Role of *Salmonella enterica* Typhimurium secreted effector Protein, SteA in Host Cell Infection

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Certificate of Examination

This is to certify that the dissertation titled “Role of *Salmonella enterica* Typhimurium secreted effector Protein, SteA in Host Cell Infection” submitted by Mr. Shivansh, registration no MS15198 for the partial fulfilment of BS-MS dual degree programme of the institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommend that the report is accepted.

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Dated: 3rd May, 2020

Declaration

The work presented in the dissertation has been carried out by me under the guidance of Dr. Arunika Mukopadhaya at the Indian institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate it clearly, with due acknowledgement of collaborative research and discussions. This thesis is bonafide record of original work done by me and all sources listed within have been detailed in the references.

Shivansh

Dated: May 4, 2020

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Arunika Mukopadhaya

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List of Figures

Figure	page no	legend title
1	10	SPI-1 mediated processes in S. Typhimurium
2	13	SPI-2 mediated processes in S. Typhimurium
3	14	modulation of Immune responses upon SteA-Cullin interaction
4	15	modulation of Immune responses upon SteA-Cullin interaction
5	20	Bacterial invasions in WT and ∆SteA
6	21	Cytotoxicity% in WT and ∆SteA at 16 hours post infection
7	21	Cytotoxicity% in WT and ∆SteA at 24 hours post infection
8	22	Conclusive comparision of cytotoxicity at 16 hours and 24 hours
9	23	Flow cytometric analysis of cell cycle

Notations

T3SS	Type-3 secretion system
SCF complex	Skp, Cullin, F-box containing complex
SteA	Salmonella translocated effector A
∆SteA	SteA deletion mutant of Wild Type bacteria
WT	Wild Type strain of bacterium (*S*. Typhimurium SL1344)
WRC	WAVE Regulatory Complex
N-WASP	Wiskott-Aldrich Syndrome protein
GEF	Guanine nucleotide Exchange Factors

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Abstract

Salmonella enterica Typhimurium is a gram-negative bacterium that enters the body through contaminated food and water and causes salmonellosis to more than 550 million people each year. S. Typhimurium enters the gut where it interacts with epithelial cells and enters inside it in an endocytic fashion but it prevents its digestion by lysosomes and lives inside the vacuole. In the *Salmonella* containing vacuole (SCV), it multiplies feeding on the host nutrients and eventually invades into other epithelial cells as well as macrophages in the peyer's patches which are just beneath the epithelial cells. *S*. Typhimurium is able to do so with the help of effectors regulated by Salmonella pathogenicity island I (SPI-1) and SPI-2. Type three Secretion System-1 (T3SS-1) and Type three Secretion System-2 (T3SS-2) translocate the effector proteins essential for its virulence, directly into host cell and has acquired such machinery through horizontal gene transfer through its course of evolution. Throughout its life inside the cell, *S.* Typhimurium uses the endocytic vacuole SCV that essentially help it to survive and replicate within the host cell while avoiding exposure and to live undetected. SteA is an effector molecule secreted by both T3SS-1 and T3SS-2 and is shown to be crucial for suppression of host's innate immune responses in SPI-1 condition. Correlating with the work already done on the role of this effector molecule in SPI-1 conditions, we aim to see how it modulates host immune responses in SPI-2 conditions and affects cytotoxicity upon infection in healthy cells. In this study towards exploring whether SteA plays any role on host cell death we observed that SteA promotes cytotoxicity in macrophages.

TABLE OF CONTENTS

List of figures 3

Notations 4

Acknowledgement 5

Abstract 6

1. Introduction 8
2. Salmonella’s invasion of cells 8-9
3. T3SS, SPI-1 and SPI-2 effects 9-14
4. Work already done so far 14-15
5. Materials and Methods 16-19
6. Results 20-23
7. Discussion 23-24
8. References 24-28

INTRODUCTION

Salmonellae are motile, gram-negative bacteria that are known to be responsible for causing enteric diseases in a broad spectrum of animals. *Salmonella enterica* consists over 2,500 serovars and among those, *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium), are pathogens that cause acute self-limiting gastroenteritis in humans and many other animals. In severe cases, it can cause systemic infection in immunosuppressed hosts, in very young, older individuals, occasionally in healthy adult humans and animals (1).

Salmonella Typhimurium enters human body upon intake of contaminated food or water then it moves to the stomach where it survives gastric acidity and moves to the intestinal epithelium. Upon bacterial entry into epithelium, it results into induction of inflammation in the intestinal epithelium which typically results in the infiltration of neutrophils and accumulation of fluid into the intestinal lumen which reportedly results in inflammatory diarrhoea (2). Due to inflammation, cells release reactive oxygen species into the lumen which then reacts with thiosulfate (a respiratory by-product generated by the microbiota and the intestinal lumen) to generate tetrathionate which turnout to be essential for the bacteria as a nutritional source and provide the pathogen nutritional advantage over the other intestinal microbiota (3).

