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Role of *Salmonella enterica* Lipopolysaccharide in Activation of Dendritic Cell Functions and Bacterial Containment

Sebastian F. Zenk,* Jonathan Jantsch,† and Michael Hensel2*

In contrast to nonpathogenic bacteria, the Gram-negative pathogen *Salmonella enterica* is not eradicated, but persists in murine dendritic cells (DC). The molecular basis of this phenotype is unknown. We set out to characterize bacterial and DC functions that are involved in *Salmonella* persistence. Our data prove that neither bacterial nor host cell de novo protein biosynthesis is required for *Salmonella* persistence in DC. We identified the *Salmonella* O-Ag of the LPS of *Salmonella* as an important factor for controlling the intracellular fate of *Salmonella* in DC. A *Salmonella* strain with entirely absent O-Ag showed an increased rate of uptake by DC, altered intracellular processing, and increased degradation, and also boosted the activation of immune functions of DC. These novel findings demonstrate that in addition to the multiple functions of the bacterial LPS in adaptation to the intestinal environment and protection against innate immune function, this molecule also has an important role in interaction of *Salmonella* with DC.

Dendritic cells (DC) are a population of phagocytic cells that provide an important link between innate and adaptive immunity (reviewed in Ref. 1). Intestinal DC, especially enriched in the subepithelial dome of the Peyer’s patches, sample and phagocytose luminal Ags (2) and are capable of killing internalized microbes. In particular, the phagocytosis of intestinal pathogens such as *Salmonella enterica* by DC is supposed to occur within the lamina propria after overcoming the gastrointestinal epithelial layer via M cells or enforced epithelial uptake. After phagocytosis of Ags, DC are thought to mature and migrate to secondary lymphoid organs (in this study: mesenteric lymph nodes), where processed antigenic material is presented to naïve CD4+ or CD8+ T cells in the context of MHC II or I molecules, respectively (reviewed in Ref. 3). Despite the central role of DC in mediating adaptive immune responses, there is accumulating evidence that specific properties of DC might also be used by bacterial pathogens as “Trojan horse” or vehicle to systematically disseminate within an infected host (4–6).

*S. enterica* is an important, Gram-negative, gastrointestinal pathogen of humans and animals with the ability to colonize different niches in the host organism, resulting in different disease outcomes (7). Human infections with *S. enterica* serovars such as Typhimurium or Enteritidis result in gastrointestinal diseases with localized inflammation and diarrheal symptoms. In contrast, *S. enterica* Serovar Typhi is highly human adapted and can cause a life-threatening systemic infection known as typhoid fever. Infection of mice with *S. enterica* Serovar Typhimurium mimics human typhoid fever in many aspects of pathogenesis. *Salmonella* is a facultative intracellular pathogen that resides in a specialized compartment within eukaryotic host cells, termed *Salmonella*-containing vacuole (reviewed in Ref. 8). The ability to survive and eventually replicate within mammalian cells is considered as one important virulence strategy in the pathogenesis of systemic infections by *Salmonella* (9). Intracellular survival and proliferation of *S. enterica* in host cells is a multifactorial process that involves diverse bacterial traits as composition of the bacterial cell envelope, activation of stress response systems, and the function of highly evolved virulence factors.

Due to adaption to the intestinal habitat, Gram-negative bacteria as *S. enterica* as well as the closely related commensal *Escherichia coli* possess LPS as a main constituent of the outer membrane. LPS consists of a conserved lipid A moiety with strong immunomodulatory properties responsible for its endotoxin effects, as well as a conserved inner and outer core oligosaccharide (reviewed in Ref. 10). The more exposed parts of the LPS are composed of the O-Ag, which is a modular assembly of oligosaccharide units that can vary with respect to the sugar composition, as well as in the number of their modul repeats. The O-Ag of *S. enterica* is found in short, long, and very long forms with ~16, up to 35, and more than 100 modul repeats of the oligosaccharide units. It has also been observed that the LPS of *Salmonella* is modified during intracellular life, for example with respect to the lipid A moiety (11–13) and the down-regulation of the number of O-Ag modul repeats (14–16).

Among the specific virulence factors for intracellular life, the type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island 2 (SPI2) is of special interest, because effector proteins translocated by this system allow intracellular *Salmonella* to modify host cell transport processes and to maintain the *Salmonella*-containing vacuole (reviewed in Ref. 17).

We and others have previously reported that *S. enterica* is able to survive within DC (18, 19). Other phagocytosed bacteria, however, are killed by DC and processed. In contrast to other host cells, *Salmonella* does not proliferate within DC. Intracellular survival of *S. Typhimurium* in murine DC was independent from the

1 Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; LB, Luria-Bertani; MOI, multiplicity of infection; SP, spacious phagosome; SPI2, *Salmonella* pathogenicity island 2; T3SS, type III secretion system; WT, wild type.

