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CELLULAR MICROBIOLOGY:
PATHOGEN-HOST CELL MOLECULAR INTERACTIONS

Salmonella enterica Serovar Typhimurium Interacts with CD209 Receptors To Promote Host Dissemination and Infection

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ABSTRACT *Salmonella enterica* serovar Typhimurium, a Gram-negative bacterium, can cause infectious diseases ranging from gastroenteritis to systemic dissemination and infection. However, the molecular mechanisms underlying this bacterial dissemination have yet to be elucidated. A study indicated that using the lipopolysaccharide (LPS) core as a ligand, *S.* Typhimurium was able to bind human dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (hCD209a), an HIV receptor that promotes viral dissemination by hijacking antigen-presenting cells (APCs). In this study, we showed that *S.* Typhimurium interacted with CD209s, leading to the invasion of APCs and potentially the dissemination to regional lymph nodes, spleen, and liver in mice. Shielding of the exposed LPS core through the expression of O-antigen reduces dissemination and infection. Thus, we propose that similar to HIV, *S.* Typhimurium may also utilize APCs via interactions with CD209s as a way to disseminate to the lymph nodes, spleen, and liver to initiate host infection.

KEYWORDS CD209, *S.* Typhimurium, dendritic cell, dissemination, lipooligosaccharide, macrophages

Salmonella enterica serovar Typhimurium is a Gram-negative bacterium that causes infectious gastroenteritis and systemic infection in both humans and animals. *S.* Typhimurium infection is usually called mouse typhoid fever, which apparently corresponds to human typhoid fever caused by *S. enterica* serovar Typhi. The ability to disseminate to and infect distal organs is a characteristic property of *Salmonella* infection (1, 2). However, the detailed molecular mechanisms and host cells involved in the dissemination of *S.* Typhimurium remain to be determined.

Citation Ye C, Li Q, Li X, Park CG, He Y, Zhang Y, Wu B, Xue Y, Yang K, Lv Y, Ying X-L, Ding H-H, Cai H, Alkraiem AA, Njiri O, Tembo J, Huang H-P, Li A-Y, Gong J, Qin J, Cheng B, Wei X, Sun Z, Zhang S-S, Zhang P, Zheng G-X, Li W, Kan B, Yan M, Xiding X, Huo X, Zeng Y, Peng H, Fu Y, Klena JD, Skurnik M, Jiang L-Y, Chen T. 2019. *Salmonella enterica* serovar Typhimurium interacts with CD209 receptors to promote host dissemination and infection. Infect Immun 87:e00100-19. https://doi.org/10.1128/IAI.00100-19.

Editor Manuela Raffatellu, University of California San Diego School of Medicine

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Received 7 February 2019
Returned for modification 28 February 2019
Accepted 4 May 2019
Accepted manuscript posted online 13 May 2019
Published 23 July 2019

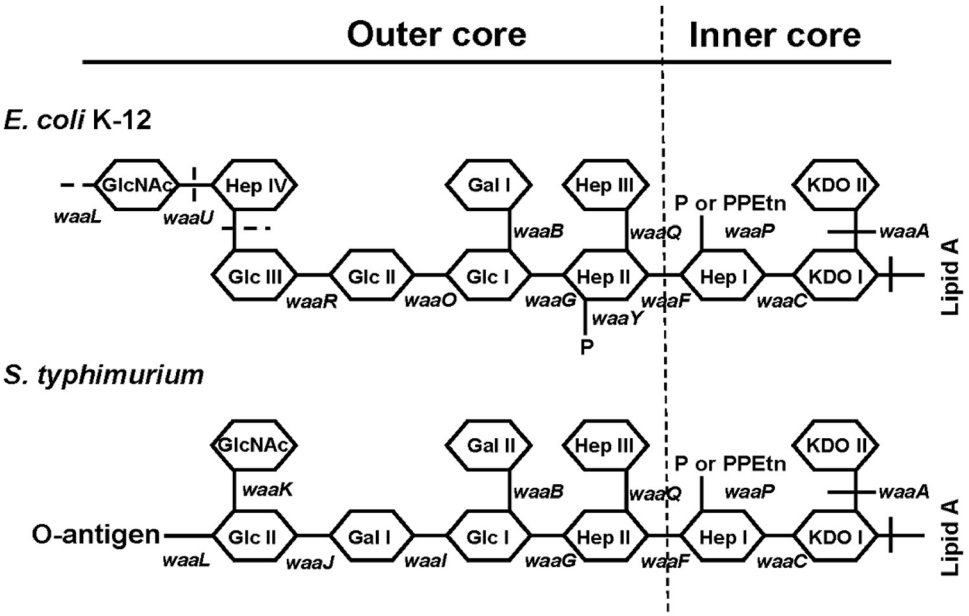

FIG 1 Structures of the inner core and outer core regions of the LPS of *E. coli* K-12 and *S.* Typhimurium and the genes involved in biosynthesis. Genes involved in the biosynthesis of the LPS core are shown at their approximate sites of action (solid lines). The sites, which are variably substituted or still under investigation, are indicated by dashed lines. Abbreviations: GlcNAc, *N*-acetylglucosamine; Glc, glucose; Hep, L-glycero-D-manno-heptose; Gal, galactose; P, phosphate; PPEtn, phosphoethanolamine; KDO, 2-keto-3-deoxyoctonate. It should be noted that *E. coli* K-12 and *Y. pestis* do not naturally possess an O-antigen. Studies have shown that the exposed GlcNAc residue in the LPS core might play an important role in the interaction with DC-SIGN (10, 26–28). (Republished from reference 12.)

