Activation of the Classical Mitogen-Activated Protein Kinases Is Part of the Shiga Toxin-Induced Ribotoxic Stress Response and May Contribute to Shiga Toxin-Induced Inflammation

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Infection with enterohemorrhagic *Escherichia coli* (EHEC) can result in severe disease, including hemorrhagic colitis and the hemolytic uremic syndrome. Shiga toxins (Stx) are the key EHEC virulence determinant contributing to severe disease. Despite inhibiting protein synthesis, Shiga toxins paradoxically induce the expression of proinflammatory cytokines from various cell types *in vitro*, including intestinal epithelial cells (IECs). This effect is mediated in large part by the ribotoxic stress response (RSR). The Shiga toxin-induced RSR is known to involve the activation of the stress-activated protein kinases (SAPKs) p38 and JNK. In some cell types, Stx also can induce the classical mitogen-activated protein kinases (MAPKs) or ERK1/2, but the mechanism(s) by which this activation occurs is unknown. In this study, we investigated the mechanism by which Stx activates ERK1/2s in IECs and the contribution of ERK1/2 activation to interleukin-8 (IL-8) expression. We demonstrate that Stx1 activates ERK1/2 in a biphasic manner: the first phase occurs in response to StxB1 subunit, while the second phase requires StxA1 subunit activity. We show that the A subunit-dependent ERK1/2 activation is mediated through ZAK-dependent signaling, and inhibition of ERK1/2 activation via the MEK1/2 inhibitors U0126 and PD98059 results in decreased Stx1-mediated IL-8 mRNA. Finally, we demonstrate that ERK1/2 are activated *in vivo* in the colon of Stx2-intoxicated infant rabbits, a model in which Stx2 induces a primarily neutrophilic inflammatory response. Together, our data support a role for ERK1/2 activation in the development of Stx-mediated intestinal inflammation.

Infection of humans with enterohemorrhagic *Escherichia coli* (EHEC) can cause severe disease, including hemorrhagic colitis and the hemolytic uremic syndrome (HUS) (1). As EHEC-associated disease usually is not accompanied by bacteremia (2), EHEC strains are thought to be noninvasive, with infection limited to the intestinal lumen and mucosa. Therefore, the systemic uptake of Shiga toxin (Stx) from the intestine during the diarrheal phase of illness may be the key event promoting systemic disease, including HUS. However, our understanding of exactly how and where Stx exerts its pathogenic effects in the human host is incomplete.

Shiga toxins are ribosome-inactivating proteins (RIPs) that damage 28S rRNA, thereby inhibiting new protein synthesis (reviewed in Vanaja et al. [3]). Stxs are approximately 70-kDa protein complexes comprised of one enzymatically active (ribosome depurinating) A subunit (~32 kDa) and five B subunits (~7.7 kDa each). The holotoxin binds via a B subunit pentamer to the glycosphingolipid receptor globotriaosylceramide (Gb₃) located on the plasma membrane of target cells, resulting in toxin internalization, retrograde trafficking through the Golgi apparatus to the endoplasmic reticulum, and translocation of the enzymatically active A subunit to the cytoplasm, where it cleaves a specific adenine from the 28S rRNA, thus inactivating the ribosome.

Stx and other toxins that damage the 28S rRNA also can induce the ribotoxic stress response, or RSR. The ribotoxic stress response originally was defined as the activation of c-jun N-terminal kinases (JNK) by certain agents that damage the 28S rRNA, including ricin, anisomycin, and alpha-sarcin (4), but the RSR also may include the activation of other mitogen-activated protein kinases (MAPKs), including p38 and the extracellular receptor kinases 1

and 2 (ERK1/2) (5–7). It should be noted that protein synthesis itself does not appear to be the key stimulus, as not all protein synthesis inhibitors activate the RSR. Furthermore, in order for the ribotoxic stress response to occur, ribosomes must be actively engaged in protein synthesis, as pretreatment with protein synthesis inhibitors such as emetine, a protein synthesis inhibitor that inactivates ribosomes by stabilizing the 80S polysome (8), or pactamycin, which blocks translational initiation, prevents the RSR (4, 9). Concomitant with the Stx-induced RSR is an upregulation of proinflammatory mRNAs, as well as a paradoxical expression of proinflammatory cytokine proteins, and it is well established that blocking host MAPKs blocks Stx-induced proinflammatory cytokine expression (6, 10–14).

Different groups (including ours) have observed Stx-induced ERK1/2 activation (12–15), but the mechanism(s) by which this occurs has not been studied in depth, and the potential contribu-

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tion of ERK1/2 signaling to disease has not been well studied. Some cell lines respond to Stx with ERK1/2 activation, but others do not (D. M. Jandhyala and C. M. Thorpe, unpublished data) (6, 15, 16). The extent to which Stx-induced ERK1/2 activation contributes to proinflammatory cytokine expression in the intestinal epithelia, or whether Stx-induced ERK1/2 activation is even part of the ribotoxic stress response, is not known but may be relevant in assessing the benefit of specific anti-inflammatory strategies in HUS prevention. Here, we investigate the roles of Stx binding and catalytic activity in ERK1/2 activation *in vitro* and *in vivo* and evaluate the contribution of Stx-induced ERK1/2 activation to interleukin-8 (IL-8) mRNA expression in intestinal epithelial cells (IECs). We also explore upstream signaling events leading to Stx-associated ERK1/2 activation, including protein synthesis inhibition, activation of mTOR (mammalian target of rapamycin), and signaling through the mitogen activated protein 3-kinase (MAP3K) ZAK.

MATERIALS AND METHODS

Cell culture, materials, and solutions. The human colonic epithelium-derived HCT-8 cell line was obtained from the American Type Culture Collection and cultured at 37°C in 5% CO₂ in RPMI 1640 medium with L-glutamine, 10% fetal bovine serum, 100 U of penicillin G sodium per ml, 100 µg of streptomycin sulfate per ml, 1 mM sodium pyruvate, and 10 mM HEPES, as previously described (11). Cell culture media and additives were from Invitrogen/Life Technologies (Grand Island, NY). QIAshredder cell homogenization spin columns and RNeasy kits were from Qiagen (Valencia, CA). Northern blotting glyoxal gel preparation, transfer reagents, and nylon membranes were from Ambion, Inc. (Austin, TX). [³H]leucine and [α-³²P]dCTP were from New England Nuclear (Boston, MA). Bio-Rad protein assay reagent was from Bio-Rad (Hercules, CA). Immobilion-P transfer membranes used for Western blotting were from Millipore (Billerica, MA). Dithiothreitol (DTT), β-glycerol phosphate, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and sodium orthovanadate were obtained from Sigma Chemical (St. Louis, MO). The MEK1/2 inhibitors PD98059 and U0126 were from Calbiochem (La Jolla, CA), and rapamycin was from Cell Signaling Technology (Beverly, MA). DHP-2 was synthesized by Albany Molecular Research Inc. (Albany, NY).

