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The Salmonella Pathogenicity Island (SPI)-2 and SPI-1 Type III Secretion Systems Allow Salmonella Serovar typhimurium to Trigger Colitis via MyD88-Dependent and MyD88-Independent Mechanisms

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Salmonella typhimurium can colonize the gut, invade intestinal tissues, and cause enterocolitis. In vitro studies suggest different mechanisms leading to mucosal inflammation, including 1) direct modulation of proinflammatory signaling by bacterial type III effector proteins and 2) disruption or penetration of the intestinal epithelium so that penetrating bacteria or bacterial products can trigger innate immunity (i.e., TLR signaling). We studied these mechanisms in vivo using streptomycin-pretreated wild-type and knockout mice including MyD88−/− animals lacking an adaptor molecule required for signaling via most TLRs. The Salmonella SPI-1 and the SPI-2 type III secretion systems (TTSS) contributed to inflammation. Mutants that retain only a functional SPI-1 (M556; sseD::aphT) or a SPI-2 TTSS (SB161; ΔinvG) caused attenuated colitis, which reflected distinct aspects of the colitis caused by wild-type S. typhimurium: M556 caused diffuse cecal inflammation that did not require MyD88 signaling. In contrast, SB161 induced focal mucosal inflammation requiring MyD88. M556 but not SB161 was found in intestinal epithelial cells. In the lamina propria, M556 and SB161 appeared to reside in different leukocyte cell populations as indicated by differential CD11c staining. Only the SPI-2-dependent inflammatory pathway required aroA-dependent intracellular growth. Thus, S. typhimurium can use two independent mechanisms to elicit colitis in vivo: SPI-1-dependent and MyD88-independent signaling to epithelial cells and SPI-2-dependent intracellular proliferation in the lamina propria triggering MyD88-dependent innate immune responses. The Journal of Immunology, 2005, 174: 1675–1685.

The facultative intracellular pathogen, Salmonella enterica subspecies 1 serovar typhimurium (S. typhimurium), ranges among the most prevalent agents of foodborne diseases. In the gut, S. typhimurium interacts with the intestinal mucosa, invades the intestinal tissue, provokes severe inflammatory responses and induces diarrhea. The pathogenetic mechanisms triggering intestinal inflammation in the infected mammalian host are still a matter of debate (1–3).

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4 Abbreviations used in this paper: TTSS, type III secretion system; PMN, polymorphonuclear leukocyte; PAMP, pathogen-associated molecular pattern; TRITC, tetramethylrhodamine isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; F-actin, filamentous actin; GALT, gut-associated lymphoid tissue; DC, dendritic cell.
metabolic enzymes (e.g., aroA) (19, 20), and most importantly the SPI-1 and SPI-2 TTSS (20–22) (for a review see Refs. 2, 23). However, it is still unclear how these virulence factors allow S. typhimurium to trigger intestinal inflammation.

Hypothetically, several different mechanisms are conceivable, including 1) translocation of SPI-1 effector proteins into enterocytes, which triggers expression and release of proinflammatory cytokines, and 2) Salmonella virulence factors might simply compromise epithelial barrier function or enhance bacterial colonization of epithelial cells and the lamina propria; elevated local PAMP concentrations might activate innate immune responses and trigger inflammation, e.g., via MyD88-dependent TLR signaling (1, 18). It is still unclear which of these mechanisms trigger inflammation, e.g., via MyD88-dependent TLR signaling.

In the present study we found that disruption of the SPI-1 TTSS does not completely abolish colitis in C57BL/6 mice but still allows induction of an ameliorated delayed form of the disease that required the SPI-2 TTSS and is strictly dependent on MyD88 signaling. Our data demonstrate how S. typhimurium uses its two TTSSs in two mechanistically different and largely independent pathways for induction of full blown intestinal inflammation.

Materials and Methods

Bacterial strains and growth conditions

*S. typhimurium* strains were grown for 12 h at 37°C in Luria-Bertani broth (0.3 M NaCl), subcultured for 4 h, washed twice in PBS and suspended in cold PBS (5 × 10^6 CFU/50 μl) as described (26).

The *S. typhimurium* wild-type strain SL1344 (Str^R^) (28) and the isogenic derivatives SB161 (∆invG) (29), M556 (sxtA::aphT) and M557 (∆invG sseD::aphT) (26), χ3262 (ΔaroA), (28), and SB566 (invC::aphT) (30) have been described. Strains M318 (invC::aphT ssaV::cat), M319 (∆aroA invC::aphT), and M324 (∆aroA invC::aphT ssaV::cat) were constructed by phage P22-mediated transduction of alleles invC::aphT (Cam^R^) and ssaV::cat (Cam^R^) from SB566 and HH110 (31), respectively. All gene disruptions and deletions were verified by PCR.

