Enterohemorrhagic *Escherichia coli* O157:H7 Shiga Toxins Inhibit Gamma Interferon-Mediated Cellular Activation

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Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is a food-borne pathogen that causes significant morbidity and mortality in developing and industrialized nations. EHEC infection of host epithelial cells is capable of inhibiting the gamma interferon (IFN- γ) proinflammatory pathway through the inhibition of Stat-1 phosphorylation, which is important for host defense against microbial pathogens. The aim of this study was to determine the bacterial factors involved in the inhibition of Stat-1 tyrosine phosphorylation. Human HEP-2 and Caco-2 epithelial cells were challenged directly with either EHEC or bacterial culture supernatants and stimulated with IFN- γ , and then the protein extracts were analyzed by immunoblotting. The data showed that IFN- γ -mediated Stat-1 tyrosine phosphorylation was inhibited by EHEC secreted proteins. Using two-dimensional difference gel electrophoresis, EHEC Shiga toxins were identified as candidate inhibitory factors. EHEC Shiga toxin mutants were then generated and complemented in *trans*, and mutant culture supernatant was supplemented with purified Stx to confirm their ability to subvert IFN- γ -mediated cell activation. We conclude that while other factors are likely involved in the suppression of IFN- γ -mediated Stat-1 tyrosine phosphorylation, *E. coli*-derived Shiga toxins represent a novel mechanism by which EHEC evades the host immune system.

Enterohemorrhagic *Escherichia coli* (EHEC), including the most common serotype, O157:H7, is a noninvasive enteric bacterial pathogen that causes both sporadic cases and outbreaks of diarrheal disease in humans as well as hemorrhagic colitis and hemolytic-uremic syndrome (14). Human infections with EHEC occur through the ingestion of contaminated foodstuffs and water supplies, as well as from person-to-person transmission of the organism (10).

One of the first lines of host defense against bacterial insult is through activation of the innate immune system (27). Proinflammatory cytokines, including gamma interferon (IFN- γ), are secreted into the extracellular environment and activate an antibacterial state in the body (45). IFN- γ production by macrophages, natural killer (NK) T cells, and activated T cells triggers an antimicrobial state in host cells by tyrosine phosphorylation of the signal transducer and activator of transcription 1 (Stat-1) molecule, leading to dimerization, translocation to the nucleus, binding to the gamma-activating sequence (GAS), and downstream upregulation of up to 2,000 proinflammatory genes, such as those for inducible nitric oxide synthase (iNOS), monocyte chemoattractant protein 1 (MCP-1), and lymphocyte adhesion protein ICAM-1, as well as increased major histocompatibility complex (MHC) class II expression (43, 44). An intact IFN- γ pathway is essential to fight off infection initiated from a wide range of microbial pathogens, with patients harboring genetic defects in Stat-1 signaling being prone to infection (3, 7, 15). IFN- γ levels are elevated in a mouse model of infection with *Citrobacter rodentium*, a murine-specific homolog of enterohemorrhagic *Escherichia coli* (20), and in humans following *E. coli* infection (32).

Subversion of the IFN- γ pathway by microbial pathogens promotes bacterial colonization and prevents bacterial clearance from the host (27, 36). EHEC has evolved a method to subvert the IFN- γ pathway, through a still unknown factor(s) (6). Therefore, the aim of this study was to determine how EHEC infection disrupts IFN- γ signal transduction in human epithelial cells. The findings reveal that the IFN- γ signal transduction pathway, which

is important for host defense, is compromised at the level of Stat-1 activation, at least in part, by EHEC-derived Shiga toxins (Stx).

MATERIALS AND METHODS

Tissue culture. HEP-2 epithelial cells (ATCC CCL-23) were used as a model epithelial cell line, as previously described (25). Briefly, cells were grown in minimal essential medium (MEM) containing 15% (vol/vol) fetal bovine serum (FBS), 2% (vol/vol) sodium bicarbonate, 2.5% (vol/vol) penicillin-streptomycin, and 1% (vol/vol) amphotericin B (all from Invitrogen, Burlington, Ontario, Canada). Cells were grown in T75 flasks (Corning Inc., Corning, NY) at 37°C with 5% CO₂ until confluent (8 × 10⁶ cells/flask). Confluent cells were trypsinized using 0.05% trypsin (Invitrogen) for 5 min at 37°C in 5% CO₂. Trypsinized cells were then pelleted by centrifugation at 40 × g for 5 min (Beckman Coulter, Mississauga, Ontario, Canada), resuspended in MEM, and reseeded into either 6-well (Becton Dickinson Labware, NJ) or 24-well (Corning Inc.) dishes and grown at 37°C in 5% CO₂ until confluent. Prior to bacterial infection, cells were incubated in MEM without antibiotics for 16 h at 37°C in 5% CO₂.

Caco-2-bbe human colonic adenocarcinoma cells (ATCC CRL-2102) were used as a model polarized epithelial cell line. These cells form confluent, polarized epithelial monolayers with well-differentiated intercellular tight-junction (TJ) structures and a pattern of brush border protein expression that is comparable to that of primary human enterocytes (37). Briefly, cells were grown in Dulbecco's modified Eagle medium (DMEM), 10% FBS, 0.01 mg/ml human transferrin, 1 mM sodium pyruvate, 200 U/ml penicillin, and 200 μg/ml streptomycin (all reagents were from GIBCO). The cell culture medium was changed to antibiotic-free culture

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TABLE 1 Primers employed in this study

