O-Antigen Delays Lipopolysaccharide Recognition and Impairs Antibacterial Host Defense in Murine Intestinal Epithelial Cells

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Abstract

Although Toll-like receptor (TLR) 4 signals from the cell surface of myeloid cells, it is restricted to an intracellular compartment and requires ligand internalization in intestinal epithelial cells (IECs). Yet, the functional consequence of cell-type specific receptor localization and uptake-dependent lipopolysaccharide (LPS) recognition is unknown. Here, we demonstrate a strikingly delayed activation of IECs but not macrophages by wildtype Salmonella enterica subsp. enterica sv. (S.) Typhimurium as compared to isogenic O-antigen deficient mutants. Delayed epithelial activation is associated with impaired LPS internalization and retarded TLR4-mediated immune recognition. The O-antigen-mediated evasion from early epithelial innate immune activation significantly enhances intraepithelial bacterial survival in vitro and in vivo following oral challenge. These data identify O-antigen expression as an innate immune evasion mechanism during apical intestinal epithelial invasion and illustrate the importance of early innate immune recognition for efficient host defense against invading Salmonella.

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Introduction

Lipopolysaccharide (LPS) is an obligate constituent of the outer membrane of gram-negative bacteria. It is composed of three parts - a conserved lipid A, a short core carbohydrate, and the O-antigen assembled by a variable number of highly polymorphic carbohydrate subunits [1]. The lipid A consists of a hexa-acylated disaccharide. It is the ligand of the innate immune receptor Toll-like receptor (TLR) 4 and represents one of the most potent immunostimulatory molecules. TLR4-mediated LPS recognition provides an important signal for activation of the antimicrobial host defense during bacterial infection [2,3]. The O-antigen confers resistance to serum complement activation during systemic infection [13,14]. The intracellular localization of TLR4 in epithelial cells is not well characterized [15,17,19–22]. In contrast TLR4 is restricted to an intracellular compartment in IEGCs [13,15]. The intracellular localization of TLR4 has been confirmed in pulmonary, renal and corneal epithelial cells as well as endothelial cells [15,17,19–22], the functional consequence of the different cellular localization of the TLR4 molecule and the functional role of ligand internalization is unknown.

Transcription, transcriptional activation, and the production and secretion of proinflammatory mediators. Beside professional immune cells, also other cell types such as epithelial cells express functionally active innate immune receptors [8,9]. Lack of TLR4 signaling has been associated with enhanced susceptibility to microbial challenge, increased tissue destruction during mucosal injury and cancerogenesis within the intestinal tract [10,11,12]. Strikingly, the subcellular localization of TLR4 has been demonstrated to differ between macrophages and intestinal epithelial cells (IEC) [13]. Myeloid cells harbor TLR4 on the cell surface and ligand recognition and signaling occur from the plasma membrane [14]. In contrast TLR4 is restricted to an intracellular compartment in IEGCs [13,15]. LPS is rapidly internalized, reaches the TLR4-positive compartment and initiates signal transduction [16]. Although LPS internalization has been noted since many years [13,14,17,18] and the intracellular TLR4 localization has been confirmed in pulmonary, renal and corneal epithelial cells as well as endothelial cells [15,17,19–22], the functional consequence of the different cellular localization of the TLR4 molecule and the functional role of ligand internalization is unknown.

Here we report a strikingly delayed recognition of wildtype Salmonella as compared to O-antigen deficient Salmonella by IEGCs but not macrophages. Delayed recognition of wildtype Salmonella is caused by lack of early TLR4-mediated cell activation associated
Author Summary

The mammalian host recognizes infection by the detection of particular microbial structures. Recognition of these structures leads to activation of host defense effector mechanisms that in turn combat infection. A very potent activating microbial structure is lipopolysaccharide, a cell wall component released by many bacteria such as Salmonella, one of the most frequent causative agents of foodborne infection of the gut. We previously showed that cells lining the gut surface require uptake of bacterial lipopolysaccharide for its detection. The functional consequence of lipopolysaccharide uptake, however, was unknown. Here, we demonstrate that the uptake of lipopolysaccharide released by Salmonella is impaired by its extensive sugar modification. Impaired lipopolysaccharide uptake prevents early activation of host defense mechanisms and thereby allows Salmonella to better survive and proliferate within the host’s intestinal cells. Thus, this lipopolysaccharide modification represents a mechanism by which Salmonella impairs recognition by the mammalian host to more efficiently cause infection of the intestinal mucosa.

with impaired LPS internalization. Importantly, lack of early epithelial activation significantly promotes intraepithelial bacterial survival and O-antigen expression is linked to enhanced numbers of intraepithelial Salmonella after oral infection in vivo. The data show that O-antigen expression contributes to bacterial virulence during apical epithelial invasion prior to contact with serum complement and illustrate the susceptibility of Salmonella to antibacterial defense activation before it reaches and establishes its protected intracellular niche.

