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Cytotoxic *Vibrio* T3SS1 Rewires Host Gene Expression to Subvert Cell Death Signaling and Activate Cell Survival Networks

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Abstract

Bacterial effectors are potent manipulators of host signaling pathways. The marine bacterium *Vibrio parahaemolyticus* (*V. para*), delivers effectors into host cells through two type three secretion systems (T3SS). The ubiquitous T3SS1 is vital for *V. para* survival in the environment, whereas T3SS2 causes acute gastroenteritis in human hosts. Although the natural host is undefined, T3SS1 effectors attack highly conserved cellular processes and pathways to orchestrate non-apoptotic cell death. Much is known about how T3SS1 effectors function in isolation, but we wanted to understand how their concerted action globally affects host cell signaling. To assess the host response to T3SS1, we compared gene expression changes over time in primary fibroblasts infected with *V. para* that have a functional T3SS1 (T3SS1⁺) to those in cells infected with *V. para* lacking T3SS1 (T3SS1⁻). Overall, the host transcriptional response to both T3SS1⁺ and T3SS1⁻ *V. para* was rapid, robust, and temporally dynamic. T3SS1 re-wired host gene expression by specifically altering the expression of 398 genes. Although T3SS1 effectors target host cells at the posttranslational level to cause cytotoxicity, network analysis indicated that *V. para* T3SS1 also precipitates a host transcriptional response that initially activates cell survival and represses cell

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death networks. The increased expression of several key pro-survival transcripts mediated by T3SS1 was dependent on a host signaling pathway that is silenced later in infection by the posttranslational action of T3SS1. Taken together, our analysis reveals a complex interplay between roles of T3SS1 as both a transcriptional and posttranslational manipulator of host cell signaling.

Introduction

Viral and bacterial toxins have proven to be vital tools for studying the cell biology of eukaryotic hosts because they have evolved to become potent and specific inhibitors and activators of host signaling pathways that control cellular proliferation, cytoskeleton and protein trafficking, intra-and extra-cellular signaling pathways, and protein homeostasis (1). An important toxin-delivery system utilized by Gram-negative bacteria is the type three secretion system (T3SS) (2). Derived from the flagellar apparatus, the T3SS is a needle-like structure that transfers specialized toxins (effectors) directly from the bacterial cytosol into the host cell cytoplasm (3). Within the host cell, effectors target and manipulate specific proteins to alter host cell processes for the benefit of the bacterium (1). *Vibrio parahaemolyticus* (*V. para*), a Gram-negative bacterium found in warm marine and estuarine environments, utilizes T3SSs for both its survival in the environment and within human hosts (4, 5). With the effects of global warming expanding its ecological niche, *V. para* has become the leading cause of acute seafood-borne gastroenteritis worldwide (4, 6). In addition to causing a self-limiting gastroenteritis, *V. para* can infect wounds that have been exposed to contaminated seawater (7, 8),

The *V. para* genome encodes several virulence factors, including thermostable hemolysins (TDH and TRH), polar and lateral flagella, adhesins, and two T3SSs (9). T3SS1 is found in all sequenced clinical and environmental isolates of *V. para*, whereas T3SS2 is found only in clinical isolates (9, 10). Because T3SS2 mediates enterotoxicity and invasion of host cells, it is the principal virulence factor responsible for gastroenteritis in both humans and animal models (5, 11, 12). The focus of this study is T3SS1, which is believed to be necessary for *V. para*'s survival in the environment but does not cause clinical gastroenteritis and does not contribute to gastroenteritis in animal models (9, 11). T3SS1 orchestrates an efficient, non-apoptotic cell death by targeting cell processes and pathways that are highly conserved from yeast to humans (13, 14). Environmental *V. para* is found in various marine organisms including finfish, codfish, bivalve shellfish, and seaweed and within marine sediments, but the specific cell type that T3SS1 has evolved to target remains undefined (15, 16). However, several human and mouse cell lines have been key in uncovering the conserved biochemical functions of the T3SS1 effectors (14, 17).

T3SS1-mediated cell death occurs in distinct and highly reproducible stages due to the temporal action of the effectors it delivers (14) (Fig. 1A). As early as 15 minutes after infection, the effector VopQ (VP1680) forms an outward-rectifying channel that neutralizes the lysosome and inhibits autophagic flux, resulting in massive autophagosome accumulation within the host cell (18, 19). Within the first hour after infection, the secreted phosphatase VPA0450 hydrolyses the 5' phosphate on phosphatidyl inositol 4,5 phosphate

(PI(4,5)P₂) at the plasma membrane, which causes blebbing by dislodging the membrane-associated actin cytoskeleton and reducing membrane stability (20). After one hour of infection, the Fic (filamentation induced by cAMP) domain-containing protein VopS (VP1686) covalently attaches an adenosine monophosphate (AMP) to the hydroxyl group of a threonine residue in the switch 1 region of Rho guanosine triphosphatases (GTPases). This posttranslational modification, termed AMPylation, inactivates Rho GTPases and precipitates cytoskeletal collapse and cell rounding (21, 22). At least one additional effector, VopR (VP1683), operates through a yet uncharacterized mechanism (9, 23). By two to three hours post-infection, the host cell ruptures and releases its cellular contents (14) (Fig. 1A).

Although T3SS effectors are most often studied in isolation, they work in concert to manipulate host cell signaling to benefit the bacterium. Previous work has demonstrated that disruption of autophagic flux by VopQ antagonizes phagocytosis of *V. para* and prevents cell death by apoptosis (24). VopQ has also been implicated as a modulator of c-Fos and c-Jun protein abundance and IL-8 secretion in *V. para*-infected Caco-2 cells (25). In addition, VopS, through AMPylation of its target proteins Rho, Rac, and Cdc42, inactivates nuclear factor-kappaB (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling pathways, which are critical pathways through which the cell responds to stimuli such as bacterial infection (22, 26). Indeed, both VopQ and VopS interfere with activation of the host NLRC4 (NOD-like receptor CARD domain-containing 4) inflammasome during *V. para* infection (27).

