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Review

Cellular microbiology of intracellular *Salmonella enterica*: functions of the type III secretion system encoded by *Salmonella* pathogenicity island 2

V. Kuhle and M. Hensel*

Institut für Klinische Mikrobiologie, Immunologie und Hygiene, FAU Erlangen-Nürnberg, Wasserturmstr. 3–5, 91054 Erlangen (Germany), Fax: +49 9131 8522531, e-mail: hensel@mikrobio.med.uni-erlangen.de

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Abstract. The facultative intracellular pathogen *Salmonella enterica* resides in a special membrane compartment of the host cell and modifies its host to achieve intracellular survival and proliferation. The type III secretion system encoded by *Salmonella* pathogenicity island 2 (SPI2) has a central role in the interference of intracellular *Salmonella* with host cell functions. SPI2 function affects antimicrobial defense mechanisms of the host, intracellular transport processes, integrity and function of the cytoskeleton and host cell death. These modifications are mediated by translocation of a large number of effector proteins by the SPI2 system. In this review, we summarize recent work on the cellular phenotypes related to SPI2 function and contribution of SPI2 effector proteins to these manipulations. These studies reveal a complex set of pathogenic interferences between intracellular *Salmonella* and its host cells.

Key words. *Salmonella*; intracellular pathogen; type III secretion system; SPI2.

Introduction

Infections by *Salmonella* spp., a food-borne Gram-negative pathogen of the family of enterobacteriaceae, are a continuing risk for human health. Historically, more than 2000 *Salmonella* 'species' have been described, but it is now established that most of these are serotypes of the species *Salmonella enterica*. Very diverse disease outcomes following *Salmonella enterica* infection can be observed. Human diseases range from a generally mild, self-limiting gastroenteritis, caused mainly by *S. enterica* serovar Typhimurium (*S. typhimurium*) and serovar Enteritidis (*S. enteritidis*), to the severe, systemic infection of typhoid fever caused by *S. enterica* serovar Typhi (*S. typhi*) or serovar Paratyphi (*S. paratyphi*). Although the incidence for typhoid fever in developed countries has been reduced drastically by improved hygiene, *S. typhi* is still a major threat in developing countries, with rates of incidence reaching up to 200 cases per 100,000 inhabitants in Vietnam or 1000 cases per 100,000 inhabitants in New Delhi, India. Worldwide, typhoid fever affects over 16 million people every year, with approximately 600,000 deaths (for review see [1]). *S. typhi* infections are transmitted mainly via contaminated water or food. The infectious dose ranges from 10^3 to 10^6 bacteria.

S. typhi is host-specific for humans and higher primates. Although it does not perfectly reflect human disease, *S. typhimurium* infection of susceptible (Nramp1-negative) mice is widely used as a model for systemic salmonellosis. In contrast to humans, where *S. typhimurium* leads to a generally mild gastroenteritis, susceptible mice develop symptoms similar to typhoid fever after infection.

In general, a relative high inoculum is required to overcome the acidic pH of the stomach and to compete with

* Corresponding author.

the resident microbiota of the gut. After reaching the small intestine, *Salmonella* adhere to mucosal cells and traverse the M-cells to reach the intestinal lymph follicles of the Peyer's patches. Here, *Salmonella* are taken up by phagocytic cells and are transported to the mesenteric lymph nodes, liver and spleen. In these organs, *Salmonella* can survive and replicate inside macrophages in a special membrane compartment, the *Salmonella*-containing vacuole (SCV). Depending on the number of bacteria, the virulence and the host cell response, intracellular *Salmonella* are released into the bloodstream, followed by bacteremia and systemic dissemination of the pathogen [1].

Virulence factors of *Salmonella enterica*

The complex pathogenesis of systemic *S. enterica* infections correlates with the presence of a large number of defensive as well as offensive virulence factors (reviewed in [2]). In non-host environments and during colonization of host habitats as diverse as the intestinal lumen, epithelial cells and professional phagocytes, the pathogen has to respond to changing nutritional limitations as well as stress and damage imposed by host defense mechanisms. As a facultative intracellular pathogen, *S. enterica* can adapt to the intracellular environment, and the two-component regulatory system PhoPQ appears to be an important sensor for the transition between extra- and intracellular life [3].

A remarkable feature of *S. enterica* is the presence of a large number of pathogenicity islands (PAI). PAI are distinct, relatively large chromosomal regions harboring virulence genes that are present in pathogens but absent in benign relatives. PAI are characterized by a base composition different from the core genome and are often associated with transfer RNA (tRNA) genes and mobile genetic elements, like insertion sequence (IS) elements, transposons or phage genes (for review see [4]).

Two hallmarks of *Salmonella* pathogenesis, i.e. host invasion and intracellular proliferation, are directly linked to genes in PAI. *Salmonella* pathogenicity island 1 (SPI1) contains invasion genes, while *Salmonella* pathogenicity island 2 (SPI2) is required for intracellular pathogenesis and has a crucial role for systemic *S. enterica* infections (reviewed in [5]). Both SPI1 and SPI2 encode type III secretion systems (T3SS).

Type III secretion systems

Although termed secretion systems, the biological function of T3SS is the translocation of proteins from the bacterial cytoplasm into the host cell, thus functioning as 'molecular syringes' (for review see [6]). In contrast to the type II secretion system, translocation via the T3SS occurs independent of an N-terminal conserved *sec*-sequence that is cleaved after secretion. T3SS are complex molecular machines consisting of more than 20 different protein subunits. The core structure of the T3SS shows homology to the flagella-assembly system. T3SS are restricted to Gram-negative bacteria and are present in a number of different species, where they fulfill distinct functions ranging from antiphagocytic and cytotoxic effects on host cells (Ysc/Yop system of *Yersinia* spp.), invasion of host cells (*S. enterica* SPI1 system, *Shigella* spp. Mxi/Spa system), intracellular pathogenesis (*S. enterica* SPI2 system, *Chlamydia* spp. T3SS) to the establishment of symbiotic relationships such as that observed for the insect endosymbiont *Sodalis glossinidius* and the plant symbiont *Rhizobium* spp.

S. enterica possesses two distinct T3SS with roles in different phases of pathogenesis. Although *S. enterica* was the first example of a pathogen employing two T3SS, genome sequencing revealed that multiple T3SS also occur in other species such as *Yersinia* spp., *Vibrio parahaemolyticus* and *Burkholderia pseudomallei* (reviewed in [7]).

SPI1

Genes within SPI1 were first identified by characterization of mutant strains deficient in host cell invasion [8]. It subsequently became evident that a large number of invasion genes are clustered in the SPI1 locus, a 40-kb region located at centisome 63 of the *S. typhimurium* chromosome, which encodes a T3SS, several effector proteins and regulators of gene expression (reviewed in [9]). In addition to the SPI1-encoded effectors, further proteins translocated by the SPI1-encoded T3SS are present on distinct loci scattered over the whole chromosome. SPI1 genes are expressed under conditions resembling those found in the intestinal environment and are repressed by intracellular *Salmonella*.