Results

Early activation of IECs after exposure to O-antigen deficient Salmonella

In order to evaluate a possible biological effect of LPS glycosylation on epithelial cell stimulation, differentiated and polarized intestinal epithelial m-ICc12 cells were cocultivated with wildtype Salmonella, isogenic O-antigen deficient mutants, or their respective complemented strains. The waaG (f6G) gene encodes a UDP-glucose:heptosyl-LPS 1,3-glucosyltransferase and mutants exhibit a rough RdL1 LPS phenotype with only the inner core sugars attached to the lipid A molecule [23,24]. waaL (f6L) encodes the O-antigen ligase, the last step in the LPS biosynthesis. WaaL functions within the periplasmic space at the cytoplasmic membrane to ligate the presynthesized O-antigen chain onto the lipid A core molecule [1,24]. waaL mutants therefore express the complete core sugars but completely lack the O-antigen (Ra LPS). O-antigen expression was confirmed using silver staining of LPS extracts (Fig. S1A).

Cellular activation was evaluated using (i) visualization of nuclear translocation of the NF-kB subunit p65/RelA, (ii) a stably transfected transcriptional NF-kB luciferase reporter construct, and (iii) quantification of the secreted proinflammatory chemokine MIP-2. Strikingly, a significant difference in the kinetics of cellular activation was recognized after challenge with wildtype and LPS mutant strains. Whereas no difference in the overall magnitude of epithelial cell activation was noted, waaL mutants induced a significantly earlier p65/RelA translocation (Fig. 1A, the earliest detectable p65/RelA translocation is marked with arrows) and an accelerated course of chemokine secretion and NF-kB reporter gene transcription in epithelial cells as compared to wildtype Salmonella (Fig. 1B and C). This difference in p65/RelA translocation (Fig. 1D), chemokine secretion (Fig. 1E), and NF-kB reporter gene activation (Fig. 1F) was similarly observed using waaG- and waaL-deficient mutants and reversed by the complemented strains carrying an expression plasmid encoding the waaG and waaL gene, respectively. Thus lack of O-antigen expression leads to a significantly accelerated recognition of Salmonella by IECs.

TLR4 significantly contributes to innate recognition of Salmonella by intestinal epithelial cells

A similar delay in epithelial activation by wildtype Salmonella was also noted using heat-killed or UV-treated Salmonella suggesting structural impairment of LPS recognition by the O-antigen rather than O-antigen-mediated active inhibition of epithelial cell activation (Fig. S1B and data not shown). To examine the contribution of TLR4-mediated epithelial cell activation and exclude indirect effects of the O-antigen on cellular activation, the role of TLR4 in Salmonella recognition during coculture with IECs over 6 hours was examined. First the stimulatory activity released in the cell culture medium at bacterial numbers corresponding to a multiplicity of infection (MOI) of 10:1 (10⁵ CFU/mL) and 1:1 (10⁵ CFU/mL) during one hour was completely inhibited by addition of the LPS-inhibiting agent polymyxin B (Fig. 2A). Also, inhibition of Tlr4 (Fig. 2B) or Myd88 (Fig. 2C) expression by small interfering (si) RNA technique inhibited epithelial activation by Salmonella to a similar degree as epithelial activation by LPS. In fact, early recognition of the O-antigen deficient waaL mutant Salmonella was almost abolished in epithelial cells treated with Tlr4 siRNA (Fig. 2D). In contrast, inhibition of Tlr2, Tlr5, or Tlr9 expression did not reduce the epithelial response to bacterial exposure (Fig. 2E). Consistently, no early epithelial stimulation was observed after apical exposure to other innate immune receptor ligands released by gram-negative bacteria such as flagellin, di- or tri-acylated lipopeptides, or CpG oligonucleotides (data not shown). The important role of LPS for the observed effect of delayed recognition of wildtype Salmonella was finally confirmed using LPS purified from O-antigen positive (smooth-type LPS, sLPS) as well as O-antigen negative (rough-type LPS, rLPS) Salmonella. Indeed, a similar pattern as compared to exposure to whole wildtype and mutant Salmonella with delayed epithelial activation in response to smooth LPS at early time points (Fig. 2F and G) but similar levels of epithelial activation at later time points (Fig. 2H) was observed. Thus epithelial activation early during the time course of coculture is predominantly caused by TLR4-mediated cell stimulation. The observed delay in the recognition of smooth Salmonella is not related to an O-antigen-mediated suppressive effect on early epithelial activation but rather caused by an inhibitory effect of the O-antigen on LPS recognition by epithelial TLR4.