Salmonella's Invasion of Cells

S. Typhimurium is reported to invade through basolateral surface of the epithelial cells. *S*. Typhimurium in the intestinal epithelial layer interacts with Microfold cells (M cells), which actively transcytoses the bacteria through the epithelial barrier and gain access to the basolateral surface as well as intestinal lymphoid tissue known as Peyer's patches (4).

It has been widely reported that the bacterium can invade and also survive in wide range of mammalian cells which also includes the immune warrior and antigen presenting cell such as macrophages but is rapidly cleared by the specialised phagocytes called neutrophils (5,6). To avoid neutrophils, S. Typhimurium has adapted intracellular lifestyle. It lives within intestinal epithelial cells and macrophages which is essential for avoiding neutrophil mediated killing and for its pathogenesis. Intracellular lifestyle here also denotes to the lifestyle that aims to recognise and resist the components of the hosts innate immune system and the acidic pH of the phagocytic vacuole. When salmonellae recognise the mediators or inducers of host innate immunity, it transcriptionally activates the necessary genes important for remodelling the bacterial cell surface by which it eventually avoids the host innate immune system and facilitates it's intracellular survival (7). To respond timely in response to such

environmental changes, it is essential to sense the intracellular environment and only after that it can initiate the remodelling of its membrane. For this purpose *Salmonella* Typhimurium is reported to be dependent on regulatory proteins such as PhoP-PhoQ, OmpR-EnvZ, PmrAPmrB, RcsB-RcsC, and Cya-Crp (8,9). Upon sensing the intracellular environment by regulatory proteins, to initiate changes such as remodelling of host system and remodelling itself, it depends on Type-3 secretion system (T3SS) which is reportedly encoded on the Salmonella pathogenicity islands (SPI) 1 and 2, which efficiently transport effector proteins into the host cell cytoplasm and leads to invasion and intracellular survival of S. Typhimurium (10).

T3SS

T3SS utilize some conserved genes to assemble a nano-syringe capable enough to transverse through plasma membranes, peptidoglycan layer and extracellular space that act as barrier for the direct delivery of proteins from the bacterium to the host. Upon interaction with the host cells, SPI-1-mediated T3SS transports effector proteins directly through the nano-syringe like structure (also called the injectosome), which penetrates the protective host cell membrane to enable bacterial invasion. Such invasion into the host cell is also capable enough to induce inflammatory responses. The endocytic vacuole in which salmonella enters and survive is referred to as *Salmonella* containing vacuole (SCV) (9). SPI-2-mediated T3SS transports effector proteins that are essential for the intracellular survival, multiplication and the movement of SCV across the membrane of the endocytic vacuole. SCV helps the pathogen in avoiding the defence mechanism of host cell which can be triggered upon direct contact with the bacteria or bacterial components. It has also been shown that many effector proteins translocated through the SPI-1 T3SS, remains within the host's cells even when the bacterium is internalised and such effectors are important for the intracellular survival of bacteria inside the SCV and modulation of host proteins (11–16).

SPI-1 and Invasion

When the bacteria interact with the host cell and senses the anaerobic environment, basic pH and high osmolarity inside the gut (46), it induces T3SS-1 mediated release

of effector proteins or molecules such as SopB, SpvD, SteA, SopE, SptP, SipA, SipC which modulates the host machinery to facilitate the entry of S. Typhimurium into the host cell enclosed in the SCV. The effector molecules released through T3SS-1 are known to perform different functions and some of them have overlapping functions which ensure invasion. Among these effector molecules some are essential for the virulence of bacterium and one of those is SopB, functioning of this effector is responsible for the recruitment of RAB5 and annexinA2 which account for recruitment of host ingestion machinery at the surface of cell wall and functions as a platform for actin rearrangements (11,12). SopB also influences the ability of the bacterium to transform follicular associated epithelial cells into M-cells by activating Wnt/β-catenin signalling. Activation of Wnt signalling results in the activation of the receptor-activator of NF-κB ligand (RANKL) and its receptor RANK, which initiate M-cell development and promote invasion. (13)