Elegant molecular analyses revealed that one subset of SPI1 effector proteins modifies small GTPases of the host cell, resulting in rearrangements of the host cell actin cytoskeleton subsequently leading to the formation of membrane ruffles and uptake of *Salmonella* by a process known as macropinocytosis [10]. SPI1-mediated invasion is not required for pathogenesis of systemic infections. A second role for the SPI1 system in enteritis and gastrointestinal inflammation has been established (reviewed in [11]). Here, an additional subset of effector proteins is translocated. This subset includes the inositol phosphate phosphatase SopB, which mediates loss of electrolytes and fluid secretion, but is also involved in invasion.

SPI2

SPI2 was identified by the characterization of mutant strains that were highly attenuated in virulence in murine

salmonellosis. The mutations clustered in a 40-kb region located between centisome 30 and 31 of the *Salmonella* chromosome with typical features of PAI. Further analyses indicated that a second T3SS is encoded by one subset of SPI2 genes. These 31 genes are organized in two operons encoding components of the secretion system apparatus (*ssa*), one transcriptional unit encoding secretion system effectors (*sse*) and chaperones (*ssc*), and an operon encoding the two-component regulatory system SsrAB (secretion system regulator, *ssr*) [12]. The genetic organization of SPI2 is shown in figure 1. In this review, we will use 'SPI2-encoded T3SS' or 'SPI2 system' to refer to these virulence genes.

S. typhimurium strains deficient in the SPI2-encoded T3SS are highly attenuated in murine salmonellosis, and a 10^4-fold higher LD50 (lethal dose 50) was reported [12]. SPI2 mutant strains were unable to proliferate within host organs [13], and on the cellular level these strains showed reduced survival and proliferation inside host cells [14, 15].

Expression of structural genes of the SPI2-encoded T3SS, as well as various loci encoding effectors, is activated after uptake by host cells and is dependent on the regulatory system SsrAB. The precise signals inducing SPI2 gene expression are not known in detail. However, the composition of minimal media inducing SPI2 expres-

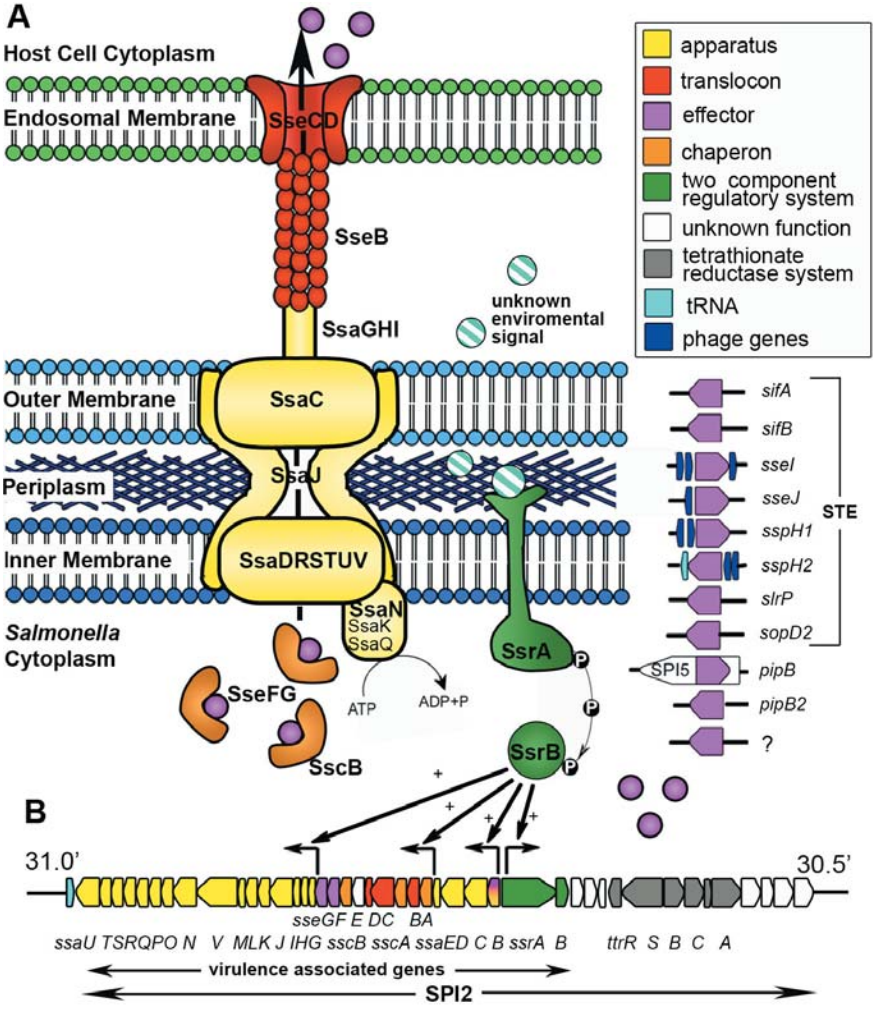

Figure 1. *Salmonella* pathogenicity island 2 (SPI2) and model of the SPI2-encoded T3SS. (*A*) A hypothetical model of the SPI2-encoded T3SS is shown and the subcellular localization of subunits indicated is based on experimental analyses [105] or studies on orthologs in T3SS of other bacteria [106]. The T3SS consists of the apparatus, a complex assembly of proteins (yellow) in the cell envelope, and the translocon. SseBCD (red) assemble to form a translocon with a putative pore for the translocation of effector proteins (violet) across the endosomal membrane. (*B*) Genetic organization of the SPI2 locus is depicted. Only *ssaU* to *ssrB* are required for virulence functions. Several effector proteins of the SPI2 system are encoded on separate loci outside of SPI2. Eight proteins of the family of *Salmonella*-translocated effectors (STE) share conserved N-terminal sequences. STE loci are in part associated with bacteriophage genes, while PipB is encoded by SPI5. Expression of T3SS genes in SPI2 as well as several effectors encoded outside this locus is under control of the SsrAB two-component regulatory system, although the precise signal sensed by SsrAB is not known.

sion in vitro suggest that nutritional limitations might be sensed. There is also evidence for the role of global regulatory systems such as PhoPQ and OmpR/EnvZ in controlling SPI2 gene expression [16, 17].
The structure of the SPI2-encoded T3SS has not been analyzed in detail, and the function of many subunits has been deduced from orthologs of better-studied systems. A model for the proposed localization and function of SPI2 proteins is shown in figure 1.