Recombinant DNA techniques

The GFPmut3b coding sequence in pM946 (27), a promoterless variant of pWK530 (32) was replaced by GFPmut2 coding sequence, yielding pM967.

The promoter of the SPI-2 *S. typhimurium* ssaG gene was PCR amplified (primers: 5′-CAT TCT AGA CGG TAG ATT AGC CCT TAC CGC-3′ and 5′-CAT GGA TCC AAT GCT TTT CCT TAA AAT AAA-3′) and cloned into XbaI/BamHI digested pM967, yielding pM973.

Animals

Specified-pathogen-free C57BL/6 mice (6- to 10-week-old) were from Harlan Breeders. Lymphotoxin β receptor (LTBR)−/− mice (33) and MyD88−/− mice (34) (6- to 10-week-old), both on C57BL/6 background, were maintained at the Biologisches Zentrallabor (BZL) Zürich under barrier conditions. Genotypes of knockout mice were verified by PCR typing.

Animal experiments

Infections were performed in individually ventilated cages (Tecniplast) at the BZL Zürich as described (27). Briefly, mice were pretreated by gavage with 20 mg of streptomycin as described (27). Twenty-four hours after streptomycin pretreatment, the mice were intragastrically inoculated with 5 × 10^7 CFU of S. typhimurium. Animal experiments were approved by the Swiss authorities and performed according to the legal requirements.

Analysis of S. typhimurium loads in cecal content, mesenteric lymph nodes (MLN), spleen, and liver

MLN, spleen, and liver were removed aseptically and homogenized in cold PBS (0.5% Tergitol, 0.5% BSA). Cecum content was suspended in 500 μl of cold PBS. The bacterial loads were determined by plating on MacConkey agar plates (50 μg/ml streptomycin) as described recently (24).

Histopathological evaluation

Tissues were embedded in OCT (Sakura) and snap-frozen in liquid nitrogen. Cryosections (5 μm; cross-sectional) were stained with H&E. Cecum pathology was evaluated by two pathologists in a blinded manner using a histopathological scoring scheme as previously described (27). Briefly, 1) the extent of submucosal edema was deduced from the extension of the submucosa and scored by morphometric analysis of the average thickness of the submucosa relative to the thickness of the complete cecal wall. The scores were: 0 = no pathological changes; 1 = detectable edema (<10%); 2 = moderate edema (10–40%); 3 = profound edema (>40%); 2) PMN infiltration into the lamina propria was scored as: 0 = <5 PMN per high power field (HPF; diameter 420 μm); 1 = 5–20 PMN per HPF; 2 = 21–60 PMN; 3 = 61–100 per HPF; 4 = >100 per HPF. 3) loss of goblet cells was scored as: 0 = >28 goblet cells per HPF (×400); 1 = 11–28 goblet cells per HPF; 2 = 1–10 goblet cells per HPF; 3 = <1 goblet cell per HPF; and 4) epithelial integrity was scored as: 0 = no pathological changes detectable; 1 = epithelial desquamation; 2 = epithelial erosion; 3 = epithelial ulceration associated with formation of granulation tissue.

The combined pathological score for each tissue sample was determined as the sum of these individual scores: 0 = no signs of inflammation; 1–2 = minimal signs of inflammation that are normally found in untreated specific-pathogen-free mice and therefore considered as nonpathological; 3–4 = slight inflammation; 5–8 = moderate inflammation; 9–13 = profound inflammation.

Immunohistology

If not indicated otherwise, 5-μm cryosections were fixed with paraformaldehyde (4% in PBS, 1 h, room temperature), blocked with goat serum (10% in PBS, 1 h, room temperature), and incubated in anti-cytokeratin (class I and class II) polyonal rabbit antisierum (Biomedical Technologies), anti-proliferator marker Ki-67 polyclonal antisierum (Abcam), rat monoclonal anti-B220 (clone RA3-6B2), and anti-CDC11 (clone M1/70, biotinylated; all from Becton Dickinson), and/or biotinylated hamster monoclonal anti-CDC11c Ab (clone HL3; Becton Dickinson) in PBS containing 10% goat serum and 0.1% Triton X-100 (overnight, 4°C). Next, biotinylated Abs (CD11b and CD11c staining) were detected with Alexa 647-conjugated streptavidin (Molecular Probes), rabbit Abs with FITC-conjugated (Cytokeratin staining) or tetramethylrhodamine isothiocyanate (TRITC)-conjugated (Ki-67 staining) goat anti-rabbit antisierum (Jackson ImmunoResearch), and rat Abs with Cy3-conjugated (B220 staining) goat anti-rat antisierum diluted in PBS containing 10% goat serum and 0.1% Triton X-100 (1 h, room temperature). For staining of DNA, 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), and for staining of filamentous actin (F-actin), FITC-conjugated (Ki-67 stain) or TRITC-conjugated (all other stainings) phalloidin (Sigma-Aldrich) were added. Coverslips were mounted over Vectashield (Vector Laboratories). Controls using appropriate species- and isotype-matched mAbs (Becton Dickinson) were performed to ensure specificity.