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For detection of LPS, 1.5-ml bacterial cultures were grown to stationary phase, cells from 1.5 ml of culture were harvested by centrifugation, and the pellet was dissolved in 50 μl of lysine buffer per OD_{600} −1.6 and lysed at 100°C for 10 min. Lysates were subsequently digested at 37°C for 1 h with proteinase K (25 μg/ml). Lysates (15 μl) were separated by electrophoresis on a 12% SDS-polyacrylamide gel at 80 V, followed by an overnight fixation in 25% isopropanol, 7% acetic acid. After oxidation with 0.3 M periodic acid, gels were washed six times for 20 min with ddH₂O and then stained with an alkaline, AgNO₃-saturated solution. Before development with a solution consisting of 1 mM citric acid, 0.1% formaldehyde, gels were washed four times with ddH₂O. The staining reaction was stopped with 0.35% acetic acid.

Preparation and culture of DC and macrophages

DC were generated from bone marrow (BM) of C57BL/6 or BALB/c mice (Charles River Laboratories), according to the protocol of Lutz et al. (28), with modifications, as described (18, 29). Before infection experiments, cells were allowed to adhere to culture plates for at least 6 h. MACS technique using CD11c MicroBeads (Miltenyi Biotec) was used to enhance the purity of 11c-positive compartment. These enriched cells were used for uptake/survival experiments with LPS mutant strains, where indicated.

The RAW264.7 macrophage-like cell line was obtained from American Type Culture Collection, and the cells between passage numbers 8 and 25 were used for the experiments. BM-DC and RAW264.7 Macrophages were cultured in RPMI 1640 and DMEM, respectively, each containing 10% FCS (PAA).

Macrophages from murine BM were generated essentially as previously described (30). Briefly, total BM cells were cultured in hydrophobic Teflon bags (DuPont, purchased via Cadillac Plastic) with 10% CO₂/90% humidified air in DMEM (Invitrogen) supplemented with 0.05 mM L-2-ME, 1% nonessential amino acids, 10% FCS (PAA), 5% horse serum (Cell Concepts), and 10% L929 cell-conditioned medium. At day 8, the cells were harvested, routinely yielding a population of >90% CD11b^{high}, F4/80^{high} positive cells.

Abs, immunofluorescence, and molecular tracers

For immunostaining, the following Abs were used at the indicated dilutions: Armenian hamster anti-CD11c mAb (BD Biosciences), dilution 1/100; rat anti-hemagglutinin epitope mAb (Roche), dilution 1/250; and rabbit anti-Salmonella O4.5 antiserum (BD Biosciences), dilution 1/500. Abs used were: anti-CD11c (H100.19), anti-CD11b (M1/70), anti-CD11c (HL3), anti-F4/80 (CI:A3-1; Serotec), anti-MHC II (2G9), anti-CD80 (16-10A1), and anti-CD86 (GL1). The specificity of the staining was verified by the use of isotype control mAbs. All analyses were performed on a FACSCalibur (BD Biosciences), applying the CellQuest Pro software. For the stimulation of BM-derived macrophages or DC, PMA (10 ng/ml) was added to the culture medium for 30 min, followed by washing for removal of noninternalized marker and incubation for 2 h before bacterial infection, to allow the partitioning of markers to the compartments.

For immunostaining, cells were fixed in 3% paraformaldehyde, stained, and subsequently mounted with Fluoprep (bioMérieux). Microscopy images were recorded with a Zeiss Axiovert 200M equipped with ApoTome, Xicam MRm, and 63 Plan Neofluar oil immersion objectives (Carl Zeiss). Taken images were processed with AXIOVISION 4.7 software (Carl Zeiss).

For surface phenotyping, the following fluorochrome (FITC, PE, or allophyocyanin)-labeled Abs were used (all from BD Biosciences, unless otherwise stated): anti-FcRγI (2.4G2), anti-CD11b (M170), anti-CD11c (HL3), anti-F4/80 (CI:A3-1; Serotec), anti-MHC II (2G9), anti-CD80 (16-10A1), and anti-CD86 (GL1). The specificity of the staining was verified by the use of isotype control mAbs. All analyses were performed on a FACSComp (BD Biosciences), applying the CellQuest Pro software. For the stimulation of BM-derived macrophages or DC, PMA (Sigma-Aldrich) was added at final concentrations of 6 or 12 ng/ml.