Translocon and effector proteins of the SPI2 system
Substrate proteins transported by the SPI2-encoded T3SS can be divided into two subgroups. Three SPI2-encoded proteins, SseBCD, function as a translocon (translocator) for the group of effector proteins that are translocated into the host cell. SseBCD are encoded within SPI2 and show a certain similarity to the translocon proteins EspABD of enteropathogenic *Escherichia coli* (EPEC). Functional analyses suggested that SseBCD fulfill similar functions within the SPI2-encoded T3SS [18]. *S. typhimurium* strains with a mutation in one of these genes were previously shown to be attenuated in mouse virulence and intramacrophage replication to the same degree as an apparatus mutant [19, 20]. These mutants were also unable to translocate other SPI2 effectors into the host cell [18, 21]. After secretion in vitro, all three proteins were found loosely attached to the bacterial surface [20, 22]. While SseB secretion was independent of SseC and SseD [22], efficient secretion of these two proteins occurred only in the presence of SseB [18]. Microscopic analysis revealed a polar localization of SseB at one end of the bacterial cell [18].
There is evidence from different groups [23–25] that SseA, a protein encoded directly upstream of *sseB*, acts as a chaperone for SseB and SseD.
At present, 13 effector proteins translocated by the SPI2-encoded T3SS have been identified (table 1). Ten of these effector proteins are encoded outside SPI2 by genes that are, in part, localized on distinct pathogenicity islets or islands, or are associated with prophages or cryptic bacteriophages [26], indicating independent acquisition by horizontal gene transfer. Eight proteins belong to the group of *Salmonella* translocated effectors (STE) that are characterized by a conserved N-terminal secretion domain [27–29]. The presence of a conserved N-terminal domain is specific for SPI2 effector proteins and was not reported for effector proteins of other T3SS. PipB and PipB2, two further SPI2 effectors encoded outside SPI2, lack this conserved domain. Interestingly, *pipB* is encoded on *Salmonella* pathogenicity island 5 (SPI5) together with the gene for the SPI1 effector protein SopB (also referred to as SigD) [30]. At least three effector proteins are located on SPI2: SpiC (also referred to as SsaB) [31], SseF and SseG [21]. Like PipB/B2, they lack the conserved N-terminal secretion domain. SscB, a protein encoded by a gene upstream of *sseF,* is thought to act as a chaperone for SseF and SseG. Our experimental analysis did not indicate that *sseE,* a further gene located on SPI2, encodes a translocated effector protein [unpublished observations] and the role of this gene has to be clarified. Mutations in most of the effector genes have no or little effect on intracellular replication or mouse virulence of the respective *Salmonella* strains. This may be due to redundant functions of the distinct effector proteins, although it is also possible that the currently applied infection models are not suitable to detect attenuation of these strains.

Cellular phenotypes mediated by the SPI2-encoded T3SS

In the following sections, the different functions of the SPI2 system inside host cells will be discussed and, if possible, be related to the respective effector proteins. A model for the different functions, as well as examples for the SPI2-dependent effects on host cells, can be found in figure 2.
It should be mentioned that cellular phenotypes were in part analyzed in cell culture models with murine macrophages and in part in infection models of human epithelial cell lines. Although some SPI2-mediated phenotypes such as intracellular replication can be observed in either model, other phenotypes such as avoidance of antimicrobial effectors are limited to macrophages. Furthermore, infection of epithelial cell lines depends on SPI1-mediated invasion, possibly resulting in a temporary overlap of SPI1- and SPI2-mediated phenotypes. Although epithelial cell lines such as HeLa are well-suited tools for studying cell biology, the cellular microbiology of *Salmonella* in epithelial cells might be of limited relevance for understanding the pathogenesis of systemic infections.

The intracellular lifestyle of *Salmonella enterica*
Inside their host cells, *S. typhimurium* resides in the SCV. After phagocytosis or induced uptake via SPI1-mediated invasion, *S. typhimurium* deviates the SCV from the degradative endosomal pathway and promotes fusion with yet unknown membrane compartments. Several stages of maturation of the SCV are initially similar in macrophages and epithelial cells. Shortly after invasion, the SCV acquired the early-endosomal antigen 1 (EEA1) and the transferrin receptor (TfR), two marker proteins for early endosomes and recycling endosomes, respectively [32–34]. Both proteins were only transiently present on the SCV and were rapidly replaced by proteins characteristic of the late endosomal/lysosomal compart-

Table 1. Effector proteins of the SPI2-encoded T3SS.

Effector protein	Chrom. location	Conserved N-terminal domain	Localization in host cells	Function	Host cell partner	Comment	Reference
SpiC	SPI2	no	cytoplasm	interference with vesicle trafficking; induction of IL-10 expression	TassC; Hook3	required for translocation of other effectors	[31, 56, 57, 102]
SseF	SPI2	no	SCV, SIF	SIF formation; microtubule bundling	ND		[21, 76]
SseG	SPI2	no	SCV, SIF, Golgi	SIF formation; microtubule bundling; Golgi targeting	ND		[21, 76, 80, 90]
SifA	pathogenicity islet	yes	SCV, SIF	SIF formation; SCV integrity	ND		[72, 73]
SifB	pathogenicity islet	yes	SCV, SIF	ND	ND		[81]
SseI	*Gifsy-2* prophage	yes	polymerizing actin cytoskeleton (after transfection)	ND	filamin		[28, 83]
SseJ	phage associated	yes	SCV, SIF	SCV integrity	ND	similarity to GDSL family of lipolytic enzymes	[79]
SspH1	*Gifsy-3* prophage	yes	nucleus (cytoplasm)	repression of NFκB-dependent gene expression	ND	translocation via SPI1 and SPI2; not under control of SsrAB	[77]
SspH2	phage associated	yes	polymerizing actin cytoskeleton (after transfection)	inhibition of actin polymerization	profilin, filamin		[28, 83]
SlrP	pathogenicity islet	yes	ND	ND	ND	translocation via SPI1 and SPI2; not under control of SsrAB	[104]
SopD2	pathogenicity islet	yes	SCV, SIF	SIF formation	ND		[29]
PipB	SPI5	no	SCV, SIF	ND	ND	enriched in DRMs	[30, 66]
PipB2	ND	no	SCV, SIF, peripheral $LAMP^+$ vesicles	ND	ND	enriched in DRMs	[66]

ND, not determined; DRMs, detergent-resistant microdomains; SCV, *Salmonella*-containing vacuole; SIF, *Salmonella*-induced filaments.

ment, such as the lgps (lysosomal glycoproteins) LAMP-1, LAMP-2, LAMP-3 (also referred to as LIMP-1, CD63) and the V-ATPase. V-ATPase activity led to acidification of the SCV to a final pH of 4.0–5.0 [35]. Fusion of early endosomes with the SCV seemed to be dependent on the presence of Rab5 and NSF (*N*-ethylmaleimide-sensitive fusion protein) [34, 36–38], two proteins involved in the regulation of docking and fusion events of early endosomes, on the SCV. Another study [39] reported that the presence of Rab7 on the SCV was necessary for TfR recycling and acquisition of lgps. Independent work from two groups [40, 41] further showed that coupling of Rab7 to dynein via RILP (Rab7 interacting lysosomal protein) led to transport of the SCV along microtubules to the MOC (microtubule organizing center) located in the perinuclear region. This was in contrast to observations by Hashim et al. [34], showing that live *Salmonella* actively depleted Rab7 from the vacuole, thereby inhibiting the transport to late endosomes. In contrast to phagosomes containing latex beads or dead *Salmonella,* the accessibility of the SCV for fluid phase tracers such as ovalbumin-TexasRed, fluorescein-dextran and avidin-HRP was clearly reduced [32, 34, 42, 43]. The acquisition of the cation-dependent and cation-independent mannose-6-phospate receptors (M6PR) was also blocked in vacuoles containing live *Salmonella* when compared to phagosomes harboring latex beads or dead *Salmonella* [32–34, 42]. Normally, M6PR was found on late endosomes [44, 45] and directed delivery of mannose-6-phosphate-containing proteins, such as cathepsin D or cathepsin L, two

