Penetration and Activation of Brain Endothelium by *Salmonella enterica* Serovar Typhimurium

Nina M. van Sorge,[1,a] Patricia A. Zialcita,[1,a] Sara H. Browne,[2] Darin Quach,[3,b] Donald G. Guiney,[2] and Kelly S. Doran[1,3]

[1]Department of Pediatrics; and [2]Medicine School of Medicine, University of California, San Diego, La Jolla; and [3]Department of Biology and Center for Microbial Sciences San Diego State University, San Diego, California

***Salmonella* meningitis is a serious disease of the central nervous system, common particularly in Africa. Here, we show that *Salmonella enterica* serovar Typhimurium is able to adhere, invade, and penetrate human brain microvascular endothelial cells (hBMECs), the single-cell layer constituting the blood–brain barrier (BBB). Cellular invasion was dependent on host actin cytoskeleton rearrangements, while expression of a functional type III secretion system was not essential. In addition, *Salmonella* infection activated a proinflammatory immune response targeting neutrophil signaling and recruitment. *Salmonella* invasion and immune activation may represent a crucial step in the penetration of the BBB and development of *Salmonella* meningitis.**

Salmonella is a Gram-negative, facultative intracellular pathogen capable of producing a diverse array of human diseases ranging from mild food poisoning to life-threatening systemic infections, including bacteremia, meningitis, septic arthritis, and osteomyelitis. These infections are associated with a significant global burden in both developed and developing countries. In addition, there is an alarming increase in multidrug-resistant *Salmonella* isolates (up to 90% in some parts of Africa) [1, 2]. Nontyphoidal *Salmonella* spp are a leading cause of meningitis in Latin America and Africa, which is associated with a very high (up to 60%) mortality [1, 3]. Various serovars of *S enterica* have been reported to cause meningitis, including Paratyphi, Enteritidis, and Typhimurium [3]. Brain infection is most common in neonates and infants, although cases of *Salmonella* meningitis have been reported in adults with underlying immunodeficiencies [4]. Complications of *Salmonella* central nervous system (CNS) infection include bacterial colonization, brain abscesses, edema, cerebral infarction, pus collection in cerebral cavities, and brain inflammation [5].

Bacterial meningitis occurs when blood-borne pathogens interact with cerebral endothelial cells and cross the blood–brain barrier (BBB); subsequent bacterial replication within the CNS may provoke an overwhelming host inflammatory response. The BBB, responsible for maintaining biochemical homeostasis within the CNS, consists principally of a single layer of specialized brain microvascular endothelial cells (BMECs). Penetration of the BBB by a bacterial pathogen reflects a complex interplay between the host endothelium and microbial surface components. A recent study demonstrated that oral infection of mice with *S enterica* serovar Typhimurium resulted in meningitis and brain infection [6]; however, the mechanism(s) whereby the bacterium leaves the bloodstream and gains access to the CNS has not been examined.

We hypothesized that direct interaction with human BMECs (hBMECs) is a primary and essential step in the pathogenesis of *Salmonella* meningitis, whereupon a combination of bacterial transcytosis and inflammatory mechanisms combine to disrupt BBB integrity. Using our in-vitro BBB model, we demonstrate that *S* Typhimurium efficiently adheres to, invades, and persists within hBMECs. We also assess the acute response of the BBB to *Salmonella* infection using microarray, real-time reverse-transcription polymerase chain reaction (RT-PCR), and protein analysis. We identify that components of the *Salmonella* SPI-1 invasion locus are not required for induction of proinflammatory chemokines IL-8, CXCL1, and CXCL2.

METHODS

Bacterial Strains and Mutants

The wild-type (WT) bacterium used in this study was *S. enterica* serovar Typhimurium 14028S 1/9, and the isogenic mutant strains lacking the *invA* and *sipB* genes were invA::aphT clone1 [7] and sipB::aphT clone 1 [8], respectively. *Salmonella* strains were grown on Luria Broth (LB), supplemented with 50 μg/mL kanamycin for mutant strains at 37°C. For infection assays,

Received 30 April 2010; accepted 25 October 2010.

[a]These authors contributed equally to this work. [b]Present affiliation: Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan.

Potential conflicts of interest: none reported.

Reprints or correspondence: Dr Kelly S. Doran, Assistant Professor of Biology, San Diego State University, 5500 Campanile Dr, Mail Code 4614, San Diego, CA 92182 (kdoran@sciences.sdsu.edu).