Mucosal localization of GFP-expressing *S. typhimurium*

The cecal tissue was fixed in paraformaldehyde (4% in PBS, 4°C, overnight), incubated in PBS (20% sucrose, 24 h, 4°C) and embedded in OCT. Cross-sectional 20-μm cryosections were dried for 2 h at room temperature and stained with phalloidin-TRITC and DAPI. GFP-expressing bacteria were counted with respect to localization in epithelium or lamina propria.

Imaging and image processing

Images were recorded with a PerkinElmer Ultraview confocal head and a Zeiss Axiovert 200 microscope; infrared, red, and green fluorescence was recorded confocally, blue fluorescence by conventional epifluorescence microscopy. For optimal color reproduction the actual fluorescence colors were replaced by false colors as indicated in the figures. The single layered images were superimposed, colorized, and processed in Adobe Photoshop 7.0. Three-dimensional reconstruction of confocal image stacks was performed with Volocity 2.6.1. Deconvolution of the nonconfocally captured image stacks (DAPI, blue fluorescence) was done using the fast restoration algorithm of Volocity 2.6.1.
Statistical analysis

Statistical analysis was performed using the exact Mann-Whitney U test and the SPSS Version 11.0 software. Values of $p < 0.05$ (two-tailed) were considered as statistically significant. For statistical analysis of bacterial loads, values of 0 CFU were set to the minimal detectable value (10 CFU/MLN, 20 CFU/spleen, 150 CFU/liver, 150 CFU/cecil content sample).

Results

**SPI-1 and SPI-2 TTSS contribute to intestinal inflammation and sustained intestinal colonization**

Wild-type *S. typhimurium* SL1344 (SB300) induces a severe colitis in streptomycin pretreated mice within the first 2 days of infection. This requires a functional SPI-1 TTSS (24). The SPI-2 TTSS does not significantly affect intestinal inflammation by day 2 postinfection (p.i.) (26). However, additional experiments revealed that a *S. typhimurium* strain with a disrupted SPI-1 TTSS (SB161) was not entirely attenuated but caused a delayed colitis, which became evident by day 3 and 4 p.i. (data not shown; see SB161 in Fig. 2). The virulence functions provoking this delayed inflammatory response were unknown.

Previous studies performed in the calf infection model had found that the SPI-2 TTSS can contribute, via unknown mechanisms, to enterocolitis (20, 22). Thus, we analyzed the contribution to later stages (day 3 and 4 p.i.) of *S. typhimurium* colitis in streptomycin pretreated mice and compared the contributions of the SPI-1 and SPI-2 TTSS. We used the *S. typhimurium* wild-type strain SB300 (SL1344) and the isogenic mutant strains SB161 (ΔinvG), M556 (sseD::aphT), and M557 (ΔinvG sseD::aphT). InvG is a core component of the SPI-1 TTSS apparatus, and InvG deficiency abolishes secretion of any effector protein via the SPI-1 TTSS (1, 29). SseD is secreted via the SPI-2 TTSS and represents an important component of the SPI-2 "translocon," which is thought to mediate translocation of effector proteins across the host cell membrane. Lack of SseD abolishes translocation of any effector protein via the SPI-2 TTSS into the host cell cytoplasm (35, 36). Thus, SB161 and M556 can be used to study the pathobiological functions of SPI-1 and SPI-2 TTSS, respectively. M557 is incapable of translocating any SPI-1 or SPI-2 effector proteins and can serve as a negative control.

Streptomycin pretreated C57BL/6 mice were infected for 2, 3, or 4 days with SB300, SB161, M556, or M557. We determined intestinal colonization, systemic infection and cecal histopathology (Figs. 1 and 2).

Systemic infection by SB300 and SB161 resulted in bacterial loads in liver and spleen that increased by ~30-fold per day. In line with previous reports (22, 26, 36, 37), systemic disease strongly depended on the SPI-2 TTSS (Fig. 1, C and D; M556 and M557). In approximately one-third of cases we found unexpectedly high loads of M556 and M557 in spleen and liver. However, these animals did not show symptoms of severe systemic disease and displayed only sporadic mild granulomatous lesions in the liver (data not shown). Thus, M556 and M557 did not persist and could be controlled in liver and spleen of these animals. The presence of M556 and M557 in spleens and livers of some animals might be attributable to transient leakage from the densely colonized intestine. Long term (12 day) infection experiments with M556 lent further support to this hypothesis (data not shown).