IECs but not macrophages show delayed immune activation by wildtype Salmonella

Myeloid cells like macrophages carry the TLR4 receptor complex on the cell surface and signaling is initiated at the plasma membrane [14]. This is in contrast to IEC lines and isolated primary IECs that exhibit restriction of the TLR4 molecule to an intracellular compartment [13,15]. In these cells, receptor activation requires ligand internalization and signaling is initiated at the intracellular TLR4-positive compartment [17]. Using the protein delivery reagent PULSin in combination with TLR4/MD2 blocking antibodies, the different receptor localization could be

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functionally demonstrated. Whereas activation of myeloid cells was readily blocked by addition of the blocking anti-TLR4 antibody MTS510 to the cell culture medium, antibody-mediated inhibition of epithelial activation was only observed in the presence of the protein delivery reagent PULSin (Fig. 3A and B). Interestingly, both sLPS - as well as rLPS - stimulated RAW 264.7 cells at early time points to a similar degree and with very similar kinetics (Fig. 3C and D). Also, early p65/RelA nuclear translocation was similarly induced in macrophages by all strains, wildtype as well as \( \Delta \text{waaL} \) and \( \Delta \text{waaG} \) mutant Salmonella as well as the respective complemented-

**Figure 1. Expression of O-antigen confers delayed epithelial activation.** (A) m-ICcl2 cells were exposed to wildtype S. Typhimurium and to isogenic O-antigen deficient \( \text{waaL} \) mutants, both carrying a constitutive GFP plasmid. Nuclear translocation of the NF-κB subunit p65/RelA was visualized by immunostaining. The arrows mark the earliest detectable p65/RelA translocation. Bar, 5 μm. (B) m-ICcl2 cells or (C) m-ICcl2 cells stably transfected with a NF-κB-luciferase construct were exposed to S. Typhimurium wildtype, or an isogenic O-antigen deficient \( \text{waaL} \) mutant, and the secretion of MIP-2, or luciferase synthesis, respectively, was quantified after the indicated time. (D) m-ICcl2 cells were exposed to wildtype S. Typhimurium, two isogenic O-antigen deficient mutants (\( \text{waaL} \) and \( \text{waaG} \)), as well as their respective complemented controls for 1 h and the nuclear translocation of the NF-κB subunit p65/RelA was visualized by immunostaining. Bar, 5 μm. (E) m-ICcl2 cells, or (F) m-ICcl2 cells stably transfected with a NF-κB-luciferase construct were exposed to wildtype S. Typhimurium, the isogenic O-antigen deficient \( \text{waaL} \) and \( \text{waaG} \) mutants, and their respective complemented control strains, and secretion of MIP-2, or luciferase synthesis, respectively, was quantified after the indicated time points. A multiplicity of infection (MOI) of 10 was chosen for all experiments. All data presented are representative for at least three independent experiments. The asterisks indicate a significant difference between the respective rough LPS mutant (\( \Delta \text{waaL} \) or \( \Delta \text{waaG} \)) as compared to all wildtype and complemented Salmonella strains; **, p<0.01.

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ed strains (Fig. 3E and F). Thus the delayed recognition of wildtype sLPS as compared to rLPS is restricted to IECs that are devoid of plasma membrane expression of TLR4 and rely on ligand internalization. Yet we cannot exclude that macrophage activation additionally occurs by LPS release during phagocytosis.

The kinetics of *Salmonella* recognition is not influenced by bacterial invasion

Genes encoded by the so called *Salmonella* pathogenicity island 1 (SPI-1) confer the ability to invade epithelial cells. Bacterial invasion is induced by direct translocation of effector proteins into the host cell cytoplasm which causes actin polymerization and membrane protrusions. Within one hour, this mechanism leads to bacterial internalization and localization within an endosomal compartment named *Salmonella* containing vacuole (SCV). Initially, the kinetics of *Salmonella* invasion was examined using constitutive GFP-positive wildtype *Salmonella* followed by immunostaining with anti-O-antigen *Salmonella* O4/O5 antibodies without prior cell membrane permeabilization. This technique allows the differentiation of extracellular (simultaneously green and red = orange) and intracellular (green) *Salmonella*. Exposure of confluent polarized m-IC cl2 cells revealed bacterial invasion starting approximately 20 minutes after challenge with significant numbers of intracellular bacteria at 2 hours after infection (Fig. 4A). Fig. 4B provides a more detailed illustration of the actin-dependent mode of *Salmonella* invasion at 30 minutes after...
infection (left panel) and the intracellular localization after 2 hours (right panel). Importantly, the O-antigen-mediated delay in LPS recognition was also observed in invasion-mutants: An isogenic pair of smooth and rough *invC*-mutants exhibited a similar difference in the kinetics of epithelial stimulation as compared to invasion-competent *Salmonella* (Fig. 4C and D). Also, *hilA* and *pho24* (PhoP') deficient smooth *Salmonella*, both significantly impaired in epithelial invasion, exhibited a similar pattern of reduced activation at early time points but cellular stimulation at later time points after infection (Fig. S2A and B). Thus the observed delay in
epithelial activation by wildtype bacteria is not dependent on their ability to exhibit an epithelial cell-invasive phenotype but rather result from extracellular ligand exposure.