The Journal of Infectious Diseases 2011;203:401–405

© The Author 2010. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com

1537-6613/2011/2033-0001$15.00
DOI: 10.1093/infdis/jiq048

strains were grown without aeration in LB with 0.3 M sodium chloride at room temperature until they reached an OD_{600} of .4 ($\sim 2 \times 10^8$ CFU/mL).

Cell Culture and Infection Assays

A well-characterized hBMEC line, immortalized by transfection by SV40 large T antigen, was generously provided by Kwang Sik Kim (Johns Hopkins University). Cell maintenance and assays for hBMEC adherence, invasion, intracellular survival, and bacterial transcytosis were performed as described previously [9–11]. In brief, hBMECs were grown to confluency and infected with *Salmonella* at indicated multiplicity of infection (MOI). To quantify intracellular bacteria, extracellular bacteria were killed by the addition of 100 μg/mL gentamicin. Following 2 hours, or at indicated time points, intracellular bacteria were liberated by the addition of 0.1 mL 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) solution followed by 0.4 mL of 0.025% Triton X-100 and quantified by plating serial dilutions on LB agar plates. Total cell-associated bacteria were quantified prior to addition of antibiotics. For inhibition studies, cytochalasin D (Sigma) was added to hBMEC monolayers at the indicated amount 1 h prior to the addition of bacteria, and remained during the experiment. Transmission electron microscopy was performed as described previously [11] following *S* Typhimurium infection for 1 h.

RNA Isolation, cDNA Preparation, Microarray Analysis, and qPCR

Monolayers of hBMEC were infected with *S* Typhimurium for 6 h. RNA extraction, complementary DNA (cDNA) transcription and microarray analysis (Sentrix Human-8 Expression BeadChips, Illumina) were performed as described previously [11]. Quantitative PCR (qPCR) conditions and primers for IL-6, IL-8, CXCL1, CXCL2, CCL20, and adrenomedullin (ADM) are described in [11], the following primer set was used to analyze ADM mRNA levels: 5′- CGTCGGAGTTTCGAAAGAAG -3′ and 5′- CCCTGGAGGTTGTTCATGCT -3′. Relative gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase transcription using the $\Delta\Delta$ C_T method.

Chemokine Secretion in hBMEC Supernatants

Supernatants were collected after hBMEC infection with *S* Typhimurium, Δ*invA*, and Δ*sipB* mutants after 6 h. Concentrations of IL-8, CXCL1 (R&D systems), and CXCL2 (BioSupply UK) were measured using enzyme-linked immunosorbent assays (ELISA) according to manufacturer's instructions.

Statistical Analysis

GraphPad Prism version 4.03 was used for statistical analyses. Differences in bacterial colony forming units (CFU) were analyzed using the Wilcoxon signed rank test. Differences in percentage of adherence/invasion were analyzed using the Friedman test with Dunn multiple comparison correction. Differences in or cytokine production were tested using one-way ANOVA with Tukey multiple comparison correction. Significance was accepted at $P < .05$.

RESULTS

Invasion of hBMEC by *Salmonella*

To test the hypothesis that *Salmonella* directly invades brain endothelium, we optimized our previously established quantitative hBMEC adherence and invasion assays [9] for *S* Typhimurium. Confluent monolayers of hBMEC were infected with increasing concentrations (multiplicities of infection, MOI) of *S* Typhimurium (MOI 1 represents $\sim 1 \times 10^5$ CFU). The percentage of adherent and intracellular bacteria ranged from 4.5%–6% for adherence and from 2%–3% for invasion compared with the original inoculum (Fig. 1A). At the standard inoculum used for subsequent comparative assays (MOI = 1), approximately 35% of total hBMEC-associated *Salmonella* had invaded the intracellular compartment within the 2 h incubation period, suggesting highly efficient endocytic uptake of surface-adherent organisms. *Salmonella* invasion required rearrangement of actin cytoskeleton, as we observed a dose-dependent decrease in *Salmonella* invasion in hBMECs in the presence of cytochalasin D, a potent actin microfilament aggregation inhibitor (Fig. 1B).