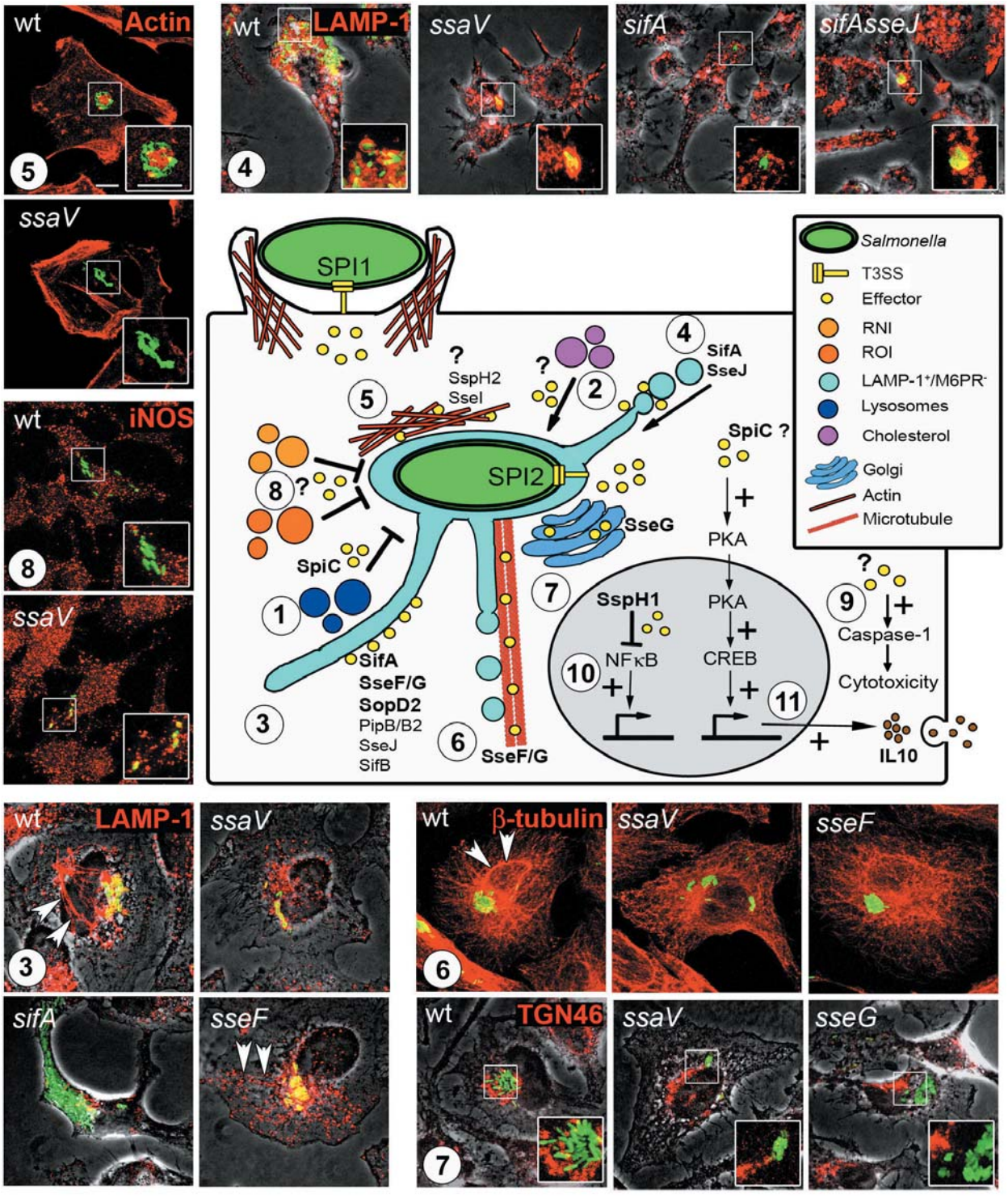

Figure 2. Cellular phenotypes associated with the function of SPI2. The interactions of intracellular *S. enterica* with host cell functions via the SPI2-encoded T3SS are shown in a model, and examples for representative host cell phenotypes after infection with *Salmonella* wild-type (wt) or relevant mutant strains are shown in micrographs. While SPI1 is activated by extracellular bacteria and triggers invasion, the SPI2 system is activated by bacteria residing within a special membrane compartment termed the *Salmonella*-containing vacuole (SCV). SPI2 function interferes with a variety of different host cell processes: (1) modification of cellular trafficking and alteration of SCV maturation; (2) recruitment of cholesterol to the SCV; (3) formation of *Salmonella*-induced filaments (SIF) in HeLa cells (indicated by arrows). Infection with an *ssaV* or *sifA* strain did not induce SIF, while infection with the *sseF* strain results in 'pseudo-SIF' formation; (4) maintenance of SCV integrity by combined fusion and scission events. In the presence of SseJ, a *sifA*-deficient strain escapes the SCV and is killed or replicates in the cytoplasm of macrophages or HeLa cells, respectively; (5) actin accumulation in the vicinity of the SCV; (6) bundling of microtubules and associated SIF-formation; (7) association of the trans-Golgi network (TGN) with the SCV; (8) inhibition of

lysosomal hydrolases, from the TGN (trans-Golgi network) to the late endosomes [46–49]. Consistent with these data, mature cathepsins D and L were found only in a small percentage of the SCV [32, 34, 42]. However, contradictory results by Brumell et al. [50] stated that the majority of the SCV at 6 h post infection were positive for cathepsin D. Vesicles negative for M6PR and cathepsin D, but positive for Rab7 and lgp, were found in HeLa cells in the vicinity of the SCV between 20 and 60 min post infection [39]. These vesicles might represent yet unknown membrane compartments recruited to the SCV by intracellular *Salmonella*. Previous studies have shown that the VacA toxin of *Helicobacter pylori* induced the formation of a similar compartment after entry into the host cell [51, 52].