Primer	Sequence (5' → 3')
pBAD-stx2-F	GCGCGAATTCGAAAGAAACCAATTGTCCATATTGCATCA
pBAD-stx2-R	GAATGGTACCCGCCCTTTTATTTACCCGTTGTATATA
pBAD-stx1-F	GCGCGGTACCGAAGATCCCTTTTGGATAATCTCATGACCA
pBAD-stx1-R	GCGCGAATTCATCTAAAGTATATATGAGTAACTTGGTCTGA
EscNKOP1	ATGATTTTCAGAGCATGATTCTGTATTGGAATAATACCCACGTGTAGGCTGGAGCTGCTTC
EscNKOP2	GGCAACCACTTTGAATAGGCTTTCAATCGTTTTTCGTAACATATGAATATCCTCCTTAG
EspAKOP1	ATGGATACATCAAATGCAACATCCGTTGTTAATGTGAGTGGTGTAGGCTGGAGCTGCTTC
EspAKOP2	TTATTTACCAAGGGATATTGCTGAAATAGTTCTATATTGTCTATGAATATCCTCCTTAG
EspBKOP1	ATGAATACTATTGATAATACTCAAGTAACGATGGTTAATTGTGTAGGCTGGAGCTGCTTC
EspBKOP2	TTACCCAGCTAAGCGACCCGATTGCCCCATACGATCTGGCATATGAATATCCTCCTTAG
EspDKOP1	ATGCTTAACGTAAATAACGATACCTGTCTGTAAACGTCTGGTGTAGGCTGGAGCTGCTTC
EspDKOP2	TTAAATTCGGCCACTAACAATACGACTATTTACCCGTGCTCATATGAATATCCTCCTTAG
EspFKOP1	ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGTGTAGGCTGGAGCTGCTTC
EspFKOP2	TTACCCCTTCTTCGATTGCTCATAGGCAGCTAAATGATCTCATATGAATATCCTCCTTAG
EspFuKOP1	ATGATTAACAATGTTTCTTCACTTTTTTCCAACCGTCAACCGTGTAGGCTGGAGCTGCTTC
EspFuKOP2	TCACGAGCGTTAGATGATTAATGCCATGCTCTGCAAGACATATGAATATCCTCCTTAG
FliCKOP1	ATGGCACAAGTCATTAATAACCAACAGCCTCTCGCTGATCAGTGTAGGCTGGAGCTGCTTC
FliCKOP2	TTAACCCCTGCAGCAGAGACAGAACCTGCTGCGGTACCTGGCATATGAATATCCTCCTTAG
Stx1KOP1	ATGAAAATAATTATTTTGTAGTGTCTAATCTTTTCTTTGGTGTAGGCTGGAGCTGCTTC
Stx1KOP2	TCAACTGCTAATAGTTCTGCGCATCAGAAATGCCCCAGACATATGAATATCCTCCTTAG
Stx2KOP1	ATGAAGTGTATATTATTTAAATGGGTACTGTGCCTGTTACGTGTAGGCTGGAGCTGCTTC
Stx2KOP2	TTATTTACCCGTTGTATATAAAAACTGTGACTTTCTGTTCCATATGAATATCCTCCTTAG
Stx1A-C'-P1	GAGGAATAATAAATGAAAATAATTATTTTGAAGTGTAACTTTTTT
Stx1A-C'-P2	TCAACTGCTAATAGTTCTGCGCA
Stx2A-C'-P1	GAGGAATAATAAATGAAGTGTATATTATTTAAATGGGTACTGTGC
Stx2A-C'-P2	TTATTTACCCGTTGTATATAAAAACTGTGACT
StcEKOP1	ATGAACACTAAAAATGAATGAGAGATGGAGAACACCGATGAAATTAAGTAGTGTAGGCTGGAGCTGCTTC
StcEKOP2	TTATTTATATACAACCCCTCATTGACCTAGGTTTACTGAAGTCCAAATACCTCATATGAATATCCTCCTTAG
TirKOP1	ATGCCATTATTGGTAATCTTGGTCAATCCCAATGTGAATAGTGTAGGCTGGAGCTGCTTC
TirKOP2	TTAGACGAAACGATGGGATCCCGCGCTGGTGGGTATTCCATATGAATATCCTCCTTAG
Z1787KOP1	GTGCTTATGTGGATTGTGTTAGTACTGTCACTGTCAACTCGTGTAGGCTGGAGCTGCTTC
Z1787KOP2	TCAATTATGTTTTAAATGGATAGGTAAAGAATAACAAGCATATGAATATCCTCCTTAG

medium prior to experimental trials using conditions equivalent to those with HEp-2 cells.

Bacterial strains and growth conditions. Enterohemorrhagic *E. coli* O157:H7 strain EDL933 (EHEC) (accession number [AE005174.2](#)) and enteropathogenic *E. coli* O127:H6 strain E2348/69 (EPEC) (accession number NC_011601.1) were used in this study. Strains were cultured on 5% sheep blood agar plates (Becton, Dickinson Co., Sparks, MD) at 37°C for 16 h and stored at 4°C until use. Prior to infecting epithelial cells, bacteria were grown in 10 ml Penassay broth (Becton, Dickinson Co.) overnight at 37°C.

Isogenic mutant strains. Isogenic mutants were generated from EHEC O157:H7 strain EDL933 using a one-step inactivation technique (11) and primers listed in Table 1. Briefly, EDL933 was transformed with pKD46 and λ-red recombinase expression induced with L-arabinose at 30°C on a shaker until the optical density at 600 nm (OD₆₀₀) reached 0.6. Mutants were generated by electroporating linear DNA fragments containing a kanamycin or chloramphenicol resistance cassette with 5' and 3' flanking regions homologous to the gene target using primers targetKOP1 and targetKOP2 on plasmid pKD4 (template plasmid with FLP recognition target sites flanking a kanamycin resistance gene) or pKD3 containing the chloramphenicol resistance gene. Double knockouts were generated from a previously created mutant after removal of the kanamycin resistance cassette using pCP20. All mutants generated were verified by PCR.

Bacterial complementation. The *stx*_{1A} and *stx*_{2A} mutants were complemented in *trans* by the introduction of the pBAD-TOPO plasmid (Invitrogen) containing either *stx*_{1A} or *stx*_{2A} under the control of the arabinose promoter. Briefly, the *stx*_{1A} gene was PCR amplified using primers Stx1A-C'-P1 and Stx1A-C'P2, while the *stx*_{2A} gene was amplified using

primers Stx2A-C'-P1 and Stx2A-C'-P2 to generate plasmids pBAD-stx1A and pBAD-stx2A, respectively. The Stx double gene knockout (*stx*_{DKO}) mutant was complemented in *trans* by cloning the pBAD promoter and downstream *stx*_{2A} gene from pBAD-stx2 using primers pBAD-stx2-F and pBAD-stx2-R into the pBAD-stx1A inverse PCR product, using primers pBAD-stx1-F and pBAD-stx1-R. All constructs were verified using PCR and DNA sequencing (Centre for Applied Genomics, Hospital for Sick Children, Toronto, Ontario, Canada). Genes were induced in the complements with 0.02% arabinose while generating bacterial culture supernatants (CS).

Bacterial CS. To collect sterile culture supernatants (CS), ~1 × 10⁹ CFU/ml of EHEC O157:H7 strain EDL933 or EPEC O127:H6 strain E2348/69 culture was centrifuged (3,000 × g, 15 min) and resuspended in 10 ml of serum-free MEM without antibiotics. After 24 h of growth at 37°C in 5% CO₂, the medium was centrifuged (3,000 × g, 15 min), filtered (0.22 μm), and stored at 4°C. Sterility was confirmed by lack of bacterial growth of 0.1 ml of culture supernatant plated onto 5% sheep blood agar plates and incubated overnight at 37°C.

