Loss of the virulence plasmid by *Shigella sonnei* promotes its interactions with CD207 and CD209 receptors

Bi-cong Wu¹,², Njiri A. Olivia¹,³, John Mambwe Tembo¹,⁴, Ying-xia He¹,⁵, Ying-miao Zhang¹,⁶, Ying Xue¹, Cheng-lin Ye¹, Yin Lv¹, Wen-jin Li¹, Ling-Yu Jiang¹, Xi-xiang Huo⁷, Zi-yong Sun⁸, Zhong-ju Chen⁸, Ji-chao Qin⁹, An-yi Li¹, Chae Gyu Park¹⁰, John D. Klena¹¹, Hong-hui Ding¹* and Tie Chen¹*

Abstract

**Introduction.** *Shigella sonnei*, the cause of bacillary dysentery, belongs to Gram-negative enteropathogenic bacteria. *S. sonnei* contains a 210 kb virulence plasmid that encodes an O-antigen gene cluster of LPSs. However, this virulence plasmid is frequently lost during replication. It is well-documented that after losing the O-antigen and becoming rough strains, the Gram-negative bacteria may express an LPS core on its surface. Previous studies have suggested that by using the LPS core, Gram-negative bacteria can interact with several C-type lectin receptors that are expressed on antigen-presenting cells (APCs).

**Hypothesis/Gap Statement.** *S. sonnei* by losing the virulence plasmid may hijack APCs via the interactions of LPS-CD209/CD207.

**Aim.** This study aimed to investigate if the *S. sonnei* rough strain, by losing the virulence plasmid, interacted with APCs that express C-type lectins of human CD207, human CD209a and mouse CD209b.

**Methodology.** SDS-PAGE silver staining was used to examine the O-antigen expression of *S. sonnei* WT and its rough strain. Invasion assays and inhibition assays were used to examine the ability of *S. sonnei* WT and its rough strain to invade APCs and investigate whether CD209 and CD207 are receptors for phagocytosis of rough *S. sonnei*. Animal assays were used to observe the dissemination of *S. sonnei*.

**Results.** *S. sonnei* did not express O-antigens after losing the virulence plasmid. The *S. sonnei* rough strain invades with APCs, including human dendritic cells (DCs) and mouse macrophages. CD209 and CD207 are receptors for phagocytosis of rough *S. sonnei*. Expression of the O-antigen reduces the ability of the *S. sonnei* rough strain to be disseminated to mesenteric lymph nodes and spleens.

**Conclusion.** This work demonstrated that *S. sonnei* rough strains – by losing the virulence plasmid – invaded APCs through interactions with CD209 and CD207 receptors.
**INTRODUCTION**

*Shigella* spp., the cause of bacillary dysentery, belong to Gram-negative invasive enteropathogenic bacteria that can penetrate mucosal surfaces of guts. The ingestion of as few as 100 bacteria is enough to cause bacillary dysentery [1]. Shigellosis represents a significant public health burden in developing countries, with about 160 million cases occurring annually, predominantly in children under the age of 5 years [2]. The *Shigella* genus comprises four species, including *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*, and *S. flexneri* and *S. sonnei* cause the most infections [3]. The ratio of species dominance is highly dependent on the socio-economic conditions of an area. In developing countries, including those of sub-Saharan Africa and certain countries in Asia, *S. flexneri* is the dominant cause of shigellosis, responsible for over 60% of infections [4]. In developed countries, such as Europe and North America, *S. sonnei* causes around 80% of shigellosis cases [5]. Countries undergoing socio-economic improvements are also experiencing a change in infections from *S. flexneri* to *S. sonnei* [6]. It appears that the frequency of *S. sonnei* isolation directly correlates with per capita gross domestic product (GDP) [7]. The primary cause of this association is not fully understood. One hypothesis is that exposure to unsanitized drinking water systems in developing countries results in *Plesiomonas shigelloides* infection, and hence in natural immunity against *S. sonnei*. *P. shigelloides* serotype O17 has a lipopolysaccharide O-antigen identical to that of *S. sonnei* [8]. Also, *S. sonnei* has been shown to possess a functional type 6 secretion system (T6SS), which provides a niche-specific competitive advantage for *S. sonnei* over *S. flexneri* [6].

