MOLECULAR PATHOGENESIS OF *Salmonella enterica* SEROTYP*E* Typhimurium-INDUCED INFLAMMATORY RESPONSES

A Dissertation

by

JOSELY FERREIRA FIGUEIREDO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Veterinary Microbiology

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ABSTRACT

Molecular Pathogenesis of *Salmonella enterica* Serotype Typhimurium-Induced Inflammatory Responses. (May 2007)

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We demonstrated that infection of HeLa cells, which are non-responsive to flagellin, with wild type *Salmonella enterica* serotype Typhimurium (*S. typhimurium)* activated chemokine expression at higher level than *S. typhimurium* lacking *sipAsopABDE2*, indicating that the corresponding effector proteins (SipA, SopA, SopB, SopD and SopE2) are required to induce chemokines independent of flagellin. The *S. typhimurium sipAsopABDE2* mutant complemented with *sipA* activated IL-8 expression at significantly higher level than a *S. typhimurium sipAsopABDE2* mutant. However, extracellular addition of recombinant SipA failed to induce IL-8. Phosphorylation analyses demonstrated that *S. typhimurium* carrying a chromosomal copy of *sipA* (*sopABDE2* mutant) induced phosphorylation of CREB1, JUN and p38MAPK, which are proteins involved in IL-8 expression.

The contribution of effector proteins to *S. typhimurium*-induced intracellular Ca^{2+} mobilization and its role in IL-8 expression and bacterial

internalization were also investigated. Our results demonstrated that wild type *S. typhimurium* significantly increased the amplitude of intracellular Ca^{2+} beginning 30 sec after infection. However, further analyses of intracellular Ca^{2+} changes in HeLa cells infected with *S. typhimurium* mutants indicated no correlation between increased intracellular Ca^{2+} and IL-8 expression or bacterial internalization.

To analyze specific cell populations targeted by wild type *S. typhimurium* or *S. typhimurium* carrying a chromosomal copy of *sipA* (*sopABDE2* mutant), laser capture microdissection was performed. Our data indicated that in wild type *S. typhimurium*-infected bovine Peyer's patches, high levels of IL-8 were expressed in enterocytes of crypts, whereas Gro-α was expressed in enterocytes of both crypts and absorptive villi. A strain carrying a chromosomal copy of *sipA* colonized the same cell population as wild type, but induced IL8 and Gro-α in enterocytes of both crypts and absorptive villi.

In conclusion, we demonstrated that *in vitro S. typhimurium* effector proteins induce chemokine expression independent of Ca^{2+} changes through phosphorylation of proteins related to IL-8 pathway. *In vivo*, we found higher levels of IL-8 expression in enterocytes of crypts than enterocytes of absorptive villi, although both cell populations contributed to Gro-α expression. These data extend the knowledge of the molecular mechanism by which *S. typhimurium* induces inflammatory genes by identifying pathogen and host molecules involved in inflammation.

This work is dedicated to the memory of my father

Joaquim Inocêncio Figueiredo

and to my mother Elisa, my lovely husband Jairo, and our child that has changed our lives.

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INTRODUCTION

Salmonella

Salmonella is a gram-negative intracellular bacillus, belonging to the Enterobacteriaceae family, that colonizes a variety of hosts including human and other mammals, birds, reptiles and insects (21, 181). The only two *Salmonella* species, *S. enterica*, which is divided into six subspecies, and *S. bongori* contain 2541 serotypes, of which 1504 belong to *S. enterica* subsp. *enterica,* 22 belong to *S. bongori* and the remaining are distributed among other subspecies of *S. enterica* (130). *S. bongori* is associated mainly with cold-blooded animals, and is found rarely in humans who have close contact with reptiles (2, 181). In contrast, serotypes of *S. enterica* have a broad host preference and cause a broad spectrum of diseases (164).

The majority of cases of salmonellosis in warm-blood animals is caused by *S. enterica* subsp *enterica*. In humans, *Salmonella*-caused diseases can range from asymptomatic carriage and gastroenteritis to bacteremia and enteric fever (164). The bacteremia and enteric fever are usually caused by host-adapted strains, *S. enterica* subsp. *enterica* serotype Typhi (*S. typhi*) and *S. enterica* subsp. *enterica*

This dissertation follows the style of *Infection and Immunity*.

serotype Paratyphi. On the other hand, the most common non-typhoidal strains, *S. enterica* subsp. *enterica* serotype Typhimurium (*S. typhimurium*) and *S. enterica* subsp. *enterica* serotype Enteritidis (*S. enteritidis*) are non-host specific and cause gastroenteritis (164).

Enteric fever is still a serious public-health problem, specially in developing countries (28). Approximately, 16.6 million cases of typhoid fever with 600,000 deaths are estimated to occur every year in all over the world (125). In USA, about 300 to 400 cases are reported each year to the Center for Disease Control and Prevention (CDC) due to travel to endemic areas (78). The number reported cases of typhoid fever is alarming, considering the economic losses, including medical care and loss of productivity and the increased transmission and risk factors (9).

Worldly, there are about 1.3 billion non-typhoidal salmonellosis cases each year, with 3 million deaths (125). In USA, since 1993, *S. typhimurium* and *S. enteritidis* are ranked as the two most frequent reported isolates in human, followed by *S. enterica* subsp. *enterica* serotype Newport (*S. newport*). Each year about 40,000 cases of enteric salmonellosis with about 40 deaths are reported to CDC. Although salmonellosis is a notifiable disease, the number of reported cases is underestimated even in countries with a well-organized case report system. CDC estimates that for every case reported to national surveillance, about 38 unreported cases occur throughout USA (78).

Similar to human populations, salmonellosis is a major concern in agriculture. The host-adapted strains including *S. enterica* subsp. *enterica* serotype Gallinarum and *S. enterica* subsp. *enterica* serotype Abortusovis, cause systemic disease

specifically in fowl (150) and ovine (165), respectively. On the other hand, *S. enterica* subsp. *enterica* serotype Choleraesus and *S. enterica* subsp. *enterica* serotype Dublin (*S. dublin*), although regarded as being host-specific serotypes for swine and bovine, respectively, may eventually be associated with disease in other animals including humans (17, 38, 98).

The 2004 CDC report found that the most frequent serotype associated with nonhuman clinical sources in USA is predominantly *S. newport* affecting most of the cases cattle, followed by *S. typhimurium* and *S. enterica* subsp. *enterica* serotype Agona (*S. agona*). Moreover, cattle were the most commonly identified source for *Salmonella* spp., including *S. typhimurium*, the most frequent serotypes identified in human in 2004. These facts identify cattle as a source for *Salmonella*-induced human illness (16).

Salmonella-induced disease in cattle is associated with *S. newport*, *S. typhimurium* and *S. dublin* (16). The disease has high mortality rate, especially in young calves (less than one month of age) where it manifests as either enteritis or bacteremia, characterized by fever, anorexia, diarrhea, polyarthritis, meningitis or uveitis. *Salmonella* can also affect adult cattle, causing diarrhea, reduced milk yield and reproductive disorders including abortion (4, 164).

Salmonella-infected cattle develop into chronic carriers that harbor the bacteria in their lymph nodes and mammary gland. It is assumed that for every one animal presenting clinical manifestations, five to twenty animals are in the subclinical stage of the disease, spreading the bacteria over the herd through urine and oronasal secretions, feces and milk (4, 119, 164). In addition to transmitting

Salmonella to other animals within the herd, *Salmonella*-carrier animals are indicated as potential source of carcass contamination during slaughter and outbreaks of human salmonellosis, including outbreaks with multidrug-resistant *Salmonella* strains (15, 32, 35). The decreased animal productivity, increased labor and animal care expenses coupled with the zoonotic potential of bovine salmonellosis make this disease one of the major concerns in the cattle industry (119).

Salmonella Pathogenicity Islands

Genes encoding virulence factors of *Salmonella* are predominantly located in 12 chromosomal regions, called *Salmonella* Pathogenicity Islands (SPI) (147). SPI are large gene clusters, 10 to 200 Kb, present exclusively in the genomes of pathogenic bacteria and carrying one or more virulence genes. Some SPI are present in the entire *Salmonella* genus, e.g. SPI-1, SPI-3, SPI-4 and SPI-5. However, other SPI are present in specific serotypes, e.g. SPI-8 is present in the *S. typhi* genome; SPI-7 or the major pathogenicity island is present in some strains of *S. typhi*, *S. dublin*, *S. typhimurium* and *S. paratyphi*, encoding Vi antigen, pilus assembly and SopE; and *Salmonella* genomic island I (SGI-1) carrying multidrug resistance region and present in the genomes of *S. typhimurium* and *S. agona* (64, 147).

During *S. typhimurium* infection, SPI-1 and SPI-2 play major roles in *S. typhimurium* pathogenesis. Both of them carry genes encoding type III-secretion

systems (TTSS) that translocate effector proteins encoded in the indicated islands or in other SPI that are responsible for *S. typhimurium*-induced disease.

TTSS are present in other human pathogenic bacteria, including *Yersinia* (176), *Shigella* (90), *Escherichia coli* 0157:H7 (129), as well as in the insect endosymbiont *Sodalis glossinidius* (29), the plant symbiotic bacteria *Rhizobium* sp (169) and the plant pathogenic bacteria *Pseudomonas syringae* (84). Although the TTSS structure is conserved between bacterial species, the proteins secreted through the structure are not conserved and their functions differ for each pathogen (57, 76, 94, 156). In *Yersinia*, the TTSS is used to evade bacteria phagocytosis (26), whereas in *P. syringae*, the TTSS mediates release of nutrients from host cells and counter host defenses (84). In *Salmonella*, the TTSSs encoded on SPI-1 and SPI-2 are essential to promote bacteria invasion, cytoskeletal changes, intracellular survival, and inflammatory responses (8, 48, 60, 68, 187)

SPI-1 is a 40 Kb long pathogenicity island, located at centisome 63 (114). It is present in all serotypes of both *S. bongori* and *S. enterica* (67, 121) and encodes genes related to iron uptake and TTSS. The iron transport system, SitABCD, is induced upon invasion of epithelial cells, when the availability of iron is scarce. This system allows the bacteria to acquire iron from the host cell, which is required during the rapid bacterial multiplication stage (83, 191). Due to redundancy of other iron transport systems present in the *Salmonella* genome, the relevance of SitABCD system for *Salmonella* pathogenesis has not yet been understood (191).

The TTSS system of SPI-1 is responsible for bacteria invasion of host cells, a major function of SPI-1 in *S. typhimurium* pathogenesis. The TTSS is composed of

about 20 proteins that assemble to form the needle-like structure, which spans the inner and outer bacterial membranes and translocates bacteria proteins into the cytosol of eukaryotic cells (47, 48, 92, 94). Host cell contact is required to activate the TTSS, which involves at least four proteins (InvJ, SpaO, PrgI, PrgJ) for the secretion process itself, and three proteins (SipB, SipC and SipD) to translocate effectors encoded in either SPI-1, including, SipA, SipB, SipC, SptP and AvrA (45, 61, 69, 108, 193), or elsewhere in the bacteria chromosome, SopA, SopB, SopD, SopE, SopE2, SspH1 and SlrP (51, 62, 85, 111, 154, 161, 179, 180). To invade host cells, *S. typhimurium* induces actin-rich membrane ruffles, cytoplasmic projections from the host cells, which surround the bacteria, promoting their internalization into membrane-bound vacuoles (42). The *S. typhimurium*-induced cytoskeletal changes are mediated by the TTSS-translocated effector proteins, SipA, SipC, SopB, SopE, SopE2 and SptP (190).

SopE is a 30 KDa protein (180) present in some strains of *S. typhimurium* and in some other serotypes of *S. enterica*, including *S. typhi*, *S. newport* (62), *S. dublin* (62, 180)*, S. enteritidis* and *S. pullorum* (62). Although SopE is secreted by SPI-1 TTSS, it is encoded outside of SPI-1 at centisome 61 in SPI-7, within a phage genome (62). The SopE2-encoded gene is located at centisome 41 of the *Salmonella* chromosome (154). Analysis of SopE2 amino acid sequence revealed 64% identity to that of SopE, however SopE2 is conserved throughout *S. enterica* serotypes (3, 154). SopE and SopE2 have the same mechanism of action, both act as a guanine nucleotide exchange factor (GEF) (43, 60, 135, 145, 177), activating components of the host signaling pathway of the Rho subfamily. However, more

detailed study of SopE and SopE2 functions found that both activate the (guanosine triphosphatases) GTPases Cdc42, whereas only SopE activated Rac1 GTPase (43). Once small Rho GTPases are activated (GTP-bound RhoGTPases), they interact with specific downstream effectors and induce cellular responses, including cytoskeletal reorganization and gene expression (19, 60). Cdc42 was demonstrated to interact with WASP (Wiskott-Alrich syndrome protein) at its CRIB domain (Cdc42/Rac interactive binding), which binds to the Arp2/3 complex, nucleating actin polymerization (100, 101). Rac-1 interacts with POR (partner of Rac) and Arf6 (ADP-ribosylation factor) inducing membrane ruffles (77, 166). Cells expressing constitutively activated versions of Rac-1 and CdC42 had activation of JNK (Jun NH2-terminal kinase) and p38MAP kinase (115). Moreover, in *S. typhimurium* infected COS cells, Cdc42 and Rac-1 were demonstrated to play a role in cytoskeletal rearrangement and phosphorylation of JNK, since transfection of dominant negative forms of Cdc42 and Rac-1 prevented both *S. typhimurium*-mediated events (19, 60).

SopB or SigD is an inositol phosphate phosphatase (120) of 60 KDa, delivered into the host cell cytosol by SPI-1 TTSS (51), but encoded in SPI5 at centisome 20 (178). This protein also regulates membrane ruffling mediated by Cdc42 and changes in phospholipids and inositol phosphate levels (102, 157, 189). The mechanism by which SopB stimulate Cdc42 activation is not completely understood. However, it is known, that in cells expressing a dominant-negative Cdc42, SopB-mediated *S. typhimurium* internalization was effectively reduced (189).

In addition, SopB dephosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) contributing to membrane fission and *Salmonella*-containing vacuole (SCV) formation (157). Phosphatidylinositol, the most abundant phosphoinositide in mammalian cells, is composed of a myo-inositol headgroup connected to a diacylglycerol (DAG) by phosphodiester bond. The phosphorylation of the inositol ring at different positions allows the generation of seven other phosphoinositides, including PIP2, that produce important cell signaling precursors (128). For example, PIP2 serves as substrate for phospholipase C (PLC), generating DAG and inositol 1,4,5-trisphosphate (IP3), which releases calcium from intracellular stores (151). *Salmonella*-induced actin rearrangement activates increased level of PIP2 at specific membrane ruffling areas (157), which binds to the pleckstrin homology (PH) domain of WASP. The interaction of PIP2-WASP together with binding of GTP-Cdc42 at CRIB domain of WASP, stabilize this molecule allowing it to activate the Arp2/3 complex inducing actin polymerization (71, 100, 101). However, the level of PIP2 decreases at the basal region of the membrane ruffling due to SopB-mediated PIP_2 hydrolyzes, which is essential to promote membrane fission and generate the *Salmonella*-containing vacuoles (157).

The cystokeletal rearrangements promoted by SopB, SopE and SopE2 are enhanced by secretion of SipA or SspA (193), a 80 KDa protein encoded at SPI-1 downstream of *sipB* and *sipC* genes (86). After delivery of SipA into the host cell cytosol through SPI-1 TTSS, which begins at 10sec after bacteria-host contact (146), the protein localizes in the host cell plasma membrane (13) where it acts as an actin-binding protein (99, 108, 193). SipA was demonstrated to alter actin

dynamics by directly binding to actin (99) or actin-binding proteins (192) or by inhibiting actin depolymerization (134).

Crystal structure analysis of *S. typhimurium* SipA revealed a globular domain, which contributes to the binding of pre-existing filamentous actin (F-actin) (99) and two opposite non-globular arms that binds to distant actin molecules (99), stabilizing actin filaments (99, 193). In addition, SipA interacts with T-plastin, a member of the plastin/fimbrin family of actin-binding proteins, increasing its actin bundling-induced activity (192). SipA also affects actin dynamics by decreasing the critical concentration of actin monomers (G-actin) required for actin polymerization (193).

Additionally, SipA was found to prevent actin-depolymerizing factor (ADF)/cofilin and gelsolin-triggered actin disassembly. ADF/cofilin binds to G and F-actin (134) altering the F-actin twist (109) and destabilizing F-actin, which increases the monomer dissociation from the pointed end (50). Likewise, gelsolin controls actin organization by severing F-actin and capping filament ends allowing monomers dissociation and F-actin disassembly (110). SipA was found to prevent ADF/cofilin function by inhibiting ADF/cofilin-actin binding as well as by displacing pre-bound ADF/cofilin from F-actin. Gelsolin-actin binding was not displaced by SipA, however SipA inhibited gelsolin-induced actin severing and re-annealing of gelsolin-severed F-actin fragments (107).

SipA not only directly affects actin polymerization, but also stimulates SipC-mediated cytoskeletal rearrangements (108). SipC is a SPI1-encoded protein translocated though TTSS-SPI1 and necessary for translocation of other TTSS-secreted proteins (23, 46, 87). Studies performed *in vitro* and *in vivo* demonstrated

that SipC induces actin polymerization by stimulating F-actin bundling mediated by the N-terminal domain and actin nucleation directed by residues 201-220 of its C-terminal domain (18, 63).

The *Salmonella*-induced actin-rearrangement is antagonized by a protein encoded on SPI-1 called SptP (45, 88), allowing the cell membrane to return to its original shape from 80 min to 3 hr after infection (45). SptP acts as a GTPase activating protein (GAP) that specifically inactivates Cdc42 and Rac by binding to their active forms and increasing their intrinsic inactivation activity, which switches them to the GDP-bound conformation. In addition, SptP downregulates JNK activation mediated by either SopE microinjection or *S. typhimurium* infection (45).

Once inside the host cells, *Salmonella* resides and proliferates in *Salmonella*-containing vacuoles (SCV), in which it is protected against the host immunity by SPI-2 functions. SPI-2 is located at centisome 30 of the *Salmonella* chromosome, a 40 Kb gene cluster (148) that encodes metabolic functions such as tetrathionate reductase (65), and the second *Salmonella* TTSS (148). The tetrathionate reductase complex is a cluster of the three tetrathionate reductase structural genes (ttrBCA) and two-component regulatory system (ttrRS) (65, 66). This system is encoded in a SPI-2-subset of 15 Kb that does not contribute to SPI-2-virulence functions and is present in both *S. enterica* and *S. bongori* (66).

The SPI-2-TTSS is encoded within the 25 Kb fragment of the SPI-2 region that is only present in *S. enterica* (67) and is essential for intracellular bacteria replication within the SCV, the major function of SPI-2 (8, 68, 122). SPI-2-TTSS is composed of proteins that form the secretion system apparatus (Ssa), translocon

and effectors (SseB-E, SseF-G and SpiC), chaperones (SscA, SscB and SseA) and regulatory system (SsrA and SsrB) (173). Similarly to SPI-1-TTSS, the majority of proteins translocated by SPI-2-TTSS are encoded outside the island and some are within bacteriophage genomes (25).

In vitro studies mimicking intracellular environment conditions or conditions within SCV found that low pH, Mg^{2+}, Ca^{2+}, Fe^{2+} or phosphate concentration are sensed by *Salmonella* global regulatory systems OmpR-EnvZ (96), PhoPQ (113) and local regulatory system SsrAB (7), inducing expression of SPI-2-TTSS (33). Once induced, the SPI-2 TTSS is responsible for secreting proteins across the membrane of SCV required for inhibition of endocytic pathways, such as SpiC-mediated-inhibition of lysosomes and SCV fusion (163), Sse-mediated inhibition of reactive oxygen damage (168) and SseJ and SifA-mediated SCV membrane dynamics (136) and others. Due to its ability to survive and replicate within phagosome, *Salmonella* systemically spread inside phagocytic cells to host organs, causing the late stage of the infection (138).