The mechanisms used by *Salmonella* for dissemination have been studied, as outlined in excellent review articles (3–5). After passing through the Peyer's patches, *Salmonella* spp. appear to utilize antigen-presenting cells (APCs), such as dendritic cells (DCs), as a carrier to reach the mesenteric lymph nodes (MLNs) (1, 3). It is suggested that a subset of DCs in the lamina propria are the first DCs to transport pathogenic *Salmonella* from the intestinal tract to the MLNs (6). Recent studies further indicated that the migratory DCs in the gut control the host dissemination of *Salmonella* (7). It is therefore proposed that *Salmonella* species use DCs or APCs as Trojan horses to promote bacterial dissemination (4).

HIV binds the human DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) (hCD209a) receptor on DCs and hijacks the infected DCs as Trojan horses to promote viral dissemination to target cells such as CD4 lymphocytes in lymph nodes (8, 9). Interestingly, the exposed lipopolysaccharide core (LPS core) of *S.* Typhimurium was able to interact with the human DC-SIGN (hCD209a) receptor, leading to the invasion of host cells (10). Two very recent studies further showed that using the LPS core, *Yersinia* spp. may interact with CD209s to promote host dissemination and infection (11, 12).

The LPS of many Gram-negative bacterial pathogens promotes resistance to serum killing and/or phagocytosis (13–17). Over 70% of the surface of Gram-negative bacteria, including *Salmonella*, is occupied by fully expressed LPS that generally consists of three structural regions: (i) lipid A, (ii) the oligosaccharide core, and (iii) the O-polysaccharide (OPS) or O-antigen (Fig. 1). The LPS produced by wild-type bacteria is known as smooth LPS, while LPS lacking the O-antigen is known as rough LPS or lipooligosaccharide (LOS).

Functioning as a shield, the O-antigen is regarded as a virulence factor for most Gram-negative bacterial pathogens (15, 16, 18, 19). However, the expression of O-antigen of *S.* Typhimurium is curbed inside macrophages (20), suggesting that its LPS core would be exposed *in vivo*. We therefore asked if the interaction between the LPS core of *S.* Typhimurium and CD209s (10) would lead to its dissemination.

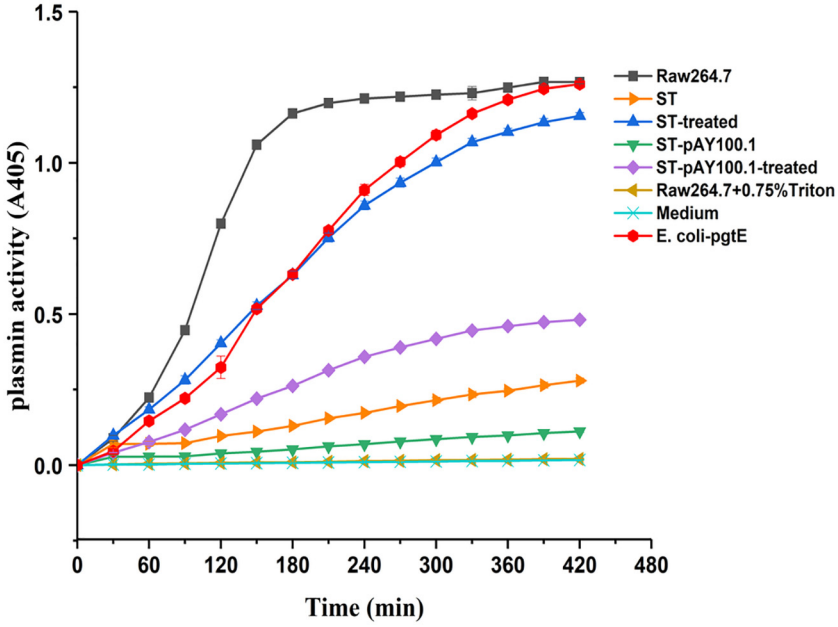

FIG 2 LPS core of *S.* Typhimurium may be exposed after phagocytosis by the macrophage cell line RAW264.7. The examination of plasminogen enzyme activity was used to evaluate the level of LPS core exposure after phagocytosis by and release from the macrophage cell line RAW264.7 (23, 24). The samples were RAW264.7 cells, *S.* Typhimurium LT2, treated *S.* Typhimurium LT2, ST-pAY100.1, ST-pAY100.1-treated (ST-pAY100.1 released from RAW264.7 cells), *E. coli*-pgtE, RAW264.7 + 0.75% Triton (RAW264.7 cells lysed with 0.75% Triton X-100), and medium. Data are representative of three independent experiments.

RESULTS

The LPS core of *S.* Typhimurium LT2 may be exposed after phagocytosis by the macrophage cell line RAW264.7. The proteolytic enzyme PgtE can activate plasminogen, the plasma proenzyme, to become plasmin (21). Thus, we examined plasmin activity to evaluate the activity of PgtE. Testing of PgtE activity was used as an index to assess the degree of exposure of the LPS core in *S.* Typhimurium (20, 22–24), i.e., the higher the activity of PgtE, the greater the exposure of the LPS core (detailed in Discussion). In this study, *S.* Typhimurium LT2, used in our previous publication (10), was studied for the activity of PgtE using a protocol identical to that described for *S.* Typhimurium strain 14028 (24). ST-pAY100.1 is *S.* Typhimurium LT2 transformed with the plasmid pAY100.1 (the cloning vector pBR322 carrying the O-antigen operon of *Y. enterocolitica* O:3) (25). Previous studies showed that the expression of this O-antigen blocks the interactions between the LPS core and CD209s (11, 12, 26).

As shown in Fig. 2, the addition of 0.75% Triton X-100 in the RAW264.7 plus Triton X-100 group resulted in the absence of plasmin activity. This might be caused by the lysis of Triton X-100 to the RAW264.7 cells. This suggests that the macrophages do not interfere with the plasmin activity. *S.* Typhimurium LT2 expressed active PgtE after phagocytosis by murine macrophage-like RAW264.7 cells (termed treated *S.* Typhimurium LT2). Meanwhile, PgtE activity on ST-pAY100.1 was inhibited regardless of whether *S.* Typhimurium was phagocytosed by RAW264.7 cells. *E. coli*-pgtE, an *Escherichia coli* K-12 strain that contains the *pgtE* gene, was used as the positive control (23). Notably, most *E. coli* K-12 strains are rough (i.e., O-antigen-negative) strains. This result indicates that, similar to that of *S.* Typhimurium 14028, the LPS core of *S.* Typhimurium LT2 is exposed after phagocytosis by macrophages (24).