Bacterial infection of cultured cells and pharmacological inhibitors

BM-DC and macrophages were infected with bacterial strains grown to stationary phase. The multiplicity of infection (MOI) was adjusted by resuspending the culture density at the OD of 600 nm. The actual MOI of each experiment was determined by plating dilutions of the inoculum onto agar plates for the determination of the number of CFU. For synchronization of

\[ \text{Table I. Bacterial strains and plasmids used in this study} \]

<table>
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<th>Strain</th>
<th>Genotype, Relevant Characteristics</th>
<th>Source/Reference</th>
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<td></td>
<td></td>
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<tr>
<td>NCTC12023 WT</td>
<td>Laboratory stock</td>
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<tr>
<td>NCTC12023 WT Na⁺</td>
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<td>thyP::aph Km²</td>
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<tr>
<td>MVPP750</td>
<td>XbaI::tnr5, SPI2 defective</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>P2D6c</td>
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<td>Laboratory stock</td>
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<td></td>
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<td>Probe strain, semirough LPS</td>
<td>DSM strain 6601</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Provided by S. Schubert, Munich, Germany)</td>
</tr>
<tr>
<td>HB101</td>
<td>Laboratory strain, rough LPS</td>
<td>Laboratory stock</td>
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<td>Expression of A Red gene</td>
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<td>Constitutively expressing mCherry, ampicillin resistance</td>
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\[ ^4 \text{The online version of this article contains supplemental material.} \]
infection, a centrifugation at 500 × g for 5 min was performed. If not otherwise stated, BM-DC were infected at a MOI 25 for 60 min, and RAW264.7 macrophages were infected at a MOI 10 for 30 min at 37°C, 5% CO₂. If indicated, infected bacteria were opsonized by incubation with 20% normal mouse serum (The Jackson Laboratory, Dianova) for 30 min at 37°C. After infection, cells were washed to remove noninternalized bacteria. Gentamicin was added to prevent replication of remaining extracellular bacteria. For BM-DC, gentamicin was added for 1 h at 100 μg/ml, followed by 25 μg/ml gentamicin for the rest of the experiment. For RAW264.7 macrophages, gentamicin was added at 100 μg/ml for 1 h, followed by 10 μg/ml gentamicin for the rest of the experiment. Infection was terminated by cellular lysis using 0.5% Triton X-100 in PBS, and the number of intracellular bacteria was determined by serial dilution in 0.05% Tween 20 in PBS and subsequent plating.

Inhibition of prokaryotic and eukaryotic protein biosynthesis was conducted with 5–80 μg/ml tetracycline and 0.01–10.0 μg/ml cytochalasin (both used at the indicated concentrations and ordered from Sigma-Aldrich), respectively.

TFN in cell culture supernatant was quantified with the BD OptEIA Mouse TNF (Mono/Poly ELISA Kit II (BD Biosciences), according to the manufacturer’s instructions.

To assay cell viability, the Live/Dead viability/cytotoxicity kit (Molecular Probes, Invitrogen) was used, according to the manufacturer’s instructions.

Statistical analyses
Statistical significance was calculated using Prism 4.0 software. One-way ANOVA was used for comparison of multiple data sets, and Student’s t test for use of pairwise comparison. Statistical significance was indicated as follows: p ≈ 0.05; NS; p < 0.05; *, p < 0.01; **; p < 0.001; ***.

Results
The intracellular survival of Salmonella in DC is independent from bacterial and host cell de novo protein biosynthesis

We set out to identify the factor(s) that allows the intracellular survival and persistence of S. Typhimurium within murine DC. Because previous approaches failed to identify mutual strains with reduced survival in DC, we applied a more general approach and investigated whether bacterial proteins synthesized after bacterial uptake are required for the adaptation to intracellular life in DC. In this study, we infected BM-DC with S. Typhimurium WT and added the antibiotic tetracycline for various periods of time during the infection to inhibit bacterial protein biosynthesis. For comparison with a cell type that is permissive for intracellular replication, we used the murine macrophage-like cell line RAW264.7. In non-activated RAW264.7 cells, Salmonella WT consistently showed a 20- to 50-fold increase in viable intracellular bacteria over a period of 15 h (27) (this study), whereas the increase in bacterial counts in BM-DC was 1- to 2-fold.

To inhibit bacterial protein biosynthesis, the antibiotic tetracycline was applied at various concentrations (Figs. S1 and S2). If added 2 h after infection for 1 h, followed by subsequent removal, increasing concentrations of tetracycline did not inhibit replication of Salmonella WT in macrophages (up to 40 μg/ml) and the survival in DC (Fig. S1). The presence of 20 or 40 μg/ml tetracycline for 1 h, followed by removal of the antibiotic, only led to minor reduction of intracellular proliferation in macrophages and had no significant effect on the survival of Salmonella in BM-DC (Fig. 1), or the synthesis and translocation of SseJ as a representative effector protein of the SPI2-T3SS (Fig. S2). The presence of tetracycline for a prolonged duration from 2 to 15 h after infection inhibited proliferation of Salmonella in macrophages, led to reduced intracellular survival in BM-DC (Fig. 1), and completely ablated translocation of SseJ (Fig. S2). By contrast, the same treatment of BM-DC with tetracycline 2–15 h after infection only had a minor effect on the intracellular survival, i.e., a 0.35- to 0.45-fold reduced bacterial recovery compared with the mock-treated control (Fig. 1C).