Production and use of wild-type and mutant Shiga toxins. Wild-type Stx1, StxA_{Y77S,E167Q}B1, Stx2, StxA_{Y77S,E167Q}B2, and StxB1 were prepared by the Phoenix Laboratory at Tufts Medical Center (Boston, MA) using receptor analog affinity chromatography as previously described (17, 18). To heat inactivate wild-type holotoxin, an aliquot of active reconstituted toxin was set aside and a paired sample was inactivated by boiling in a water bath for 8 h. Boiled toxin was checked for biological activity and did not inhibit [³H]leucine incorporation in HCT-8 cells (11). The expression plasmids encoding StxA_{Y77S,E167Q}B1 (pSW09) and StxA_{Y77S,E167Q}B2 (pNR100) were provided by Tom O'Brig of the University of Maryland (Baltimore, MD); the plasmids were constructed in the laboratory of Alison O'Brien at the Uniformed Services University of the Health Sciences (Bethesda, MD) as previously described (18). All toxin/toxoid-producing bacterial strains were deposited in the Phoenix Laboratory. The concentration of Shiga toxins employed in HCT-8 studies (i.e., 1 µg/ml) is based on demonstrated effects of this concentration on [³H]leucine incorporation assays, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell viability assays, and IL-8 enzyme-linked immunosorbent assay (ELISA) as previously published (11, 19).

RNA isolation and Northern blotting. Following cell treatments, HCT-8 cells were disrupted using QIAshredders, and total RNA was prepared using RNeasy RNA purification kits according to the manufacturer's instructions (Qiagen, Valencia, CA). For Northern blotting, 10 to 30 µg of total RNA was separated on a glyoxyl agarose gel and transferred to a nylon membrane according to the manufacturer's instructions (Ambion). [α-³²P]dCTP-labeled *il-8* and *gapdh* probes were generated and

TABLE 1 shRNA constructs used in this study

shRNA name/target	Clone ID ^a	Sequence
<i>zak</i> 69	V3LHS_336769	TCTCGTTCTTTAAGCTCCT
<i>gapdh</i>	RHS4371	CTCATTTCTCGGTATGACA

^a GE Healthcare/Dharmacon clone identifier (ID).

used as previously described (11). The blots were hybridized overnight at 65°C and washed using phosphate-buffered hybridization solutions and wash solutions (20), and hybridized probe was detected via exposure to film.

Preparation of whole-cell extracts, MAPK immunoprecipitation assays, and Western blotting. Following treatment and removal of cell culture media, cell culture plates were transferred to ice, and cells were washed with cold phosphate-buffered saline (PBS) and then scraped off the culture dishes using cold PBS containing 10 µg/ml leupeptin, 1 mM PMSF, and 0.5 mM DTT. Cells were pelleted by centrifugation at 4°C, the supernatant was removed, and pelleted cells were resuspended in lysis buffer consisting of 0.1% Triton X-100, 25 mM HEPES (pH 7.5), 300 mM NaCl, 1.5 mM MgCl₂, 200 µM EDTA (pH 8.0), 0.1 mM Na₃VO₄, 20 mM β-glycerol phosphate, 10 µg/ml leupeptin, 1 mM PMSF, and 0.5 mM DTT. The resuspended cells were gently rocked at 4°C for 30 min; cellular debris was removed by centrifugation at 4°C, and supernatants were collected and frozen at -80°C. Protein concentrations of cell extracts were estimated using Bio-Rad (Hercules, CA) protein assay reagent according to the manufacturer's instructions.

For loading whole-cell lysates onto SDS-PAGE gels for Western blotting, equal amounts of protein were added to each lane. The non-radioactive MAPK precipitation assay kit 8794s or 9800 (Cell Signaling Technology, Beverly, MA) was used to assay phosphorylated p44/42 (phospho-ERK1/2) or phosphorylated JNK (phospho-JNK), respectively. Assays were performed per the manufacturer's protocol, except that we used the previously described lysis buffer instead of that supplied by the kit. Briefly, for each sample, phospho-ERK1/2 or phospho-JNK were immunoprecipitated from whole-cell lysate equivalent to 200 µg total protein. Once washed, the samples were treated with kinase buffer containing 0.5 µg kinase substrate (Elk-1 fusion protein for phospho-ERK1/2 or a c-jun fusion protein for phospho-JNK) and 200 µM ATP, followed by incubation at 30°C for 30 min. Western blots were used to visualize relative amounts of phosphorylated substrate, an indicator of phospho-ERK1/2 or phospho-JNK activity. Antibodies used for other Western blot experiments or as loading controls included phosphorylated p44/42 (no. 9101), p44/42 (no. 9102), and phosphorylated mTOR (no. 2971) from Cell Signaling Technology, SC25778 anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody from Santa Cruz Biotechnology Inc. (Dallas, TX), and A2228-200UL anti-β-actin from Sigma-Aldrich (St. Louis, MO).

Lentiviral shRNA knockdown. *gapdh* and *zak* short hairpin RNA (shRNA)-expressing plasmids (pGipZ) and corresponding lentiviral packaging systems were purchased from Thermo Scientific (now sold by GE Healthcare/Dharmacon) and transfected along with helper plasmids into HEK293T cells (HCL4517; Thermo Scientific) according to the manufacturer's protocol. shRNA constructs used in this study are shown in Table 1.

Resulting lentivirus-containing supernatants were used to transduce HCT-8 cells according to the manufacturer's protocols. In brief, the night before transduction, HCT-8 cells were plated on 10-cm tissue culture plates at cell densities of 0.8 × 10⁶ to 3.2 × 10⁶. The following day, the culture medium of each plate was replaced with 3 ml of HCT-8 medium without antibiotics and FBS and mixed with 1 ml of nonconcentrated lentiviral particles and 4 µl of 8 mg/ml Polybrene (final concentration of 8 µg/ml Polybrene). Cells then were incubated for 4 h, after which media containing the lentiviral particles was replaced with 10 ml of fresh growth media. Transductants produced green fluorescent protein (GFP) that was

detected by fluorescence microscopy. Forty-eight hours posttransduction, growth media was removed and transduced cell populations were selected by the addition of fresh media supplemented with 7 $\mu\text{g/ml}$ puromycin (for more stringent selection, we increased the puromycin concentration to 10 $\mu\text{g/ml}$). Semiclonal populations were isolated and plated in the absence of puromycin prior to treatment with Stx. Knockdown was verified by quantitative real-time PCR (qRT-PCR) (see Fig. 5A).

qRT-PCR. Purification of RNA was performed using the Qiagen (Valencia, CA) RNeasy plus minikit (no. 74134) according to the manufacturer's instructions. qRT-PCR was performed on RNA using Power SYBR green RNA-to- C_T (no. 4389986; Thermo Fisher Scientific, Grand Island, NY). All reactions were performed on a CFX Connect real-time system (Bio-Rad, Hercules, CA). Five nanograms of total cellular RNA was used for each reaction. Qiagen Quantitect primer assays (QT00058282, QT01192646, and QT00095431) were used to quantitate the expression of *zak*, *gapdh*, and β -*actin*, respectively.