To confirm that *Salmonella* was indeed present inside hBMECs, we performed electron microscopy. Already after 1 h, *Salmonella* was present inside hBMECs in membrane-bound intracellular vacuoles (Fig. 1C). Similar vacuoles have not been observed in noninfected monolayers [11]. This observation prompted us to test whether *Salmonella* may persist or replicate intracellularly after invasion of hBMECs. Statistically similar amounts of intracellular organisms were recovered 2, 4, 8, and 24 h post addition of antibiotics, indicating that *S* Typhimurium persists within hBMECs following invasion (Fig. 1D). Finally, using a transwell assay [11], we observed that *Salmonella* was able to transcytose across a confluent hBMEC monolayer from the luminal to basolateral side (Fig. 1E).

Requirement of *Salmonella* SPI-1 Genes *invA* and *sipB* for hBMEC Invasion

The ability of *Salmonella* to invade epithelial cells requires a cluster of genes termed the *Salmonella* pathogenicity island 1 (SPI-1) locus. This locus encodes a type III secretion system (TTSS) that delivers bacterial effector proteins into the host cell cytoplasm, inducing actin cytoskeletal rearrangements that facilitate uptake of the organism. We hypothesized that the SPI-1 components *invA*, a needle complex export protein, and *sipB*, a translocation machinery component, would contribute to *Salmonella* hBMEC invasion. Using quantitative hBMEC invasion assays, *invA* and *sipB* deficient strains exhibited a 4-fold decrease in hBMEC invasion compared with the WT strain