Next, we investigated the role of host cell protein biosynthesis and performed similar experiments with cycloheximide, an inhibitor of eukaryotic protein biosynthesis. Treatment of macrophages with cycloheximide resulted in increased intracellular proliferation (1.4- to 1.5-fold; Fig. 2A). By contrast, no difference in proliferation of intracellular Salmonella was observed in BM-DC treated with cycloheximide, and a slightly decreased bacterial recovery was observed (0.19- to 0.23-fold; Fig. 2B). The effect of the inhibitor was confirmed by quantification of the release of the TNF by BM-DC (Fig. 2D). Higher concentrations of cycloheximide caused eukaryotic cell death (data not shown). Application of cycloheximide at various time points before infection also resulted in increased Salmonella replication in RAW264.7 macrophages, but did not affect the intracellular bacterial survival in BM-DC (data not shown).

Taken together, inhibitor experiments suggest that neither bacterial de novo protein biosynthesis nor de novo host cell protein biosynthesis affects the intracellular fate of Salmonella in BM-DC. This unexpected result prompted us to investigate in more detail the role of preformed bacterial factors for intracellular survival in BM-DC.

FIGURE 1. Bacterial de novo protein biosynthesis is dispensable for intracellular survival of Salmonella in BM-DC, but not for replication in macrophages. A, Schematic representation of the experimental set-up of assays for intracellular survival. RAW264.7 macrophages (B) or BM-DC (C) were infected with S. Typhimurium WT at a MOI of 10 or 25, respectively. To kill residual extracellular bacteria, gentamicin (G) was added at 100 μg/ml for 1 h and maintained at 10 μg/ml for the rest of the experiment. Tetracycline (Tet) was added at a final concentration of 20 or 40 μg/ml at various time points, as indicated. The number of viable intracellular bacteria at 2 and 15 h post-infection was determined by lysis of host cells by addition of 0.1% Triton X-100 for 10 min and plating of serial dilutions of the lysate onto agar plates to enumerate CFU. Relative survival or replication is determined as the ratio of CFU at 15 and 2 h after infection. Intracellular replication of S. Typhimurium WT in RAW264.7 macrophages (B) and survival in BM-DC (C) without or with addition of various amounts of tetracycline was quantified, and means and SD of three assays are shown. The inhibitory effect of tetracycline on bacterial protein biosynthesis was controlled by analyzing translocation of a Salmonella effector protein (SseJ) at various antibiotic concentrations and application time point conditions (Fig. S2). Statistical analysis, antibiotic-treated cells vs mock-treated cells: *, p < 0.05; ***, p < 0.001.
The intracellular fate of bacteria in DC is determined by the bacterial cell envelope

To approach the specific adaptation of Salmonella enterica to the intracellular life in DC experimentally, we first compared the uptake and intracellular fate of S. enterica with closely (E. coli, Shigella flexneri) or distantly (Yersinia enterocolitica) related Gram-negative bacteria, as well as typical Gram-positive pathogens (Staphylococcus aureus, Streptococcus pyogenes). We observed that the intracellular survival in BM-DC of the various bacteria varied substantially (data not shown). We also observed that the rates of uptake and intracellular survival varied between closely related S. Typhimurium and E. coli strains. Although genetically closely related, the E. coli strains used for comparison of the intracellular fate are commonly laboratory strains that differ from S. Typhimurium in the absence of a complete O-Ag. For a more detailed analyses, the fate of S. Typhimurium and two representative E. coli strains, i.e., the laboratory strain HB101 and the probiotic strain Nissle Mutaflor, was analyzed (Fig. 3). The phagocytic uptake of E. coli Mutaflor by macrophages and BM-DC in general was lower than that of Salmonella or E. coli HB101 (Fig. 3, A and B). In macrophages, a massive intracellular replication of Salmonella was observed, whereas phagocytosed E. coli were killed. The rate of killing of E. coli HB101 was higher than that of E. coli Mutaflor (Fig. 3, C and D). In accordance with previous results, we observed that the number of viable Salmonella in BM-DC remained constant between 2 and 15 h after infection. A decrease of viable E. coli after phagocytosis was observed, and E. coli HB101 was killed with the highest rate (Fig. 3D). Because de...
new synthesized factors as well as known virulence factors (19) were not required for the survival of *Salmonella* in BM-DC, we questioned which preformed bacterial factors might be responsible for the different outcome of the infection by *S. Typhimurium* and the *E. coli* strains. Laboratory strains of *E. coli* such as HB101 are known to harbor various mutations, including the lack of O-Ag. Furthermore, the probiotic strain Mutaflox has been characterized as possessing a semimough LPS, i.e., partially lacking the polymeric O-Ag of the LPS (31). Our analysis indicated a strong difference in the LPS structure of the three strains (Fig. 3E), with *E. coli* Mutaflox and HB101 lacking the polymeric O-Ag, as well as the outer core oligosaccharides, respectively. These data gave a first indication for the role of the O-Ag in bacterial survival in BM-DC.

