Host-cell modulation by T3SS effectors of Enteropathogenic *E. coli*

Thesis for the degree of "Doctor of Philosophy"

by

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Abstract

EPEC is an important enteric pathogen for children in developing countries. This pathogen employs a Type 3 secretion system (T3SS) to inject the host cells with dozens of proteins, termed effectors, which subvert host processes to benefit the pathogen. How these effectors function is only partially understood and the aim of this work was to elucidate the function of some of these effector proteins and the impact of the T3SS on the fate of the host cells. Specifically, in this work I examined the following aspects of the T3SS activity:

1. NleD is an injected metalloprotease effector which cleaves MAPKs in the host cells. How does NleD manipulate the MAPK signaling pathway in the host cells during infection?
2. Some of the injected effectors have opposed impact on the survival of infected cells. How are these effects integrated and how does EPEC regulate the host cell viability during infection?
3. Which host genes are required for efficient infection by EPEC?

I show here an in-depth molecular analysis of the function of the effector NleD and its modulation of a key signaling pathway in eukaryotic cells – the MAPK pathway. NleD is a metalloprotease which cleaves within the TXY motif of the p38 and JNK MAP kinases (MAPKs). I showed that this translocated metalloprotease is a dual-function effector which has two seemingly contradicting roles: (1) It cleaves specifically un-phosphorylated p38 and JNK, and (2) it binds the phosphatase PPM1A which can dephosphorylate the TXY of p38 and JNK and restore their susceptibility to cleavage, but the binding of NleD inhibits its activity. This way EPEC can, with the action of a single effector, both promote and attenuate MAPK signaling. The inhibition of the catalytic activity of PPM1A is achieved by binding of NleD which physically blocks the entrance of large substrates into the catalytic pocket of the phosphatase, yet the inherent activity of PPM1A is not inhibited as it is able to dephosphorylate small substrates which infiltrate the NleD-mediated blockage.

I then analyzed factors regulating the survival of host cells during infection, which requires the integration of signaling from several pathways. Here I show that EPEC institutes a fragile state of equilibrium of signals affecting the viability of the host cells. Alterations in this equilibrium which impair the ability of EPEC to translocate the protective effector EspZ lead to rapid death of the host cells. The specific factor that initiates the death of the host cells was not identified but I showed that it is not one of the known effectors of EPEC. It may be a previously un-characterized effector or some other molecule such as a metabolite which leaks into the host cells via the T3SS needle.

Taking a wider look on the infection process, I performed a genome-wide CRISPR-Cas9 screen to identify genes in the host cells which promote EPEC infection. The screen identified a few dozens of host cell genes which are involved in the infection process. Mutations in these genes conferred resistance to the death induced following EPEC infection. Notable among the results are genes such as MGAT1 and TMEM165 which are involved in protein glycosilation, and I postulate that this glycosilation enables the initial attachment of the bacteria to the cells. Other prominent genes in the screen are members and regulators of the Arp-2/3 complex. This complex is responsible for the dynamics of the actin cytoskeleton of the host cells and is required for processes such as cell migration and phagocytosis. The role of the Arp-2/3 complex during EPEC infection is yet to be discovered and based on previous works I hypothesize that polymerization of actin, which is regulated by this complex, is required for translocation of effectors into the host cells.

Overall, my study highlights the complex modes of action of bacterial type three secretion system effectors and the complexity of the host-pathogen interaction during infection and dissects several aspects of this process. I performed a thorough analysis of the mechanism by which EPEC controls a key signaling pathway in the host – the MAPK signaling. Zooming out, I took a look at how EPEC manipulates the viability of the host cells during infection – the result of integration of several signaling pathways. Finally, I performed a genome-wide screen to identify genes of the host which are involved in any stage of the infection. Thus I put my share in gaining a better understanding of

the processes underlying infection, which can lead to development of new tools to prevent and treat devastating diseases caused by bacterial pathogens.

Contents

1. Introduction – Virulence mechanisms and regulation in EPEC

1.1 EPEC

Escherichia coli (*E. coli*) is a facultative un-aerobic type of bacteria which is widely spread as a member of the normal intestinal microbiome of healthy people (Lozupone *et al*, 2012). Some strains of *E. coli* acquired various virulence factors during evolution, enabling them to cause a range of intestinal and systemic diseases (Kaper *et al*, 2004). Examples of such processes include uropathogenic *E. coli* (UPEC) which acquired several elements, such as fimbriae, to allow it to bind to epithelial cells in the urinary tract and cause Urinary Tract Infections (UTIs) (Subashchandrabose & Mobley, 2015). Enterotoxigenic *E. coli* (ETEC) possesses two toxins – heat labile toxin and heat stable toxin which induce inflammation, leading to gastrointestinal disease (Fleckenstein & Kuhlmann, 2019). Enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) share a prominent virulence factor – a type three secretion system (T3SS) for translocation of proteins to the environment and into host cells (Wong *et al*, 2011).

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of persistent infantile diarrhea with high rates of associated mortality, mainly in developing countries (Kotloff *et al*, 2013). It is not one of the most common intestinal pathogens, but it is the pathogen with the highest case fatality ratio among them (Asare *et al*, 2022). An animal model to study the process of disease is lacking since EPEC is a human-specific pathogen, therefore the closely related murine pathogen *Citrobacter rodentium* serves as the best alternative.

1.2 T3SS

The major virulence factor employed by EPEC is the Type 3 secretion system (T3SS), a needle-like structure termed injectisome (Clements *et al*, 2012). The T3SS is a structure composed of over twenty protein components and is evolutionarily related to the flagella (Deng *et al*, 2017). The length of the needle of the T3SS is tightly regulated and in EPEC it measures approximately 50 nm (Monjarás Feria *et al*, 2012). The translocation of

effectors by the T3SS requires ATP and involves denaturation of the translocated protein which is re-folded once it is inside the host cells (Gaytán *et al*, 2016). EPEC employs the T3SS to deliver dozens of bacterial effector proteins into the cytoplasm of host cells. The T3SS is encoded on a 35-kbp pathogenicity island termed "Locus of Enterocyte Effacement" (LEE). The LEE encodes the structural components of the T3SS along with several effector proteins and related chaperones and regulators (Wong *et al*, 2011). The injected effectors target different host-cell processes and signaling cascades to allow efficient colonization (Pearson *et al*, 2016).

1.2.1 T3SS regulation

Production of the T3SS consumes a substantial portion of the bacterial energetic and metabolic resources and therefore this process is tightly regulated. The LEE is composed of five operons termed LEE1-LEE5, along with several additional independent transcriptional units (Mellies *et al*, 2007). The first gene in the LEE1 operon encodes the LEE Encoded Regulator (Ler), which is the master regulator of the LEE expression. Ler has autoinhibitory effect as it down-regulates the expression of LEE1, but it strongly activates the expression of the other LEE operons (Yerushalmi *et al*, 2008). In conditions which are not suitable for virulence, such as low temperature, the expression of the LEE1 and therefore Ler is repressed by the histone-like protein H-NS (Umanski *et al*, 2002). When conditions are permissive, the negative regulation of H-NS is relieved and a positive regulator activates the expression of Ler. The activator can be either the LEE-encoded GrlA or the plasmid-encoded PerC which are functionally redundant (Bustamante *et al*, 2011). Together with additional regulators involving quorum sensing and chemosensing, a complex regulatory network is formed.

1.2.2 T3SS effectors and chaperons

EPEC translocates dozens of effectors into the host cell. These effectors influence many aspects of cell biology such as cytoskeleton modification, cellular trafficking, signaling and cell cycle and survival (Pearson *et al*, 2016). One of the characteristics of EPEC infection of the intestine is that infection leads to the effacement of microvilli on the apical surface of the enterocytes and formation of actin-rich pedestals underneath the attached bacteria

(Cepeda-Molero *et al*, 2017). Therefore EPEC belongs to a group of bacteria which are known as "attaching and effacing" pathogens (AE pathogens). The attachment and effacement process is mediated mainly by the effector Tir. The translocation of many effectors is assisted by different chaperon proteins which specifically bind them in the cytoplasm of the bacteria and direct them to the T3SS apparatus for translocation. For example, the multi-cargo chaperon protein CesT supports the translocation of Tir as well as other effectors and another chaperon protein, CesF, assists the translocation of the effector EspF (Mills *et al*, 2013). The injected effectors act together in order to silence the immune response and modify the host cell biology, allowing for successful establishment of host colonization.

2. Objectives

In this work I aimed to study the host cell manipulation during EPEC infection from several perspectives. First, focusing on a single signaling pathway in the host I studied the manipulation of the MAPK signaling pathway during EPEC infection. Looking at a more complex signaling network, I dissected the host cell death in response to infection. Finally, I took a broad-sight approach in order to identify host cell genes which are required for the different stages of EPEC infection. The following specific questions were addressed:

1. NleD is an injected metalloprotease effector which cleaves MAPKs in the host cells. How does NleD manipulate the MAPK signaling pathway in the host cells during infection?
2. Some of the injected effectors have opposed impact on the survival of infected cells. How are these effects integrated and how does EPEC regulate the host cell viability during infection?
3. Which host genes are required for efficient infection by EPEC?

For the convenience of the reader the work performed to answer these questions is presented here as three separate chapters, each containing its own specific introduction. In the end of the work, a comprehensive discussion is presented.

3. Materials and methods

3.1 Bacterial strains, plasmids, and primers

Bacterial strains and plasmids used in this study are listed in Supplementary Tables S1 and S2, respectively. Cloning was performed using either restriction enzymes or Gibson assembly. Inserts were amplified by PCR using the primers listed in Supplementary Table S3.

3.2 Cell lines

Several cell lines were used in this work: HeLa (ATCC CCL-2, from prof. Banin, BIU), HEK293T (ATCC CRL-3216, from prof. Mandelboim, HUJI) and HT-29 (ATCC HTB-38, from prof. Ben-Neriah). General handling was similar: Cells were grown in DMEM + 10% fetal-bovine serum in T75 flasks, in 37ºC humidified incubator with 5% CO_2. The specific cell-line to use in each experiment was chosen based on parameters such as ease of handling and protein production (HEK293T), good visualization with microscopy (HeLa) and resemblance to the natural infection site – the intestine (HT-29).

3.3 Protein production in *E. coli* BL21

To produce SBP-tagged NleD, an overnight culture of *E. coli* BL21 containing plasmids encoding the desired form of NleD was diluted 1:100 in LB supplemented with the appropriate antibiotics and grown for 2.5-3 h at 37ºC to reach an OD_{600} of ~0.6. The bacteria were then transferred to 16ºC, and after 30 minutes, IPTG was added (0.2 mM). Expression was allowed for 18-20 h. The culture was centrifuged, and the pellet was resuspended in 1xPBS supplemented with 150 mM NaCl, 0.1% Triton X-100, 2 mM $MgCl_2$, and DNaseI. Bacteria were then lysed using a microfluidizer, and the lysate was cleared by centrifugation for 30 min at 20,000 RCF. Streptavidin-agarose beads were used to capture NleD, and proteolysis and pull-down assays were performed while the protein was bound to the beads.

For the production of p38 and P-p38, we used overnight cultures of *E. coli* BL21 containing a plasmid expressing 6xHis-tagged p38 alone, or co-expressed with a constitutively active form of MKK6, a dual-specificity MAP2K that phosphorylates both the T and Y residues of the p38 TXY motif (Huang *et al*, 1997). Cultures were grown to an OD_{600} of ~0.4 in LB, 37ºC. The bacteria

were then transferred to 30ºC, and after 30 minutes, IPTG (1 mM) was added. Expression was allowed for 5 h. The culture was centrifuged, and the pellet was resuspended in buffer containing 50 mM Tris-HCl pH 7.4, 0.5 M NaCl, 10 mM imidazole, 2 mM $MgCl_2$, and DNaseI. Lysis of the bacteria was performed using a microfluidizer. The lysate was cleared by centrifugation for 30 min at 20,000 RCF. Proteins were purified in an AKTA machine using a His-trap column and eluted with an imidazole gradient up to 300 mM. Protein-containing fractions were desalted and equilibrated with a buffer containing 12.5 mM HEPES pH 7.5, 100 mM KCl and 1 mM dithiothreitol (DTT). For storage, glycerol was added to a final concentration of 6.25%. Purified proteins were stored at -80ºC.

3.4 Transfection for PPM1A production

Expi293 cells in suspension were transfected using Expifectamine 293 (Thermo) according to the manufacturer's protocol. Expression was allowed for 72 h after transfection.

3.5 Protein production in HEK293T cells

For the production of myristoylated PPM1A and its mutants, HEK293T were transfected with a plasmid expressing the desired 3xFLAG-tagged version of PPM1A. Cells were seeded into 15-cm plates. The following day transfection was performed: Plasmid DNA was diluted in 150 mM NaCl, and polyethylenimine (PEI, Polyscience 24765-1) was added at a ratio of 4 µl of 1mg/ml PEI solution per 1 µg DNA. The tube was thoroughly vortexed and incubated for 30 minutes at room temperature to allow transfection complexes to form. The complexes were added to the cells, and after 5 h, the cells were washed twice, and the medium was changed to DMEM supplemented with 5% FCS. Expression was allowed for 72 h. Cells were scraped and resuspended in 20 mM Tris pH 7.4. A short sonication was performed, and then 150 mM NaCl and 1% Triton X-100 were added. The lysate was cleared by centrifugation for 30 min at 20,000 RCF. Protein purification was performed on anti-FLAG beads with elution using 3xFLAG peptide (Sigma) according to the manufacturer's protocol. 40% glycerol was added, and the protein was transferred to -20ºC for long-term storage. To preserve the enzymatic activity of PPM1A, the protein was never frozen before the enzymatic assay.

3.6 Protein-protein interactions (pull-downs and mass-spectrometry)

Lysates cleared as described above (section 3.3) were incubated with streptavidin-agarose (Sigma 51638) or glutathione-agarose (Sigma 4510) beads for 1 h at 4ºC, washed 3 times with 1xPBS, and incubated for another 1 h with HeLa/HEK293 cell lysates or other bacteria lysates. After incubation, beads were washed 3 times with 1xPBS and frozen for analysis by mass-spectrometry or boiled in Laemmli sample buffer (Bio-rad #1610747) for western blot analysis.

For MS analysis, the immobilized proteins were denatured, reduced, alkylated, and digested by standard procedures employing 8M urea, dithiothreitol, iodoacetamide, and trypsin. Analysis of the resulting peptides was performed using a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA USA) coupled online to a nanoflow UHPLC instrument, Ultimate 3000 Dionex (Thermo Fisher Scientific, Waltham, MA USA), using a 1 – 80% acetonitrile gradient on a reverse phase 25-cm-long C18 column (Thermo Scientific, PepMapRSLC). Mass spectra data were processed using the MaxQuant computational platform, version 1.5.3.12, and searched against translated coding sequences of the human proteome obtained from Uniprot. Relative protein quantification in MaxQuant was performed using the label-free quantification (LFQ) algorithm.

3.7 *In vitro* proteolysis and dephosphorylation assays

Cleavage of p38 and JNK by NleD: SBP-NleD was captured on streptavidin-agarose beads as described above. The beads and p38 or P-p38 were incubated for 1 h at 37ºC in a buffer of 50 mM Tris pH 8.0, 50 mM NaCl and 2 mM $CaCl_2$.

Assays of P-p38 dephosphorylation by PPM1A were performed by addition of the indicated purified proteins to a reaction buffer composed of 50 mM Tris, 50 mM Bis-Tris and 100 mM Acetate, pH 8.0. When needed, the reaction tube was incubated for 1 hour at 4ºC to allow protein binding and complex formation. The tubes were then transferred to 37ºC, and the reaction was allowed for 1 hour. In all cases, the reaction was stopped by boiling in the Laemmli sample buffer.

3.8 pNPP assay for PPM1A activity

As described above, purified PPM1A and NleD were added to the reaction buffer and incubated for 1 h at 4ºC to allow protein binding and complex formation. pNPP (1 mg/ml) was added, and the samples were transferred to 37ºC. Cleavage of pNPP was assessed by serial absorbance measurements at 410 nm using a TECAN Spark 10M plate reader. The results were plotted, and the slope was calculated to give the relative activity of PPM1A in each experimental condition.

3.9 SDS PAGE and western blot analysis

Protein extracts were separated on 12% GTX gels (Bio-Rad 456-8046) by SDS-PAGE run for ~60 min with voltage of 120V. Semi-dry system (Trans-Blot Turbo, Bio-Rad) was used to transfer the separated proteins to nitrocellulose membranes according to the manufacturer protocol (i.e., 3 min, 25V). Membranes were probed by western blot analysis using the antibodies listed in Table S4.

3.10 Infections

Cells seeded in 35 mm or 100 mm dishes were washed with 1xPBS and infected with 2 ml or 8 ml DMEM supplemented with EPEC diluted 1:100 from an overnight standing culture at 37ºC for the desired time (3 h unless mentioned otherwise). When mentioned, 0.05-0.5 mM IPTG were added to the cells. For Western blot analysis after infection, cells were washed and lysed with RIPA buffer (Sigma R0278) or 1xPBS supplemented with 1% Triton X-100, centrifuged, and the supernatant was collected. For toxicity assay see section.

3.11 Live microscopy

HT-29 cells were seeded on ibidi µ-Dish 35 mm plates in a density of 10^5 cells/plate. The next day, a pre-activation step was performed: Bacterial over-night standing culture was diluted 1:100 in DMEM and incubated for 3 hr in 37°C to allow for activation of virulence gene expression. The cells were then washed once with 1xPBS and the DMEM containing the pre-activated bacteria was added. Live microscopy was performed using a Zeiss Axio observer Z1 inverted microscope in 37°C and analyzed the ZEN software.

3.12 Fluorescence microscopy

After infection the cells were fixed in 3.7% formaldehyde in 1xPBS for 10 min at room temperature, washed once with 1xPBS, perforated with 0.25% Triton X-100 in 1xTBST for 10 min at room temperature, washed once with 1xPBS. Phalloidin-rhodamine (Sigma) was used to stain F-actin and DAPI (Molecular Probes) to strain Host and bacterial DNA. Stained cells were analyzed by fluorescence microscopy using Zeiss Axio observer Z1 inverted microscope and the ZEN software.

3.13 PI staining

10 minutes before the end of the desired infection time, propidium iodide (PI, 10 µM) was added to the infection medium to allow staining. After this the medium was collected and centrifuged at 700 rcf to collect the detached cells. To collect the population of attached cells the plate was washed with 1xPBS and the cells were scraped. Both attached and detached cell samples were washed with 1xPBS by centrifugation and fixed in 3.7% formaldehyde. Analysis was performed using pictures taken in a Bio-Rad ZOE microscope and analyzed with the CellProfiler software to detect cells and measure the intensity of PI staining.

3.14 Toxicity assay

HEK293-T stably expressing GFP were seeded in 100 mm plates at a density of $5x10^6$ cells per plate and grown overnight in DMEM medium supplemented with 10% fetal calf serum (FCS; Biological Industries) and antibiotics (penicillin-streptomycin solution; Biological Industries) at 37°C in 5% CO_2 incubator. For infection, DMEM was replaced with fresh DMEM + FCS lacking antibiotics and cells were infected with bacteria that had been statically grown overnight at 37°C (multiplicity of infection 100), for the indicated times. Where indicated, IPTG was added to induce CesT expression. To terminate the infection cells were washed with 1xPBS, the remaining attached cells were scraped and GFP intensity was measured by fluorimetry using Spark 10M microplate reader (Tecan). The toxicity relative to non-infected cells (NI) was calculated as $1 - \frac{\text{fluorescence level(Infected cells)}}{\text{fluorescence level (NI)}}$.