Because a growing body of evidence suggests the *V. para* T3SS1 is not only cytotoxic to the host cell, but actively subverts host cellular immune responses, we postulated that the T3SS1 may alter host gene expression as a critical part of its subversive mechanism. Here, utilizing genome-wide RNA sequencing methods, we demonstrate that *V. para* lacking T3SS1 induced the expected host cell response to pathogen-associated molecular patterns (PAMPs), whereas *V. para* with intact T3SS1 rewired the temporal expression patterns of hundreds of host genes. Furthermore, our systems-level analysis revealed that the host transcriptional response manipulated by T3SS1 altered signaling through several vital host pathways, which resulted in the activation of cell survival and repression of cell death networks. Our data suggest that altering host gene expression by the posttranslational targeting of critical cell processes, such as autophagy and Rho GTPase signaling, is an important mechanism by which T3SS1 subverts host immune responses to a Gram-negative bacterial infection.

Results

V. para T3SS1 orchestrates death of primary human dermal fibroblasts

Although the identity of the organism or cell type in which *V. para* T3SS1 evolved to act is unknown, T3SS1-orchestrated cell death has primarily been characterized in HeLa cells and in RAW267.4 macrophages (14, 24). However, both cell lines are transformed and their high genetic heterogeneity may influence their transcriptional response to infection. Consequently, we sought to characterize *V. para* infection in primary human dermal fibroblasts (ATCC[®] PCS-201-01). To study the effects of T3SS1 alone, we utilized a *V. para* strain, POR3, which is a derivative of the clinical strain RIMD2210633. This POR3:T3SS1⁺ strain (*tdhA*S⁻*vcrD*2⁻) does not produce functional hemolysins or a functional T3SS2, but

maintains an active T3SS1 as its primary virulence factor (5, 28). As a control for host cell response to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), we also infected primary fibroblasts with the non-cytotoxic strain POR4:T3SS1⁻. This POR4:T3SS1⁻ strain was derived from POR3 (*tdhA*S⁻ *vcrD1*⁻ *vcrD2*⁻) and does not produce a functional T3SS1 (28).

As previously reported in HeLa and RAW264.7 cells, POR3:T3SS1⁺ induced both cell rounding and rupture in primary fibroblasts (Fig. 1B) whereas POR4:T3SS1⁻ induced no toxic effects (fig. S1) (14). We observed a lag in cytotoxicity in primary fibroblasts, with maximal cell death occurring at 240 minutes after infection compared to 150 minutes after infection in HeLa cells (Fig. 1C). Correspondingly, we have previously observed that maximal lysis occurs only 120 minutes after POR3 infection in RAW264.7 macrophages, supporting the idea that cell type-specific factors govern the timeline of cell lysis in response to T3SS1 (14).

V. *para* infection provokes a rapid and robust host transcriptional response

After characterizing the cellular response to a POR3:T3SS1⁺ infection in primary fibroblasts, we employed genome-wide transcriptional profiling methods (RNA-seq) to measure changes in gene expression over time in response to infection with POR4:T3SS1⁻ or POR3:T3SS1⁺. Time points were collected 45, 60, 75 and 90 minutes post-infection. In total, libraries from 27 samples (triplicates of uninfected cells and of cells infected with POR3 and POR4 at t=45, 60, 75, and 90 minutes) were sequenced and mapped to the human genome. The sequencing data passed all statistical quality control tests (fastqc and fastq_screen), and principal component analysis revealed tight clustering of technical replicates (fig. S2). We performed differential expression analysis across the time points for the two comparisons: uninfected vs. POR4:T3SS1⁻ and uninfected vs. POR3:T3SS1⁺. Applying statistical cutoffs of false discovery rate (FDR) 0.01, log₂counts-per-million (log₂CPM) 0 and fold change (FC) cutoffs of -1.5 FC 1.5 we found that 1205 genes were differentially regulated between uninfected vs. POR4:T3SS1⁻-infected fibroblasts and 1385 genes between uninfected vs. POR3:T3SS1⁺-infected fibroblasts (Tables S1 and S2).

Hierarchical clustering of the differentially expressed genes revealed a distinct temporal pattern in gene expression changes in both comparisons (Fig. 2). By grouping genes with similar temporal differential expression patterns, we pinpointed groups of transcripts that were dynamically regulated during specific phases of the infection. Two major clusters resulting from both the uninfected vs. POR4:T3SS1⁻ and POR3:T3SS1⁺ differential expression analyses were the “early response” (Fig. 2A, clusters 3–5) and “late response” (Fig. 2A, cluster 6) clusters. Transcript abundance of genes in early response clusters was maximal at 45 minutes post-infection, whereas late response genes were maximally expressed at 90 minutes post-infection. For example, the abundance of the transcripts of many cytokines, including *IL6*, *CXCL1*, *CXCL2*, *CXCL3*, *CXCL6*, and *IL8*, increased (log₂FC>2) in response to infection with both POR4:T3SS1⁻ and POR3:T3SS1⁺ (Tables S1 and S2). All of these cytokines followed the late response expression pattern and were maximally expressed at 90 minutes post-infection (Fig. 2A, cluster 4; Fig. 2B, cluster 6).

The T3SS1-induced expression of several cytokines is cell type-specific

It was previously reported that *V. para*-induced expression and secretion of interleukin 8 (IL8) depends on the T3SS1 effector VopQ in Caco-2 cells, but we did not observe a significant difference in *IL8* expression between POR4:T3SS1⁻-infected and POR3:T3SS1⁺-infected fibroblasts (Tables S1 and S2) (25). To resolve this discrepancy, we used quantitative RT-PCR (qPCR) to measure *IL8* mRNA in POR4:T3SS1⁻ and POR3:T3SS1⁺-infected HeLa cells at 75 minutes post-infection. We observed that the increase in *IL8* expression in HeLa cells was indeed T3SS1-dependent (fig. S3A). Taken together, these data suggest that the wiring of signaling networks regulating *IL8* expression differs per cell type. Another important inflammatory cytokine, *IL11*, fell into the late response clusters (Fig. 2A, cluster 6; Fig. 2B, cluster 4). However, its expression was repressed by both POR4:T3SS1⁻ and POR3:T3SS1⁺ early in infection and although the repression was gradually alleviated in later time points, the *IL11* transcript was never significantly more abundant in infected fibroblasts than in uninfected fibroblasts (Tables S1 and S2). Because this repression of *IL11* expression was unexpected, we measured *IL11* mRNA in HeLa cells and RAW264.7 macrophages by qPCR at 75 and 60 minutes post-infection, respectively. We found that *IL11* expression was specifically induced by POR3:T3SS1⁺ infection in HeLa cells and RAW264.7 macrophages (fig. S3B). Consistent with *IL8* data, the expression of *IL11* in response to POR4:T3SS1⁻ or POR3:T3SS1⁺ was cell type-specific, which may be due to the differences in TLR expression between species and cell types (29–31).