**LPS O-Ag determines uptake and intracellular fate of *Salmonella***

To further investigate the role of the O-Ag for the intracellular survival of *Salmonella* in BM-DC, we generated an isogenic set of mutant strains with defined defects in the O-Ag biosynthesis. The LPS of *S. Typhimurium* has been studied in detail, and gene functions for the addition of the O-Ag and for the regulation of the O-Ag chain length are known (see Fig. 4A for a model). To generate a set of isogenic mutant strains with defined defects in the O-Ag, *rfaP* (O-Ag transferase; NP_461027) or *rfaL* (O-Ag ligase; NP_462613) was deleted, and the resulting strains should be defective in the addition of the polymeric O-Ag to the outer core. Deletion of *rfaG* (glucosyltransferase I; NP_462622) should result in a highly truncated LPS, because no outer core can be added to the essential inner core. *rfaH* (transcriptional activator; NP_462862) encodes a function for the stabilization of the long mRNA for LPS biosynthesis genes, and an overall reduction on the amount of the LPS species with polymeric O-Ag was anticipated (32). To analyze the effect of the various deletions on LPS structure, *S. Typhimurium* WT and various mutant strains were grown in liquid medium and processed for analyses of the LPS. The LPS profiles indicated that all mutant strains had the expected phenotypes with respect to O-Ag length (Fig. 4B). To restore O-Ag biosynthesis, the WT alleles of *rfal* and *rfag* were cloned in low copy number plasmids, and mutant strains harboring complementing plasmids showed restored O-Ag length of the LPS (Fig. 4B). We also analyzed growth rate of the various strains and did not observe differences in the growth in rich medium (LB broth) or in minimal medium (phosphate/carbon/nitrogen (pH 7.4 or 5.8); data not shown).

We next quantified phagocytic uptake of *S. Typhimurium* WT and various mutant strains by macrophages and BM-DC (Fig. S3; Fig. 4C). Both cell types phagocytosed the *rfag* strain with the highest rate, and uptake of the *rfaP* and *rfal* mutant was also higher than that of the WT strain. For the intracellular replication, we observed a clear difference between macrophages and BM-DC. In macrophages, replication for *rfaP*, *rfal*, and *rfah* strains was only slightly reduced compared with *Salmonella* WT, whereas the *rfag* strain was ~10-fold reduced in intracellular proliferation. In BM-DC, *rfaP* and *rfal* were slightly reduced in survival (~2- to 3-fold), and a strong reduction of the intracellular survival of *rfah* and *rfag* mutants (7.6- and 8.6-fold, respectively) was evident (Fig. 4D). A more detailed analysis of the survival early after phagocytosis showed that both WT and *rfag* *Salmonella* were not killed by BM-DC within the first 2 h of intracellular life (Fig. S3C; data for other LPS mutant strains not shown). The increased uptake of *rfag* and *rfal* strains by BM-DC and the reduced intracellular survival in BM-DC of the *rfag* strain could be restored by addition of the plasmid for the expression of *rfal* or *rfag* (Fig. 4E).

We next analyzed whether the intracellular fate of *Salmonella* in DC is affected by opsonization before uptake (Fig. 4F). As expected, the rates of uptake were 3- to 5-fold higher if bacteria were opsonized with mouse normal serum. The intracellular survival was slightly reduced for all strains after opsonization. However, the defect in intracellular survival of the *rfag* strain was not affected by opsonization. Although uptake of opsonized and nonopsonized bacteria will result in different form in processing by DC, our data show that the defect of strains lacking the O-Ag remains present.

Infection of murine macrophages with *Salmonella* induces the formation of spacious phagosomes (SP) (33). A previous study showed that uptake of *Salmonella* by SP and the maintenance of SP in macrophages were required for intracellular survival of *Salmonella* (34). *Salmonella* serovars not adapted to murine hosts as well as attenuated mutant strains showed reduced induction of SP (33, 34). We analyzed whether SP formation was also induced in BM-DC and whether the LPS defect of the *rfag* strain affects SP formation (Fig. S4). The infection with *Salmonella* WT or *rfag* strains induced SP formation in BM-derived macrophages, as well as in BM-DC (Fig. S4, A and B). The number of SP per cell was similar for both strains in BM-DC and in the range of stimulation by PMA. In macrophages, higher numbers of SP were detected after infection with the *rfag* strains. In accordance with our previous observations, the uptake of the *rfag* strain was ~10-fold higher by primary macrophages as well as by BM-DC (Fig. S4C). In macrophages, only low numbers of *Salmonella*-containing SP were observed per cell, whereas the number of SP containing the *rfag* strain was much higher (Fig. S4D). This difference was less pronounced in BM-DC, and at 60 min after infection the number of SP containing the WT or the *rfag* strain was similar (Fig. S4D).

