated with DC-SIGN blocking Ab. To further confirm these findings, DCs were pretreated with mannan, which specifically binds mannose related receptors, including DC-SIGN and ES pretreated with His-Mermaid, which is a newly identified C-type lectin (40), which has been shown to compete with DC-SIGN for the binding to *E. coli* CS180, *Yersinia pestis*, and HIV-1 (31–38). Anti-CD64 Ab was also used to examine the role of other receptors in ES internalization. Pretreatment of DCs with these compounds completely inhibited the survival of OmpA+ ES in DCs; however, pretreatment with dextran (used as control for mannan), anti-CD64 Abs, or isotype-matched control Abs had no effect. Of note, the concentrations of anti-DC-SIGN Ab, mannan, and His-Mermaid used in this study were found to have no effect on viability of either bacteria or DCs (data not shown). Lack of survival of OmpA+ ES in DCs pretreated with anti-DC-SIGN Abs was due to absence of entry of the bacteria into DCs (data not shown). OmpA+ ES also could not enter DCs, indicating that both these strains are using DC-SIGN to enter DCs; however, OmpA expression is important for the survival of the bacteria inside DCs. The inhibitory effect of His-Mermaid on ES entry into DC is due to the binding of His-Mermaid to both OmpA+ and OmpA- ES as evaluated by flow cytometry (Fig. 5B). To confirm whether the interaction of OmpA with DCs is required for distinct DC phenotype, OmpA+ ES was

![FIGURE 6. OmpA+ ES suppresses the expression of DC-SIGN upon infection of DCs. The expression of DC-SIGN following infection with ES was assessed at different time points by immunocytochemistry (A) and flow cytometry (B). The error bars represent SDs from the means of triplicate samples. The results are representative of three independent experiments. The decrease in the expression of DC-SIGN in DCs infected with OmpA+ ES was significantly lower compared with OmpA- ES infected DCs at 90 and 120 min postinfection. *, p < 0.001 by two-tailed t test.](http://www.jimmunol.org/content/jimmunol/193/5/3514/F6.large.jpg)

A  Bright Field  DC-SIGN

B

<table>
<thead>
<tr>
<th>Mean Fluorescence Intensity</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>OmpA+ ES</td>
<td>100</td>
<td>200</td>
<td>300</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>OmpA- ES</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>200</td>
<td>250</td>
</tr>
</tbody>
</table>

**Post-infection time (min)**
pretreated with anti-OmpA Abs and cultured with DCs. Blocking of OmpA binding to DCs led to activation as shown by up-regulation of CD40, HLA-DR, and CD86 (Fig. 5, C–E), indicating that OmpA might interact with DC-SIGN to suppress the maturation of DCs infected with OmpA+/H11001 ES. In addition, the spatial relationship of ES binding with DC-SIGN was examined by immunocytochemistry. DCs were stained using PE-conjugated anti-DC-SIGN Ab, and ES were visualized for the presence of GFP. As shown in the Fig. 5F and supplemental video 1, DC-SIGN was colocalized at the site of entry of bacteria. In addition, no bacteria were observed inside DCs following treatment of DCs with DC-SIGN blocking Ab, mannan, or His-mermaid, indicating that DC-SIGN is a receptor for ES (data not shown). Of note, the entry of OmpA+/H11001 ES into DCs increased the expression of DC-SIGN up to 60 min postinfection, which was reduced to basal levels by 120 min postinfection as shown by both immunocytochemistry and flow cytometry (Fig. 6, A and B). DC-SIGN expression was also observed with OmpA− ES infection of DCs by 60 min postinfection, however, stayed at the similar level even after 120 min postinfection. These data suggest that the presence of OmpA+/ES in DCs suppresses the maturation and DC-SIGN expression on the surface of the cells.

**DC-SIGN expression is sufficient to increase ES uptake by HeLa cells and intestinal epithelial cells**

To determine whether the expression of DC-SIGN is sufficient to allow the invasion of ES, a mammalian expression plasmid containing DC-SIGN cDNA was introduced into HeLa cells. The transfected cells were examined for the expression of DC-SIGN by flow cytometry using anti-DC-SIGN Abs (A). In addition, the ability of OmpA+ and OmpA− ES to bind to and invade DC-SIGN-expressing HeLa and IEC-6 cells was determined and compared with nontransfected cells (B–E). The error bars represent SDs of triplicate samples. The results are representative of three independent experiments. The increase in the binding or invasion of ES was significantly greater compared with control cells. *, p < 0.001 calculated by Student’s t test.