Proteinase K and heat inactivation treatment of culture supernatants. Bacterial culture supernatants from EHEC were incubated with proteinase K conjugated to agarose beads (10 to 1,000 μg/ml, 1 h, shaking, 37°C) (Sigma-Aldrich, Oakville, Ontario, Canada). Culture supernatants incubated with agarose beads and preincubated with 5% bovine serum albumin (BSA) were used as a negative control. After incubation, agarose beads were removed from the solution by centrifugation (3,000 × g, 1 min) before incubation with HEp-2 cells. Culture supernatants from EHEC were also heat inactivated by boiling (100°C, 0.5 h) and cooled to 37°C before incubation with HEp-2 cells.

Epithelial cell infection. Infection of HEP-2 or Caco-2 epithelial cells was performed at a multiplicity of infection (MOI) of 100:1. Overnight bacterial culture (10 ml) was centrifuged ($3,000 \times g$, 10 min), the supernatant decanted, and bacterial pellets resuspended in 1.0 ml of antibiotic- and serum-free MEM. An aliquot of this bacterial suspension ($\sim 1 \times 10^8$ CFU in 0.1 ml) was then used to infect either confluent HEP-2 or Caco-2 monolayers grown in 6-well plates (roughly 1×10^6 cells/well). The cells were infected with either EHEC or EPEC for 6 h at 37°C in 5% CO₂. Infections at an MOI of 10:1 required approximately 8 h for robust Stat-1 suppression. Cells were then washed with phosphate-buffered saline (PBS) and stimulated with IFN- γ (50 ng/ml; 0.5 h at 37°C in 5% CO₂), followed by preparation of whole-cell protein extracts for immunoblotting. To determine if active bacterial protein synthesis was required to inhibit the phosphorylation of Stat-1, in a subset of experiments epithelial cells and EHEC were incubated with chloramphenicol (100 μ g/ml) at 0 to 4 h after infectious challenge (MOI of 100:1, 6 h).

Immunoblotting. Whole-cell protein extracts were collected by resuspending epithelial cells in radioimmunoprecipitation assay (RIPA) buffer (1% Nonidet P-40, 0.5% sodium deoxyolate, and 0.1% sodium dodecyl sulfate [SDS] in PBS) supplemented with 150 mM NaCl, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 20 μ g/ml phenylmethylsulfonyl fluoride, 15 μ g/ml aprotinin, 2 μ g/ml leupeptin, and 2 μ g/ml pepstatin A (all from Sigma-Aldrich). Aliquots were applied directly onto cells, mixed, and left on ice for 0.5 h. Resuspended pellets were centrifuged at $20,000 \times g$ for 1 min at 4°C. Supernatants were collected and stored at -80°C until further analysis by Western blotting.

Immunoblotting was conducted by combining whole-cell protein extracts with SDS-PAGE loading buffer in a 1:1 (vol/vol) ratio, incubating at 100°C for 3 min, and loading into precast 10% polyacrylamide gels (Ready Gel; Bio-Rad Laboratories, Hercules, CA). Gels were electrophoresed (150 V for 1 h at room temperature), followed by protein transfer onto nitrocellulose membranes (BioTrace NT; Pall Corporation, Ann Arbor, MI) (110 V for 1 h at 4°C). Membranes were incubated in Odyssey blocking buffer (Mandel Scientific Company Inc., Guelph, Ontario, Canada) for 0.5 h at room temperature on a shaker, followed by incubation with primary antibodies (4°C overnight on a shaker). Primary antibodies included rabbit anti-native Stat-1 (1 in 1,000 dilution; Cell Signaling, Beverly, MA), rabbit anti-phospho-Stat-1 (1 in 1,000 dilution; Cell Signaling), rabbit anti-IRF1 (1 in 2,000 dilution; Sigma-Aldrich), and mouse anti- β -actin (1 in 5,000 dilution; Sigma). Membranes were washed 3 times with PBS plus 0.1% Tween (5 min per wash) and then incubated with secondary antibodies (1 h at room temperature on a shaker). Secondary antibodies included IRDye 800 goat anti-rabbit IgG (1 in 20,000 dilution; Rockland Immunochemicals, Gilbertsville, PA) and Alexa Fluor 680 goat anti-mouse IgG (1 in 20,000 dilution; Molecular Probes, Eugene, OR).

Immunoblots were scanned into an infrared imaging system (Odyssey; Li-Cor Biosciences, Lincoln, NE), using both the 700-nm and 800-nm channels, and immunoblots were scanned at a resolution of 169 μ m. Using automated software (Li-Cor Biosciences), densitometry was performed to obtain the integrative intensity of positively stained bands. Integrative intensity values for each phospho-Stat-1 were normalized to the integrative intensity values obtained for the corresponding β -actin bands as they were run on the same gel; results for native Stat-1 are presented to confirm that its levels are unaffected by incubation with EHEC, EPEC, or sterile culture supernatants. Uninfected cells stimulated with IFN- γ were used as positive controls and standardized to 100%. Densitometry values obtained from samples incubated with live bacteria or sterile culture supernatants were then calculated as a percentage of the positive uninfected control value.

Cell cytotoxicity assay. Lactate dehydrogenase (LDH) released into the tissue culture medium from HEP-2 cells challenged with bacteria or culture supernatants was quantified using Cytoscan (G-Biosciences, St. Louis, MO). Briefly, HEP-2 cells were incubated with either live bacteria (MOI, 100:1) or culture supernatants for 6 h. Supernatants were then transferred to a new 96-well plate and incubated with a commercial sub-

strate mixture in the dark for 30 min at room temperature. A stop solution was added to samples and absorbance at 490 nm measured in a plate reader (Victor³ reader; Perkin-Elmer, Ontario, Canada). The level of LDH released from the infected cells was calculated as a percentage, compared to LDH activity measured in total cell lysates.

2D gel electrophoresis. Two-dimensional (2D) difference gel electrophoresis (DIGE) and protein identification were performed by Applied Biomic Inc. (Hayward, CA). Briefly, 1 ml of 2D lysis buffer {30 mM Tris-HCl [pH 8.8], 7 M urea, 2 M thiourea, and 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS]} and EDTA (5 mM final concentration) were added to each sample, and the mixtures were transferred to 3,000-molecular-weight-cutoff (MWCO) Amicon filters (Millipore). The samples were spun in a GS-6KR centrifuge (Beckman Coulter) at $5,000 \times g$ until the volume was concentrated to 400 μ l per sample. Samples were then transferred to 5,000-MWCO spin columns (Sartorius Biolab Products, Goettingen, Germany) and spun at $15,000 \times g$ until the volume was concentrated to 50 μ l per sample. The samples were precipitated with 100% methanol and resuspended in 30 μ l of 2D cell lysis buffer. The samples were sonicated at 4°C, followed by shaking for 30 min at room temperature and centrifugation for 30 min at $15,000 \times g$, and the supernatants were collected. The protein concentration was measured by Bradford assay (Bio-Rad).