We consistently found significant numbers of M556 and M557 in the MLN ($\sim 10^3$ CFU/MLN at days 2–4 p.i.). The loads of SB300 and SB161 were significantly higher: $\sim 10^4$ CFU/MLN at day 2 p.i., and $\sim 10^5$ CFU/MLN at days 3 and 4 p.i. (Fig. 1B).
In conclusion, the combined functions of the SPI-1 and SPI-2 TTSS are required for full blown colitis, yet each TTSS can exert its function in absence of the other: when only the SPI-1 TTSS is functional (M556), a slightly attenuated colitis develops early on, when only the SPI-2 TTSS is functional (SB161), colitis is attenuated and delayed by ~2 days. These observations suggested that M556 and SB161 are useful to study the functions of the SPI-1 or the SPI-2 TTSS in murine colitis without interferences caused by inflammatory stimuli attributable to the other TTSS.

**Distinct histopathological features characterize SB161- and M556-induced colitis**

The general signs of inflammation induced by SB161 and M556 were quite similar, but detailed analyses revealed marked histopathological differences: M556 elicited an acute diffuse (phlegmonous) inflammation of the mucosa characterized by diffuse influx of PMNs (H&E stain; cell morphology), CD11b+ (including PMNs and macrophages/monocytes), and B220+ (predominantly B cell-like) cells into the lamina propria (Fig. 3C). M556 induced broad epithelial lesions (desquamation, erosion, and marked PMN transmigration at the villus tips; crypt abscesses), marked epithelial regeneration (expanded proliferative zone of Ki-67+ cells), and loss of goblet cells. In contrast, SB161 induced focal inflammatory lesions (Fig. 3B, upper panels), which were interspersed with wide areas of the mucosa showing little inflammation (Fig. 3B, lower panels). These mucosal lesions were located basally and were characterized by pronounced local infiltration of PMNs and other CD11b+ cells and local loss of crypt epithelium (Fig. 3B, upper panels). The histopathology induced by SB300 combined features of both pathologies: at day 3 p.i. SB300-induced histopathology included areas of pronounced M556-like inflammation (Fig. 3A, lower panels) and focal (SB161-like) lesions marked by profound loss of crypt epithelium (Fig. 3A, upper panels), which often culminated in ulceration by day 4 p.i.

**Neither induction of SB161-mediated nor of M556-mediated intestinal inflammation requires gut-associated lymphoid tissues (GALT) organs**

The GALT organs are essential inductive sites for immune responses toward organisms colonizing the gut and are involved in inflammatory bowel diseases (38). A previous study had shown that SPI-1-dependent *S. typhimurium* colitis (day 2 p.i.) (24) was not diminished in LTβR-deficient mice that lack MLN, Peyer’s patches, isolated lymphoid follicles, MOMA-1+, and metallophilic marginal zone macrophages of the spleen, germinal centers, and follicular dendritic cell networks (33, 39). Peyer’s patches and possibly isolated lymphoid follicles are regarded as the preferred sites of epithelial translocation for *S. typhimurium* mutants with a disrupted SPI-1 TTSS (40–42) as well as for gut commensals (43, 44). It was unknown whether Peyer’s patches/GALT organs are required for SB161-induced colitis.

To test these sites, streptomycin pretreated LTβR+/− mice were infected for 3 days with SB300, SB161, M556, or M557. Wild-type C57BL/6 mice served as controls. In the LTβR+/− mice the bacterial loads in liver (Fig. 4B) and spleen (data not shown) were ~10-fold higher than in the wild-type control mice. This is consistent with the observation that MLN (lacking in LTβR−/− mice) have a barrier function for dendritic cells (DCs) harboring intestinal bacteria (44). Despite the more pronounced systemic infection, colonization of the cecal lumen (10^7–10^10 CFU/g; Fig. 4A) and SB161-induced colitis were not affected in LTβR−/− mice (Fig. 4C).
Thus, the GALT organs, a normal splenic architecture, or the extent of spleen and liver colonization are not crucial for the induction of cecal inflammation caused by wild-type *S. typhimurium* SB300, a *S. typhimurium* strain retaining only a functional SPI-1 TTSS (M556) (26) or a strain retaining only a functional SPI-2 TTSS (SB161; this study). This is in line with data from B cell- and T cell-deficient Rag1−/−H11002 mice, which indicated that cecal inflammation induced either by SB300, SB161, or M556 does not require functional B cells and T cells (data not shown). This led us to focus our further analyses on the cecal absorptive mucosa.