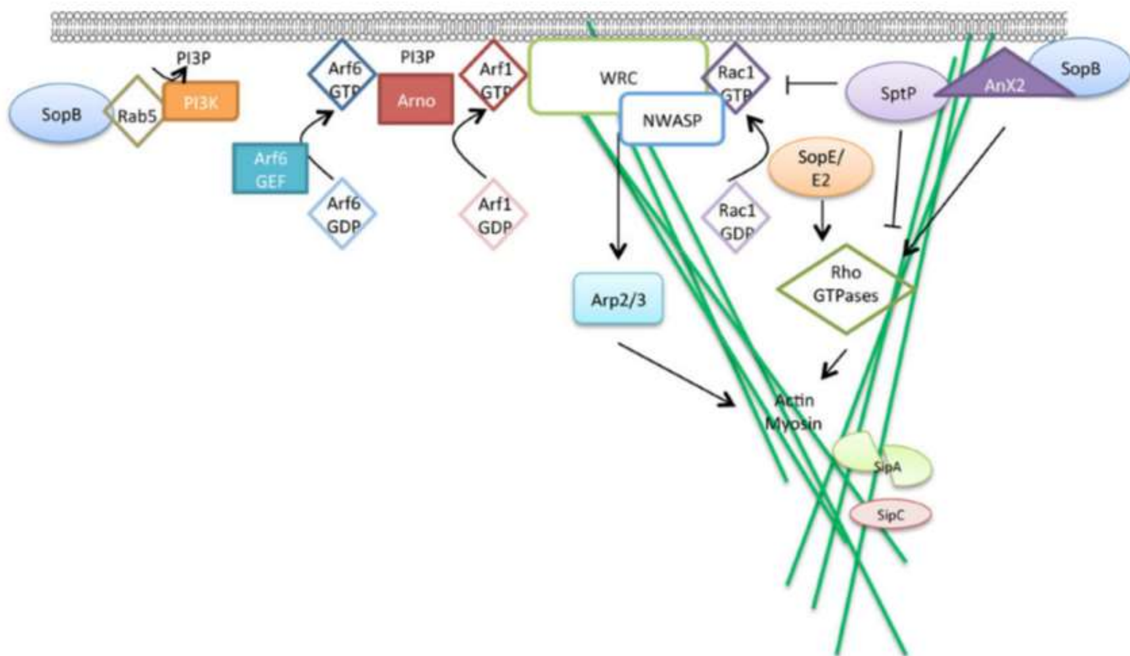


Figure 1 Diagrammatic representation of **SPI-1 mediated processes in S. Typhimurium** highlighting important steps followed by SCV and bacterium to promote its virulence. (adapted from Doris L. LaRock et al 2015, Nature Reviews)

SipA and SipC are also involved in bacterial internalisation as they bind directly with actin at the site of insertion of the T3SS translocon, of which SipC is a component. SipA is known to inhibit actin depolymerization and also for increasing actin bundling at entry point of the bacteria (14,15). SipC is necessary as it bundles and nucleates actin upon translocon insertion for invasion (16,17). As soon as the bacteria enters the host cells, the cell architecture needs to be restored and that is done by another such effector known as SptP which reverses the activation of Rac1 and Cdc42 by SopE,

SopE2 and SopB, restoring the architecture of epithelial cells (18). SopE, due to its GEF activity, recruits WRC and N-WASP and as a result of this activity, it leads to the activation of an actin related protein Arp2/3 at the membranous end of the cell. As a result of actin related protein Arp2/3 activation, further actin polymerisation and membrane ruffling takes place which is essential for the bacterial uptake inside a phagosome into the epithelial cell (48). Following all of these essential processes, the bacteria is finally internalised. During the invasion the host defence system gets activated which aims to eliminate S. Typhimurium. In reaction to bacteria the host cells produce localised inflammation which attracts components of host immune system to clear out the foreign antigens and protect the host. In the next section we will briefly discuss how salmonella survives the host's defence.

SPI-1 and Inflammation

Inflammation of the intestinal tract is an essential step as it provides a growth advantage for S. Typhimurium, which is necessary for invading cells and for inflammation that enhances bacterial transmission through multiple mechanisms. Many of the SPI-1 effectors including SopE, SopE2, SopB, SipA, SipC and SopA contribute to intestinal inflammation by inducing the synthesis of proinflammatory cytokines such as IL-8 through the mitogen-activated protein kinase (MAPK) and NF-κB pathways while destabilizing the tight junctions (19). Toll-like receptors (TLRs) present on the host cell are activated when they encounter the salmonellae components which can be lipopolysaccharide (LPS) leading to macrophage activation and also leading to upregulated phagocytosis. Eventually such encounters with TLR's leads to transcriptional activation of inflammatory caspase genes (20). Caspase-1 is reportedly activated in macrophages by flagellar filament protein FliC and PrgJ, which is a rod protein (21). SopE, due to its GEF activity for the Rho GTPases Rac and Cdc42 activates caspase-1 in stromal cells leading to upregulation of inflammation upon infection (22). With all these events leading to heightened inflammatory response against the bacteria we also see some effectors that are reported to have the ability to supress the inflammation and promote the survival of bacterium. *S*. Typhimurium have such inflammation suppressors like tyrosine phosphatase SptP, which reverses the MAPK-mediated inflammation and reportedly promotes IL-8 secretion (23). The SPI-1 mediated effector AvrA and the SPI-1/2 effector SspH1 have been shown to downregulate invasion induced by IL-8 production by epithelial cells and also inhibits NF-κB activity (24,25). SteA which is also an effector protein secreted by both SPI-1 and SPI-2 have been shown to upregulate gene expression of genes that regulate ECM organization, cell proliferation and serine/threonine kinase signalling pathways it has shown to be essential for the downregulation of genes involved in regulating immune processes (26).SteA has also been shown to supress the immune responses of the host by supressing the degradation of IκB which is an inhibitor of NF-κB. SteA binds to Cullin-1 which is an essential component of IκB ubiquitinating complex and eventually decreases the inflammatory response by NF-κB pathway.