LPS core-dependent invasion of *S.* Typhimurium into human and mouse APCs. Given the possible exposure of the LPS core after phagocytosis by macrophages, we examined bacterial invasion of APCs, human DCs from the lamina propria, and mouse peritoneal macrophages, with and without phagocytosis of *S.* Typhimurium LT2. The

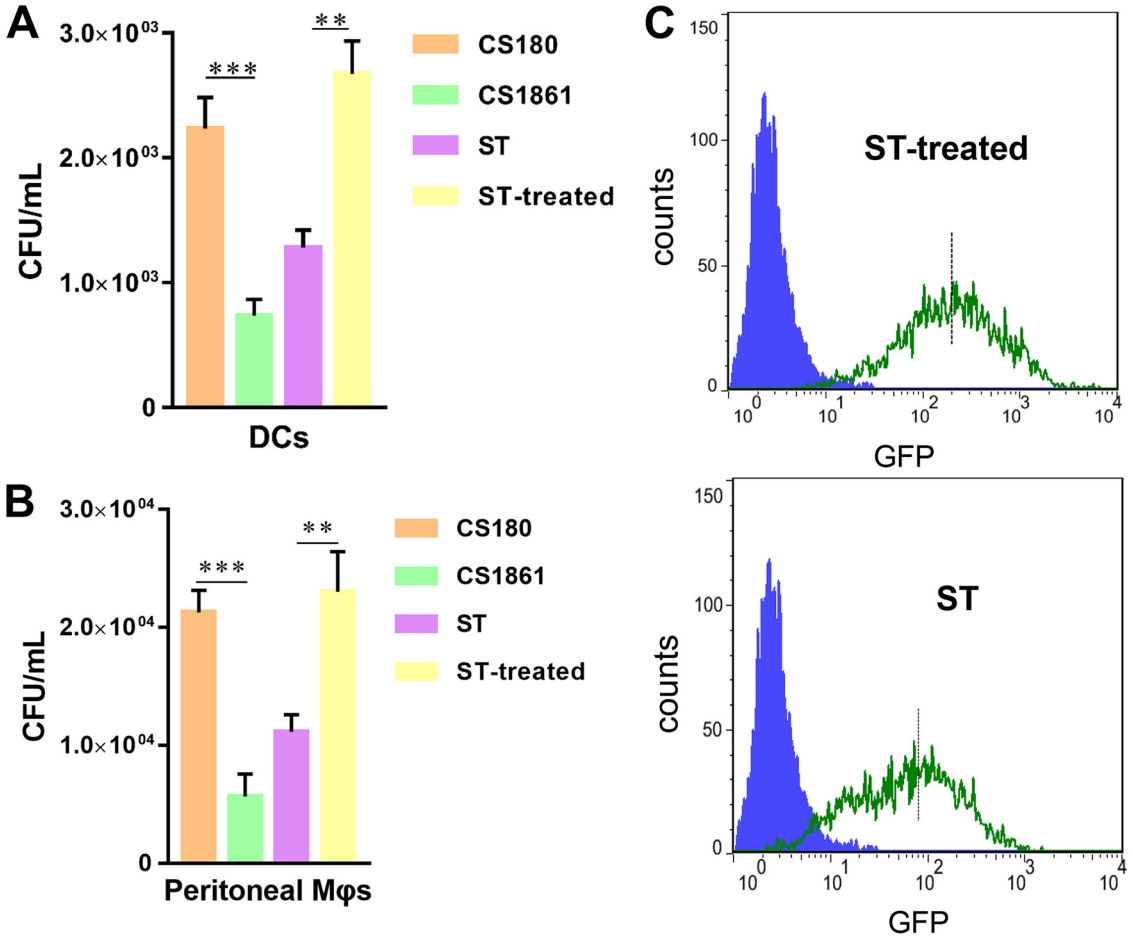

FIG 3 *S.* Typhimurium released from macrophages invaded human DCs from the lamina propria and mouse peritoneal macrophages. Gentamicin protection and flow cytometry-based assays were used to determine the ability of *S.* Typhimurium LT2 and treated *S.* Typhimurium LT2 to invade purified human DCs from the lamina propria and mouse peritoneal macrophages. *E. coli* K-12 CS180 and CS1861 were used as controls. (A and B) Data from the gentamicin protection assay. (C) Results of cytometry according to the ability of *S.* Typhimurium LT2 and treated *S.* Typhimurium LT2 to invade mouse peritoneal macrophages. The blue curve indicates uninfected macrophages, and the green curve indicates macrophages infected by *Salmonella* containing pAcGFP1. The data shown were obtained from three independent experiments (**, $P \leq 0.01$; ***, $P \leq 0.001$; both by Student's *t* test).

E. coli K-12 strains CS180 (with rough LPS) and CS1861 (a CS180 derivative with smooth LPS) were used as controls. We used this pair of strains as controls for the LPS core-CD209 interaction in many of our previous studies (10, 12, 26–30). Gentamicin protection (Fig. 3A and B) and flow cytometry (Fig. 3C) assays were used to determine the rate of invasion. As shown in Fig. 3A and B, *S.* Typhimurium LT2 released from the RAW264.7 macrophage cell line after phagocytosis (treated *S.* Typhimurium LT2) invaded primary macrophages and human gut DCs much more effectively than *S.* Typhimurium without phagocytosis. On the basis of the study regarding *Yersinia pseudotuberculosis* (12), in which the modification of the expression of O-antigen influenced the interaction between the LPS core and APCs, we hypothesized that the exposed LPS core of *S.* Typhimurium contributed to the invasion of primary mouse macrophages and human DCs from guts. Flow cytometry was conducted on DCs because the number of isolated DCs was too low to be detected in this setting.