**The O-Ag structure modulates immunological functions of DC**

The response of BM-DC to infection with *Salmonella* WT and various mutant strains with altered O-Ag was analyzed. As a representative costimulatory molecule, the expression of CD86 was quantified (Fig. 5A). The infection with strains defective in *rfaP*, *rfag*, *rfal*, or *rfah* resulted in higher levels of CD86 compared with BM-DC infected with the same amount of *Salmonella* WT. Similar data were obtained for other costimulatory molecules (CD80 and MHC-II; data not shown). As a further parameter for DC activation, the secretion of TNF was analyzed. Infection with any of the four strains with defects in the O-Ag biosynthesis induced higher levels of TNF secretion than infection with the WT (Fig. 5B). Highest TNF release was observed for BM-DC infected with the *rfag* strain. To test whether the different activation of WT and mutant-infected BM-DC has consequences for the capacity of DC to internalize Ag, we infected BM-DC with *Salmonella*-expressing GFP and simultaneously added fluorescently labeled OVA (Fig. 5C). As expected from the results shown in Fig. 4, the internalization of the *rfag* strain was higher than that of *Salmonella* WT. A higher rate of internalization of OVA was observed in cells infected with the *rfag* strain, indicating increased activation. To control whether the increased uptake of *rfag* *Salmonella* results in the uptake of OVA by the same phagocytic event, the fluorescence microscopy was performed (Fig. 5D). We found that phagocytosed bacteria and the internalized soluble Ags were located in separate compartments and only occasionally colocalized. This separation was observed in BM-DC infected with WT as well as with *rfag* bacteria. This result indicates that the ability of BM-DC to internalize soluble Ags is not altered although the degree of activation is dependent on the structure of O-Ag.
Increased stimulation of DC does not enhance antimicrobial capacity

We performed infection experiments of BM-DC with mixtures of Salmonella WT and rfaG or rfaH mutant bacteria (Fig. 6). Either strain was tagged by different antibiotic resistance markers, thus allowing the quantification of the relative uptake and intracellular survival of each strain. The ratios of WT to mutant strain were 1:10, 1:1, and 10:1. As anticipated from the previous results, the internalization of the rfaG and rfaH strains was generally higher, and a 10-fold excess of the WT strains was required to achieve a higher amount of internalized WT Salmonella (Fig. 6A). The activation of infected DC as quantified by the CD86 expression was dependent on the amount of the mutant strains, but not affected by varying amounts of the WT strain (Fig. 6B). The level of activation was closely correlated to the amount of rfaG bacteria, either used for individual infection (Fig. 6B, □) or in mixture with WT Salmonella (Fig. 6B, ▪). Similar results were observed with mixed infections with the rfaH strain and for the quantification of other activation markers of activation (data not shown). These observations show that LPS with lacking or highly truncated O-Ag is the major factor for the activation of DC.
The subcellular localization of WT and LPS-deficient strains was analyzed by immunofluorescence microscopy (Fig. 6D). Strains were labeled by the expression of distinct fluorescent proteins. After simultaneous infection of BM-DC with a mixture of WT (GFP) and WT (mCherry), we observed that compartments harboring the bacteria were frequently colocalized. By contrast, the WT (GFP) and WT (mCherry), we observed that compartments harboring the bacteria were frequently colocalized. The simultaneous presence of bacteria with defective LPS had no apparent effect on the intracellular survival of WT strains present in the mixture.

The subcellular localization of WT and LPS-deficient strains was analyzed by immunofluorescence microscopy (Fig. 6D). Strains were labeled by the expression of distinct fluorescent proteins. After simultaneous infection of BM-DC with a mixture of WT (GFP) and WT (mCherry), we observed that compartments harboring the bacteria were frequently colocalized. By contrast, the distribution of the WT and the rfaG strain was different, and events of colocalization of compartments harboring WT and rfaG bacteria were very rare. This observation shows that the intracellular fate of Salmonella with intact and defective LPS is different. The simultaneous presence of bacteria with defective LPS had no effect on the fate of WT bacteria present within the same DC and did not affect their survival.