3.15 Bacterial adherence assay

HEK-293T cells stably expressing GFP were infected by mCherry-expressing bacteria. At 3 h post infection the infected cells were washed from unattached bacteria and detached host cells and the levels of remaining mCherry (bacteria) and GFP (cells) were measured. The mCherry/GFP ratio was used as readout for bacterial attachment.

3.16 Genome-wide CRISPR-Cas9 screen

HT-29-Cas9 CRISPR libraries were constructed as described previously (Blondel et al, 2016) using the Avana sgRNA library (from prof. Waldor, Harvard medical school), which contains four different sgRNAs targeting each human protein-coding gene (Doench et al, 2016). The HT-29 cell-line was chosen as it closely resembles the actual biological infection site – the intestine. There are complex considerations in using these cells, as being cancerous these cells may harbor gene duplications. Although this may lead to certain biases in the screen, having several alleles is an advantage in case of essential genes, allowing for only partial deletions (practically a knock-down). An additional advantage of using the HT-29 cell-line is the ability to compare our results to the results of previously performed screens (Blondel *et al*, 2016; Pacheco *et al*, 2018; Zhang *et al*, 2020). Two T225 flasks (Corning) were seeded with $40x10^6$ cells per flask and then incubated for 24 h. At the time of the screen, there were ~$80x10^6$ cells, corresponding to ~1000x coverage per sgRNA. Cells were at ~80-90% confluence at the time of infection. The infection was performed as described above with minor modifications. Briefly, HT-29 libraries were infected with pre-activated EPEC/pMgrR at an MOI of 100 for 90 min. After infection, the libraries were expanded in McCoy's 5A + FBS containing 50 µg/mL gentamycin which kills almost 100% of the bacteria (i.e. no detectable bacteria in the medium but bacterial re-growth occurs if gentamycin is cleared at any point). Flasks were checked daily to monitor recovery of survivor cells; when 80-90% confluence was achieved, cells were trypsinized, pooled, and reseeded for the next round of infection. In total, four rounds of infection were conducted.

To sequence the CRISPR libraries, gDNA was prepared with the GeneGet DNA PCR purification kit from ~$10x10^6$ cells. Guide sequences were amplified

as previously performed (Zhang et al, 2020) and sequenced for 78 cycles with a MiSeq V3 150 cycle kit, to depth of 0.4-2.7 million reads/sample. Fastq files were trimmed in CLC Genomics workbench and then mapped with MaGeCk (Li et al, 2014). Comparisons between input and output libraries were performed using the -test command and control non targeting guide RNAs were used for normalization.

3.17 Knock-down of candidate genes

Plasmids encoding shRNA targeting the desired genes were transfected to HEK293T cells: Plasmid DNA was diluted in 150 mM NaCl, and polyethylenimine (PEI) was added at a ratio of 4 µl PEI solution per 1 µg DNA. The tube was thoroughly vortexed and incubated for 30 minutes at room temperature to allow transfection complexes to form. The complexes were added to the cells, and after 5 h, the cells were washed twice, and the medium was changed to DMEM supplemented with 10% FCS. After allowing for 48 hr recovery, 2 µg/ml Puromycin was added to select for stably expressing cells. Knock-down was confirmed using antibodies recognizing the proteins encoded by the target genes.

Supplementary Table S1– Strains used in this study

Name	Description	Reference or source
E2348/69	EPEC wild-type	J. Kaper
SN1961	E2348/69 *escV*::Tn5*kan*	(Nadler *et al*, 2006)
BL21(DE3)	F-, *ompT*, *hsdSβ*(rβ-mβ-), *dcm*, *gal*, (DE3) *tonA*	Novagen
EM2018	EPEC *cesT:Kn*	(Li *et al*, 2006)
EM3325	EPEC *PP4*:*Kn*	Erez Mills (HUJI)
EM3715	EPEC *grlRA*:*Kn*	(Padavannil *et al*, 2013)
SK5590	EPEC *espZ*:*Kn*	(Berger *et al*, 2012)
SS7291	EPEC *ΔnleBECD ΔespB*	(Litvak *et al*, 2017)
SS7292	EPEC *ΔnleBECD espB* K179	(Litvak *et al*, 2017)
SS7293	EPEC *ΔnleBECD espB* E203	(Litvak *et al*, 2017)
SS7294	EPEC *ΔnleBECD espB* T239	(Litvak *et al*, 2017)
SS7295	EPEC *ΔnleBECD espB* L241	(Litvak *et al*, 2017)
SS7296	EPEC *ΔnleBECD espB* K282	(Litvak *et al*, 2017)
YS7606	EPEC *Δ*cesT	This study
YS7607	EPEC *Δ*cesT *espZ*:*Kn*	This study
NE8810	EPEC cesT*	(Pearl Mizrahi *et al*, 2021)
EPEC2	EPEC deleted of all effectors except Tir and EspZ	(Cepeda-Molero *et al*, 2017)
EPEC0	EPEC deleted of all effectors	(Cepeda-Molero *et al*, 2017)

Supplementary Table S2 – Plasmids used in this study

Name	Description	Reference or source
pLG4821	pET15b p38α	David Engelberg (HUJI)
pYS8540	pACYC184-lacIq-MKK6(EE)	This study
36943	pET28 SBP TEV	Addgene
pLG5877	pET28 SBP-*nleD* EPEC	This study
pLG5976	pET28 SBP-*nleD* EPEC E143A	This study
pLG5878	pET28 SBP-*nleD* Salmonella enterica ser. Arizona	This study
pLG5880	pET28 SBP-*nleD* Citrobacter rodentium	This study
pNC4775	pGEX2T-NleE	This study
pLG5859	pGEX2T-NleD$_{EPEC}$	This study
pEGFP_N1	Expression vector	Clontech
pLG6626	pEGFP-N1 PPM1A	This study
pSK7210	pEGFP-N1 PPM1A R174G	This study
pSK7212	pEGFP-N1 PPM1A D239N	This study
pYS8994	pEGFP-N1 PPM1A-3xFLAG	This study
pYS8996	pEGFP-N1 PPM1A D239N-3xFLAG	This study
pSA10	pKK177-3 containing *lacI*^{q}	(Schlosser-Silverman *et al*, 2000)
pYS7878	pZE12-MgrR	Hannah Margalit (HUJI)
pEM2063	pACYC184-lacIq-His-CesT	Erez Mills (HUJI)
TRCN0000000408	shRNA targeting STK11	Sigma
TRCN0000000409	shRNA targeting STK11	Sigma
TRCN0000035194	shRNA targeting MGAT1	Sigma
TRCN0000035198	shRNA targeting MGAT1	Sigma
TRCN0000123061	shRNA targeting WASL	Sigma
TRCN0000123062	shRNA targeting WASL	Sigma
TRCN0000151349	shRNA targeting TMEM165	Sigma
TRCN0000151701	shRNA targeting TMEM165	Sigma

Supplementary Table S3 – Primers used in this study

Plasmid/ strain	Primer sequence
pYS8540	cttttgtaaaactgattcttggagactaaTAAGCTTATCGATGATAAGCTGTCAAAC
	CTTATCGTCGTCATCCTTGTAATCCATAGTTAATTTCTCCTCTTTAATGAATTCTGTGTG
	ATGGATTACAAGGATGACGACGATAAGATGGCGTCTCAGTCGAAAGGC
	TTAGTCTCCAAGAATCAGTTTTACAAAAG
pLG5976	GACTATTGTTTTTCATGCGTTGCTCCATGTTTTCC
	GGAAAACATGGAGCAACGCATGAAAAACAATAGTC
pLG6626	CATCAACAGATGATATGTGGTAAAGCGGCCGCGACTCTAG
	CTAGAGTCGCGGCCGCTTTACCACATATCATCTGTTGATG
pSK7210	CCAAGTAATCCGCTGGAGAAAGAAGGAATTCAGAATGCAGGTGGCTCTGTAATG
	CTTCTTTCTCCAGCGGATTACTTGG
pSK7212	GATCAGTTCATTATCCTTGCATGTAATGGTATCTGGGATGTTATGGGAAATG
	ACATGCAAGGATAATGAACTGATCATC
YS7606	A GCGTTATTCA GAATTTTTCA GGGA
	CGGTCATGTTGCTTTTGGTC
YS7607	GCATGGATCCTCATGATGTCATCCTGCGAACG
pYS8994	GATCATGATGGCGATTATAAAGATCATGATATTGATTATAAAGATGATGATGATAAATAAAGCGGCCGCGACTCTAG
	CATGATCTTTATAATCGCCATCATGATCTTTATAATCCCACATATCATCTGTTGATGTAGAGTCAG

Table S4 – Antibodies used in this study

Antibody	Company	Serial number
Anti-JNK	BD Pharmingen	554285
Anti-phospho-JNK	Cell signaling	9251
Anti-p38	Santa Cruz	SC-535
Anti-phospho-p38	Cell signaling	9216
PY20	Sigma	P4110
Anti-SBP	Santa Cruz	101595
Anti-PPM1A	Cell Signaling	D18C10 XP

4. NleD is a dual-function T3SS effector executing simultaneous host MAPK cleavage and inhibition of PPM1A protein phosphatase

4.1 Introduction

4.1.1 MAPK Signaling

Following pathogen infection, bacterial components activate signaling cascades that induce the host inflammatory response. The mitogen-activated protein kinase (MAPK) signaling pathway is conserved in all eukaryotes and is involved in fundamental cell processes, including a pivotal role in inflammation signaling (Cargnello & Roux, 2011). MAPKs are serine/threonine kinases that can be divided into three main subfamilies, each containing several isoforms; the Jun N-terminal Kinase (JNK), Extracellular signal-Regulated Kinase (ERK), and p38. Following diverse stimuli such as pathogen infection, host cell receptors are activated by bacterial components or cytokines such as IL-1. In other cases, the MAPK cascade is initiated in response to specific intracellular signals such as the stress induced by the translation inhibitor anisomycin. The MAPK cascade transduces the signal through three tiers of protein kinases. The first tier includes MAPK kinase kinases (MAP3K) such as TAK1 or MLK7, which phosphorylate the downstream tier of MAPK kinases (MAP2K). Consequently, MAP2Ks phosphorylate adjacent threonine and tyrosine residues at a conserved TXY motif of MAPKs, where T and Y are threonine and tyrosine, respectively, and X is either proline, glycine or glutamic acid. TXY phosphorylation leads to activation of the MAPKs which then phosphorylate downstream proteins including other kinases and transcription or translation factors (Arthur & Ley, 2013).

The MAPK signaling is negatively regulated by protein phosphatases such as members of the metal-dependent protein phosphatases (PPM) family. The PPM family is comprised of several serine/threonine protein phosphatases, which share a conserved catalytic domain and have various substrates, which are frequently shared by several phosphatases (Kamada *et al*, 2020). PPM1A

and PPM1B are involved in regulation of cell cycle and inflammation through the MAPK and NF-κB cascades.

4.1.2 PPM1A

Both PPM1A and PPM1B undergo irreversible N-myristoylation, i.e. elimination of the first methionine and ligation of a 14-carbon saturated fatty myristic acid to a glycine residue at position 2. This myristoyl group is predicted to be located in proximity to the opening of the phosphatase catalytic site. Due to its composition, the myristoyl group in some cases acts as an anchor which docks proteins to the membrane but this appears not to be the case here. It was shown that this modification is needed to render PPM1A its substrate specificity to p38 and JNK. In its absence the phosphatase activity is diminished when p38 was used as a substrate, but increased when the synthetic substrate pNPP was used (Chida *et al*, 2013).

In addition to its role in halting MAPK signaling, PPM1A is also a modulator of the Transforming Growth Factor beta (TGF-β) and Bone Morphogenetic Protein (BMP) signaling via dephosphorylation of SMAD signal transducers (Lin *et al*, 2006). Additional PPM1A substrate is the adenosine monophosphate (AMP)-activated protein kinase α (AMPKα) (Chida *et al*, 2013), an important sensor of energy requirements within the cell. Thus, PPM1A is an important regulator of host cell processes, affecting cell cycle, cellular metabolism and its response to various external as well as internal stimuli – from hormonal signaling to pathogen infection.

4.1.3 EPEC detection by host cells

Host cells possess an intrinsic ability to detect the insertion of the T3SS needle of EPEC into the membrane and in response activate the NF-κB signaling pathway. However, the bacteria inject effectors such as NleC and NleE that intercept the T3SS sensing and NF-κB signaling (Litvak *et al*, 2017). NleC cleaves NF-κB proteins (Baruch *et al*, 2011; Pearson *et al*, 2011), whereas NleE blocks the activation of the MAP3K TAK1, and thus is expected to block both NF-κB and MAPK signaling (Nadler *et al*, 2010; Zhang *et al*, 2012; Newton *et al*, 2010). Concomitantly, the effector NleD blocks MAPK signaling via the direct cleavage of JNK and p38. The cleavage site is

precisely between the X and Y residues of the TXY motif in the activation loop of the substrate MAPKs (Baruch *et al*, 2011).

4.1.4 Hypothesis, rationale and aim

Given that the MAPK phosphorylation site and the NleD cleavage site are overlapping it is plausible that cleavage of the MAPKs by NleD is affected by their phosphorylation state. If this is the case, one may assume that EPEC developed strategies to optimize the cleavage of the MAPKs. In order to do so, NleD may interact with other host proteins.

We hypothesized that the phosphorylation state of MAPKs affects the ability of NleD to cleave them, and in order to achieve optimal cleavage of the MAPKs NleD recruits other host proteins.

Specifically, we asked the following questions:

1. Does the phosphorylation state of the MAPKs affect their susceptibility to cleavage by NleD?
2. Does NleD interact with other host proteins and if yes, what are the consequences of this interaction?

4.2 Results

4.2.1 Phosphorylated p38 is resistant to cleavage by NleD

Given that the MAPK phosphorylation site and the NleD cleavage site are overlapping (i.e both are at the TXY motif), we asked whether NleD can cleave phosphorylated p38 and JNK. To directly test whether NleD can cleave phospho-p38, I set up an *in vitro* system composed of NleD or catalytically dead NleD mutant, incubated with purified p38 or P-p38. To obtain phosphorylated p38, I co-expressed it in *E. coli* with a constitutively active form of its cognate MAP2K, MKK6 (Huang *et al*, 1997). I then analyzed the cleavage products and phosphorylation levels using anti-p38 (total p38), anti-phospho-p38 (to detect dually-phosphorylated p38) and anti-phospho-tyrosine antibodies (to detect tyrosine-phosphorylated p38). I found that NleD cleaved p38, but not phosphorylated p38 (Fig. 4.2.1). These results show that phosphorylation of the TXY motif interferes with NleD activity, demonstrating that NleD specifically targets the un-phosphorylated forms of p38.

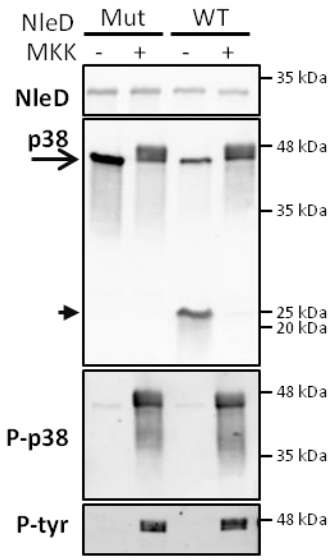

Figure 4.2.1 Phosphorylated p38 is resistant to cleavage by NleD.
Purified NleD-SBP was incubated with purified recombinant p38. For purification, p38 was either expressed alone or co-expressed with constitutively active MKK6 that phosphorylates p38. Intact and degraded p38 were detected using anti-p38 and anti phospho-p38 antibodies. Arrows point to full-length proteins. Arrowheads point to cleavage products. Molecular weight marker is shown on the right-hand side.

4.2.2 NleD binds PPM1A

To gain a better understanding of the function of NleD, we used Mass Spectrometry (MS) to identify interacting partners of NleD in extracts of HEK293T host cells. Immobilized NleD, N-terminally fused to GST, was incubated with extract of HEK293T cells, and the captured proteins were subjected to MS analysis. As control we performed parallel analysis using GST-NleE. Notably, the MS analysis failed to identify the known NleD substrates p38 and JNK, presumably due to the very transient nature of enzyme-substrate interaction (Table 4.2.2). Nevertheless, NleD exhibited robust binding to protein metallophosphatase 1A and 1B (PPM1A and PPM1B, respectively). Intriguingly, NleD and PPM1A share the same substrates, the TXY motif of p38 and JNK; NleD cleaves it and PPM1A dephosphorylates it (Kamada *et al*, 2020).

Protein name	Gene name	Unique peptides NleD	Unique peptides NleE	LFQ intensity NleD	LFQ intensity NleE
Protein phosphatase 1A	PPM1A	17/18	0/0	$4.58*10^7$	0
Protein phosphatase 1B	PPM1B	14/15	0/0	$2.86*10^7$	0
Coatomer subunit beta	COPB2;Copb2	3/1	26/26	$1.39*10^5$	$2.54*10^7$
Coatomer subunit epsilon	COPE	1/0	9/9	0	$1.79*10^7$
Dynactin subunit 4	DCTN4	0/1	4/4	0	$8.77*10^6$

Table 4.2.2 Major identified interacting partners of NleE and NleD.
Data were obtained from two independent experiments, and the number of unique peptides from each experiment is shown together with an average LFQ intensity score.

4.2.3 Catalytically dead mutants of PPM1A and NleD still interact

I next tested whether the catalytic activity of PPM1A or that of NleD are required for their interaction. I examined the capacity of recombinant NleD immobilized on beads to bind to two reported inactive mutants of PPM1A, ectopically expressed in HEK293T cells. I found that mutating the arginine residue in position 174 of PPM1A (R174G) drastically diminished the NleD-PPM1A interaction (Fig. 4.2.3). Of note, this residue is reported to form salt-bridges that stabilize the structure of the catalytic domain of PPM1A (Pan *et al*, 2015). In contrast, a PPM1A mutant in the aspartic acid residue at position 239 (D239N), which is also required for the catalytic activity, but lacks the structural significance of the former variant, still binds NleD similarly to the wild type PPM1A (Fig. 4.2.3). Likewise, the catalytically dead NleD E143A efficiently binds wild type PPM1A and PPM1A D239N mutant. These results demonstrate that the catalytic activities of NleD and PPM1A are not required for their physical interaction.

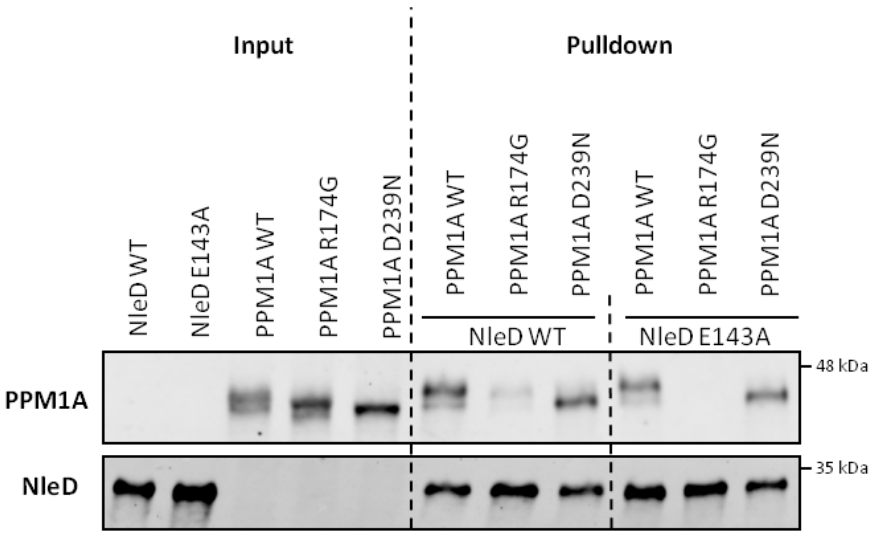

Figure 4.2.3 NleD binds PPM1A regardless of catalytic activity.
SBP-tagged wild type NleD or catalytically dead $NleD_{E143A}$ mutant were immobilized on streptavidin beads, and subsequently incubated with lysates of HEK293T cells transfected with plasmids expressing wild type or mutated $PPM1A_{R174G}$ and $PPM1A_{D239N}$. Bound proteins were analyzed by western blot with antibodies against SBP (to detect NleD) or PPM1A as indicated. Molecular weight marker is shown on.