Delayed recognition of smooth LPS is associated with retarded ligand internalization

Viable bacteria continuously release LPS in the surrounding medium. In accordance, significant amounts of 10^7 EU/mL (approximately 10 ng/mL) LPS were found to be released from viable wildtype Salmonella into the cell culture medium within 30 minutes. The concentration increased up to 10^8 EU/mL (approximately 100 ng/mL) during the observed time period of 2 hours. No significant difference in the degree of endotoxin release between wildtype and waaL- and waaG-mutants or their respective complemented Salmonella strains was noted (Fig. 5A). As expected, inhibition of CD14 and LBP expression by siRNA significantly impaired LPS and Salmonella-mediated activation of m-iC<sub>C10</sub> cells in accordance with the literature (Fig 5B) [25]. Strikingly, LPS internalization studies using biotinylated rLPS or sLPS preparations revealed a marked difference in the kinetics of ligand uptake. Whereas detectable amounts of rLPS were observed after 30 minutes, wildtype LPS remained undetectable until many hours after exposure (Fig 5C). Previous characterization of intestinal epithelial stimulation with rLPS identified a clathrin- and lipid raft-dependent pathway of LPS internalization and receptor activation [15]. Inhibition of lipid raft formation with filipin previously linked to recognition of rLPS abolished early recognition of rLPS but left the more delayed cellular activation induced by wildtype sLPS unaffected (Fig. 5D). Also, a significant inhibitory effect of clathrin siRNA on early recognition of rLPS was noted (data not shown) and dynamin inhibition by dynasore significantly reduced activation by rough, ΔwaaL, Salmonella consistent with this rapid internalization pathway for rLPS uptake (Fig. 5E). These data suggest that qualitative differences in the uptake and intracellular transport mechanism between sLPS and rLPS exist and account for the observed delay in the epithelial recognition of wildtype, O-antigen-positive invasive Salmonella. Similar results obtained using viable invasive and non-invasive ΔinvC waaL double mutant Salmonella [MOI 10:1], ***, p<0.01, doi:10.1371/journal.ppat.1000567.g004

Early innate immune activation restricts the number of intracellular bacteria in vitro and in vivo

To examine the functional consequences of early epithelial activation, a standard Gentamicin protection-assay was performed. Strikingly, both O-antigen negative mutants but not their respective complemented strains exhibited a significantly reduced number of viable intracellular bacteria two hours after infection (Fig. 6A). This was confirmed by immunofluorescence (Fig. 6B) as well as by flow cytometry (Fig. 6C) using GFP expressing wildtype and waaL-deficient Salmonella. Both, the relative number of Salmonella-positive epithelial cells (6.3±0.5% versus 1.7±0.1%, p<0.01) as well as the mean fluorescence intensity (MFI) of positive cells indicating the number of bacteria per cell [MFI 487.8±14.1 vs MFI 349.9±7.4, p<0.01] were significantly enhanced 2 hours after infection with
wildtype as compared to waaL-deficient Salmonella (Fig. 6C). Notably, this difference in the number of viable Salmonella was not due to impaired invasion of waaL-deficient Salmonella since similar numbers of intracellular bacteria were obtained 30 minutes after infection (1.4±0.1% versus 1.9±0.1%) (Fig. 6D). In fact, flow cytometric quantification of intracellular bacteria after epithelial cell lysis revealed an approximately 2-fold enhanced invasion rate of the O-antigen deficient waaL mutant Salmonella as compared to wildtype bacteria (Fig. S1C and D). The increase of the number and fluorescence intensity of wildtype Salmonella-infected epithelial cells together with the marked clusters of intracellular wildtype Salmonella 2 hours after infection suggest significant intraepithelial proliferation early after invasion. In contrast, no signs of bacterial growth were noted for the waaL-deficient Salmonella strain. In addition, reduced bacterial numbers in epithelial cells did not appear to result from general growth or viability defects of O-antigen deficient Salmonella. Wildtype, ΔwaaL, and ΔwaaG Salmonella as well as the complemented mutants exhibited comparable growth rates in LB medium, or m-