Expression of a family of zinc transcription factors is induced during the early response to *V. para*

For both fibroblasts infected with POR4:T3SS1⁻ and fibroblasts infected with POR3:T3SS1⁺, the early response genes were dominated by genes encoding zinc finger transcription factors, for example *ZNF554*, *ZNF681*, *ZNF665*, *ZKSCAN3*, that are largely uncharacterized (Fig. 2A, clusters 3–5). The expression of over 20 *ZNF* and *ZKSCAN* genes was induced with $\log_2\text{FC} > 1.1$ at 45 minutes post infection compared to uninfected cells (Table S1). At later infection time points, the expression of these genes resembled that in uninfected cells. The conservation of the expression patterns of these genes in both infection conditions suggests that expression of these transcripts is activated by *V. para* PAMPs and not by the specific action of the T3SS1 (Fig. 2B, clusters 6 and 7; Table S2).

398 host genes are differentially expressed in response to *V. para* T3SS1

To understand specifically how T3SS1 affects host cell gene expression, we performed differential expression analysis on RNA-seq datasets from POR4:T3SS1⁻-infected versus POR3:T3SS1⁺-infected fibroblasts. Applying fold change (FC) cutoffs of $-1.5 \leq \text{FC} \leq 1.5$ and statistical cutoffs of $\text{FDR} \leq 0.01$, $\log_2\text{CPM} \geq 0$, we found that 398 genes were differentially expressed between POR4:T3SS1⁻-infected and POR3:T3SS1⁺-infected fibroblasts (Table S3). We used hierarchical clustering to uncover the major temporal patterns of T3SS1-specific gene expression and found that the 398 genes grouped into six distinct clusters (Fig. 3A).

Cluster 4, which contains genes that are strongly activated late in infection, was the largest cluster and contained the most significantly differentially expressed genes (Fig. 3A). Notably, the transcripts of *FOS*, *RGS2*, *KLF6*, and *TSC22D3* followed the “early response” pattern in POR4:T3SS1⁻ infected fibroblasts, where expression of these genes increased early (45 minutes) but then returned to baseline (Fig. 3B). In contrast, transcripts of these genes in POR3:T3SS1⁺-infected cells followed a similar pattern early but then maintained a steady or late maximal induction over the course of the infection (Fig. 3B). For example, the transcript abundance of *TSC22D3*, which encodes an anti-inflammatory leucine zipper protein, increased in both POR4:T3SS1⁻-infected and POR3:T3SS1⁺-infected cells early in infection (2.3 fold), but after 90 minutes of infection was 11.9 fold higher in POR3:T3SS1⁺-infected fibroblasts than in POR4:T3SS1⁻-infected fibroblasts (Tables S1–3).

Cluster 4 also contained the gene encoding the GTPase RhoB (*RHOB*), which was only differentially expressed during T3SS1-mediated infection. The abundance of *RHOB* mRNA was 8.5 fold higher at 75 minutes post-infection and 18 fold higher at 90 minutes post-infection in POR3:T3SS1⁺-infected versus POR4:T3SS1⁻-infected fibroblasts (Fig. 3B, Table S3). *GEM*, which encodes an inner membrane GTP-binding protein of unknown function, also fell into cluster 4. *GEM* expression was very low in fibroblasts throughout POR4:T3SS1⁻ infection, but steadily increased over the course of POR3:T3SS1⁺ infection (Fig. 3B).

Several genes encoding transcription factors displayed increased expression in POR3:T3SS1⁺-infected fibroblasts compared to POR4:T3SS1⁻-infected fibroblasts. These transcription factors primarily fell into clusters 4 and 6. The most significant T3SS1-induced transcription factors in cluster 4 include the Kruppel-like factors *KLF2*, *KLF6*, and *KLF4*, the cAMP dependent transcription factor *ATF3*, and AP-1 complex members *FOS* and *JUND* (Fig. 3A, cluster 4; 3B). The AP-1 complex member *JUN*, however, fell into cluster 6 (Fig. 3A, cluster 6; 3B). The expression of most genes in cluster 6, including *JUN*, increased earlier in infection than genes in cluster 4.

In addition to *JUN*, cluster 6 contained the inducible prostaglandin-endoperoxide synthase *PTGS2* (*COX2*) (Fig. 3A, cluster 6; Table S3). Starting at 60 minutes post-infection, *PTGS2* expression increased steadily in POR3:T3SS1⁺-infected fibroblasts and at 90 minutes was 39-fold higher than uninfected and 9-fold higher than POR4:T3SS1⁻-infected fibroblasts (Fig. 3B; Tables S2 and S3). Cluster 6 also contained the early growth response transcription factor *EGR1*. We found that POR4:T3SS1⁻ also significantly induced *EGR1* expression in fibroblasts early during the infection time course, but this increase was not sustained through later stages of infection. However, POR3:T3SS1⁺ induction of *EGR1* expression was sustained throughout the infection, and was higher than in POR4:T3SS1⁻ infected fibroblasts later in the time course (Fig. 3B).

T3SS1-induced expression of several genes is conserved in different cell types

To test the conservation of the host transcriptional response to T3SS1 in other cell types, we measured the mRNA abundance of several of the genes whose transcription was strongly activated by T3SS1 in primary fibroblasts in infected HeLa cells and RAW264.7 macrophages by qPCR. *RHOB* and *KLF2* expression was greatly increased during

POR3:T3SS1⁺ infection, but not during POR4:T3SS1⁻ infection, in both HeLa cells and RAW264.7 macrophages (fig. S4A,B). *KLF6* and *FOS* also showed similar expression patterns in HeLa cells and RAW264.7 macrophages as detected in primary fibroblasts, because the abundance of both transcripts was significantly increased upon POR3:T3SS1⁺ infection, but not POR4:T3SS1⁻ infection (fig. S4C,D).