Mechanism of *S. typhimurium*-induced Enteritis

Humans naturally infected with *S. typhimurium* manifest abdominal pain, vomiting and diarrhea beginning 8 to 48 hr after infection. In most cases, the disease is self-limiting and symptoms last for less than one week (82). In immunocompromised, infant and elderly patients, however, the disease can be presented as protracted diarrhea, lasting more than 21 days and can be fatal due to

rapid progressive weight loss, decreased absorption of nutrients and dehydration (91). Biopsies of clinical *Salmonella* cases indicated that the bacteria induce mucosal damage localized at small intestine and colon characterized by mucosal edema, infiltration of inflammatory cells, predominantly polymorphonuclear neutrophilic leukocytes (PMN), and erosion of the epithelial surface (31).

Several experimental models have been used to characterize and understand the pathogenesis of enteropathogenic *Salmonella* (142, 160). In calves, which develop clinical disease manifestations similar to humans, *S. typhimurium* was found to be associated with M cells of the Peyer's patches, enterocytes (11, 44, 141) and goblet cells (141). *S. typhimurium*-infected bovine tissues had fibrinopurulent necrotizing enteritis with infiltration of PMN through the follicle associated epithelium (FAE) into the lumen, depletion of lymphoid cells, detachment of epithelial cells, stunted and shortened villi, and fluid accumulation (44, 141). Molecular analysis of *S. typhimurium*-induced gene expression changes in bovine ligated ileal loops revealed increased expression of chemokines and cytokines, including IL-8, growth-related oncogenes (GRO) α and β, granulocyte chemotactic protein (GCP)2 and IL-1β, which correlated temporally with PMN migration (141).

S. typhimurium TTSS-SPI1 was demonstrated to be involved in the inflammatory response induced by the bacteria in the host tissue. Mutation of *sipB*, *invH* or combined mutations of *sipA*, *sopA*, *sopB*, *sopD* and *sopE2* significantly reduced inflammatory responses and fluid secretion in the bovine ligated ileal loop model (174, 187). Furthermore, oral infection of calves with *S. typhimurium* Δ*sipAsopABDE2*, Δ*hilA*, Δ*prgH*, or Δ*invH* reduced pathological lesions, mortality,

and/or bacterial shedding (159, 174, 187). Although *S. typhimurium* induces typhoid–like systemic infection in mice, recent studies found that mice orally treated with streptomycin are predisposed to *Salmonella*-induced gastroenteritis. Using this model the relevance of SPI-1-TTSS as well as translocated effector proteins, SipA, SopE and SopE2, the *S. typhimurium*-induced inflammatory response was supported (5, 59). The involvement of SPI-1 TTSS-translocated proteins on inflammatory response is not attributable to all effector proteins, since mutation of *sptP*, *avrA*, *sspH1* or *slrP* had no effect on fluid secretion or inflammation in bovine ligated ileal loops (187). These findings substantiate SPI1 TTSS and specific effector proteins being involved in *S. typhimurium* enteropathogenicity.

In orally infected cattle, in bovine ligated ileal loops and in streptomycin pre-treated mice, SipA was demonstrated to contribute significantly to inflammation (59, 187). The mechanism, however, by which SipA induced inflammatory response was not addressed in these models due to difficulties in investigating the molecular events in such complex systems. Using polarized monolayers of epithelial cells, a model that mimics the morphology of enterocytes (81), *S. typhimurium* was found to induce PMN transepithelial migration independently of bacterial internalization, but dependent on bacteria-cell contact (54). The *S. typhimurium*-induced PMN migration involved both basolateral secretion of IL-8 (103) mediated by cytosolic calcium and NFκB translocation (53), and a NFκB-independent apical secretion of a pathogen-elicited epithelial chemoattractant (PEEC), identified as the eicosanoid hepoxilin A_3, which directs PMNs through intestinal epithelial cells (54, 106, 116). The polarized secretion of PEEC was induced by SipA through an Arf6-mediated lipid signaling

pathway and consequent redistribution and phosphorylation of protein kinase-C (PKC)α (27, 97, 152).

The PMN transepithelial migration is stimulated by enteric *Salmonella* serotypes, including *S. typhimurium* and *S. enteritidis*, as opposed to *S. pullorum*, *S. arizonae*, *S. typhi* and *S. paratyphi*, which although as invasive as the enteric serotypes, fail to stimulate PMN migration in polarized cells. Therefore in cell culture, the ability of *Salmonella* to induce a signaling cascade that stimulates PMN transepithelial migration seems to be required for *Salmonella*-induced enteritis (105). Likewise, PMN infiltration into the lamina propria and villi is the major histological finding in *S. typhimurium* infected bovine ligated ileal loops (44, 141), as opposed to the predominant mononuclear-macrophage migration in the *S. typhi*-infected mucosa (93). Moreover, in *S. typhimurium* infected bovine ligated ileal loops, the severity of tissue damage and fluid secretion corresponded to the intensity of PMN migrating toward the *Salmonella* infected area (140, 141).

S. typhimurium translocated effector protein, SopB, has also been shown to mediate fluid secretion in epithelial cells. Once in the host cell, SopB hydrolyzes inositol 1,3,4,5,6 pentakisphosphate [Ins(1,3,4,5,6)P_5] producing inositol 1,4,5,6-tetrakisphosphate [Ins(1,4,5,6)P_4], which in turn increases chloride secretion. SopB also hydrolyzes phosphatidylinositol 3,4,5-trisphosphate (PIP_3), an inhibitor of Ca^{2+}-dependent chloride secretion. In this way, SopB induces chloride secretion followed by a net flow of water into the lumen and consequently watery diarrhea (120).

SPI-2 is predominantly involved in intracellular survival and systemic disease (8, 68). However, the contribution of SPI-2 genes in inflammatory disease has been

questioned and further studies are required to define its involvement in enteritis. Mutation in a SPI-2 gene, *spiB*, reduced the severity of *S. typhimurium*-induced lesions but had no effect on mortality and diarrhea (159). On the other hand, mutation of *sseD* or *ssaT* reduced the ability of *S. dublin* to induce PMN influx and fluid secretion in bovine ligated ileal loops, probably by affecting intracellular growth (10). Likewise, oral infection of streptomycin-pretreated mice with *S. typhimurium* SPI-2 encoded TTSS (TTSS-2) mutants significantly reduced intestinal inflammation by reducing bacteria persistence in the intestine and by interfering with the expression of adhesion molecules in the endothelium and consequently decreasing leukocyte migration (22, 24).

Therefore, *S. typhimurium* induced diarrhea is a vastly complex event with cascades involving both host (i.e. PMNs) and bacterial (i.e. SopB or SipA or SPI-2-TTSS) molecules that cooperatively promote tissue damage and fluid secretion. The specific contribution of each one of these mediators in the context of inflammation needs to be further elucidated through a combination of *in vivo* and *in vitro* studies.

Objectives

The goal of this study is to generate new information regarding the signal cascade activated during *S. typhimurium* infection that initiates and propagates *S. typhimurium*-induced inflammatory responses in epithelial cells. The identification of molecules activated during the process of host-pathogen interaction, as well as specific cell types responsible for chemokine expression will provide potential targets

either in the host or in the pathogen that may be used in therapeutic approaches. The Chapter II demonstrates the involvement of TTSS-effector proteins in chemokine expression independent of TLR-5 activation, as well as, pathways induced by SipA in epithelial cells. The Chapter III extends the study of effector proteins in *S. typhimurium*-induced cytosolic Ca^{2+} increase and its importance to inflammation. The Chapter IV identifies cell types targeted by *S. typhimurium* SipA and their chemokine profiles *in vivo*.

CHAPTER II

Salmonella typhimurium SIPA INDUCES CXC-CHEMOKINE EXPRESSION AND PROTEIN PHOSPHORYLATION INDEPENDENT OF TLR-5

Introduction

Salmonella enterica serotype Typhimurium (*S. typhimurium*) activates expression of chemokines in humans and cattle, stimulating polymorphonuclear neutrophilic leukocyte (PMN) migration, enteritis and diarrhea (141, 142, 159, 171). In bovine ligated ileal loops, wild type *S. typhimurium* activates CXC chemokines at higher levels than *S. typhimurium* lacking *sipAsopABDE2* in the early stage of infection (186). Moreover, the severity of infection is higher in wild type *S. typhimurium*-infected tissues as compared to *sipAsopABDE2* mutant-infected tissues either in the bovine ligated ileal loop model or after *per os* infection (187). These findings indicate that the corresponding effector proteins (SipA, SopA, SopB, SopD and SopE2) translocated through the *Salmonella* Pathogenicity Island-1 (SPI-1) encoded T3SS (T3SS-1) play roles in the activation of inflammatory response and the induction of diarrhea.

The functions of some effector proteins in *Salmonella* pathogenesis have been described. For example, SopB alters the cellular inositol phosphate levels, thereby interfering with chloride secretion (120). SipA induces PMN migration in polarized cells through PKC-α-mediated secretion of a pathogen-elicited epithelial

chemoattractant (PEEC) (97, 152). SopA interacts with a ubiquitin E3 ligase, promoting bacterial escape from the *Salmonella*-containing vacuoles into the cytosol (188). Finally, SopE2 induces phosphorylation of JNK through activation of the guanosine triphosphatase Cdc42 (19, 60, 115). However, the mechanism by which *S. typhimurium* effector proteins induce chemokine expression is not yet clearly defined. Infection of intestinal epithelial cell lines with *S. typhimurium* usually conceals the effect of effector proteins on chemokine expression, because *S. typhimurium* flagellin behaves as a pathogen-associated molecular pattern (PAMP), triggering nuclear responses and pro-inflammatory gene expression (80, 185). *S. typhimurium* translocates flagellin across epithelia (55), activating the Toll-like receptor-5 (TLR-5) pathway on the basolateral surface of the cell (52). TLR-5 activation has been implicated in the pathogenesis of other infectious agents, including *Pseudomonas aeruginosa* (1), by inducing nuclear translocation of NFκB and consequently chemokine gene expression (1, 52, 153).

The relevance of TLR-5 activation in *S. typhimurium*-induced diarrhea has not been elucidated *in vivo*. The basolateral localization of TLR-5 protects intestinal epithelial cells from the constant exposure of commensal bacteria expressing flagella present on the luminal intestinal surface (55). For this reason, either *S. typhimurium*-induced translocation of flagellin or TLR-5 activation mediated by bacterial internalization should be required for *S. typhimurium* to activate inflammatory responses through TLR-5 *in vivo*.

Since bovine ligated ileal loops infected with *S. typhimurium* lacking T3SS-1-translocated effector proteins induce a much less severe inflammatory response

than the wild type (187), this study was designed to further elucidate the role of T3SS-1-translocated effector proteins on activation of chemokine expression in a cell line non-responsive to flagella to avoid the predominant signal of TLR-5 and consequent activation of cellular responses. Although activation of TLR-5 by flagellin is the dominant pathway of *S. typhimurium*-mediated cell signaling events in some cell lines, we provide evidence that *S. typhimurium* activates pro-inflammatory gene expression by a TLR-5 independent pathway via T3SS-secreted effector proteins and that SipA induces phosphorylation of proteins that affect IL-8 expression.

Materials and Methods

Cell line and bacterial strains

HeLa S3 cells (human cervical epithelial cells – ATCC, Manassas, VA) were grown in Kaighn's modification of Ham's F12 medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum. T84 cells (human colonic epithelial cells – ATCC, Manassas, VA) were grown in Ham's F12 medium and Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. HeLa and T84 cells were grown to a concentration of 0.2 x 10^5 cells/cm^2 in 24 well polystyrene plates (Coning Incorporated, Acton, MA) for 48 hr before performing assays. Strains of bacteria used in this study are listed in Table 1. *S. typhimurium* strains were grown in Luria-Bertani (LB) medium overnight then sub-cultured in LB for 4 hr with appropriated antibiotics at the following concentrations:

kanamycin 100 mg/l, ampicillin 100 mg/liter; nalidixic acid 50 mg/liter, tetracycline 20 mg/liter (187). The bacterial inoculum was washed with phosphate buffered saline (PBS) and diluted in cell culture media, so that ~ 200 bacteria were added per cell.

Gene expression

Expression levels of IL-8, growth related oncogene-α (GRO-α) and TLR-5 in uninfected, *Salmonella*-infected or flagellin-treated cells were measured by quantitative real time-PCR (qRT-PCR). Briefly, HeLa S3 and/or T84 cells were grown in 24 well polystyrene plates for 48 hr and then infected with *Salmonella* strains (Table 1) for either 1 hr or 5 hr or treated with *S. typhimurium* flagellin (InvivoGen, San Diego, CA) at 1000 ng/ml for 5 hr (52). Total RNA was extracted using Tri reagent (Molecular Research Center, Cincinnati, OH). Reverse transcription of 2 μg of total RNA was performed at 48°C for 30 min with random hexamer primers using Multiscribe Reverse Transcriptase (Applied Biosystem, Foster City, CA). The qRT-PCR was performed in a Smart Cycler II (Cepheid, Sunnyvale, CA) by using 40 ηg of cDNA mixed with OmniMix beads (Cepheid, Sunnyvale, CA), SYBR Green I 0.2 x (Invitrogen Corporation, Carlsbad, CA) and 500 nM of each primer set (Table 2). The cDNA concentration of each sample was normalized to an internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The normalized levels of gene expression in infected cells were calculated relative to uninfected cells as previously described (186).

Protein purification

E. coli F18 carrying pAK68C (97) was grown to exponential phase at 37°C in LB medium containing ampicillin 100 mg/liter. Expression of SipA-human influenza hemagglutinin (SipA-HA) was induced using 1 mM IPTG, and cells were harvested after growth for 12 hr at 30°C. The bacterial cultures were centrifuged and lysed for 1 hr at room temperature with 20 mM Tris pH 8, 5 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 1% Triton X-100 and 10 mg/ml lysozyme. Bacterial extracts were French pressed twice, sonicated three times for 30 sec and centrifuged at 12,000 rpm for 1 hr. In the clear supernatant saturated ammonium sulfate was added to a final concentration of 25%, and centrifuged 12,000 rpm for 30 min. The pellet was discarded, and soluble proteins in the supernatant were precipitated with saturated ammonium sulfate solution to a final concentration of 75%. Precipitated proteins were solubilized and dialyzed overnight against equilibration buffer (20 mM Tris pH 7.5, 0.1 M NaCl, 0.1 mM EDTA). The dialyzed sample was applied on a HA-column and SipA-HA was purified according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN).

Table 1 – Bacterial strains and plasmids used to study the role of SipA in chemokine expression and protein phosphorylation.

Strain and plasmid	**Genotype**	**Reference**
Escherichia coli F18	str^r and rif^r	(104)
S. typhimurium strains		
IR715	ATCC 14028 wild type, *nal*^{r}	(155)
ZA10	ATCC 14028 *nal*^{r} Δ*sipA*	(187)
ZA19	ATCC 14028 *nal*^{r} Δ*sopA*	(187)
ZA15	ATCC 14028 *nal*^{r} Δ*sopB*	(187)
ZA17	ATCC 14028 *nal*^{r} Δ*sopD*	(187)
ZA9	ATCC 14028 *nal*^{r} Δ*sopE2*	(187)
ZA21	ATCC 14028 *nal*^{r} Δ*sipA*Δ*sopAsopBDE2*	(187)
ZA20	ATCC 14028 *nal*^{r} Δ*sopAsopBDE2*	(187)
Plasmids		
pWSK29	Cloning vector	(172)
PMR15	pWSK29 carrying the *sopD* gene	(131)
pMR17	pWSK29 carrying the *sopE2* gene	(131)
pMR26	pWSK29 carrying the *sopB* gene	(131)
pMR28	pWSK29 carrying the *sopA* gene	(131)
pMR29	pWSK29 carrying the *sipA* gene	(131)
PBH	Cloning vector – Roche, IN	(97)
pAK68C	pBH carrying the *sipA* gene	(97)

Table 2- Primers used in qRT-PCR to study the role of SipA in chemokine expression and protein phosphorylation.

Human target	**Primer sequence**[a]	**Product size (bp)**
IL-8	AAG GAA CCA TCT CAC TGT GTG TAA AC	70
	ATC AGG AAG GCT GCC AAG AG	
Gro-α	TCT GAG GAG CCT GCA ACA TG	66
	CAT TGG CCA TTT GCT TGG A	
GAPDH	GAAGGTGAAGGTCGGAGTC	226
	GAAGATGGTGATGGGATTTC	
TLR-5	ACCTAAGGTAGCCTACATTGATTTGC	150
	GAAGATATCGGGTATGCTTGGAA	

[a] Sequences for human genes were obtained from GenBank. Top row: forward primer; bottom row: reverse primer.

Localization of SipA in HeLa cells

The functionality of purified SipA-HA was analyzed as previously described (70). Briefly, HeLa cells were grown at concentration of 0.2 x 10^5 cells/cm^2 in 0.1 mg of collagen type I (Sigma, St. Louis, MO)-treated chamber slide (Nalge Nunc International, Naperville, IL) for 48 hr. Cell were fixed with 2% paraformaldehyde and permeabilized with 1% Triton X-100 in PBS, followed by 30 min incubation with 20 μg of purified SipA-HA or HA peptide (Roche Applied Science, Indianapolis, IN). Cells were washed three times with 0.3% Tween-PBS, and incubated overnight at 4°C with anti-HA fluorescein-conjugated antibody (Roche Applied Science, Indianapolis IN). After incubation, cells were washed three times with 0.3% Tween-PBS and incubated for 20 min with Texas Red-labeled phalloidin (Invitrogen Corporation, Carlsbad, CA). SipA and F-actin were separately visualized with appropriate filter sets using a Zeiss Axioplan-2 microscope interfaced with a Zeis Axiocam HR high resolution color CCD camera. Axiovision V 4.2 software was used to evaluate the co-distribution of these proteins in image overlays.

Protein phosphorylation assay

Cell lysates were prepared as recommended by Kinexus Bioinformatics Corporation (Vancouver, British Columbia, Canada). HeLa cells were infected with *S. typhimurium* wild type, ZA21, and ZA20 for 1 hr at MOI 1:200. After infection, total cellular proteins were extracted using ice-cold lysis buffer containing detergent and

protease inhibitors, sonicated twice for 15 seconds and centrifuged 30 min at 13,000 rpm. The supernatant was assayed for protein concentration using a commercial Bradford reagent (Sigma, St. Louis, MO), diluted in 20 % sodium dodecyl sulfate (SDS) sample buffer and boiled for 4 min at 100°C. The protein phosphorylation screen of 37 phosphorylation sites of replicate samples was performed by Kinexus using the KPSS-1.3 – Phospho-Site 1.3 Kinetworks Screen type (Table 3), which entails resolution of a single lysate sample by SDS-polyacrylamide gel electrophoresis (PAGE), immunoblotting with highly validated phospho-specific antibodies, and protein visualization via enhanced chemiluminescence (ECL). Band quantification of the resulting immune complexes was visualized using Quantity One Software (Bio-Rad, Hercules, CA). Kinexus provided the analysis of normalized data using a 25% increase or decrease of fluorescence intensity values as significant of the averaged values of two independent experiments (49). Targeted kinases not detected on the immunoblots or those for which the average normalized ECL signal in counts per minute (CPM) was less than 100 were not considered in the analysis.

Statistical analysis

Geometric means and standard deviation were determined and statistical significance of differences was calculated using analysis of variance and Student's *t* test at $p < 0.05$.

Table 3- Phosphorylation sites in phosphoproteins tracked by Kinetworks KPSS1.3.