Stx2 intoxication of infant rabbits and immunohistochemistry for phospho-ERK1/2. All animal experiments were preapproved by our institutional animal care and use committee. The treatment of infant rabbits was performed as previously described (21). Briefly, 3-day-old rabbits were given Stx2, StxA_{Y77S, E167Q}B2, or heat-inactivated Stx2 intragastrically at a dose of 1.0 mg/kg of body weight daily for 2 consecutive days. Initial treatment was noted as day 0, and on day 2, the infant rabbits underwent euthanasia and necropsy. Distal colon tissue was fixed in 10% buffered formalin followed by embedding in paraffin. Five-micrometer-thick sections were deparaffinized and then hydrated. Microwave antigen retrieval was performed at pH 10.00 using an antigen retrieval solution (Dako, Carpinteria, CA). Sections were blocked for endogenous peroxidase activity using 3% H_2O_2 - H_2O . To prevent nonspecific protein binding, a serum-free protein block (Dako) was used. Sections then were incubated overnight at 4°C with antibody against phosphorylated ERK1/2 (no. 7383; Santa Cruz Biotechnology, Dallas, TX), followed by incubation with biotinylated anti-mouse secondary antibody. Following treatment with peroxidase-conjugated streptavidin (Dako), antibody binding was detected by using the 3-amino-9-ethylcarazole peroxidase substrate chromogen. Sections then were counterstained with Mayer's hematoxylin. The specificity of staining was confirmed by omitting the primary antibody (negative controls).

RESULTS

Shiga toxin 1 induces ERK1/2 in a biphasic manner in IECs. Previously, we reported that Stx1-induced ERK1/2 activation in HCT-8 cells contributed to the phosphorylation of the eukaryotic initiation factor eIF4E and, as a result, translational reprogramming (12). However, in that study we did not further characterize the ERK1/2 activation by Stx1. Here, we show the effects of Stx1 holotoxin on ERK1/2 phosphorylation for a period of up to 6 h posttreatment. Whole-cell lysates from untreated HCT-8 intestinal epithelial cells, or cells treated with Stx1 for 15 min to 6 h, were immunoblotted to detect phosphorylated ERK1/2. Results of a representative experiment are shown in Fig. 1. Stx1 induced ERK1/2 in a biphasic manner. ERK1/2 activation was seen as early as 15 min following Stx1 treatment (Fig. 1). This early induction was transient, returning to baseline levels by 45 min, and was not consistently observed in all experiments. ERK1/2 activation consistently recurred at approximately 1 h postexposure and was sustained for a longer period. We next tested the StxB1 subunit and the catalytically inactive mutant StxA_{Y77S, E167Q}B1 to determine the relative contributions of binding and *N*-glycosidase activity to Stx1-induced ERK1/2 activation. In this experiment, as an alternative to probing immunoblots directly to detect phosphorylated ERK1/2, we immunoprecipitated phosphorylated ERK1/2 and then used it to phosphorylate ERK1/2 substrate Elk-1 fusion pro-

FIG 1 Western blot for phosphorylated ERK1/2 in whole-cell lysates of HCT-8 cells treated with Stx1 for zero minutes (C_0) to 6 h. Lanes are labeled C (untreated), S_1 (Stx1), or HI (heat-inactivated Stx1). Ladder refers to the molecular mass ladder. Anti-total ERK1/2 (α -ERK1/2) were used for loading controls. The graph displays the relative phospho-ERK1/2 band intensity normalized to the average from all untreated controls (no treatment). The error bar represents the standard deviations for the untreated controls. Stx1 and heat-inactivated Stx1 were used at a final concentration of 1 $\mu\text{g/ml}$.

tein. We then performed a Western blot on the reaction mixture and probed for phosphorylated Elk-1 fusion protein as an indicator of ERK1/2 activation. This indirect method is a more sensitive indicator of ERK1/2 activation. As with Stx1 holotoxin treatment, an early, transient ERK1/2 activation also was detected following treatment with only purified StxB1 subunit (Fig. 2A). However, the later and sustained ERK1/2 activation required Stx1 catalytic activity: unlike Stx1 wild-type toxin, the *N*-glycosidase mutant StxA_{Y77S, E167Q}B1 (18) did not induce ERK1/2 phosphorylation at 6 h (Fig. 2B). As an additional positive control, cells were treated for 30 min with fresh media containing 60% FBS to induce ERK1/2 activation, or as a negative control, cells were treated for 30 min with fresh media containing 60% FBS plus the MEK1/2 inhibitor U0126 (Fig. 2B, lanes labeled GF₃₀ and U0126 + GF₃₀, respectively).

Other protein synthesis inhibitors, some with differing modes of action, also induce ERK1/2 in IECs. The stimulus re-

FIG 2 (A) Western blot of phosphorylated Elk-1 fusion protein as an indicator of ERK1/2 activity in immunoprecipitates from whole-cell lysates of HCT-8 cells treated from 5 min to 60 min with Stx1, StxB1 subunit only, heat-inactivated Stx1 (HI), or the ribotoxic stressor anisomycin (500 ng/ml). Briefly, phosphorylated-ERK1/2 was immunoprecipitated from 200 μ g of whole-cell lysate. To each immunoprecipitation sample, buffer containing 0.5 μ g Elk-1 fusion protein (i.e., ERK1/2 substrate) and 200 μ M ATP was added, followed by incubation at 30°C for 30 min. (B) Western blot of phosphorylated ERK1/2 in whole-cell lysates from untreated samples (C) or samples treated with Stx1 (S) or StxA_{Y77S,E167Q}B1 (M). Growth medium was replaced with fresh medium containing 60% FBS (GF₃₀) as a positive control. Cells were treated with the MEK1/2 inhibitor U0126 (10 μ M) and placed in fresh medium containing 60% fresh FBS as a negative control. Cells were incubated for 6 h posttreatment for samples C, S, and M and for 30 min posttreatment for samples GF₃₀ and U0126 plus GF₃₀. Graphs beneath blots show the relative band intensity for corresponding blot lanes. Stx1, mutant, and HI treatments were applied at a final protein concentration of 1 μ g/ml for all experiments.

sulting in the RSR has been demonstrated to not be protein synthesis inhibition specifically but instead disruption or damage to the 28S rRNA of actively translating ribosome (4). To determine whether Stx was activating ERK1/2 through the RSR, we compared ERK1/2 activation by ricin, puromycin, and emetine to assess whether ERK1/2 activation is restricted to protein synthesis inhibitors causing 28S rRNA damage or, in contrast, could result from protein synthesis inhibition *per se*, irrespective of the mechanism by which protein synthesis is abrogated. We compared Stx1, ricin (both of which are known ribotoxic stressors depuri-

nating 28S rRNA at the same site), puromycin (which promotes the premature release of the nascent polypeptide chain [9] and does not induce a ribotoxic stress response in Rat-1 cells [4]), and emetine (which halts protein elongation, possibly through interactions with the 40S ribosomal subunit at the E site) (8, 22). Concentrations used are noted in the legend to Fig. 3, and all were above the approximate 50% inhibitory concentration (IC₅₀) as determined by measuring protein synthesis via [³H]leucine incorporation (data not shown). Interestingly, as with Stx1, puromycin and ricin demonstrated sustained ERK1/2 activation (Fig. 3A),

FIG 3 (A) Western blot for phosphorylated ERK1/2 in whole-cell lysates from HCT-8 cells treated with 300 μg/ml puromycin (P) or 10 ng/ml ricin (R) for 2, 4, and 6 h or Stx1 for 4 h. As a positive control, media were replaced with fresh media containing 60% FBS (GF₃₀). (B) Western blot for phosphorylated mTOR in whole-cell lysates from HCT-8 cells treated with 1 μg/ml Stx1 following a pretreatment with vehicle (dimethyl sulfoxide [DMSO]) or the MEK1/2 inhibitor U0126 (10 μM) or PD98059 (20 μM). (C) Western blot for Phospho-ERK1/2 in whole-cell HCT-8 lysates with no pretreatment, pretreatment for 1 h with rapamycin (10 nM), or pretreatment with the MEK1/2 inhibitor U0126 (10 nM) for 1 h followed by no treatment or treatment with Stx1 (1 μg/ml) for 6 h.

but emetine at 100 ng/ml showed no sustained ERK1/2 activation (see Fig. S1 in the supplemental material). However, very strong ERK1/2 activation was noted following treatment with a 1,000-fold higher dose (100 μg/ml) of emetine (data not shown). Thus, the ability to activate ERK1/2 in HCT-8 cells appeared not to be limited to only classic ribotoxic stressors.