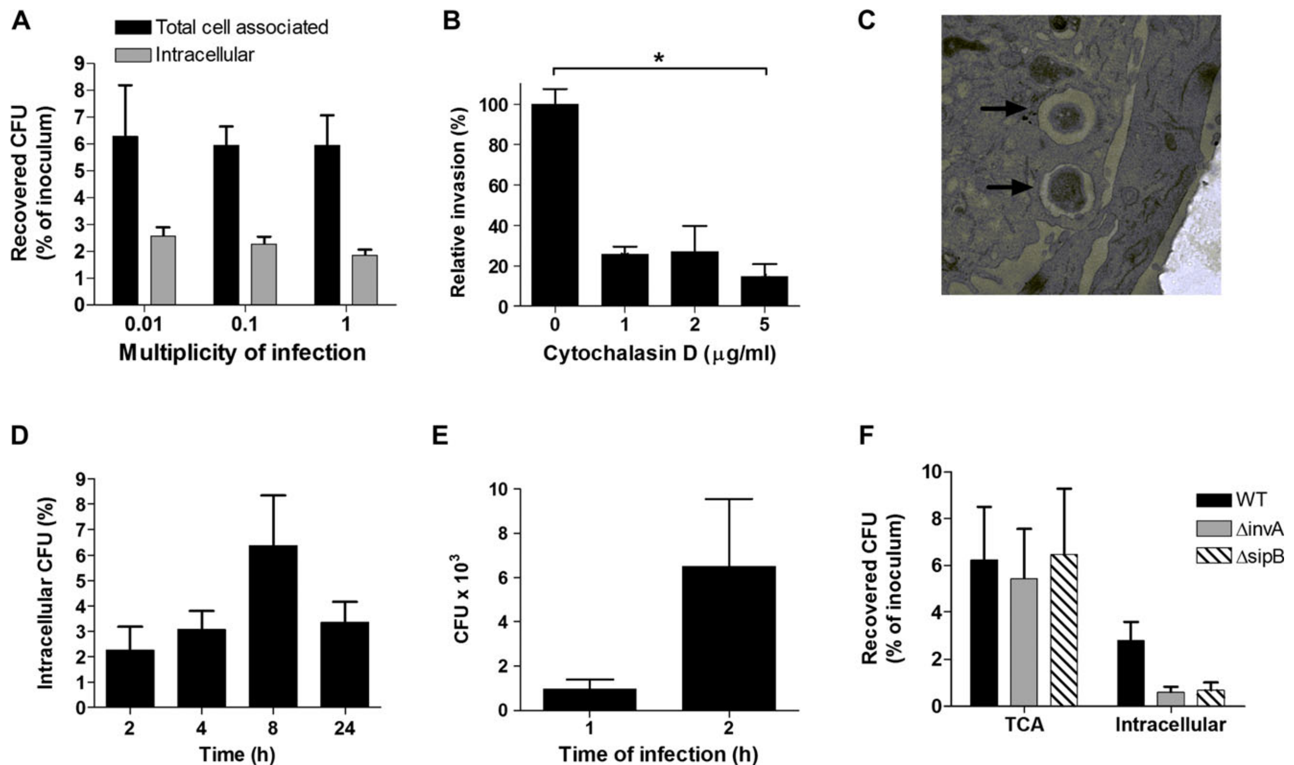

Figure 1. *S* Typhimurium interaction with brain endothelium. (a) *S* Typhimurium adheres to (total cell-associated, TCA) and invades hBMECs. The number of recovered bacteria is expressed as a percentage of the initial inoculum. MOI of 0.01, 0.1, and 1 represents approximately 10^3, 10^4, and 10^5 CFU, respectively. (b) Dose-dependent inhibition of *S* Typhimurium invasion of hBMECs by cytochalasin D. * $P < .05$. (c) Transmission electron microscopy of intracellular *S* Typhimurium 1 h after hBMEC infection (MOI = 1). Bacteria present in intracellular compartments are indicated by arrowheads. (d) *S* Typhimurium survival and persistence inside hBMECs over time (MOI = 1). (e) Transcytosis of *S* Typhimurium across confluent hBMEC monolayers seeded on transwells after 1 and 2 h (MOI = .1). (f) Invasion and total cell-associated (TCA; surface adherent plus intercellular bacteria) percentage of WT and SPI-1 mutants Δ*invA* and Δ*sipB* (MOI = 1). Pooled data from three independent experiments are shown for Fig 1A, B, D, and E, where bars represent mean and error bars represent standard error of mean (SEM). Pooled data from two independent experiments are shown in Fig. 1F; bars represent mean and error bars represent standard deviation.

(Fig. 1F), while affinity for the hBMEC surface was similar (Fig. 1F). However, this difference was not statistically significant by nonparametric statistical analysis, suggesting that these components are not essential to the invasion process.

Response of Brain Endothelium to *Salmonella* Infection

As the response of the BBB to bacterial infection may impact bacterial CNS penetration and the progression of *Salmonella* meningitis, we further examined the initial transcriptional responses of hBMECs to WT *Salmonella* infection using microarray analysis. By 6 h post infection, ~40 hBMEC genes exhibited a greater than 2-fold change in transcript abundance (data not shown), including those involved in stress response, signal transduction, and angiogenesis pathways, as well those regulating innate immunity. Expression of some of the most highly induced cytokines and peptides, including IL-8, CXCL-1 and CXCL-2, IL-6, CCL20, and ADM were confirmed in independent experiments by quantitative RT-PCR (Fig. 2A). Overall, the relative abundance of the different transcripts correlated with the fold increases observed by microarray analysis (data not shown). We also analyzed hBMEC supernatants for the presence of IL-8, CXCL-1, and CXCL-2 proteins following infection with the WT strain or Δ*invA* and Δ*sipB* mutants. Chemokine secretion increased upon infection with WT *S* Typhimurium, compared with the uninfected control (Fig. 2B–D). Protein expression did not require SPI-1 effectors, as infection with the isogenic Δ*invA* and Δ*sipB* mutants induced chemokine activation and subsequent protein secretion similar to *Salmonella* WT (Fig. 2B–D).

DISCUSSION

Salmonella is a common cause of meningitis in neonates and infants in developing countries such as Malawi, Brazil, Thailand, and Taiwan. Moreover, when it occurs, *Salmonella* meningitis is associated with high mortality and complications, including significant neurological sequelae and a high relapse rate in surviving patients [1]. The presence of *Salmonella* in the cerebrospinal fluid clearly indicates bloodstream dissemination to the CNS; however, the basic pathogenic mechanisms by which