The O-Ag controls degradation of internalized bacteria in DC

Finally, we followed the intracellular fate of S. Typhimurium and E. coli strains after internalization by BM-DC. To investigate whether the internalized bacteria enter the lysosomal degradation pathway, BM-DC were first pulsed with dextran-Texas Red for 2 h to load lysosomal compartments. Infection with various strains was performed, and after intracellular presence of the bacteria for 8 h, the colocalization of the tracer and the intracellular bacteria was quantified (Fig. 7). There was a clear difference between bacteria with an intact O-Ag and those with truncated or entirely absent O-Ag (Fig. 7, B and C). Approximately 80 and 90% of S. Typhimurium rfaG and E. coli HB101 were closely colocalized with the lysosomal marker, indicating that these bacteria rapidly entered the lysosomal degradation pathway. In contrast, only 20% of S. Typhimurium WT and of the complemented rfaG strain were associated with dextran-Texas Red (Fig. 7, B and C). These observations indicated that intact O-Ag is a crucial component of the bacterial cell envelope that enables Salmonella to control its intracellular fate after uptake by DC. We have previously reported that intracellular Salmonella in epithelial cells controls their fate by the function of the SPI2-encoded T3SS (18). To control whether the association of the rfaG strain with the lysosomal marker is a result of a defective SPI2-T3SS function due to outer membrane defects, we followed the translocation of a representative effector protein. Both the WT and the rfaG strain translocated an SseJ-HA fusion protein into BM-DC, indicating that the T3SS function is not affected by the lack of the O-Ag in the rfaG strains (Fig. S5). Taken together, these data show that in DC the integrity of the LPS, but not the function of the SPI2-T3SS, is essential to control intracellular survival of Salmonella.
**Discussion**

Persistence of *Salmonella* in DC is considered as an important prerequisite for the development of systemic infections by *Salmonella*. DC might act as vehicles for the spread of *Salmonella* and other pathogenic microbes, and the ability to persist within DC would enable the efficient use of DC as vehicle.

Previous work reported that BM-DC possess only a low capacity of killing and degradation of internalized *S. Typhimurium* and allow the pathogen to form a static intracellular population (18, 19, 35). Surprisingly, the intracellular persistence of *Salmonella* in DC was independent from the function of virulence factors known to control the intracellular survival and replication in other types of host cells (18, 19). In this study, we demonstrate that the intracellular survival is even independent from the synthesis of new proteins of intracellular *Salmonella* as well as of the infected host cell.

Because preformed components were required, this prompted us to investigate the role of the bacterial envelope. We noted a marked difference in the intracellular survival of *Salmonella* and related *E. coli* strains with defects in the LPS. The in-depth characterization of isogenic *Salmonella* strains with defined defects in LPS biosynthesis shows that the integrity of the core oligosaccharides and the polymeric O-Ag is important to prevent the activation of DC after phagocytosis of *Salmonella* and the intracellular survival. A model summarizing these events is shown in Fig. S6.
In addition to the protection against killing of intracellular bacteria, LPS integrity appears important to reduce or delay the immune responses toward a bacterial infection. A recent analysis in a model with intestinal epithelial cells demonstrated a similar role of the O-Ag in delaying the activation of antimicrobial functions of intestinal epithelial cells (C. Duerr, S. Zenk, C. Chassin, J. Pott, D. Gütle, M. Hensel, and M. Hornef, unpublished observations). The work proposed that intact O-Ag delayed the recognition of the lipid A moiety of LPS by TLR4. Such delayed response may be crucial to enable Salmonella to breach barriers of the host before an efficient immune response is mounted.

The mechanisms of killing of internalized microbes by DC are only partially understood. There are several indications that phagosomes in DC and macrophages have distinct compositions. Studies with soluble Ags revealed the presence of a mildly acidified compartment in DC that mediates retention of the Ag (36). More recently, the role of the NADPH oxidase NOX2 in maintaining an alkaline phagosomal environment was reported (37). NOX2 activity limits Ags degradation and allows cross-presentation by MHC II pathways in DC. It is not known to what extent these events also take place in phagosomes harboring particulate Ags, but the different fate of pathogens such as Salmonella in macrophages and DC suggests that distinct intracellular environments exist in either cell type. Our results indicate that intact LPS of S. Typhimurium either directs the formation of such compartment, or protects the pathogen against the specific antimicrobial effectors present in the phagosome of DC.

The LPS has multiple roles in the adaptation of the bacteria to life in mammalian hosts in mediating protection against antimicrobial peptides, complement, and further antimicrobial molecules of the innate immune system (10). Although the role of LPS for the survival of extracellular bacteria within host environments has been exemplified for various bacterial species, including Salmonella (38), the contribution of the LPS to adaptation to an intracellular lifestyle has only been studied partially. The role of LPS with intact O-Ag (smooth LPS) for the intracellular replication of Brucella spp. was observed, and bacteria with rough LPS lacking the O-Ag were deficient in replication in monocytes (39) and induced to a higher extent the maturation of DC (40). Increased presentation of Ags expressed by Salmonella strains with rough LPS was observed after uptake by macrophages (41), indicating increased degradation. Infection with Salmonella mutant strains expressing LPS without O-Ag resulted in increased activation of DC functions, such as expression of costimulatory molecules.