SPI2-mediated modification of the *Salmonella*-containing vacuole

Only little is known about the bacterial factors responsible for alteration of the normal phagosomal maturation pathway. Garvis et al. [43] have shown that the *phoPQ* regulon played an important role in inhibition of fusion of the SCV with late endosomal/lysosomal compartments. The authors did not observe significant differences in the composition of the SCV harboring a SPI2 or SPV (*Salmonella* plasmid virulence) mutant strain compared to vacuoles containing wild-type *S. typhimurium*. However, at late time points after infection of RAW 264.7 macrophages with a *phoP* strain, they observed fusion of the SCV with ovalbumin-TexasRed-preloaded lysosomes, as well as association with cathepsin D and LBPA (lysobisphosphatidic acid), a component of the internal lamellar membranes of late endosomes [53]. Segregation of the SCV from the endocytic pathway was dependent on bacterial protein synthesis and was irreversibly established between 15 min and 4 h after infection.

There is also evidence for the involvement of the SPI1-encoded T3SS in SCV maturation. Infection of HeLa cells with a non-invasive *invA* strain (via *Yersinia* invasin-mediated uptake or co-infection with wild-type *S. typhimurium*) showed a drastic replication defect of the SPI1 mutant. Invasin-mediated uptake of the SPI1 mutant also led to reduced EEA1 acquisition and transient co-localization of M6PR with the SCV, which yet was considered to be due to the different uptake mechanisms and not as a SPI1-specific effect. Co-infection with wild-type *Salmonella,* in contrast, did not alter the acquisition of EEA1, but significantly delayed LAMP-1 acquisition [54]. Another hint at an involvement of SPI1 in SCV maturation came from Mukherjee et al. [55], who showed that the SPI1 effector SopE played an important role in the rapid recruitment of Rab5 to the SCV.

The role of SPI2 in modifying the SCV is still unclear. While Garvis et al. [43] did not find evidence for involvement of SPI2 in the segregation of the SCV from the endocytic pathway, Uchiya et al. [31] reported that function of SpiC was required to block fusion of the SCV with lysosomes and incoming endosomes and for interference with general trafficking events in the host cell. Purified SpiC also inhibited endosome-endosome fusion in vitro. SPI2-dependent translocation of SpiC into the host cell was shown by immunofluorescence, SDS-polyacrylamide gel electrophoresis (PAGE) [31, 56] and CyaA reporter assays [57]. Two target proteins of SpiC have been identified to date, namely TassC (target for *Salmonella* secreted protein C) [56] and Hook3 [57]. The NIPSNAP-like protein TassC was identified by yeast-two-hybrid (YTH) analysis. In *Caenorhabditis elegans,* NIPSNAP has been implicated in vesicular trafficking. After infection of J774 macrophages with *S. typhimurium*, TassC was excluded from the SCV in a SpiC-dependent manner [56]. The authors speculated that TassC might be required for fusion of distinct endosomal compartments with the phagosome. SpiC-TassC interaction might inhibit delivery of vesicles with detrimental cargo, allowing survival of *Salmonella* inside macrophages. Indeed, suppression of cellular TassC production restored intracellular replication of a *spiC* strain in macrophages. Hook3 was identified by pull-down experiments with SpiC. It is a member of a protein family involved in cellular traffic and suggested to link specific organelles (in case of Hook3 the Golgi complex) to microtubules [58]. SpiC transfection led to fragmentation of the Golgi (similar to cells expressing a C-terminal truncated form of Hook3) and an aberrant distribution of late endosomes/lysosomes [57].

However, the function of SpiC as translocated effector is controversial. Attenuation of the *spiC* strain in mouse virulence and intracellular replication in macrophages was similar to strains deficient in the T3SS, whereas mutations in most other effectors showed only a slight or no attenuation in these models. Two groups have shown inde-

Figure 2 (continued)

delivery of reactive oxygen (ROI) and nitrogen intermediates (RNI) to the SCV; (9) delayed cell death; (10) inhibition of NFκB-dependent gene expression and (11) induction of IL10 expression. Bold typeface indicates involvement of SPI2 effectors in the respective phenotype; regular typeface indicates localization of the effector to the respective compartment. For infection experiments shown in micrographs, RAW264.7 (4, 8) and HeLa cells (3, 5, 6, 7) were infected at a multiplicity of infection of 5 with the indicated strains expressing green fluorescent protein. The cells were fixed 16 h after infection and stained with the indicated primary antibodies and Cy3-coupled secondary antibodies. Actin was stained by Phalloidin-Texas-Red. Scale bars represent 8 μm. Infected host cells are shown that are representative for published SPI2-dependent phenotypes.

pendently that SpiC was essential for secretion of the translocon proteins SseBCD under SPI2-inducing conditions, but was not secreted itself [59, 60]. In contrast to the data from Uchiya et al. [31] and Shotland et al. [57], Freeman et al. [60] could not detect translocation of SpiC into the host cell cytoplasm. Although a *spiC* strain did not translocate other SPI2 effectors into the host cell, in vitro secretion of SseJ was not inhibited in this strain [60]. From these data the authors concluded that SpiC might act as a chaperone for, or regulator of, the translocon proteins SseBC. A *spiC* mutant lacking a functional translocator would be unable to translocate any SPI2 effectors into the host cell, which would explain the strong phenotype of the *spiC* strain. However, it remains possible that SpiC has a dual function as translocated effector and component of the T3SS.

Cholesterol accumulation

Recent studies by different groups demonstrated that cholesterol accumulation plays an important role in bacterial invasion and modification of the SCV. Visualization of cellular cholesterol by filipin staining revealed enrichment of this compound in membrane ruffles at early time points after infection and increasing cholesterol accumulation on the SCV during a time course of 20 h [50, 61, 62]. At late time points after infection, the SCV represented the major site of cholesterol accumulation in the host cell. Redistribution of cholesterol to the membrane ruffles at the bacterial entry site and subsequent accumulation around the ingested bacteria was dependent on a functional SPI1-encoded T3SS. While localization of cholesterol on the SCV up to 6 h post infection was independent of the function of the SPI2-system [61], cholesterol recruitment at later time points post infection seemed to be SPI2-dependent, as vacuoles harboring an *ssaT* mutant showed no filipin staining at 20 h post infection [62]. While cholesterol accumulation on membrane ruffles was shown to be essential for efficient invasion of *Salmonella,* the function of this compound on late SCV remains speculative. Cholesterol recruitment may suggest a role for detergent-resistant membrane microdomains (DRM) or 'lipid rafts' in SCV signaling or fusion events with other host cell membranes. DRM are sphingolipid- and cholesterol-enriched moving platforms (~50 nm) found in plasma and endosomal membranes. These are rich in GPI (glycosyl-phosphatidylinositol)-anchored proteins and different receptor and signaling molecules (e.g. tyrosine kinases, MAP kinases, adenylate cyclase) (for reviews see [63–65]). Indeed, PipB and PipB2, two further effector proteins translocated by the SPI2 T3SS, were shown to be targeted to DRM in infected HeLa cells [66]. After translocation, both proteins localized to the membrane of SCV and *Salmonella*-induced filaments, indicating the presence of lipid rafts on the SCV. PipB2 was also found in LAMP-1-positive vesicles in the periphery of the cell. Previous studies have revealed several examples how pathogens use lipid rafts for efficient entry into the cells or to utilize host-signaling cascades (for reviews see [67–69]).