SPI-2 induced Effects

Once the bacteria have invaded into the cell in an endocytic vacuole called the SCV, the environment has now changed from the alkaline pH to acidic pH of the endocytic vacuole and concentration of phosphate ions is now reduced. The basic aim of SPI-2 is to promote survival of bacteria and help the bacteria to replicate and disseminate eventually to infect other cells. In order to do so It initiates SPI-2 encoded T3SS which secretes effector proteins that function to achieve its aim. The essential proteins that confers its virulence were experimentally determined and they are sifA, spvB, sseF, sseJ and SteA (27). Among these effectors the most studied effector is sifA which is shown to be crucial for the formation of Salmonella induced filaments (28). The SCV following the endosomal pathway matures and maturation of the SCV denotes transient interactions with early endosomes, as a result of which it recruits early endosomal markers. The early endosomal markers are known to be transferrin receptor, early endosomal antigen-1 and the small GTPase Rab5 (29). Slowely, the early endosomal markers are replaced with late endosomal markers and lysosomal markers that includes lysosome-associated membrane proteins (LAMPs), the GTPase Rab7, RILP and mannose-6-phosphate receptor (MPR) (30,31). Rab7-interacting lysosomal protein (RILP) is shown to link active Rab7 to the motor protein dynein which makes a complex that is important for the centripetal movement of the SCV at earlier stages of infection (45). Among the effector proteins secreted by SPI-2, SseJ localise onto the SCV to activate its glycerophospholipid cholesterol acyltransferase activity by binding to Rhoa and modifies the composition of cholesterol in the membrane such that it directly increases the concentration of cholesterol esters on membrane and affect the proteins that associate with the SCV (32) by this it keeps the host proteins like endosomal markers away from SCV. Effector protein SifA, when recruited at the surface of SCV, it is reported to connect the SCV to the microtubular network (33) and also recruits Rab7 to displace the Rab7-RILP-dynein motor complex essential for the peripheral movement of the SCV. Kinesin, which is microtubular protein is shown to induce extension of the endosomal tubules with the help of PipB2 and the host Arf GTPase Arl8B (34). SifA-SKIP (SKI- interacting Protein) complex is shown to sequester Rab9 which blocks Rab9 dependent trafficking of mannose-6-phosphate receptors. The mannose-6-phosphate receptor trafficking is essential for the lysosomal activity and hence the SCV is able to avoid lysosomal degradation (35). Through the microtubular network, SCV moves to the perinuclear region of non-polarized host depending on SPI-2 effectors (36). Towards the later stage of infection, the SCV moves to the peripheral regions to disseminate and infect other healthy cells (42). Other SPI-2 effectors, which help in the movement of SCV are PipB2 and SseF by interacting with microtubule proteins (37). SspH2 is another such protein that localize to the SCV and aid in the removal of host proteins through ubiquitination. SPI-2 effector, SteA has been shown to control the dynamics of the SCV (38) along with other effectors SifA,

PipB2, SseF, SseG, SseJ, SpvB, and SopD2 contribute to ET dynamics and SCV movement (39, 40, 41).