Human DC-SIGN and mouse SIGN-R1 are receptors for *S.* Typhimurium phagocytosis by macrophages. We have previously shown that human DC-SIGN (hCD209a) is a receptor for the LPS core of *S.* Typhimurium (10). However, our laboratory has evidence showing that mouse SIGN-R1 (mSIGN-R1/mCD209b), not mouse DC-SIGN (mCD209a) or mouse langerin (mCD207), serves as a receptor for the LPS core of other Gram-negative bacteria, such as *Yersinia* spp. (10, 12, 26–28, 30). To investigate whether

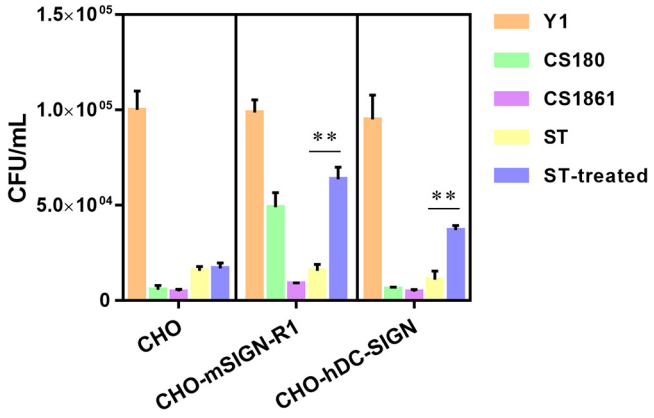

FIG 4 Human DC-SIGN and mouse SIGN-R1 are receptors for *S.* Typhimurium phagocytosis by macrophages. The three sets of bacteria, *E. coli* K-12 strains (CS180 and CS1861), *Y. pseudotuberculosis* cultured at 26°C (Y1), and *S.* Typhimurium (*S.* Typhimurium LT2 [ST] and treated *S.* Typhimurium LT2 [ST-treated]), were used to determine the rate of invasion into CHO/CHO-mSIGN-R1/CHO-hDC-SIGN cells. Data are representative of three independent experiments (**, $P \leq 0.01$ by Student's *t* test).

hDC-SIGN and mSIGN-R1 were responsible for the interactions (Fig. 4), the stably transfected cell lines expressing hDC-SIGN (CHO-hDC-SIGN) and mSIGN-R1 (CHO-mSIGN-R1) were tested for their ability to phagocytose *S.* Typhimurium and *S.* Typhimurium released from macrophages. Again, *E. coli* CS180 and CS1861 bacteria were used as controls. *Y. pseudotuberculosis* grown at 26°C (Y1) was used as a positive control in this experiment because it invades most epithelial cell lines, including CHO (31), via the invasion-integrin interaction (32, 33).

S. Typhimurium released from macrophages invaded the CHO-hDC-SIGN and CHO-mSIGN-R1 cells more effectively than CHO cells (Fig. 4). We then concluded that, similar to hDC-SIGN (10), mSIGN-R1 functions as a receptor for *S.* Typhimurium in mice.

The hDC-SIGN- and mSIGN-R1-mediated phagocytosis of *S.* Typhimurium released from macrophages was inhibited by mannan oligosaccharides. To verify the specificity of the interaction of *S.* Typhimurium with hDC-SIGN and mSIGN-R1, we examined whether the LPS core-CD209 interaction could be inhibited by mannan oligosaccharides, which inhibit the HIV-CD209 and LPS core-CD209 interactions from other Gram-negative bacteria (10, 12, 26–28, 30). *E. coli* K-12 CS180 and Y1, mediating SIGN-R1-dependent and SIGN-R1-independent interactions, respectively, were again utilized as control strains. However, the mannan can only inhibit mSIGN-R1-mediated interaction in a limited fashion (Fig. 5A and B), indicating that

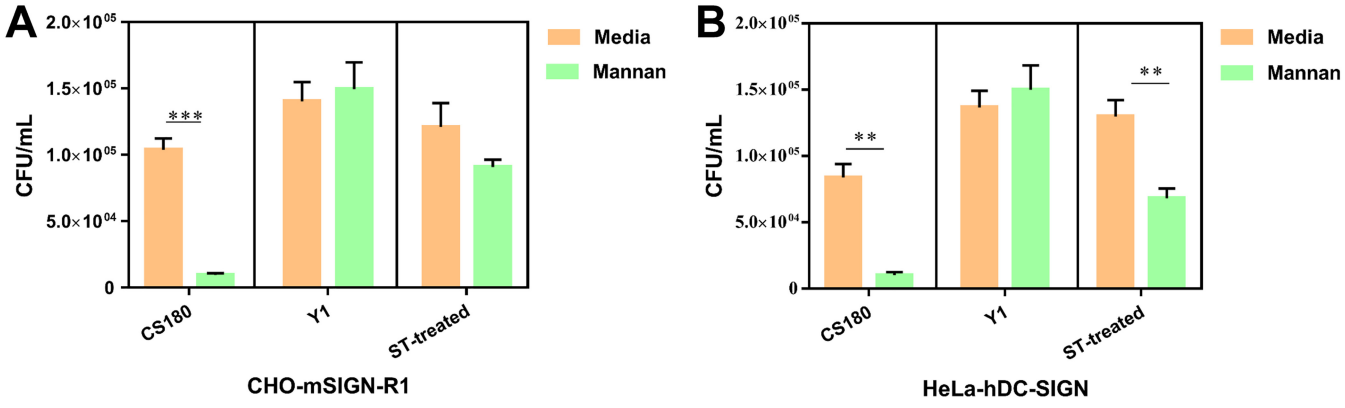

FIG 5 Addition of mannan oligosaccharide inhibits *S.* Typhimurium infection. (A) Treated *S.* Typhimurium LT2 bacteria were incubated with CHO-mSIGN-R1 cells with or without mannan. The rate of phagocytosis of treated *S.* Typhimurium LT2 bacteria was evaluated by the recovery of bacteria from gentamicin protection. *E. coli* K-12 CS180 and Y1 were used as control strains. (B) Mannan was also evaluated for its ability to inhibit the interaction of HeLa-hDC-SIGN cells with treated *S.* Typhimurium LT2 bacteria. Data are representative of three independent experiments (**, $P \leq 0.01$; ***, $P \leq 0.001$; both by Student's *t* test).