Stx-induced ERK1/2 activation is unaffected by mTOR. Regulation of Stx1-induced cytokine production by phosphatidylinositol 3-kinase/Akt/mTOR signaling has been observed in macrophage-like THP-1 cells (23). However, direct activation (phosphorylation) of mTOR by Stx1 treatment was not investigated. Furthermore, cross talk between ERK1/2- and mTOR-associated signaling pathways may result in one pathway inhibiting or activating the other (24). Since puromycin and emetine, non-ribotoxic stressors (4), are able to induce ERK1/2 activation (Fig. 3A and data not shown) and mTOR can act as a sensor of amino acid accumulation (25), we hypothesized that Stx1-induced protein synthesis inhibition results in amino acid accumulation with subsequent mTOR phosphorylation and possible cross talk with ERK1/2 pathways. Figure 3B shows that Stx1 treatment results in mTOR phosphorylation, and that the inhibition of ERK1/2 activation with the MEK1/2 inhibitor U0126 or PD98059 did not prevent Stx1-mediated activation of mTOR. Furthermore, blocking the mTOR pathway with rapamycin had no effect on Stx1-induced activation of ERK1/2 (Fig. 3C). Pretreatment of cells with U0126 (Fig. 3C) was used as an additional negative control to demonstrate blocking of Stx1-induced ERK1/2 phosphorylation. Together, these data suggest that Stx1-mediated ERK1/2 activation and mTOR activation are distinct in this model system.

Stx-induced ERK1/2 activation is dependent on the MAP3K ZAK. We previously identified the mixed-lineage kinase ZAK as a MAP3K that transduces the Stx- and ricin-induced ribotoxic stress responses, resulting in p38 and JNK activation (19). Therefore, we tested the hypothesis that Stx1- and ricin-induced sustained ERK1/2 activation is mediated by ZAK and can be consid-

ered part of the ribotoxic stress response to these agents. After pretreatment with the ZAK inhibitor DHP-2, cells were exposed to Stx1 or ricin for 4 h. DHP-2 blocked both Stx1- and ricin-induced ERK1/2 activation (Fig. 4A). As Stx2 is more often associated with severe EHEC-associated disease than Stx1 and, as is the case for ricin and Stx1, Stx2 damages ribosomes via depurination of the same site on the sarcin/ricin loop of the 28S rRNA, we tested (i) whether Stx2 activates ERK1/2 and, if so, (ii) whether inhibition of ZAK blocks this ERK1/2 activation. As with Stx1 and ricin, Stx2 activated ERK1/2 (Fig. 4B), and DHP-2 pretreatment blocked this activation by Stx2.

DHP-2 has been shown to have a high specificity for ZAK (26). However, we sought to verify that ZAK was indeed the signaling intermediary required for ERK1/2 activation by Stx. While we have been able to detect ZAK by Western blotting in the Vero cell line, we have been unable to detect it with confidence in HCT-8 cells, possibly due to low-level expression in this cell line. Therefore, we employed a pGipZ lentiviral shRNA delivery system (GE Healthcare/Dharmacon) to generate a stable cell line that could be validated for *zak* knockdown by qRT-PCR. HCT-8 cells transduced with pGipZ expressing a *zak* shRNA (Fig. 5A) had decreased *zak* expression compared to that of HCT-8 cells expressing a *gapdh* shRNA or nontransduced HCT-8 cells. We evaluated whether the *zak* knockdown cell line showed a predicted decrease in the ribotoxic stress response, as measured by Stx2-mediated JNK activation (4, 19). Indeed, compared to HCT-8 cells expressing the *gapdh* shRNA, those expressing the pGipZ *zak* shRNA construct showed decreased JNK activation (Fig. 5B). As our *zak* shRNA-expressing pGipZ construct showed decreased *zak* expression resulting in a decreased Stx2-induced RSR, we evaluated the cell lysates for ERK1/2 activation. As with DHP-2-treated cells, HCT-8s expressing the *zak* shRNA construct showed decreased Stx2-induced ERK1/2 activation (Fig. 5C). Therefore, like Stx-induced JNK and p38 activation, Stx-induced ERK1/2 activation

FIG 4 Western blot of whole-cell lysates of HCT-8 cells pretreated with the vehicle DMSO (–) or the specific ZAK inhibitor DHP-2 at 200 nM (+), followed by treatment with Stx1 1 μ g/ml or ricin 100 ng/ml for 6 h (A) or Stx2 1 μ g/ml for 5 h (B). The graph beneath each blot shows the relative band intensity for corresponding lanes.

is part of the ribotoxic stress response and is mediated through ZAK signaling.

Stx1 induces IL-8 mRNA in an MEK1/2-dependent manner *in vitro*. We have previously shown that Stx induces IL-8, and that blocking ZAK or p38 decreases Stx-induced IL-8 gene expression and protein production in HCT-8 cells (11, 19, 27). Therefore, we assessed the contribution of Stx1-induced ERK1/2 activation to IL-8 mRNA expression. We used the small chemical inhibitors of MEK1/2, U0126 and PD98059, to block ERK1/2 activation. MEK1/2 inhibition with either U0126 or PD98059 resulted in the abrogation of Stx1-induced IL-8 mRNA expression (Fig. 6A).

Oral challenge with Stx2 in infant rabbits results in colonic epithelial ERK1/2 activation. Because the ERK1/2 pathways can be dysregulated in cancer cells (28), we also chose to assess whether ERK1/2 activation could be observed *in vivo* following oral administration of Stx2 in an infant rabbit model of intoxication. In this model, the administration of Stx2 by oral gavage at a dose of 100 μ g/kg to rabbits on days 3 and 4 of life results in mid-to distal colonic inflammation according to necropsy on day 5 (21, 29). Distal colonic tissue was obtained from animals intoxicated with Stx2, heat-inactivated (HI) Stx2, or the A subunit mutant StxA_{Y77S,E167Q}B2, fixed in formalin, paraffin embedded, cut, and then subjected to immunohistochemistry for phosphorylated ERK1/2, as outlined in Materials and Methods.

Immunohistochemistry slides were assessed by a veterinary pathologist. Representative section images are shown in Fig. 6B. As noted by brown staining, phosphorylated ERK1/2 are not readily noted in tissue from the HI-treated animal, slight staining can be seen in the section from the StxA_{Y77S,E167Q}B2-treated subject, and profound staining occurs in the sample from the Stx2-treated subject. Together, these data suggest that Stx2 treatment results in increased phosphorylation of ERK1/2 *in vivo*.