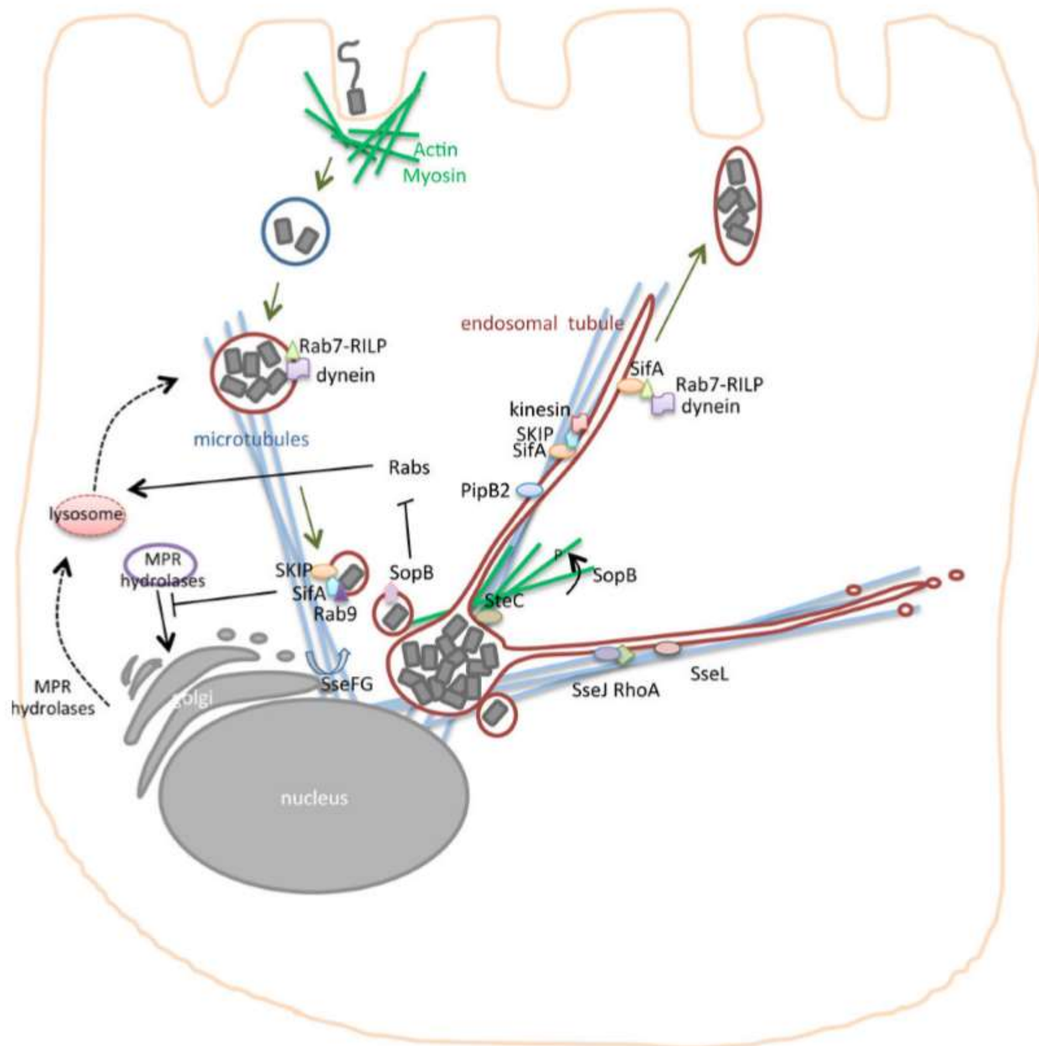


Figure 2 Diagrammatic representation of **SPI-2 mediated processes in S. Typhimurium** highlighting important steps followed by SCV and bacterium to promote its virulence. (adapted from Doris L. LaRock et al 2015, Nature Reviews)

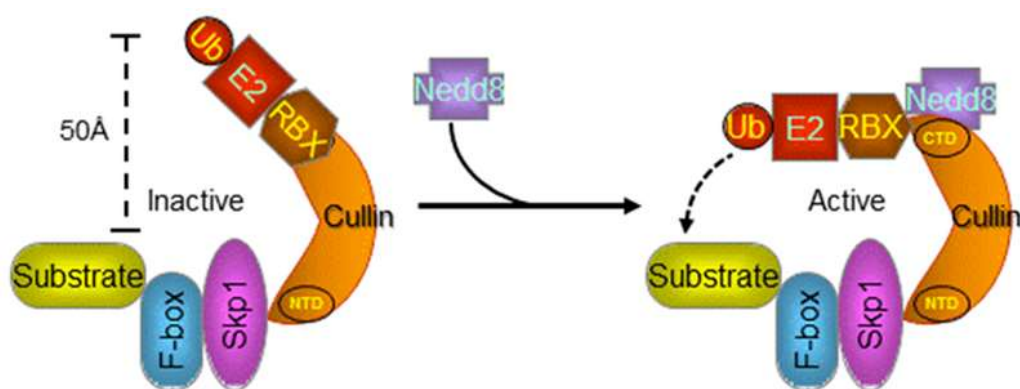
In this study we have focused on one of these effector protein SteA and now we will elaborately discuss about it in next section where we will also discuss and correlate with the work already done on this effector protein.

WORK ALREADY DONE SO FAR

So far in our laboratory itself we have shown that SteA an essential effector molecule for the virulence of *S*. Typhimurium binds to Cullin-1 and modulates immune responses in the host cells in SPI-1 mediated conditions (46).

Background and Hypothesis

Cullin-1 is an important and integral component of SCF E3 ubiquitin ligases which is a complex of proteins essential for the regulation of cell proliferation, apoptosis and degradation of many cellular proteins which are cell cycle regulators and transcription factors (45). Upon Interaction of SteA with Cullin-1, the function of Cullin-1 gets compromised. As Cullin-1 is shown to be involved in cell cycle regulation and it is also shown to regulate cell proliferation (43), if the cullin-1 function is compromised there is ample chance that host-cell survival can be jeopardized. Role of Cullin-1 is to activate the ubiquitin activity of SCF E3 complex upon attachment of NEDD-8 to Cullin-1 which function to bring the complex in close proximity with the ubiquitinating enzymes present in the complex and the process is called Neddylation (Figure 3). In the case when SteA is bound to Cullin-1, there is no room for NEDD-8 to bind to the Cullin-1 which was otherwise necessary to ubiquitinate and degrade IκB which is an inhibitor of NF-κB and hence very critical for modulating immune response in host cell (46).



http://mpmp.huji.ac.il/maps/SCF.html

Figure 3 Diagramatic representation of Neddylation (adapted from Malaria Parasite Metabolic Pathways, http://mpmp.huji.ac.il/maps/SCF.html)