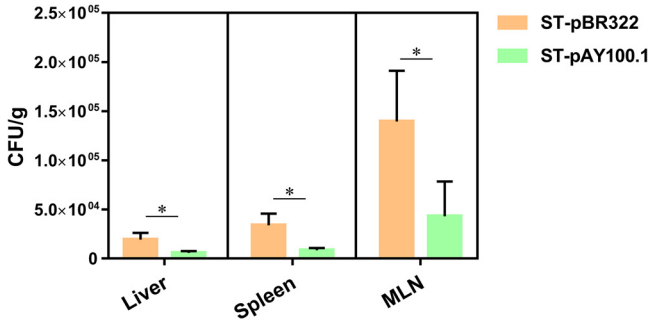

FIG 6 Expression of O-antigen reduced the capacity of *S.* Typhimurium to disseminate to the MLNs, spleen, and liver. ST-pBR322 and ST-pAY100.1 were examined for their ability to disseminate to the lymph node, spleen, and liver. After intragastric inoculation of ST-pBR322 or ST-pAY100.1, mice were euthanized on day 4 postinfection. The spleen, liver, and MLNs of mice then were separated, homogenized, and spread onto LB agar plates. The recovered CFU value was regarded as the rate of bacterial dissemination. The data shown were obtained from three independent experiments (*, $P \leq 0.05$ by Student's *t* test).

hDC-SIGN (hCD209a) and mSIGN-R1 (mCD209b) vary in their interactions with *S.* Typhimurium (10).

Expression of the O-antigen inhibits the dissemination of *S.* Typhimurium. We have shown that after phagocytosis by and release from macrophages, *S.* Typhimurium interacted with hDC-SIGN and mSIGN-R1 receptors, potentially leading to the invasion of APCs. We therefore hypothesized that dissemination would also be a result of this host-pathogen interaction. Subsequently, if the exposed LPS core of *S.* Typhimurium could be shielded, the dissemination of the bacteria to the spleen and liver should be reduced.

ST-pBR322 is *S.* Typhimurium LT2 transformed with the cloning vector plasmid pBR322 (25). Mice were infected via the intragastric route with ST-pAY100.1 and ST-pBR322 bacteria and sacrificed. Liver, MLNs, and spleen were then recovered and homogenized. The dissemination rates of the bacteria into the different organs were calculated by counting CFU. Figure 6 shows that higher numbers of ST-pBR322 than ST-pAY100.1 were isolated from the spleens, MLNs, and livers.

The expression of O-antigen and the addition of mannan reduce the infectivity of *S.* Typhimurium. The LPS core-CD209 interaction in *E. coli* and *Yersinia* spp. can be blocked *in vitro* by several oligosaccharides, including mannan (10). Given that the aforementioned interactions were mediated by the LPS core oligosaccharide, we hypothesized that shielding the exposed LPS core with CD209-interacting oligosaccharides could reduce bacterial infectivity in a murine model. To this end, we tested the influence of mannan on the infectivity of *S.* Typhimurium. The mortality of the mice was monitored. The results clearly demonstrated that the mortality rate (Fig. 7A and B) of mice infected with *S.* Typhimurium in the presence of mannan oligosaccharides was significantly reduced. In addition, the mortality rate did not differ by peritoneal infection (Fig. 7C). It should be noted

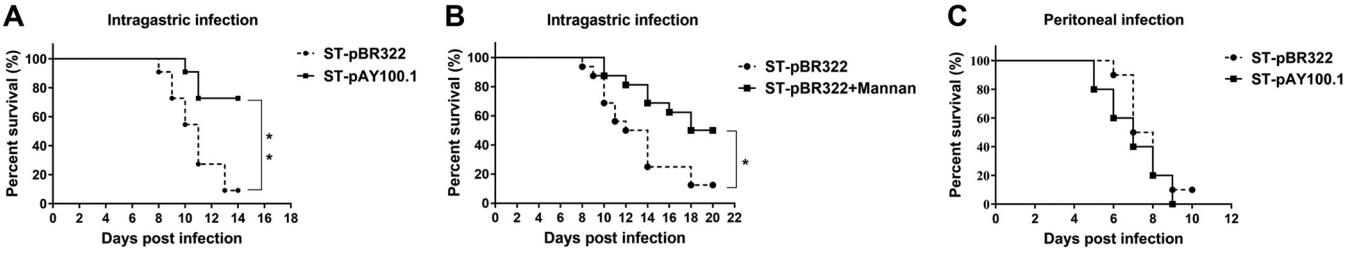

FIG 7 Expression of O-antigen and addition of mannan reduced the mortality of mice caused by *S.* Typhimurium infection. (A) Survival rates of mice ($n = 11$) after intragastric infection with ST-pBR322 or ST-pAY100.1 cultured at 37°C. (B) Survival rates of mice ($n = 16$) after intragastric infection of ST-pBR322 with or without mannan. Up to 10 mg of the C-type lectin receptor antagonist mannan was orally administered to the mice. (C) Survival rates of mice ($n = 10$) after peritoneal infection with ST-pBR322 or ST-pAY100.1 cultured at 37°C. The data shown were obtained from three independent experiments (*, $P \leq 0.05$; **, $P \leq 0.01$; both by the log-rank test).