For CyDye labeling, 30 μ g of protein was mixed with 1.0 μ l of diluted Cy2 or Cy3 and kept in the dark on ice for 0.5 h. The labeling reaction was stopped by adding 1 μ l of 10 mM lysine to each sample, and the mixture was incubated in the dark on ice for an additional 15 min. The labeled samples were then mixed together, and $2 \times$ 2D sample buffer (8 M urea, 4% CHAPS, 20 mg/ml dithiothreitol [DTT], 2% pharmalytes, and a trace amount of bromophenol blue), destreak solution (GE Healthcare), and rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes, and a trace amount of bromophenol blue) were added to a final volume of 260 μ l. The samples were mixed well and spun down before being loaded onto a strip holder. After labeled samples were loaded onto pH 3 to 10 linear immobilized pH gradient (IPG) strips (GE Healthcare), isoelectric focusing was run for 12 h of rehydration at 20°C, followed by 500 V for 1,000 voltage hours (Vh), 1,000 V for 2,000 Vh, and 8,000 V for 24,000 Vh. IPG strips were then incubated in freshly made equilibration buffer 1 (50 mM Tris-HCl [pH 8.8] containing 6 M urea, 30% glycerol, 2% SDS, a trace amount of bromophenol blue, and 10 mg/ml DTT) for 15 min with gentle shaking. Strips were then rinsed in freshly made equilibration buffer 2 (50 mM Tris-HCl [pH 8.8] containing 6 M urea, 30% glycerol, 2% SDS, a trace amount of bromophenol blue, and 45 mg/ml iodoacetamide) for 10 min with gentle shaking. Next, the IPG strips were rinsed in SDS gel running buffer before being transferred into 12% SDS gels (18 cm by 16 cm), followed by sealing with 0.5% agarose (Bio-Rad) in SDS-PAGE running buffer. The SDS gels were run at 15°C.

Image scan and data analysis. Gel images were scanned immediately following SDS-PAGE using Typhoon TRIO (Amersham Biosciences). The scanned images were then analyzed with Image Quant software version 6.0 (Amersham Biosciences), followed by in-gel analysis using DeCyder software version 6.0 (Amersham Biosciences). The fold change of protein expression levels was obtained from in-gel DeCyder analysis, with a set cutoff of 1.5.

Shiga toxin immunoassay. The concentrations of Stx1 and Stx2 present in culture supernatants of both wild-type (WT) and mutant bacteria were determined by a Stx capture enzyme-linked immunosorbent assay (ELISA) as described previously (2, 8, 50) at the Laboratory for Food-Borne Zoonoses (LFZ), Guelph, Ontario, Canada. Briefly, flat-bottom wells of eight-well immunostrips (Nunc Maxisorb Immuno-Module; Thermo Scientific) in 96-well frames were coated with 100 μ l of 2 μ g/ml rabbit anti-Stx antibodies (LFZ) in carbonate-bicarbonate buffer (pH 9.6) and then postcoated by the addition of 200 μ l/well of a stabilizing/blocking solution (LFZ). The culture supernatants were added to each well, incubated at 22°C for 30 min, and then washed five times with 300 μ l of

FIG 1 EHEC, but not EPEC, inhibits IFN- γ -mediated Stat-1 tyrosine phosphorylation in a noncytotoxic manner. (A) Whole-cell protein extracts from HEp-2 cells analyzed by immunoblotting showed that IFN- γ -mediated (50 ng/ml, 0.5 h) Stat-1 tyrosine phosphorylation is suppressed by enterohemorrhagic *Escherichia coli* O157:H7, strain EDL933 (EHEC), but not by enteropathogenic *Escherichia coli* O127:H6, strain E2348/69 (EPEC) (MOI of 100:1; 6 h). (B) Incubation with chloramphenicol (100 μ g/ml) up to 1 h after infection with EHEC (MOI of 100:1; 6 h) prevented EHEC suppression of IFN- γ -mediated Stat-1 tyrosine phosphorylation. (C) LDH cytotoxicity assay was performed on HEp-2 cells after infection with EHEC or EPEC (MOI of 100:1) or sterile EHEC culture supernatants (CS) for 6 h ($n = 3$; one-way ANOVA, $P > 0.05$). (D) Incubation with EHEC, but not EPEC, culture supernatants (6 h) suppressed IFN- γ -mediated tyrosine phosphorylation of Stat-1. (E) Caco-2-bbe cells infected with EHEC or with sterile EHEC CS, but not EPEC (MOI of 100:1) suppressed IFN- γ -mediated tyrosine phosphorylation of Stat-1.

0.01 M PBS (pH 7.4) containing 0.1% Tween 20. Bound Stx in the wells was detected by sequential incubations at 22°C with monoclonal antibodies directed against either Stx1 or Stx2/2c (2 μ g/ml; LFZ) for 30 min, followed by horseradish peroxidase-labeled anti-mouse IgG (Jackson ImmunoResearch, PA) at 0.6 μ g/ml for 30 min. Then, 100 μ l of 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich, Oakville, Ontario, Canada) was added and left for 10 min to induce color development, and the reaction was stopped with 100 μ l 0.2 M sulfuric acid. Plates were read in a microplate spectrophotometer (ELx 808 Ultra microplate reader; Bio-Tek Instruments) at dual wavelengths of 450 and 630 nm. The mean optical densities of Stx standards were used to generate a standard curve for each toxin, which was then used to calculate the amounts of Stx1 and Stx2 present in the bacterially derived culture supernatants.

Shiga toxin purification. Purified Shiga toxins were kindly provided by Clifford Lingwood, Hospital for Sick Children. Shiga toxin 1 was purified from *E. coli* strain JB28 (23) as previously described (31), and Shiga toxin 2 was purified from *E. coli* R82pJES120DH5 α as previously described (48).

Statistics. Results are expressed as means $\pm$ standard errors (SE). One-way analysis of variance (ANOVA) with Tukey's multiple-comparison test was performed to analyze the statistical significance of the results between groups of treatments from multiple independent experiments. Analyses were performed using Prism4 (GraphPad, San Diego, CA). P values of <0.05 were considered significant.

RESULTS

EHEC, but not EPEC, inhibits IFN- γ -mediated Stat-1 tyrosine phosphorylation in a noncytotoxic manner. To demonstrate that EHEC, but not EPEC, was able to subvert the IFN- γ pathway,

we assessed the phosphorylation state of the Stat-1 molecule. Under unstimulated circumstances, Stat-1 is normally not tyrosine phosphorylated, but it becomes activated in response to IFN- γ stimulation (43). Infection with EHEC prevented Stat-1 tyrosine phosphorylation in response to cytokine stimulation (Fig. 1A) (21, 26). In contrast, infection with EPEC did not inhibit IFN- γ -mediated Stat-1 tyrosine phosphorylation, indicating that the ability to subvert IFN- γ signaling is a specific ability of EHEC and not all pathogenic *E. coli* strains. Levels of native Stat-1 were not affected by either pathogen (Fig. 1A). Both pathogens have been demonstrated to adhere at similar levels to HEp-2 cells (42).