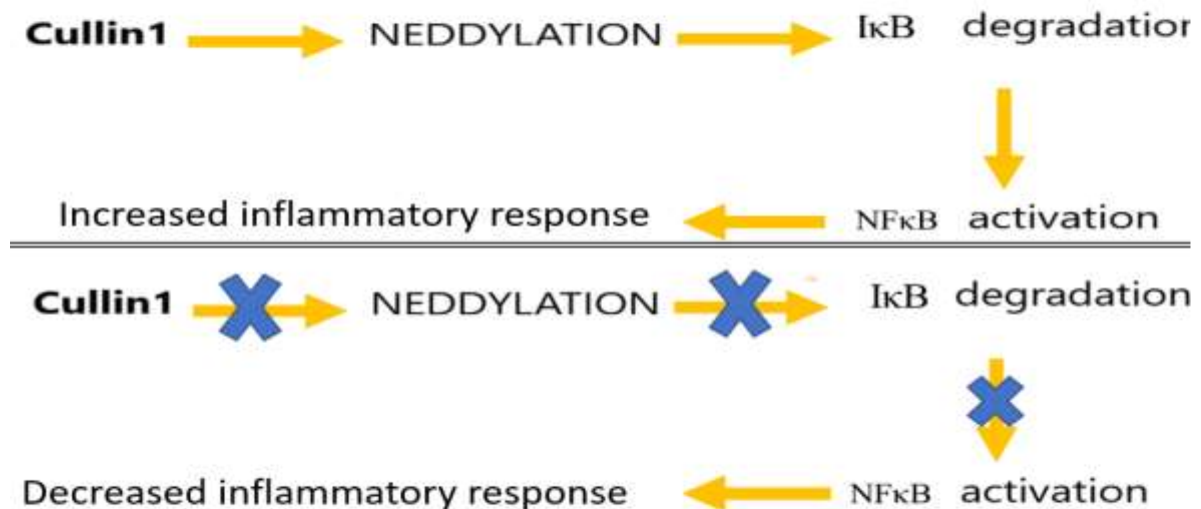


Figure 4 Diagrammatic representation of **modulation of host's Immune responses upon SteA-Cullin-1 interaction** (46).

Further, Cullin-1 regulation of cell-cycle progression have been reported to be due to increased expression of p21 and p27, and decreased expression of cyclin D1 and cyclin E (43). After Cullin-1 knockdown, cells had significantly higher G1 population than control cells in both the cell lines using fluorescence activated cell sorting which suggested cell cycle arrest (43). Cell cycle arrest is known to stimulate different cascade of processes to facilitate the removal of arrest and if not possible, such cells are eliminated through programmed cell death. Interestingly, SteA released by SPI-2 mediated machinery has been reported to be associated with T3SS-1 independent cytotoxicity in macrophages (27).

Based on this background our hypothesis was *S*. Typhimurium effector protein SteA can induce host cell death.

MATERIALS AND METHODS

Bacterial strains and Culture Conditions

Salmonella enterica typhimurium SL1344 (WT) is the bacteria used in this study and ΔSteA (SteA deletion mutant of S. typhimurium SL1344) had been derived from the same strain of bacteria (46) replacing the *steA* gene with kanamycin cassette by one step inactivation method following the protocol by Datsenko and Warner (46).

Since SteA is produced in both SPI-1 and SPI-2 mediated conditions. As our previous observation suggested that in SPI-1 condition SteA does not affect have much effect on host cell death, we wanted to explore what happens in the SPI-2 condition. To ensure that we are analysing the SPI-2-mediated effects only, we provide external environment that induces SPI-2 mediated release of effector proteins which are different in both the environments and which also serves as cue for the induction of either of the machinery.

SPI-2 INDUCING CONDITIONS

To induce SPI-2 machinery, we have grown the bacteria on LB culture and after we have bacteria in LB culture, bacteria were washed from LB culture and suspended in low phosphate, low magnesium minimal media (LPM) culture at a pH of 5.8 for overnight incubation.

LPM constituents:

0.5 mM K2SO4

5 mM KCL

0.1% casamino acids

7.5 mM (NH4)2SO4

80 mM 2-(N-morpholino) ethane sulfonic acid (pH 5.8)

38 mM glycerol

Cell Lines and Culture Conditions

RAW 264.7, a murine macrophage cell line is used in this study and the cells were maintained at 37°C and 5% CO_2 in RPMI medium supplemented with 10% FBS.

Infection of Cells

Bacteria was inoculated in LB medium and was grown overnight in an incubator at 37°C and 5% CO_2. After that the culture was centrifuged at 10000 rpm for 3 minutes and supernatant was removed and the bacterial pellet was suspended into 1ml of LPM medium and left to adjust to new medium overnight at same conditions so that it senses the SPI-2 mimicking conditions. Next day the bacteria in LPM medium was centrifuged at 10000 Rotations Per Minute (RPM) for 3 minutes and the supernatant was carefully removed and the bacterial pellet was dissolved in 1ml PBS and then with the help of spectrophotometer the optical density denoting the density of bacteria in the solution was adjusted to 1 to ensure equal number of bacteria in all the samples. RAW 264.7 cells plated at a density of 0.5 Million cells was infected with a MOI of 50:1 i.e. 50 bacteria invading a single cell. After 30 minutes of infection the media rich in bacteria was removed and fresh media containing 100µg of gentamycin was provided to the cells for a period of 1 hour and after that fresh media containing 20µg gentamycin was provided to the cells for the rest of the experiment. To ensure equal invasion of bacteria in all the samples subject to comparison we lysed the cells with 0.1% Triton-X-100 in PBS after two hours of infection and plated on LB agar plates and then the number of bacterial colonies were counted and compared and only when the number of colonies was similar we analysed the samples.