4.2.4 NleD-PPM1A interaction is resilient

In order to characterize the nature of NleD-PPM1A binding I tested how stable is the interaction under different strains such as increasing concentration of urea or high salinity. In addition, since Zn ions are crucial for the structural stability of NleD I also tested whether de-stabilization of NleD using EDTA might interfere with PPM1A binding.

SBP-NleD was captured on beads and incubated with lysates of HEK293T cells expressing PPM1A to allow NleD-PPM1A complex formation. The bead-bound NleD-PPM1A complex was then placed in buffers of the desired composition. After incubation, a centrifugation was performed and the beads and supernatant ('eluate') were analyzed using western blot with antibodies against SBP (to detect NleD) or PPM1A. The results show that none of the tested conditions resulted in a significant perturbation of NleD-PPM1A complex; either they both were found in the beads fraction or both were eluted from the beads and found in the supernatant (Fig. 4.2.4A and 4.2.4B). These results show that the nature of NleD-PPM1A binding is not only specific but also stable across wide strenuous conditions.

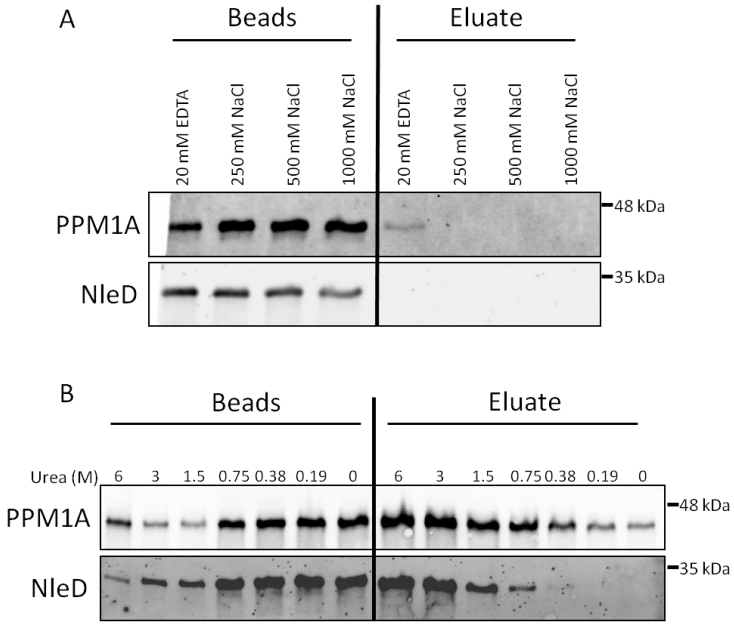

Figure 4.2.4 NleD-PPM1A interaction is resilient.
Bead-bound NleD-PPM1A complex was placed in different conditions and possible elution of the proteins was analyzed using western blot of the beads and supernatant after centrifugation. Molecular weight marker is shown.

4.2.5 NleD homologs differ in their ability to bind PPM1A

The NleDs of EPEC, *Salmonella enterica* subsp. arizonae and the murine pathogen *Citrobacter rodentium* (CR) share nearly 75% sequence identity (Fig. 4.2.5A). To test whether the ability to bind human PPM1A is conserved among these homologs, I expressed SBP-tagged $NleD_{EPEC}$, $NleD_{Sal}$ and $NleD_{CR}$, and used them for capturing PPM1A expressed in HEK293T cells. As before, $NleD_{EPEC}$ showed robust interaction with PPM1A. $NleD_{Sal}$ bound PPM1A as well, but $NleD_{CR}$, showed only poor binding to PPM1A (Fig. 4.2.5B). These results show that not all the members of the NleD family evolved to interact with PPM1A, suggesting that the inability of $NleD_{CR}$ to bind to PPM1A might reflect a difference in the infection strategies of EPEC and CR.

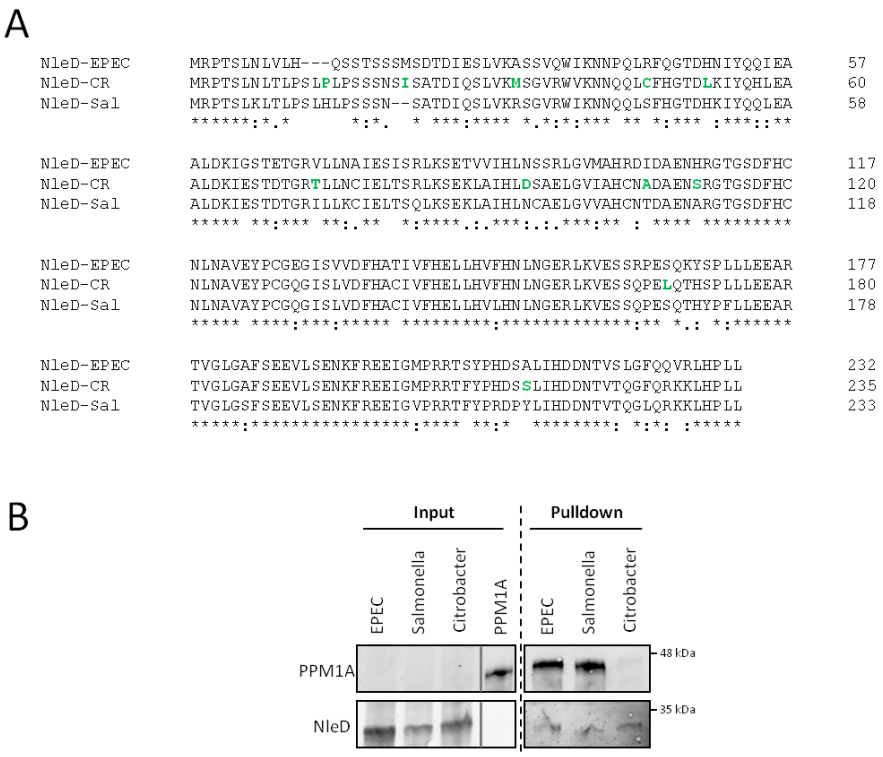

Figure 4.2.5 Binding of NleD homologs to PPM1A.
(A) Sequence alignment of NleD from EPEC ($NleD_{EPEC}$), Citrobacter rodentium ($NleD_{CR}$), and Salmonella Arizona ($NleD_{Sal}$). When comparing the $NleD_{CR}$ and $NleD_{Sal}$, they are ~75% and ~78% identical to the sequence of $NleD_{EPEC}$, respectively. (B) SBP-tagged NleD of EPEC, *Salmonella arizona* or *Citrobacter rodentium* were immobilized on streptavidin beads, and incubated with purified PPM1A-FLAG. Proteins were then extracted from the washed beads and subjected to western blot analysis using anti-PPM1A and anti SBP (to detect SPB-NleD). Molecular weight marker is shown.

4.2.6 PPM1A dephosphorylates P-p38, priming it to cleavage by NleD

Given the inability of NleD to cleave phospho-p38/JNK and its interaction with PPM1A, we speculated that the PPM1A-NleD complex might function as a team by initial dephosphorylation of p38/JNK by PPM1A, priming them for the subsequent cleavage by NleD. To test this premise, I examined whether the inherent activity of recombinant PPM1A purified from HEK293T cells is sufficient to dephosphorylate the TGY motif of p38. Indeed, my data show that incubation of purified PPM1A with P-p38 resulted in dephosphorylation of the p38 TGY motif. Notably, despite being considered as serine/threonine phosphatase, the data show that PPM1A dephosphorylated also the tyrosine residues of the motif. Moreover, the dephosphorylation of p38 by PPM1A enabled cleavage by NleD, when the latter was subsequently added to the reaction (Fig. 4.2.6). Collectively, these results showed that when P-p38 was treated sequentially by PPM1A and then by NleD, it was dephosphorylated and then cleaved.

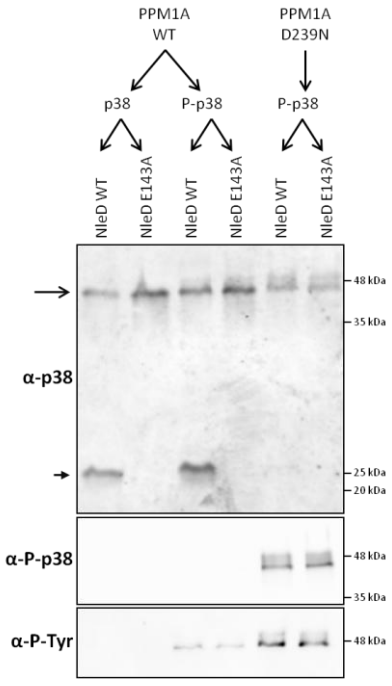

Figure 4.2.6 PPM1A dephosphorylates both T and Y residues of the p38 TXY motif.
Wild type PPM1A or the inactive mutant $PPM1A_{D239N}$ were incubated for one hour with p38 or phospho-p38 (P-p38) as indicated. Then, NleD, or catalytically dead $NleD_{E143A}$, was added to the reaction mix, followed by incubation for an additional hour. p38 dephosphorylation and p38 cleavage were assessed using western blot and antibodies against p38, phospho-p38 and phospho-tyrosine. Arrow and arrowhead point to intact p38 and cleavage products, respectively. Molecular weight marker is shown.

4.2.7 In the context of pre-formed complexes, bound NleD inhibits dephosphorylation of phospho-p38 by PPM1A

I next aimed at testing the simultaneous activity of NleD and PPM1A when in complex. To avoid interference between their activities, I also used complexes containing catalytically-dead forms of PPM1A ($PPM1A_{D239N}$) and NleD ($NleD_{E143A}$), which are proficient in complex formation (Fig. 4.2.3). I incubated SBP-NleD or SBP-$NleD_{E143A}$ with purified PPM1A or $PPM1A_{D239N}$ expressed in HEK293 cells. Thus, four different purified complexes were formed; PPM1A-NleD, $PPM1A_{D239N}$-NleD, PPM1A-$NleD_{E143A}$, and $PPM1A_{D239N}$-$NleD_{E143A}$. I then mixed each of these complexes with either p38 or P-p38, and tested their capacity to dephosphorylate the TGY motif of the added recombinant P-p38, and/or to cleave added p38 or P-p38. The results show that NleD remains active when in complex, clipping the unphosphorylated p38, but not the phosphorylated form (Fig. 4.2.7). Surprisingly however, when associated with NleD, PPM1A fails to dephosphorylate P-p38 and to prime it for cleavage by NleD. Similar results were obtained when using PPM1A in complex with the catalytically inactive $NleD_{E143A}$, indicating that the catalytic activity of NleD is not responsible for inhibiting PPM1A capacity to dephosphorylate P-p38. These results indicate that bound NleD inhibits the capacity of PPM1A to dephosphorylate p38. Since the proteolytic activity of NleD was dispensable for PPM1A inhibition, a plausible mechanism for the effect of bound NleD is that it might interfere with P-p38 recognition by PPM1A. In contrast, PPM1A does not interfere with the proteolytic activity of the bound NleD.

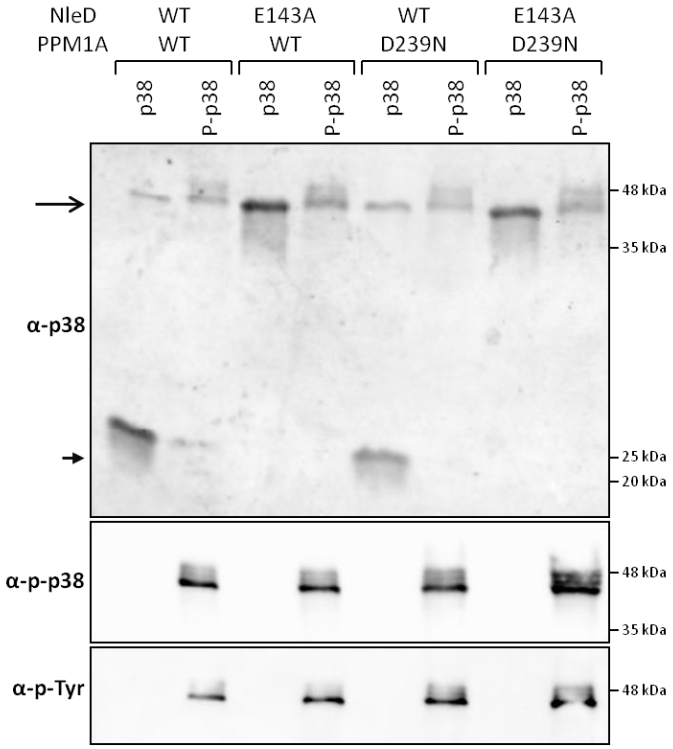

Figure 4.2.7 In a pre-formed comlex, bound NleD inhibits p38 dephosphorylation by PPM1A.
p38 or phospho-p38 (P-p38) were treated for one hour with different NleD-PPM1A pre-formed complexes including all combination of wild type and mutated, catalytically inactive, NleD and PPM1A (E143A and D239N, respectively) as indicated above the lanes. Then, dephosphorylation and cleavage of p38 and phospho-p38 were assessed using western blot with antibodies against p38, phospho-p38 and phospho-tyrosine. Arrow and arrowhead point to intact p38 and cleavage products, respectively. Molecular weight marker is shown.

4.2.8 PPM1A in complex with NleD can still hydrolyze small substrates

A reasonable explanation for the inability of PPM1A to dephosphorylate p38 when in complex with NleD is that the bound NleD blocks the catalytic site of PPM1A. Such a model of the NleD-PPM1A complex would suggest that the restricted access to the PPM1A catalytic site depends on the size of the substrate. While the access of large substrates such as P-p38 is denied, small substrates such as p-nitrophenyl phosphate (pNPP, molar mass 219 dalton) might diffuse into its catalytic site. To test this prediction, I incubated the NleD-PPM1A complex with pNPP and found that in this case NleD did not inhibit, but in fact enhanced the activity of PPM1A (Fig. 4.2.8). NleD-bound PPM1A exhibited almost 3-fold higher efficiency of pNPP hydrolysis. Importantly, similar results were observed when a catalytically inactive form of NleD was used, further confirming that the catalytic activity of NleD is not required for its action on PPM1A. These results reinforce the premise that NleD prevents the dephosphorylation of p38 by masking the catalytic site of PPM1A, while the catalytic capacity remains intact.

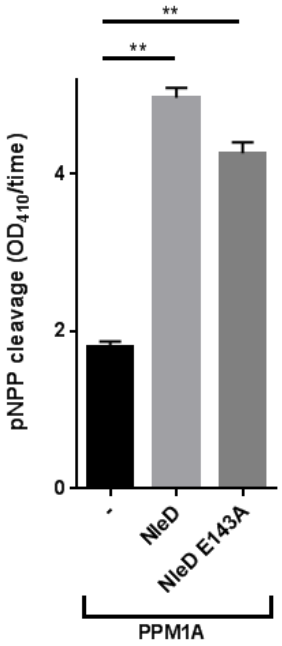

Figure 4.2.8 NleD doesn't inhibit pNPP hydrolysis by PPM1A.
The colorimetric phosphatase substrate pNPP was treated with PPM1A or PPM1A in complex with wild type NleD or $NleD_{E143A}$ mutant as indicated. Hydrolysis of pNPP was assessed using absorbance at 410 nm. The rate of pNPP cleavage, represented by the slope of OD_{410} over time is shown. Error bars represent the standard deviation from two independent experiments. Statistical analysis was performed using student's t-test. **p<0.01

4.3 Discussion

T3SS and the associated effector proteins represent a major strategy used by bacteria to manipulate eukaryotic host cells. In many cases the T3SS effectors employ innovative strategies to target specific host factors, including those involved in MAPK signaling. For example, *Salmonella*, *Shigella* and *Psudomonas syringae* employ the homologues SpvC, OspF and HopAII effectors to inactivate MAPKs via their phosphothreonine lyase activity (Li *et al*, 2007; Arbibe *et al*, 2006; Kramer *et al*, 2007; Zhang *et al*, 2007). Likewise, the *Salmonella*, *Yersinia* and *Vibrio parahaemolyticus* effectors AvrA, YopJ and VopA, inactivate host MAP2Ks by acetylating their targets (Jones *et al*, 2008; Du & Galán, 2009; Mukherjee *et al*, 2006; Trosky *et al*, 2007). Notably, pathogens deliver into the host a cohort of co-translocated effectors, which functions in a coordinated fashion within the host to attain the desired impact. These two fundamental features of T3SS effectors; novel activities and coordinated action of effectors groups, are nicely exemplified in the case of attaching and effacing (AE) pathogens (Pearson *et al*, 2016; Ruano-Gallego *et al*, 2021). Yet, in most cases knowledge of the effectors activities and how these activities are controlled and coordinated remained limited (Ruano-Gallego *et al*, 2021). Here I report the surprisingly complex nature of the NleD effector and its multifaceted interactions with host targets.

NleD cleaves JNK and p38 within the TXY motif between the X and Y. Here I show that when the T and Y of the motif are phosphorylated, NleD can no longer cleave these MAPKs (Fig. 4.2.1). How the phosphorylation prevents the cleavage is not clear. It is possible that the phospho group deters NleD, reducing it accessibility to the cleavage site. Alternatively, the stiff nature of the entire phosphorylated activation-loop might render it immune to NleD. The latter suggestion stems from a recent observation that NleD cleaves various MAPKs as long as their activation loop is flexible, but fails to cleave the stiff activation loop of ERK, regardless of its phosphorylation status (Gur-Arie *et al*, 2020).

I unexpectedly found that NleD interacts with PPM1A (Table 4.2.2 and Fig. 4.2.3). Notably, NleD and PPM1A share the activation loops and TXY motif of JNK and p38 as prime-substrates. This motivated me to examine the NleD-

PPM1A relationship, leading to several interesting observations. First, although PPM1A was defined as serine/threonine phosphatase (Chida *et al*, 2013), I found that it effectively dephosphorylated both the threonine and tyrosine residues of the TXY motif of p38 and JNK, converting them to the unphosphorylated, NleD-sensitive form (Fig. 4.2.6). The second and most surprising observation was that the bound NleD inhibited the activity of PPM1A (Fig. 4.2.7). To understand how NleD inhibits PPM1A I further investigated the NleD-PPM1A interaction. I found that the binding of these proteins is very tight, being able to withstand harsh conditions (Fig. 4.2.4). In addition, I found that small substrates such as pNPP can still diffuse into the PPM1A catalytic site and be hydrolyzed (Fig. 4.2.8). This suggests that the mechanism of PPM1A inhibition by NleD is masking of its catalytic site, blocking the entrance of large substrates such as p38.

The biological logic of NleD having two seemingly counteracting functions; clipping exclusively the unphosphorylated MAPKs, and inhibiting dephosphorylation of MAPKs by PPM1A, remains mysterious. Yet, this logic should be considered in the context of the intricate effector networks of EPEC (Ruano-Gallego *et al*, 2021). Attached EPEC is detected by the infected cells leading to activation of the NF-κB pathway and p38/JNK phosphorylation (Litvak *et al*, 2017). However, the pathogen employs NleE, NleC and NleD to intercept these defense signaling cascades. NleE, a methyl-cysteine transferase, targets the TAB2/3 components of the TAK1 complex, thereby preventing TAK1 activation (Nadler *et al*, 2010; Zhang *et al*, 2012) and the consequent downstream NF-κB activation and MAPKs phosphorylation. NleC and NleD act downstream to NleE by cleaving NF-κB and p38/JNK, respectively (Baruch *et al*, 2011). Thus, the activity of NleE, which prevents p38/JNK phosphorylation, synergizes with that of NleD. Furthermore, by preventing JNK/p38 phosphorylation, NleE makes the dephosphorylation activity of PPM1A dispensable for their cleavage by NleD. Hence, what is the significance of PPM1A inhibition by NleD? I speculate that NleD may block the capacity of PPM1A to dephosphorylate substrates other than p38 and JNK. Notable primary PPM1A substrates are SMAD proteins (Lin *et al*, 2006), essential components of bone morphogenetic proteins (BMPs) signaling,

which control the development of the intestinal epithelium. Thus, if EPEC infects the intestinal stem cells and affects the SMAD/BMP signaling pathway it can have a profound effect on the development and morphology of the intestine. This hypothesis is the subject of further work.