Figure 5. Delayed recognition of wildtype LPS is associated with reduced internalization. (A) Endotoxin released in the cell culture medium from wildtype S. Typhimurium, two isogenic O-antigen deficient mutants (waaG and waaG), as well as their respective complemented controls at a concentration of 10^6 CFU/mL was quantified using a Limulus assay. (B) MIP-2 secretion by m-iC02 cells treated with control-, Cd14-specific, or Lbp-specific siRNA 2 and 6 h after exposure to rough or smooth LPS (10 ng/mL), or 2 h after infection with wildtype and waaL-deficient S. Typhimurium at a MOI of 10:1. (C) m-iC02 cells were exposed to biotin-conjugated rough or smooth LPS for the indicated time points and the internalized LPS was visualized by immunostaining. Counterstaining was performed with MFP488-phalloidin and Dapi. Bar, 5 μm. (D) Stably transfected m-iC02 cells expressing a NF-κB-luciferase construct were stimulated with rough (rLPS), or smooth (sLPS) in the absence, or presence of filipin for the indicated time points and the amount of luciferase was quantified. (E) MIP-2 secretion by m-iC02 cells exposed to wildtype or waaL-deficient S. Typhimurium in the presence or absence of the dynamin inhibitor dynasore. **, p<0.01.
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ICcl2 cell lysate (Fig. S1E). Also, both wildtype and ΔwaaL or ΔwaaG Salmonella were able to induce persistent intracellular infection in m-ICcl2 cells (Fig. S1F). In accordance with the different phenotype observed in epithelial cells and macrophages (Fig. 1 versus Fig. 3), the intracellular survival illustrated by enhanced fluorescence of infected cells with wildtype Salmonella was only found in epithelial cells. In contrast, the fluorescence of Salmonella-infected RAW 264.7 macrophages was not significantly altered during the first 2 hours of infection, irrespective of the Salmonella strain used (Fig. S2C). Of note, an enhanced internalization of the waaL-deficient Salmonella mutant was observed after macrophage infection in accordance with Ilg et al. [26] (Fig. S2C).

To confirm that the observed antibacterial effect was directly linked to early innate immune recognition and cell activation, epithelial cells were infected with wildtype bacteria in the presence or absence of rLPS. Indeed, the number of intracellular bacteria as measured by invasion assay (Fig. 6E), or immunofluorescence (Fig. 6F) was significantly reduced in rLPS-stimulated epithelial cells illustrating the critical importance of early cell activation to restrict intracellular bacterial growth. Wildtype Salmonella in rLPS-stimulated epithelial cells were significantly less affected (Fig. S2D). The dramatic nature of this antibacterial effect was illustrated by flow cytometric quantification of intracellular bacteria in cell lysate between 30 and 60 minutes after infection. Whereas invasion of naive epithelial cells allowed immediate intracellular bacterial growth, rLPS-stimulated epithelial cells were able to restrict the number of Salmonella (Fig. 6G and H). Thus early activation of intestinal epithelial cells by O-antigen-deficient Salmonella is associated with significantly reduced intraepithelial survival.

Salmonella has been shown to invade IECs in vivo after oral challenge [27]. Intestinal epithelial invasion from the luminal side occurs without prior contact with tissue macrophages or complement. To examine a possible effect of O-antigen expression...
on intraepithelial survival in vivo, mice were orally challenged and highly pure IECs were isolated and examined for the presence of viable Salmonella. Similar numbers of intracellular wildtype and waaL-deficient Salmonella were noted at early time points following infection (Fig. 7A). Interestingly, a significant reduction of O-antigen-deficient (waaL) Salmonella as compared to wildtype as well as the respective complemented Salmonella was detected in highly pure IECs later during the course of infection (Fig. 7B). The presence of intraepithelial wildtype Salmonella after oral challenge was also confirmed by immunohistology (Fig. 7C). Thus, lack of O-antigen expression does not influence intestinal epithelial invasion but intraepithelial survival of Salmonella in vitro and in vivo. These results identify O-antigen expression as an innate immune evasion strategy to enhance intraepithelial survival. O-antigen expression might thereby promote intraepithelial proliferation and mucosal spread.

Discussion

S. Typhimurium is one of the leading causative agents of enteritis in humans. Infection is acquired by oral ingestion of contaminated food. In the intestine, Salmonella firmly attaches to the epithelial surface and induces membrane protrusions that surround the bacterium and form an endosomal vesicle called Salmonella-containing vacuole (SCV). This process has been extensively studied in vitro but also confirmed in vivo [27,28]. Intestinal epithelial invasion from the enteric lumen occurs prior to contact with serum complement or professional phagocytes such as macrophages. It plays an important role in the induction of enteritis and mucosal damage in vivo and thus represents an essential step in Salmonella pathogenesis [29,30].

Similar to professional immune cells, also intestinal epithelial cells express receptors of the innate immune system, and thus might contribute to recognition of microbial infection and antibacterial host defense during the initial phase of infection. Indeed, the LPS structure was shown to significantly influence epithelial invasion [26,31]. Also, innate immune recognition via TLR4 was reported to play a significant role in the host defense against Salmonella infection in vivo [10,32–33]. Strikingly, the subcellular localization of TLR4 in myeloid versus epithelial cells is markedly different. Whereas the receptor molecule is situated on the cell surface of macrophages and ligand recognition and cell signaling occurs at the cell membrane, TLR4 in IECs is restricted to the intracellular compartment and ligand recognition requires uptake and intact cell traffic [13,15,16]. We could previously show that internalization of rLPS results in significant intracellular accumulation within minutes after exposure [16]. In the present study we show that qualitative differences in the uptake and intracellular transport mechanism between rLPS and sLPS might significantly contribute to immune evasion of wildtype Salmonella during the early phase of mucosal infection. Thus our results for the first time report on a biological consequence of intracellular TLR4 localization in IECs. Since apical invasion of enterocytes by Salmonella occurs prior to contact with serum complement or professional phagocytes such as tissue macrophages, the epithelial specific delay in wildtype Salmonella LPS recognition significantly contributes to bacterial virulence at the mucosal surface in addition to what has been described as serum resistance during systemic spread of the bacteria (Fig. S2E).