The addition of chloramphenicol, a specific bacterial protein synthesis inhibitor, prevented bacterial inhibition of Stat-1 tyrosine phosphorylation in response to IFN- γ within 1 h of EHEC infection (Fig. 1B), suggesting that subversion of IFN- γ -mediated Stat-1 tyrosine phosphorylation is time dependent and requires new and active bacterial protein synthesis. HEp-2 cell cytotoxicity analysis confirmed that infection with EHEC or EPEC did not cause a significant difference in cell death compared to that of uninfected controls (Fig. 1C), indicating that the lack of Stat-1 tyrosine phosphorylation due to IFN- γ stimulation was not due to cell death.

Inhibitor of IFN- γ -mediated Stat-1 tyrosine phosphorylation is a secreted factor and affects different cell lines. As shown in Fig. 1D, EHEC, but not EPEC, culture supernatants inhibited

FIG 2 A secreted factor(s) has protein-like qualities. Panels A, C, and E show representative Western blots of densitometry analyses shown in panels B, D, and F, respectively. (A and B) Whole-cell protein extracts from HEp-2 cells analyzed by immunoblotting showed that incubation with sterile culture supernatants (CS) from EHEC, but not EPEC, (1, 3, and 6 h), increases suppression of IFN- γ -mediated (50 ng/ml, 0.5 h) Stat-1 tyrosine phosphorylation over time ($n = 3$; one-way ANOVA, $P < 0.05$ [*]). (C and D) Incubation with heat-treated (100°C, 0.5 h) EHEC CS did not suppress IFN- γ -mediated Stat-1 tyrosine phosphorylation. (E and F) Incubation with EHEC CS (6 h) pretreated with proteinase K conjugated to agarose beads (0 to 1,000 mg/ml, 37°C, 1 h) progressively lost its ability to suppress IFN- γ -induced tyrosine phosphorylation of Stat-1 ($n = 3$; one-way ANOVA, $P < 0.05$ [*]).

Stat-1 tyrosine phosphorylation in response to IFN- γ stimulation. Culture supernatants harvested from bacteria while in exponential growth phase (6 h) showed less inhibition than those generated at stationary phase (24 h) (data not shown). EHEC has been shown to successfully adhere and form attaching and effacing (A/E) lesions with Caco-2-bbe cells, a human polarized epithelial cell line (47). Incubation of Caco-2 cells with EHEC (or culture supernatants) or EPEC demonstrated results similar to those with the HEp-2 cell line (Fig. 1E), indicating that the inhibitory factor secreted by EHEC affects multiple cell lines. As shown in Fig. 2A and B, the inhibitory effects of EHEC-derived culture supernatants occurred in a time-dependent manner. Taken together, these findings demonstrate that a bacterial factor was secreted specifically from EHEC and that direct contact of the pathogen

with host epithelia was not required for subverting IFN- γ -mediated Stat-1 tyrosine phosphorylation.

Secreted inhibitory factors are proteins. To verify that the inhibitory effects of EHEC were not due to heat-resistant factors, such as lipopolysaccharide (40, 41), culture supernatants were heated to 100°C for 30 min and then assessed for the ability to inhibit Stat-1 tyrosine phosphorylation. Boiled culture supernatants did not inhibit Stat-1 tyrosine phosphorylation, even after 6 h of incubation with tissue culture epithelial cells (Fig. 2C and D).

To confirm that the inhibitory factors were protein in nature, bacterial culture supernatants were treated with proteinase K and then tested for their ability to inhibit Stat-1 tyrosine phosphorylation. Culture supernatants treated with agarose beads as a negative control still blocked signaling responses to IFN- γ , while

FIG 3 Comparative proteomic profiles of EHEC and EPEC secreted proteins. (A and B) Culture supernatants from EHEC (A) or EPEC (B) were separated by 2D difference gel electrophoresis (DIGE) and labeled with Cy3 and Cy2, respectively. (C) The resulting gels were overlaid, and candidate spots were then picked and identified using mass spectrometry.

those treated with increasing concentrations of proteinase K progressively lost the ability to subvert IFN- γ -mediated Stat-1 tyrosine phosphorylation (Fig. 2E and F).

Comparative proteomic profiles of EHEC and EPEC secreted proteins. To identify the EHEC factor involved in subverting IFN- γ -mediated Stat-1 phosphorylation, the secreted protein profile of EHEC O157:H7 (Fig. 3A, green) was compared with that of EPEC O126:H7 (Fig. 3B, red). Secreted proteins from both enteric pathogens were differentially labeled and separated using 2D difference gel electrophoresis (2D DIGE), and the gels were overlaid to compare the profiles of the two pathogens (Fig. 3C). Densitometry

analyses revealed increased, decreased, and no changes in 124, 216, and 190 proteins, respectively, in EHEC culture supernatants compared to EPEC culture supernatants. Subsequently, proteins that were overexpressed in the EHEC fraction compared to the EPEC fraction were identified using mass spectrometry, and 14 isogenic EHEC mutants were generated using the λ -red gene deletion technique (11) to determine if the proteins identified were involved in subverting the IFN- γ signal transduction pathway (Table 2).

Stx1A and Stx2A suppress IFN- γ -mediated Stat-1 tyrosine phosphorylation. Of the isogenic mutants generated, only culture supernatants from the *stx*_{1A} and *stx*_{2A} isogenic mutants partially