DISCUSSION

MAPKs are evolutionarily conserved signaling molecules that influence eukaryotic cellular regulation in response to diverse stim-

uli, including that associated with environmental stress (e.g., osmotic stress and oxidative stress), with growth and differentiation (hormones and growth factors), and with exposure to pathogens. However, the majority of MAPK pathways identified are associated with stress and inflammatory stimuli as opposed to mitogens (30). MAPK activation can result in a variety of downstream processes, including transcriptional activation, chromatin remodeling, translational reprogramming, and cytoskeletal rearrangement (31). We have previously demonstrated that Stx-mediated activation of p38 and JNK in intestinal epithelial cells is mediated as part of the RSR (27) and is dependent on activation of the MAP3K ZAK (19).

While ERK1/2 activation by Stx1 previously has been demonstrated in macrophage-like THP-1 cells (14), in the ACHN renal tubule-derived cell line (32), and by members of our group in HCT-8 cells (12), the mechanism by which ERK1/2 activation occurred in these studies was not investigated further. *In vitro* studies have suggested that intestinal inflammation promotes the systemic uptake of Stx through disruption of the epithelial tight junction during neutrophil influx into the lumen (33). Since Stx has been shown to promote IL-8 production, a potent neutrophil chemoattractant, and MAPK activation, including that of ERK1/2, may effect IL-8 production through transcriptional and posttranscriptional mechanisms, we investigated the ERK1/2 activation by Stx to determine if it is mediated as part of the RSR and if it is associated with Stx-induced inflammation. Knowing if classical MAPKs also contribute to the proinflammatory effects of Stx may be important, as therapeutics selectively targeting only one subset of MAPKs may not be fully effective in limiting inflammation.

Based on serological profiling, Shiga toxins are classified into two main types, Stx1 and Stx2 (34). Within each type there are several subtypes, and for reasons not completely understood, EHEC strains that express only Stx2 are more likely to be associated with HUS and severe disease in humans than strains

FIG 5 (A) qRT-PCR for *zak* expression in HCT-8 cells that were not transduced or were transduced with the lentiviral vector pGipZ expressing shRNAs against *gapdh* or *zak* (*zak* 69). Expression was normalized to the expression of the gene for β -actin. Error bars represent the standard errors of the means (SEM). (B) Western blot of phosphorylated c-jun, which is indicative of JNK activation, in immunoprecipitates from HCT-8 cells transduced with pGipZ expressing shRNAs against *gapdh* or *zak* (*zak* 69) and following no treatment or treatment with 1 μ g/ml Stx2. (C) Phospho-ERK1/2 in whole-cell lysates from HCT-8 cells transduced with lentiviral pGipZ vectors expressing shRNAs for *gapdh* or *zak* (*zak* 69) following no treatment or treatment with 1 μ g/ml Stx2. The graph beneath each blot shows the relative band intensity for corresponding lanes.

encoding only Stx1 or both Stx1 and Stx2 (1). Supporting this epidemiology are studies that show Stx2 is more cytotoxic *in vitro* (35) and is associated with more severe pathology *in vivo* (36, 37) than Stx1.

We demonstrate that both StxA1 and StxB1 subunits can contribute to ERK1/2 activation in intestinal epithelial cells *in vitro*. In response to holotoxin, there is a biphasic activation of ERK1/2. Early ERK1/2 activation can be observed within minutes of toxin treatment but is not sustained and can be reproduced by treatment with the StxB1 subunit alone, suggesting that this early ERK1/2 activation is triggered by binding of the toxin to its cell surface receptor, Gb₃, and/or subsequent retrotranslocation to the Golgi apparatus. That the StxB subunit can promote cellular signaling is supported by studies of other cell types, demonstrating that the StxB subunit is sufficient for the activation of Syk tyrosine kinase in HeLa cells (38), increasing intracellular Ca^{2+} in BL-Ramos cells (39), activation of protein kinase C δ in HeLa cells (40), transiently activating p38 in HeLa and HEp-2 cells (41), and promoting von Willebrand factor secretion by human um-

bilical vein endothelial cells and inducing platelet adhesion following treatment of human glomerular microendothelial cells (42). While earlier studies have suggested that StxB1 subunit, StxB2 subunit, or anti-Gb₃ antibody can induce apoptotic signaling in BL-Ramos cells (39, 43), previous *in vivo* studies by our group suggest that N-glycosidase activity is critical to Stx2-mediated intestinal pathology in infant rabbits, although we have not investigated Stx1 in this model (21).

We also observed a later sustained activation of ERK1/2 by Stx1. Unlike early ERK1/2 activation, late activation (beginning 1 to 2 h posttreatment and sustained up to 6 h) required StxA subunit activity and was dependent on ZAK signaling. It should be noted that the timing of ERK1/2 activation parallels the timing of Stx-induced p38 and JNK activation (27) and protein synthesis inhibition (11). A similar ZAK-dependent ERK1/2 activation also was observed for Stx2 and ricin. Since we have previously shown that ZAK MAP3K activity occurs upstream of Stx2- and ricin-induced p38 and JNK activation (19), our current data suggest that, together with p38 and JNK activation, the late sustained

FIG 6 (A) Northern blot of IL-8 mRNA from HCT-8 cells following no treatment or treatment with 1 μ g/ml Stx1, StxA_{Y77S,E167Q}B1, StxB1 subunit, or 1 μ g/ml Stx1 following pretreatment with DMSO or the MEK1/2 inhibitor PD98059 (20 μ M) or U0126 (10 μ M). The graph beneath each blot shows the relative band intensity for corresponding lanes. (B) Immunohistochemistry for phosphorylated ERK1/2 in sections of distal colon from infant rabbits treated twice over 2 days with 1.0 mg/kg heat-inactivated Stx2, active-site mutant StxA_{Y77S,E167Q}B2, or wild-type Stx2.

ERK1/2 activation by Stx1, Stx2, and ricin are components of the ribotoxic stress response.

ZAK, also called AZK, MLK7, MRK, or MLTK, belongs to the family of mixed-lineage serine/threonine kinases. The kinase domains of this family contain subdomains, some of which resemble those of serine/threonine kinases, and some of which resemble those of receptor tyrosine kinases (44). The *zak* gene encodes two splice variants resulting in ~91-kDa and ~55-kDa isoforms termed ZAK- α and ZAK- β , respectively. Both isoforms share an identical N-terminal 329 amino acids which contain the kinase domain. Although we are able to do so in Vero cells (1), we have not been able to reliably detect either ZAK isoform in HCT-8 cells by Western blotting, possibly due to low *zak* expression in HCT-8 cells. As we do not know which isoform(s) is required for the ribotoxic stress response, we used shRNA to target both isoforms.

In addition to being activated by Stx- and ricin-mediated ribotoxic stress, ZAK signaling and/or expression also has been associated with cardiac hypertrophy (45, 46); G₂/M cell cycle arrest in response to gamma irradiation (47); metastasis (48); gastric, colon, and bladder cancers (49, 50); doxorubicin toxicity (51); anisomycin treatment; exposure to UV irradiation (26); osmotic shock (52); and oxidative stress (53), suggesting that ZAK transduces a number of different stress responses.