Aggregation of endosomes and *Salmonella*-induced filaments

Around 4–6 h after infection of epithelial cells, *Salmonella*-induced reorganization of endosomal membrane becomes evident by the appearance of long filamentous membrane structures called *Salmonella*-induced filaments (SIF). SIF formation was initially observed in epithelial cells, but a recent study on interferon (IFN)-γ-primed macrophages infected with *S. typhimurium* also revealed SIF in this cell type [66]. SIF originate from the SCV and extend throughout the entire cell, eventually connecting separate SCV. Like the SCV, these structures are rich in lgps and V-ATPase, but lack M6PR [70]. LBPA, cholesterol and cathepsin D also localized to SIF [50], indicating involvement of late endosomes in SIF formation. Formation of SIF occurred on a scaffold of microtubules [71], and depolymerization of microtubules with nocodazole inhibited the formation and maintenance of these filaments [70]. Recent work by Harrison et al. [40] showed that kinesin, a microtubule motor protein, was required for SIF formation. SIF formation was also dependent on the presence of functional Rab7 [47]. There is evidence that the STE protein SifA [28] binds to Rab7, leading to dislocation of RILP and dissociation of the SCV from the dynein motor protein. This seemed to be one prerequisite for SIF formation. SifA was identified before as the main bacterial effector necessary for the induction of these structures [72, 73]. A *sifA* mutant was drastically attenuated in mouse virulence and proliferation in macrophages, but showed normal, or even increased, rates of replication in epithelial cells [72–74]. Inside the host cell, SifA localized to SCV membrane and to SIF [71]. Transfection of HeLa cells with a *sifA*::GFP construct was sufficient to induce vacuolation of LAMP-1-positive compartments and formation of SIF-like structures [50]. A cysteine-rich hexapeptide at the C-terminus of SifA was shown to function as a membrane anchor and to be essential for the biological activity of the protein. Deletion of this domain led to attenuation in the mouse virulence model and intramacrophage proliferation similar to a *sifA* knockout strain [75]. In addition to SifA, further SPI2 effectors are involved in the formation of SIF. Guy et al. [76] screened 11,520 transposon mutants of *S. typhimurium* for loss of SIF formation. In addition to mutants with a transposon insertion in genes encoding structural proteins of the SPI2 system, the screening identified SpiC, SseF and SseG as playing a role in SIF formation. SpiC was previously shown to alter endocytic trafficking

and SCV maturation. Due to the requirement of SpiC for SPI2 effector translocation, the observed effect on SIF formation could also be due to a defect in translocation of SifA. SseF and SseG are without similarity to any protein in the databases. Strains deficient in either protein showed moderate attenuation in systemic pathogenesis and intracellular proliferation [19]. We could show that SseF and SseG were translocated into host cells in a SPI2-dependent manner and localized to membranes of SCV and SIF. In accordance with the results of Guy et al. [76] SIF formation in HeLa cells was reduced after infection with a *sseF* or *sseG* mutant. However, immunofluorescence analysis of translocated SseJ in these cells revealed another form of filamentous structure termed 'pseudo-SIF'. Translocated SseJ was distributed continuously along pseudo-SIF but only a punctuated, 'string of pearls'-like pattern was observed for lgps [21]. While the composition of these structures is still unclear, we showed that similar to SIF, pseudo-SIF assembled along a scaffold of microtubules [77]. This observation raised the hypothesis that pseudo-SIF do not represent a continuous membrane compartment, but rather individual vesicles threaded along microtubule filaments.

A structure similar to pseudo-SIF was also observed in HeLa cells infected with a *sopD2* mutant strain [78]. SopD2 is a further member of the STE family that shows high similarity to SopD, a SPI1 effector. The conserved N-terminal secretion domain of SopD2 not only mediated SPI2-dependent secretion, but also targeted the protein to the membrane of the SCV and SIF. Little is known about the exact function of SopD2 inside the host cell. Transfection experiments with a *sopD2*::GFP construct revealed association of the protein with late endocytic compartments, positive for LAMP-1, LBPA, Rab7, cathepsin D and fluid tracer-loaded lysosomes. These data suggested that SopD2 might play a role in blocking fusion of the SCV with these late endocytic compartments [29].

Maintenance of the membrane integrity of the SCV

As mentioned before, a *sifA*-deficient strain showed a severe replication defect in macrophages but normal, or even increased, proliferation in epithelial cells. This contrary behavior in two cell types could be explained by the fact that a *sifA* mutant was unable to maintain the membrane integrity of the SCV and was released into the host cell cytosol. While the macrophage cytoplasm contains factors detrimental for *Salmonella* and leading to rapid killing of the bacteria, proliferation is enhanced in the nutrient-rich environment of the cytoplasm of epithelial cells. A *sifA* mutant strain could be complemented by ectopic expression of *sifA*::GFP, maintaining the integrity of the SCV [73]. Although SifA was shown to be translocated via the SPI2-encoded T3SS, infection with a SPI2 T3SS-deficient strain (*ssaV*) did not lead to loss of the vacuolar membrane and release of this strain into the cytoplasm. The same was found in the case of a *ssaV sifA* double mutant [73]. Ruiz-Albert et al. [79] showed that translocation of a second SPI2 effector, SseJ, was responsible for the loss of vacuolar membrane integrity. An *sseJ sifA* double mutant retained the vacuolar membrane after invasion of HeLa cells [79]. However, this phenotype was not observed in another study [80], possibly due to different infection protocols. SseJ contains a domain with similarity to an acyltransferase/lipase from *Aeromonas hydrophila*, a secreted toxin catalyzing acyl-group transfer from glycerophospholipids to cholesterol. The *sseJ* strain was slightly attenuated in mouse virulence and intracellular replication in peritoneal macrophages [79, 81]. After SPI2-dependent translocation, SseJ localized to the SCV membrane and SIF [21, 81]. Ectopic expression of SseJ led to globular aggregation of LAMP-1-positive compartments and inhibited SIF formation and the intracellular replication of *S. typhimurium* after infection of HeLa cells [79]. The authors suggested that SseJ might modify SCV membrane lipids, thereby facilitating fusion, scission or budding events with selected vesicular compartments. However, the proposed catalytic activity of SseJ needs to be demonstrated experimentally.