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Effect on IFN- γ signaling	Reference
<i>E. coli</i> strains			
O127:H6 strain E2348/69	Wild-type EPEC strain used in this study	—	38
O157:H7 strain EDL933	Wild-type EHEC strain used in this study	+	28
EDL933 mutants			
<i>espA</i>	<i>espA</i> disrupted by kanamycin resistance cassette	—	This study
<i>espB</i>	<i>espB</i> disrupted by kanamycin resistance cassette	—	This study
<i>espD</i>	<i>espD</i> disrupted by kanamycin resistance cassette	—	This study
<i>espF</i>	<i>espF</i> disrupted by kanamycin resistance cassette	—	This study
<i>espFu</i>	<i>espFu</i> disrupted by kanamycin resistance cassette	—	This study
<i>espF/Fu</i>	<i>espFu</i> disrupted by kanamycin resistance cassette of <i>espF</i> kanamycin cassette-cured mutant	—	This study
<i>fliC</i>	<i>fliC</i> disrupted by kanamycin resistance cassette	—	This study
pO157 cured	pO157 plasmid-cured mutant obtained using pCURE2 kit (18)	—	This study
<i>stx</i> _{1A}	<i>stx</i> _{1A} disrupted by kanamycin resistance cassette	+	This study
<i>stx</i> _{2A}	<i>stx</i> _{2A} disrupted by kanamycin resistance cassette	+	This study
<i>stx</i> _{DKO}	<i>stx</i> _{1A} disrupted by kanamycin resistance cassette of <i>stx</i> _{2A} kanamycin cassette-cured mutant	+	This study
<i>stx</i> _{DKO} <i>escN</i>	<i>stx</i> _{DKO} mutant with <i>escN</i> gene disrupted by a chloramphenicol cassette	+	This study
<i>stcE</i>	<i>stcE</i> disrupted by kanamycin resistance cassette	—	This study
Tir	Tir disrupted by kanamycin resistance cassette	—	This study
Z1787	Z1787 disrupted by kanamycin resistance cassette	—	This study
Plasmids			
pBAD- <i>stx</i> _{1A}	<i>stx</i> _{1A} gene inserted downstream of the pBAD-TOPO plasmid	+	This study
pBAD- <i>stx</i> _{2A}	<i>stx</i> _{2A} gene inserted downstream of the pBAD-TOPO plasmid	+	This study
pBAD- <i>stx</i> ₁₂	pBAD promoter and downstream <i>stx</i> _{2A} gene inserted in the vector backbone of the pBAD- <i>stx</i> _{1A} plasmid	+	This study

FIG 4 Shiga-like toxins from EHEC suppress IFN- γ -mediated activation of Stat-1. Whole-cell protein extracts from HEP-2 cells analyzed by immunoblotting showed that incubation with sterile culture supernatants (CS) from EHEC Shiga-like toxin single mutants partially suppressed IFN- γ -mediated (50 ng/ml, 0.5 h) tyrosine phosphorylation of Stat-1, while the suppressive ability from the *stx*_{DKO} mutant was completely ablated. (A) A representative Western blot showing CS from single and double mutants inhibiting Stat-1 tyrosine phosphorylation less than WT CS and CS from bacterial complements returning suppression to WT levels. (B) A representative Western blot showing CS from single and double mutants supplemented with either purified Stx1 (5 ng/ml), Stx2 (50 ng/ml), or both suppressing IFN- γ -mediated Stat-1 tyrosine phosphorylation at levels comparable to that for WT CS. (C) Densitometry analysis of Western blotting ($n = 3$ to 5; one-way ANOVA, $P < 0.05$ [*]). (D) Whole-cell protein extracts from HEP-2 cells analyzed by immunoblotting showed that incubation with EHEC, but not EPEC or EHEC *stx*_{DKO}, culture supernatants (6 h) suppressed IFN- γ -mediated (50 ng/ml, 2 h) expression of IRF1.

($n = 5$; $P < 0.05$) recovered Stat-1 tyrosine phosphorylation in response to IFN- γ (Fig. 4A and B). Culture supernatants prepared from an Stx double gene knockout (*stx*_{DKO}) mutant completely recovered Stat-1 phosphorylation ($n = 5$; $P < 0.05$) compared to culture supernatants derived from the parent strain (Fig. 4A to C), indicating that secreted Shiga toxins mediate EHEC suppression of the IFN- γ signaling pathway. The amounts of Stx1 and Stx2 present in culture supernatants were measured using an established ELISA (50), and the levels were confirmed to be reduced by the λ -red gene deletion technique (Table 3). Culture supernatants from the complemented mutants returned Stat-1 tyrosine phosphorylation to levels comparable to wild type, indicating that each of the Shiga toxins contributes to the suppression of IFN- γ -mediated Stat-1 tyrosine phosphorylation (Fig. 4A to C). To further confirm that Shiga toxins are involved in the suppression of IFN- γ -mediated Stat-1 tyrosine phosphorylation, epithelial cells were incubated with mutant culture supernatants supplemented with purified Stx1 and Stx2. Incubation with these supernatants

showed suppressive effects that were comparable to those of wild-type culture supernatant (Fig. 4B and C). However, purified Stx alone did not inhibit Stat-1 tyrosine phosphorylation (data not shown). These results indicate that while Stx1 and Stx2 play a role in inhibiting Stat-1 phosphorylation, it is possible that other bacterially derived factors are also involved.

To confirm that the IFN- γ signaling pathway is subverted due to EHEC Stx, IRF1 expression was assessed. IRF1 expression is induced only after IFN- γ stimulation (43). Similar to the case for Stat-1 tyrosine phosphorylation, incubation of cells with EHEC culture supernatants prior to IFN- γ stimulation suppressed IRF1 expression, whereas incubation with EPEC or the *stx*_{DKO} culture supernatants did not have an inhibitory effect (Fig. 4D).

DISCUSSION

In the present study, we show that EHEC, but not EPEC, subverts the IFN- γ signaling pathway at the level of Stat-1 tyrosine phosphorylation in different human epithelial cell lines. Direct contact of this pathogen with host cells is not required to mediate the effect, and the subversive factor is secreted into the extracellular culture medium. Through a series of complementary biochemical tests, we have now established that at least one of the secreted factors is protein in nature, and through genetic manipulation of the EHEC chromosome and the use of purified Stx, we show that EHEC suppresses IFN- γ -mediated Stat-1 phosphorylation at least in part through elaboration of Shiga toxins.

While EHEC produces an array of virulence factors that aid in infection, such as lipopolysaccharide (30) and flagella (4), one of the virulence factors classically associated with EHEC infection is

TABLE 3 Quantification of Stx1 and Stx2 present in culture supernatants obtained from WT *E. coli* O157:H7 strain EDL933, and corresponding isogenic mutants

Bacterial strain	Concn, ng/ml (mean $\pm$ SD; $n = 3$)	
	Stx1	Stx2
WT	3.84 $\pm$ 0.28	33.59 $\pm$ 1.58
<i>stx</i> _{1A} mutant	1.21 $\pm$ 0.05	28.66 $\pm$ 0.93
<i>stx</i> _{2A} mutant	0.96 $\pm$ 0.11	5.60 $\pm$ 0.23
<i>stx</i> _{DKO} mutant	0.32 $\pm$ 0.01	5.06 $\pm$ 0.46

its Shiga toxins, which can cause systemic complications, including hemorrhagic colitis and the hemolytic-uremic syndrome (35). The German *E. coli* O104:H4 outbreak in May 2011, where approximately 4,000 cases of infection and 50 deaths were reported, was due to an enteroaggregative *E. coli* strain that acquired the ability to produce Stx2 (5, 39). The importance of Stx2 mediating infection severity stems from the fact that *E. coli* O104:H4 without Stx2 was previously associated with sporadic cases of human disease but not with large-scale outbreaks (16, 29).