LPS is composed of the hydrophobic lipid A, the core polysaccharides, and the highly polymorphic and hydrophilic immunodominant O-antigen [1]. The LPS receptor TLR4 specifically interacts with and recognizes the lipid A part of the LPS molecule. Therefore, even small variations observed in the lipid A structure and their influence on TLR4-mediated recognition have extensively been studied [36]. The O-antigen is not required for the immunostimulatory activity of LPS and variations of the O-antigen and their impact on TLR4-mediated recognition have only recently attracted attention [37,38]. The O-antigen is composed of up to 100 repetitive structurally variable carbohydrate subunits and the distinction of different O-antigen subunits has been used in the serotyping of various gram-negative bacteria. It is synthesized separately from the rest of the LPS molecule on a lipid carrier by enzymes encoded by the fliH/waa locus. The O-antigen chain is subsequently transferred to the periplasmic space where ligation to the lipid A-core polysaccharide precursor takes place. Only then, the completed LPS molecule is transferred to the bacterial cell surface [39]. The O-antigen is the major determinant of complement resistance and thus represents an important virulence factor [40]. Indeed, gram-negative enteropathogenic bacteria isolated from fecal samples of diseased patients such as Enterinna verotoxica, Salmonella enterica, Shigella dysenteriae, as well as enterohemorrhagie (EHEC) or enteropathogenic (EPEC) Escherichia coli exhibit long O-antigen chains on their respective LPS molecule. Modifications within the lipid A portion of the molecule have been described to alter the stimulatory potential of LPS [41]. The presence or absence of the O-antigen,

Figure 7. Early epithelial stimulation restricts the number of intraepithelial Salmonella in vivo. Colony forming units (CFU) cultured from isolated highly pure (>98% E-cadherin/C45+) intestinal epithelial cells (IEC) (A) 4 h (n = 8 mice per group) and (B) 24 h (n = 16 mice per group) after oral infection of Balb/c mice with 1 x 10^8 CFU wildtype (wt), waaL-deficient (waaL), and complemented wildtype (waaL) Salmonella. * p < 0.05. (C) Immunostaining for S. Typhimurium O-antigen (anti-O4 and anti-O5) in intestinal tissue sections obtained from orally infected mice. Magnification, Bar 50 μm (left panel) and 5 μm (right panel).

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however, has not been linked to alterations in the TLR4-mediated signaling cascade leading to MAP kinase and NF-κB activation [30].

Using X-ray diffraction of dried LPS, Kastowsky and colleagues estimated the size of the lipid A molecule to measure approximately 2.4 nm in length [42]. Addition of the inner core carbohydrates (corresponding to the LPS produced by the waaG mutant Salmonella) would result in a length of approximately 3.5 nm, addition of the outer core carbohydrates (corresponding to the LPS produced by the waaL mutant Salmonella) in a length of approximately 4.4 nm. Their analysis further suggested an additional length of 1.1 to 1.6 nm per repeating carbohydrate unit of the O-antigen. A full length O-antigen with up to 100 repeating units may therefore extend the molecular length to more than 100 nm. The tertiary structure and orientation of long chain O-antigen in respect to the outer cell membrane is not fully understood [42]. Although the O-antigen might be heavily coiled and allow (and actually favor) some degree of lateral bending [42,43], addition of this long chain hydrophilic residue might dramatically enhance the spacial extension of the LPS molecule [42–44]. In accordance, electron microscopic images from the membrane of gram-negative bacteria suggest that the O-antigen extends from the outer cell membrane for 40–100 nm [43,44]. The length of the extending O-antigen structures are also illustrated by reports on O-antigen mediated impairment of efficient type III secretion in enteropathogenic Shigella [45]. Taking into account that the inner diameter of clathrin coated vesicles is strictly defined; one explanation for the delayed internalization of smooth LPS by epithelial cells might therefore be its physical size.

Both in vitro as well as in vivo experiments revealed comparable intestinal epithelial invasion by wildtype and O-antigen-deficient bacteria at early time points after challenge. Once inside the epithelial cell, Salmonella is able to interfere with cellular processes of endosomal maturation altering the molecular composition of its surrounding membranous compartment for its own benefit [46]. Well-established virulence determinants such as the PhoPQ regulon and the SPI-2 type III secretion system contribute to this immune evasive behavior [47]. Avoidance of epithelial activation might significantly contribute to bacterial survival since innate immune signaling has been suggested to promote maturation of endosomal compartments and to influence intracellular bacterial proliferation [48,49]. Indeed the intracellular viability of O-antigen-deficient Salmonella in intestinal epithelial cells in vitro and in vivo was significantly reduced. Previous animal studies have indicated a significant effect of Salmonella O-antigen expression after oral but not intraperitoneal or intravenous infection [50,51]. Our results provide an explanation for these findings and demonstrate that the O-antigen-modification of LPS significantly contributes to mucosal immune evasion and thus bacterial virulence in the intestine. Our data further point towards a role of intestinal epithelial infection for enteric bacterial multiplication and fecal excretion and thereby transmission of enteropathogenic bacteria like Salmonella.