No.	Abrev.	Epitope	No.	Abrev.	Epitope
1	Adducin α	S726	20	PKB α (Akt1)	T308
2	Adducin γ	S693	21	PKB α (Akt1)	S473
3	B23 (NPM)	S4	22	PKC α	S657
4	CDK1/2	T14+Y15	23	PKC α/β2	T638/T641
5	CREB1	S133	24	PKC δ	T507
6	Erk1	T202+Y204	25	PKC ε	S729
7	Erk2	T185+Y187	26	PKR1	T451
8	GSK3 α	S21	27	RAF1	S259
9	GSK3 α	Y279	28	Rb	S780
10	GSK3 β	S9	29	Rb	S807+S811
11	GSK3 β	Y216	30	RSK1/2	T359/S365
12	JNK	T183+Y185	31	S6K α	T389
13	JUN	S73	32	SMAD1/5/9	S463+S465/S463+S465/S465+S467
14	MEK1	S217/S221	33	SRC	Y418
15	MEK3	S189	34	SRC	Y529
16	MEK6	S207	35	STAT1	Y701
17	MSK1	S376	36	STAT3	S727
18	NR1	S896	37	STAT5A	Y694
19	P38 α MAPK	T180+Y182			

Y = tyrosine

S = serine

T = threonine

Results

S. typhimurium *activates gene expression independent of TLR-5*

To demonstrate that HeLa cells were an acceptable model to study *S. typhimurium* pathogenesis in the absence of TLR-5 activation, we treated HeLa and T84 cell lines with *S. typhimurium* flagellin (52) and *S. typhimurium* wild type for 5 hr and performed qRT-PCR for IL-8 expression. In HeLa cells, *S. typhimurium* significantly increased IL-8 expression as compared to flagellin. On the other hand, *S. typhimurium*-infected T84 cells produced similar level of IL-8 expression (27.68 fold increase) as compared to flagellin-treated cells (28.24 fold) (Figure 1A). To determine if the lack of response to flagella in HeLa cells was due to the absence of TLR-5, we performed qRT-PCR for TLR-5 in HeLa and T84 cell lines. The TLR-5 mRNA levels were 70% down-regulated in HeLa cells as compared to T84 cells (Figure 1B). These results suggest that HeLa cells are non-responsive to flagella due to a down-regulation of TLR-5 expression and that *S. typhimurium* may activate IL-8 expression in HeLa cells through TLR-5 independent pathways. Therefore, HeLa cells were deemed adequate to study the action of effector proteins on gene expression and protein phosphorylation.

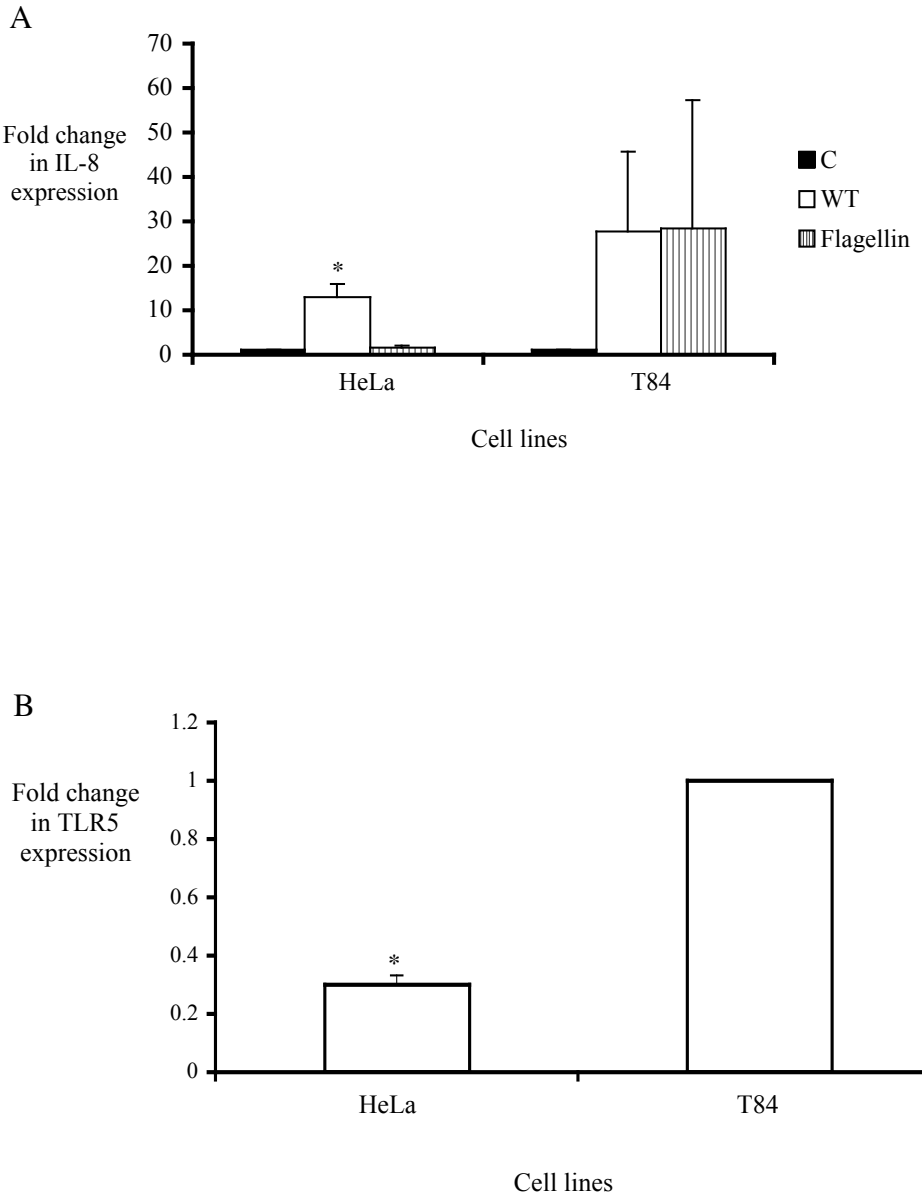


Figure 1: Gene expression in HeLa and T84 cells. HeLa and T84 cells were treated with 1000 ng/ml of *S. typhimurium* flagellin or *S. typhimurium* wild type for 5 hr. IL-8 gene expression was analyzed by qRT-PCR (A). Asterisk indicates a significant increase in IL-8 mRNA level in cells infected with *S. typhimurium* compared to cells treated with flagellin ($P < 0.05$). C: Control (uninfected cells), WT: *S. typhimurium* wild type. TLR-5 expression in untreated HeLa and T84 cells (B). Asterisk indicates a significant down regulation of TLR-5 mRNA level in HeLa compared to T84 cells ($P < 0.05$).

Additive effect of effector proteins on chemokine gene expression

To determine whether T3SS-1-translocated effector proteins are required for the induction of chemokine gene expression in flagellin-non-responsive cells, HeLa cells were infected with *S. typhimurium* wild type or *S. typhimurium* mutants lacking *sipA* (ZA10), *sopA* (ZA19), *sopB* (ZA15), *sopD* (ZA17), *sopE2* (ZA9) and *sipAsopABDE2* (ZA21) for 1 hr. RNA was extracted and IL-8 expression was measured by qRT-PCR. *S. typhimurium* induced a 9.4-fold increase in IL-8 expression as compared to an uninfected control sample (Figure 2A). *S. typhimurium* lacking *sipAsopABDE2* (ZA21), however, did not activate CXC chemokine expression (Figure 2A), indicating that *S. typhimurium*-mediated activation of IL-8 expression in HeLa cells is dependent on T3SS-1-secreted effector proteins. *S. typhimurium* strains carrying mutations in *sipA*, *sopA*, *sopB*, *sopD*, or *sopE2* did not show defects in inducing IL-8 expression, suggesting that effector proteins may act in concert during induction of IL-8 expression (Figure 2A).

SipA induces chemokine expression in HeLa cells

Since *S. typhimurium* strains carrying single gene mutations did not provide information related to gene expression, we complemented the *S. typhimurium sipAsopABDE2* mutant (ZA21) with each individual effector gene. Only HeLa cells infected with ZA21 complemented with *sipA* or with a *S. typhimurium* strain carrying a chromosomal copy of *sipA* (*sopABDE2* mutant, ZA20) expressed IL-8 at

significantly higher levels than the *sipAsopABDE2* mutant (ZA21). This same profile was found for Gro-α gene expression (Figure 2B).

Extracellular SipA failed to induce IL-8 expression in flagella non-responsive cells

To analyze whether SipA internalization is required to induce IL-8 expression, HeLa cells were treated with *S. typhimurium* wild type, a *sipAsopABDE2* mutant (ZA21), a *sopABDE2* mutant (ZA20), purified SipA-HA or HA peptide for 1 hr. RNA extraction was performed and IL-8 expression was analyzed by qRT-PCR. Different doses of SipA-HA (20 μg, 40 μg and 80 μg) failed to induce IL-8 expression above levels seen in infected with the *sipAsopABDE2* mutant (ZA21) or treated with HA peptide (Figure 3). To verify that purified SipA-HA protein was active, permeabilized HeLa cells were treated with 20 μg of SipA-HA or HA peptide and co-localization with F-actin was evaluated using fluorescence microscopy. Purified SipA-HA, but not HA-peptide, colocalized with Texas Red-labeled phalloidin (Figure 4A-D), indicating that purified SipA-HA was functionally active but was not able to induce IL-8 expression when added extracellularly.

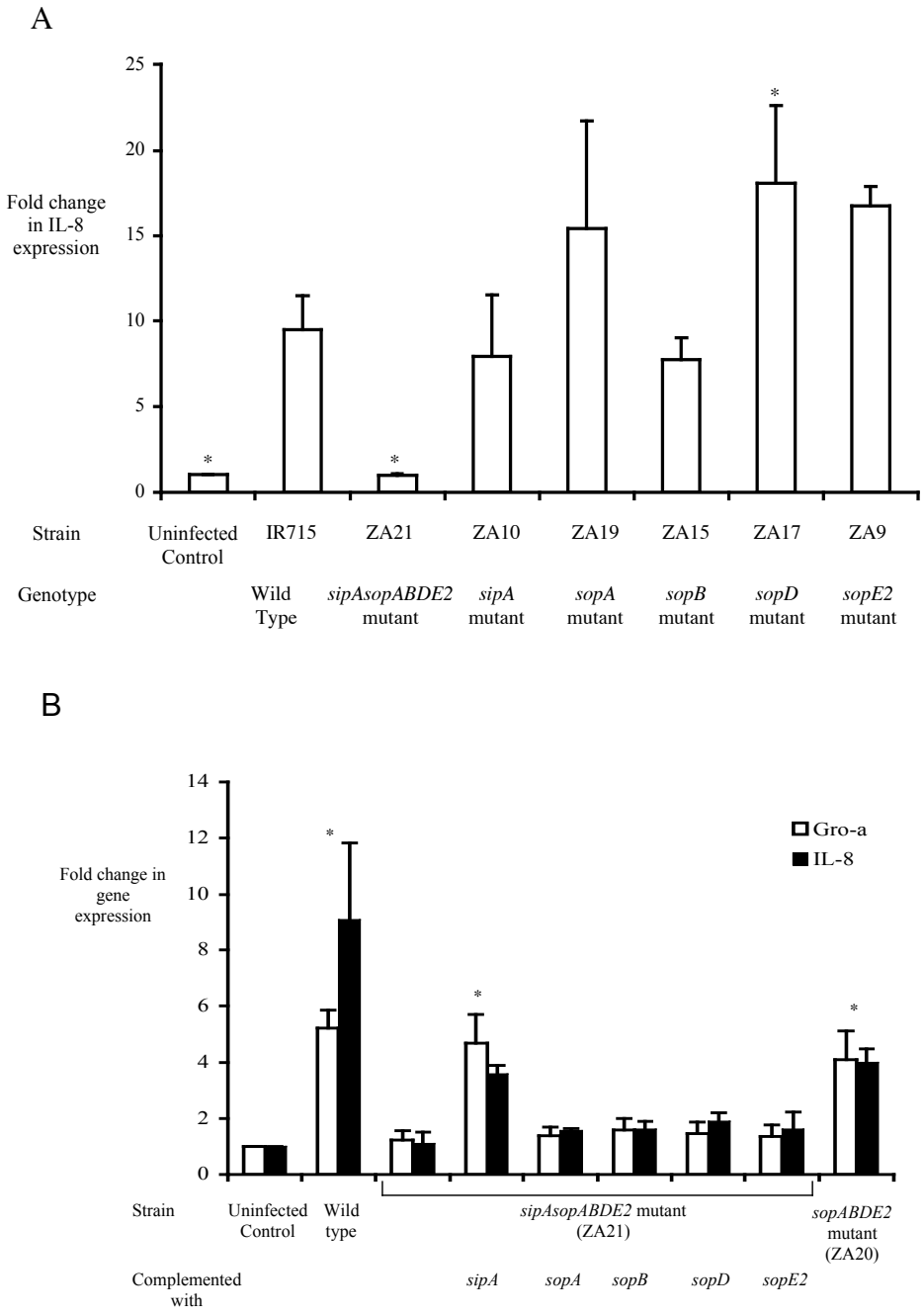


Figure 2: Gene expression profile in *S. typhimurium* infected HeLa cells. HeLa cells were infected with *S. typhimurium* strains for 1 hr and IL-8 (A and B) and Gro-α (B) expression analyzed by qRT-PCR. Asterisk indicates that expression level induced by the strains is statistically different from that induced by ZA21 ($P < 0.05$).

Protein phosphorylation analysis of S. typhimurium *infected cells*

Because only SipA had a measurable effect on IL-8 expression, we performed protein phosphorylation analysis of HeLa cells infected with the *S. typhimurium* wild type, the *S. typhimurium sipAsopABDE2* mutant (ZA21) and *S. typhimurium* carrying a chromosomal copy of *sipA* (*sopABDE2* mutant, ZA20) to identify SipA-activated pathways related to chemokine expression.

Protein phosphorylation comparison of averages from normalized CPM values from two independent experiments is shown in Table 4. *S. typhimurium* wild type induced significant phosphorylation of seven proteins relative to an uninfected control, including B23, CREB1, ERK1, JUN, p38MAPK, NR1, and RAF1-60 (Figure 5A and 6C). Six of these proteins, B23, CREB1, ERK1, JUN, p38MAPK and NR1, were not significantly phosphorylated in ZA21 (*sipAsopABDE2* mutant) infected cells, indicating that their phosphorylation is dependent on T3SS-1-translocated effector proteins (Figure 5A and 6D). Of the six proteins that were phosphorylated in a T3SS-1-dependent manner, the phosphorylation of four, including B23, CREB1, JUN, and p38MAPK, was induced by a *S. typhimurium* strain carrying a chromosomal copy of *sipA* (*sopABDE2* mutant, ZA20) (Figure 5A-B and 6E). ZA20 (*sopABDE2* mutant) also induced phosphorylation of proteins (GSK3a and GSK3b, PKCa/b2 and STAT3) that were not phosphorylated in cells infected with the *S. typhimurium* wild type (Figure 5B).

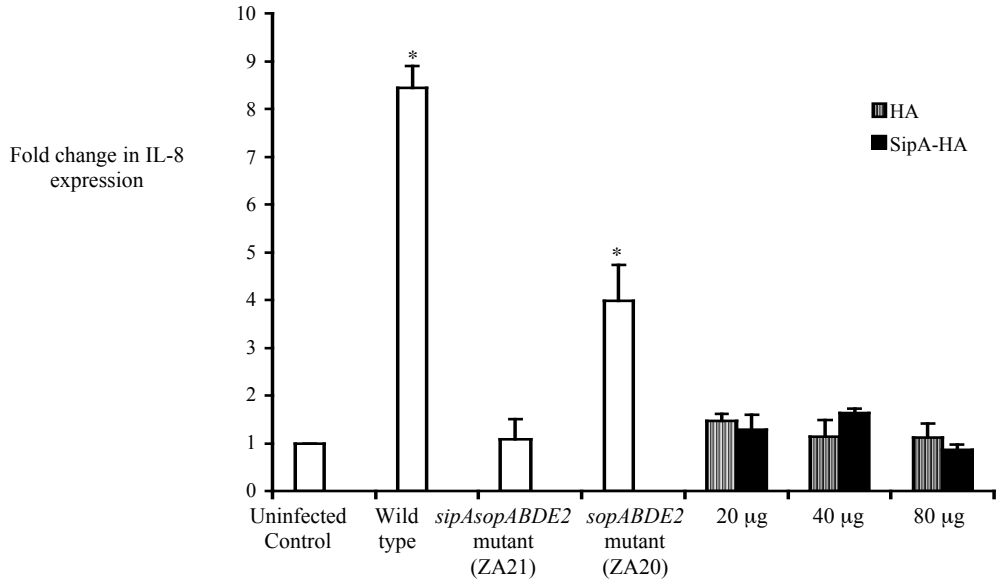


Figure 3: IL-8 expression in *S. typhimurium* and SipA-HA treated HeLa cells. HeLa cells were infected with *S. typhimurium* strains or treated with SipA-HA or HA peptide for 1 hr and IL-8 gene expression analyzed by qRT-PCR. Asterisks indicate significant difference in gene expression relative to ZA21 ($P < 0.05$).

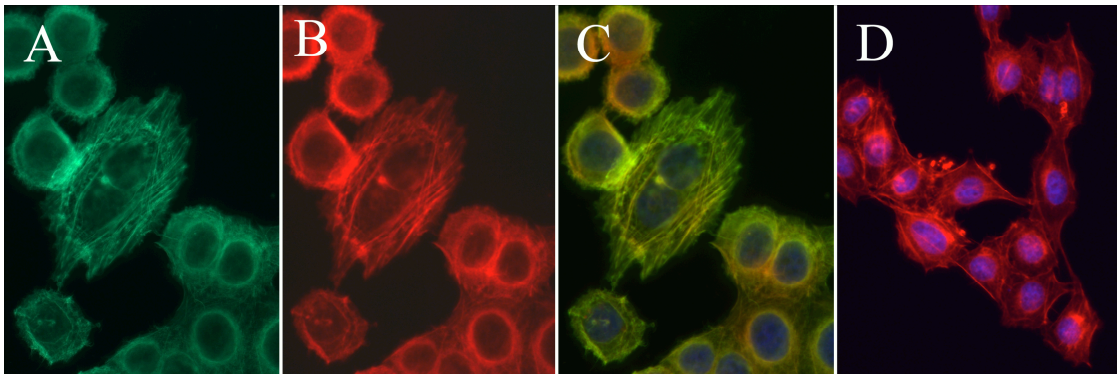


Figure 4: Immunofluorescence microscopy of HeLa cells treated with 20 μg of SipA-HA (A-C) or HA peptide (D) for 1 hr. SipA-HA was visualized using anti-HA FITC (A). F-actin labeling of cells with Texas Red-phalloidin (B). Overlay image of SipA-HA and F-actin shows co-localization of proteins (C). Nuclei in overlay were counterstained with DAPI. Comparable overlay image in cells treated with HA peptide, which reveals absence of HA-peptide binding with F-actin (negative control) (D). Width of each field is 110 μm.

Table 4- Protein phosphorylation comparison of the Kinetworks KPSS1.3 analysis.

	Normalized CPM[a]			
Protein	**Control**	**WT**	**ZA21**	**ZA20**
Adducin α	919	897	1044	1024
Adducin γ	2528	2093	2644	1468
B23 (NPM)	1398	2028	1523	1808
CREB1	854	1203	693	1097
CDK1/2 (27)	4001	2835	2970	4318
PKR (65)	7352	7723	6323	8226
PKR (73)	5101	6250	4814	6370
Erk1	1029	850	1318	487
Erk2	3281	2705	3309	1682
GSK3 α	182	104	102	108
GSK3 α (44)	345	350	247	560
GSK3 β (39)	657	758	565	950
Jun (39)	340	807	409	789
MEK1/2	1384	825	941	686
P38 α MAPK (36)	721	622	620	1238
NR1	703	1253	1036	562
S6K α p70	198	142	166	203
PKC α	3977	4039	5346	4262
PKC α/β2	291	318	527	472
Raf1 (60)	549	1045	1003	776
Raf1 (63)	415	526	667	375
STAT3	529	631	682	691
Src (44)	162	109	179	114
Src (46)	439	361	531	418

[a] CPM is the trace quantity of the band corrected to a scan time of 60 seconds. Values represent averages of two independent experiments.