Most Gram-negative enteropathogenic bacteria contain LPSs located in the outer membrane. The LPSs are composed of three covalently linked domains: lipid A, which is embedded in the outer membrane; the oligosaccharide core (Fig. 1); and the O-polysaccharide or O-antigen, which cover the bacterial surface [9]. The O-antigen is one of the essential components for bacterial survival during infection. For example, *Salmonella enterica*, *Francisella tularensis* and *Burkholderia cepacia* utilize the O-antigen to avoid phagocytosis and to resist lytic action of the complement system [10–12]. Unlike *S. flexneri*, the genes for O-antigen in *S. sonnei* are located on the large 210 kb virulence plasmid.

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**Fig. 1.** Structures of inner- and outer-core regions of the LPS or LOS of *E. coli* K12, *S. typhimurium* and *S. sonnei* and the genes involved in their synthesis. Genes involved in the biosynthesis of core LPS are shown at their approximate site of action (solid line). The sites, which are variably substituted or still under investigation, are indicated by dashed lines. The abbreviations in this figure are as follows: GlcNAc, N-Acetylglucosamine; Glc, glucose; Hep, heptose; Gal, galactose; P, phosphate; PPEtn, phosphoethanolamine; KDO, 2-keto-3-deoxyoctonate. It should be noted that *E. coli* K12 does not possess O-Ag.
Table 1. Bacterial strains and cell lines used in this study

| Strains               | Genotypes (phenotypes) | Refs                      |
|-----------------------|------------------------|---------------------------|
| **E. coli K12**       |                        |                           |
| CS180                 | WT (rough)             | [29, 53, 54]              |
| CS1861                | CS180-O antigen (smooth)|                          |
| **Y. pseudotuberculosis** |                        |                           |
| Y1                    | O:1a, WT expressing invasin but with PYV plasmid naturally cured (smooth) | [30–32, 55]          |
| **S. sonnei**         |                        |                           |
| Ss-WT                 | WT (smooth)            | This study                |
| Ss-rough              | without 210 kb virulence plasmid (rough) | This study            |
| Rough O+              | S. sonnei-rough with pSS37 (smooth) | This study            |
| **Cell lines**        |                        |                           |
| CHO-NEO cells         | Control cell line, which expresses the neomycin resistance gene only |                      |
| CHO-mSIGNR1           | Generated by transfecting CHO cells with mSIGNR1 cDNAs for stable surface expression |                      |
| CHO-hDC-SIGN          | Generated by transfecting CHO cells with hDC-SIGN cDNAs for stable surface expression |                      |
| CHO-hLangerin         | Generated by transfecting CHO cells with hLangerin cDNAs for stable surface expression |                      |

involved in invasion [13]. However, the large plasmid of S. sonnei is unstable and easily lost. Why does S. sonnei carry genes that control the synthesis of the somatic antigen and the expression of virulence on an unstable plasmid, and what are selective pressures to maintain the virulent state [14]? Maybe, S. sonnei has evolved to lose the virulence plasmid as an advantage during infection [15].

Rough Gram-negative bacteria bear shortened LPSs – referred to as lipooligosaccharides (LOS) – for which the oligosaccharide core is exposed to the extracellular environment [9]. Previous studies suggested that by using the LPS core, Gram-negative bacteria can interact with human Langerin (CD207) and human DC-SIGN (CD209), expressed on antigen-presenting cells (APCs) [16–22]. It is well-documented that human DC-SIGN is also a receptor for HIV GP-120 that hijacks DC-SIGN to be captured by APCs and trafficked to target cells such as CD4 lymphocytes [23–25].

*Shigella* initially crosses the epithelial layer of the colorectal mucosa via M cells. M cells are capable of transcytosing luminal antigens into the subepithelial space where gut-associated lymphoid tissue and/or APCs, such as dendritic cells (DCs) and macrophages, are located [26]. The APCs of colonic and rectal mucosa can act as ‘Trojan horses’ allowing crossing of the intestinal epithelial barrier [27, 28].

In this study, we investigated if *S. sonnei* rough, rather than smooth, strains interacted with human DCs from guts, murine primary macrophages and the CHO cell lines that express C-type lectins of human CD207, human CD209a and mouse CD209b.