Another EHEC virulence factor is its pathogenicity island-encoded type three secretion system (T3SS), which allows the bacterium to disrupt host processes by injecting protein effectors directly into the host cell (46). Studies on the T3SS and its effectors have found that many of these bacterially derived proteins have redundant and overlapping functions, each of which appears to have multiple roles in subverting eukaryotic cellular processes and thereby aiding in EHEC-mediated pathogenesis (12). For example, EspF in EHEC and EPEC plays multiple roles in the subversion of host cellular pathways, such as effacing the microvilli of infected cells, disrupting the nucleolus, and preventing phagocytosis, to name a few (22). Furthermore, EspF, Tir, and Map all contribute to SGLT-1 inactivation (49). Our novel finding that EHEC Shiga toxins possess the ability to subvert IFN- γ -mediated Stat-1 tyrosine phosphorylation, supports previous findings that EHEC virulence proteins can be multifunctional, cooperative, and redundant (12).

Recent studies have found that subversion of innate immune pathways is a theme common to multiple pathogens, illustrating the important role of the IFN- γ signal transduction pathway in fighting off microbial infections (27). For example, viruses of the *Paramyxoviridae* family subvert the IFN- γ pathway by degrading intracellular Stat-1 (13), while the parasite *Leishmania donovani* prevents the phosphorylation of Stat-1 (34). The ability of EHEC O157:H7 to suppress the IFN- γ pathway could promote its ability to colonize the gut (27).

While previous studies from our laboratory suggested that culture supernatants were not capable of suppressing IFN- γ -mediated Stat-1 tyrosine phosphorylation (24), it has been demonstrated that bicarbonate ions stimulate gene expression in EHEC (1), and we were able to induce expression of the inhibitory factors through the supplementation of sodium bicarbonate in the medium (21). Furthermore, our laboratory has also published studies showing that Shiga toxins were not involved in the suppression of IFN- γ -mediated Stat-1 tyrosine phosphorylation (6, 24). In the present study, we show through the use of knockout mutagenesis, genetic complementation *in trans*, and supplementation with purified Stx that these toxins are involved in the suppression of Stat-1 activation. While Stx classically enters the cell through the globotriaosylceramide (Gb3) receptor present in HEp-2 cells (9), the role of the glycolipid receptor in mediating the suppression of Stat-1 tyrosine phosphorylation is uncertain, because EHEC O157:H7 also suppresses IFN- γ -mediated Stat-1 activation in Gb3-negative T84 epithelial cells (24) in which Stx still undergoes retrograde trafficking (33). Furthermore, no suppression of Stat-1 activation was observed when purified Stx was added without culture supernatant (data not shown). Indeed, direct infection of the *stx*_{DKO} mutant, or even a triple mutant with an inactivated T3SS (*stx*_{DKO} *escN*) (17), suppressed IFN- γ -mediated Stat-1 tyrosine phosphorylation completely (data not shown), indicating that there are likely to be additional factors involved in the suppression of Stat-1

tyrosine phosphorylation, which may explain why WT culture supernatants did not fully suppress Stat-1 tyrosine phosphorylation.

To our knowledge, this is the first study to show that Shiga toxins modulate IFN- γ signaling, and it supports recent findings that each of EHEC's many effectors has multiple and overlapping roles in subverting host cell processes (12, 19). Further research should elucidate the mechanisms by which these Shiga toxins are able to suppress Stat-1 tyrosine phosphorylation. Such information then could be used to restore host signaling responses to EHEC infection and thereby reduce the severity and spread of this infectious agent.

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REFERENCES

1. Abe H, Tatsuno I, Tobe T, Okutani A, Sasakawa C. 2002. Bicarbonate ion stimulates the expression of locus of enterocyte effacement-encoded genes in enterohemorrhagic *Escherichia coli* O157:H7. *Infect. Immun.* 70:3500–3509.
2. Atalla HN, Johnson R, McEwen S, Usborne RW, Gyles CL. 2000. Use of a Shiga toxin (Stx)-enzyme-linked immunosorbent assay and immunoblot for detection and isolation of Stx-producing *Escherichia coli* from naturally contaminated beef. *J. Food Prot.* 63:1167–1172.
3. Averbuch D, Chappier A, Boisson-Dupuis S, Casanova JL, Engelhard D. 2011. The clinical spectrum of patients with deficiency of signal transducer and activator of transcription-1. *Pediatr. Infect. Dis. J.* 30:352–355.
4. Bellmeyer A, et al. 2009. Enterohemorrhagic *Escherichia coli* suppresses inflammatory response to cytokines and its own toxin. *Am. J. Physiol. Gastrointest. Liver Physiol.* 297:G576–581.
5. Bielaszewska M, et al. 2011. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *Lancet Infect. Dis.* 11:671–676.
6. Ceponis PJ, McKay DM, Ching JC, Pereira P, Sherman PM. 2003. Enterohemorrhagic *Escherichia coli* O157:H7 disrupts Stat1-mediated gamma interferon signal transduction in epithelial cells. *Infect. Immun.* 71:1396–1404.
7. Chappier A, et al. 2006. Human complete Stat-1 deficiency is associated with defective type I and II IFN responses *in vitro* but immunity to some low virulence viruses *in vivo*. *J. Immunol.* 176:5078–5083.
8. Chen J, Johnson R, Griffiths M. 1998. Detection of verotoxigenic *Escherichia coli* by magnetic capture-hybridization PCR. *Appl. Environ. Microbiol.* 64:147–152.
9. Ching JC, Jones NL, Ceponis PJ, Karmali MA, Sherman PM. 2002. *Escherichia coli* Shiga-like toxins induce apoptosis and cleavage of poly-(ADP-ribose) polymerase via *in vitro* activation of caspases. *Infect. Immun.* 70:4669–4677.
10. Croxen MA, Finlay BB. 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. *Nat. Rev. Microbiol.* 8:26–38.
11. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97:6640–6645.
12. Dean P, Kenny B. 2009. The effector repertoire of enteropathogenic *E. coli*: ganging up on the host cell. *Curr. Opin. Microbiol.* 12:101–109.
13. Didcock L, Young DF, Goodbourn S, Randall RE. 1999. The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. *J. Virol.* 73:9928–9933.