The SPI-1 TTSS mediates epithelial cell invasion and cooperates with the SPI-2 TTSS for colonization of the lamina propria

We hypothesized that the differences in histopathology and onset of inflammation caused by SB161 and M556 might correlate with differences in the pattern of tissue colonization. To analyze this we applied a microscopic approach: SB300, SB161, M556, and M557 were transformed with plasmid pM973 that encodes GFP under control of the SPI-2 promoter pssaG. GFP was not expressed in culture broth used for growing the bacterial inocula or in the intestinal lumen (data not shown), but allowed reliable detection of intracellular (GFP+) bacteria (SB300, SB161, M556, and M557) as early as 1–4 h after invasion (data not shown). Pilot animal experiments verified that the GFP expression plasmid was stably propagated (>95% recovery at 4 days p.i.), did not significantly attenuate virulence of the tested strains (data not shown), and allowed in situ detection of intracellular bacteria.

Streptomycin pretreated C57BL/6 mice were infected for 3 days with SB300(pM973), SB161(pM973), M556(pM973), or M557(pM973). Histopathology and colonization of the cecal lumen and internal organs were equivalent to earlier experiments (data not shown; see Figs. 1 and 2). Cecum 20-μm cross-sections were stained and we determined the localization of GFP-expressing bacteria in epithelial cells and the lamina propria of the cecal mucosa (Fig. 5, A–C; data not shown).

SB300(pM973) was observed in epithelial cells and in the lamina propria (Fig. 5 C). The numbers of M556(pM973) in epithelial cells were similar to those of SB300(pM973), but the numbers of bacteria in the lamina propria cells were 10- to 100-fold lower (Fig. 5, B and C). SB161(pM973) was virtually absent from the epithelial cells, but present in lamina propria cells in similar numbers as M556(pM973) (Fig. 5, A and C). Over 90% of these bacteria were confined to the characteristic inflammatory foci in the lamina propria, which were observed in SB161-infected mice (Figs. 5A and 3B, upper panels). M557(pM973) was not detected in any of the over 20 cecal tissue sections analyzed (Fig. 5C).

These data indicated that SPI-1 and SPI-2 play different roles in the colonization of the cecum of streptomycin pretreated mice; the SPI-1 TTSS (but not the SPI-2 TTSS) is required for colonization of the cecal epithelium. In contrast, efficient colonization of cells...
Bacterial loads in MLN were ~100-fold reduced by disruption of either *aroA* (*p = 0.008; SB300 vs *χ*3625 or SB566 vs M319) or the SPI-2 TTSS (*p = 0.016; SB566 vs M318; compare Fig. 1B for SB300 vs M556) and were further reduced when both were disrupted (Fig. 6B). These data indicated that the role of the SPI-2 TTSS in spread to and/or colonization of the MLN is attributable primarily to enhanced intracellular proliferation. Disruption of SPI-1 TTSS had no significant effect (*p > 0.05; SB300 vs SB566, *χ*3625 vs M319).

Strains carrying a single mutation in *aroA*, the SPI-1, or the SPI-2 TTSS (*χ*3625, SB566, Fig. 6D; SB161, M556, Fig. 2B) caused less severe inflammation than SB300. Intriguingly, the strains with a combined disruption of *aroA* and the SPI-1 TTSS were unable to cause significant colitis (*p < 0.05; M319 vs SB300, SB566 or *χ*3625). This resembled the attenuation caused by combined disruption of the SPI-1 and SPI-2 TTSS (Fig. 6D; *p > 0.05; M318 vs M319). In contrast, deletion of *aroA* only slightly affected colitis when a functional SPI-1 TTSS was present (i.e., in *χ*3625) and the histopathology induced by *χ*3625 was similar to that caused by M556 (data not shown; see Fig. 3).

Thus, the *aroA* mutation affected *S. typhimurium*-induced colitis in a manner very similar to disruption of the SPI-2 TTSS. This indicated that abrogation of *aroA*-dependent intracellular growth specifically abrogates the inflammatory pathway that requires the SPI-2 TTSS; the function of the SPI-2 TTSS in murine colitis is closely linked to intracellular proliferation. Yet, the impairment of intracellular growth still allows induction of inflammation via the SPI-1 TTSS.

**SB161 and M556 may reside in different leukocyte populations of the lamina propria**

The data already presented suggested that the lamina propria cells harboring *S. typhimurium* (especially SB161) in the murine gut might play a role in the colonic inflammation. We characterized these cells by immunohistology using Abs to CD11c, the α8β2-integrin (CD11c/CD18) expressed on DCs and activated CD8 intraepithelial and lymph node T cells. Abs to CD18 (integrin β2-chain expressed on most infiltrating leukocytes notably NK cells, Mac-1/CD11b+ PMNs, and macrophages, and CD11c+ DCs) were also used (data not shown).