The expression of several genes induced by POR4:T3SS1⁻ infection differed between HeLa cells and RAW264.7 macrophages. In both cell types, we measured significantly increased transcript abundance for *JUN*, *EGR1*, *ATF3*, *PTGS2* and *GEM* upon POR3:T3SS1⁺ infection (fig. S4D–I). However, the relative expression of all these genes was also somewhat increased in POR4:T3SS1⁻-infected RAW264.7 macrophages, but not in POR4:T3SS1⁻-infected HeLa cells (fig. S4D–I). This ability of POR4:T3SS1⁻ to induce significant expression of these transcripts in RAW264.7 macrophages, but not in HeLa cells highlights the differential ability of these cell types to respond to environmental cues like bacterial PAMPs.

T3SS1 mediates differential activation or repression of core pathways over time

To understand how the observed differential gene expression in response to *V. para* T3SS1 changed host cell signaling events, we employed Ingenuity Pathway Analysis (IPA, Qiagen) of our two sets of temporal differential expression data [uninfected vs. POR4:T3SS1⁻, uninfected vs. POR3:T3SS1⁺ (Fig. 2, 3)]. To maximize the expression data available to the IPA software, we loosened the inclusion criteria by applying filters of $-0.3 \leq \log_2\text{FC} \leq 0.3$, $\log_2\text{CPM} \geq 0$, and $\text{FDR} \leq 0.05$. IPA Canonical pathway analysis, which compares well-established signaling pathways, predicted that several host signaling pathways were significantly activated or repressed in each comparison (Tables S4 and S5). Two pathways that were predicted to be strongly affected in POR4:T3SS1⁻-infected, but not in POR3:T3SS1⁺-infected, primary fibroblasts were signaling through TREM1 (triggering receptor expressed on myeloid cells 1) and GαS (G-protein subunit alpha S) (Fig. 4). In response to the stimulus of POR4:T3SS1⁻, the expression of genes encoding cytokines that are transcriptional targets of TREM1 signaling as well as co-stimulatory proteins ICAM1 and CD83 was activated in all time points. Conversely, GαS signaling was predicted to be strongly repressed by POR4:T3SS1⁻ due to the decreased expression of genes encoding components of GαS signaling and increased expression of the gene encoding RGS2, a negative regulator of GαS signaling, in POR4:T3SS1⁻-infected fibroblasts.

Several pathways were predicted to be activated or repressed specifically in POR3:T3SS1⁺-infected fibroblasts, but not in POR4:T3SS1⁻-infected fibroblasts. Based on expression of genes encoding components of these pathways, signaling by Rho Family GTPases, including RhoA and Rac, was predicted to be activated in response to POR3:T3SS1⁺ starting at 60 minutes post-infection (Fig. 4). Correspondingly, RhoGDI (Rho GDP dissociation inhibitor) signaling, which inhibits Rho GTPases, was repressed by POR3:T3SS1⁺ starting at 60 minutes post-infection (Fig. 4)(32). MAPK pathways were also differentially affected by POR4:T3SS1⁻ and POR3:T3SS1⁺ infection. The IPA software predicted extracellular signal-regulated kinase (ERK) and MAPK signaling to be moderately activated at 60 minutes post-infection during POR4:T3SS1⁻ infection, but not during POR3:T3SS1⁺

infection (Fig. 4, Tables S4 and S5). In fact, ERK and MAPK signaling were repressed in POR3:T3SS1⁺-infected fibroblasts 90 minutes post-infection (Fig. 4). Additionally, p38 MAPK signaling was differentially affected by POR3:T3SS1⁺ and POR4:T3SS1⁻. The action of T3SS1 greatly repressed p38 MAPK signaling from 45 to 75 minutes post-infection, but at 90 minutes post-infection the pathway was suddenly activated (Fig. 4). Given the role of p38 MAPK signaling in cytokine-induced mRNA stability, it is an intriguing hypothesis that the activation of this pathway contributes to the observed increase in mRNA abundance of some genes late in POR3:T3SS1⁺ infection (Fig. 3A, cluster 4)(33).

T3SS1 manipulates host gene expression through the MAPK pathway

In all cell lines tested, *RHOB* expression was specifically increased by T3SS1. Because many of the pathways affected by T3SS1 in our IPA canonical pathway analysis center on MAPK signaling, we tested whether the T3SS1-specific activation of *RHOB* expression was induced by MAPK signaling. We treated primary fibroblasts and RAW264.7 macrophages with either the mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) inhibitor U0126 or DMSO (vehicle), infected with POR3:T3SS1⁺, and then monitored *RHOB* expression by qPCR. Following U0126 pre-treatment, POR3:T3SS1⁺ infection did not increase *RHOB* expression in fibroblasts or macrophages, and *RHOB* transcript abundance was similar to quantities observed in DMSO-pretreated POR4:T3SS1⁻-infected cells (Fig. 5A,B; fig. S5A,B). These data indicate that the T3SS1-induced expression of *RHOB* depends on MEK1/2 activity in these cell types. We next tested whether other transcripts induced by T3SS1 also depended on MEK1/2 activity for their T3SS1-specific increased expression. As reported previously in Caco-2 cells (25), the T3SS1-specific induction of *FOS* expression in primary fibroblasts was inhibited by U0126 treatment (Fig. 5C). However, in contrast to previous observations in Caco-2 cells (25), we found that the T3SS1-triggered increase in *JUN* expression was not significantly inhibited by U0126 treatment in primary fibroblasts (Fig. 5C). Whereas U0126 treatment attenuated the T3SS1-induced expression of *KLF2*, *PTGS2*, and *EGR1* in primary fibroblasts, it did not significantly alter *ATF3* expression (Fig. 5D,E).