Invasion estimation

To ensure equal invasion of bacteria in all the samples subject to comparison, we lysed the cells (Raw 264.7) after two hours post infection with 0.1% Triton-X-100 in PBS and plated on LB agar plates so the all the bacteria that has invaded the cells would come out of cells after the cells are lysed and we then collected the solution. After collecting the solution, we diluted the bacteria with PBS (Phosphate Buffer Saline) four times while diluting the bacterial concentration 10 times in each. After diluting, we plated the bacteria suspended in PBS media on LB Agar plates and left the plates in incubator overnight. Next day the number of bacterial colonies grown on LB Agar plates were enumerated. No. of bacterial colonies grown were multiplied by 10^4 as we diluted the bacterial load 10^4 times before plating.

Lactate dehydrogenase (LDH) release assay for quantifying cell cytotoxicity

Lactate dehydrogenase is an enzyme that is involved in energy production in nearly all living cells as it catalyses the conversion of lactate to pyruvate which makes it important in cellular respiration, a process by which food (glucose) is converted to usable energy for our cells. Upon tissue damage or damage to cell, it is released to cell surrounding. Due to its high stability it serves as a reliable marker for assessing cell damage. So, to evaluate the cytotoxicity levels, RAW 264.7 (0.5 $x10^6$) cells were infected with wild type and ∆steA strain (MOI 50:1) under SPI-2-induced condition. LDH release by the cell into the surrounding medium was detected and quantified using LDH release assay. To do the assay, culture media from the infected (RAW264.7) cells were isolated at 16 h and 24 h post infection. The media were the centrifuged at 3000 rpm for 3 minutes and supernatants were collected. As a positive control RAW 264.7 cells were lysed to ensure 100% cell death. To estimate the cell cytotoxicity yellow coloured tetrazolium salt was added and incubated for 10-15 minutes at room temperature till the wine-red colour of the formazan salt was developed. The NADH produced by LDH during conversion of pyruvate to lactate reduces the tetrazolium and converts it into formazan salt. Optical density was determined at a wavelength of 490 nm using spectrophotometer and the results were analysed with the principle formula:

$$\%cytotoxicity = \ [(\ Test - Blank) \div (positive\ control - blank)] * 100$$

Cell Cycle Analysis

After the infection, when the invasion of bacteria was almost equal, we centrifuged the solution containing infected RAW 264.7 cells at 3000 rpm and discarded the supernatant while dissolving the pellet in PBS and then after a PBS wash, we fixed the cells with chilled 70% ethanol for 10 minutes and washed with PBS twice. After washing with PBS, cells were suspended in binding buffer, RNAse and Propidium Iodide as DNA staining dye and incubate the mixture on ice for 15 minutes before the samples were analysed in BD Accuri Flow Cytometer and the cell Cycle analysis was done in FlowJo X.

Statistical Analysis

Data was analysed in Microsoft Excel, we calculated standard error in our data using the formula:

$$\text{Standard error} = \frac{Standard\ Deviation}{\sqrt{n}}$$

Where n = number of observations

Results

Cell cytotoxicity was downregulated in ∆steA in RAW 264.7 cells

To understand the effect of SteA deletion on cell health, we infected the RAW 264.7 macrophages with WT and ∆steA and observed whether there is any difference in cytotoxicity. To have an estimate of cell cytotoxicity caused by both types of bacterial strains i.e. Wild Type and ∆steA, we kept uninfected cells as negative control and Triton-X 100 treated cells (Triton-X is a strong detergent that capable enough to lyse all the cells) as positive control which shows 100% cytotoxicity. Upon infection, at first we checked whether there is equal invasion of both the bacterial strains after 2 h of infection (Figure 5). Further, wiith equal bacterial invasion in each sample, we checked cell at death in terms of cell cytotoxicity at two different time at 16 hours post infection and at 24 hours post infection (Figure 6-8). We observed a decrease in cell cytotoxicity in ∆steA in comparison to Wild Type in both the time points, further we repeated the experiment more times to confirm the trend.