Previous studies indicated that intracellular fate of S. Typhimurium in murine DC is different after Fcγ-dependent or -independent uptake (42). Increased presentation of a model Ag expressed by Salmonella was observed if opsonized bacteria were internalized by Fcγ-mediated phagocytosis. Microscopic analyses revealed increased targeting of bacteria to lysosomes and increased degradation after Fcγ-mediated uptake (42). Reduced intracellular survival after FcγRIII-mediated uptake was reported (43), although the assay conditions did not address the different rates of uptake. Our data indicate increased uptake, but not decreased intracellular survival in DC after opsonization of Salmonella. The uptake into SP and the maintenance of SP have been reported to contribute to intracellular survival of Salmonella in murine macrophages (33). From our analyses of SP formation, we conclude

![Image](http://www.jimmunol.org/)

**FIGURE 7.** Effect of the O-Ag on the intracellular fate of bacteria in DC. A, BM-DC were pulsed with dextran-Texas Red (red) for 30 min, and noninternalized marker was removed by washing 2 h before infection with various strains constitutively expressing GFP (green). Infection was conducted for 1 h, and noninternalized bacteria were removed by washing and treatment with gentamicin (G), as indicated. Cells were fixed 8 h after infection and immunostained for CD11c (blue), and the colocalization of the internalized bacteria and dextran-Texas Red was analyzed by confocal microscopy (B). Micrographs of representative infected BM-DC are shown. The XY images show Z projections of 8–15 sections, and XZ and YZ sections for individual intracellular bacteria are shown. Scale bar, 10 μm. C, The percentage of colocalization of bacteria and dextran-Texas Red was quantified. Means and SDs from five independent experiments for the quantification of 50–200 intracellular bacteria per strain, are displayed. Statistical analysis, mutant strains vs S. Typhimurium WT: ***, p < 0.01; ***, p < 0.001.
that *Salmonella* is able to induce SP in BM-DC, and that the lack of O-Ag and outer core of the LPS does not affect the induction of SP. The similar number of SP containing WT or rfaG bacteria indicates that SP formation is unlikely to contribute to the reduced survival of the rfaG strain in BM-DC.

We have recently analyzed the influence of the composition of the O-Ag on the function of the TSSS of *Salmonella* and found that variation of the LPS O-Ag (S. Hölder, M. Schlumberger, and M. Hensel, unpublished observation) affected the function of the SPI1-TSSS during adhesion and invasion by intracellular *Salmo-

nella*, but had no detectable effect on the function of the SPI2-

TSSS of intracellular *Salmonella*.

There is only little knowledge on the early events of intracellular life of *Salmonella* in host cells. Because the activation of virulence factors for intracellular survival, such as the SPI2-TSSS, occurs with a delay for at least 1 h (18), it is likely that preformed factors control the early phase after phagocytosis. Although no bacterial replication is detectable within the first few hours of intracellular presence of *Salmonella* in phagocytic cells, this phase is likely to be important for the decision between life and death of the pathogen (44). We propose that the specific composition of the cell envelope allows phagocyted *Salmonella* to prevent entering the degradative and bactericidal pathway in the host cell. We propose that this phenotype is also decisive for the fate in macrophages. The rapid killing of *Salmonella* mutants with defective LPS in macrophages is usually considered as a consequence of increased sensitivity to antimicrobial effectors. In addition, the LPS structure might be important means of preventing the phagolysosomal pathway after uptake. Such function would be similar to the contribution of the cell envelope of *Mycobacterium tuberculosis* (45).

Bueno et al. (46) recently reported differences in the intracellu-

lar fate of human-adapted *S*. Typhi and murine-adapted *S*. Typhimurium dependent on the species of the DC used for infec-

tion. *S*. Typhi, but not *S*. Typhimurium, was able to proliferate in human DC, whereas the situation was inverse in murine DC. Analy-

ses by electron microscopy indicated increased signs of degrada-

tion of *S*. Typhi in murine DC and *S*. Typhimurium human DC (46). Although the molecular mechanisms leading to degradation were not elucidated, the different fate in DC is an interesting explanation of the host specificity of *Salmonella* serovars. In addition to dif-

ferent compositions of the O-Ag of LPS, highly human adapted *S*. Typhi also possesses a polysaccharide capsule that shields the LPS.

Although LPS is an obligate constituent of the outer membrane of enterobacteria, this molecule has evolved toward an optimal function after uptake. Such function would be similar to the contribu-

tion of the SPI2-T3SS encoded by *Salmonella* pathogenicity island 2 towards the chemokines CCL19 and CCL21.

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**Disclosures**

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**References**