5. EPEC-induced toxicity to host cells

5.1 Introduction

5.1.1 Regulated cell death

Currently there are 12 known pathways of regulated cell death (Galluzzi et al, 2018). These pathways differ not only in their initiating triggers and executioner proteins, but also in the degree to which the contents of the dying cell are exposed to the environment. For example, apoptosis is a type of programmed cell death in which the dying cells efficiently fractionate into small particles that are eliminated by neighboring phagocytic cells, a tidy process that doesn't induce inflammation in the tissue (Elmore, 2007). Programs such as pyroptosis and necroptosis, in contrast, result in spilling of cellular contents into the extra-cellular fluid, a process which activates inflammatory cascades in the adjacent cells. Pyroptosis occurs when caspase activation leads to cleavage of gasdermin proteins. This cleavage liberates the N-terminal domain of the protein which undergoes olygomerization and creates large pores in the membrane (Shi *et al*, 2017). Necroptosis resembles pyroptosis in many regards, but the pore-forming protein is MLKL and the trigger for its oligomerization is phosphorylarion by RIPK3 (Pasparakis & Vandenabeele, 2015). The natural urge to define distinct programs of cell death stands in contrast to the current inclination to see the pathways as a spectrum of inflammatory to non-inflammatory cell death, where apoptosis represents the non-inflammatory cell death edge and pyroptosis and necroptosis represent the highly inflammatory one (Galluzzi *et al*, 2018). Regulated cell death programs have roles in the normal physiology of organism development and aging, as well as cardinal roles as central pillars of the innate immunity of multi-cellular organisms (Elmore, 2007). In the context of innate immunity the regulation is typically exerted by cytosolic multiprotein oligomers termed inflammasomes (Rathinam & Fitzgerald, 2016). The inflammasomes consist of a sensor protein, an adaptor protein and an executioner – typically caspase-1. Assembly of the inflammasome occurs in response to intrinsic signals (i.e. cell damage) or extrinsic ones (i.e. pathogen detection). Assembly and activation of the inflammasome can result in

maturation of pro-inflamatory cytokines as well as execution of a cell death program such as apoptosis, pyroptosis or necroptosis (Tsuchiya, 2020).

5.1.2 Evolutionary arms race

Pathogens and their target organisms are in a constant state of evolutionary arms race against one another. During this race host organisms develop novel tools to detect the attacking pathogens, which in their turn produce additional strategies to avoid them. In Lewis Carrol's classic *'Through the looking glass'* the red queen tells Alice that 'here, you see, it takes all the running you can do, to keep in the same place'. This phrase caught the attention of the evolutionary biologist Leigh Van Valen who in 1973 proposed the 'Red queen hypothesis' which states that in the evolutionary arms race between host and pathogens both must constantly evolve and create new means of detection or evasion in order to avoid extinction (Van Valen, 1973).

This is nicely exemplified by the programmed cell death response to bacterial infection in mammalian cells. Cells utilize a plethora of intra- and extra-cellular receptors to sense the presence of Pathogen-Associated Molecular Patterns (PAMPs). For example TLR4 is an extra-cellular sensor of bacterial LPS which triggers a transcriptional response to infection, while intracellular LPS is detected by Caspase-4/5 which can trigger pyroptotic cell death (Chow *et al*, 1999; Shi *et al*, 2015). Several tiers of response which act as consecutive lines of defense can be activated as a result of PAMP recognition. Typically, the first and dominant response is a transcriptional response such as the NF-κB or MAPK pathways which lead to production of cytokines and activation of the innate immune system. When the transcriptional response is silenced due to the actions of the infecting pathogen, a different response can occur in the form of apoptotic (*i.e.* non-inflammatory) or conversely necroptotic (*i.e.* inflammatory) cell death. A clear example of this concept and the efforts of the pathogen to overcome it is the TNF receptor signaling during EPEC infection. RIPK1 is the coordinator of TNF repector signaling. When this pathway is activated RIPK1 interacts with the TAK1 complex to initiate transcriptional response (Dondelinger *et al*, 2016a). If this interaction doesn't occur, RIPK1 is able to interact with Fas-associated death domain (FADD) which in turn recruits caspase-8 to initiate apoptosis (Dondelinger *et al*, 2016b). If the

RIPK1-FADD interaction fails as well, RIPK1 binds RIPK3 which then phosphorylates MLKL, leading to death of the cell by necroptosis (Lacey & Miao, 2020).

EPEC infection may lead to TNF signaling but translocated effectors can inhibit almost every component of this pathway. NleE methylates the TAB2/3 components of the TAK1 complex, preventing their interaction with RIPK1 (Zhang *et al*, 2012). In addition, EPEC employs the metalloproteases NleC and NleD to directly cleave NF-κB and JNK and p38 MAPKs, respectively, and prevent the transcriptional response (Baruch *et al*, 2011). As discussed before, the perturbation of RIPK1-TAK1 interaction would lead to apoptosis of the infected cell but this is inhibited by NleB – an effector that catalyzes arginine GlcNAcylation of FADD and thus prevents the apoptotic cell death (Li *et al*, 2013). Still, RIPK1 can bind RIPK3 and lead to necroptosis, but the effector EspL is a cysteine protease able to cleave these proteins to prevent this outcome (Pearson *et al*, 2017). Thus all tiers of response are blunted and EPEC avoids a full-blown response of the innate immune system.

5.1.3 EPEC infection and host cell viability

In addition to what was discussed earlier, some other effectors injected by EPEC into the host cells interfere with signaling pathways that activate or repress host cell death. The pro-apoptotic effectors of EPEC include EspF and Map which localize to the mitochondria upon translocation and compromise its integrity and membrane potential, thus activating intrinsic apoptotic pathways (Pearson *et al*, 2016). In contrast, NleF opposes programmed cell death through interactions with caspase-4/5 (Pearson *et al*, 2016), while it was suggested that NleH1 and NleH2 do so by interacting with host proteins such as Bax inhibitors, although this interaction was questioned lately (Pollock *et al*, 2022). Additionally, EspZ antagonizes EPEC-induced toxicity in a mechanism which is still elusive (Shames *et al*, 2010, 2011; Roxas *et al*, 2012; Berger *et al*, 2012). Moreover, the bacterial metabolite Haptose Bi-Phosphate (HBP) acts to enhance host cell survival by activating the NF-κB pathway in a T3SS-dependent manner, presumably due to leakage of HBP through the T3SS needle (Zhou *et al*, 2018). Thus, there is an intricate balance between signals modulating cell viability during EPEC infection.

5.1.4 Tir, CesT and MgrR

Tir is the major EPEC effector, the first to be injected into host cells and the most abundant one (Mills *et al*, 2013). Upon translocation, Tir is integrated into the host-cell membrane and serves as the receptor for a LEE-encoded adhesin named intimin (Kenny *et al*, 1997). Binding of intimin to Tir promotes phosphorylation of the latter by host cell kinases (Rosenshine *et al*, 1996). This in turn initiates cytoskeletal rearrangements, resulting in formation of the characteristic AE lesions and actin-rich structures termed 'pedestals' which are associated with EPEC infection (Campellone & Leong, 2003). Tir was implicated in several other EPEC-associated phenotypes such as inhibition of TLR signaling in the host cell (Yan *et al*, 2012) and it was suggested that it interacts with many other host cell proteins (Blasche *et al*, 2015), although its precise role in such processes is yet to be elucidated.

Delivery of 11 EPEC effectors, including Tir and EspZ, is dependent on the chaperone CesT (Mills *et al*, 2013) and it is plausible that additional effectors yet to be discovered are also delivered in a CesT-dependent manner. CesT binding to effectors both stabilizes them and directs them to the T3SS export apparatus. Remarkably, CesT functions also as a key regulator by the virtue of its capacity to bind and inhibit CsrA, which is an RNA-binding protein and post-transcriptional regulator of hundreds of metabolic and virulence genes (Katsowich *et al*, 2017). CesT appears to regulate CsrA activity, thus facilitating adaptation of infecting bacteria to a host-attached state. Mutants lacking CesT exhibit severe deficiency in effector stability, dramatic reduction in effector translocation and fail to colonize the host and to cause disease in an in-vivo mouse model using the related pathogen *Citrobacter rodentium* (Runte *et al*, 2018). The levels of CesT in the infecting bacteria are tightly regulated to ensure suitable free-CesT:CsrA ratio (Elbaz *et al*, 2019). One such regulator is MgrR which acts to reduce CesT levels in the bacteria (Pearl Mizrahi *et al*, 2021). MgrR is a conserved sRNA present in commensal *E. Coli* which can have positive or negative effects on the stability and translation levels of multiple mRNAs. With the assistance of the protein Hfq, MgrR binds through base-pairing to specific sequences in its target mRNAs, which can have positive or negative effects on the stability and expression level of the

target mRNA (Vogel & Luisi, 2011). Highly expressed in conditions of low environment concentrations of Mg^{2+} ions, MgrR serves as a regulator of bacterial metabolism (Moon *et al*, 2013). The role of MgrR in the regulation of virulence was studied in EPEC, with some interesting findings. MgrR inhibits the translation of the negative regulator GrlR, leading to up-regulation of the activity of the T3SS (Bhatt *et al*, 2017). In a recent work from our lab it was shown that among other functions in regulating the virulence of EPEC, MgrR specifically inhibits the translation of CesT (Pearl Mizrahi *et al*, 2021).

5.1.5 Hypothesis, rationale and aims

EPEC translocates effectors with opposing effects on the survival of host cells, and since the expression and translocation of effectors are subjected to strict regulation, one may assume that interfering with this regulation will result in a dis-balance between the pro- and anti-death signals delivered during infection and will thus affect the survival of host cells during EPEC infection.

We hypothesized that regulators of EPEC virulence, including sRNAs and CesT, have roles in regulating toxicity to host cells during infection.

Specifically, we asked the following questions:

1. How does the sRNA MgrR affect host cell survival during infection?
2. How many mechanisms of host cell death are affected by the sRNA MgrR?

5.2 Results

5.2.1 Over-expression of MgrR makes EPEC toxic

To investigate the role of MgrR as a regulator of virulence I over-expressed it in EPEC and used this strain to infect HT-29 cells and monitored the infection process with time lapse microscopy. Starting already at 1.5 hours of infection a very clear phenotype was observed: the cells swelled and rounded, concurrent with condensation of the nucleus, resulting in detachment of the cells from the plate (Fig. 5.2.1A). This phenotype was observed in several additional cell lines including HeLa and HEK293T. Of note, this morphology resembles that of cells undergoing lytic death program such as pyroptosis or necroptosis.

To further characterize the result of infection, the viability of the infected cells was assessed using propidium iodide (PI) staining. The round, detached cell population was collected after 3 hours of infection and the ability of PI to penetrate the cell membrane and stain the nuclear DNA was assessed separately for both the attached and detached cell populations. In agreement with the cells undergoing some sort of death process, all the detached cells were stained with PI while the cells that remained attached to the plate have retained their membrane integrity and remained unstained (Fig. 5.2.1B). This shows that infection of cells with an EPEC strain over-expressing MgrR leads to a rapid process of death and detachment of the host epithelial cells.

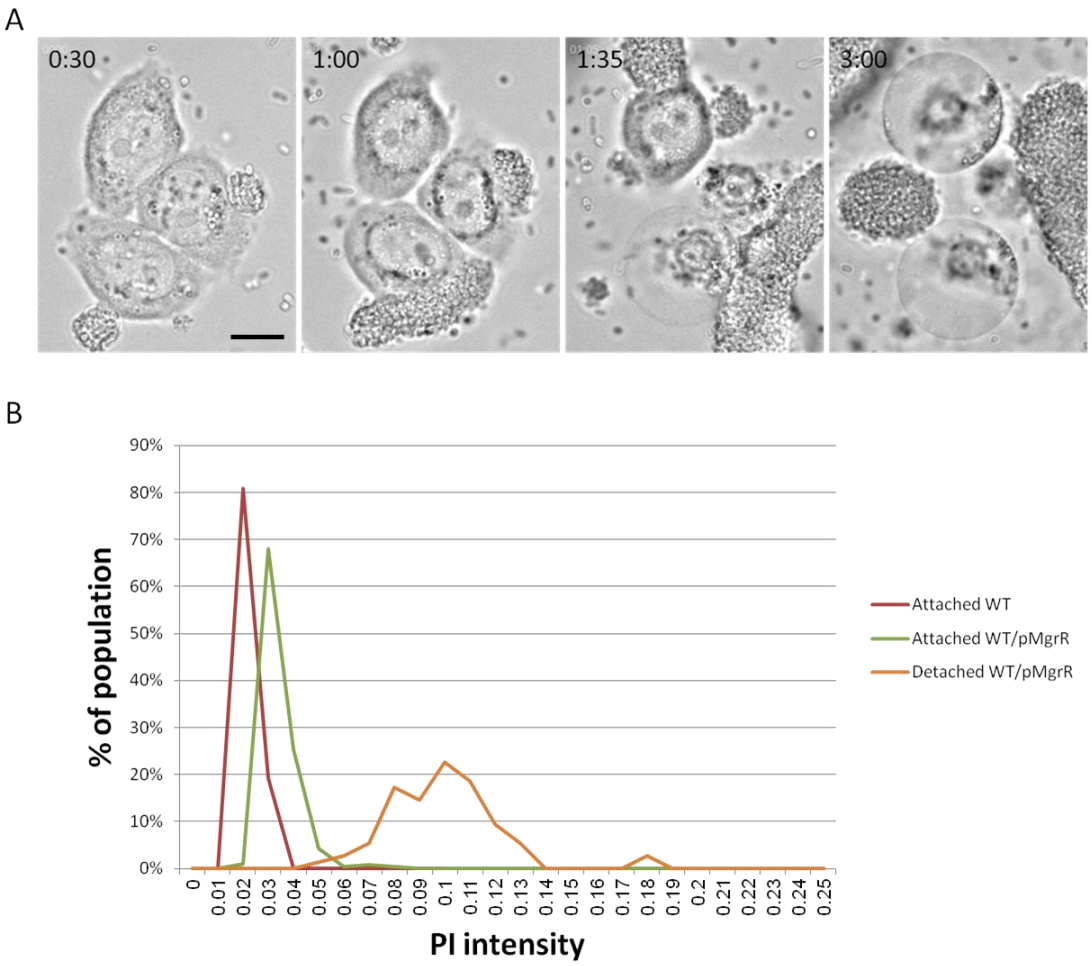

Figure 5.2.1 EPEC over-expressing MgrR is higly toxic to host cells.
(A) HT-29 cells were infected with EPEC containing a plasmid for constitutive over-expression of MgrR. Time lapse microscopy was used to monitor the process of infection (scale bar 10 µm). (B) HEK293T cells were infected with WT EPEC or with a strain over-expressing MgrR. After 3 hours propidium iodide (PI) was added and 10 minute incubation was performed to allow for staining. Then the medium was collected to analyze the detached cell population along with the cells that remained attached to the plate. Microscopy followed by measurement of PI fluorescence intensity was used.

5.2.2 MgrR-induced toxicity is T3SS-dependent

I next wanted to confirm that the toxicity to the host cells is indeed a feature of EPEC pathogenicity. To this end I utilized an EPEC mutant in which *escV*, a core component of the T3SS, is deleted so the T3SS cannot be assembled. A special experimental system was constructed in order to reliably quantify the detachment of host cells during infection as proxy for host cell death: I infected HEK293T cells with a lentivirus encoding EGFP, producing a cell-line constitutively expressing EGFP (HEK-GFP). After infection of this cell line with the desired strains for the desired time the plate is washed to remove the detached cells. The cells that remain attached to the plate are retrieved and the level of GFP in the sample, which is directly correlated to the number of cells, is measured. Of note, some fluctuations in the results of the toxicity experiments is observed, stemming from numerous factors such as the passage of the cell line, the exact level of confluence of the cell culture and the precise duration of infection. Such fluctuations are typical to such toxicity experiments, and therefore comparison of the level of toxicity induced by the different EPEC strains should only be made based directly on experiments comparing the desired strains.

Using this system, I confirmed the previous results and saw that over-expression of MgrR in EPEC WT resulted in a dramatic increase in the toxicity to the host cells, reaching 90% killing after 3 hours. In sharp contrast, over-expression of MgrR in EPEC *escV* mutant didn't show any increase in the toxicity level (Fig. 5.2.2). These results show that the toxicity to the host cells which is induced by EPEC over-expressing MgrR is indeed attributed to the virulence machinery of the pathogen and requires an active T3SS.

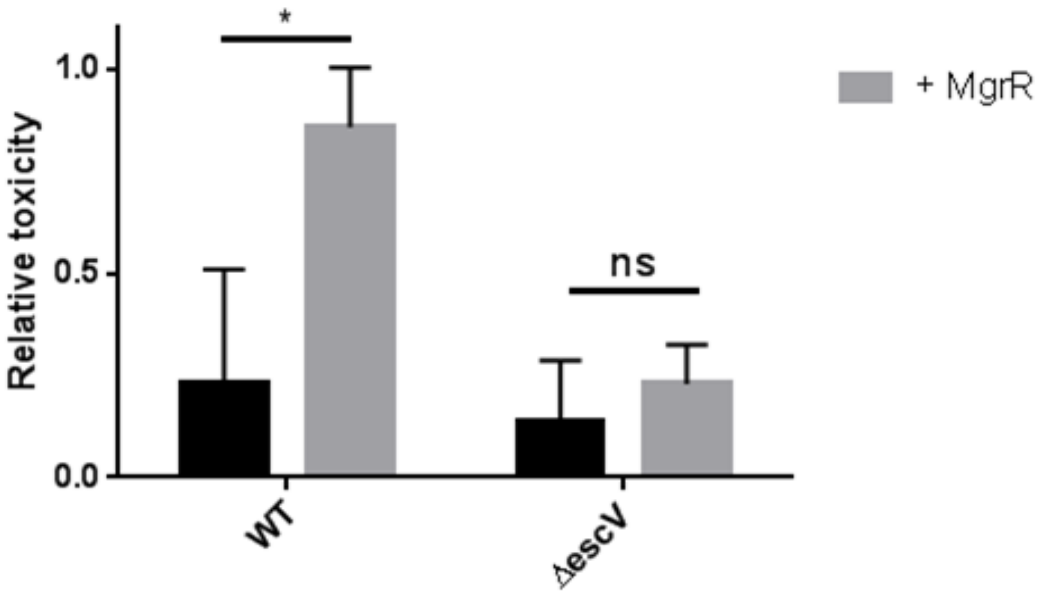

Figure 5.2.2 MgrR-induced toxicity is T3SS-dependent.
EPEC WT or *escV* mutant (T3SS defective), each containing or not the pZE12-MgrR plasmid for constitutive over-expression of MgrR, were used to infect HEK-GFP cells. After 3.5 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. Error bars represent the standard deviation from three independent experiments. Statistical analysis was performed using student's t-test. *p<0.05, ns=non-significant.