In conclusion, we for the first time provide a functional consequence of internalization-dependent ligand recognition by TLR4 as compared to surface recognition in myeloid cells. We demonstrate that O-antigen modification of Salmonella LPS hinders rapid epithelial internalization and delays TLR4-mediated recognition. Evasion from early innate immune activation of IECs markedly enhances intracellular proliferation of wildtype Salmonella. This novel immune evasion mechanism might thus significantly contribute to mucosal virulence of enteropathogenic bacteria.

Materials and Methods

Ethics statement

Animals were handled in strict accordance with good animal practice as defined by the relevant local animal welfare bodies, and all animal work was approved by the appropriate committee (Landesamt für Lebensmittelsicherheit und Verbraucherschutz, Oldenburg, 07/1334).

Antibodies and reagents

Antibodies against β3/β4/MD-2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rat monoclonal anti-TLR4/MD-2 antibody (MT510) and the mouse monoclonal anti-O4 and anti-O5 antigen antibody were kindly provided by K. Miyaki (Saga Medical School, Nabeshima, Saga, Japan) and M. Kim (Kim Laboratories Inc., Champaign, IL), respectively. All fluorophore-conjugated secondary antibodies and Cy5-conjugated streptavidin were from Jackson Immunoresearch (West Grove, PA). Filipin, dynasore, and polymyxin B were purchased from Sigma (Taufkirchen, Germany). Plasmid DNA was prepared using the EndoFree Plasmid kit from Qiagen (Hilden, Germany). High purity grade smooth and rough form LPS were purchased from List Biochemicals (Campbell, CA) and Alexis Biochemicals (Lausen, Switzerland) and tested for its TLR4-specific activity using Tlr4-deficient C57BL/10ScN-Tlr4−/− [Jul] mice (The Jackson Laboratory, Bar Harbor, USA) (Fig. S1G–I). LPS was biotinylated using EZ link biotinylation kit from Thermo Scientific (Rockford, IL). Endotoxin was quantified using the chromogenic QCL-1000 Limulus amebocyte lysate system from Lonza (Basel, Switzerland). All siRNA probes used (Tie2, Tie4, Tgfb, Tgfβ, Myd88, Cd14, Lbp, Clchitin and control siRNA) were from Qiagen (Hilden, Germany). For plasmid transfection, siRNA transfection, and intracellular antibody delivery Lipofectamine 2000 (Invitrogen, Carlsbad, CA), INTERFERin (Polyplus Transfection, New York, NY) and PULSin (Polyplus Transfection), respectively, were used according to the manufacturer’s instructions. Cell culture reagents were purchased from Invitrogen. All other reagents were obtained from Sigma (Taufkirchen, Germany) if not stated otherwise.

Bacterial strains and cell culture

Salmonella enterica subsp. enterica sv. Typhimurium (S. Typhimurium) ATCC 14028 was used as wildtype strain. Isogenic mutant strains (ΔwaaL and ΔwaaG) were generated by Red recombinase mediated deletion and chromosomal insertion of a Kanamycin antibiotic resistance cassette as described elsewhere (Zenk et al., submitted). The construction of plasmids for the complementation of mutant strains is described in (Zenk et al. submitted). The LPS profiles of the various strains were analyzed using SDS-PAGE and silver staining (Fig. S1A). Bacteria were incubated at 70°C for 10 min to produce heat-killed Salmonella. The non-invasive isogenic pho-24 (PhoP constitutive) and ΔhilA mutants were a generous gift from Mikhail Rhen (Karolinska Institute, Stockholm) and the isogenic ΔinvC and ΔinvC ΔwaaL, double mutants were generated as described above. HilA is a central regulator of SPI-1 mediated epithelial cell invasion, the PhoP/PhoQ two component system is a central regulator in Salmonella virulence, and InvC is required for type III secretion of SPI-1-encoded virulence determinants. The phenotype of the non-invasive pho-24 mutant is designated PhoP−constitutive (PhoP+). All three mutants exhibit strongly impaired epithelial invasion. Fluorescent bacteria were generated by transformation with a constitutively GFP expressing plasmid. For all experiments, bacteria were routinely grown in Luria-Bertani (LB) broth, supplemented with antibiotics if required. Murine small intestinal epithelial m-ICcl2 cells and m-
IC_{142} cells stably expressing a NF-κB luciferase reporter construct were cultured as described previously [32]. RAW 264.7 macrophages were purchased from ATCC and cultured in RPMI 1640 medium (Invitrogen) supplemented with 20 mM HEPES, 2 mM L-glutamine, and 10% FCS.