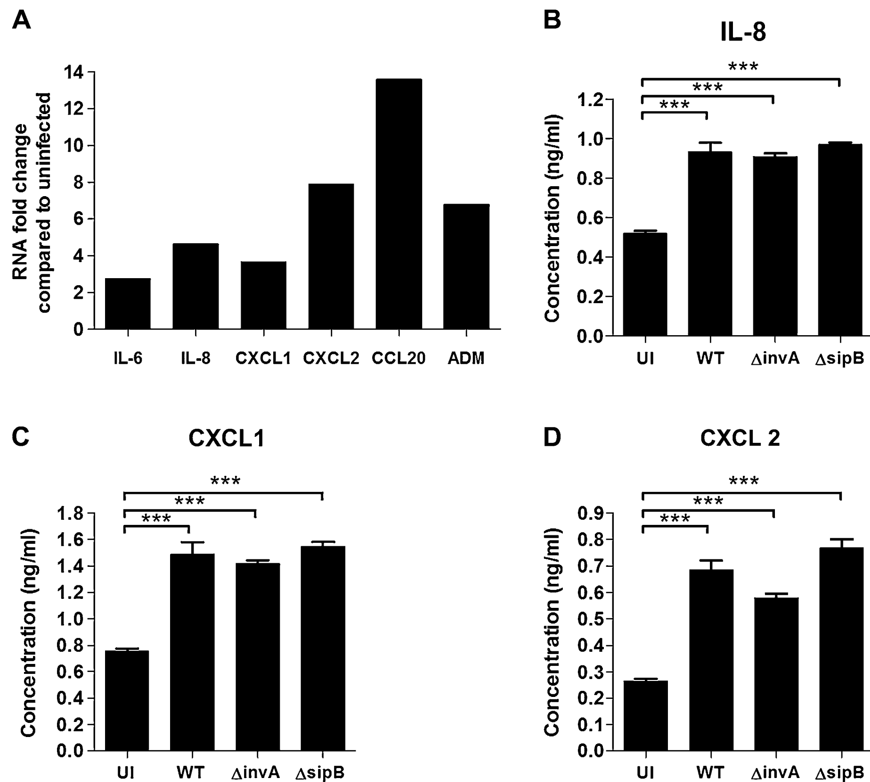

Figure 2. Transcriptional response and protein secretion by hBMECs during *S* Typhimurium infection. (a) mRNA expression levels for IL-8, CXCL1, CXCL2, CCL20, IL-6, and adrenomedullin (ADM) in hBMECs 6 h post infection with *S* Typhimurium analyzed by quantitative reverse-transcription polymerase chain reaction (RT-PCR). Protein secretion of (b) IL-8, (c) CXCL1, and (d) CXCL2 in hBMEC supernatants 6 h after infection with *S* Typhimurium wild-type (WT) or the isogenic Δ*invA* and Δ*sipB* mutants. This experiment was repeated 2 times in triplicate; data from a representative experiment are shown. Bars represent mean and error bars represent standard deviations of 3 wells. UI, uninfected hBMEC control. *** $P < .001$.

Salmonella species are able to gain access to the CNS and brain have not been studied. We have demonstrated for the first time the ability of *Salmonella* to invade the single-cell layer of specialized brain microvascular endothelial cells comprising the BBB. *Salmonella* penetration of brain endothelium requires rearrangement of the actin cytoskeleton, suggesting specific cellular mechanisms underlying the uptake process. We have routinely visualized pathogen–hBMEC interactions by electron microscope (EM) analysis [11], and intracellular *Salmonella* were similarly observed within membrane-bound vesicles as soon as 1 h after infection. Finally, we demonstrate that *Salmonella* persist intracellularly and traverse across hBMEC monolayers.

The SPI loci are major virulence determinants of *S enterica*. SPI-1 encodes a TTSS that injects effector proteins directly into the cytosol of host cells that modulate actin cytoskeleton dynamics, enabling bacterial uptake into intestinal epithelial cells. Our experiments suggest that bacteria were able to invade the BBB in an SPI-1–independent fashion, in contrast to uptake in epithelial cells [12]. This raises the possibility that an additional, as yet undiscovered mechanism mediates *Salmonella* uptake into the specialized BBB endothelium. *Salmonella* adherence to and intracellular persistence (data not shown) in hBMECs were independent of the SPI-1 locus. Intracellular survival mechanisms in hBMECs are likely encoded by SPI-2, as genes within SPI-2 promote *Salmonella* survival in other cell types [13]. However, further studies are required to identify the adhesion molecule(s) that mediates *Salmonella* association with hBMECs.

Salmonella interaction with the BBB induced an increased expression of chemokines IL-8, CXCL-1, and CXCL-2. The CXC subfamily of chemokines share a high affinity for receptors on neutrophils, and are known to act as strong neutrophil chemoattractants. In addition, another highly induced chemokine, CCL-20, is chemotactic for both lymphocytes and neutrophils. These results correlate with our previous data demonstrating that the BBB plays an active role in initiating neutrophil recruitment in response to bacterial infection [10]. This first line of CNS defense against bacterial infection may be particularly important during *Salmonella* infection as neutrophils are cytotoxic to *Salmonella*, in contrast to macrophages in which *Salmonella* are able to replicate [14]. Chemokine induction did not require an intact TTSS, as production of IL-8, CXCL-1 and CXCL-2 was similarly induced by the WT and Δ*invA* and Δ*sipB* mutant strains. This is in contrast to studies in intestinal epithelial cells where SPI-1 encoded proteins, such as *sipB*, initiate substantial inflammatory responses in the intestinal epithelium [15].