---

*The online version of this article contains supplemental material.*

---

**FIGURE 7.** ES binds and invades HeLa and IEC-6 cells expressing DC-SIGN. A mammalian expression plasmid containing DC-SIGN cDNA was introduced into HeLa and IEC-6 cells. The transfected cells were examined for the expression of DC-SIGN by flow cytometry using anti-DC-SIGN Abs (A). In addition, the ability of OmpA+ and OmpA− ES to bind to and invade DC-SIGN-expressing HeLa and IEC-6 cells was determined and compared with nontransfected cells (B–E). The error bars represent SDs of triplicate samples. The results are representative of three independent experiments. The increase in the binding or invasion of ES was significantly greater compared with control cells. *, p < 0.001 calculated by Student’s t test.
Therefore, the influence of ES on various MAPKs in DCs immune response, including the activation and maturation of DCs. The MAPKs have been shown to be involved in all aspects of the maturation of DCs independent experiments. The phosphorylation of MAPKs in OmpA was plotted for each MAPK (Fig. 8). For quantitative measurements, the geometric mean fluorescence intensity was plotted for each MAPK (B). The results are representative of five independent experiments. The phosphorylation of MAPKs in OmpA ES-infected DCs was significantly lower compared with OmpA ES-infected or LPS-treated cells. * p < 0.001 calculated by Student’s t test.

**FIGURE 8.** ES impairs phosphorylation of p38, ERK1/2, and JNK in DCs. DCs were cultured in the presence of OmpA− or OmpA+ for 6 h, washed, and stimulated with LPS for 30 min. The cells were washed and fixed and then phosphorylation of p38, ERK1/2, and JNK was determined by flow cytometry after staining with respective phospho-specific Abs (A). For quantitative measurements, the geometric mean fluorescence intensity was plotted for each MAPK (B). The results are representative of five independent experiments. The phosphorylation of MAPKs in OmpA+ ES-infected DCs was significantly lower compared with OmpA+ ES-infected or LPS-treated cells. * p < 0.001 calculated by Student’s t test.

also transfected with DC-SIGN expressing plasmid construct, and the resulting cells were examined for ES binding to and invasion (Fig. 7, D and E). Significant increase in binding and invasion of both OmpA+ and OmpA− ES to IEC-6-DC-SIGN cells was observed, indicating that ES directly interacts with DC-SIGN receptor. However, since OmpA+ ES also invades DC-SIGN-transfected cells, we conclude that OmpA does not play a significant role in the invasion of DCs; however, it is necessary for the survival inside DCs.

OmpA+ ES suppresses phosphorylation of MAPKs involved in maturation of DCs

The MAPKs have been shown to be involved in all aspects of the immune response, including the activation and maturation of DCs (55–58). Therefore, the influence of ES on various MAPKs in DCs was determined. DCs infected with OmpA+ or OmpA− ES or LPS were stained with Abs to phospho-p38, -ERK1/2, -JNK and then subjected to flow cytometry. As shown in Fig. 8, DCs infected with OmpA+ ES showed basal level phosphorylation of p38, ERK1/2, and JNK in comparison to OmpA− ES in which all these molecules were phosphorylated. LPS also showed similar increase in phosphorylation of MAPKs, indicating that OmpA+ ES suppresses the activation of MAPK pathway. The expression of non-phosphorylated MAPKs was similar in all three treatments (data not shown). To determine whether the activation of MAPKs is necessary for the entry of ES into DCs, the cells were pretreated with MAPK inhibitors SB203580 (p38 kinase inhibitor), PD 98059 (MEK1 inhibitor), U0126 (inhibitor of MEK1 and MEK2, which are upstream of JNK), or with a JNK inhibitor SP600125 or with a combination of these inhibitors. The intracellular survival of ES was not affected by pretreating the cells with MAPK inhibitors (Fig. 9A). In contrast, no up-regulation of maturation markers was observed in DCs treated with MAPK inhibitors, followed by LPS treatment or OmpA− ES infection similar to that of OmpA+ ES-induced levels (Fig. 9, B–D). Maximum inhibitory effect was observed when DCs were pretreated with all the three inhibitors. Similarly, the production of proinflammatory cytokines was also significantly reduced in DCs pretreated with MAPK inhibitors, followed by LPS stimulation in comparison to LPS treated DCs (Fig. 10). These data demonstrate that ES prevents the maturation of DCs by interfering with MAPK pathway, which is distinct from entry mechanisms.