Network analysis predicts that cell survival pathways are activated and cell death pathways are repressed during T3SS1-induced cell death

Next, we sought to understand how the observed changes in gene expression and pathway activation in response to POR3:T3SS1⁺ infection affected broader cell signaling networks and cellular functions by performing network analysis using IPA (Table S6). Surprisingly, two of the most highly activated biological functions at 90 minutes post-POR3:T3SS1⁺ infection were organismal cell survival and cell viability (Fig. 6, Fig. 7A). The majority of differentially expressed genes in fibroblasts infected with POR3:T3SS1⁺ promote cell survival. These genes encode cytokines, transcription factors, receptors, kinases, peptidases, enzymes, and phosphatases and include genes focal to this study, such as, *KLFs*, *FOS*, *JUN*, *EGR1*, and *PTGS2* (Fig. 6). Although the expression state of some genes (*ATF3* and *TSC22D3*) is predicted to repress cell survival, the expression state of the majority of the genes promotes cell survival. Collectively, this expression pattern activates the cell survival network. Conversely, cell death networks were predicted to be strongly repressed during POR3:T3SS1⁺ infection (Fig. 7A). These data suggest that by posttranslational targeting of

key cellular functions, the T3SS1 effectors alter host gene expression to enhance pro-survival signals within the host, thereby masking their cytotoxic effects and perhaps dampening the host immune response (Fig. 7B).

Many additional interesting disease and biological function networks were predicted to be activated or repressed during POR3:T3SS1⁺ infection (Table S6), especially the expression and transactivation of RNA (Fig. 7A). These transcription-centered networks are both maximally activated later in infection, which is consistent with the high number of genes that we found to be activated by T3SS1 late in infection (Fig. 3A, clusters 4 and 6). The activation of these networks suggests that even as the T3SS1 toxins perturb vital cellular processes, T3SS1 does not shut down host transcription, but activates it (Fig. 7B).

DISCUSSION

V. para–induced transcripts reveal new players in bacteria-induced innate immunity

Herein, we have discovered previously unrecognized genes whose expression increases in response to a Gram-negative bacterial pathogen. By measuring the temporal gene expression changes in host fibroblasts infected with a non-cytotoxic strain of *V. para* (POR4:T3SS1[−]) lacking the three major virulence factors (*tdhA*[−] *vcrD1*[−] *vcrD2*[−]), we identified genes whose expression was primarily modulated by general bacterial PAMPs and not the specific action of *V. para* T3SS1 effectors. We identified a class of uncharacterized zinc finger transcription factors that are transcriptionally induced within 45 minutes post-infection by either POR4:T3SS1[−] or POR3:T3SS1⁺ (Fig. 2A, clusters 3–5; Fig. 2B, clusters 6–7). This pattern of regulation suggests that these uncharacterized transcription factors may be involved in the early innate immune response to Gram-negative bacteria. The cellular function of most of these transcription factors is unknown, but ZKSCAN3 has been shown to repress autophagy (34). Perhaps POR3:T3SS1⁺ counteracts this repression by injecting the effector VopQ, which acts early during infection to interrupt autophagic flux and induce autophagosome accumulation by a posttranslational mechanism (18, 19, 24).

The expression of *CXCL1*, *CXCL2*, and *CXCL3*, which encode chemokines that promote antimicrobial innate immune responses, was induced in fibroblasts infected by both POR4:T3SS1[−] and POR3:T3SS1⁺ starting at 45 minutes post-infection ($\log_2\text{FC} > 1$) and rose steadily as the infection proceeded (Fig. 2A, cluster 6; Fig. 2B, cluster 4)(35). This kinetic profile indicates that primary fibroblasts can quickly increase the expression of these transcripts in response to bacterial PAMPs and sustain them even through the cytotoxic action of T3SS1 effectors. Although Gram-negative bacteria, like *V. para*, are especially susceptible to the defense responses elicited by these chemokines, the *V. para* T3SS1 does not dampen their expression (35). It is possible, however, that these secreted chemokines might be subverted on the post-transcriptional level by a yet to be discovered T3SS1 effector.

T3SS1 rewires host gene expression during infection

The primary aim of this study was to determine the extent to which the action of T3SS1 specifically alters host gene expression. Through differential expression analysis of

POR4:T3SS1⁻-infected versus POR3:T3SS1⁺-infected primary fibroblasts, we found that the expression of 398 genes was specifically affected by T3SS1 (Fig. 3A). Most of the affected genes had not been previously linked to the to *V. para* infection or T3SS1-mediated cytotoxicity. For example, expression of *EGR1*, encoding the transcription factor early growth response 1, is transiently activated in epithelial cells and macrophages by bacterial pathogens (*Helicobacter pylori*) and PAMPs (LPS, sPGN), but we observed that T3SS1 enhanced and extended *EGR1* expression throughout *V. para* infection (36, 37). Our data suggest that *EGR1* expression is not only activated by generic Gram-negative PAMPs, but also by the specific action of the T3SS1. We observed that signaling through MEK1/2 is required for the increase in *EGR1* expression in response to *V. para* T3SS1, which has been previously noted in response to Gram-negative PAMPs during *H. pylori* infection (Fig. 5D) (36).

The expression of *prostaglandin-endoperoxide synthase 2* (*PTGS2*, also known as *COX2*) is induced by both viral and bacterial PAMPs (38). We show that *PTGS2* expression is more rapidly and robustly induced by POR3:T3SS1⁺ than by POR4:T3SS1⁻ (Fig. 3A,B) and that *PTGS2* expression is induced by POR3:T3SS1⁺ through MEK1/2. Another study demonstrated that *PTGS2*-dependent inflammation allows uropathogenic *E. coli* to circumvent host resistance to colonization and cause severe recurrent urinary tract infections (39). Clearly, *PTGS2* plays an important role in Gram-negative infections, but overexpression of *PTGS2* is also associated with resistance to apoptosis and promotion of cancer cell growth and survival (40). Deciphering the exact mechanism of MEK1/2-dependent increase in *PTGS2* transcript abundance during POR3:T3SS1⁺ will provide important insight into how this gene is regulated in other disease states.