5.2.3 MgrR-induced toxicity is independent of GrlR targeting

The first role of MgrR in regulating EPEC virulence to be discovered was targeting of GrlR. The GrlRA system is composed of a bi-cistronic operon which encodes the LEE positive regulator GrlA and the negative regulator GrlR which acts through direct binding of GrlA (Iyoda *et al*, 2006). A specific decrease of the levels of GrlR leads to higher amount of free GrlA, producing the effect of up-regulation of LEE transcription (Iyoda & Watanabe, 2005). MgrR specifically inhibits the translation of GrlR, leading to higher levels of free GrlA, resulting in activation of LEE transcription (Bhatt *et al*, 2017). Such hyper-activity may result in hyper-translocation of T3SS effectors into the host cells and cause the observed effect of increased toxicity when the bacteria over-express MgrR.

To check if this is the case I used an EPEC strain in which the *grlRA* bi-cistronic operon was deleted, so expression of the LEE is driven by PerC and not the GrlRA system. I over-expressed MgrR in this strain and tested the toxicity it induced in the infected host cells. The results showed that similarly to wiled type EPEC, expression of MgrR induced high levels of toxicity even in the *grlRA* mutant (Fig. 5.2.3). This suggests that the mechanism by which expression of MgrR in the bacteria leads to toxicity in the infected host cells is not through inhibition of GrlR translation and general up-regulation of T3SS activity.

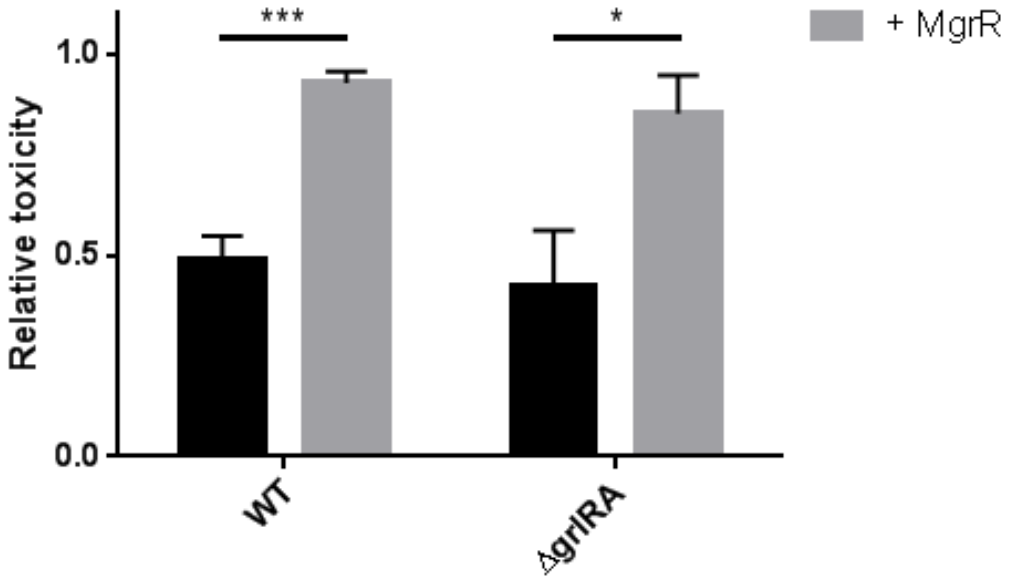

Figure 5.2.3 Over-expression of MgrR in a *grlRA* mutant still leads to toxicity.
EPEC WT or *grlRA* mutant, each containing or not the pZE12-MgrR plasmid for constitutive over-expression of MgrR, were used to infect HEK-GFP cells. After 3.5 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. Error bars represent the standard deviation from three independent experiments. Statistical analysis was performed using student's t-test. *p<0.05, ***p<0.001

5.2.4 EPEC *ΔcesT* is toxic to host cells

Another role for MgrR in regulating EPEC virulence was recently discovered in our lab. We found that MgrR binds to the mRNA of *cesT* and inhibits its translation, leading to profoundly low levels of CesT (Pearl Mizrahi *et al*, 2021). This lead us to hypothesize that the low levels of CesT are what is actually responsible for the MgrR-induced toxicity. If this is really the case, an EPEC strain lacking *cesT* should be toxic to host cells even without over-expression of MgrR. CesT has a central role during infection, promoting several key processes such as Tir translocation and subsequent actin rearrangement and pedestal formation, and finally infection itself. The result is that mutants lacking CesT are severely impaired in their infection ability (Runte *et al*, 2018). It was thus counterintuitive and surprising when I found that deletion of *cesT* had a 'gain of function' effect and the mutant was highly toxic to host cells, substantiating the idea that the low levels of CesT in the MgrR over-expressing strain are promoting toxicity to the host cells. Importantly, the hyper-toxicity phenotype was reverted in a dose-dependent manner when CesT was ectopically expressed from a plasmid (Fig. 5.2.4).

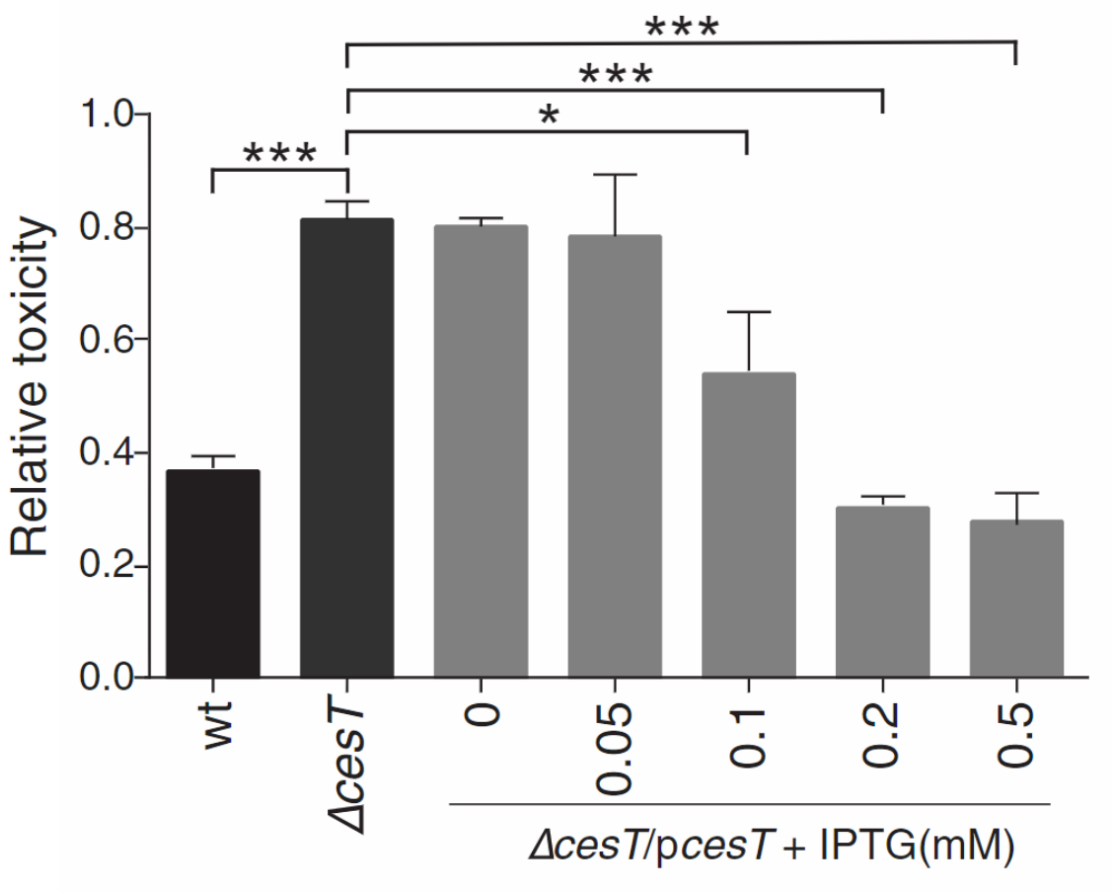

Figure 5.2.4 EPEC *ΔcesT* is toxic to host cells.
EPEC WT, *ΔcesT* mutant or *ΔcesT* complemented with a plasmid expressing CesT were used to infect HEK-GFP cells. At 90 min after infection IPTG was added to induce expression of CesT. After a total of 3.5 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. Error bars represent the standard deviation from three independent experiments. Statistical analysis was performed using student's t-test. *p<0.05, ***p<0.001. This figure was published in Pearl Mizrahi *et al*, 2021.

5.2.5 MgrR regulates toxicity during infection

Thus far the putative role of MgrR in regulating the toxicity induced by EPEC towards infected cells was postulated based on the effects which were observed when it was ectopically over-expressed. To directly study the role of MgrR in wild type EPEC I took advantage of the fact that the specific binding site (i.e. the base paring sequence) of MgrR to the mRNA of CesT was elucidated. In the EPEC strain in which this site in the 5' UTR of *cesT* was mutated by converting two residues of the binding site from CG to GC (designated *cesT**), binding of MgrR is abolished and MgrR can no longer affect the levels of CesT while it maintains its other capabilities (Pearl Mizrahi *et al*, 2021). Using this strain in infection for 3 hours didn't produce striking effects as at this time point both the WT and mutant showed low levels of toxicity. However, extending the infection time up to 6 hours allowed the WT strain to demonstrate high level of toxicity. Under these conditions, the *cesT** strain was significantly less toxic to the host cells (Fig. 5.2.5A). Importantly, the mutation in the *cesT** strain didn't have a global effect on EPEC virulence, exhibiting similar general characteristics of EPEC such as attachment to host cells (Fig. 5.2.5B). These results show that MgrR, through regulation of CesT levels, controls the toxicity of EPEC during infection even when natively expressed.

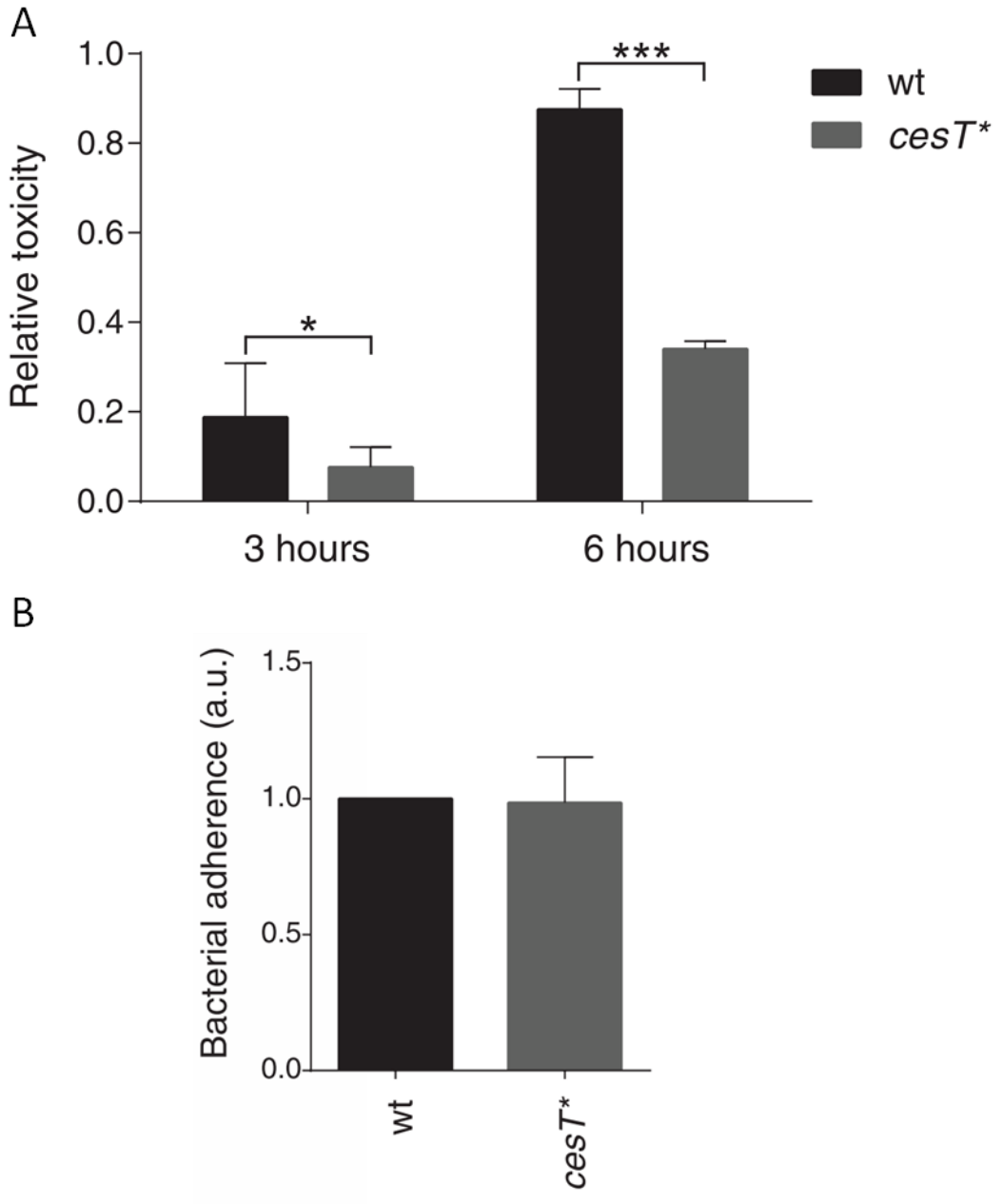

Figure 5.2.5 MgrR regulates toxicity during infection.
(A) EPEC WT or *cesT** mutant were used to infect HEK-GFP cells. After 3 or 6 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. (B) EPEC WT or *cesT** mutant, both containing a plasmid expressing mCherry, were used to infect HEK-GFP cells. After 3 hours the cells were washed, scraped and the ratio of mCherry to GFP (representing the amount of adhered bacteria per host cell) was measured. Error bars represent the standard deviation from three independent experiments. Statistical analysis was performed using student's t-test. *p<0.05, ***p<0.001. This figure was published in Pearl Mizrahi *et al*, 2021.

5.2.6 Additional minor mechanisms for MgrR-induced toxicity

The previous results showed that over-expression of MgrR leads to low levels of CesT in the infecting bacteria, which in turn lead to toxicity towards the infected cells. Since MgrR is a major regulator of EPEC virulence I speculated that there can be additional mechanisms besides down-regulation of CesT by which over-expression of MgrR renders EPEC toxic. If this is the case, over-expression of MgrR in a *cesT* mutant would activate these mechanisms, which should increase the toxicity beyond the already known hyper-toxicity of the *cesT* mutant. If, however, MgrR only makes EPEC toxic because of lowering the levels of CesT, no additional effect should be observed.

The results of this experiment showed that while the *cesT* mutation is sufficient to dramatically increase the level of toxicity induced by EPEC, over-expression of MgrR further slightly, yet significantly, increased this level (Fig. 5.2.6). This shows that while the main mechanism by which over-expression of MgrR makes EPEC toxic is the down-regulation of CesT levels, there are additional effects of MgrR which increase the toxicity to infected cells. This increased toxicity might be related to the elevation of free-GrlA which was mentioned earlier, or reflect an additional mechanism of MgrR activity.

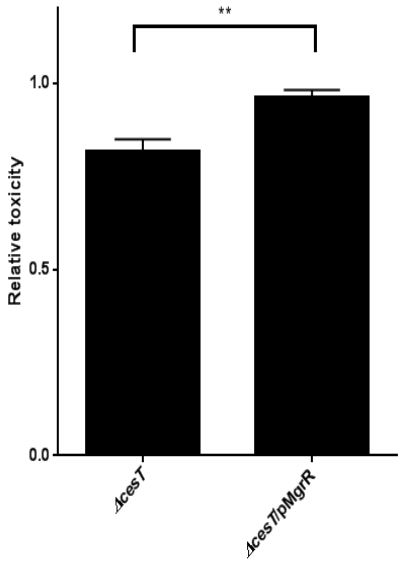

Figure 5.2.6 CesT-independent MgrR-induced toxicity.
EPEC *cesT* mutant or *cesT* mutant constitutively over-expressing MgrR were used to infect HEK-GFP cells. After 3 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. Error bars represent the standard deviation from three independent experiments. Statistical analysis was performed using student's t-test. **p<0.01

5.2.7 EPEC translocates effectors to protect cells during infections

I next aimed to gain a better mechanistic insight into the cytotoxicity induced by EPEC *ΔcesT*. Schematically, there can be two types of mechanisms by which the hyper-toxicity can take place: First, the *cesT* mutant might deliver to the host cell a toxic substance (an effector or some other molecule) which is not translocated, or translocated to a lesser extent, by the wild type strain ('over-killing mechanism'). Such an effect can be attributed to the role of CesT in the post-transcriptional regulation exerted by CsrA. The second type is a mechanism in which the *cesT* mutant fails to deliver an effector which prevents the death of the host cell, a process that is triggered by another, CesT-independent, effector or substance ('protection mechanism'). Such a mechanism seems more straight-forward because of the role of CesT as a T3SS chaperon. In order to distinguish between the two types of mechanisms an experiment of co-infection was designed in which I infected the host cells with a mixed population of EPEC strains, which may complement each other *in trans*. I reasoned that when co-infecting with EPEC WT and *cesT* mutant, in case of over-killing mechanism the mutant will translocate the toxic factor and cytotoxicity will be observed similarly to infection with *cesT* mutant alone. In case of a protection mechanism one would expect that the WT bacteria will translocate the protective effector, thus complementing *in-trans* the mutant infection and preventing excessive toxicity.

In agreement with a protection mechanism, the results of the experiment showed that co-infection with EPEC WT and *cesT* mutant strains, mixed in a 1:1 ratio, resulted in levels of toxicity which are similar to infection with WT alone. Importantly, this effect was not observed when I used a T3SS-defficeint mutant which is incapable of translocating any effectors as the complementing strain (Fig. 5.2.7.1).

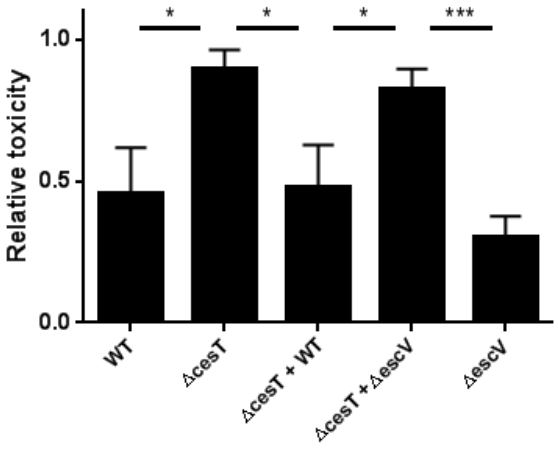

Figure 5.2.7.1 Co-infection with EPEC WT and *cesT* mutant.
EPEC WT, *cesT* mutant or *escV* mutant (T3SS-defective) were used to infect HEK-GFP cells alone or in a 1:1 ratio mixed culture. After 3 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. Error bars represent the standard deviation from three independent experiments. Statistical analysis was performed using student's t-test. *p<0.05, ***p<0.001

I conducted several experiments in order to confirm that the WT EPEC indeed protected the cells by translocating a protecting effector and not by interfering with the infection of the toxic *cesT* mutant. I first verified that both strains are attached to the cells during infection. A mixed WT-*cesT* mutant culture, where the *cesT* mutant also expresses GFP, was used to infect HeLa cells. Microscopy images of the infection show that both strains infect simultaneously and create mixed microcolonies (Fig. 5.2.7.2).