Modulation of the host cell cytoskeleton

Salmonella has evolved strategies to actively modify its intracellular habitat that also involve modifications of the host cell cytoskeleton. Meresse et al. [82] demonstrated a frequent accumulation of actin around the SCV in different cell types. This process was SPI2-dependent and occurred by *de novo* polymerization of actin monomers. The formation of an actin meshwork around the SCV was important for maintenance of the membrane integrity of the SCV as well as for intracellular replication of *Salmonella* inside macrophages. Two effector proteins of the SPI2 system interact with the actin cytoskeleton [83]. YTH analysis showed binding of SspH2 to α-filamin and profilin via its N- and C-terminus, respectively. Dimers of α-filamin crosslink F-actin in areas of active polymerization. Profilin interacts directly with G-actin, thereby enhancing actin polymerization. Screening of further SPI2 effectors revealed an interaction of SseI with filamin. Consistent with this finding, the N-terminal domain of SseI shows 97% identity to the N-terminal 60 amino acids of SspH2. After transfection, both SPI2 effectors were highly enriched in membrane ruffles and co-localized with filamin. SspH2, but not SseI, inhibited actin polymerization in vitro. Although SspH2 and filamin were associated with the SPI2 T3SS-induced vacuole-associated actin polymerization (VAP), neither SspH2 nor SseI were necessary for the formation of these

structures. However, SpvB, a protein encoded by the *Salmonella* virulence plasmid, contributed to this phenotype, as cells infected with a *spvB* mutant showed a clear increase in VAP formation. It was shown before that SpvB was expressed by intracellular *Salmonella* [84] and disrupted the actin cytoskeleton by ADP ribosylation of monomeric actin [85, 86].

Although studies of the interaction of pathogens with the host cell cytoskeleton have mainly focused on actin filaments, there is growing evidence that pathogenic bacteria also alter the microtubule network of their host cells (for review see [87]). The importance of an intact microtubule skeleton for *Salmonella* infection becomes evident by the fact that intracellular replication of *Salmonella* inside HeLa cells is clearly reduced after treatment of cells with the microtubule-depolymerizing drug nocodazole [70]. Brumell et al. [71] showed that microtubules served as a scaffold for SIF formation in *S. typhimurium*-infected HeLa cells. This is consistent with an earlier finding that SIF formation was abolished in cells treated with nocodazole [70]. An intact microtubule network, but not SIF formation, was necessary for transport of PipB2-positive vesicles to the periphery of the cell [66]. Recently, we found that the effectors SseF and SseG colocalized with microtubules in infected HeLa cells [80]. SseF and SseG were responsible for the induction of massive bundling of microtubules at late time points after infection. We could further show that these bundles served as a scaffold for SIF formation. Co-localization of SseF or SseG with the microtubule skeleton was also observed in cells infected with *sseG*- or *sseF*-deficient strains, respectively ([80] and unpublished data). This showed that the association with microtubules is not only due to the localization to SIF, but an intrinsic property of these proteins. A direct interaction with tubulin was not observed and the mechanism by which *Salmonella* induces microtubule bundling is still unclear. We hypothesize that, by inducing microtubule bundling, *Salmonella* can increase the acquisition of different membrane compartments, which results in the formation of SIF. Due to the inability to alter the microtubule skeleton, *sseF* or *sseG* mutant strains may recruit fewer membrane compartments to the SCV, resulting in the punctuate staining pattern of the pseudo-SIF. *Salmonella*-induced microtubule accumulation was also described by Guignot et al. [88]. However, these authors did not report bundling of microtubules, but a tubulin meshwork around *S. typhimurium* micro-colonies in different cell types. This accumulation was independent of the actin accumulation observed by the same group [82] and seemed to depend on both systems, SPI1 and SPI2. The microtubule motor proteins dynein and kinesin were recruited to the area of microtubule accumulation and played an important role in regulating membrane dynamics during maturation of the SCV. Dynein and kinesin activity were required for bacterial replication, SIF formation and loss of the vacuolar membrane around *sifA* mutants. Dynein was recruited to the SCV in a Rab7-dependent manner. Infected HeLa cells expressing p50/dynamitin, which uncouples the dynactin complex and disrupts dynein function [89], showed an aberrant SCV morphology. In contrast to non-transfected cells, individual bacteria were not separated by LAMP-1 membranes, but enlarged vacuoles were formed containing several bacteria. From these observations the authors concluded that SCV-associated dynein might drive the process of membrane detachment that occurs during the segregation of vacuoles [88].

Targeting of SCV to the Golgi network

Beside its function in microtubule bundling, SseG was also shown to target the SCV to the vicinity of the Golgi network in infected epithelial cells [90]. More than 80% of the SCV harboring wild-type *Salmonella*, but less than 20% of the vacuoles containing an *sseG* mutant, were associated with Golgi membranes. In contrast to our observations [77], SseG localization to the trans-Golgi network (TGN) was not only found in HeLa cells transfected with a myc::*sseG* construct but also after immunostaining for SseG in *S. typhimurium*-infected cells. Golgi targeting was dependent on a 55-amino acid region containing the predicted transmembrane domain HR2 of SseG. Treatment of infected cells with brefeldin A (BFA), which leads to rapid redistribution of the Golgi network into the endoplasmic reticulum [91], strongly inhibited intracellular growth of wild-type *S. typhimurium*, but not of a SPI2 mutant. BFA had no noticeable effect on the maturation of the SCV. This indicated that an intact and functional Golgi network is required for intracellular replication of *S. typhimurium*. The authors suggested that the close contact between SCV and Golgi membranes might facilitate transient fusion events between these two compartments, resulting in the acquisition of molecules required for *Salmonella* proliferation.

Evasion of antimicrobial radicals

S. enterica has evolved different mechanisms to evade damage caused by antimicrobial compounds such as reactive oxygen (ROI) or nitrogen intermediates (RNI). In addition to mechanisms that are also present in non-pathogenic bacteria, *Salmonella* possesses specific mechanisms to exclude ROI- and RNI-producing enzymes from the SCV. Vazquez-Torres et al. [92] showed that SPI2 mutants caused lethal infections in a *gp91phox* knockout mouse and that the clearance of SPI2 mutants in *gp91phox*$^{-/-}$ macrophages was similar to wild-type *Salmonella.* This effect was not achieved by suppression of NADPH oxidase expression. Co-localization studies indicated that SPI2 function prevented co-localization of

the NADPH oxidase with the SCV and by that exposure of *Salmonella* to ROI. Gallois et al. [93] showed that wild-type *Salmonella* prevented localization of cytochrome b_{558} to the SCV in a SPI2-dependent manner. Cytochrome b_{558} is the membranous part of the NADPH oxidase that, by functioning as docking site for further components of the enzyme complex, is essential for the assembly of a functional enzyme.

Similar findings were made for the RNI-generating enzyme iNOS (Ca^{2+}-independent, inducible nitric oxide synthase) [94]. Although there was no difference in iNOS expression and synthesis of NO, the distribution of iNOS in macrophages differed between cells infected with wild-type *S. typhimurium* or a SPI2 mutant strain. While iNOS was distributed equally in host cells infected with wild-type *Salmonella* and showed little or no co-localization with the SCV, around 60% of the intracellular SPI2-deficient bacteria co-localized with iNO*S*. A similar pattern was observed for nitrotyrosine residues, a reaction product of peroxynitrite. Peroxynitrite is a highly potent cytotoxic molecule that results from the reaction of NO with O_2^-. Nitrotyrosine formation was frequently observed on intracellular SPI2-deficient bacteria, but rarely on wild-type *Salmonella*. Further experiments showed that iNOS was essential for the control of *Salmonella* proliferation in infected macrophages as well as in the mouse model, although iNOS activity was important only at late stages of pathogenesis [94].