As EHEC strains are thought to be noninvasive (2), the onset of extraintestinal disease (e.g., HUS) requires the systemic uptake of Stx from the intestinal lumen. *In vitro* studies have demonstrated that the recruitment of neutrophils across epithelial tight junctions can promote the basolateral uptake of Stx (33). Members of our group have previously demonstrated *in vitro* in HCT-8 cells that despite the inhibition of protein synthesis, Stx paradoxically induces IL-8 production. Furthermore, oral Stx intoxication results in neutrophil influx in the colon of infant rabbits (10–12, 21). We demonstrate here that late RSR-dependent ERK1/2 activation is required for Stx1-mediated IL-8 expression. Stx previously has been shown to promote synergistic MAPK activation and superinduction of IL-8 when coadministered with other proinflammatory stimuli, such as tumor necrosis factor alpha (TNF- α) (11) and flagellin (54). Therefore, through the increased production of neutrophil chemoattractants such as IL-8, Stx-mediated ERK1/2 activation may act to promote the systemic uptake of Stx, leading to the onset of HUS.

That ERK1/2 also are activated *in vivo* in the infant rabbit further validates a role for this pathway in Stx-associated disease. However, a direct role for Stx in mediating damage to the human colonic epithelium remains controversial, as some reports have suggested that both normal and inflamed human colonic epithelium are devoid of the Stx receptor Gb₃ (55, 56). The colon of infant rabbits has been shown to contain Gb₃ (21), and in a more recent report, both Gb₃ and another Stx receptor, Gb₄, were detected on human colonic epithelium and HCT-8 cells (57). In this study, however, Gb₃ levels were found to be very low in both human colonic epithelium and in HCT-8 cells, with only ~0.01% of HCT-8 cells being positive for Gb₃. Conversely, Gb₄ was found to be abundant in both human tissue and HCT-8 cell culture. Together, these data lend support to the use of HCT-8 cells as a model for Stx-associated disease.

While Stx can induce IL-8 *in vitro* in HCT-8 cells (11), Miyamoto et al. demonstrated that the major EHEC determinant contributing to chemokine production in human intestinal ep-

FIG 7 (A) Shiga toxins depurinate the 28S rRNA subunit, which results in inhibition of protein synthesis (61). (B) Following Stx-mediated 28S rRNA damage on actively translating ribosomes, one or more isoforms of ZAK are activated. (C) Stx-activated ZAK results in activation of MAP2Ks that subsequently phosphorylate and activate JNK and p38 (19). (D) Phosphorylation of the MAP2Ks MEK1/2 results in activation of ERK1/2. (E) Activation of MAPKs ERK1/2, JNK, and p38 contributes to increased proinflammatory cytokine production (6, 10–14, 19, 27).

ithelium was flagellin (58), suggesting that Stx is not a major participant in promoting intestinal inflammation. However, Stx has been shown to act in synergy with other proinflammatory stimuli (11, 14, 54, 59, 60), so it is plausible that Stx exacerbates epithelial inflammation induced by other more potent EHEC virulence factors. Alternatively, Stx may not play a major role in damaging epithelial cells directly during EHEC infection; instead, Stx may intoxicate and activate ERK1/2 signaling in other cells of the human colon, including stromal cells, neuroendocrine cells, lamina propria endothelium, and pericyptal fibroblasts, on which Gb₃ surface expression has been demonstrated (55, 57).

In summary, Stx can activate ERK1/2 *in vitro* in human-derived intestinal epithelial cells, leading to the increased expression of IL-8 mRNA, and ERK1/2 activation can be detected *in vivo* in the infant rabbit colon following Stx2 exposure. In the infant rabbit model, Stx2 exposure alone results in diarrhea with a proinflammatory histopathology (21), and Stx2 contributes to the inflammation and diarrhea observed following exposure to EHEC (21, 29). Taken together, these data suggest a role for Stx-induced ERK1/2 activation in promoting inflammation. Mechanistically, sustained late ERK1/2 activation is StxA subunit dependent and requires MAP3K ZAK. Thus, along with the activation of the stress-activated protein kinases (SAPKs) p38 and JNK (Fig. 7), Stx-induced ERK1/2 activation can be considered part of the ribotoxic stress response. Blocking the effects of Stx on proinflammatory pathways may be beneficial in preventing the late sequelae of EHEC-associated infection by preventing inflammation-induced intestinal damage and subsequent systemic uptake of Stx. Finally, therapeutic modalities aimed at inhibiting the proinflammatory effects of Stx through targeting ZAK may be more efficacious than those targeting individual downstream MAPK/SAPKs.