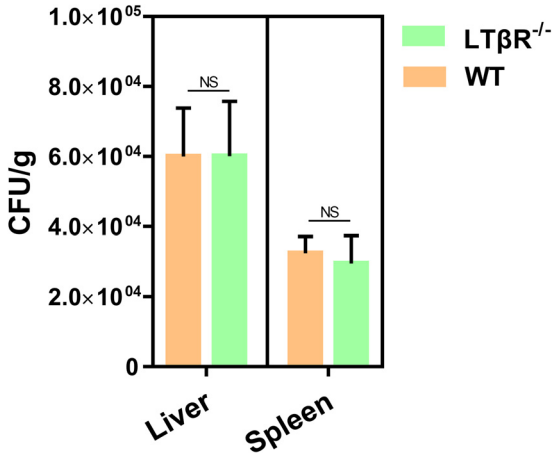

FIG 8 *S.* Typhimurium was able to disseminate to the liver and spleen in Peyer's patch-deficient mice. ST-pBR322 was examined for its ability to disseminate to the spleen and liver of wild-type or lymphotoxin-LTβR-1-deleted mice. After intragastric inoculation of ST-pBR322 or ST-pAY100.1, mice were euthanized on day 4 postinfection. The spleen and liver of the mice were then separated, homogenized, and spread onto LB agar plates. Recovered CFU was considered the rate of bacterial dissemination. The data shown were obtained from three independent experiments (NS, not significant by Student's *t* test).

that the growth rate of *S.* Typhimurium was not influenced by the mannan oligosaccharide, as is the case with other Gram-negative bacteria (10).

In conclusion, the result can potentially elucidate how and why oligosaccharide-based preventive approaches and treatments for certain diarrheal infections work.

Peyer's patches may not play a major role in the dissemination of *S.* Typhimurium. The involvement of Peyer's patches in the host dissemination of *S.* Typhimurium has been well documented (1, 3). However, it is also possible that host APCs, such as macrophages, phagocytose the invading *S.* Typhimurium, facilitating penetration of the first line of defense of the epithelial mucosa. Therefore, we used a Peyer's patch-deficient mouse model carrying a deletion of the lymphotoxin-LTβR-1 gene to examine whether the host dissemination of *S.* Typhimurium involved Peyer's patches (34). Figure 8 shows that the dissemination of *S.* Typhimurium to the liver and spleen was not reduced in Peyer's patch-deficient mice, suggesting that Peyer's patches do not play a major role in the dissemination of *S.* Typhimurium. It should be noted that MLNs are also underdeveloped in LTβR-1 knockout mice (34).

DISCUSSION

As described in textbooks, *Salmonella* spp. are able to disseminate to distal mammalian organs and cause systemic infections (2, 16, 35). However, the molecular mechanisms for *Salmonella* dissemination are not fully understood. In the current study, the data obtained suggest that similar to HIV, *Salmonella* spp. are able to use a mechanism to hijack APCs by interacting with CD209 for bacterial dissemination.

A decade ago, using a series of LPS core mutants of several Gram-negative bacteria, we demonstrated that *S.* Typhimurium utilized its LPS core to interact with human DC-SIGN, resulting in phagocytosis by host cells (10). However, the biological significance of the LPS core–DC-SIGN interaction in *S.* Typhimurium has been a puzzle to us. This is because unlike wild-type *Y. pseudotuberculosis*, which was able to modify expression of its full O-antigen LPS at 37°C (12), *S.* Typhimurium does not expose its LPS core at 37°C (23), and the rough O-antigen knockout strain does not cause infection *in vivo* (unpublished data). This was addressed in studies from T. K. Korhonen's laboratory (23, 24): the expression of O-antigen in *S.* Typhimurium is inhibited inside the macrophages, suggesting that the LPS core of *S.* Typhimurium is exposed during the *in vivo* infection (23, 24). However, this conclusion might be deduced from indirect evidence.

S. Typhimurium possesses the surface protease PgtE, which is not functional until its phagocytosis by macrophages (20, 22–24). While inside macrophages, the PhoP/Q

regulatory system of *S*. Typhimurium is activated, which can lead to the downregulation of O-antigen synthesis *rfb* genes (20). PgtE requires rough LPS for activity but is sterically inhibited by the O-antigen present in smooth LPS (23). Consequently, the shortened O-antigen chain can expose and activate the PgtE enzyme. Hence, *S*. Typhimurium released from macrophages produces an elevated level of PgtE because of a reduced O-antigen chain length.

DC-SIGN (CD209a) and langerin (CD207) have been shown to be receptors for the LPS core ligands of several Gram-negative bacterial species, thereby promoting bacterial adherence and phagocytosis (10–12, 26–28, 30). The present study provides another instance of the biological significance of the interactions. Moreover, its possible biological significance will contribute to future studies in broad fields, as shown below.

(i) We would like to borrow one comment from a reviewer for our recent publication (12): "The work potentially carries a broad application for the dissemination of other enteric pathogens from mucosal tissues to systemic sites." In this context, similar to *Y. pseudotuberculosis*, *Salmonella* spp. rather than *Shigella* spp. are able to modify their expression of full O-antigen LPS into LPS core during infection. Consequently, *Salmonella* spp. may be able to hijack APCs, similar to the infection caused by *Y. pseudotuberculosis*, as described in our recent publication (12). Interestingly, a recent report indicates that *Shigella* spp. are able to disseminate if the expression of the O-antigen or O-antigen-expressing capsule is genetically modified (35).

(ii) *Y. pestis*, the cause of bubonic and pneumonic plague, has directly evolved from *Y. pseudotuberculosis* within the last 10,000 to 20,000 years (36–38). It is well documented that a distinguishing characteristic between the two *Yersinia* species is that *Y. pseudotuberculosis* strains possess an O-antigen (full LPS expression), which was lost by *Y. pestis* during evolution. As a result, after entering the skin via an infected flea, *Y. pestis*, with its LPS core, can directly interact with APCs, leading to phagocytosis of the pathogen (26). As reported in our recent publication (11), the infected APCs consequently serve as "taxis" to deliver *Y. pestis* to the lymph nodes and initiate plague infection (39).