**METHODS**

**Bacterial strains**

Bacterial strains used in this study are listed in Table 1. *E. coli* K12 strain CS180 contains core oligosaccharides but lacks the O-antigen. CS1861 is the strain of CS180 harbouring pSS37, a plasmid containing all genes necessary for expression of the *Shigella dysenteriae* serotype 1 O-antigen [29]. *E. coli* strains were cultured on Luria–Bertani (LB) agar at 37 °C overnight. *Y. pseudotuberculosis* (Y1) is a serotype O:1a strain, lacking the virulence plasmid (pYV); the strain was obtained from the Centers for Disease Control (GA, USA), and used as a control strain for invasion since this bacterium invades almost all epithelial cell lines via an invasin-integrin interaction [30–32]. This strain has been used as a positive invasion control previously and was cultured on LB agar at 26 °C overnight [33]. *S. sonnei*, a clinical strain isolated from a patient with dysentery in Wuhan, PR China, was incubated in tryptic soy broth (TSB) at 37 °C overnight. *S. sonnei* rough strain without the 210 kb virulence plasmid was obtained from TS broth containing 0.6% yeast extract with 1.5% agar and 0.003% Congo red [15, 34]. Rough O+ is a derivative of *S. sonnei* rough strains that harbour pSS37, a plasmid containing all of the genes necessary for expression of the *S. dysenteriae* serotype 1 O-antigen [29]. The three *S. sonnei* strains are isogenic. *S. sonnei* rough and rough O+ strains were cultured on LB agar with the corresponding antibiotic at 37 °C overnight.

**Cell lines**

Cell lines used in this study are listed in Table 1. CHO-mSIGNR1, CHO-hDC-SIGN and CHO-hLangerin cell lines were generated by transfecting CHO cells with corresponding C-type lectin cDNAs. Transfection was followed by G418 (1.5 mg ml−1) selection and screening for stable surface expression [35]. CHO-NEO cells were used as a control cell line that expresses the neomycin resistance gene only.

**LPS isolation and SDS-PAGE silver staining**

The LPS extracts were isolated using the Lipopolysaccharide Extraction Kit (iNtRON Biotechnology, Korea),
Isolation of mouse peritoneal macrophages

The experiments for isolating mouse peritoneal macrophages have been described previously [20]. After the mice were euthanized, abdomens were immediately exposed, cleaned with 75% ethanol and opened with scissors; 5 ml of RPMI was injected into the intraperitoneal cavity. The mouse abdomen was gently massaged for 2 min and then lavage fluid was collected. The suspension containing the macrophages was seeded onto six-well plates, in which each well contained a 1.5 cm diameter glass cover-slide, and placed in a CO₂ incubator for 1.5 h. The cell layers were washed three times to remove non-adherent cells. Macrophages were then removed from the plastic surface by incubating with citrate saline and re-seeded for interaction assays.

Isolation of human gut DCs

Human intestinal segments were obtained from patients who were undergoing gastrointestinal surgery and provided informed consent. Purification of gut DCs has been described previously [22, 36]. Samples were collected in ice-cold Dutch modification of RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, gentamycin (25 μg ml⁻¹), and penicillin/streptomycin (100 U ml⁻¹). The samples were incubated for 20 min at room temperature in calcium and magnesium-free HBSS containing 1 mM EDTA. To remove the epithelium, biopsies 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⁻¹ collagenase D in HEPES-buffered RPMI 1640 containing 20 μg ml⁻¹ DNase I and 2% FCS at 37°C on a shaker for 90–180 min. Mononuclear cells were separated (650 g, 20 min, room temperature) on Ficoll-Paque and washed in complete medium. The isolated cells were labelled with anti-CD11c⁺ (Biolegend) and anti-hDC-SIGN antibodies (Pharmingen) and then examined by flow cytometry.

Invasion assays

The invasion assays have been described previously [37]. Briefly, host cells (CHO, hLangerin, mSIGNR1 and hDC-SIGN) were plated in a cell culture flask. The degree of cell fusion was 80%. The cells were suspended in RPMI medium supplemented with 2% foetal calf serum at a concentration of 1×10⁶ ml⁻¹ and were added to 24-well plates; 12–24 h later, cells were washed three times. The 500 μl RPMI medium supplemented with 2% foetal calf serum was added to 24-well plates. After the addition of 50 μl of bacterial suspensions at a concentration of 5×10⁶ c.f.u. ml⁻¹, cells were incubated for 2 h at 37°C in the presence of 5% CO₂.

To determine the internalization of bacteria, gentamicin – which kills extracellular bacteria but cannot penetrate host cells – was added to each well at a final concentration of 100 μg ml⁻¹ and the cultures were incubated for 60 min. The cells were washed three times to remove the gentamicin. Cells were suspended in phosphate-buffered saline containing 0.25% Triton, after which the cells were diluted and plated on LB plates with corresponding antibiotics. The level of internalization of bacteria in the host cells was calculated by determining the c.f.u. recovered from lysed cells. All experiments were performed in triplicate, and the data are expressed as means±standard errors.