Penetration of the BBB by a bacterial pathogen reflects a complex interplay between the host endothelium and microbial surface components. Our results build upon the recent development of a mouse model for *Salmonella* meningitis [6], and demonstrate for the first time that *Salmonella* penetrates the brain endothelial cells that constitute the BBB. We speculate that the combination of direct bacterial invasion and the induction of proinflammatory signaling pathways contribute to the severity of *Salmonella* meningitis. Ongoing studies on the elucidation of BBB penetration as well as the modulation of BBB gene expression and innate defense mechanisms by *Salmonella* will be critical for developing preventative therapies for CNS infection.

Funding

This work was supported by the National Institute of Neurological Disorders and Stroke/National Institutes of Health (grant AI062758 to S.H.B. and R01NS051247 to K.S.D).

Acknowledgments

We thank Monique Stins and Kwang Sik Kim, for providing hBMEC, and Marilyn Farquhar and Timo Meerloo, for assistance with electron microscopy. The microarray analysis was performed at the Biogem Core Facility of the University of California San Diego, director Gary Hardiman.

References

1. Owusu-Ofori A, Scheld WM. Treatment of *Salmonella* meningitis: two case reports and a review of the literature. Int J Infect Dis. **2003**; 7:53–60.
2. Gordon MA, Graham SM, Walsh AL, et al. Epidemics of invasive *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium infection associated with multidrug resistance among adults and children in Malawi. Clin Infect Dis **2008**; 46:963–9.
3. Graham SM, Molyneux EM, Walsh AL, Cheesbrough JS, Molyneux ME, Hart CA. Nontyphoidal *Salmonella* infections of children in tropical Africa. Pediatr Infect Dis J **2000**; 19:1189–96.
4. Swe KS, Nagel G, Van der Westhuizen M, Hoosen AA. *Salmonella typhimurium* meningitis in an adult patient with AIDS. J Clin Pathol **2008**; 61:138–9.
5. Chang CJ, Chang WN, Huang LT, et al. Bacterial meningitis in infants: the epidemiology, clinical features, and prognostic factors. Brain Dev **2004**; 26:168–75.
6. Wickham ME, Brown NF, Provias J, Finlay BB, Coombes BK. Oral infection of mice with *Salmonella enterica* serovar typhimurium causes meningitis and infection of the brain. BMC Infect Dis **2007**; 7:65.
7. Galan JE, Curtiss R. 3rd. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc Natl Acad Sci USA **1989**; 86:6383–7.
8. Kaniga K, Tucker S, Trollinger D, Galan JE. Homologs of the Shigella IpaB and IpaC invasins are required for *Salmonella typhimurium* entry into cultured epithelial cells. J Bacteriol **1995**; 177:3965–71.
9. Doran KS, Engelson EJ, Khosravi A, et al. Blood–brain barrier invasion by group B Streptococcus depends upon proper cell-surface anchoring of lipoteichoic acid. J Clin Invest **2005**; 115:2499–507.
10. Doran KS, Liu GY, Nizet V. Group B streptococcal beta-hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. J Clin Invest **2003**; 112:736–44.
11. van Sorge NM, Ebrahimi CM, McGillivray SM, et al. Anthrax toxins inhibit neutrophil signaling pathways in brain endothelium and contribute to the pathogenesis of meningitis. PLoS One **2008**; 3:e2964.
12. Galan JE, Ginocchio C, Costeas P. Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of *invA* to members of a new protein family. J Bacteriol **1992**; 174:4338–49.
13. Kuhle V, Hensel M. Cellular microbiology of intracellular *Salmonella enterica*: functions of the type III secretion system encoded by *Salmonella* pathogenicity island 2. Cell Mol Life Sci **2004**; 61:2812–26.
14. Browne SH, Lesnick ML, Guiney DG. Genetic requirements for *Salmonella*-induced cytopathology in human monocyte-derived macrophages. Infect Immun **2002**; 70:7126–35.
15. Coombes BK, Coburn BA, Potter AA, et al. Analysis of the contribution of *Salmonella* pathogenicity islands 1 and 2 to enteric disease progression using a novel bovine ileal loop model and a murine model of infectious enterocolitis. Infect Immun **2005**; 73:7161–9.