(iii) This work helps to understand the molecular mechanisms by which some methods from tradition or folklore and human milk can prevent diarrheal diseases. Whether they are myth or fact, traditional methods have been practiced to prevent or treat diarrhea for centuries, regardless of culture and country. A quick check of 50 such traditional methods from the Internet reveals that almost all of these recipes contain carbohydrates. Moreover, human breast milk that contains certain oligosaccharides is capable of protecting infants from certain infectious diseases (40, 41). It is well documented that oligosaccharide-based prevention and treatment for infectious diseases have been proposed for many years (42). In this study and our previous publications (10–12, 26), certain oligosaccharides, including the purified LPS core and mannan, are able to block this LPS core-mediated host-pathogen interaction. For instance, blockage of this interaction by certain oligosaccharides is able to reduce the mortality rates attributed to *Y. pseudotuberculosis* and *Salmonella* spp. (this study). As a result, certain oligosaccharides and carbohydrates can prevent diarrhea caused by Gram-negative bacteria, which is why traditional methods are occasionally effective.

(iv) The LPS core-CD209 interaction may contribute to the persistent infections caused by Gram-negative bacteria. The most significant contribution of this study is to reveal a potential new molecular mechanism of several persistent infections, including urinary tract infection (UTI) caused by *E. coli*, Gram-negative bacterium-associated Crohn's disease, typhoid fever, *Helicobacter pylori*-associated chronic atrophic gastritis (ChAG), and chronic infection caused by *Klebsiella* spp. We expect that the loss of O-antigen or modification of LPS during infection renders Gram-negative bacteria less virulent but promotes phagocytosis by APCs. Thus, the phagocytosed bacteria may hide themselves from attacks of various host clearance systems, as shown in *Salmonella* (43).

In summary, the evidence presented in this study suggests that changes in the LPS structure in *S*. Typhimurium lead to the exposure of the LPS core oligosaccharide. The exposed LPS core of *S*. Typhimurium then interacts with CD209s from APCs, such as gut

TABLE 1 Cell lines used in this work

Cell	Characteristic
CHO	Control cell line, which expresses the neomycin resistance gene only
CHO-mSIGNR1	Generated by transfecting CHO cells with mouse SIGNR1 cDNAs for stable surface expression
CHO-hDC-SIGN	Generated by transfecting CHO cells with human DC-SIGN cDNAs for stable surface expression
Hela-hDC-SIGN	Generated by transfecting HeLa cells with human DC-SIGN cDNAs for stable surface expression
Primary macrophage	Primary macrophages from mouse peritoneal cavity
RAW264.7	Macrophage cell line

DCs. As a result, *S.* Typhimurium is carried by the infected APCs to the lymph nodes, spleen, and liver, facilitating bacterial colonization of these tissues and the establishment of host infection. In addition, an important corollary to this study is that certain pathogens may develop mechanisms for exploiting the very host defenses designed to eliminate them, resulting in the expanded ability to disseminate. This host-pathogen interaction is not restricted to one species; thus, the findings presented in this study may carry broad applications and understanding of host dissemination. Most importantly, as suggested in the commentary article to our publication in 2015 (30, 44), our work may start a new therapy or prevention strategy for certain Gram-negative bacterial infections by simply blocking the interactions between C-type lectins and LPS core with certain oligosaccharides or carbohydrates.

MATERIALS AND METHODS

Ethics statement. All animal procedures were carried out in strict accordance with the standards of the People's Republic of China. The handling of the mice and all experimental procedures were specifically approved for this study by the Medical Ethics Committee of Tongji Hospital (IRB ID TJ-A20141220 for animal experiments). All procedures on mice were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Reagents. Mannan, the ligand antagonist of the human mannose receptor, was purchased from Sigma (St. Louis, MO). Collagenase D, Ficoll-Paque, human Glu-plasminogen (Hematologic Technologies), and the chromogenic plasmin substrate S-2251 (Chromogenix) were also used.

Bacterial strains. *Salmonella enterica* serovar Typhimurium, specifically *S.* Typhimurium LT2, was described previously (10). ST-pBR322 is *S.* Typhimurium LT2 transformed with the plasmid pBR322. ST-pAY100.1 was constructed by transforming *S.* Typhimurium with the pAY100.1 plasmid, which carries the genes necessary for *Y. enterocolitica* O-antigen expression. ST GFP is *S.* Typhimurium transformed with the green fluorescent protein-expressing plasmid pAcGFP1 (Clontech).

CS180 is an *E. coli* K-12 strain with no O-antigen expression. CS1861 is an *E. coli* K-12 strain with the pss37 plasmid, which is conjugated with genes encoding *Shigella dysenteriae* 1 O-antigen (45, 46).

Y. pseudotuberculosis Y1 is a serotype O:1a strain lacking the virulence plasmid (pYV). This strain originates from the Centers for Disease Control and Prevention (CDC) in the United States and has been used as a positive control for invasion previously (47).

Host cell lines. CHO-mSIGN-R1 cells stably transfected with mouse C-type lectin were constructed by transfecting CHO cells (purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) with a mouse SIGN-R1 plasmid (Table 1). Positive transfected cells were selected using G418 according to previous procedures. The cell lines were recently used for the identification of LPS core from several Gram-negative bacteria as ligands for the DC-SIGN receptor (12, 29).

Mice. Female C57BL/6J mice, used at 8 to 12 weeks of age, were purchased from the Wuhan University Animal Center. Mice were housed in the animal facilities of Tongji Hospital in direct accordance with the guidelines drafted by the Animal Care Committees of Tongji Hospital.