Molecular weight of different isoforms is indicated in parentheses

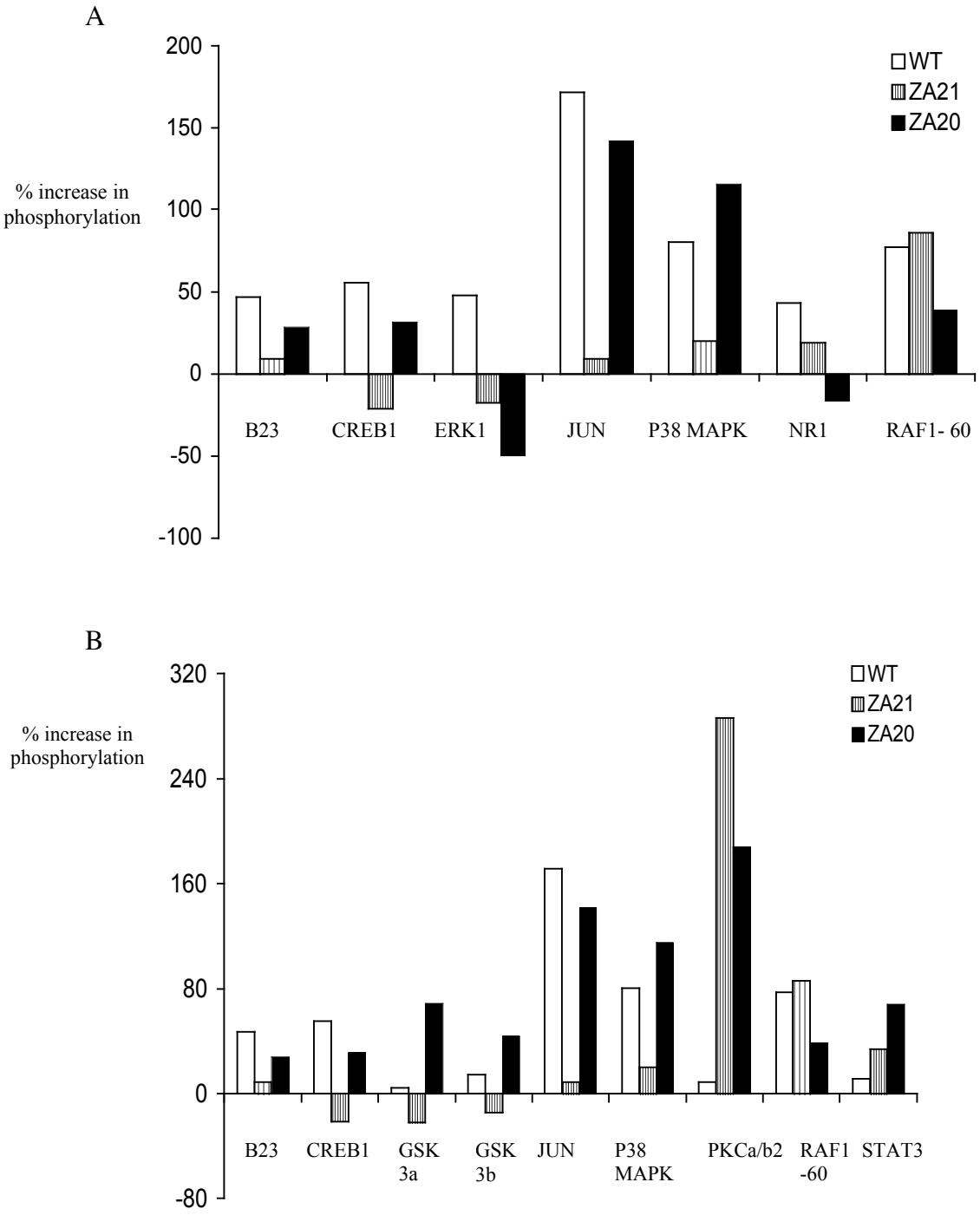


Figure 5: Kinetworks KPSS1.3 phosphoprotein analysis. Graphic representation of percent increases in phosphorylation induced by *S. typhimurium* wild type (A) or ZA20 (*S. typhimurium sopABDE2* mutant) (B). Percent increase was calculated by averaging normalized CPM values of two independent experiments.

A

Lane	Band	Abbreviation	Epitope	~MW	Lane	Band	Abbreviation	Epitope	~MW
2	1	NR1	S896	115	12	2	Raf1	S259	67
2	2	Unclassified	Unclassified	38	12	3	Raf1	S259	62
2	3	Unclassified	Unclassified	25	14	1	Unclassified	Unclassified	76
3	1	Adducin a	S726	121	14	2	PKBa (Akt1)	S473	58
3	2	Adducin g	S693	78	15	1	GSK3a	S21	44
3	3	Src	Y529	53	15	2	GSK3b	S9	40
3	4	Src	Y529	48	16	1	PKR	T451	74
3	5	CDK1/2	Y15	26	16	2	PKR	T451	68
5	1	PKCa	S657	79	17	1	GSK3a	Y279	44
6	1	Unclassified	Unclassified	37	17	2	GSK3b	Y216	40
7	1	PKCa/b2	T638/T641	79	18	1	Rb	S780	127
7	2	Unclassified	Unclassified	56	18	2	Unclassified	Unclassified	111
8	1	S6Ka p70	T389	69	18	3	Unclassified	Unclassified	58
8	2	Erk1	T202+Y204	41	18	4	p38a MAPK	T180+Y182	38
8	3	Erk2	T185+Y187	40	19	1	Unclassified	Unclassified	91
8	4	Unclassified	Unclassified	37	19	2	MEK1/2	S217/S221	42
9	1	Unclassified	Unclassified	60	19	3	B23 (NPM)	S4	37
9	2	Unclassified	Unclassified	56	20	1	Unclassified	Unclassified	81
10	1	STAT3	S727	83	20	2	CREB1	S133	44
11	1	Jun	S73	40	20	3	Unclassified	Unclassified	38
12	1	Unclassified	Unclassified	88					

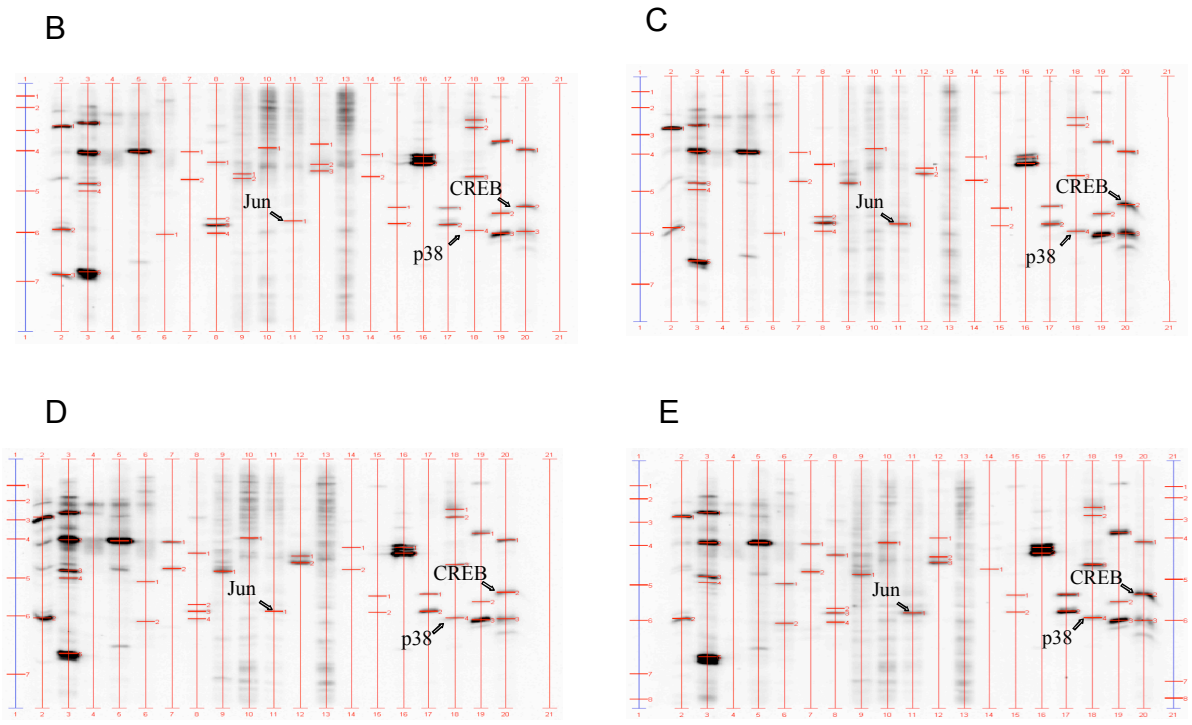


Figure 6: Phosphoproteins analyzed by Kinetworks KPSS1.3. List of phosphoproteins detected by Kinetworks KPSS1.3 in HeLa cells (A) Column 1 and 2 (lane and band) indicate the location of each phosphoprotein on immunoblots. Examples of immunoblots of control cells (B), or *S. typhimurium* wild type-infected HeLa cells (C), *S. typhimurium sipAsopABDE2* mutant (ZA21)-infected HeLa cells (D) and *S. typhimurium sopABDE2* mutant (ZA20)-infected HeLa cells (E). In all immunoblots lane 1 indicates standard molecular marker.

Discussion

The mechanism by which SipA, SopA, SopB, SopD and SopE2 induce chemokine expression is not well defined in part because activation of TLR5 by flagella is the predominant signal for inducing expression of chemokines in intestinal epithelial cell lines. In this study, we analyzed the effect of T3SS-1-translocated effector proteins in flagellin non-responsive cell lines to further elucidate the function of effector proteins on induction of chemokines and protein phosphorylation. We demonstrated that *S. typhimurium* SipA activates chemokine expression and induces phosphorylation of CREB1, Jun and p38MAPK, proteins that have been previously described to be involved in inducing IL-8 expression (74, 89, 126).

Our studies demonstrate that the *S. typhimurium sipAsopABDE2* mutant complemented with *sipA* activated chemokine expression, however, strains carrying a mutation in *sipA* also activated chemokine gene expression at wild type levels, which was likely due to compensatory effects of SopA, SopB, SopD and SopE2. This additive effect of *S. typhimurium* effector proteins has previously been observed during studies in the bovine ligated ileal loop model (187).

Our results indicate that *S. typhimurium* induces phosphorylation of B23, CREB1, ERK1, JUN, p38MAPK, NR1 and RAF1-60. Phosphorylation of all these proteins except RAF1-60, was dependent on T3SS-1 effector proteins (SipA, SopA, SopB, SopD and SopE2). Of these T3SS-1-dependent-phosphorylated proteins, SipA induced significant phosphorylation of B23, CREB1, JUN and p38MAPK. B23 is a RNA binding protein involved in cell growth, DNA replication and apoptosis (14,

41), whereas CREB, JUN and p38MAPK have been implicated as potential stimuli of pathways resulting in IL-8 production. CREB is a transcription factor that, once phosphorylated by protein kinase A (PKA), binds to the co-activator protein CBP (CREB-binding protein) (20) and its homolog p300 resulting in histone hyperacetylation, chromatin remodeling, and initiation of IL-8 transcription (74). JUN is part of the transcriptional regulator activating protein-1 (AP-1), which is constitutively bound to its cognate DNA, the AP-1 binding site. Phosphorylation of JUN by MAPK, such as JNK and the ERKs (118) regulates its stability and hence the AP-1 transcriptional activity. Moreover phosphorylated JUN binds to transcriptional co-activator CBP, enhancing its transcription activity (74, 89). Studies of p38MAPK revealed that it can control IL-8 gene expression either at the post-transcriptional level by stabilizing mRNA (74), or at the transcriptional level by activation of the IL-8 promoter (126).

S. typhimurium porins and LPS stimulate human monocytic cells to release IL-8 through several pathways, including PTK, ERK 1/2 and p38MAPK, NFκB and AP-1 pathways (170). In Henle-407 cells, *S. typhimurium* induces activation of ERK, JNK and p38MAPK in an invasion dependent manner, which leads to activation of NFκB and AP-1 transcription factors (72). However, to our knowledge, our data comprise the first report demonstrating the role of SipA on phosphorylation of CREB, JUN and p38MAPK.

Our data suggest that SipA mediates phosphorylation of CREB, JUN and p38MAPK, however, expression of SipA in a strain lacking *sopA*, *sopB*, *sopD* and *sopE2* (ZA20) also induced phosphorylation of proteins that were not significantly

induced in *S. typhimurium* wild type infected cells, such as GSK3 a/b, PKCa/b2 and Stat 3. GSK3 phosphorylates β-catenin leading either to its degradation or to nuclear translocation and binding to transcription factors (Tcf/Lef), thereby activating transcription of many genes, including IL-8 (58). Stat 3 is predominantly involved in cell proliferation, differentiation and apoptosis (30, 132), however, there is indication that it may also be involved in regulation of IL-8 gene expression (183)

Recombinant *S. typhimurium* SipA has been demonstrated to activate PKC-α, PKC-δ and PKC-ω, although only PKC-α has been implicated in being involved in triggering *S. typhimurium* induced PMN migration across monolayers of T84 polarized cells (152). In our study, PKC-δ and PKC-ε were not detected on immunoblots. PKC-α production was induced by the *S. typhimurium sipAsopABDE2* mutant and a *S. typhimurium* strain expressing *sipA* (*sopABDE2* mutant), however it was not significantly phosphorylated in wild type infected cells. The different response found in T84 cells as compared to our findings in HeLa cells may be due to heterogeneous expression profiles of individual PKC family members in different cell types.

In previous studies, *S. typhimurium* was determined to activate IL-8 gene expression in polarized epithelial cells expressing dominant-negative Rac1 or Cdc42 (73), indicating that activation of Rho GTPases is not required for inducing IL-8 expression. In the same study, it was found that *Salmonella* flagellin could activate IL-8 gene expression. Our study supports the idea that Cdc42 is not involved in IL-8 expression, because no change in expression was found in cells infected with *S. typhimurium sipAsopABDE2* mutant complemented with *sopE2*. We also found

that, in the absence of flagellin-induced gene expression, SipA mediates significant chemokine activation.

We demonstrate by qRT-PCR that T84 cells expressed the same level of IL-8 transcripts upon stimulation with either flagellin or *S. typhimurium*, indicating that these cells are an appropriate model to study the function of the TLR-5 pathway in host-pathogen interactions. In contrast, HeLa cells express very low levels of TLR-5 and were non-responsive to flagellin. Therefore, HeLa cells represent a useful model to study the mechanism by which *S. typhimurium* effector proteins activate an inflammatory response. Although both mechanisms (TLR-5 and effector proteins) may be involved during *in vivo S. typhimurium*-induced inflammatory responses and diarrhea, the relevance of flagellin in *S. typhimurium* pathogenesis remains to be further elucidated (40). Our data provide further support for the idea that T3SS-1-translocated effector proteins play major roles in inducing an inflammatory response during the first 1 hr post-infection. Importantly, our experiments performed with a flagella non-responsive cell line suggest that activation of chemokine expression and protein phosphorylation was independent of TLR-5 and flagella, but dependent on effector proteins.

CHAPTER III

Salmonella typhimurium-INDUCED INTERNALIZATION AND IL-8 EXPRESSION IN HeLa CELLS DO NOT CORRELATE WITH INTRACELLULAR Ca^{2+} LEVELS

Introduction

Salmonella enterica subsp. *enterica* serotype Typhimurium (*S. typhimurium*) activates inflammatory responses in humans and calves characterized by severe, acute, fibrinopurulent, necrotizing enteritis with blunting of absorptive villi, detachment of epithelial cells, fluid accumulation in the intestinal lumen, and infiltration of polymorphonuclear neutrophilic leukocytes (PMN) at the site of infection (141, 142, 159, 171). The mechanisms by which *S. typhimurium* induces inflammation *in vivo* are not completely understood. Numerous studies have been performed to identify *S. typhimurium* pathogen-associated molecular patterns (PAMPs) related to inflammation, as well as host molecules involved with host-agent interaction that initiate and propagate the inflammatory response (72, 152, 162, 185).

The *S. typhimurium* effector proteins SipA, SopA, SopB, SopD and SopE2, that are translocated through the *Salmonella* Pathogenicity Island-1 (SPI-1) encoded T3SS-1, were demonstrated to play important roles in generating an inflammatory response in the bovine ligated ileal loop model. A *S. typhimurium* strain carrying mutations in *sipA*, *sopA*, *sopB*, *sopD* and *sopE2* (ZA21) causes 60% less

inflammatory responsiveness and decreased fluid accumulation relative to wild type infected loops. On the other hand, mutations in the *slrP*, *avrA*, *sspH1* or *sptP* genes that encode other T3SS-1 effector proteins did not reduce fluid accumulation or the degree of inflammation in bovine ligated ileal loops. The degree of attenuation observed in a *sipAsopABDE2* mutant was similar to that of a *S. typhimurium sipB* mutant (187), a strain deficient for translocating T3SS-1 effector proteins into host cells. These findings suggest that SipA, SopA, SopB, SopD and SopE2 are the major effector proteins required for *S. typhimurium*-induced enteritis in calves.

S. typhimurium T3SS-1-translocated effector proteins, SipA, SopA, SopB, SopD and SopE2, induce cytoskeletal rearrangements leading to bacterial internalization (131, 190). SopE2, is a guanine nucleotide exchange factor (43, 60, 135, 145, 177), and SopB, is an inositol phosphate phosphatase (120). Phosphatidylinositol phosphates (generated by SopB) and activated Rho-family GTPases (produced by SopE2) act in concert to activate WASP/Scar proteins, which in turn recruit the Arp2/3 complex to initiate the formation of new branches on actin filaments (117, 149). SipA, which is delivered into host cells as quickly as 10 sec after exposure (146), localizes in the host cell plasma membrane (13) where it acts as an actin-binding protein (99, 108, 193).

Ca^{2+} is an important intracellular messenger involved in many cellular functions, including vesicular trafficking (184), cytoskeletal rearrangements (6) and changes in gene expression (167). Therefore, changes in intracellular Ca^{2+} level, depending on the amplitude of transients, may influence different cell signaling pathways (6). In cell culture systems, *S. typhimurium* alters cytosolic Ca^{2+}

concentrations within 2 to 4 minutes after infection (53, 56, 123, 124), which precedes an induction of IL-8 gene expression (53).

Bacterial pathogens have been found to alter Ca^{2+} homeostasis in host cells. Although intracellular Ca^{2+} has been implicated in mediating cytoskeletal rearrangements, the dependence of bacterial invasion on changes in Ca^{2+} fluxes is controversial. Invasion of *Shigella flexneri* in epithelial cells induces an increase in intracellular Ca^{2+} that is dependent on a functional T3SS, but cytoskeletal rearrangements are also observed in cells with no detectable Ca^{2+} response (158). *Listeria monocytogenes* and *Campylobacter jejuni* induce intracellular Ca^{2+} changes in host cells (34, 79). While chelation of intracellular Ca^{2+} interferes with *C. jejuni* internalization (79) it has no effect on *L. monocytogenes* invasion (34). Previous studies suggest that *S. typhimurium* invasion is dependent on the induction of cytosolic Ca^{2+} increases (123), and that chelation of Ca^{2+} inhibits bacterial internalization (137). However, a non-invasive *S. typhimurium ΔhilA* mutant induces cytosolic Ca^{2+} changes (53), indicating that host cell internalization is not a prerequisite for inducing Ca^{2+} mobilization.