Generally, GFP-expressing SB300(pM973), SB161(pM973), and M556(pM973) residing in the lamina propria were detected in close association with (probably inside) CD18+ cells (>80%; data not shown). Most (81%) of GFP-expressing M556(pM973) but only 1% of SB161(pM973) were found in association with CD11c+ lamina propria cells (Fig. 5, D–F). Thus, SB161 and M556 may reside in different lamina propria lymphocyte populations; SB161 in CD18+CD11c+ cells and M556 in CD18+CD11c− cells. This might be connected to the distinct inflammatory responses caused by SB161 and M556.

**Delayed SB161 inflammation strongly depends on MyD88, M556 inflammation does not**

Two distinct pathogenetic mechanisms might contribute to the inflammatory response to *S. typhimurium*. Inflammation might be primarily triggered actively by translocated *S. typhimurium* effector proteins, which activate proinflammatory signaling. Alternatively, SPI-1 and SPI-2 effector protein functions might simply enhance colonization of the deeper intestinal tissue. In the latter case, an inflammatory response could be triggered by elevated concentrations of bacterial PAMPs, which is sensed by the TLRs of the innate immune system (18). In an attempt to distinguish between these two mechanisms, we used knockout mice lacking MyD88, a key adaptor protein in the signal transduction cascades...
shared by most of the TLRs. MyD88 deficiency abrogates or delays proinflammatory responses to many TLR ligands including LPS, peptidoglycan and other cell wall components, flagellin, and CpG (18).

Streptomycin pretreated MyD88−/−/− mice and MyD88+/−/− littermates were infected for 3 days with SB300, SB161, M556, or M557. We determined bacterial loads in cecal contents, MLN, spleens, and livers and evaluated the cecal histopathology. We found that all four bacterial strains efficiently colonized the cecal lumen of MyD88−/−/− mice and MyD88+/−/− littermates (Fig. 7A). An increased susceptibility of MyD88−/−/− mice to systemic salmonellosis was described recently (46). Similarly, we observed elevated bacterial loads in MLN, spleens, and livers of MyD88−/−/− mice (Fig. 7, B–D). Only in the case of the wild-type strain SB300 we did not observe significant differences (p > 0.05), probably due to high animal to animal variations.

SB300 caused the same level of inflammation in MyD88−/−/− and MyD88+/−/− mice (Fig. 7E). M556 induced a slightly but significantly (p = 0.008) ameliorated cecal inflammation in MyD88−/−/− animals. In contrast, SB161 failed to induce marked inflammation in MyD88−/−/− mice (p = 0.002), even though analysis of SB161(pM973)-infected MyD88−/−/− mice revealed high numbers of SB161 in the lamina propria (Fig. 7F).

In conclusion, wild-type S. typhimurium can efficiently induce cecal inflammation even in the absence of MyD88 signaling. M556-induced inflammation is only slightly affected in MyD88−/−/− mice, whereas MyD88-dependent signaling is essential for the delayed inflammation caused by SB161. This is the first in vivo evidence for MyD88-dependent and MyD88-independent pathways in S. typhimurium colitis.

Discussion

The underlying mechanism of S. typhimurium colitis has been a matter of debate. We used the streptomycin pretreated mouse model to study the mechanisms of Salmonella enterocolitis. In this model, the SPI-1 TTSS and namely the SPI-1 effector proteins SipA, SopE, and SopE2 are of key importance during the early phase of colitis (1–2 days) (24, 26). In this study we have shown that the SPI-2 TTSS also contributes to S. typhimurium colitis in streptomycin pretreated mice and that this strictly requires MyD88 signaling.

Although both TTSS are required to cause full blown inflammation, S. typhimurium strains lacking either the SPI-1 or the SPI-2 TTSS can cause colitis. This allowed studying the functions of each TTSS in murine colitis without interferences caused by functional overlaps between the SPI-1 and SPI-2 TTSS.
FIGURE 6. Role of intracellular proliferation in *S. typhimurium* colitis. Streptomycin pretreated mice were infected with SB300, c3625 (ΔaroA), SB566 (invC::aphT), M319 (ΔaroA invC::aphT), M318 (invC::aphT ssaV::cat) or M324 (ΔaroA invC::aphT ssaV::cat). At day 3 p.i. we determined the bacterial loads in the cecal lumen (A), MLN (B), and livers (C). D. Histopathological evaluation of H&E stained sections of cecal tissue. Each stacked bar represents one animal. NS, Not statistically significant; dotted line, detection limit; arrows, statistical analysis of the difference between the indicated animal groups.