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REFERENCES

- Paton JC, Paton AW. 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin Microbiol Rev* 11:450–479.
- Tarr PI, Gordon CA, Chandler WL. 2005. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* 365:1073–1086.
- Vanaja SK, Jandhyala DM, Mallick EM, Leong JM, Balasubramanian S. 2013. Enterohemorrhagic and other Shiga-toxin-producing *Escherichia coli*. In: Donnenberg MS (ed), *Escherichia coli*, pathotypes and principles of pathogenesis, 2nd ed. Elsevier, Boston, MA.
- Iordanov MS, Pribnow D, Magun JL, Dinh TH, Pearson JA, Chen SL, Magun BE. 1997. Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. *Mol Cell Biol* 17:3373–3381. <http://dx.doi.org/10.1128/MCB.17.6.3373>.
- Iordanov MS, Pribnow D, Magun JL, Dinh TH, Pearson JA, Magun BE. 1998. Ultraviolet radiation triggers the ribotoxic stress response in mammalian cells. *J Biol Chem* 273:15794–15803. <http://dx.doi.org/10.1074/jbc.273.25.15794>.
- Foster GH, Tesh VL. 2002. Shiga toxin 1-induced activation of c-Jun NH(2)-terminal kinase and p38 in the human monocytic cell line THP-1: possible involvement in the production of TNF-alpha. *J Leukoc Biol* 71:107–114.
- Zhou HR, Islam Z, Pestka JJ. 2003. Rapid, sequential activation of mitogen-activated protein kinases and transcription factors precedes proinflammatory cytokine mRNA expression in spleens of mice exposed to the trichothecene vomitoxin. *Toxicol Sci* 72:130–142. <http://dx.doi.org/10.1093/toxsci/kfg006>.
- Pestka S, Hintikka H. 1971. Studies on the formation of ribonucleic acid-ribosome complexes. XVI. Effect of ribosomal translocation inhibitors on polyribosomes. *J Biol Chem* 246:7723–7730.
- Pestka S. 1971. Inhibitors of ribosome functions. *Annu Rev Microbiol* 25:487–562. <http://dx.doi.org/10.1146/annurev.mi.25.100171.002415>.
- Thorpe CM, Smith WE, Hurley BP, Acheson DW. 2001. Shiga toxins induce, superinduce, and stabilize a variety of C-X-C chemokine mRNAs in intestinal epithelial cells, resulting in increased chemokine expression. *Infect Immun* 69:6140–6147. <http://dx.doi.org/10.1128/IAI.69.10.6140-6147.2001>.
- Thorpe CM, Hurley BP, Lincicome LL, Jacewicz MS, Keusch GT, Acheson DW. 1999. Shiga toxins stimulate secretion of interleukin-8 from intestinal epithelial cells. *Infect Immun* 67:5985–5993.
- Colpoys WE, Cochran BH, Carducci TM, Thorpe CM. 2005. Shiga toxins activate translational regulation pathways in intestinal epithelial cells. *Cell Signal* 17:891–899. <http://dx.doi.org/10.1016/j.cellsig.2004.11.014>.
- Leyva-Illades D, Cherla RP, Lee MS, Tesh VL. 2012. Regulation of cytokine and chemokine expression by the ribotoxic stress response elicited by Shiga toxin type 1 in human macrophage-like THP-1 cells. *Infect Immun* 80:2109–2120. <http://dx.doi.org/10.1128/IAI.06025-11>.
- Cherla RP, Lee SY, Mees PL, Tesh VL. 2006. Shiga toxin 1-induced cytokine production is mediated by MAP kinase pathways and translation initiation factor eIF4E in the macrophage-like THP-1 cell line. *J Leukoc Biol* 79:397–407.
- Cameron P, Smith SJ, Gienbycz MA, Rotondo D, Plevin R. 2003. Verotoxin activates mitogen-activated protein kinase in human peripheral blood monocytes: role in apoptosis and proinflammatory cytokine release. *Br J Pharmacol* 140:1320–1330. <http://dx.doi.org/10.1038/sj.bjp.0705560>.
- Cameron P, Paton N, Smith DG. 2012. Verotoxin-2 activates mitogen-activated protein kinases in bovine adherent peripheral blood mononuclear cells. *J Comp Pathol* 147:20–23. <http://dx.doi.org/10.1016/j.jcpa.2011.08.010>.
- Donohue-Rolfe A, Acheson DW, Kane AV, Keusch GT. 1989. Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross-reactive monoclonal antibodies. *Infect Immun* 57:3888–3893.
- Wen SX, Teel LD, Judge NA, O'Brien AD. 2006. Genetic toxoids of Shiga toxin types 1 and 2 protect mice against homologous but not heterologous toxin challenge. *Vaccine* 24:1142–1148. <http://dx.doi.org/10.1016/j.vaccine.2005.08.094>.
- Jandhyala DM, Ahluwalia A, Obrig T, Thorpe CM. 2008. ZAK: a MAP3Kinase that transduces Shiga toxin- and ricin-induced proinflammatory cytokine expression. *Cell Microbiol* 10:1468–1477. <http://dx.doi.org/10.1111/j.1462-5822.2008.01139.x>.
- Church GM, Gilbert W. 1984. Genomic sequencing. *Proc Natl Acad Sci U S A* 81:1991–1995. <http://dx.doi.org/10.1073/pnas.81.7.1991>.
- Stone SM, Thorpe CM, Ahluwalia A, Rogers AB, Obata F, Vozenilek A, Kolling GL, Kane AV, Magun BE, Jandhyala DM. 2012. Shiga toxin 2-induced intestinal pathology in infant rabbits is a subunit dependent and responsive to the tyrosine kinase and potential ZAK inhibitor imatinib. *Front Cell Infect Microbiol* 2:135.
- Wong W, Bai XC, Brown A, Fernandez IS, Hanssen E, Condrón M, Tan YH, Baum J, Scheres SH. 2014. Cryo-EM structure of the Plasmodium falciparum 80S ribosome bound to the anti-protozoan drug emetine. *eLife* 3:e03080.
- Cherla RP, Lee SY, Mulder RA, Lee MS, Tesh VL. 2009. Shiga toxin 1-induced proinflammatory cytokine production is regulated by the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling pathway. *Infect Immun* 77:3919–3931. <http://dx.doi.org/10.1128/IAI.00738-09>.
- Mendoza MC, Er EE, Blenis J. 2011. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci* 36:320–328. <http://dx.doi.org/10.1016/j.tibs.2011.03.006>.
- Jewell JL, Russell RC, Guan KL. 2013. Amino acid signalling upstream of mTOR. *Nat Rev Mol Cell Biol* 14:133–139. <http://dx.doi.org/10.1038/nrm3522>.
- Wang X, Mader MM, Toth JE, Yu X, Jin N, Campbell RM, Smallwood JK, Christe ME, Chatterjee A, Goodson T, Jr, Vlahos CJ, Matter WF, Bloem LJ. 2005. Complete inhibition of anisomycin and UV radiation but not cytokine induced JNK and p38 activation by an aryl-substituted dihydropyrrlopyrazole quinoline and mixed lineage kinase 7 small interfering RNA. *J Biol Chem* 280:19298–19305. <http://dx.doi.org/10.1074/jbc.M413059200>.
- Smith WE, Kane AV, Campbell ST, Acheson DW, Cochran BH, Thorpe CM. 2003. Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. *Infect Immun* 71:1497–1504. <http://dx.doi.org/10.1128/IAI.71.3.1497-1504.2003>.
- Wortzel I, Seger R. 2011. The ERK cascade: distinct functions within various subcellular organelles. *Genes Cancer* 2:195–209. <http://dx.doi.org/10.1177/1947601911407328>.
- Ritchie JM, Thorpe CM, Rogers AB, Waldor MK. 2003. Critical roles for stx2, eae, and tir in enterohemorrhagic *Escherichia coli*-induced diarrhea and intestinal inflammation in infant rabbits. *Infect Immun* 71:7129–7139. <http://dx.doi.org/10.1128/IAI.71.12.7129-7139.2003>.
- Kyriakis JM, Avruch J. 2012. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiol Rev* 92:689–737. <http://dx.doi.org/10.1152/physrev.00028.2011>.
- Kyriakis JM, Avruch J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 81:807–869.
- Nakamura A, Johns EJ, Imaizumi A, Yanagawa Y, Kohsaka T. 2001. Activation of beta(2)-adrenoceptor prevents shiga toxin 2-induced TNF-alpha gene transcription. *J Am Soc Nephrol* 12:2288–2299.
- Hurley BP, Thorpe CM, Acheson DW. 2001. Shiga toxin translocation across intestinal epithelial cells is enhanced by neutrophil transmigration. *Infect Immun* 69:6148–6155. <http://dx.doi.org/10.1128/IAI.69.10.6148-6155.2001>.
- Scheut F, Teel LD, Beutin L, Pierard D, Buvens G, Karch H, Mellmann