Ca^{2+} is central to many cell signaling events, including the expression of IL-8 and the induction of cytoskeletal changes. Furthermore, the *S. typhimurium* effector proteins SipA, SopA, SopB, sopD and SopE2 induce inflammatory responses and bacterial invasion. We therefore focused our studies on the role(s) of intracellular Ca^{2+} in *S. typhimurium*-induced invasion and the induction of IL-8 gene expression. Furthermore, we investigated whether a causal relationship exists between injection of *S. typhimurium* effector proteins and a change in intracellular Ca^{2+}.

Materials and Methods

Cell line and bacterial strains

HeLa S3 cells (human cervical epithelial cells – ATCC) were grown in Kaighn's modification of Ham's F12 medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum. Before performing assays, HeLa cells were grown to a concentration of 0.2×10^5 cells/cm^2 in 24 well polystyrene plates (Corning, Acton, MA) or in 0.1 mg of collagen type I-pretreated Coverglass Chambers (Nalge Nunc International, Rochester, NY) for 48 hr. Strains of bacteria used in this study are listed in Table 5 *S. typhimurium* strains were grown in Luria-Bertani (LB) medium overnight then sub-cultured in LB for 4 hr with appropriate antibiotics at the following concentrations: kanamycin 100 mg/l, ampicillin 100 mg/liter; nalidixic acid 50 mg/liter, tetracycline 20 mg/liter (187).

Table 5- *S. typhimurium* strains and plasmid used to study the correlation of *S. typhimurium* induced-intracellular Ca^{2+} changes and bacterial internalization and IL-8 expression.

Strains or plasmids	**Genotypes**	**References**
S. typhimurium strains		
IR715	ATCC 14028 wild type, *nal*^{r}	
ZA10	ATCC 14028 *nal*^{r} *ΔsipA*	(187)
ZA21	ATCC 14028 *nal*^{r} *ΔsipAΔsopAsopBDE2*	(187)
ZA20	ATCC 14028 *nal*^{r} *ΔsopAsopBDE2*	(187)
Plasmids		
PWSK29	Cloning vector	(172)
pMR15	pWSK29 carrying the *sopD* gene	(131)
pMR17	pWSK29 carrying the *sopE2* gene	(131)
pMR26	pWSK29 carrying the *sopB* gene	(131)
pMR28	pWSK29 carrying the *sopA* gene	(131)
pMR29	pWSK29 carrying the *sipA* gene	(131)

Gene expression

Expression levels of IL-8 in *S. typhimurium* infected cells were measured by quantitative real time-PCR (qRT-PCR). HeLa cells were grown on 24 well polystyrene plates for 48 hr and then infected with *Salmonella* strains (Table 6) MOI 1:200 for 1 hr. After infection, total RNA was extracted using Tri reagent (Molecular Research Center, Cincinnati, OH). Reverse transcription of 2 μg of total RNA was performed at 48°C for 30 min with random hexamer primers using Multiscribe Reverse Transcriptase (Applied Biosystem, Foster City, CA). The qRT-PCR was performed in a Smart Cycler II (Cepheid, Sunnyvale, CA) by using 40 ng of cDNA mixed with OmniMix beads (Cepheid, Sunnyvale, CA), SYBR Green I 0.2 x (Invitrogen Corporation, Carlsbad, CA) and 500 nM of each primer set (Table 6). The cDNA concentration of each sample was normalized to an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The normalized levels of gene expression in infected cells were calculated relative to uninfected cells as previously described (186). The effect of an intracellular Ca^{2+} chelator on gene expression was performed by treating HeLa cells with 50 μM BAPTA -AM (Calbiochem, San Diego, CA) or BAPTA-AM derivative, D-BAPTA-AM, which has a reduced chelating capacity (143), in HBSS media for 45 min (53) prior to *Salmonella* infection. Gene expression analysis of BAPTA-AM or D-BAPTA-AM treated cells was performed as described above.

Expression levels of *sipA* in bacteria culture were measured by qRT-PCR as described above, using *sipA* specific primers (Table 6). *S. typhimurium* strains were grown overnight and sub-cultured for 4 hr. *S. typhimurium* RNA was extracted using the SV total RNA isolation kit (Promega Corporation, Madison, WI). The cDNA concentration of each sample was normalized to an internal control, *rrsB* 16S ribosomal RNA (Table 6) (95). The normalized levels of gene expression in ZA21 complemented with *sipA* and ZA20 were calculated relative to *S. typhimurium* wild type (186).

Table 6- Primers used in qRT-PCR to study the correlation of *S. typhimurium* induced-intracellular Ca^{2+} changes and gene expression.

Target	Primer sequence[a]	Product size (bp)
IL-8	AAG GAA CCA TCT CAC TGT GTG TAA AC	70
	ATC AGG AAG GCT GCC AAG AG	
GAPDH	GAAGGTGAAGGTCGGAGTC	226
	GAAGATGGTGATGGGATTTC	
RrsB	GGCAGGCCTAACACATGCA	150
	CTTGCGACGTTATGCGGTATT	
SipA	AAGATTGCTGCGGGTTAACG	200
	TGGCTGCCAGAAACAAAGAA	

[a] Sequences for human and bacterial genes were obtained from GenBank. Top row: forward primer; bottom row: reverse primer.

Bacterial protein analysis

Bacteria were grown in LB medium overnight then sub-cultured in LB for 4 hr with appropriate antibiotics (187). The bacteria cultures were centrifuged and the culture supernatants collected and filtered through a 0.45 μm filter. Secreted proteins were precipitated with 10% trichloroacetic acid as described previously (131). Bacteria extracts were obtained by lysing cells with 20 mM MOPS, pH 7.0, 0.5% Triton X-100, and protease inhibitor mixture. The homogenates were sonicated twice for 15 sec, centrifuged for 30 min at 16,000 x g and the supernatant removed for further assays. The protein concentration of both precipitated secreted proteins and bacteria cell extracts was determined using a commercial Bradford assay (Sigma, St. Louis, MO). An equal amount of protein obtained from supernatant and bacteria cell extract was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to immobilon transfer membrane (Millipore Corporation, Bedford, MA) and subsequently reacted with mouse anti-*S. typhimurium* SipA antibody (Center for Innovations in Medicine, Biodesign Institute, Arizona State University, Tempe, Arizona), and anti-mouse peroxidase secondary antibody (Sigma, St. Louis, MO).

Invasion assay

HeLa cells were grown on 24 well polystyrene plates for 48 hr and infected with *Salmonella* strains (Table 1) at MOI 1:200 for 1 hr. After infection, plates were centrifuged for 5 min at 1000 rpm and incubated at 37°C for 1 hr. Cells were washed three times with 500 μl/well of phosphate buffer saline (PBS) and treated with 100 μg/ml of gentamicin (Invitrogen Corporation, Carlsbad, CA) for 1 hr at 37°C. After antibiotic treatment, cells were washed again with PBS and incubated with 1% Triton X-100 for 5 min at 37°C. The number of internalized bacteria was determined by plating ten-fold serial dilutions of the cell lysates in nalidixic acid containing LB plates. To analyze the effect of BAPTA-AM on bacteria internalization, cells were treated with 50 μM BAPTA-AM in HBSS media for 45 min (53) followed by *Salmonella* infection, and invasion assay was performed as described above.

Intracellular Ca^{2+} measurements

The intracellular Ca^{2+} levels were assessed by confocal microscopy. Monolayers of HeLa cells grown on Coverglass Chamber slides (Nalge Nunc International, Rochester, NY) were loaded with 3 μM of Fluo-4 (Invitrogen Corporation, Carlsbad, CA) for 1 hr at 37°C and washed with serum- and phenol red-free medium. Fluo-4 loaded epithelial cells were then examined with Meridian Ultima confocal microscope using 488 nm wavelength for excitation and 530 nm for emission. Basal intracellular Ca^{2+} level was obtained during the first four scans, then

the cells were infected with *S. typhimurium* strains and Ca^{2+} was monitored for 5 min. The changes in intracellular Ca^{2+} after infection at any time (t) were normalized to basal Ca^{2+} level at time (0). Cells treated with thapsigargin (Sigma, St. Louis, MO) 10 μM were used as a positive control and PBS as a negative control. To chelate intracellular Ca^{2+}, HeLa cells were treated with 50 μM BAPTA (AM) in HBSS media for 45 min (53) followed by *Salmonella* infection.

Statistical analysis

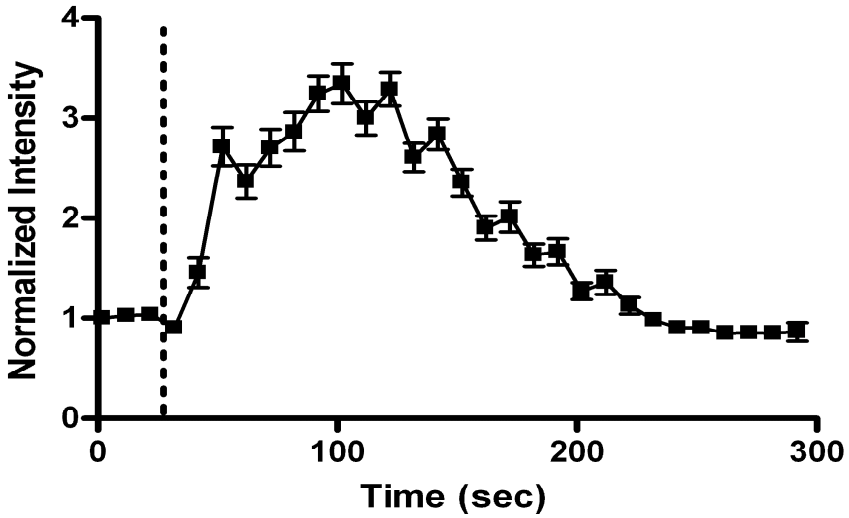
To analyze the effects of *S. typhimurium* effector proteins on intracellular Ca^{2+}, induction of gene expression, and invasion in HeLa cells, geometric means and standard deviation were determined and statistical significance was calculated using Student's *t* test at $p < 0.05$. Anova was followed by Tukey test at $p<0.05$ to evaluate differences between multiple treatments.

Results

S. typhimurium-*induced changes in intracellular Ca^{2+} are dependent on T3SS-1-translocated effector proteins*

Intracellular Ca^{2+} in HeLa cells was measured by confocal microscopy after stimulation with *S. typhimurium* wild type, a *sipAsopABDE2* mutant (ZA21), and ZA21 complemented with plasmids carrying cloned effector genes (*sipA*, *sopA*, *sopB*, *sopD* or *sopE2*). The *S. typhimurium* wild type increased intracellular Ca^{2+} in HeLa cells (Figure 7A), at higher level than *S. typhimurium sipAsopABDE2* mutant (Figure 7B). An increase in Ca^{2+} was detectable as early as 30 sec after infection with the *S. typhimurium* wild type (Figure 7A). To determine whether an individual T3SS-1 translocated effector protein was responsible for inducing the increase in Ca^{2+}, the *S. typhimurium sipAsopABDE2* mutant was complemented *in trans* with the corresponding effector gene. The *S. typhimurium sipAsopABDE2* mutant complemented with *sopD* reduced Ca^{2+} response, whereas, only the *S. typhimurium sipAsopABDE2* mutant complemented with *sipA* (ZA21pSipA) induced Ca^{2+} transients at wild type levels (Figure 7B).

A



B

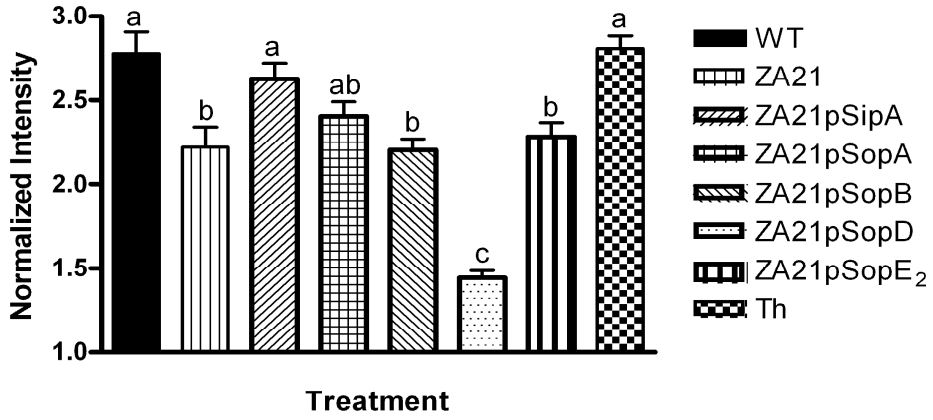


Figure 7: Evaluation of intracellular Ca^{2+} in HeLa cells by confocal microscopy. (A) An example of *S. typhimurium*-induced intracellular Ca^{2+} transients. The arrow indicates the time of addition of *S. typhimurium* (B) Fluorescence intensity of Fluo-4 in *S. typhimurium* infected HeLa cells. Values represent normalized fluorescence intensity ± standard deviation induced following addition of each treatment. For each treatment, data were recorded from at least 15 cells/area, 16 areas/well, and 4 wells/treatment. Different letters indicate significant difference at $P < 0.05$. WT: *S. typhimurium* wild type; ZA21: *S. typhimurium sipAsopABDE2* mutant; ZA21pSipA: ZA21 complemented with *sipA*, ZA21pSopA: ZA21 complemented with *sopA*; ZA21pSopB: ZA21 complemented with *sopB*; ZA21pSopD: ZA21 complemented with *sopD*; ZA21pSopE2: ZA21complemented with *sopE2*; Th: thapsigargin.

Cytosolic concentration of SipA at high levels correlates with an increase in intracellular Ca^{2+} in epithelial cells

To determine whether SipA is required to stimulate Ca^{2+} mobilization, HeLa cells were infected with ZA10 (*ΔsipA*), and cytosolic Ca^{2+} was measured. In the absence of SipA, *S. typhimurium* induced no significant increase in the concentration of cytosolic Ca^{2+} as compared to *S. typhimurium sipAsopABDE2* mutant (Figure 8A). To compare the phenotype generated by *sipA* cloned into a low copy number plasmid to that observed in a strain carrying a chromosomal copy of *sipA*, intracellular Ca^{2+} was analyzed in HeLa cells infected with ZA21pSipA (*sipAsopABDE2* mutant complemented with plasmid encoded *sipA*) and ZA20 (*sopABDE2* mutant). Although ZA21pSipA stimulated an increase in Ca^{2+}, ZA20 induced a Ca^{2+} level approximately half the Ca^{2+} level stimulated by ZA21pSipA (Figure 8A). To investigate the basis of the lower level of Ca^{2+} response in ZA20 (*sopABDE2* mutant) infected cells, *sipA* mRNA levels were measured by qRT-PCR. The *sipA* mRNA levels were increased 177.5-fold and 1.4-fold, respectively, in ZA21pSipA and ZA20 as compared to the wild type (Figure 8B). SDS-PAGE and Western blot analysis of bacterial supernatants revealed increased SipA secretion by ZA20 as compared to ZA21pSipA (Figure 8C and 8D), however Western blot analysis of the bacteria cell extract indicated a higher SipA concentration in ZA21pSipA than in ZA20 and in wild type (Figure 8E). The phenotype of the *sipA* mutant (ZA10) indicated that SipA is required for inducing increases in intracellular Ca^{2+}. Other effector proteins (SopA, SopB, SopD, SopE2) may contribute to

increasing intracellular Ca^{2+}, because wild type- intracellular concentration of SipA was not sufficient to allow a *sopABDE2* mutant (ZA20) to induce changes in intracellular Ca^{2+} levels. However, this requirement for other effector proteins (SopA, SopB, SopD, SopE2) could be overcome when expression of *sipA* was artificially increased by cloning the gene on a plasmid (Figure 8B).

Ca^{2+} mobilization is not required for S. typhimurium *SipA-induced gene expression*

To analyze whether intracellular Ca^{2+} correlates with chemokine gene expression, HeLa cells were infected with *S. typhimurium* strains for 1 hr and IL-8 gene expression was analyzed by qRT-PCR. Only ZA21pSipA (*sipAsopABDE2* mutant complemented with plasmid encoded *sipA*) stimulated IL-8 gene expression at higher levels than ZA21 (*sipAsopABDE2* mutant) (Figure 9A), which was in good agreement with the intracellular Ca^{2+} data (Table 7). However, ZA20 (*sopABDE2* mutant) and ZA10 (*sipA* mutant), which induced cytosolic Ca^{2+} changes at similar or lower level than ZA21 (*sipAsopABDE2* mutant), both activated IL-8 at higher levels than ZA21 (Figure 9B and Table 7). These results provide strong evidence that *S. typhimurium* effector proteins can induce gene expression changes independently of cytosolic Ca^{2+} levels.

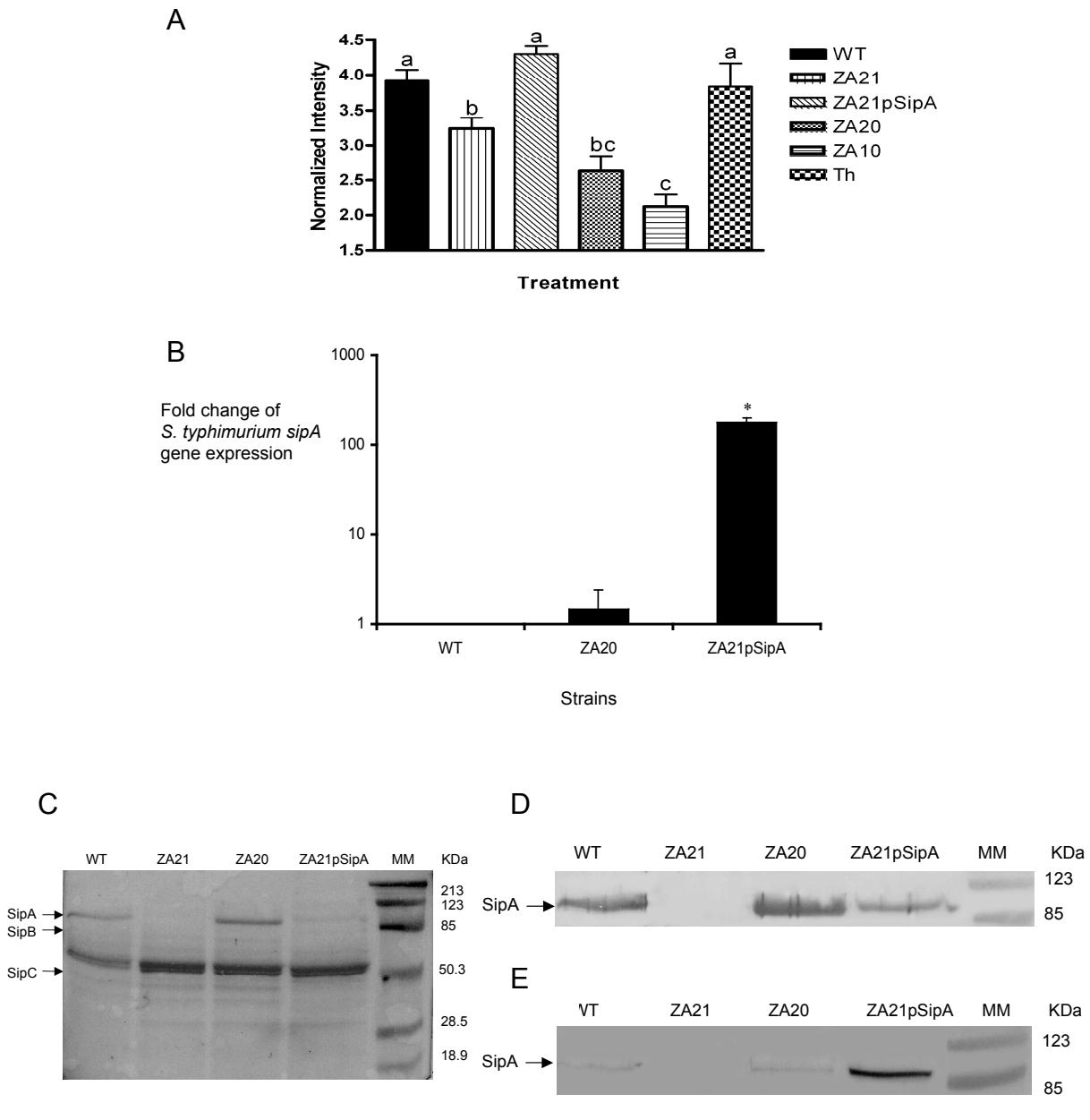


Figure 8: Effect of *S. typhimurium* SipA on intracellular Ca^{2+} levels. (A) Treatment-induced Ca^{2+} response in HeLa cells. Values represent mean normalized intensity ± standard deviation following addition of treatment. For each treatment, data were recorded from at least 15 cells/area, 16 areas/well, and 4 wells/treatment. (B) qRT-PCR of *sipA* in culture of *S. typhimurium* WT, ZA20 and ZA21pSipA. Value represent mean ± standard deviation of at least three experiments (C) SDS-PAGE of proteins secreted by *S. typhimurium* into culture supernatant. (D) Western blot analysis, using anti-SipA antibody, of proteins secreted by *S. typhimurium* into culture supernatant or (E) crude lysate. Different letters indicate significant difference $P < 0.05$. Asterisk indicates significant difference in *sipA* level compared to other strains ($P < 0.05$). WT: *S. typhimurium* wild type; ZA21: *S. typhimurium sipAsopABDE2* mutant; ZA21pSipA: ZA21 complemented with *sipA*; ZA20: *S. typhimurium sopABDE2* mutant; ZA10: *S. typhimurium sipA* mutant; Th: thapsigargin.