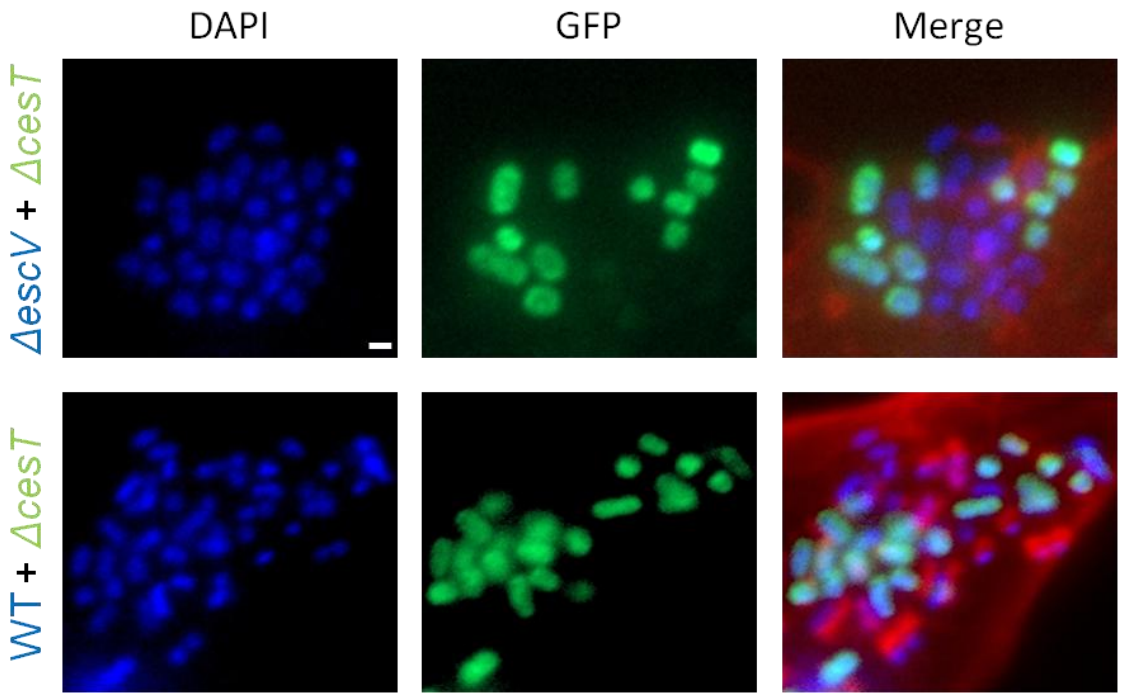

Figure 5.2.7.2 Attachement of EPEC strains in mixed infection.
EPEC WT, *escV* mutant (T3SS-defective) or *cesT* mutant expressing GFP were used to infect HeLa cells in a 1:1 ratio mixed culture. After 3 hours the cells were washed, fixed and DNA staining with DAPI and actin staining with Rhodamine-Phalloidin were performed. Red – Phalloidin (cells), Blue – DAPI (bacteria), Green – GFP (*cesT* mutant). Mixed microcolonies composed of green and blue bacteria infecting the cells simultaneously are seen (scale bar 1 µm).

Next I checked whether the T3SS of the *cesT* mutant remains functional during co-infection and is capable of effector injection. To this end I used EPEC ΔPP4, which lacks the effector NleD, as the complementing strain in co-infection with the *cesT* mutant. Cleavage of host JNK, medited by NleD, was used as a readout for T3SS functinality in the co-infecting *cesT* mutant. Notably, translocation of NleD is not dependent on CesT (Mills *et al*, 2013). I found that while infection with EPEC ΔPP4 alone didn't result in cleavage of JNK, co-infection with *cesT* mutant lead to cleavage of JNK, idicative of functional T3SS in the co-infecting *cesT* mutant (Fig. 5.2.7.3).

Taken together these results strongly support a protection mechanism for *cesT* mutant toxicity over an over-killing mechanism.

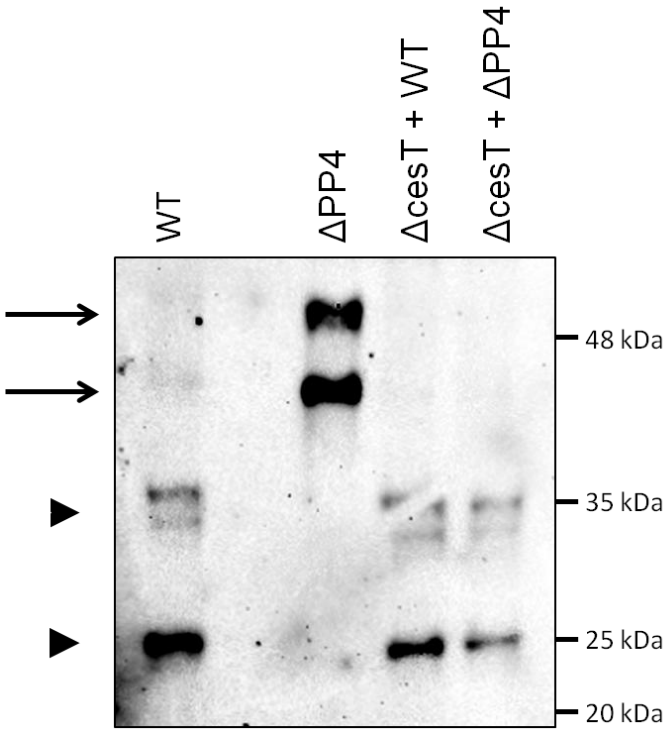

Figure 5.2.7.3 JNK cleavage by EPEC strains in mixed infection.
EPEC WT, ΔPP4 mutant (lacking NleD) or *cesT* mutant were used to infect HEK293T cells alone or in a 1:1 ratio mixed culture. After 3 hours the cells were washed and analyzed with Western blot using antibodies against JNK1/2 to observe JNK cleavage. Arrows – Intact JNK. Arrowheads – cleavage products. Molecular weight marker is shown.

5.2.8 EspZ is the protective effector

After finding that EPEC *cesT* mutant fails to deliver a protective effector I set out to find the identity of this effector. EPEC0 is a strain of EPEC deleted of all the known T3SS effectors genes (Cepeda-Molero *et al*, 2017). Introducing plasmids expressing single effectors one at a time can allow one to assess the effect of each of the effectors on the toxicity induced to the host cells. EPEC0 alone cannot protect the cells form EPEC *cesT* mutant, confirming that the protective effector is a known T3SS effector (Fig. 5.2.8). I screened several T3SS effectors and found that EPEC0 expressing EspZ is able to effectively protect the cells, showing that translocation of EspZ is sufficient for the protection (Fig. 5.2.8). Of note, CesT is known to be required for the translocation of EspZ (Mills *et al*, 2013). Together, the results suggest that *cesT* mutation impedes the translocation of EspZ which is then unable to counteract the induced toxicity in the host cells.

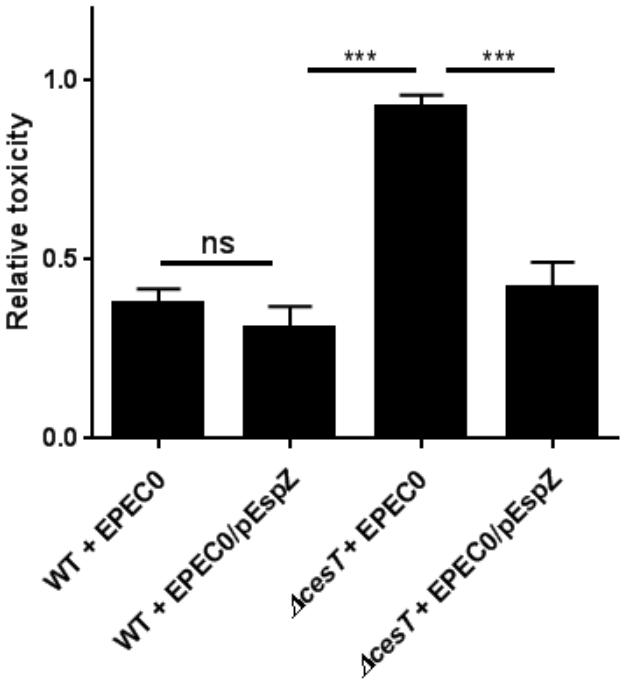

Figure 5.2.8 EspZ is the protective effector.
EPEC WT, *cesT* mutant, EPEC0 or EPEC0 expressing EspZ were used to infect HEK-GFP cells alone or in a 1:1 ratio mixed culture. After 3 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. Error bars represent the standard deviation from three independent experiments. Statistical analysis was performed using student's t-test. ***p<0.001, ns=non-significant.

5.2.9 Failure to deliver EspZ is responsible for EPEC *ΔcesT* toxicity

The previous results showed that deletion of *cesT* in the infecting bacteria renders them unable to translocate EspZ, which in turn leads to toxicity towards the infected cells. Since CesT is a chaperon for many EPEC T3SS effectors including Tir, EspZ, Map, EspH, EspJ, NleG, NleH1, NleH2 and, NleA (Mills *et al*, 2013) we speculated that additional protective effectors besides EspZ might exist. If this is the case a *cesT* mutant, in which the ability to deliver all of the protective effectors is severely impaired, should be more toxic then an *espZ* mutant in which only EspZ is not translocated. Similarly, a *cesT espZ* double mutant should also be more toxic then the *espZ* mutant. If, however, EspZ is the only relevant protective effector, no additional effect should be observed.

The results of the experiment examining this showed that the *cesT* mutant actually exhibited significantly lower levels of toxicity compared to the *espZ* mutant, although still higher than the wild type strain. The *cesT espZ* double mutant showed a level of toxicity which is intermediate between *cesT* mutant and *espZ* mutant, although this apparent trend was not statistically significant (Fig. 5.2.9). These results can be explained by some degree of translocation of EspZ even in the *cesT* mutant, which is absent in the *espZ* mutant. Alternatively, deletion of *cesT* may impede other aspects of the infection (e.g. reduced translocation due to poor Tir-dependent attachment) and somewhat impair the ability of the bacteria to fully demonstrate its toxic potential.

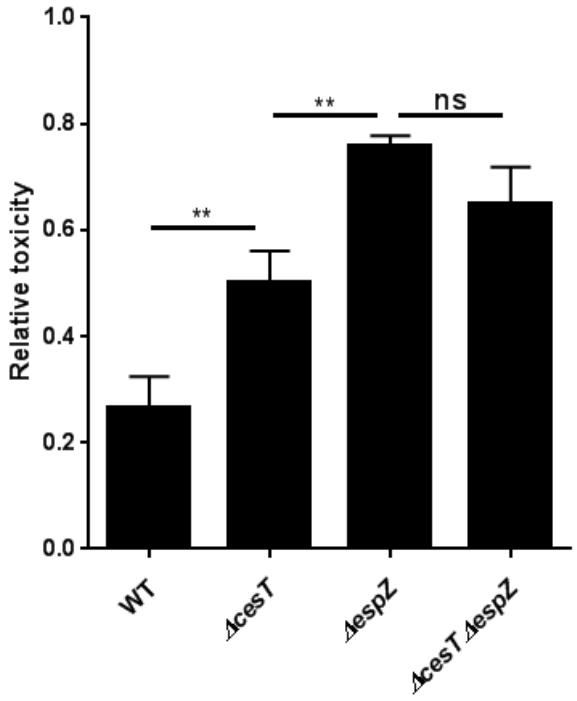

Figure 5.2.9 Failure to deliver EspZ is responsible for EPEC *ΔcesT* toxicity.
EPEC WT, *cesT* mutant, *espZ* mutant or *cesT espZ* double mutant were used to infect HEK-GFP cells. After 3 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. Error bars represent the standard deviation from three independent experiments. Statistical analysis was performed using student's t-test. **p<0.01, ns=non-significant.

5.2.10 None of the known effectors are needed for toxicity

After establishing that the protective effector is EspZ I wanted to decipher the identity of the toxic factor. I first wanted to check whether it is one of the known effectors of EPEC, or perhaps some combination of them. Therefore I used the deletion mutants EPEC2 in which all the known effectors except Tir and EspZ were deleted and the even more complete EPEC0 which is devoid of all the known effectors of EPEC (Cepeda-Molero *et al*, 2017). I used a plasmid for constitutive over-expression of MgrR in order to unmask the normally hidden potential of EPEC for cytotoxicity. Using this approach I found that similarly to WT EPEC, the EPEC2 mutant exhibited increased toxicity when over-expressing MgrR. Moreover, while after 3 hours of infection EPEC0 is not toxic, when extending the infection time to 4.5 hours, the EPEC0 mutant which lacks the protective EspZ is toxic to host cells even without over-expression of MgrR (Fig. 5.2.10). The delayed toxicity of EPEC0 might be attributed to attenuation in host attachment due to loss of the Tir-intimin interaction and the concomitant reduction in injection efficiency. These results demonstrate that none of the known T3SS effectors of EPEC are needed for the toxicity phenotype. The toxic factor is thus either a previously un-characterized effector or some other substance which penetrates the host cell via the T3SS needle.

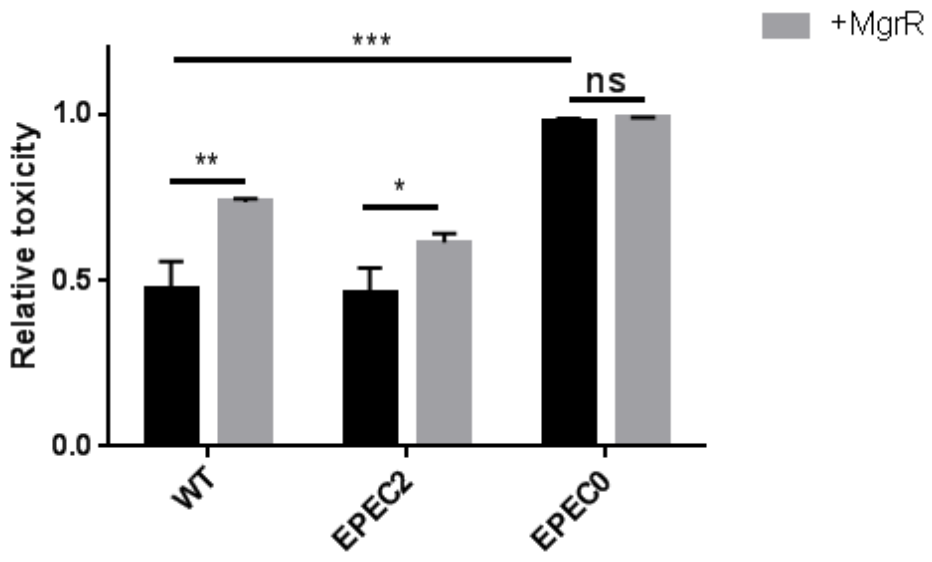

Figure 5.2.10 EPEC2 and EPEC0 have the potential for cytotoxicity.
EPEC WT, EPEC2 or EPEC0, containing or not the pZE12-MgrR plasmid for constitutive expression of MgrR, were used to infect HEK-GFP cells. After 4.5 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. Error bars represent the standard deviation from three independent experiments. Statistical analysis was performed using student's t-test. ***p<0.001, **p<0.01, * p<0.05, ns=non-significant.

5.2.11 Poor correlation between NF-κB activation and toxicity

Some components of the T3SS can activate the inflammasome in host cells (Miao *et al*, 2010). In addition, the activity of the T3SS itself and its insertion into the membrane can be sensed by the host and trigger an inflammatory response, which is usually blocked by the translocated effectors (Litvak *et al*, 2017). After learning that none of the known effectors are required for the toxicity phenomenon, I asked whether the toxicity phenomenon is the result of a bacterial component entering the cell (either actively by translocation or passively by diffusion) or is the result of the host sensing the T3SS needle. To answer this I used a set of mutants in which the ability of the T3SS to deliver effectors into the host cell or its capability to form pores in the membrane are uncoupled from its sensing by the innate immune system and subsequent activation of the NF-κB signaling pathway (Litvak *et al*, 2017). This uncoupling was achieved by inserting a small sequence in different locations along the translocon subunit EspB (Luo & Donnenberg, 2006). Among these mutants are insertions in T239 and L241 in which activation of the NF-κB signaling is preserved and even enhanced, even though pore formation and protein translocation are impaired. In other mutants containing insertions at K179 and E203, protein translocation is relatively preserved but activation of NF-κB signaling is significantly lower (Table 5.2.11). I used this set of mutants, containing a plasmid for over-expression of MgrR and compared the levels of toxicity which they induced in the host cells. I expected that if the toxicity is the result of sensing of the T3SS by the host cells, the level of toxicity will correlate with the degree of NF-κB activation and mutants which efficiently activate the NF-κB will show high level of toxicity even if their ability to translocate effectors is impaired. If, conversely, the toxicity is mediated by a translocated effector or diffusing substance I expected to see a correlation between induced toxicity and translocation or pore formation ability, respectively.

The preliminary results of this experiment failed to show clear correlation between induced toxicity and mutant characteristics as all the mutants exhibited similar levels of cytotoxicity (Fig. 5.2.11). A notable exception is mutant L282 which is severely impaired in all three traits and indeed induced somewhat decreased levels of toxicity. These results suggest that sensing of the T3SS *per se* is not the initiating trigger for cytotoxicity. Rather, it is more likely that an un-identified effector or some other molecule which passes through the T3SS needle sets off this response.

EspB insertion site	Pore formation	Protein translocation	NF-κB activation
Wild type	100	88	40
ΔespB	14	0	2
K179	31	81	17
E203	66	88	11
T239	59	28	45
L241	54	66	50
L282	15	0	10
Uninfected cells	16	0	0

Table 5.2.11 Characteristics of EspB insertion mutants.
The ability of the different EspB insertion mutants to form pores in the host cell membrane, translocate effector proteins and activate NF-κB signaling were compared. Data taken from Litvak *et al*, 2017.

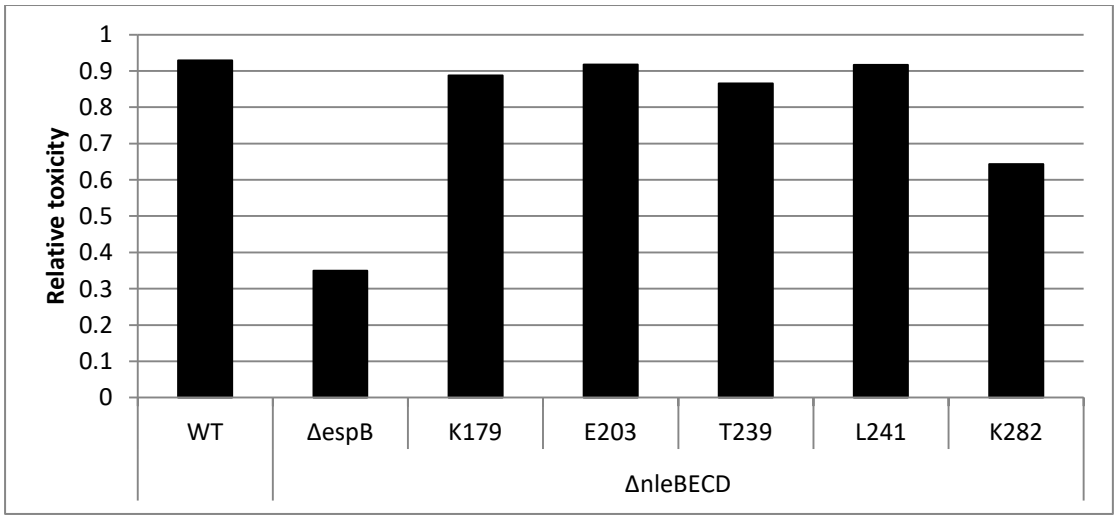

Figure 5.2.11 Toxicity levels induced by EspB insertion mutants.
EPEC strains containing short insertions in various locations along the translocon subunit EspB and over-expressing MgrR were used to infect HEK-GFP cells. After 3 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. These are preliminary results and more experiments are needed to thoroughly assess the significance of the differences between toxicity levels induced by the different strains.