Discussion

DCs play a crucial role in the initiation and modulation of pathogen-specific immune responses (18). Immature DCs in the periphery and submucosa sense the external environment and constantly monitor for pathogens (19). Once a DC recognizes and captures a pathogen, it undergoes considerable changes, resulting in DC maturation (20–23). However, some of the pathogens interfere with the maturation of DCs and exploit them as replication permissive niche. In this study, we show that exposure to ES that expresses OmpA can impair the maturation of myeloid DCs, triggering the production of IL-10 and TGF-β, the cytokines generally associated with immunosuppressive response (59–61). Remarkably, ES that lack OmpA induced the production of proinflammatory cytokines in infected DCs. Although both OmpA+ and OmpA− ES were efficiently taken up by DCs, the cells immediately killed OmpA− ES. In contrast, OmpA+ ES resists killing and multiplied in these cells, suggesting that OmpA expression is critical for the survival of ES in DCs. Complementation with OmpA gene restored the ability of OmpA− ES to persist in DCs highlighting the crucial role of OmpA for survival in DCs. Phagocytosis and the subsequent intracellular events control the generation of immune response and the fate of the pathogen. Phagocytosis of infectious organisms begins with binding of the organism to the cell. SEM and TEM studies revealed that ES was taken up by DCs in a conventional phagocytosis mechanism enclosed inside membrane-bound compartments of DCs. Two or more bacteria were also observed in a single phagosome-like organelles.

The idea that DCs use DC-SIGN to capture microbial pathogens for delivery to lymphocytes emerged with the discovery of DC-SIGN as a receptor for gp120 Ag of HIV. Several studies have established that DCs serve as the carrier for HIV-1, with DC-SIGN as the receptor for viral particles and delivering them to target cells such as CD4 lymphocytes (31–33). A similar concept also applies to ES, because it binds DC-SIGN to enter DCs. Anti-DC-SIGN Abs and mannan, which affect DC-SIGN binding ability, and His-Mermaid, which can compete with DC-SIGN for ES binding, all significantly prevented the entry of ES into DCs, indicating that ES interacts with DC-SIGN. Although several mannose C-type lectin receptors are present on DCs, including DC-SIGN, langerin, and the mannan receptor, ES binding and entry was completely
FIGURE 9. Effect of MAPK inhibitors on phagocytosis and maturation of DCs. DCs were treated with the p38 inhibitor SB203580 (SB), the ERK1/2 inhibitors U0126 (U), PD98059 (PD), the JNK inhibitor SP600125 (SP), or with a combination (SB+U+PD+SP) for 1 h and then incubated with bacteria. Percentage intracellular bacteria were assessed using gentamicin protection assay as described in Materials and Methods (A). Furthermore, bacteria treated with these MAPK inhibitors were stimulated with LPS for 24 and 48 h, and expression of maturation markers CD40 (B), HLA-DR (C), and CD86 (D) was assessed using flow cytometry. The data are presented as geometric mean fluorescence intensity of logarithmic data subtracted from isotype-matched controls. The error bars represent SDs of triplicate samples from four individual experiments. The decrease in the expression of maturation markers in DCs pretreated with various inhibitors was significantly lower compared with LPS pretreated cells. *, p < 0.001 calculated by Student’s t test.

prevented with anti-DC-SIGN Ab. Therefore, it is possible that ES interacts specifically with DC-SIGN to bind to and enter DCs. However, the absence of OmpA did not affect the ability of ES to enter DCs or to bind His-Mermaid, suggesting that ES might be entering DCs via the interaction of mannose residues present on LPS with DC-SIGN. DC-SIGN binds several mannose-containing glycoconjugates as well as fucose-containing Lewis blood group Ags (Le^a, Le^b, Le^c, and Le^d). However, the core LPS of E. coli K12, Haemophilus ducreyi, N. gonorrhoeae, and Salmonella typhimurium do not contain either mannose or fucose, but GlcNAc is part of the core region (34). Zhang et al. (34) have shown that when the GlcNAc epitopes of the core region have been removed, the ability of LPS from these bacteria to bind DC-SIGN is decreased or lost.

To further confirm that ES interacts with DC-SIGN, binding of ES to His-Mermaid, a DC-SIGN-like molecule was determined by flow cytometry. The carbohydrate recognition domain of Mermaid shares both structural and functional similarity with that of DC-SIGN (40). Strong binding of ES to His-Mermaid was observed, indicating that there is a specific interaction between DC-SIGN and ES. Additional ES was also observed to invade HeLa cells expressing DC-SIGN with a 50-fold greater efficiency in comparison to plasmid-alone transfected cells. In addition, ES was able to invade DC-SIGN-expressing IEC-6 cells, which are noninvasive by this pathogen, substantiating the evidence that engaging DC-SIGN is sufficient for the invasion. Our results clearly support the notion that ES uses the DC-SIGN receptor to invade and replicate inside DCs. Interestingly, the survival of OmpA^+ ES in DCs requires bacterial protein synthesis because chloroamphenicol-treated ES could not survive in the DCs, although entered normally, indicating that the expression of OmpA^+ ES might interact with certain cellular components to induce the secretion of bacterial proteins into phagosome. Of note, blocking of OmpA interaction with DCs by anti-OmpA Ab prevented the suppressive effects of the bacterium in DCs, indicating that OmpA might be interacting with DC-SIGN, which triggers the secretion of proteins into DCs and thereby suppresses DC function. Additional studies are needed to identify these proteins produced by ES in DCs.