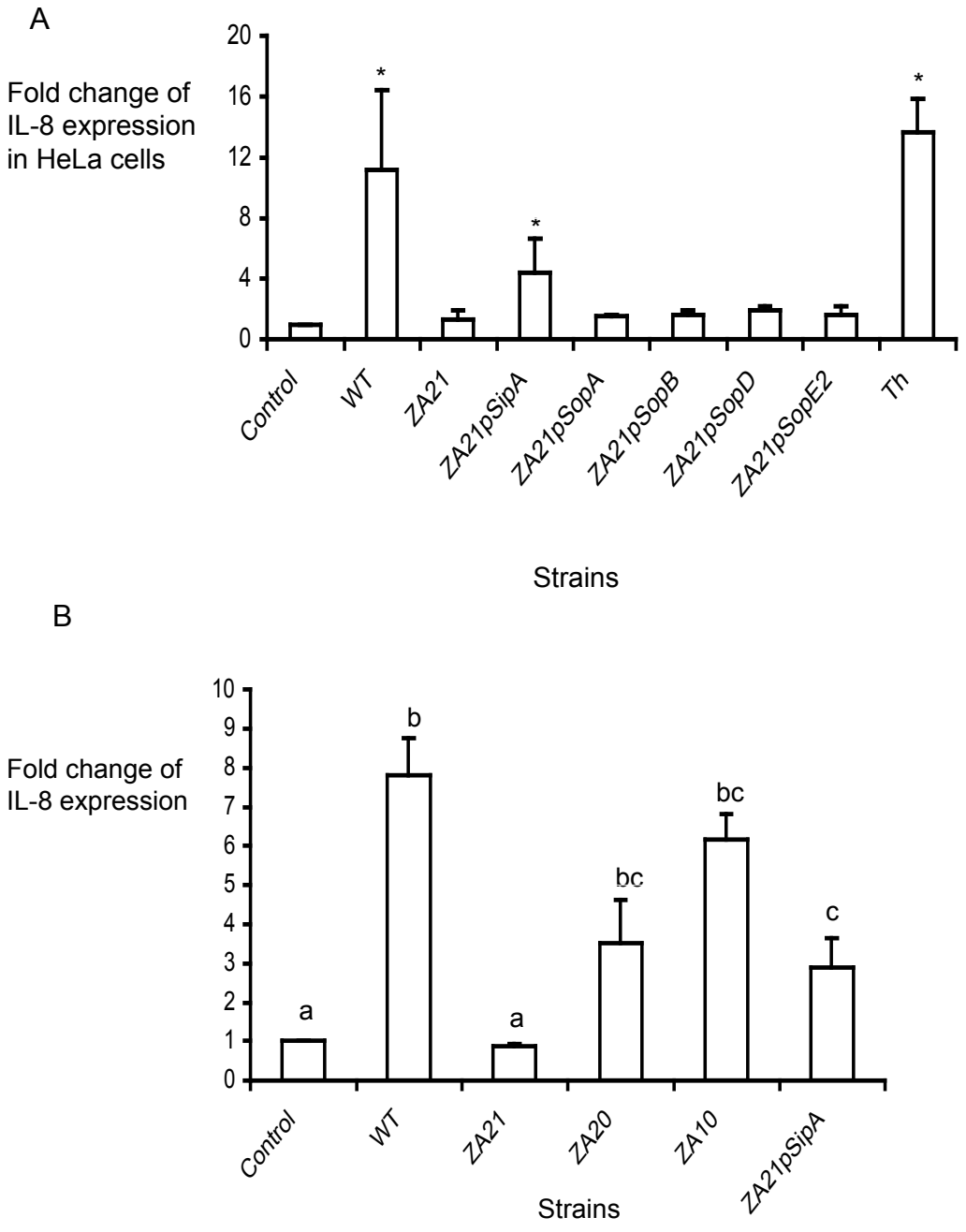


Figure 9: (A and B) qRT-PCR of IL-8 gene in uninfected (control) and *S. typhimurium*-infected HeLa cells. Value represent mean ± standard deviation of at least three experiments. Asterisk indicates significant difference in IL-8 gene expression when compared to ZA21 ($P < 0.05$). Different letters indicate significant difference between strains at $P < 0.05$. WT: *S. typhimurium* wild type; ZA21: *S. typhimurium sipAsopABDE2* mutant; ZA21pSipA: ZA21 complemented with *sipA*, ZA21pSopA: ZA21 complemented with *sopA*; ZA21pSopB: ZA21 complemented with *sopB*; ZA21pSopD: ZA21 complemented with *sopD*; ZA21pSopE2: ZA21complemented with *sopE2*; ZA20: *S. typhimurium sopABDE2* mutant; ZA10: *S. typhimurium sipA* mutant; Th: thapsigargin.

Table 7- Activation of Ca^{2+} response, IL-8 expression and invasion in HeLa cells by different *S. typhimurium* strains.

***S. typhimurium* strains**	**Ca^{2+} response***	**IL-8 expression***	**Invasion***
IR715	Yes	Yes	Yes
ZA21 (Δ*sipAsopABDE2*)	No	No	No
ZA10 (Δ*sipA*)	No	Yes	Yes
ZA20 (Δ*sopABDE2*)	No	Yes	Yes
ZA21pSipA	Yes	Yes	Yes
ZA21pSopA	No	No	No
ZA21pSopB	No	No	No
ZA21pSopD	No	No	No
ZA21pSopE2	No	No	Yes

* Cellular responses induced at significant higher level than ZA21.

ZA21pSipA: ZA21 complemented with *sipA*, ZA21pSopA: ZA21 complemented with *sopA*, ZA21pSopB: ZA21 complemented with *sopB*, ZA21pSopD: ZA21 complemented with *sopD*, ZA21pSopE2: ZA21 complemented with *sopE2*,

S. typhimurium-*induced intracellular Ca^{2+} changes are not required for invasion*

Invasion assays were performed in HeLa cells infected with *S. typhimurium* strains. The *sipAsopABDE2* mutant complemented with plasmids encoding *sipA* (ZA21pSipA) or *sopE2* (ZA21pSopE2), the *sopABDE2* mutant (ZA20) and the *sipA* mutant (ZA10) invaded HeLa cells at significantly higher levels than the *sipAsopABDE2* mutant (ZA21) (Figure 10). Of these strains, only ZA21pSipA altered intracellular Ca^{2+} at higher level than ZA21 (Table 7), thus demonstrating that there was no correlation between *S. typhimurium* invasion and increased intracellular Ca^{2+}.

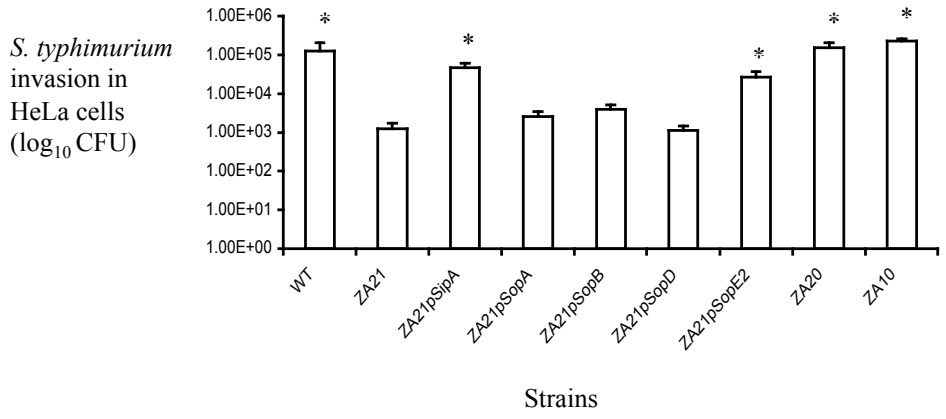


Figure 10: Invasion of *S. typhimurium* strains at HeLa cells. Value represent mean ± standard deviation of at least three experiments. Asterisk indicates significant difference from ZA21 at $P < 0.05$. WT: *S. typhimurium* wild type; ZA21: *S. typhimurium sipAsopABDE2* mutant; ZA21pSipA: ZA21 complemented with *sipA*, ZA21pSopA: ZA21 complemented with *sopA*; ZA21pSopB: ZA21 complemented with *sopB*; ZA21pSopD: ZA21 complemented with *sopD*; ZA21pSopE2: ZA21complemented with *sopE2*; ZA20: *S. typhimurium sopABDE2* mutant; ZA10: *S. typhimurium sipA* mutant.

Effect of BAPTA-AM on S. typhimurium *pathogenesis*

We did not find a correlation of *S. typhimurium*-induced Ca^{2+} mobilization with bacterial internalization and IL-8 gene expression using strains that induced different Ca^{2+} responses in epithelial cells. Previous studies, however, indicated that chelating intracellular Ca^{2+} with BAPTA-AM inhibits *S. typhimurium* invasion and IL-8 gene expression, suggesting that those cellular responses are mediated through changes in Ca^{2+} homeostasis (53, 137). Several other studies have demonstrated, however, that BAPTA-AM can interfere with cell signaling events independently of effecting changes in the Ca^{2+} concentration (127, 143). We tested the effect of BAPTA-AM on *S. typhimurium* invasion and IL-8 gene expression. As expected, chelating intracellular Ca^{2+} with 50 μM of BAPTA-AM inhibited *S. typhimurium* and thapsigargin-induced Ca^{2+} transients (Figure 11A). In addition, BAPTA-AM or D-BAPTA-AM treatment significantly reduced *S. typhimurium*-induced IL-8 gene expression in HeLa cells (Figure 11B). On the other hand, treating HeLa cells with BAPTA-AM had no significant effect on *S. typhimurium* internalization (Figure 11C).

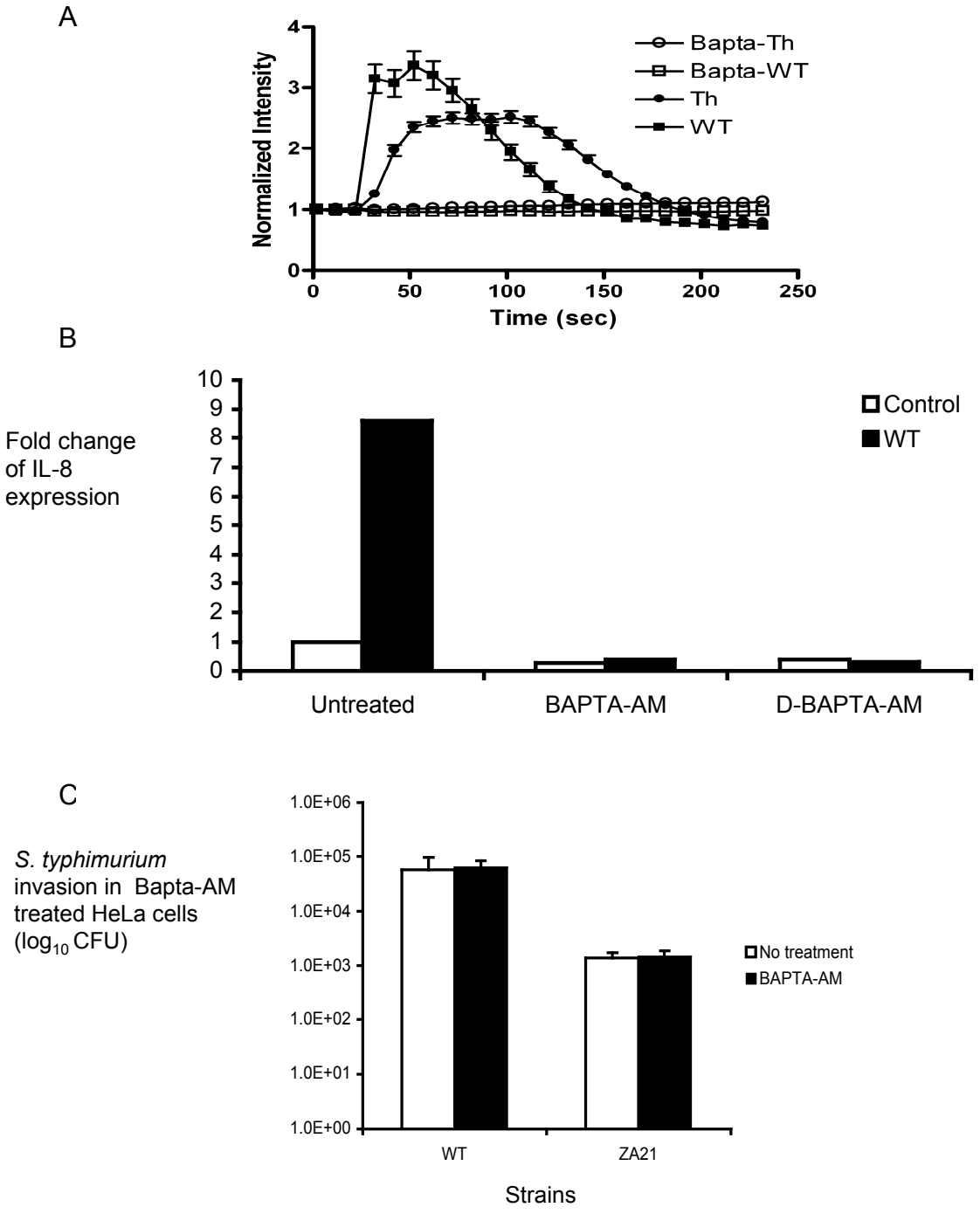


Figure 11: Analysis of *S. typhimurium* pathogenesis in BAPTA-AM treated HeLa cells. (A) Effect of BAPTA on *S. typhimurium*-induced intracellular Ca^{2+} transients. For each treatment, data were recorded from at least 15 cells/area, 16 areas/well, and 4 wells/treatment. (B) *S. typhimurium*-induced IL-8 gene expression with or without BAPTA-AM treatment. (C) *S. typhimurium*-induced internalization with or without BAPTA-AM treatment. Value represent mean $\pm$ standard deviation of at least three experiments. Asterisk indicates significant difference in IL-8 gene expression in BAPTA-treated cells when compared to non-treated cells ($P < 0.05$). WT: *S. typhimurium* wild type; ZA21: *S. typhimurium sipAsopABDE2* mutant; Th: thapsigargin.

Discussion

Because bacteria-host cell interactions induce Ca^{2+} responses at a very early stage of infection (34, 53, 56, 79, 123, 124, 158), and intracellular Ca^{2+} is involved in the activation of many cell signaling pathways (6, 167, 184), it has been presumed that once *S. typhimurium* induces increases in Ca^{2+} levels, it activates chemokine gene expression, thereby inducing an inflammatory response. Previous studies found that *S. typhimurium* stimulates Ca^{2+} mobilization in epithelial cells (53, 56, 123, 124), which was required to activate IL-8 production, since no increase in Ca^{2+} levels and IL-8 secretion were found in *S. typhimurium* infected T84 cells previously treated with BAPTA-AM (53). In our controlled experiments, we reinforced the idea that *S. typhimurium* alters Ca^{2+} levels as early as 30 sec after infection, and that the use of BAPTA-AM under the same conditions as described previously (53) inhibited cytosolic Ca^{2+} transients and *S. typhimurium*-induced IL-8 gene expression. However, using a series of defined mutants defective in secreting individual or combinations of T3SS-1 effector proteins, we found no correlations between intracellular Ca^{2+}, IL-8 gene expression and cell invasion.

A mutant lacking *sipAsopABDE2* (ZA21) failed to produce Ca^{2+} responses in epithelial cells at *S. typhimurium* wild type levels, indicating that alterations in Ca^{2+} homeostasis are dependent on T3SS-1-translocated effector proteins. Complementation of ZA21 with genes of each effector protein revealed that SopD reduced the calcium response, which is under investigation and that only ZA21pSipA stimulated increased cytosolic Ca^{2+} at levels similar to that induced by wild type

Salmonella. Furthermore, mutation of *sipA* interfered with the *S. typhimurium*-mediated Ca^{2+} response. Interestingly, it has been found that SipA is delivered into host cells through T3SS-1 beginning at 10-90 sec after bacteria-cell contact (146), which may explain the SipA-induced increases in cytosolic Ca^{2+} levels at 30 sec after infection. Therefore, SipA, once intracellular, may be a potential mediator of *S. typhimurium*-induced Ca^{2+} responses. The mechanism by which SipA induces Ca^{2+} mobilization, however, remains unclear.

Although ZA21pSipA activated increases in cytosolic Ca^{2+} at higher levels than the *sipAsopABDE2* mutant (ZA21), *sipA* present in the *S. typhimurium* chromosome of a *sopABDE2* mutant (ZA20) induced at cytosolic Ca^{2+} changes at ZA21 levels. Even though ZA20 lacks other invasion-associated genes (146), its invasiveness in HeLa cells was similar to that of ZA21pSipA, indicating that the absence of a Ca^{2+} response in ZA20 infected cells was not a consequence of reduced invasiveness. Subsequent to these observations, secreted and cytosolic SipA levels were determined in cultures of ZA21pSipA and ZA20. Our findings indicated that ZA21pSipA secreted less SipA into the culture supernatant than ZA20. However, the levels of intracellular SipA and *sipA* mRNA in ZA21pSipA were higher than those in ZA20. These results raise the possibility that immediately after cell contact, ZA21pSipA may secrete higher amounts of SipA into the host cell, thereby triggering cytosolic Ca^{2+} responses.

While only high levels of SipA (expressed from pSipA) may induce cytosolic Ca^{2+} responses at wild type levels, stimulation of IL-8 gene expression at 1 hr post-infection was observed when *sipA* was present either on the chromosome or on a

plasmid. Furthermore, a *S. typhimurium sipA* mutant, that mobilized intracellular Ca^{2+} at lower levels than wild type, activated IL-8 gene expression at wild type levels. As mentioned previously, the Ca^{2+} response occurs immediately after bacteria-cell contact (34, 53, 56, 79, 123, 124, 158), whereas IL-8 gene expression, in this study, was measured at 1 hr post infection. Therefore, even low concentrations of SipA or other *S. typhimurium* proteins (SopA, SopB, SopD and SopE2), once internalized, are able to interact with host cell signaling molecules and induce IL-8 gene expression. Activation of IL-8 gene expression independent of high Ca^{2+} increase requires further elucidation, however, it has been shown that TNF-α also induces IL-8 in a Ca^{2+} -independent manner (53). In addition, IL-8 gene expression can be regulated by the transcription factor activator protein 1 (AP-1) through activation of MAP kinases (Erk) (75).