T3SS1-induced *RHOB* expression requires activation of a pathway that is silenced later in infection

The abundance of *RHOB* protein has previously been shown to increase in macrophages during POR3:T3SS1⁺ infection (41), but the kinetics of *RHOB* expression had not been analyzed. Our data clearly demonstrate that T3SS1 quickly and robustly increases *RHOB* transcript abundance in primary fibroblasts and that T3SS1-induced expression is conserved in both RAW264.7 macrophages and HeLa cells (Fig. 3B, Fig. S4A). Previously, we identified Rho GTPases as targets of AMPylation by VopS and found that the modified GTPases could no longer activate downstream signaling molecules (21). However, VopS does not AMPylate Rho GTPases until one hour post-infection when host cells start rounding (21, 22). Additionally, AMPylation of Rho GTPases inhibits signaling by MAPK. *RHOB* transcription increased substantially prior to cell rounding in a MAPK-dependent manner, which leads us to propose that the mechanism and timing of *RHOB* transcript induction and posttranslational silencing by T3SS1 is likely more complex than predicted (22, 41, 42). Further work to understand the upstream mechanism of T3SS1-mediated induction of *RHOB* expression, as well as the downstream function of RhoB in infected cells, will be crucial to understanding the complex interplay between T3SS1 effectors and these host signaling outputs during infection.

T3SS1 specifically induces expression of genes encoding Kruppel-like transcription factors

A key observation in our analysis was the T3SS1-specific activation of transcripts encoding the Kruppel-like zinc finger transcription factors KLF2, KLF6, and KLF4. The T3SS1-induced expression of *KLF2* and *KLF6* was conserved in HeLa cells and RAW264.7 macrophages (Fig. S3B, C). KLF2 is an immunosuppressive transcription factor that induces quiescence in various T-cell lines, and KLF6 controls *transforming growth factor β* (*TGF- β*) expression and represses cell proliferation by inducing the expression of the gene encoding the cell-cycle inhibitor p21 and by interacting with Jun and Cyclin D1 (43, 44). Our work demonstrates that the T3SS1-induced expression of *KLF2* is dependent on signaling through the ERK-MAPK pathway, specifically on the activity of MEK1/2 (Fig. 5D). Rho-inactivating toxins, like YopT from *Yersinia spp*, ExoS and ExoY from *Pseudomonas aeruginosa*, and C3 exotoxin from *Clostridium botulinum* induce *KLF2* and *KLF6* expression in several cell types, such as macrophages, endothelial cells, and epithelial cells (45–47). Given that the T3SS1 effector VopS inactivates Rho GTPases, including RhoA, during T3SS1-mediated cell death, it is an attractive hypothesis that VopS is responsible for induced the expression of *KLF2*, *KLF6*, and perhaps even *KLF4* (22).

Pathway analysis reveals new signaling pathways targeted by T3SS1 effectors

By analyzing host gene expression over the course of an infection, we discovered new host signaling pathways targeted by T3SS1 effectors. Specifically, T3SS1 subverted the predicted activation of TREM1 signaling and repression of G α S signaling induced by POR4:T3SS1[−] (Fig. 4). It has been demonstrated that TREM1 can be activated by LPS, which may explain the predicted activation of the pathway in POR4-infected fibroblasts (48). TREM1 activation initiates signaling through Janus kinase 2 (JAK2), protein kinase B (PKB, also known as AKT), ERK1, and ERK2 that results in increased expression of genes involved in the inflammatory response (49). Our data suggest that, through an unknown mechanism, T3SS1 subverts TREM1 activation by LPS. It is not clear how G α S signaling is so strongly repressed in POR4:T3SS1[−]-infected fibroblasts but not in POR3:T3SS1⁺ infected fibroblasts. G α S signaling centers on signal transduction between G protein-coupled receptors and G α S to activate downstream effectors, such as protein kinase A, and downstream signaling outputs affect cell proliferation, cytoskeletal remodeling, and cell cycle regulation (50).

Conversely, signaling by Rho GTPases and RhoGDI was specifically altered by POR3:T3SS1⁺ and not POR4:T3SS1[−] (Fig. 4). Signaling by Rho GTPases was activated, whereas RhoGDI signaling was repressed. Previous studies have shown that depletion of RhoGDI constitutively activates Rho GTPases and increases *PTGS2* (*COX2*) expression, which mirrors our observations of increased expression of *PTGS2* and activation of Rho GTPase signaling in primary fibroblasts (Fig. 3B, Fig. 4) (51). Perhaps the increase of *PTGS2* expression and predicted activation of Rho GTPase signaling in POR3:T3SS1⁺-infected fibroblasts are due to a specific suppression of RhoGDI signaling by T3SS1 effectors.

The Gram-negative T3SS controls host signaling at multiple regulatory levels

Network analysis of the differential expression data provided one of the most unanticipated insights of this study: while the T3SS1 mediates a methodical, orchestrated posttranslational death through the action of an outward rectifying ion-channel (VopQ) to block autophagic flux, a PIP₂-5-phosphatase (VPA0450) to compromise the integrity of the cell membrane, and a Rho GTPase AMPylator (VopS) to inhibit cellular cytoskeletal signaling, the T3SS1 also simultaneously manipulates the host response to infection by altering the expression of hundreds of host genes to activate cell survival signaling pathways (Fig. 7) (14, 18, 20, 21). Conceptually, this study provides unique insight into the classical view of the activity of a type three secretion system and its effectors. Beyond their roles as posttranslational mediators of cell invasion or cytotoxicity, these secretion systems profoundly rewire host cell signaling at the transcriptional level, thereby altering the host environment to promote the proliferation and survival of the bacterial pathogen.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The *Vibrio parahaemolyticus* POR3 (POR1 *vcrD2*) and POR4 (POR1 *vcrD1 vcrD2*) strains were generously provided by Drs. Tetsuya Iida and Takeshi Honda of Osaka University. The POR3 and POR4 strains were cultured at 30°C in MLB (Luria-Bertani broth +3% NaCl).

Mammalian Cell Culture

Primary Adult Dermal Fibroblasts were purchased from ATCC and revived and maintained at 5% CO₂ and 37°C in Low Serum Primary Fibroblast Media (ATCC) according to ATCC instructions. HeLa cells (ATCC) and RAW264.7 macrophages were maintained at 5% CO₂ and 37°C in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich), 1% (v/v) penicillin-streptomycin-glutamine, and 1% (v/v) sodium pyruvate.