Adherence and phagocytosis assays. The assays for adherence and phagocytosis were performed in accordance with a previously described protocol (31, 48, 49). First, 0.5 ml of host cells and macrophages was plated in 24-well plates at a concentration of 4×10^5/ml, suspended in RPMI with 2% fetal bovine serum (FBS). Subsequently, 50 μl of bacterial suspension at a concentration of 1×10^7 CFU/ml was added to the cells and incubated for 2.5 h (2 h for mouse peritoneal macrophages) at 37°C. The cell monolayers were then washed 3 times with phosphate-buffered saline (PBS). Gentamicin, which kills extracellular bacteria but cannot penetrate host cells, was added into each well to a final concentration of 100 μg/ml and incubated for 60 min. To remove the antibiotics, the cell monolayers were washed twice. PBS containing 0.2% Triton X-100 was added to each well of the plate, and the samples were then diluted and plated on LB. Bacterial internalization in these host cells was calculated by determining the CFU recovered from lysed cells. All experiments were performed in triplicate, and the data are expressed as the means $\pm$ standard errors. Statistical significance was calculated using Student's *t* test.

Determination of phagocytosis by flow cytometry. *S.* Typhimurium transformed by green fluorescent protein-expressing plasmid pAcGFP1 was added to macrophage cultures for 2 h. Cell cultures were incubated with 100 μg/ml gentamicin for 1 h to remove unbound bacteria. The macrophages were fixed with 4% paraformaldehyde. The rate of bacterial internalization was determined by comparing the

intensity of fluorescence-positive macrophages with various bacteria. The higher the fluorescence intensity, the more bacteria were phagocytosed by macrophages.

Animal challenge for the dissemination assay. The dissemination rate was defined as the transport of *S.* Typhimurium to the lung, MLNs, liver, and spleen. Mortality was monitored after the inoculation of pathogens. Female C57BL/6J mice, 6 to 8 weeks of age, were randomly divided into two groups. The day before the infection, the mice were deprived of water and food for 4 h and orally given 20 mg streptomycin (200 mg/ml in 100 μl). On the day of infection, the mice were deprived of water and food for 4 h prior to the infection. Before the infection, the optical density (OD) values of ST-pBR322 and ST-pAY100.1 were adjusted to an OD at 600 nm (OD_{600}) of 0.3 in 0.9% NaCl. Thirty minutes before infection, the mice were intramuscularly injected with ampicillin at a final concentration of 50 μg/g mouse body weight to maintain the expression of O-antigen in the plasmid. After injection, the mice were infected via the intragastric route with ST-pBR322 and ST-pAY100.1, at an OD_{600} of 0.3, in 300 μl. Immediately after the infection, the mice received drinking water, and 2 h after the infection, the mice were given food. Organs, including the liver, spleen, and MLNs, were collected after 4 days of infection, homogenized, and spread on LB agar plates with 50 μg/ml ampicillin. The total number of isolated CFU of organs per mouse was considered the dissemination rate.

Plasminogen activation assay of released *S.* Typhimurium. RAW264.7 cells were grown at a concentration of 2×10^6 cells/ml in five 10-cm^2 cell culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The cells were washed with PBS, and 2 ml of DMEM with 2% FBS was added. Six ml of *S.* Typhimurium 14028 grown to stationary phase then was added to RAW264.7 cells in each well at an OD_{600} of 1 and incubated for 45 min. After washing with PBS, extracellular bacteria were killed by treatment with gentamicin (100 μg/ml in 2% FBS DMEM) for 1 h, and the cells were incubated for 20 h in 10% FBS fresh medium containing 10 μg/ml gentamicin. After washing, the cells were lysed with 0.75% Triton X-100 for 5 min, and the lysates were pelleted, washed, and suspended in PBS. The dose of bacteria was confirmed by measuring the OD_{600} and plating on LB agar following incubation at 37°C for 1 day. Plasminogen activation measurement was performed as described previously (23, 50). Briefly, *S.* Typhimurium suspended in PBS was mixed with 4 μg of human Glu-plasminogen and 0.45 mM S-2251 in 96-well plates with a 200-μl total volume at 37°C. The absorption values at 405 nm were measured at 30-min intervals. The results are presented as the difference between each measurement value and the starting value.

Purification of gut DCs. Purification of gut DCs has been described previously (51). Colonic biopsy specimens were obtained from patients with ulcerative colitis (UC) undergoing a survey. Samples were collected in an ice-cold Dutch modification of RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, gentamicin (25 μg/ml), and penicillin-streptomycin (100 U/ml). The samples were incubated for 20 min at room temperature in calcium and magnesium-free Hanks' balanced salt solution (HBSS) containing 1 mM dithiothreitol. To remove the epithelium, biopsy specimens were transferred to HBSS containing 1 mM EDTA and incubated for 30 min on a shaker at 37°C. To continue the isolation of gut DCs, the tissue was digested with 1 mg/ml collagenase D in HEPES-buffered RPMI 1640 containing 20 μg/ml DNase I and 2% fetal calf serum at 37°C on a shaker for 90 to 180 min. Mononuclear cells were separated ($650 \times g$, 20 min, room temperature) on Ficoll-Paque and washed in complete medium. The isolated cells were labeled with anti-CD11c and anti-human DC-SIGN antibodies and then examined by flow cytometry.

Statistical analyses. All statistical analyses were conducted using Prism software, version 5. Statistical analyses were performed using Student's *t* test. Survival group comparison was performed via the log-rank (Mantel-Cox) test. A *P* value of 0.05 was considered the threshold for statistically significant differences.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00100-19.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (NSFC 81271780 and 81471915 [T.C.] and 81602722 [L.-Y.J.]) and by two local grants from Tongji Hospital, Tongji Medical College (T.C.). C.G.P. was supported by grants from the National Research Foundation of Korea (NRF-2013R1A1A2058427 and NRF-2014R1A4A1008625) and a faculty research grant from Yonsei University College of Medicine for 2014 (6-2014-0062).

We deeply thank Olivier Schwartz at the Pasteur Institute, Paris, for his support in initiating the project on DC-SIGN and LPS core interactions. Finally, we specifically thank the Tongji Hospital, HUST, for support that allowed this project to continue.

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