Using strains that promoted different Ca^{2+} responses but invaded cells with similar efficiency, no correlation was found between invasion and intracellular Ca^{2+} mobilization, which was similar to results obtained in a previous study (53). Treatment of HeLa cells with 50 μM of BAPTA-AM did not affect invasion, which was consistent with results from a previous study demonstrating that 10 μM of BAPTA-AM had no effect on *S. typhimurium* or *L. monocytogenes* invasion in Hep2 cells (34). It should be mentioned that the use of BAPTA-AM to study bacteria internalization has yielded conflicting results, as others have found that 200 μM of BAPTA-AM reduces *S. typhimurium* invasion by 87% in HeLa cells (137), and also affects *C. jejuni* internalization in human intestinal cells (79).

Although Ca^{2+} chelators have been used to address the involvement of a Ca^{2+} response on bacteria internalization and cell signaling pathways, BAPTA-AM has been found to affect cell functions other than those related to Ca^{2+}. In different cell types, 10-50 μM of BAPTA-AM acts on actin and microtubule disassembly and affects mitochondrial function independent of its Ca^{2+} chelating activity (143). Additionally, BAPTA-AM has a dose-dependent-neurotoxic effect on primary neuronal cells, reduces global protein synthesis and induces an endoplasmic reticulum (ER)-specific stress response by influencing the ER Ca^{2+} homeostasis (127). Therefore, the use of BAPTA-AM to elucidate the role of Ca^{2+} on cell signaling pathways may lead to controversial results not only due to dose dependent effects, but also due to non-specific effects on metabolic pathways. In this study, we found that BAPTA-AM treatment abolished the ability of *S. typhimurium* to cause IL-8 production, however, similar results were found in *S. typhimurium*-infected HeLa cells treated with BAPTA-AM derivative, D-BAPTA AM, which has low calcium chelating capacity (143), indicating that the inhibition of IL-8 expression by BAPTA-AM is due to its side effects rather than its calcium chelating properties.

For these reasons, we based our conclusions on Ca^{2+} signaling responses on strains carrying different effector proteins while measuring IL-8 gene expression and invasion of HeLa cells. Our results support the idea that the *S. typhimurium*-induced increase in the cytosolic Ca^{2+} concentration is mediated by SipA in a dose dependent manner, however, changes in intracellular Ca^{2+} levels were not required for *S. typhimurium*-induced invasion and chemokine gene expression.

CHAPTER IV

ANALYSIS OF CHEMOKINE PROFILES OF *Salmonella typhimurium* INFECTED BOVINE PEYER'S PATCH CELLS BY LASER CAPTURE MICRODISSECTION

Introduction

Salmonella enterica subsp *enterica* serotype Typhimurium, herein *S. typhimurium* carries two type III secretion systems (TTSS) that are involved in its pathogenesis. The TTSS encoded on *Salmonella* Pathogenesis Island (SPI) –1 (TTSS-1) translocates effector proteins that contribute to bacterial invasion and enteropathogenesis (48, 60), whereas, the TTSS encoded in SPI-2 (TTSS-2) translocates effector proteins responsible for intracellular survival (8, 68).

In bovine ligated ileal loops, strains having mutations in genes encoding TTSS-1 translocated effector proteins SipA, SopA, SopB, SopD, and SopE2 induced significantly fewer pathological lesions and inflammatory responses than the isogenic *S. typhimurium* wild type strain (187). At 1 hr post-infection, the expression levels of IL-8, Gro-α, Gro-γ and GCP-2 in bovine ileal tissue infected with a strain carrying mutations in *sipAsopABDE2* were significant lower than the expression levels of *S. typhimurium* wild type infected tissue. However, at 8 hr post-infection, there was no significant difference in levels of chemokine expression induced by these two strains, except for the Gro-α gene (186), indicating that 1 hr post-infection

may be a crucial time point to characterize differences related to expression profiles of chemokines induced by *S. typhimurium* strains.

Another study found that *S. typhimurium* induces IL-8, Gro-α, and TNF-α expression as early as 1 hr post-infection, while IL-1 was induced at later time points (141). However, the gene expression analysis for this study was performed using fragments of Peyer's patches, which have several different populations of cells. Although *S. typhimurium* was localized in a variety of cell types in bovine ligated ileal loop, including goblet cells, enterocytes, and cells of the lamina propria (141), the contribution of specific cell populations to the changes in gene expression was not characterized. Further studies demonstrated that Gro-α transcripts are localized in enterocytes of absorptive villi and crypts in *S. typhimurium* infected bovine tissue as compared to uninfected control (186). The quantitative contributions of the various cell types for the *S. typhimurium* induced Gro-α gene expression are unknown.

Interestingly, infection of bovine ligated ileal loops with *S. typhimurium* strains with mutated *sipA* induced significantly less fluid secretion and less inflammation than the wild type strain, indicating that SipA is a major contributor to *S. typhimurium* pathogenesis. The cells of Peyer's patch targeted by SipA to induce inflammation *in vivo* are also unknown. Thus, our objective of this study was to measure the alterations in *S. typhimurium* induced chemokine gene expression in different cell types of Peyer's patches infected with either *S. typhimurium* wild type, *S. typhimurium* carrying mutations in *sopABDE2* (ZA20), *S. typhimurium* carrying mutations in *sipA* (ZA10), or *S. typhimurium* carrying mutations in *sipAsopABDE2* (ZA21).

Materials and Methods

Bacterial strains

Strains of bacteria used in this study are listed in Table 8. *S. typhimurium* strains were grown in Luria-Bertani (LB) medium overnight with appropriated antibiotics at the following concentrations: kanamycin (100 mg/liter); nalidixic acid (50 mg/liter), chloramphenicol, (30 mg/liter); tetracycline (20 mg/liter), then sub-cultured in LB for 4 hr.

Table 8: Bacteria strains used in bovine ligated ileal loop model.

Strain or plasmid	Genotype	Reference
S. typhimurium strains		
IR715	ATCC 14028 wild type, *nal*^{r}	(155)
ZA21	ATCC 14028 *nal*^{r} Δ*sipAsopAsopBDE2*	(187)
ZA20	ATCC 14028 *nal*^{r} Δ*sopAsopBDE2*	(187)
ZA10	ATCC 14028 *nal*^{r} Δ*sipA*	(187)

Bovine ligated ileal loop model

Bovine ligated ileal loop surgery was performed as described previously (141, 144, 187). Briefly, male beef calves, 4 weeks old, negative for *Salmonella* were fasted for 24 hr prior to the surgery. Anesthesia was induced with propofol followed by placement of an endotracheal tube and maintenance with isofluorane. A laparatomy was performed, and 8 cm loops were ligated in the distal part of the ileum, intercalated with 1 to 2 cm loops. The loops were injected with 3 ml of either PBS or PBS containing 0.75 x 10^9 cfu of *S. typhimurium* strains (Table 1). Loops were returned to abdominal cavity and loop excision was performed at 1 hr and 8 hr post infection and samples were collected for histopathology, bacteriology, and quantitative real time PCR (qRT-PCR).

Invasion assay

Two 6 mm biopsy punches from bovine Peyer's patches were collected at 1 and 8 hr post-infection, washed three times in 5 ml of PBS and homogenized in 900 μl of PBS. For enumeration of bacteria, ten-fold serial dilutions of tissue samples were plated in nalidixic acid (50 mg/liter) containing LB plates (187).

Immunohistochemistry

S. typhimurium was detected in tissue sections by using *Salmonella* O group B antiserum (BD biosciences, CA) diluted 1:150 with Da Vinci Green Antibody Diluent (Biocare Medical, Concord, CA). The detection was performed using the MACH 2 polymer detection kit (Biocare Medical, Concord, CA). DAB (3,3'-diaminobenzidine) (DakoCytomation, Carpinteria,CA) was used as the chromogen to detect the target antigen. For each pair of slides, the primary antibody was substituted with a universal rabbit control (DakoCytomation, Carpinteria, CA).

Laser capture microdissection

Bovine intestinal specimens were collected at surgery and placed in a cryomold containing Optimal Cutting Temperature (O.C.T.) compound on dry ice and stored at –80°C. Slides of frozen sections were prepared under RNAse free conditions in a cold cryostat and sectioned at 8 μm thickness. Tissue sections were dehydrated and stained using HistoGene LCM frozen section staining kit (Molecular Devices Corporation, Sunnyvale, CA). Enterocytes of crypts and tips of absorptive villi were visualized using staining solution provided in the HistoGene LCM frozen section staining kit (Molecular Devices Corporation, Sunnyvale, CA) and captured into CapSure Macro LCM caps (Molecular Devices Corporation, Sunnyvale, CA) using PixCell II instruments (Molecular Devices Corporation, Sunnyvale, CA) (12, 36, 37, 39).

RNA isolation and amplification

RNA was isolated from captured cells using PicoPure RNA Isolation Kit (Molecular Devices Corporation, Sunnyvale, CA) (112). The quality of RNA from both scraped section and captured cells was visualized by Agilent Lab-on-a-Chip System with the bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). After analysis of RNA quality, 2.5 ηg of RNA was submitted to two rounds of amplification using the RiboAmp HS RNA Amplification Kit (Molecular Devices Corporation, Sunnyvale, CA) (182).

Gene expression

Expression levels of IL-8 and GRO-α from uninfected and *Salmonella*-infected samples were measured by qRT-PCR. Briefly, 2 μg of amplified RNA was reverse transcribed at 48°C for 30 min with random hexamer primers using Multiscribe Reverse Transcriptase (Applied Biosystem, Foster City, CA). The qRT-PCR was performed in a Smart Cycler II (Cepheid, Sunnyvale, CA) by using 40 ηg of cDNA mixed with SmartMix beads (Cepheid, Sunnyvale, CA), SYBR Green-I 0.2 x (Invitrogen Corporation, Carlsbad, CA) and 500 nM of each primer set (Table 9). The cDNA concentration of each sample was normalized to an internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The normalized levels of

gene expression in infected cells were calculated relative to uninfected cells as previously described (186).

Table 9- Primers used in qRT-PCR to study the gene expression of *S. typhimurium* infected bovine Peyer's patches.

Target	**Primer sequence**[a]	**Amplicon size (bp)**
IL-8	AATCTTGAGACTGTCTTTCC	139
	TAAAGTTTATGTTCGAATACACAA	
Gro-α	CACTGTTAATGTAGGGAATGTAT	147
	TGTCCAAGGGATATTTAGATCATTG	
GAPDH	TTTCCTGGTACGACAATGAATTT	162
	CAGTGTGGCGGAGATGGGGCACG	

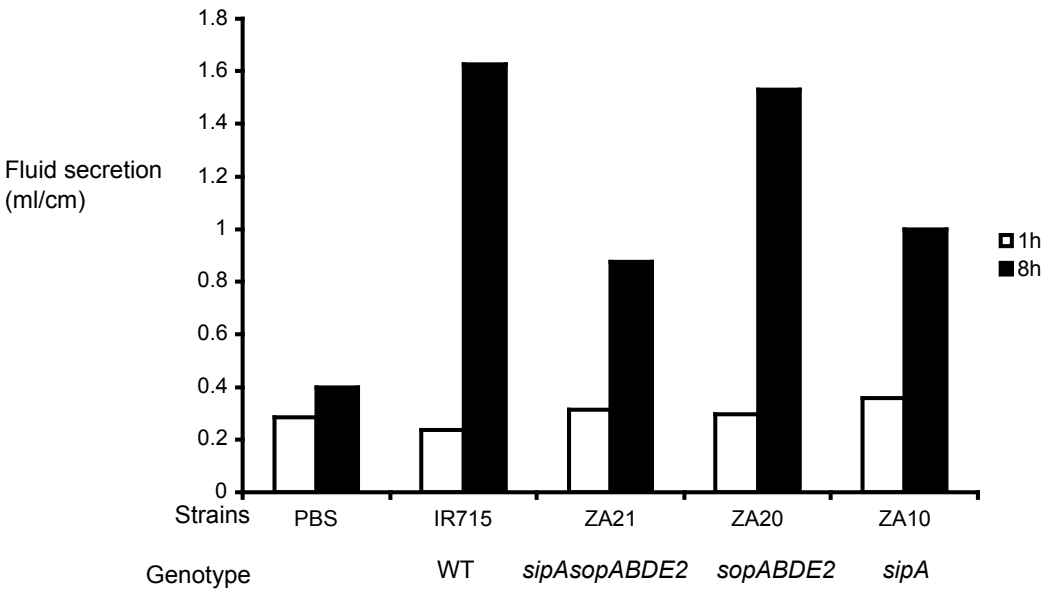
[a] Sequences for bovine genes were obtained from GenBank. Top row: forward primer; bottom row: reverse primer.

Results

S. typhimurium *SipA interferes with fluid secretion and invasion in bovine ligated ileal loop*

At 1 hr post-infection, *S. typhimurium* wild type did not induce fluid secretion as compared to control. However, at 8 hr post-infection, *S. typhimurium* wild type induced twice as much fluid as ZA21 (*sipAsopABDE2* mutant) in bovine ligated ileal loop (Figure 12A). Moreover, the *sipA* (ZA10) mutant induced about the same amount of fluid as ZA21, while ZA20 (*sopABDE2* mutant) induced fluid secretion at wild type level (Figure 12A). These findings indicate that SipA contributes to fluid secretion in the bovine ligated ileal loop assay. Mutation of *sipA* also interfered with bacteria internalization at 1 hr post-infection, however no effect was detected at later time points (Figure 12B), suggesting that internalization was delayed in the *sipA* mutant.

A



B

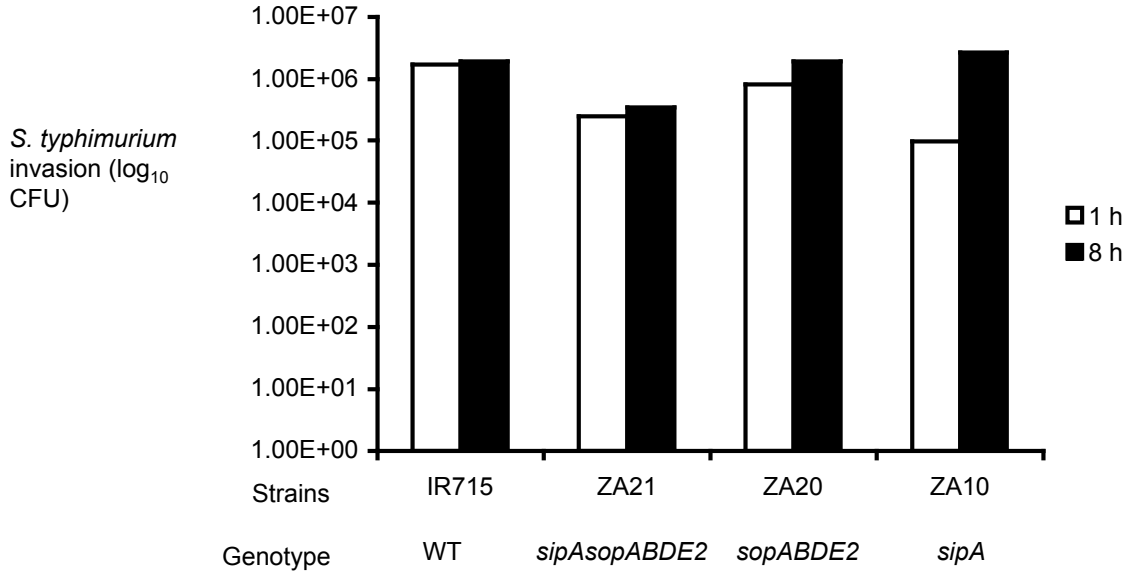


Figure 12: *S. typhimurium*-induced fluid secretion (A) and invasion (B) in bovine ligated ileal loops at 1 and 8 hr post-infection.

S. typhimurium sipA, sopABDE2 *and* sipAsopABDE2 *mutants have the same distribution in Peyer's patch as* S. typhimurium *wild type*

Analysis of the distribution of *S. typhimurium* wild type, ZA21 (*sipAsopABDE2* mutant), ZA20 (*sopABDE2* mutant) and ZA10 (*sipA* mutant) in Peyer's patch by immunohistochemistry in bovine ligated ileal loop revealed the most positive staining concentrated along the surface or within enterocytes of domed villi overlying Peyer's patches and within desquamated enterocytes or/and inflammatory cells in the lumen immediately on the surface of domed villi. Occasional positive bacteria were visualized within enterocytes from basolateral absorptive villi. Very few positive bacteria were present in the tips of villi and in scattered enterocytes outside Peyer's patches. No bacteria were found in enterocytes of crypts (Table 10 and Figure 13).

Table 10: Distribution of *S. typhimurium* strains in the Peyer's patch of bovine ligated ileal loops.

Samples	Positive staining			
	Domed villi	**Absorptive villi**	**Crypts**	**Lumen**
Control	–	–	–	–
WT	++	+	–	+
ZA21 (*sipAsopABDE2* mutant)	++	+	–	+
ZA20 (*sopABDE2* mutant)	++	+	–	+
ZA10 (*sipA* mutant)	++	+	–	+

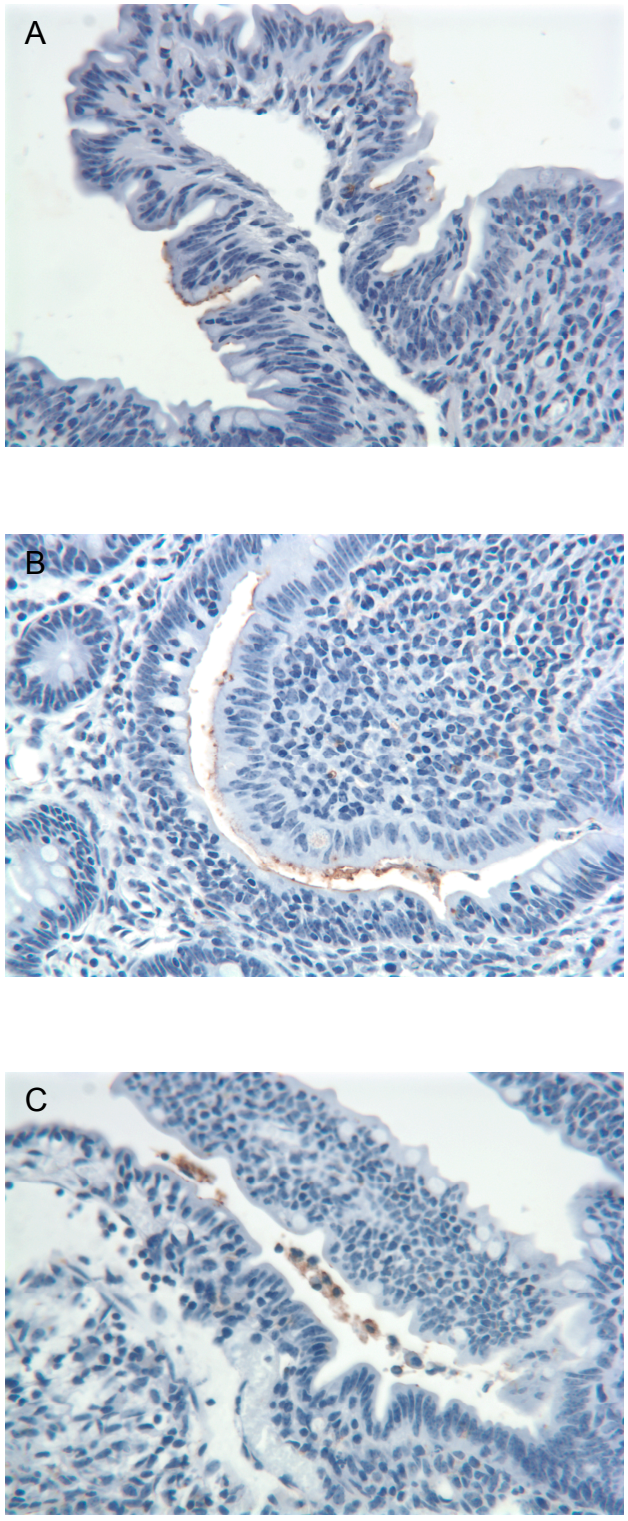


Figure 13: Immunohistochemistry of bovine ligated ileal loops infected with *S. typhimurium*. *S. typhimurium* localized within enterocytes of basolateral absorptive villi (A), along the surface or within enterocytes of domed villi (B) and within desquamated enterocytes or/and inflammatory cells in the lumen (C). Magnification: 800X.