14. DuPont HL. 2009. Clinical practice. Bacterial diarrhea. *N. Engl. J. Med.* 361:1560–1569.
15. Dupuis S, et al. 2003. Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. *Nat. Genet.* 33:388–391.
16. Frank C, et al. 2011. Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany—preliminary report. *N. Engl. J. Med.* 365:1771–1780.
17. Golan L, Gonen E, Yagel S, Rosenshine I, Shpigiel NY. 2011. Enterohemorrhagic *Escherichia coli* induce attaching and effacing lesions and hemorrhagic colitis in human and bovine intestinal xenograft models. *Dis. Model Mech.* 4:86–94.
18. Hale L, Lazos O, Haines A, Thomas C. 2010. An efficient stress-free strategy to displace stable bacterial plasmids. *Biotechniques* 48:223–228.
19. Hamada D, Hamaguchi M, Suzuki KN, Sakata I, Yanagihara I. 2010. Cytoskeleton-modulating effectors of enteropathogenic and enterohemorrhagic *Escherichia coli*: a case for EspB as an intrinsically less-ordered effector. *FEBS J.* 277:2409–2415.
20. Higgins LM, et al. 1999. Role of bacterial intimin in colonic hyperplasia and inflammation. *Science* 285:588–591.
21. Ho NK, Crandall I, Sherman PM. 2012. Identifying mechanisms by which *Escherichia coli* O157:H7 subverts interferon-gamma mediated signal transducer and activator of transcription-1 activation. *PLoS One* 7:e30145. doi:10.1371/journal.pone.0030145.
22. Holmes A, Muhlen S, Roe AJ, Dean P. 2010. The EspF effector, a bacterial pathogen's Swiss army knife. *Infect. Immun.* 78:4445–4453.
23. Huang A, et al. 1986. Cloning and expression of the genes specifying Shiga-like toxin production in *Escherichia coli* H19. *J. Bacteriol.* 166:375–379.
24. Jandu N, et al. 2006. Conditioned medium from enterohemorrhagic *Escherichia coli*-infected T84 cells inhibits signal transducer and activator of transcription 1 activation by gamma interferon. *Infect. Immun.* 74:1809–1818.
25. Jandu N, et al. 2009. Enterohemorrhagic *Escherichia coli* O157:H7 gene expression profiling in response to growth in the presence of host epithelia. *PLoS One* 4:e4889. doi:10.1371/journal.pone.0004889.
26. Jandu N, et al. 2007. Multiple seropathotypes of verotoxin-producing *Escherichia coli* (VTEC) disrupt interferon-gamma-induced tyrosine phosphorylation of signal transducer and activator of transcription (Stat)-1. *Microb. Pathog.* 42:62–71.
27. Jones RM, Neish AS. 2011. Recognition of bacterial pathogens and mucosal immunity. *Cell. Microbiol.* 13:670–676.
28. Karmali MA, et al. 2003. Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *J. Clin. Microbiol.* 41:4930–4940.
29. Kuijper EJ, Soonawala D, Vermont C, van Dissel JT. 2011. Household transmission of haemolytic uraemic syndrome associated with *Escherichia coli* O104:H4 in the Netherlands, May 2011. *Euro Surveill.* 16:pii:19897.
30. Landoni VI, et al. 2010. Shiga toxin 1-induced inflammatory response in lipopolysaccharide-sensitized astrocytes is mediated by endogenous tumor necrosis factor alpha. *Infect. Immun.* 78:1193–1201.
31. Lingwood CA, et al. 1987. Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin *in vitro*. *J. Biol. Chem.* 262:8834–8839.
32. Long KZ, et al. 2010. Associations between mucosal innate and adaptive immune responses and resolution of diarrheal pathogen infections. *Infect. Immun.* 78:1221–1228.
33. Maluykova I, et al. 2008. Latrunculin B facilitates Shiga toxin 1 transcellular transcytosis across T84 intestinal epithelial cells. *Biochim. Biophys. Acta* 1782:370–377.
34. Nandan D, Reiner NE. 1995. Attenuation of gamma interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with *Leishmania donovani*: selective inhibition of signaling through Janus kinases and Stat1. *Infect. Immun.* 63:4495–4500.
35. Obrig TG. 2010. *Escherichia coli* Shiga toxin mechanisms of action in renal disease. *Toxins (Basel)* 2:2769–2794.
36. Pedron T, Sansonetti P. 2008. Commensals, bacterial pathogens and intestinal inflammation: an intriguing menage a trois. *Cell Host Microbe* 3:344–347.
37. Peterson MD, Mooseker MS. 1992. Characterization of the enterocyte-like brush border cytoskeleton of the C2BBE clones of the human intestinal cell line, Caco-2. *J. Cell Sci.* 102:581–600.
38. Philpott DJ, McKay DM, Sherman PM, Perdue MH. 1996. Infection of T84 cells with enteropathogenic *Escherichia coli* alters barrier and transport functions. *Am. J. Physiol.* 270:G634–645.
39. Rasko DA, et al. 2011. Origins of the *E. coli* strain causing an outbreak of hemolytic-uremic syndrome in Germany. *N. Engl. J. Med.* 365:709–717.
40. Rietschel ET, Brade H. 1992. Bacterial endotoxins. *Sci. Am.* 267:54–61.
41. Rietschel ET, et al. 1993. The chemical structure of bacterial endotoxin in relation to bioactivity. *Immunobiology* 187:169–190.
42. Riff JD, Callahan JW, Sherman PM. 2005. Cholesterol-enriched membrane microdomains are required for inducing host cell cytoskeleton rearrangements in response to attaching-effacing *Escherichia coli*. *Infect. Immun.* 73:7113–7125.
43. Saha B, Jyothi Prasanna S, Chandrasekar B, Nandi D. 2010. Gene modulation and immunoregulatory roles of interferon gamma. *Cytokine* 50:1–14.
44. Schroder K, Hertzog PJ, Ravasi T, Hume DA. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* 75:163–189.
45. Shea-Donohue T, Fasano A, Smith A, Zhao A. 2010. Enteric pathogens and gut function: role of cytokines and STATs. *Gut Microbes* 1:316–324.
46. Spears KJ, Roe AJ, Gally DL. 2006. A comparison of enteropathogenic and enterohaemorrhagic *Escherichia coli* pathogenesis. *FEMS Microbiol. Lett.* 255:187–202.
47. Tatsuno I, et al. 2000. Isolation and characterization of mini-Tn5Km2 insertion mutants of enterohemorrhagic *Escherichia coli* O157:H7 deficient in adherence to Caco-2 cells. *Infect. Immun.* 68:5943–5952.
48. Tesh VL, Ramegowda B, Samuel JE. 1994. Purified Shiga-like toxins induce expression of proinflammatory cytokines from murine peritoneal macrophages. *Infect. Immun.* 62:5085–5094.
49. Wong AR, et al. 2011. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: even more subversive elements. *Mol. Microbiol.* 80:1420–1438.
50. Ziebell K, et al. 2008. Genotypic characterization and prevalence of virulence factors among Canadian *Escherichia coli* O157:H7 strains. *Appl. Environ. Microbiol.* 74:4314–4323.