**Bacterial coculture and stimulation assays**

For all coculture experiments, wildtype or mutant *Salmonella* were grown overnight at 37°C, diluted 1:10 and subcultivated with mild agitation at 37°C, until mid-logarithmic growth was reached (OD_{600nm} 0.5). Bacteria were adjusted by dilution, added to polarized and differentiated intestinal epithelial m-IC_{142} cells at a multiplicity of infection (MOI) of 10:1 and centrifuged at 300 × g for 5 min. Following incubation for one hour, the medium was replaced with fresh medium supplemented with 50 μg/mL Gentamicin. Cell culture supernatants, as well as cell lysates, were collected after the indicated periods of time and stored at −20°C. The chemokine MIP-2 was analyzed using a commercial ELISA kit (Promega, Madison, WI). Stimulation with mouse recombinant TNF (R&D Systems GmbH, Wiesbaden, Germany) was performed at 100 ng/mL. NO production was determined by measurement of nitrite in cell culture supernatant using Griess reagent [53]. Pharmacological inhibitors were added to the cell medium 30 min prior to stimulation. NO production was determined by measurement of nitrite in cell culture supernatant using Griess reagent [53].

**Statistical analysis**

All experiments were performed at least three times and results are given as the mean±SD of one representative experiment. Statistical analyses were performed using the Student’s t test. A p value<0.05 was considered significant.

**Supporting Information**

**Figure S1**

(A) Characterization of the LPS produced by the wildtype, *waaL* and *waaG* mutant, as well as the respective complemented *waaG* (*waaG*) and *waaL* (*waaL*) *Salmonella* strains used in this study. LPS extracts from equal numbers of bacterial cells (3 × 10^8 CFU) were loaded in each lane and analyzed by SDS-PAGE using a 12% acrylamide gel subsequently followed by silver staining. The relative positions of the lipid A, inner core, outer core, and O-antigen are indicated. (B) Kinetic of MIP2 secretion by m-IC_{142} cells in response to exposure to heat-killed wildtype and *waaL* mutant O-antigen-deficient *Salmonella*. (C and D) Quantitative flow cytometric analysis of intracellular GFP expressing wildtype and *waaL*-deficient *Salmonella* 30 min. after infection. (C) Dot blot analysis and (D) normalized numbers of GFP-positive bacteria detected in a defined volume of epithelial cell lysate 30 min. after infection. (E) Growth of wildtype (wt), *waaG* and *waaL*-mutant, as well as the respective complemented strains *waaG* (*waaG*) and *waaL* (*waaL*) in m-IC_{142} cell lysate diluted 1:3 in phosphate buffered saline (PBS). The number of colony forming units (CFU) normalized to the inoculum is indicated at each time point. (F) Viable intracellular wildtype or *waaL*-deficient *Salmonella* 2, 4, and 6 hours after infection of naive m-IC_{142} cells. Gentamicin (50 μg/mL) was added to the cell culture medium one hour after addition of the bacteria. (G, H, and I) Analysis of the purity of the LPS preparation used in this study. NO release (H) by LPS-stimulated [100 ng/mL] wildtype or *Tlr4*-deficient peritoneal macrophages. (I) Th2-deficient peritoneal macrophages readily responded to the proinflammatory cytokine TNF but not LPS (10 ng/mL) CFU, colony forming units; n.d., not detectable. **, p<0.01.

**Figure S2**

(A) The non-invasive phenotype of the *T. Typhimurium pho-24* (PhoP^−) and *hilC* mutants (MOI 10:1) demonstrated by fluorescent protein to visualize efficient protein transfection by lipofectamine reagent [53]. Stimulation with mouse recombinant TNF (R&D Systems GmbH, Wiesbaden, Germany) was performed at 100 ng/mL. NO production was determined by measurement of nitrite in cell culture supernatant using Griess reagent [53]. Pharmacological inhibitors were added to the cell medium 30 min prior to stimulation. NO production was determined by measurement of nitrite in cell culture supernatant using Griess reagent [53].
a Gentamicin protection-assay. (B) Stably transected m-ICc12 cells expressing a NF-κB-luciferase construct were co-incubated with wildtype Δ. Typhimurium, or isogenic non-invasive Pho–24 (PhoP–)

and hld4 mutants [MOI 10:1] for 2 hours, or 6 hours, and the amount of luciferase was quantified. **, p<0.01. (C) Flow cytometric analysis of RAW 264.7 cells left untreated or 30 and 120 min after infection with GFP-expressing wildtype or waaL-deficient Salmonella. The left lower panel illustrates the number of Salmonella-positive RAW 264.7 macrophages [%] at 30 and 120 min after infection. Note the significantly enhanced invasion rate of rough as compared to smooth Salmonella in macrophages. (D) Viable intracellular wildtype Salmonella two hours after infection of m-ICc12 cells [MOI 10:1] as measured by Gentamicin.

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