For the inhibition assay, the concentration of cells was 2×10⁶ ml⁻¹. Anti-mSIGNR1 (5 μg ml⁻¹) antibody, anti-hLangerin (5 μg ml⁻¹) antibody (Pharmingen), and mannann (500 μg ml⁻¹) (Sigma-Aldrich) were added 20 min before the addition of bacteria. The concentrations used were determined based on our preliminary data and were selected based on the fact that, at these concentrations, the compounds exerted no effects on the survival of bacteria or host cells, as previously shown [17, 18].

Statistical analyses

All statistical analyses were performed using Prism software, version 6 (Graph Pad, San Diego, CA, USA). Statistical significance was assessed using Student’s t-test for the univariate analysis of two sets of data and two-way ANOVA for multiple comparisons. P <0.05 was considered statistically significant.
RESULTS

*S. sonnei* without the virulence plasmid did not express the O-antigen

SDS-PAGE silver staining was used to examine the O-antigen expression of *S. sonnei* WT and its rough strain. The *E. coli* strains K-12 CS180 (lacking the O-antigen) and CS1861 (CS180 expressing an O-antigen) were used as controls [29]. As shown in Fig. 2, the LPS extract of *S. sonnei* WT had a typical LPS ladder with a predominant chain length of 20 to 25 O-Ag repeating units; *S. sonnei* rough strain (lacking the O-antigen-encoding virulence plasmid) had only the low molecular weight band corresponding to the LPS core-lipid A moieties. For rough O+, which harboured the pSS37 derivative of CS1861, the O-Ag repeating units were identical to CS1861.

Rough *S. sonnei* invades human DCs and mouse macrophages

We examined the ability of *S. sonnei* WT and its rough strain to invade DCs. The *E. coli* strains K-12 CS180 (an avirulent strain with the core LPS exposed) and CS1861 (CS180 expressing an O-antigen) were used as controls [17, 39]. Results from the gentamicin protection assays (Fig. 3a, b) showed that all of these *S. sonnei* strains were phagocytosed by DCs to some extent, and *S. sonnei* rough strains were taken up more than WT. Previously, it has been reported that the central hydrophobic portion of IpaC on virulence plasmids, the membrane-spanning domain, was critical for entry of *Shigella* into macrophages [40], suggesting that *S.
sonnei rough strains use different invasive mechanisms to hijack DCs.

**CD209 and CD207 are receptors for phagocytosis of rough S. sonnei**

To further study the interaction of DCs with S. sonnei rough strains, stably transfected mSIGNR1/hDC-SIGN/hLangerin CHO cell lines were used to test their ability to bind and internalize S. sonnei rough strains. *Y. pseudotuberculosis* (Y1) was used as a control since it invaded almost all epithelial cell lines via an invasion-integrin interaction [18, 31, 33]. Results showed that both CS180 and S. sonnei rough strains effectively invaded CHO-mSIGNR1 (Fig. 4a), CHO-hDC-SIGN (Fig. 4b) and CHO-hLangerin (Fig. 4c), but not the CHO. This demonstrated that S. sonnei rough strains interacted with mSIGNR1, hDC-SIGN and hLangerin, as the S. sonnei rough-mediated invasion was also blocked by the expression of O-antigen.

**Inhibition of receptor-mediated phagocytosis of S. sonnei rough strain by an anti-mSIGNR1/anti-hLangerin antibody**

To verify specific interactions of S. sonnei rough and hCD207/CD209 receptors, anti-mSIGNR1/anti-hLangerin antibodies and mannan (an antagonist of mannose receptors) were used. As shown in Fig. 5, when antibodies were applied, the phagocytosis of S. sonnei rough strains by transfected CHO cell lines was significantly reduced. This suggests that the mSIGNR1 and hLangerin have a role in the interaction between DCs and S. sonnei rough strains. Mannan still inhibited the interactions of CS180 with CD209 and hCD207 (Fig. 5a), as shown in our previous publications [18–20]. However, mannan did reduce the interaction of S. sonnei rough strains and CD209, but not the hCD207 receptor (Fig. 5b), suggesting that in addition to the LPS core other sugar residues exposed on the surfaces of S. sonnei rough strains may also mediate their interactions.
Expression of O-antigen reduces the ability of S. sonnei rough strain to be disseminated to mesenteric lymph nodes and spleens

To determine whether S. sonnei can be disseminated to local lymph nodes and spleens, mice were infected via the intraperitoneal route. Fig. 6 shows that all S. sonnei WT, rough and rough O+ strains were isolated in spleens and MLNs, but S. sonnei rough strains were recovered from spleens and MLNs in higher numbers than rough O+ strains. indicating that the expression of O-antigen reduced the ability of S. sonnei to be disseminated. Most likely, because of the invasive genes on virulence plasmids, S. sonnei WT had a strong ability to be disseminated.