- A, Caprioli A, Tozzoli R, Morabito S, Strockbine NA, Melton-Celsa AR, Sanchez M, Persson S, O'Brien AD. 2012. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *J Clin Microbiol* 50:2951–2963. <http://dx.doi.org/10.1128/JCM.00860-12>.
35. Louise CB, Obrig TG. 1995. Specific interaction of *Escherichia coli* O157: H7-derived Shiga-like toxin II with human renal endothelial cells. *J Infect Dis* 172:1397–1401. <http://dx.doi.org/10.1093/infdis/172.5.1397>.
36. Siegler RL, Obrig TG, Pysher TJ, Tesh VL, Denkers ND, Taylor FB. 2003. Response to Shiga toxin 1 and 2 in a baboon model of hemolytic uremic syndrome. *Pediatr Nephrol* 18:92–96.
37. Tesh VL, Burris JA, Owens JW, Gordon VM, Wadolkowski EA, O'Brien AD, Samuel JE. 1993. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect Immun* 61:3392–3402.
38. Lauvrak SU, Walchli S, Iversen TG, Slagsvold HH, Torgersen ML, Spilberg B, Sandvig K. 2006. Shiga toxin regulates its entry in a Syk-dependent manner. *Mol Biol Cell* 17:1096–1109.
39. Taga S, Carlier K, Mishal Z, Capoulade C, Mangeney M, Lecluse Y, Coulaud D, Tetaud C, Pritchard LL, Tursz T, Wiels J. 1997. Intracellular signaling events in CD77-mediated apoptosis of Burkitt's lymphoma cells. *Blood* 90:2757–2767.
40. Torgersen ML, Walchli S, Grimmer S, Skanland SS, Sandvig K. 2007. Protein kinase Cdelta is activated by Shiga toxin and regulates its transport. *J Biol Chem* 282:16317–16328. <http://dx.doi.org/10.1074/jbc.M610886200>.
41. Walchli S, Skanland SS, Gregers TF, Lauvrak SU, Torgersen ML, Ying M, Kuroda S, Maturana A, Sandvig K. 2008. The mitogen-activated protein kinase p38 links Shiga toxin-dependent signaling and trafficking. *Mol Biol Cell* 19:95–104. <http://dx.doi.org/10.1091/mbc.E07-06-0565>.
42. Huang J, Motto DG, Bundle DR, Sadler JE. 2010. Shiga toxin B subunits induce VWF secretion by human endothelial cells and thrombotic microangiopathy in ADAMTS13-deficient mice. *Blood* 116:3653–3659. <http://dx.doi.org/10.1182/blood-2010-02-271957>.
43. Marcato P, Mulvey G, Armstrong GD. 2002. Cloned Shiga toxin 2 B subunit induces apoptosis in Ramos Burkitt's lymphoma B cells. *Infect Immun* 70:1279–1286. <http://dx.doi.org/10.1128/IAI.70.3.1279-1286.2002>.
44. Gallo KA, Johnson GL. 2002. Mixed-lineage kinase control of JNK and p38 MAPK pathways. *Nat Rev Mol Cell Biol* 3:663–672. <http://dx.doi.org/10.1038/nrm906>.
45. Bloem LJ, Pickard TR, Acton S, Donoghue M, Beavis RC, Knierman MD, Wang X. 2001. Tissue distribution and functional expression of a cDNA encoding a novel mixed lineage kinase. *J Mol Cell Cardiol* 33:1739–1750. <http://dx.doi.org/10.1006/jmcc.2001.1437>.
46. Hsieh YL, Tsai YL, Shibu MA, Su CC, Chung LC, Pai P, Kuo CH, Yeh YL, Viswanadha VP, Huang CY. 2015. ZAK induces cardiomyocyte hypertrophy and brain natriuretic peptide expression via p38/JNK signaling and GATA4/c-Jun transcriptional factor activation. *Mol Cell Biochem* 405:1–9. <http://dx.doi.org/10.1007/s11010-015-2389-z>.
47. Gross EA, Callow MG, Waldbaum L, Thomas S, Ruggieri R. 2002. MRK, a mixed lineage kinase-related molecule that plays a role in gamma-radiation-induced cell cycle arrest. *J Biol Chem* 277:13873–13882. <http://dx.doi.org/10.1074/jbc.M111994200>.
48. Korkina O, Dong Z, Marullo A, Warshaw G, Symons M, Ruggieri R. 2013. The MLK-related kinase (MRK) is a novel RhoC effector that mediates lysophosphatidic acid (LPA)-stimulated tumor cell invasion. *J Biol Chem* 288:5364–5373. <http://dx.doi.org/10.1074/jbc.M112.414060>.
49. Adler AS, McClelland ML, Yee S, Yaylaoglu M, Hussain S, Cosino E, Quinones G, Modrusan Z, Seshagiri S, Torres E, Chopra VS, Haley B, Zhang Z, Blackwood EM, Singh M, Junttila M, Stephan JP, Liu J, Pau G, Fearon ER, Jiang Z, Firestein R. 2014. An integrative analysis of colon cancer identifies an essential function for PRPF6 in tumor growth. *Genes Dev* 28:1068–1084. <http://dx.doi.org/10.1101/gad.237206.113>.
50. Liu J, McClelland M, Stawiski EW, Gnad F, Mayba O, Haverty PM, Durinck S, Chen YJ, Klijn C, Jhunjhunwala S, Lawrence M, Liu H, Wan Y, Chopra V, Yaylaoglu MB, Yuan W, Ha C, Gilbert HN, Reeder J, Pau G, Stinson J, Stern HM, Manning G, Wu TD, Neve RM, de Sauvage FJ, Modrusan Z, Seshagiri S, Firestein R, Zhang Z. 2014. Integrated exome and transcriptome sequencing reveals ZAK isoform usage in gastric cancer. *Nat Commun* 5:3830.
51. Wong J, Smith LB, Magun EA, Engstrom T, Kelley-Howard K, Jandhyala DM, Thorpe CM, Magun BE, Wood LJ. 2013. Small molecule kinase inhibitors block the ZAK-dependent inflammatory effects of doxorubicin. *Cancer Biol Ther* 14:56–63. <http://dx.doi.org/10.4161/cbt.22628>.
52. Gotoh I, Adachi M, Nishida E. 2001. Identification and characterization of a novel MAP kinase kinase kinase, MLTK. *J Biol Chem* 276:4276–4286. <http://dx.doi.org/10.1074/jbc.M008595200>.
53. Wang C, Weerapana E, Blewett MM, Cravatt BF. 2014. A chemoproteomic platform to quantitatively map targets of lipid-derived electrophiles. *Nat Methods* 11:79–85.
54. Jandhyala DM, Rogers TJ, Kane A, Paton AW, Paton JC, Thorpe CM. 2010. Shiga toxin 2 and flagellin from Shiga-toxigenic *Escherichia coli* superinduce interleukin-8 through synergistic effects on host stress-activated protein kinase activation. *Infect Immun* 78:2984–2994. <http://dx.doi.org/10.1128/IAI.00383-10>.
55. Schuller S, Heuschkel R, Torrente F, Kaper JB, Phillips AD. 2007. Shiga toxin binding in normal and inflamed human intestinal mucosa. *Microbes Infect* 9:35–39. <http://dx.doi.org/10.1016/j.micinf.2006.10.005>.
56. Schuller S, Frankel G, Phillips AD. 2004. Interaction of Shiga toxin from *Escherichia coli* with human intestinal epithelial cell lines and explants: Stx2 induces epithelial damage in organ culture. *Cell Microbiol* 6:289–301. <http://dx.doi.org/10.1046/j.1462-5822.2004.00370.x>.
57. Zumbun SD, Hanson L, Sinclair JF, Freedy J, Melton-Celsa AR, Rodriguez-Canales J, Hanson JC, O'Brien AD. 2010. Human intestinal tissue and cultured colonic cells contain globotriaosylceramide synthase mRNA and the alternate Shiga toxin receptor globotetraosylceramide. *Infect Immun* 78:4488–4499. <http://dx.doi.org/10.1128/IAI.00620-10>.
58. Miyamoto Y, Iimura M, Kaper JB, Torres AG, Kagnoff MF. 2006. Role of Shiga toxin versus H7 flagellin in enterohaemorrhagic *Escherichia coli* signalling of human colon epithelium in vivo. *Cell Microbiol* 8:869–879. <http://dx.doi.org/10.1111/j.1462-5822.2005.00673.x>.
59. Taylor CM, Williams JM, Lote CJ, Howie AJ, Thewles A, Wood JA, Milford DV, Raafat F, Chant I, Rose PE. 1999. A laboratory model of toxin-induced hemolytic uremic syndrome. *Kidney Int* 55:1367–1374. <http://dx.doi.org/10.1046/j.1523-1755.1999.00387.x>.
60. Louise CB, Obrig TG. 1992. Shiga toxin-associated hemolytic uremic syndrome: combined cytotoxic effects of Shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells in vitro. *Infect Immun* 60:1536–1543.
61. Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K. 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. *Eur J Biochem* 171:45–50.