Infection of Primary and Transformed Cell lines

For RNA-seq, primary human fibroblasts were seeded onto 6-well plates at a density of 1×10^5 cells per mL and grown for 18–20 hours to ~80% confluency. Overnight *V. para* cultures were normalized to an OD₆₀₀ of 0.2 and subcultured to OD₆₀₀ = 0.6. Bacteria were pelleted, resuspended in unsupplemented DMEM and grown at 37°C for 30 minutes to induce T3SS1 expression (20). Primary Fibroblasts were washed with unsupplemented DMEM and then infected with pre-induced POR3 or POR4 strains at a multiplicity of infection (MOI) of 10. Plates were centrifuged at 1000xg for 5 minutes to synchronize infection and incubated at 37°C, 5% CO₂. At 45, 60, 75 and 90 minutes post-infection, RNeasy Protect Cell Reagent (Qiagen) was added to halt the infection and preserve the RNA. Cells were harvested by scraping, pooled and pellets were resuspended in RLT-plus buffer (Qiagen) and stored at –80°C. The same infection protocol was followed for qPCR experiments with primary fibroblasts, HeLa cells and RAW264.7 macrophages except macrophages were seeded at a density of 5×10^4 cells per mL. For MEK1/2 inhibitor

experiments, cells were starved in plain DMEM for 30 minutes and then incubated with 10 μ M U0126 (Cell Signaling) or 10 μ M DMSO (vehicle) for one hour prior to infection.

RNA Isolation and Gene Expression RNA-seq

RNA isolation was performed the same for all cell types: primary fibroblasts, HeLa cells and RAW264.7 macrophages. Cells were lysed with 27G^{1/2} needles before homogenization with QiAshredder columns (Qiagen). Total RNA was then purified using the RNeasy Plus Kit (Qiagen). Purified total RNA samples were run on the Agilent 2100 Bioanalyzer to determine RNA quality. Only samples with RIN score 9 or higher were used. RNA concentration was determined with a Qubit fluorimeter prior to library prep. 4 μ g of total DNase treated RNA were prepared with the TruSeq Stranded Total RNA LT Sample Prep Kit from Illumina. In brief, polyadenylated RNA was purified and fragmented before strand specific cDNA synthesis. cDNA was A-tailed and indexed adapters were ligated. After adapter ligation, samples were PCR amplified and purified with AmpureXP beads, then validated again on the Agilent 2100 Bioanalyzer. Samples were quantified with the Qubit fluorimeter before being normalized, pooled, and then sequenced on the Illumina HiSeq 2500 using SBS v3 reagents. Read lengths were 50 nucleotide single-end and the sequencing depth was at least 25 million reads per sample.

RNA-seq Analysis Methods

First, Fastq files were analyzed using fastqc v0.11.2 (52) and fastq_screen v0.4.4 (53) and reads were quality-trimmed using fastq-mcf (ea-utils/1.1.2-806) (54). The trimmed reads were mapped to the hg19 assembly of the human genome (UCSC version from igenomes) using TopHat (55). Duplicated reads were marked using picard-tools (v1.127 <https://broadinstitute.github.io/picard/>), read counts were generated using featureCounts (56) and differential expression analysis was performed using edgeR (57). For differential expression analysis statistical cutoffs of false discovery rate (FDR) 0.01, log₂counts-per-million (log₂CPM) 0 and fold change (FC) cutoffs of -1.5 FC 1.5 were used to identify statistically significant and possibly biologically relevant differentially regulated transcripts. Pathway and network analysis were conducted using QIAGEN's Ingenuity® Pathway Analysis tool (IPA®, QIAGEN Redwood City, <http://www.qiagen.com/ingenuity>). Differentially expressed gene heat maps were clustered by hierarchical clustering using R (<http://www.R-project.org>).

Quantitative RT-PCR

Primers were designed to amplify a 100–150bp of each target gene (Table S7), and tested for efficiency. RNA was isolated from infected primary fibroblasts and HeLa cells 75 and 90 minutes post infection, and from infected RAW264.7 macrophages 60 minutes post infection by the methods described above. RNA concentration was measured via NanoDrop and cDNA was generated using the iScript cDNA Synthesis kit (BioRad). Transcripts were quantified on a CFX384 Touch Real-Time PCR Detection System using iTaq Universal SYBR Green Supermix (BioRad) and 500nM primers. Relative gene expression for each target gene was calculated by the Cq method with respect to transcript levels in uninfected cells using the reference gene IPO8 for normalization (58).

Confocal Microscopy

HeLa cells and primary fibroblasts were seeded at 1×10^5 cells/well onto UV-sterilized glass coverslips in 6-well plates. Following the infection protocol described above, samples were first washed with 1X PBS and then fixed with 3.2% (v/v) ρ -formaldehyde for 10 minutes at room temperature. Cells were permeabilized with 0.5% (w/v) saponin for 4 minutes at room temperature and then washed in 0.1% saponin. F-actin was stained with 2 units/mL Alexa Fluor 488-phalloidin (Thermo Fisher Scientific) and DNA with 1 μ g/mL Hoechst A33342 (Invitrogen). Coverslips were mounted on glass slides with Prolong Gold anti-fade mounting media (Thermo Fisher Scientific) and imaged on a Zeiss LSM800 confocal microscope. Images were converted using ZEN 2 software (Zeiss).

Lactate dehydrogenase (LDH)-release Assay

HeLa cells and primary fibroblasts were seeded in 24-well plates. Prior to infection mammalian cells were washed in unsupplemented DMEM (-phenol red). Infection was performed at a MOI=10 with pre-induced POR3 or POR4 as described above. At each time point triplicate medium samples were collected in 96-well plates and the activity of LDH was measured using a Cytotoxicity Detection kit (Takara). Percent LDH-release was calculated compared to cells lysed in 1% Triton X-100.