FIGURE 7. Analysis of MyD88-deficient mice. Streptomycin pretreated MyD88-deficient (-/-) and C57BL/6 littermate control (+/+ ) mice were infected with SB300, SB161, M556, or M557. At day 3 p.i., we determined the bacterial loads in the cecal lumen (A), MLN (B), spleens (C), and liver (D) of MyD88-/- (○) and wild-type (●) mice. E. Histopathological evaluation of H&E-stained sections of cecal tissue. Each stacked bar represents one animal. Dotted line, detection limit; NS, Not statistically significant. F. MyD88-/- mice were infected for 3 days with SB161(pM973). Cecal tissue was stained with TRITC-phalloidin (red: F-actin at the cell periphery) and DAPI (blue: nuclei). The white box (inset left panel) indicates the region shown at higher magnification and scale bars are 20 μm.
We found marked differences between colitis caused by SB161 (functional SPI-1 but disrupted SPI-1 TTSS) and M556 (functional SPI-1 but disrupted SPI-2 TTSS) in streptomycin pretreated mice: 1) Disease progression: M556 caused cecal inflammation early on after infection, while SB161 caused a delayed colitis; 2) Histopathology: SB161 induced pronounced inflammatory foci in the lamina propria, whereas M556 infection caused uniformly distributed epithelial lesions and inflammatory infiltrates throughout the mucosa; 3) Localization: M556 was found in equal numbers in epithelial cells and in the lamina propria (mostly CD18\(^+\)/CD11c\(^+\) cells), SB161 was preferentially located in CD18\(^+\)/CD11c\(^+\) cells in the inflammatory foci in the lamina propria but absent from epithelial cells; 4) aroA-dependence: strains with a disrupted SPI-1 TTSS require AroA to cause colitis. A wild-type strain with intact SPI-1 and SPI-2 TTSS does not; 5) MyD88-dependence: M556, but not SB161, caused colitis in MyD88\(^{−/−}\) mice.

These observations support the notion that the SPI-1 and the SPI-2 TTSS allow \textit{S. typhimurium} to trigger cecal inflammation via two different pathways.

**TLR signaling triggered by bacteria in the lamina propria**

The results obtained with SB161 indicate that the SPI-2 TTSS allows \textit{S. typhimurium} to survive and replicate in the lamina propria. SB161 resided preferentially in CD18\(^+\)/CD11c\(^+\) cells in characteristic inflammatory foci at the base of the lamina propria. When intracellular replication was impaired by another mutation (aroA, a metabolic gene), inflammation did not occur via this pathway. SB161 colitis was also impaired when MyD88 signaling was disrupted, even though SB161 was still present in high numbers in the lamina propria (Fig. 7F). MyD88 is a central adaptor protein in the signal transduction cascades downstream of most TLRs (notably those specific for bacterial PAMP) and of the two cytokine receptors IL-1R and IL-18R. Especially IL-1R is involved in many inflammatory processes (47, 48). Hypothetically, disruption of IL-1 signaling could also explain the attenuation of SB161 colitis in MyD88\(^{−/−}\) mice. However, SB161-induced colitis was not affected in IL-1 receptor-deficient mice (our unpublished data). These observations suggest that the attenuation of SB161-colitis in MyD88\(^{−/−}\) mice was attributable to disrupted TLR signaling.

Thus, the SPI-2 TTSS contributes to murine \textit{S. typhimurium} colitis by preventing bacterial killing by innate effector cells and/or enhancing intracellular bacterial replication in the lamina propria. As a consequence thereof, innate immune responses to the increased loads of bacteria and/or released PAMPs provide the primary proinflammatory stimulus.

**MyD88-independent signaling via the SPI-1 TTSS**

The results obtained with M556 indicate that the SPI-1 TTSS allows \textit{S. typhimurium} to interact with epithelial cells and cells in the lamina propria and to cause inflammation in a MyD88-independent fashion. Efficient intracellular replication in the mucosa is not required for this.

How does the SPI-1 TTSS contribute to colitis? In principle, the interaction with epithelial cells or cells of the lamina propria (possibly DCs as judged by CD11c staining) might provide the primary proinflammatory stimulus. In the former case, inflammation might be triggered directly by injection of SPI-1 effector proteins into epithelial cells. Several SPI-1 effector protein genes are expressed in the cecal lumen of streptomycin pretreated mice (our unpublished data). This would resemble the situation in tissue culture experiments, where effector proteins like SopE, SpIP, SspH1/2, or AvrA were found to directly manipulate JNK and NF-kB signaling and thereby the release of proinflammatory cytokines (9, 10, 49–51). Alternatively, the SPI-1 effector proteins might simply trigger bacterial entry into cecal epithelial cells (and possibly also into the lamina propria), which might increase the loads of bacterial components (PAMPs) presented to the innate immune system. In this case the primary trigger for inflammation would be the recognition of PAMPs by specific receptors, i.e., TLRs. In fact, recognition of internalized LPS by intracellular TLR pools has recently been described in intestinal epithelial cells (52, 53). However, M556 causes colitis in MyD88\(^{−/−}\) mice. This excludes many of the “classical” TLR triggered signaling cascades as the primary trigger for inflammation (even though some MyD88-dependent signaling may well have occurred). However, MyD88-independent TLR signaling pathways have been identified, recently (54–56). Future work will have to determine the relative contribution of MyD88-independent TLR signaling and proinflammatory signaling directly triggered by the SPI-1 effector proteins to M556-induced colitis.