Salmonella-induced cell death

As observed for a variety of other pathogens, infection with *S. typhimurium* can induce host cell death, and three different forms of cytotoxicity were observed in infected macrophages. A rapid SPI1-dependent form of cell death took place within 40 min after infection and was mediated by interaction of the SPI1 effector protein SipB with the host cysteine protease caspase-1 (for reviews see [95, 96]). A delayed mechanism of *Salmonella*-induced cell death was described in caspase-1-deficient macrophages at 4–6 h after infection, involved activation of caspase-2, -3, -6 and -8 and was associated with cytochrome c release from mitochondria. Again, SPI1 and SipB were required for mediating the cytotoxic response [97]. The third, delayed and SPI1-independent mechanism of cytotoxicity started around 6 h after infection, as seen by TUNEL (terminal deoxytransferase-mediated dUTP nick and labeling) staining, and became morphologically apparent by 12–14 h post infection. It was shown that *ompR* and the SPI2 system were required for induction of delayed cytotoxicity [80, 98, 99]. As seen for the rapid mechanism, caspase-1 activation was, in part, also involved in the delayed SPI2-dependent cell death and led to maturation and release of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 [99]. There is evidence that in addition to *ompR* and SPI2 genes, the SPV may also be involved in delayed macrophage killing [100, 101]. It is most likely that the different cytotoxicity mechanisms take place at different points during infection. In the Peyer's patches, rapid killing of macrophages and subsequent release of pro-inflammatory cytokines could be advantageous for *Salmonella* by attracting additional phagocytic cells and allowing more efficient systemic spread. At the sites of systemic infection, delayed *Salmonella*-induced cell death would allow intercellular spread within apoptotic bodies. SPI2 effectors involved in *Salmonella*-induced killing of macrophages have not been defined [98, 99]. It should be noted that SPI2-dependent cytotoxicity might also be a consequence of replication of intracellular *Salmonella* and perturbation of normal host cell processes.

Alteration of host cell gene expression

Two recent studies described SPI2-dependent changes in expression of host cell genes. Haraga and Miller [77] stated a role of effector SspH1 in inhibition of nuclear factor kappa B (NFκB)-dependent gene expression. SspH1 is a member of the *Salmonella*-translocated effectors (STE) family and is translocated via the SPI1 and the SPI2 systems. SspH1 contains a subtype of the leucine-rich repeat motif called LPX repeat and shows 69% identity to the SPI2 effector SspH2 [27]. After infection of the human epithelial cell line INT-407, SspH1 localized predominantly to the nucleus. Reporter assays with the firefly luciferase gene under control of a NFκB-inducible promoter showed a dose-dependent down regulation of gene expression in cells expressing *sspH1*. This subsequently led to inhibition of IL-8 production by tumor necrosis factor (TNF)-α-activated epithelial cells. Preceding experiments [27] showed that expression of *sspH1* is constitutive, allowing secretion under SPI1- and SPI2-inducing conditions. However, the authors mainly discussed the importance of the anti-inflammatory function of SspH1 for the survival and colonization of *S. typhimurium* in the intestinal tract, which is thought to be independent of SPI2. Further studies will have to reveal the exact role that the different secretion systems play in translocation of SspH1 during the course of infection.

Uchiya et al. [102] observed an upregulation of the expression of the anti-inflammatory cytokine IL-10 by infected macrophages. This effect was dependent on the function of SpiC. Further studies indicated that *Salmonella* activated the protein kinase A (PKA) signaling pathway that leads to IL-10 upregulation. Specific inhibition of PKA, but not of IL-10 expression, resulted in reduced intracellular proliferation. In future studies, it would be of interest to test whether addition of IL-10 would rescue the intracellular defect of the *spiC* strain.

Conclusions and outlook

Work by several groups has identified a variety of distinct functions related to the activity of the SPI2 system within infected host cells. The effects on host cells include the modification of intracellular transport and the endosomal system, alteration of the actin and microtubule cytoskeleton, host cell death as well as changes in gene expression. Future work has to demonstrate which of the cellular phenotypes related to the SPI2 system are linked to the most obvious SPI2 function, i.e. the proliferation of *Salmonella* within infected host tissues.

A complex group of 13 effector proteins that are translocated by the SPI2 system have been defined. Few host cell phenotypes could be clearly related to the function of individual effectors, such as modification of the endosomal system by SifA. The presence of a conserved N-terminal domain in 8 effectors and the observation of duplicate effectors such as SifA/SifB, SspH1/SspH2 and PipB/PipB2 could be an indication of ongoing evolution. Such duplication may allow the development of new virulence functions and adaptation to new hosts species or new niches in present host species. Keeping in mind that *S. enterica* can cause different disease outcomes in a variety of hosts, currently applied animal models and cell culture models might only reveal a subset of the SPI2 functions.

The molecular mechanisms of most cellular phenotypes of the SPI2 system require further clarification. There is probably a need to reevaluate the role of SPI2 in interaction of *Salmonella* with the endosomal system and with maturation of the SCV. Studies in other host pathogen systems such as *Legionella pneumophila* indicated the rapid and transient nature of such interactions. The availability of an array of cell biology tools could make it possible to analyze intracellular fate in living host cells in temporal and spatial resolution.

Such investigation might also help to understand the mechanism of SPI2-mediated evasion of iNOS and NADPH oxidase activities. One possibility is that *Salmonella* can selectively block the association of antimicrobial activities with the SCV. It is known that the NADPH oxidase is selectively assembled on the phagosomal membrane, but the basic cellular mechanism of selective localization of iNOS is not known. Further studies are needed to clarify the exact mechanism by which *Salmonella* can prevent targeting of ROI and RNI to the SCV as well as to identify the effector proteins involved in these processes.

It will also be of interest to analyze the role of SPI2 during interaction with antigen-presenting cells in more detail. Initial observations indicated that in dendritic cells SPI2 function is not required for survival, but affects the processing of the phagocytozed bacteria [103]. Interference with the cytoskeleton and endosomal system of such cells might also affect their capability of antigenpresentation.

The SPI2-encoded T3SS is an excellent example of the efficacy and complexity of adaptation of the pathogen to a specific host environment, such as the intraphagosomal lifestyle of *S. enterica*. Although work by various groups has revealed several important SPI2-dependent phenotypes, we are just starting to unravel the intricacy of manipulations of normal host cell processes by SPI2 effector proteins. We expect that the knowledge of the pathogenic interference will significantly contribute to our understanding of basic eukaryotic cell functions and the molecular pathogenesis of *Salmonella* as an important human pathogen.

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