DCs are the most potent APCs capable of activating naïve T lymphocytes and, hence, play a central role in the induction of adaptive immunity (18–20). Immature DCs sample and process Ags, and efficiently sense a large variety of signals from the surrounding environment. ES-infected DCs fail to present Ag to T cells as indicated by the inability of T cells to proliferate in MLR. Strong T cell immune responses are instrumental in controlling microbial infections. Our studies support the notion that interference with DC function is a mechanism of pathogenicity used by ES to evade T cell recognition. The inability of DCs to present Ag to T cells can have serious consequences like chronicity and recurrence of infection. The suppression of T cell immune responses also could be due to the production of anti-inflammatory cytokine production in microbial infections. The nature of microbial stimuli exerts a potent influence on the ability of DCs to produce distinct cytokines and to induce Th1 vs Th2 responses (53, 54). The cytokine profile of DCs infected with OmpA^+ ES showed higher production of IL-10 and TGF-β and very low levels of proinflammatory cytokines, a phenotype associated with tolerogenic DCs (59–61). In contrast, infection of DCs with OmpA^- ES led to higher production of proinflammatory cytokines, indicating...
and subsequently cause meningitis in neonates. A high degree of bacteremia required to cross the blood-brain barrier provides ES with a niche to multiply. This may help in reaching a pivotal side, the suppression of MAPK activation and subsequent pre-

ative effects thereupon by targeting MAPK activity. On the bacte-

mediates ES access to DCs and exerts profound immunosuppres-

SIGN and the ES outer membrane protein OmpA. Host DC-SIGN

phosphorylation cascades in the intracellular-signaling pathways

beneficial strategy used by many pathogens is to interfere with the

activation and maturation of DCs. Thus, ES could exploit IL-10-

and TGF-β-producing tolerogenic DCs to escape potent host immune
defense mechanisms.

Signals emanating from many cell-surface receptors and envi-

ronmental cues converge on MAPKs, which in turn phosphorylate and activate various transcription factors and other molecular ef-
factors (62–64). MAPKs comprise three major groups: the ERKs (ERK1 and ERK2), the JNKs, and the p38 MAPKs (65–67). A

bacterial strategy used by many pathogens is to interfere with the

phosphorylation cascades in the intracellular-signaling pathways

of the host cell. Our studies revealed that ES severely impairs the

phosphorylation of p38, ERK1/2, and c-JNK. On par with these

findings, pretreatment of DCs with inhibitors of MAPK pathway

prevented the activation and maturation of DCs stimulated by LPS

or OmpA− ES as observed with OmpA− ES. Thus, ES prevents

the activation and maturation of DCs by compromising the MAPK

pathway. Nevertheless, MAPK inhibitor pretreatment did not af-
dict the entry of ES in DCs.

The present study highlights the fundamental role of two mol-

ecules in ES pathogenesis: the DC-specific immunoreceptor DC-

SIGN and the ES outer membrane protein OmpA. Host DC-SIGN

mediates ES access to DCs and exerts profound immunosuppres-
sive effects thereupon by targeting MAPK activity. On the bacte-

rial side, the suppression of MAPK activation and subsequent pre-

vention of the expression of maturation markers require the

expression of OmpA in ES. The absence of presentation of bac-

terial Ags to T cells ensures ES survival inside DCs and likely

provides ES with a niche to multiply. This may help in reaching a

high degree of bacteremia required to cross the blood-brain barrier

and subsequently cause meningitis in neonates.

Acknowledgments

The following reagents were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: pcDNA3-DC-SIGN (catalog no. 5444) from Drs. S. Pohmann, F. Baribaud, F. Kirchhoff, and R. W. Doms and DC-SIGN Ab (clone 120526, catalog no. 6886). We thank Ernesto Barron and Douglas Hauser for their help in scanning and transmission electron microscopy at University of Southern California School of Medicine, Los Angeles.

Disclosures

The authors have no financial conflict of interest.

References


5. Drudy, D., N. R. Mullane, T. Quinn, P. G. Wall, and S. Fanning. 2006. Enter-
obacter sakazakii: an emerging pathogen in powdered infant formula. Clin. In-


prevents intestinal epithelial cell injury caused by Enterobacter sakazakii-


tomegalovirus inhibits maturation and impairs function of monocyte-derived