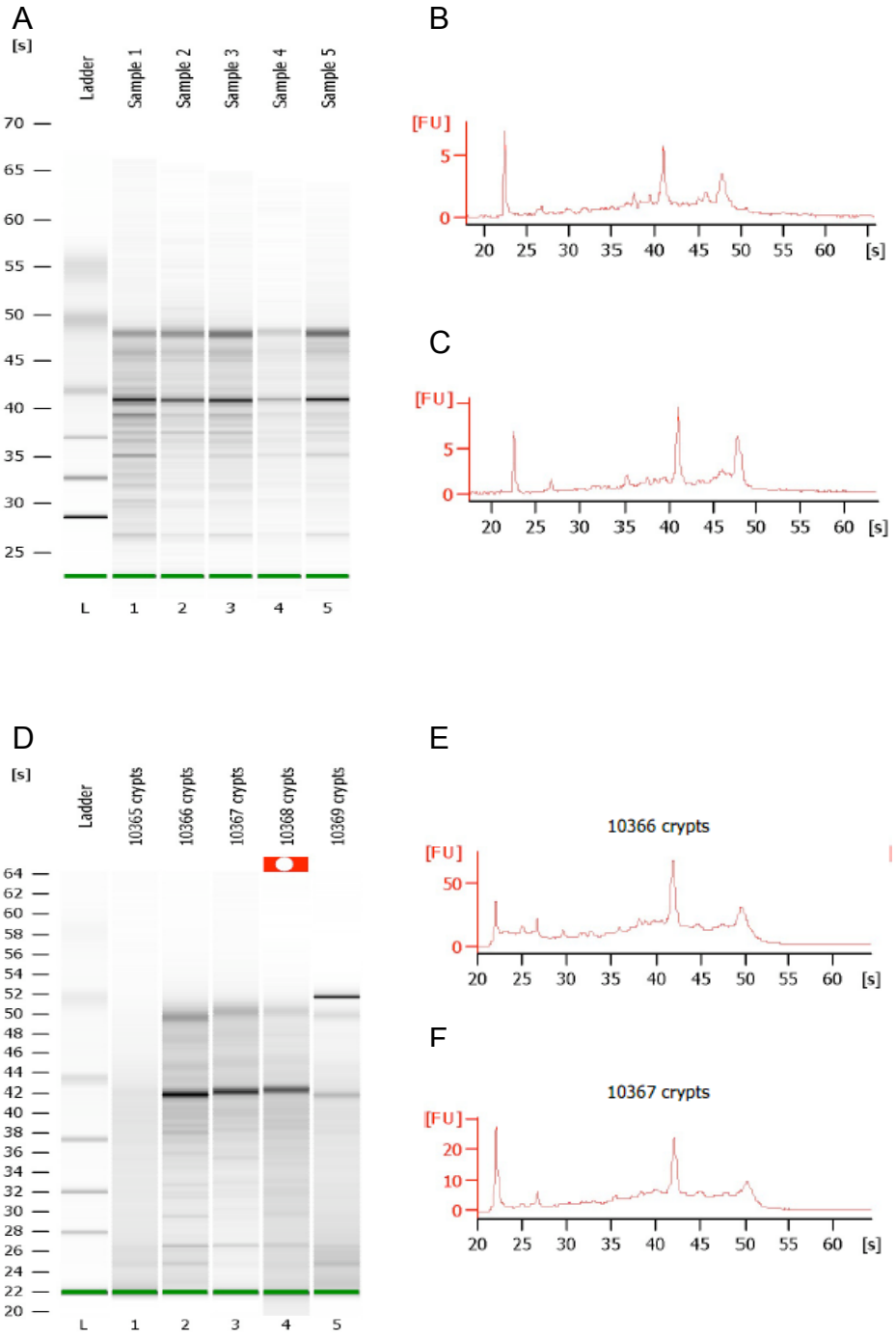


Figure 14: Bioanalyzer images of RNA from scraped sections (A-C) and from captured cells (D-F).

LCM technique and RNA quality

The bioanalyzer analysis of the RNA extracted from scraped sections demonstrated that the sections were adequate to proceed with LCM of targeted cells (Figure 14 A-C). Approximately 200 to 1000 enterocytes of crypts and 100 to 1000 enterocytes of tip villi were captured by LCM (Figure 15 A-C), which originated about 7.5 ηg to 140 ηg of total RNA (Figure 14 D-F). Amplification of 2.5 ηg of both RNA extracted from scraped sections and RNA from captured cells produced from 7 to 60 μg of mRNA.

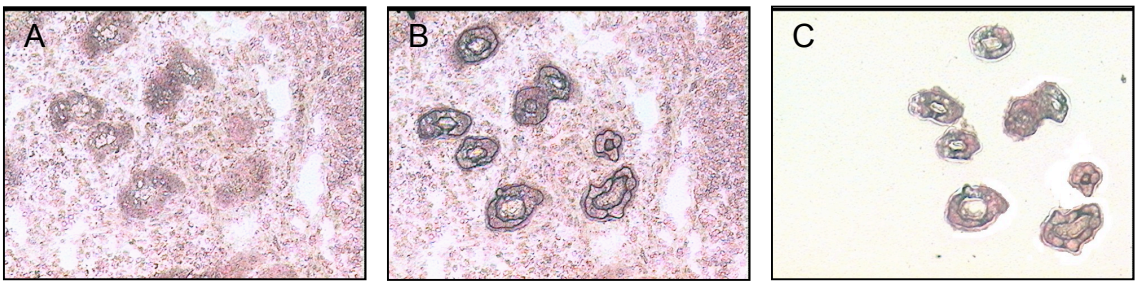


Figure 15: Examples of crypt cells captured by LCM from Peyer's patch. Localization of crypt cells (A), captured crypt cells in a tissue section (B) and captured crypt cells visualized in CapSure Macro LCM caps (C).

Real time PCR

Gene expression analysis of amplified RNA from total scraped bovine Peyer's patch samples documented that *S. typhimurium* wild type induced higher levels of IL-8 and Gro-α than *S. typhimurium sipAsopaBDE2* mutant (Figure 4A and B). Mutation of *sipA* (ZA10) failed to reduce expression of IL-8 and Gro-α, whereas a strain carrying chromosomal copy of *sipA* (ZA20) induced higher levels of IL-8 and Gro-α than ZA21 (*sipAsopABDE2* mutant) (Figure 16A and B). In wild type and ZA21 (*sipAsopABDE2* mutant) infected loops, enterocytes of crypts expressed higher levels of IL-8 than absorptive villi, however in crypts, ZA21 (*sipAsopABDE2* mutant) induced the same level of IL-8 as wild type *S. typhimurium* (Figure 16A*). S. typhimurium* wild type induced Gro-α expression in both enterocytes of crypts and absorptive villi (3.4 and 5 fold increase, respectively), however ZA20 and ZA10 strains induced higher expression of Gro-α in enterocytes of absorptive villi (Figure 16B).

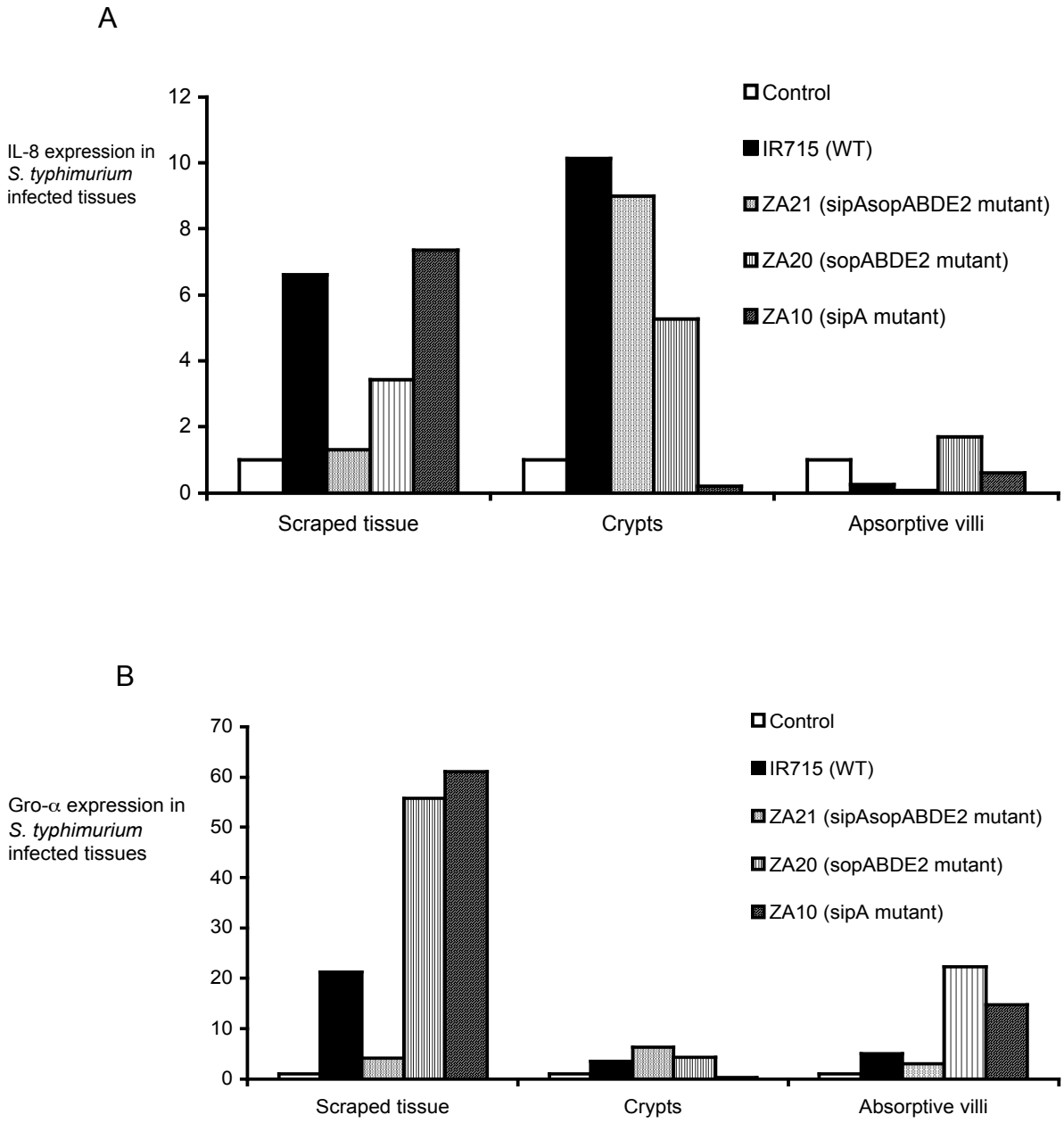


Figure 16: qRT-PCR of *S. typhimurium* infected tissues. IL-8 (A) and Gro-α (B) expression in *S. typhimurium* scraped tissue, enterocytes of crypts and enterocytes of absorptive villi compared to uninfected control samples.

Discussion

S. typhimurium invade a variety of cell types and induce expression of chemokines, resulting in infiltration of neutrophils to the lamina propria, fluid accumulation, and diarrhea (139, 141, 175, 187). In the bovine ligated ileal loop model, which resembles the *S. typhimurium* induced enteritis in humans, the inflammatory response has been measured in heterogeneous *S. typhimurium* infected tissue and compared to uninfected control, since it is largely unknown which cell populations contribute to the expression of inflammatory cytokines.

In this study, *S. typhimurium* wild type and *S. typhimurium* mutants (*sipAsopABDE2* mutant, *sipA* mutant and *sopABDE2* mutant) were found to have the same tissue distribution in bovine Peyer's patches. Similar results were found previously when tissue distribution of *S. typhimurium* wild type was compared to *S. typhimurium sopB* mutant (133). Although, in this study, *S. typhimurium* mutants colonized the same cell population (enterocytes of absorptive villi and domed villi), they induced different host responses. *S. typhimurium sipAsopABDE2* mutant induced less fluid secretion than either wild type, *sipA* mutant or *sopABDE2* mutant. Moreover, analysis of gene expression by qRT-PCR from scraped tissues revealed that the *S. typhimurium sipAsopABDE2* mutant induced less expression of Gro-α and IL-8 than *S. typhimurium* wild type at 1 hr post-infection, as earlier described (186).

To better understand the contribution of enterocytes from crypts and absorptive villi to the chemokines expression, we harvested these specific cell

populations by laser capture microdissection and performed qRT-PCR of amplified RNA. Our data indicated that enterocytes of crypt are a major contributor for IL-8 expression. Epithelial cells of crypts had higher levels of IL-8 expression than scraped tissue, probably because of the lower number of other cell types being present in specific captured cells when compared to heterogeneous scraped tissue. No IL-8 expression was found in cells harvested from the absorptive villi of *S. typhimurium* wild type infected bovine Peyer's patches as compared to controls. On the other hand, enterocytes of both crypts and absorptive villi of *S. typhimurium* wild type infected bovine Peyer's patches induced Gro-α, which is in agreement with transcript localization detected previously by *in situ* hybridization (186); however none of these cells reached the same expression level of scraped tissue. These data may indicate that other cell populations could be responsible for the significant expression of Gro-α, an example is enterocytes of domed villi, which were not included in this study.

Although the *S. typhimurium sipAsopABDE2* mutant induced lower expression levels of IL-8 and Gro-α in scraped tissue than *S. typhimurium* wild type, this profile of expression was not detected in enterocytes of crypts and absorptive villi. We propose that the *S. typhimurium sipAsopABDE2* mutant activates alternative pathways such as the Toll-like receptor-5 pathway in bovine Peyer's patches that would result in activation of chemokine expression in crypts and absorptive villi, however, the absence of chemokine expression in the same cell population in *sipA* mutant-infected samples will need to be further elucidated. Strains expressing chromosomal copy of *sipA* (ZA20), on the other hand, induced

chemokine expression in all cell populations analyzed, whereas enterocytes of crypts were the major target for SipA induced IL-8 expression, and enterocytes of villi were major contributors for SipA-induced Gro-α expression.

In summary, the LCM technique allowed isolation of high quality RNA of specific cell population from heterogeneous tissues, and the qRT-PCR greatly facilitated cell-specific gene expression quantification. Our data indicate that enterocytes of crypts are the major contributors for IL-8 expression; and enterocytes of both crypts and absorptive villi contribute to Gro-α expression, however, other cells are likely involved in the induction of Gro-α expression detected during *S. typhimurium* infection. Further experiments are required to identify molecules that are targeted by either the *S. typhimurium sipAsopABDE2* mutant or the *S. typhimurium sopABDE2* mutant inducing chemokine expression in specific sub-populations of enterocytes or other cell types in the ileum.

CHAPTER V

SUMMARY AND CONCLUSIONS

Cell culture studies of *S. typhimurium* pathogenesis demonstrate that flagellin rather than effector proteins plays important role in inducing nuclear response and activation of pro-inflammatory genes. On the other hand, *in vivo* studies provide evidence that effector proteins are required for inducing inflammation at wild type induced levels, although the mechanism by which effector proteins activate inflammation is not completely elucidated. In Chapter II, we described that HeLa cells are non-responsive to *S. typhimurium* flagellin as compared to T84 cells due to relative down regulation of TLR-5. While T84 cells are an adequate model to study cellular events induced by *S. typhimurium* flagellin through TLR-5 pathway, HeLa cells are a more appropriate model to study pathways induced by *S. typhimurium* PAMPS other than flagella.

Using HeLa cells and a series of *S. typhimurium* mutants, we found that SipA alone or cooperatively SopA, SopB, SopD and SopE2 induce IL-8 and Gro-α expression. Extracellular addition of recombinant SipA failed to induce IL-8 expression, indicating that translocation of SipA through TTSS-1 is required for inducing chemokine expression. To determine pathways activated by SipA in HeLa cells that could drive the IL-8 expression, 37 phosphorylation sites in phosphoproteins were screened in HeLa cells infected with either *S. typhimurium* wild type, *S. typhimurium sipAsopABDE2* mutant or *S. typhimurium* expressing a

chromosomal copy of *sipA* (*sopABDE2* mutant). SipA was found to significantly restore phosphorylation of three phosphoproteins, CREB, p38MAPK and JUN, which are functionally linked to IL-8 pathway.

To identify the role of cytosolic Ca^{2+} in gene expression as well as the functions of effector proteins promoting Ca^{2+} changes, in Chapter III, we performed a series of experiments demonstrating that *S. typhimurium* mobilizes Ca^{2+} at 30 sec after infection, which was dependent on TTSS-1 translocation effector proteins. Infection of HeLa cells with different *S. typhimurium* strains demonstrated that SipA induces Ca^{2+} changes in a dose dependent manner. Although *S. typhimurium* mobilizes Ca^{2+} at the early stage of infection, we found no correlation between Ca^{2+} changes and activation of chemokine expression or bacteria internalization. Along the same line, bacterial internalization or bacteria-induced cytoskeletal changes were not found to be the initiation factors to induce chemokine expression in HeLa cells, since *S. typhimurium* expressing *sopE2* invaded epithelial cells with no activation of gene expression. Instead, translocation of SipA and/or translocation of SopA, SopB, SopD, and SopE2 are required to initiate the activation of pro-inflammatory genes.

To analyze whether specific cells are responsible for the high levels of chemokine expression detected during *S. typhimurium* infection *in vivo*, we infected bovine ligated ileal loops with either *S. typhimurium* wild type or *S. typhimurium sipA* mutant, or *S. typhimurium sopABDE2* mutant or *S. typhimurium sipAsopABDE2* mutant and performed laser capture microdissection of enterocytes of crypts and absorptive villi. We found that enterocytes of crypts to be a major contributor of IL-8

expression, whereas enterocytes of both crypts and absorptive villi contributed to Gro-α expression in *S. typhimurium* wild type infected Peyer's patches. However in *S. typhimurium sopABDE2* mutant infected tissue, enterocytes of both crypts and absorptive villi contributed to the expression of IL-8 and Gro-α.

In conclusion, the data presented here indicate that *S. typhimurium* is capable of activating chemokines through effector proteins, SipA, SopA, SopB, SopD and SopE2, independent of both TLR-5 pathways and Ca^{2+} mobilization. Translocation of SipA alone is adequate to induce bacteria internalization, gene expression, and phosphorylation of CREB, p38MAPK, and JUN proteins. In bovine Peyer's patches, SipA induces chemokine expression in enterocytes of crypts and absorptive villi, however the pathways involved in this process will require further investigation. These data facilitate understanding the molecular basis of host:pathogen-interactions established by *S. typhimurium* and provide potential targets for therapeutic approaches by identifying bacterial proteins required to initiate the inflammatory response as well as host proteins and host cells targeted by *S. typhimurium* at early infection.

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