DCs might also be involved in triggering cecal inflammation via the SPI-1 TTSS. Significant loads of M556 are present in lamina propria cells, DCs as judged by CD18\(^+\)/CD11c\(^+\) staining. DCs are key regulators in innate and adaptive immune responses and they are capable of sampling bacteria directly from the cecal lumen (57). SPI-1 effector proteins might directly induce proinflammatory signaling in DCs. Alternatively, DCs might be recruited and internalize \textit{S. typhimurium} in response to signals released by infected epithelial cells.

Currently, none of these possibilities can be dismissed. However, it is clear that the SPI-1-mediated inflammation does not require MyD88 signaling or massive bacterial replication in the lamina propria. Therefore, this mechanism is clearly distinct from the SPI-2-mediated inflammation observed with SB161.

Does wild-type \textit{S. typhimurium} use both of these mechanisms in parallel? It is conceivable that disruption of the SPI-1 TTSS or genes required for intracellular survival/replication (aroA, SPI-2 TTSS) might modify the chain of events leading to forms of the disease, which are normally not encountered in wild-type \textit{S. typhimurium} colitis. The latter possibility cannot be completely ruled out. However, several lines of evidence indicate that both mechanisms observed with M556 and SB161 are operational in wild-type \textit{S. typhimurium} (SB300) colitis. The histopathology of SB300-induced colitis included characteristic features of M556 and of SB161 colitis. M556 and SB300 loads in epithelial cells were similar and both induced an early onset of colitis. Deletion of specific SPI-1 effector protein genes (sopE, sopE2, and sipA) had similar effects in M556 and wild-type \textit{S. typhimurium} (26). Moreover, MyD88 signaling was dispensable in SB300 and M556 colitis. This suggests that the pathogenetic mechanisms of the M556 infection are also operational in wild-type \textit{S. typhimurium} colitis.

Like SB161, SB300 also induced and resided preferentially in characteristic inflammatory foci at the base of the lamina propria. Furthermore, M556, which lacks a functional SPI-2 TTSS, causes less severe colitis than SB300. Therefore, intracellular persistence/repetition also contribute to the inflammation caused by SB300. This is further supported by the slightly ameliorated cecal inflammation caused by an aroA strain (Fig. 6). Overall, these observations suggest that both pathways, the one used by SB161 and the one used by M556, are operational in and contribute to wild-type \textit{S. typhimurium} colitis. In fact, SB300 colitis can be regarded as a combination of the typical features of M556- and SB161-mediated colitis that is aggravated by the cooperation between the functions of the SPI-1 and SPI-2 TTSS.

What is the role of the GALT? In our analysis we found a significant difference in the spread from the gut lumen to spleen or liver in LTβR\(^{−/−}\) mice that lack GALT organs (including the cecal patch and the MLN) (33). Even the highly attenuated strains M556 and M557, which both lack a functional SPI-2 TTSS, were both...
consistently found in significant numbers in the spleens and livers of these mice. This is in line with the hypothesis that the MLN serve as barrier for intestinal bacteria, which are sampled from the intestinal lumen and transported to the MLN by DCs (44). In the initiation of T cell-dependent and -independent adaptive immune responses toward bacteria of the gut lumen, the GALT organs play an important role as “inductive sites” (58–60). However, our studies of LTβR−/− and Rag1−/− mice argue against a significant role of the GALT-dependent or T cell- and B cell-dependent mechanisms in initiation of the early (M56-induced, SPI-1-dependent) (24, 26) or delayed (SB161-induced, SPI-2-dependent; this work) intestinal inflammation.

Overall, S. typhimurium seems to use two different strategies in parallel for efficient triggering of intestinal inflammation in streptomycin pretreated mice: manipulation of epithelial or lamina propria cells (DCs?) and replication in the lamina propria, respectively. The use of appropriate mutant Salmonella strains and mouse strains allowed demonstrating that the latter strategy relies on MyD88-dependent innate immune responses whereas the former strategy does not. These observations represent an important step in the analysis of the molecular mechanisms contributing to S. typhimurium colitis.

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References