5.2.12 *de-novo* protein translation in the host during infection is not needed for cytotoxicity

Programs of regulated cell death are usually executed by pre-formed proteins. I wanted to test whether the death of infected host cells requires protein synthesis during infection and thus represents a relatively late response or is executed by pre-formed proteins already present at the beginning of the infection. To this end I used the translation inhibitor cycloheximide to block protein synthesis in the host cells during infection.

The results show that cycloheximide didn't alter the course of infection in regard to induced toxicity, suggesting that *de-novo* protein synthesis in the host is not required for the cytotoxicity and that it is performed by pre-formed proteins (Fig. 5.2.12). Alternatively, the death of the cell may represent a previously un-described mechanism resulting from the action of translocated bacterial proteins without activation of programmed cell death.

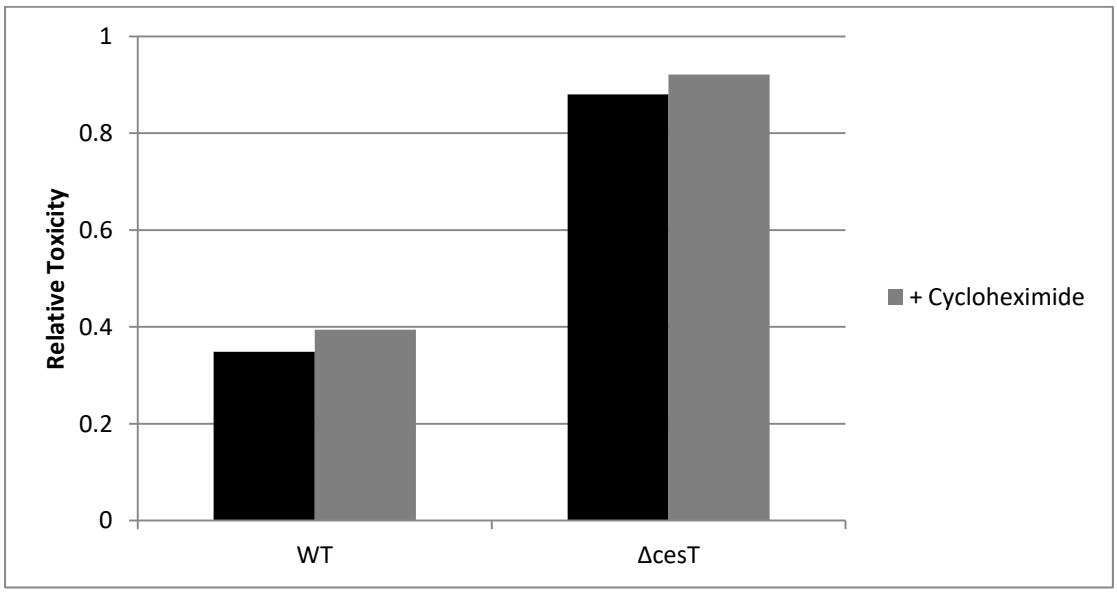

Figure 5.2.12 Cycloheximide doesn't affect EPEC-induced toxicity.
EPEC WT or *cesT* mutant were used to infect HEK-GFP cells, with or without addition of the translation inhibitor cycloheximide. After 3 hours the plate was washed to remove the detached cells, then the remaining attached cells were scraped and the amount of GFP (representing the number of cells in the sample) was measured. These are the results of a representative experiment.

5.3 Discussion

Infection of host cells by pathogenic bacteria can result in several types of response of the innate immune system. In some instances, usually when bacterial components are sensed by extracellular receptors, a transcriptional response such as the NF-κB or MAPK signalling pathway is activated (Chow *et al*, 1999). In other cases a program of regulated cell death such as apoptosis or necroptosis, frequently regulated by an inflammasome, occurs. Bacterial pathogens seek to avoid recognition by the host cells and thus an evolutionary molecular arms race led to complex signaling pathways with branching systems for sensing pathogens by the host, and unique and creative tools to avoid or silence them by the pathogens (Lacey & Miao, 2020). For example, the presence of *Shigella* can trigger apoptosis of the infected cells, which is inhibited by the effector OspC1. The inhibition of apoptosis in the presence of apoptotic triggers would lead to death of the infected cell by necroptosis, but this process is inhibited by an additional effector – OspD3 (Ashida *et al*, 2020). Similar strategies are utilized by EPEC, EHEC and other intestinal pathogens (Pearson *et al*, 2017).

PMAPs, including LPS and flagellin, are sensed by extra- and intra-cellular receptors such as TLRs and NLRs and can induce innate immune response (Takeda & Akira, 2004; Platnich & Muruve, 2019). Still, in the case of cell death in response to EPEC infection the specific triggers are poorly defined even though various bacterial strategies for its inhibition were described. Moreover, the response can be triggered in cell lines which don't possess many of the innate immune receptors and in some cases was shown to be independent of all the known signaling pathways (Litvak *et al*, 2017). A recent set of works propesed that polymerization of actin which follows translocation of Tir and its integration to the host membrane leads to calcium influx in the cells. This influx may result in internalization of LPS or another PAMP which can trigger the cell death response directly by activating caspases, bypassing the known innate immune receptors (Goddard *et al*, 2019; Zhong *et al*, 2020).

In this study both the trigger for cellular death and the exact mechanism of its inhibition remain mysterious. I have shown that this cell death is not triggered by one of the known effectors of EPEC, including Tir (Fig. 5.2.10). It is

possible that a previously undescribed effector is responsible for this cytotoxicity. Alternatively, some other substance such as a metabolite might pass through the T3SS needle and be recognized by the host cells as PAMP. Since there is no linear correlation between the ability of the bacteria to translocate effectors and their ability to induce toxicity in the host cells, the later option seems more probable (Fig. 5.2.11). A possible future line of work is to perform random mutagenesis folowed by screening for EPEC mutants that lost their toxic potential. Alternatively, as over-expression of MgrR directly induces toxicity we can take hints from RNA-seq data which we already possess from EPEC WT and EPEC over-expressing MgrR. Focus can be made on virulence-related genes whose expression level is significantlly affected by MgrR and their role in inducing toxicity in the host cells can be tested.

As for the inhibition of cytotoxicity – I have shown that EspZ is an effector which prevents the death of the host cells (Fig. 5.2.8). While it is known that EspZ is required for survival of host cells during infection its mechanism of action is yet to be discovered (Berger *et al*, 2012). Here I showed that failure to deliver EspZ to the host cells due to low levels of its chaperon CesT is sufficient to enable death of the host cells during infection. Moreover, I demonstrated that the site of action of EspZ is in the host cell rather then inside the infecting bacteria, as injection of EspZ by EPEC0 protected the cells from the toxicity induced by EPEC strains which cannot translocate it (Fig. 5.2.8). To discover the exact mechanism of action of EspZ it may be useful to perform pull-down or other assay to find its interacting partners in the host.

Finally, the exact death program which is triggered in the infected cells remains to be described. To do so it whould be helpful to use caspase inhibitors or alternatively to knock-out genes that encode for executioner proteins such as caspases, gasdermins or MLKL.

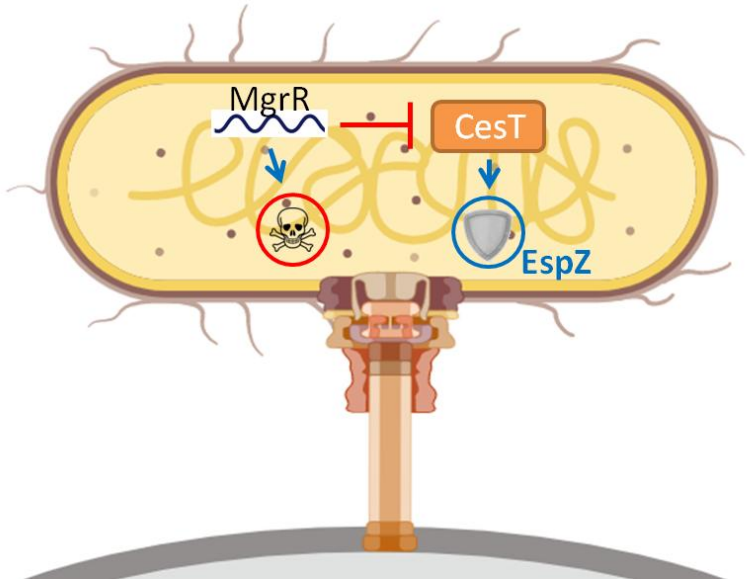

Figure 5.3 A model for EPEC-induced cytotoxicity.
A small molecule leaks through the T3SS and triggers cell death in the host. Death is prevented by EspZ which is translocated with the assistance of CesT. MgrR lowers the level of CesT and also increases toxicity directly.

Taken together the results enable us to describe the following model for EPEC-induced toxicity (Fig. 5.3): A bacterial component, likely a small molecule, leaks through the T3SS and triggers an innate immune response. This response would lead to death of the cells, but it is inhibited by EspZ which is translocated with the assistance of the chaperon protein CesT. The sRNA MgrR augments the toxicity induced by EPEC indirectly through inhibition of translation of CesT, lowering its levels, and directly due to enhancement of toxicity.

6. Genome-wide CRISPR-Cas9 screen for identification of host factors for infection

6.1 Introduction

6.1.1 Host components in EPEC infection

EPEC utilizes a unique strategy when infecting host cells. First the bacteria attach to the host cells, a process which is augmented by the bacterial Bundle-Forming Pillus (BFP) (Cleary *et al*, 2004). The BFP is an important virulence factor for EPEC, and mutations render it significantly less virulent to humans (Bieber *et al*, 1998). After the initial attachment, retraction of the BFP promotes effector translocation and host colonization (Zahavi *et al*, 2011; Aroeti *et al*, 2012). No specific receptor protein in the host was identified for the initial attachment, but this is closely followed by T3SS-mediated translocation of Tir which integrates into the membrane of the host cell and serves as the receptor for an outer membrane adhesin termed intimin (Kenny *et al*, 1997). Thus EPEC injects its own receptor to the host cells. Tir-intimin interaction leads to clustering of Tir under the bacterial attachment site. Tir undergoes phosphorylation, which enables the recruitment of the adaptor protein Nck which in turn recruits the regulator N-WASP (Lommel *et al*, 2001). N-WASP is one of the major regulators of the Arp-2/3 complex and its activation leads to polymerization of actin underneath the bacteria, resulting in the formation of characteristic actin-rich pedestals (Campellone & Leong, 2003). The Arp-2/3 complex is composed of seven subunits and plays a key role in the dynamics of the actin cytoskeleton including actin polymerization and branching (May, 2001). The role of actin pedestals during EPEC infection is poorly understood, but some works in closely related pathogens suggest that polymerization of actin is required for efficient bacterial attachment and translocation of effectors by the infecting bacteria (Battle *et al*, 2014; Russo *et al*, 2021). Additionally, it was suggested that the formation of pedestals and related actin reorganization enable the bacteria to spread to neighboring cells directly, without first detaching from the originally infected cell (Velle & Campellone, 2017). Thus the host cells participate in all stages of the infection process starting at initial attachment and effector translocation, and their role

is not limited to sensing the presence of bacteria and initiating an inflammatory response.

6.1.2 Genome-wide screens

Genome-wide screens are powerful tools to detect genes involved in various processes. Many techniques were described in prokaryotic as well as eukaryotic species which differ in the methods for mutant generation and selection, but the basic principles are similar: A population of cells is created in which each cell contains a single perturbation (insertion of transposon in case of Tn-seq, shRNA-containing plasmid in case of iRNA screen, gRNA-directed knock-out in case of CRISPR screen, etc.). The population then undergoes a selection process which can be survival of the cells in insulting conditions or Fluorescence-Assisted Cell Sorting (FACS) in case of a fluorescent reporter (genetically encoded reporter or staining after fixation). After this the abundance of different mutants is determined in both the pre- and post-selection populations using quantitative sequencing. For example, mutants in genes required for production of an amino acid can survive in rich media and will therefore be present in the initial pre-selection population, but will be eliminated after growth in media depleted from this specific amino acid. By comparing the two populations one can detect the genes involved in the metabolic pathway responsible for production of this amino acid. Similarly, the offending insult can be bacterial infection and host components involved in different stages of infection can be discovered. Several such screens were performed in the past. Previous screens identified genes in the host cells which are promoting efficient translocation of effectors by *Yersinia*, as well as genes needed for modification of surface proteins which are presumed to enable binding of bacteria to the cell, which was demonstrated for *Vibrio* and EHEC (Sheahan & Isberg, 2015; Blondel *et al*, 2016; Pacheco *et al*, 2018). Here I performed a genome-wide CRISPR screen to identify host genes which have roles in different stages during EPEC infection.

6.1.3 Hypothesis, rationale and aims

Pathogens rely on host factors for efficient infection. Viruses and bacteria use host surface proteins as receptors for attachment and entry into host cells. The surface-attached or intracellular pathogens exploit host cellular processes. In the case of EPEC host factors might be needed for the BFP-dependent attachment, for insertion of the T3SS translocon to the host membrane, and for signaling the bacteria to initiate effectors injection. Additionally, host factors that are required for toxicity might also exist.

We hypothesized that mutating the genes that encode for such host factors will result in lower susceptibility to EPEC infection, and therefore mutants in such genes will have a survival advantage during infection.

Specifically, we asked the following questions:

1. Can we use a genome-wide CRISPR-Cas9 library to identify gRNA targets and host genes that are required for efficient infection and thus high toxicity?
2. What is the role of such genes during infection?

6.2 Results

6.2.1 Genome-wide CRISPR-Cas9 screen for EPEC-resistant cells

In order to identify host genes that have a role in the different stages of EPEC infection we decided to conduct a genome-wide CRISPR screen. I utilized HT-29 cells which were transduced with the Avana sgRNA library. This library contains up to four gRNAs targeting each of the annotated human genes (Doench *et al*, 2016). I infected this population with an EPEC strain constitutively over-expressing MgrR to induce death of the infected cells. This strain was picked to enhance the robustness of the screen due to stronger selective pressure relative to the wild-type strain (Pearl Mizrahi *et al*, 2021). Following 90 minutes of infection with pre-activated bacteria, 50 µg/mL gentamycin was added to stop the infection and the cell population was allowed to re-expand before another round of infection was performed (Fig. 6.2.1, see methods for details). A total of four rounds of infection-expansion were performed and the abundance of the different gRNAs in the starting population and in the final population were analyzed using next generation sequencing.

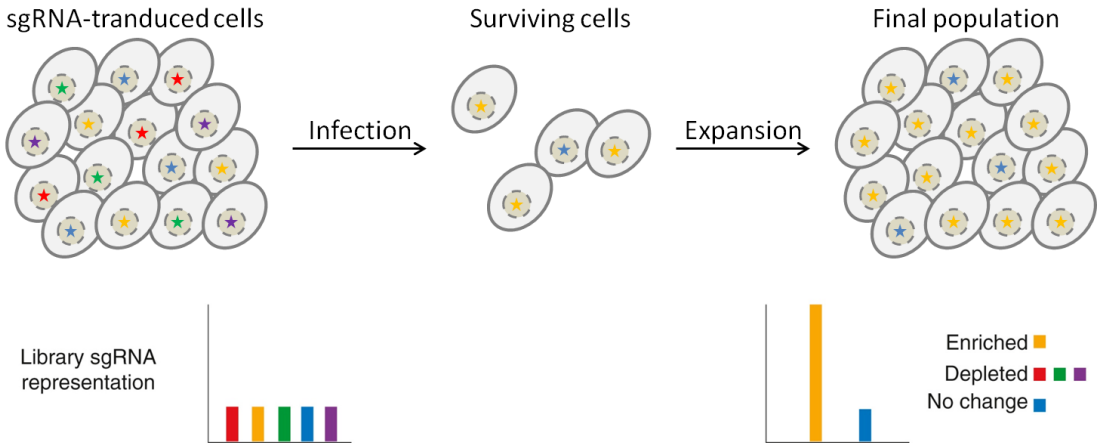

Figure 6.2.1 Design of the genome-wide CRISPR-Cas9 screen.
The Avana sgRNA library was used to transduce HT-29 cells, creating the initial population of mutant cells. This population was infected with EPEC over-expressing MgrR which causes toxicity in the host cells in a T3SS-dependent manner. The infection was stopped by addition of gentamycin and the cells were allowed 2-3 days for re-expansion, followed by another round of infection. A total of four rounds of infection and re-expansion were performed. Next generation sequencing was employed to determine the abundance of the different gRNAs in the initial and the final populations.

Comparing the relative frequencies of the different gRNAs in the populations revealed a few dozens of genes mutants in which were positively selected by the screen (Table 6.2.1). These genes are presumably involved in some stage of the infection process – from attachment of the bacteria to the specific mechanism of cell death induced by it. Interestingly, three main groups of genes can be defined based on their function. The first group is composed 13 cytoskeleton-related genes, including 5 out of 7 components of the Arp-2/3 complex and two regulators of Arp2/3. The second group contains genes annotated as expression regulators (11 genes), and the third group contains genes involved in protein glycosilation (5 genes). Thus, the screen showed its robustness and pointed to numerous host genes which are required for efficient infection by EPEC

Gene	log_2 fold-change	p value	Functional group
WASL	4.543	0.000291	Actin cytoskeleton
CDC42	4.3249	0.000291	Actin cytoskeleton
ACTR3	4.2429	0.000291	Actin cytoskeleton
MGAT1	5.0885	0.000291	Glycosilation
STK11	3.3053	0.000291	Expression regulation
TMEM165	4.2085	0.000291	Glycosilation
B3GNT2	5.4651	0.000291	Glycosilation
SLC35A2	4.3195	0.000291	Glycosilation
ARPC4	3.3115	0.000291	Actin cytoskeleton
ARPC3	3.5938	0.000291	Actin cytoskeleton
ARPC4-TTLL3	4.0469	0.000291	Actin cytoskeleton
KMT2D	2.9111	0.000291	Expression regulation
YTHDF2	2.9816	0.000291	Expression regulation
ARPC2	3.0848	0.000291	Actin cytoskeleton
SPTBN1	2.0082	0.000291	Actin cytoskeleton
SPTAN1	2.2181	0.000291	Actin cytoskeleton
TFAP4	2.2044	0.000291	Expression regulation
CBLL1	2.6997	0.000825	Expression regulation
ACTR2	3.3761	0.001303	Actin cytoskeleton
CTNND1	2.6889	0.002228	Expression regulation
PHIP	1.7498	0.008251	Actin cytoskeleton
WDR77	1.3288	0.010126	Expression regulation
BCOR	2.3461	0.014421	Expression regulation
DEFB121	1.5162	0.015644	
COMMD7	1.4894	0.015644	Expression regulation
RALGAPB	1.7598	0.025704	Expression regulation
RRBP1	1.9204	0.032087	Glycosilation
SPTBN2	1.383	0.039781	Actin cytoskeleton
WSB1	1.6976	0.040083	
ARIH2	1.6596	0.040083	Expression regulation
FITM2	1.7456	0.040083	Actin cytoskeleton

Table 6.2.1 Results of the screen.
log_2 fold-change of the relative abundance of gene-targeting gRNAs in the final population relative to the initial population is presented along with a corrected p value.

6.2.2 Knock-down of candidate genes confers some resistance from EPEC-induced toxicity

I next wanted to validate the results of the screen and investigate the role of specific genes during infection. Several genes were selected for further analysis, representing each of the functional groups which were defined. These included WASL which encodes the protein N-WASP and was selected as the representative of cytoskeleton-related genes. STK11, also known as LKB1, was picked out of the expression regulators. Finally, MGAT1 and TMEM165 were chosen to represent genes involved in protein glycosilation.