Immunoblotting

Primary fibroblasts and RAW264.7 macrophages were infected as described above and for each time point were washed with 1X ice-cold PBS. Fibroblasts were treated with 0.25% Trypsin-EDTA (Thermo Fischer) prior to collection by scraping to aid in sample collection and macrophages were collected by scraping alone. Collected cells were pelleted (1,000xg) and washed twice in ice-cold 1X PBS + protease inhibitors and lysed in 50mM Tris, pH 8.0, 150mM NaCl, 5mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with protease and phosphatase inhibitors (Roche Applied Science) for 20 minutes on ice before gel electrophoresis and immunoblotting. Total ERK1/2 and Phospho-ERK1/2 were detected with p44/42 MAPK (137F5) and P-p44/42 MAPK T202/Y204 (197G2) primary antibodies, respectively and donkey secondary antibodies against rabbit (GE Healthcare).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *V. para* T3SS1 is cytotoxic to primary fibroblasts

(A) Schematic of T3SS1-induced cell death as previously defined in HeLa cells (14). The cell death is divided into stages defined by the action of known T3SS1 effectors (VopQ, yellow; VPA02450, cyan; VopS, magenta). (B) Representative confocal micrographs showing primary human fibroblasts infected with *V. para* POR3:T3SS1⁺. DNA is shown in blue (Hoechst) and actin in green (Phalloidin-Alexa488). Scale bar, 20 μ m. N=3 independent experiments. C. Lactate dehydrogenase (LDH) released by primary fibroblasts and HeLa cells infected with POR4:T3SS1⁻ and POR3:T3SS1⁺. Percent cytotoxicity is relative to uninfected (negative) and Triton X-100 treated (positive) controls. Data represent the mean of 9 replicates (N=3 biological replicates, n=3 technical replicates) $\pm$ SD.

Fig. 2. *V. para* infection induces rapid changes in host gene expression

(A) Heatmap of normalized differential expression between uninfected and *V. para* POR4:T3SS1⁻-infected primary human fibroblasts over time. The transcripts depicted here met cutoffs of $-1.5 \leq \text{FC} \leq 1.5$, $\text{FDR} \leq 0.01$, and $\log_2\text{CPM} \geq 0$. Yellow indicates transcripts with higher abundance in POR4-infected fibroblasts compared to uninfected fibroblasts, and blue indicates transcripts with lower abundance in infected cells compared to uninfected cells. Clusters (colored bars on the left) were defined through hierarchical clustering of the differential expression data and represent groups of genes with similar temporal expression patterns. **(B)** Heatmap of normalized differential expression between uninfected and POR3:T3SS1⁺-infected fibroblasts. Cutoffs and color coding are the same as in (A), and clusters were also assigned by hierarchical clustering.

Fig. 3. T3SS1 specifically induces the differential expression of 398 genes

(A) Heatmap of normalized differential expression between primary human fibroblasts infected with *V. para* POR4:T3SS1⁻ and those infected with *V. para* POR3:T3SS1⁺. The transcripts depicted here met cutoffs of $-1.5 \leq \text{FC} \leq 1.5$, FDR ≤ 0.01 , and $\log_2\text{CPM} \geq 0$ and were assigned into clusters by hierarchical clustering. Yellow denotes transcripts with increased abundance in POR3-infected cells compared to POR4-infected cells, and blue denotes a decreased abundance. (B) Heatmap of $\log_2\text{FC}$ values of the most significantly differentially expressed genes in (A). The $\log_2\text{FC}$ between uninfected and either POR4- or POR3-infected fibroblasts is shown. Yellow indicates transcripts with increased abundance in infected cells compared to uninfected cells, and blue represents decreased abundance in infected cells compared to uninfected cells.

Fig. 4. *V. para* differentially affects core pathways based on the activity of T3SS1

Heatmap of predicted activation (yellow) and repression (blue) Z-scores calculated by QIAGEN's Ingenuity® Pathway Analysis using the temporal Uninfected vs. POR4:T3SS1⁻ and Uninfected vs. POR3:T3SS1⁺ differential expression data. The color key correlates the displayed heatmap color and calculated Z-scores. Gray denotes that a pathway was unaffected ($p < 0.05$) in the infected condition relative to uninfected.

Fig. 5. T3SS1-specific induction of several transcripts requires MEK1/2 activity

(A). Immunoblot showing phosphorylated ERK1 and ERK2 (p-ERK1/2) and total ERK1/2 in POR3:T3SS1⁺-infected primary human fibroblasts treated with DMSO (vehicle) or U0126 to demonstrate the activity of U0126 as a MEK inhibitor in primary fibroblasts. N=3 independent experiments. (B–E) qRT-PCR showing the expression of *RHOB*, *JUN*, *FOS*, *PTGS2*, *EGR1*, *KLF2*, and *ATF3* in primary human fibroblasts that were pretreated with the MEK1/2 inhibitor U0126 or DMSO before being infected with POR3:T3SS1⁺ or POR4:T3SS1⁻ compared to uninfected cells (UN). Expression was normalized to the housekeeping gene *IPO8*. Data are $2^{-Cq} \pm SD$, N = 3 experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA analysis and Tukey's multiple comparisons test; ns, not significant.

Fig. 6. Key differentially expressed genes activate cell survival in POR3-infected fibroblasts
Illustration of the action key POR3-differentially expressed genes in the cell survival network generated using Ingenuity® Pathway Analysis. The subcellular localization of the protein products of each gene and differential expression status (yellow, increased; blue, decreased) are depicted. Dotted lines denote indirect interactions. Orange lines indicate a gene expression state consistent with activation of cell survival; yellow lines indicate an expression state inconsistent with cell survival activation.

Fig. 7. *V. para* T3SS1 activates cell survival networks and represses cell death networks
(A) Heatmap of Ingenuity® Pathway Analysis Z-score prediction of activation (yellow) or repression (blue) of biological networks in response to POR3:T3SS1⁺ infection over time using the temporal Uninfected vs. POR3:T3SS1⁺ differential expression data from Fig. 2B.
(B) Predicted cell survival and organismal death network activation (>0) and repression (<0) by POR3:T3SS1⁺ over the time course of T3SS1-mediated cell death. Plotted values correspond to the Ingenuity® Pathway Analysis Z-scores from Cell Survival and Organismal Death networks in (A) and Table S6.