DISCUSSION

C-type lectins have been studied for their interactions with bacteria, viruses and parasites [41–43]. Both DC-SIGN and Langerin recognize carbohydrate structures with high mannose specificities, therefore, both bind HIV-1 gp120 [44]. Langerin induces the formation of the Birbeck granules in Langerin’ cells, and mediates HIV-1 degradation, whereas DC-SIGN mediates HIV-1 transmission [45, 46]. DC-SIGN can bind and readily internalize rough E. coli, Klebsiella pneumonia, Mycobacterium tuberculosis, S. typhimurium, Y. pestis and Y. pseudotuberculosis [17, 18, 20, 39, 47–49]. This CD209-LPS core leads to bacterial dissemination and persistent infection in general [19–22]. We, however, found that DC-SIGN/Langerin mediated the phagocytosis of S. sonnei rough strains, which was not able to cause disease after losing the virulence plasmid [14].

This virulence plasmid is essential for S. sonnei pathogenicity [50]. Specifically, infection and dissemination are tightly orchestrated by the IpaB and VirG proteins encoded on the virulence plasmid [51]. After losing the virulence plasmid, S. sonnei rough strains fail to escape from macrophages [40], and the CD209/hCD207-S. sonnei rough interactions most likely result in the clearance of rough S. sonnei during infection. As shown in Fig. 6, immune organs, such as spleens and MLNs, displayed higher levels of S. sonnei rough internalization compared to rough O+. Functioning as the shield for the O-antigen, it appears that recovery of rough O+ bacteria from spleens and MLNs is seen as an indication of partial virulence.

However, a recent paper indicated that deletion of toxin-antitoxin systems in the evolution of S. sonnei as a host-adapted pathogen reduces the metabolic burden for S. sonnei growth, reflecting the ongoing transition of S. sonnei into an obligate pathogen that is less dependent on survival outside a mammalian host than other species of Shigella [15]. Moreover, intracellular Shigella can remodel its LPS, dampen the innate immune recognition, and evade inflammasome activation [1]. Recent studies by Lugo-Villarino et al. demonstrated that DC-SIGN has an anti-inflammatory role in macrophages in response to pathogens [52]. Therefore, based on the CD209/hCD207–S. sonnei rough interactions – most likely result in the clearance of rough S. sonnei – we hypothesize that S. sonnei rough strains may provide for less immune...
system-based killing for losing the plasmid during infection (which contributes to S. sonnei WT pathogenicity). Future studies may focus on verifying the mechanisms of immune system-based killing for losing the plasmid and helping the survival of WT S. sonnei.

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### Author contributions
B.C.W., H.H.D. and T. C. designed the experiment and scrutinized the manuscript. B.C.W., N.A.O. and J.M.T. performed the experiments. Y.M.Z., Y.X.H., W.J.L., Y.X., X.X.H., Z.Y.S., Z.J.C., J.C.Q., C.G.P. and A.Y.L. contributed reagents, materials and analysis tools. B.C.W., Y.M.Z., Y.X.H., Y.X., Y.L., H.H.D. and T.C. analysed the data. B.C.W., J.D.K., H.H.D. and T.C. were involved in drafting the manuscript. The authors were given final approval of the version to be published and agreed to be accountable for all aspects of the work.

### Conflicts of interest
The authors declare that there are no conflicts of interest.

### Ethical statement
All animal procedures and human experiments were carried out in strict accordance with the Institutional Animal Care and Use Committees (IACUCs) and Institutional Review Board (IRB) of Tongji Hospital, Tongji Medical College, China. The handling of the mice and all experimental procedures were specifically approved for this study by the Medical Ethics Committee of Tongji Hospital and were conducted in accordance with the institutional guidelines (IRB ID: TJ-A20141220 for animal experiments and TJCC20140113 for human experiments). All procedures on mice were performed under sodium pentobarbital anaesthesia, and all efforts were made to minimize suffering. And all the volunteers involved in the experiment have signed informed consent.

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