To examine the role of these genes during infection I used shRNA to knock down their expression in HEK293T cells, and tested whether it confers increased resistance to EPEC infection. Indeed, knock down of all the candidate genes conferred some degree of resistance to infection with the hyper toxic EPEC over-expressing MgrR, although TMEM165 knock down showed a modest, nonsignificant resistance (Fig. 6.2.2A). Importantly, the knock-down of TMEM165 was very weak, which explains the lack of a significant effect (Fig. 6.2.2B). These data validate the results of the performed CRISPR screen, suggesting that the tested host factors are involved in the process of EPEC infection and EPEC-induced toxicity. The specific role of these genes during EPEC infection remains to be dissected in future work.

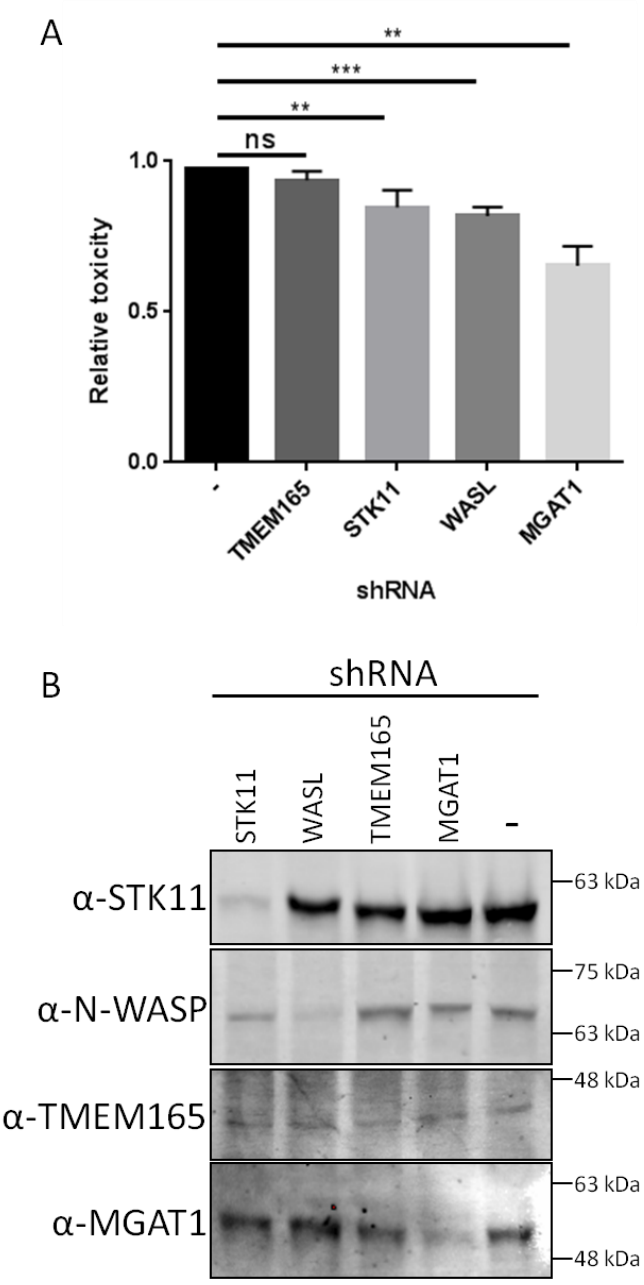

Figure 6.2.2 Knock down of WASL, STK11 and MGAT1 confers resistance to EPEC-induced toxicity.

(A) EPEC over-expressing MgrR was used to infect HEK293T expressing shRNA targeting WASL, STK11, MGAT1 or TMEM165 and toxicity after 3 hours was assessed. Error bars represent the standard deviation from three independent experiments. Statistical analysis was performed using student's t-test. ***p<0.001, **p<0.01, *p<0.05, ns=non-significant. (B) Western blot analysis was performed to verify the knock down of the candidate genes. Molecular weight marker is shown.

6.3 Discussion

Genome-wide screens proved to be powerful tools in identifying genes involved in various processes. In the context of bacterial infection previous screens revealed that host genes are needed not only for attachment of bacteria but also for efficient translocation of effector proteins (Sheahan & Isberg, 2015). The process of EPEC infection can be subdivided to several steps: First – initial recognition of potential host cells and attachment. Second – translocation of protein effectors, and third – specific results of the action of these effectors in the host cells. Here I present the results of a genome-wide CRISPR-Cas9 screen which produced a list of over thirty host genes which might be required for efficient EPEC infection. Of the list I focused on four genes: WASL, STK11, MGAT1 and TMEM165.

WASL is the gene encoding N-WASP, which is an activator of the Arp-2/3 complex (May, 2001). Notably, five out of the seven structural components of this complex, along with two of its most important regulators (N-WASP and CDC42) were identified in the screen as important for EPEC infection. The Arp2/3 complex mediates branching of actin filaments and their nucleation (i.e. creation of new filaments) in a process conserved in all eukaryotes. When activated in a specific location such as at the edge of the cell the activity of the complex can produce the mechanical force which drives cell motility. In other cases the force produced by the activity of the Arp2/3 complex results in perturbation of the membrane structure which occurs in processes such as phagocytosis and membrane blebbing during apoptosis (Martin & Suzanne, 2022). The role of these genes during infection is not clear, but N-WASP-mediated activation of the Arp2/3 complex is crucial for the formation of the hallmark feature of EPEC infection – actin-rich pedestals underneath the infecting bacteria and AE lesions in the intestinal epithelium (Campellone & Leong, 2003). It was recently proposed that the mechanical force produced by polymerization of actin during infection with the closely related *Shigella* is required to keep the translocon pores of the T3SS in an open state, thus enabling efficient translocation of effectors (Russo *et al*, 2021). If this is the case also for EPEC it can explain the relative resistance of Arp2/3-mutant cells to infection.

STK11, also known as LKB1, is a key kinase protein in the regulation of host cell metabolism, proliferation and inflammatory response to infection (Zyla *et al*, 2021). This gene acts as a tumor-suppressor and germline mutations are responsible in human patients for Peutz-Jeghers syndrome which is characterized by various over-proliferation disorders including elevated risk of developing cancer (Tacheci *et al*, 2021). The best-studied substrate of STK11 is AMPK, whose activity regulates the metabolism of the cell and its response to infection, including autophagy and formation of inflammasomes (Shaw *et al*, 2004). Interestingly, AMPK was not suggested by the screen as having a role in the infection process. Thus the role of STK11 during EPEC infection remains unsolved, and I suggest that a less-studied role of this protein, or a more complex interaction with the described substrate AMPK are conferring the relative resistance to infection.

The other genes in the top 10 list produced by the screen have roles in protein glycosilation. MGAT1 is localized to the Golgi apparatus, where it is responsible for the formation of complex N-glycans and their transfer to glycoproteins (Yip *et al*, 1997). These complex glycoproteins are required for cell-cell interactions which were previously shown to be required for embryogenesis (Ioffe & Stanley, 1994). The role of TMEM165 is poorly understood. It possesses cation-transport ability which is important for protein glycosilation in the Golgi apparatus and mutations in this gene are associated with congenital disorders of glycosilation (Stribny et al, 2020). SLC35A2 transports UDP-galactose from the cytosol to the Golgi apparatus where it is transferred to glycoproteins. Mutations in this gene were also described as causing congenital disorders of glycosilation (Quelhas *et al*, 2021). B3GNT2 is another nucleotide-sugar transporter, which is probably the most important polylactosamine synthase in human cells (Zhou *et al*, 1999). It is tempting to speculate that these genes promote protein glycosilation which supports the initial attachment of EPEC to host cells. The specific host surface proteins which are recognized by EPEC for initial attachment remain mysterious, but it was shown for EPEC and other pathogens that modifications of membrane proteins such as glycosilation are required for efficient attachment (Blondel *et al*, 2016). An appealing possibility is that the sugars themselves, rather than a

specific protein are recognized by the infecting bacteria. For example, it was shown that BfpA, the major adhesin of EPEC which is required for the initial attachment, binds N-acetyl-lactosamine (Hyland *et al*, 2007). Although the BFP is not required for colonization, it assists in the primary, not-specific attachment of the bacteria to the host cells (Cleary *et al*, 2004). Remarkably, N-acetyl-lactosamine chains are the product of the aforementioned B3GNT2 gene activity, suggesting that this sugar moiety indeed has a role in the infection process. Thus the results of the screen stand in agreement with previous works and suggest that the EPEC-host cell interaction is dependent on protein modifications rather than on protein sequences in its early stages and that EPEC binds to specific sugars on the surface of the colonized host cell.

Finally, it is worth mentioning that the screen didn't highlight specific regulators or executioner proteins of host cell death programs. This suggests that EPEC infection can result in death of the host cells by several redundant mechanisms. Therefore, only upstream perturbations in the infection process itself protect the cells from death.

7. Comprehensive discussion

The process of EPEC infection of host cells is a masterpiece of coordination of bacterial mechanisms and exploitation of host components, resulting in a perfectly orchestrated interplay between the pathogen and its host. During my doctoral project several aspects of this interaction were studied. I showed here an in-depth molecular analysis of the function of the effector NleD. I found that this translocated metalloprotease is a dual-function effector which has two seemingly contradicting roles: On the one hand it cleaves specifically un-phosphorylated p38 and JNK. On the other hand, it binds the phosphatase PPM1A which can dephosphorylate p38 and JNK and restore their susceptibility to cleavage, but the binding of NleD inhibits its activity. This way EPEC can, with the action of a single effector, both promote and attenuate MAPK signaling, one of the key signaling pathways in response to infection. The inhibition of the catalytic activity of PPM1A is achieved by binding of NleD which sterically blocks the entrance of large substrates into the catalytic pocket of the phosphatase, yet the inherent activity of PPM1A is not inhibited as it is able to dephosphorylate small substrates which can still diffuse into the catalytic site through the NleD blockage. The benefit of the bacteria from NleD-mediated inhibition of PPM1A during EPEC infection is not known. But whether this benefit is achieved by de-regulation of the MAPK signaling pathway or is related to other functions of PPM1A in the regulation of developmental and metabolic signaling pathways in the intestine, this double-activity of a single effector represents a novel and exciting mode of action for bacterial pathogens. In order to study the effect of PPM1A inhibition during infection a more complex experimental set up is needed. Work is now done in order to perform such experiments in mouse model using a related pathogen and in human organoids.

I then took a wider look on the host-pathogen interaction during EPEC infection and analyzed factors regulating the survival of host cells during infection. Programs of cell death can be initiated in infected cells in response to infection and lead to activation of the cell-autonomous immune system which is devastating to the pathogen (Lacey & Miao, 2020). To make matters

worse, some of the injected effectors have a pro-apoptotic activity (Pinaud *et al*, 2018). To compensate, EPEC injects several effectors which inhibit different steps in the various cell death programs. Here I show that EPEC institutes a fragile state of equilibrium of signals affecting the viability of the host cells. Alterations in this equilibrium which impair the ability of EPEC to translocate the protective effector EspZ lead to rapid death of the host cells. The specific factor that initiates the death of the host cells was not identified but I showed that it is not one of the known effectors of EPEC. It may be a previously un-characterized effector or some other molecule such as a metabolite which leaks into the host cells via the T3SS needle.

In the next step, I performed a genome-wide CRISPR-Cas9 screen to identify host mutants that exhibit increased resistance to EPEC-mediated toxicity. Since the toxicity is T3SS-dependent, this screen was anticipated to identify host genes which promote EPEC infection. Pathogens utilize host components in virtually every step of infection from attachment to protein translocation. In the screen I identified a few dozens of host cell genes which are putatively involved in the infection process. To demonstrate the validity of this screen I tested the role of some of these genes and showed that mutations in these genes conferred resistance to the death induced by the hyper-toxic EPEC strain over-expressing MgrR. Other genes in the list whose role is yet to be verified await experimental confirmation. Notable among the verified results are the genes MGAT1 and TMEM165 which are involved in protein glycosilation and I postulate that this glycosilation enables the initial attachment of the bacteria to the cells (Schachter, 2010; Stribny *et al*, 2020). Other prominent genes in the screen are members and regulators of the Arp-2/3 complex. This complex is responsible for the dynamics of the actin cytoskeleton of the host cells and is required for processes such as cell migration and phagocytosis (May, 2001). The role of the Arp-2/3 complex during EPEC infection is yet to be discovered and based on previous works I hypothesize that polymerization of actin, which is regulated by this complex, is required for translocation of effectors into the host cells (Russo *et al*, 2021). The screen failed to highlight a dominant cell death program in response to infection and this aspect remains vague.

Overall, my study reflects the complexity of the host-pathogen interaction during infection and dissects several aspects of this process. I performed a thorough analysis of the mechanism by which EPEC controls a key signaling pathway in the host – the MAPK signaling. Zooming out, I took a look at how EPEC manipulates the viability of the host cells during infection – the result of integration of several signaling pathways. Finally, I performed a genome-wide screen to identify genes of the host which are involved in any stage of the infection. Thus I put my share in gaining a better understanding of the processes underlying infection, which can lead to development of new tools to prevent and treat devastating diseases caused by bacterial pathogens.

List of abbreviations

AE pathogens – Attaching and effacing pathogens

CR – *Citrobacter rodentium*

EPEC – Enteropathogenic *Escherichia coli*

BFP – Bundle-forming pilus

T3SS – Type 3 secretion system

LEE – Locus of enterocyte effacement

MAPK – Mitogen-activated protein kinase

JNK – Jun N-terminal Kinase

ERK – Extracellular signal-Regulated Kinase

MS – Mass spectrometry

SBP – Streptavidin-binding peptide

pNPP – p-nitrophenyl phosphate

PI – propidium iodide

DAPI – 4′,6-diamidino-2-phenylindole

IPTG – Isopropyl β-d-1-thiogalactopyranoside

GFP – Green fluorescent protein

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תקציר

EPEC הוא פתוגן אנטרי חשוב בילדים במדינות מתפתחות. פתוגן זה משתמש במערכת הפרשה מסוג 3 כדי להזריק לתאי המאכסן עשרות חלבונים המכונים אפקטורים, המשבשים תהליכים בתאי המאכסן לטובת הפתוגן. אופן פעולת האפקטורים הללו מובן רק חלקית ומטרת עבודה זו הייתה להבהיר את תפקודם של חלק מהאפקטורים הללו ואת ההשפעה של מערכת ההפרשה מסוג 3 על גורל התאים המארחים. באופן ספציפי, בעבודה זו בדקתי את ההיבטים הבאים של פעילות מערכת ההפרשה:

1. NleD הוא מטלופרוטאז מוזרק שמבצע חיתוך של MAPKs בתאי המאכסן. כיצד NleD משפיע על מסלול ה-MAPK בתאי המאכסן במהלך ההדבקה?
2. לחלק מהאפקטורים המוזרקים יש השפעה מנוגדת על הישרדותם של תאים מודבקים. כיצד משולבות השפעות אלו וכיצד EPEC מווסת את השרידות של התא המאכסן במהלך ההדבקה?
3. אילו גנים של המאכסן נדרשים להדבקה יעילה על ידי EPEC?

אני מראה כאן ניתוח מולקולרי מעמיק של תפקוד האפקטור NleD וההשפעה שלו על מסלול איתות חשוב בתאים איקריוטיים – מסלול ה-MAPK. NleD הוא מטאלופרוטאז שפועל בתוך מוטיב ה-TXY של p38 ו-JNK. הראיתי שהמטאלופרוטאז הזה הוא אפקטור בעל תפקוד כפול בעל שני תפקידים לכאורה סותרים: (1) הוא מבקע באופן ספציפי את p38 ו-JNK בצורתם הלא-מזורחנת, ו-(2) הוא קושר את הפוספטאז PPM1A שיכול לבצע דה-פוספורילציה של מוטיב ה-TXY של p38 ו-JNK וכך להחזיר את הרגישות שלהם לחיתוך, אבל הקישור של NleD מעכב את פעילותו. בדרך זו EPEC יכול, בפעולה של אפקטור יחיד, גם להפעיל וגם להחליש איתות MAPK. עיכוב הפעילות הקטליטית של PPM1A מושג על ידי קשירה של NleD אשר חוסמת פיזית את הכניסה של סובסטרטים גדולים לכיס הקטליטי של הפוספטאז, אך הפעילות המובנית של PPM1A אינה מעוכבת מכיוון שהוא מסוגל לבצע דה-פוספורילציה של סובסטרטים קטנים אשר חודרים את החסימה הזו.

לאחר מכן ניתחתי גורמים המבקרים את הישרדותם של תאי מאכסן במהלך הדבקה, מה שדורש שילוב של איתות ממספר מסלולים. אני מראה ש-EPEC מפעיל מצב של שיווי משקל שבריר בין אותות המשפיעים על השרידות של תאי המאכסן. שינויים בשיווי המשקל הזה הפוגעים ביכולת של EPEC להזריק את האפקטור המגן EspZ מובילים למוות מהיר של תאי המאכסן. הגורם הספציפי שמוביל למוות של התאים המארחים לא זוהה אבל הראיתי שהוא לא אחד מהאפקטורים הידועים של EPEC. ייתכן שזהו אפקטור שטרם אופיין או

שמדובר במולקולה אחרת כגון מטבוליט שדולף לתאי המארחים דרך מערכת ההפרשה מסוג 3.

בהסתכלות רחבה יותר על תהליך ההדבקה, ביצעתי סקר CRISPR-Cas9 כלל גנומי כדי לזהות גנים בתאי המאכסן המקדמים הדבקה של EPEC. הסקר זיהה כמה עשרות גנים של תאי המאכסן המעורבים בתהליך ההדבקה. מוטציות בגנים אלה העניקו עמידות למוות שנגרם בעקבות הדבקה ב-EPEC. בין התוצאות הבולטות – גנים כמו MGAT1 ו-TMEM165 המעורבים בגליקוזילציה של חלבונים, שאני מניח שמאפשרת התקשרות ראשונית של החיידקים לתאים. גנים בולטים נוספים בסקר הם גורמים מבניים וכן וסתים של קומפלקס Arp-2/3. קומפלקס זה אחראי על הדינמיקה של שלד האקטין בתאי המאכסן והוא דרוש לתהליכים כמו נדידת תאים ופגוציטוזיס. תפקידו של קומפלקס Arp-2/3 במהלך ההדבקה ב-EPEC טרם התגלה במלואו, ובהתבסס על עבודות קודמות אני משער כי פולימריזציה של אקטין, המווסתת על ידי קומפלקס זה, נדרשת להזרקה של אפקטורים לתאי המאכסן.

בסך הכל, המחקר שלי מדגיש את דרכי הפעולה המורכבות של אפקטורים של מערכת ההפרשה מסוג שלוש ואת המורכבות של האינטראקציה בין הפתוגן לבין המאכסן במהלך הדבקה ומנתח מספר היבטים של תהליך זה. ביצעתי ניתוח יסודי של המנגנון שבאמצעותו EPEC שולט במסלול איתות חשוב ביותר במאכסן – מסלול ה-MAPK. באופן רוחבי יותר, הסתכלתי על האופן שבו EPEC מבקר את השרידות של תאי המאכסן במהלך ההדבקה – תוצאה של אינטגרציה של מספר מסלולי איתות. לבסוף, ביצעתי סקר כלל גנומי כדי לזהות גנים של המאכסן המעורבים בכל שלב של ההדבקה. כך תרמתי את חלקי להבנה טובה יותר של התהליכים העומדים בבסיס ההדבקה, הבנה שיכולה להוביל לפיתוח כלים חדשים למניעה וטיפול במחלות קשות הנגרמות על ידי פתוגנים חיידקיים.

עבודה זו נעשתה בהדרכתו של

פרופסור אילן רוזנשיין

השפעת החיידק Enteropathogenic *E. coli* על תאי המאכסן בעזרת אפקטורים של מערכת ההפרשה מסוג שלוש

חיבור לשם קבלת תואר דוקטור לפילוסופיה

מאת

יעקוב סוקול

הוגש לסנט האוניברסיטה העברית בירושלים

דצמבר 2022