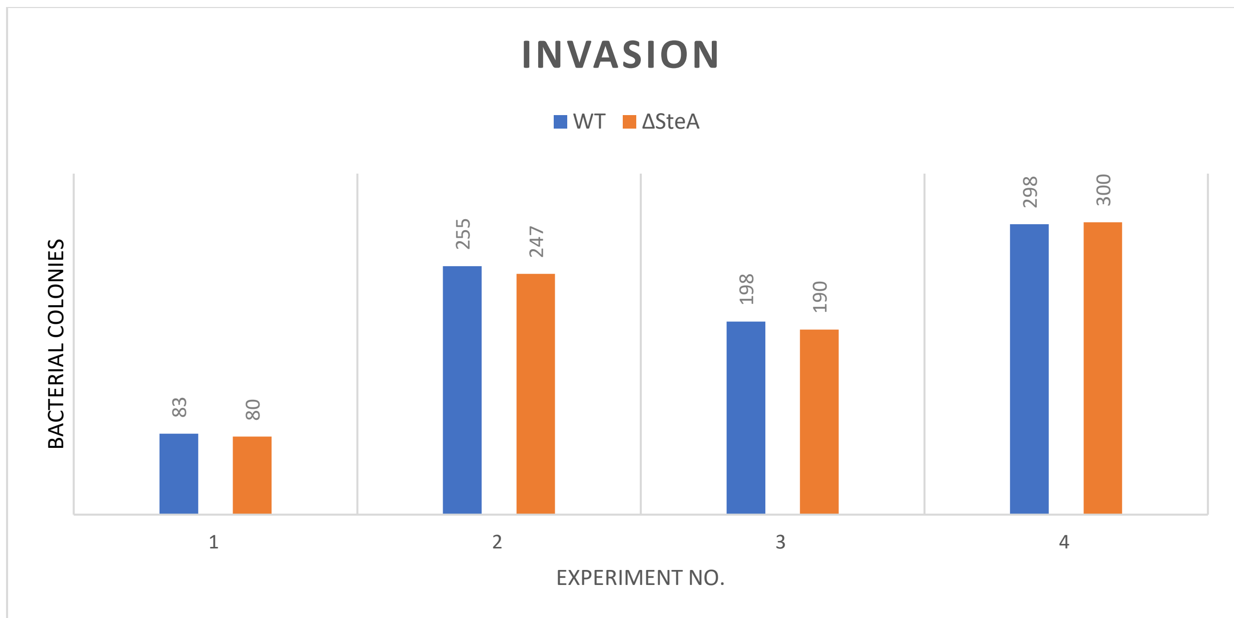


Figure 5 The above shown graph represents the number of bacterial colonies grown on LB agar plate which representing the **invasion of bacteria in WT and ΔsteA infected cells 2 h post infection.** The results of the four different experiments are shown individually and numbered as1,2,3 and 4. Only after almost equal invasion was observed further experiments were done and considered.

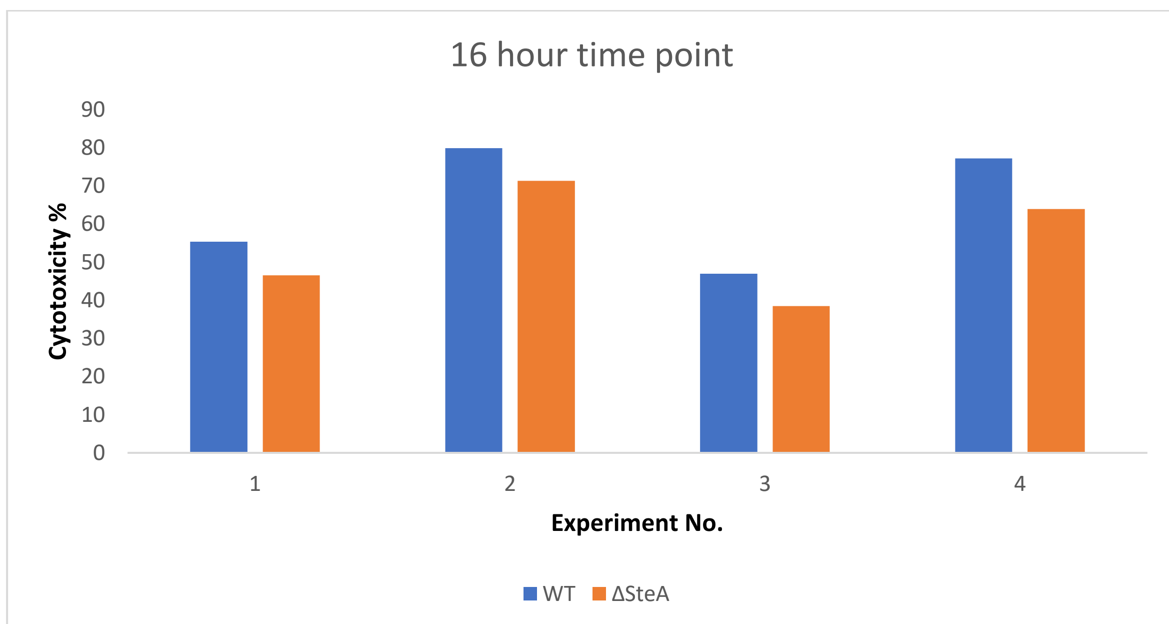


Figure 6 Bar graph represents cell cytotoxicity induced in presence and absence of SteA. Data represents **% cytotoxicity** on **Y** axis and experiment numbers on **X** axis. Cytotoxicity was determined using LDH release at 16 hours post infection **wild type and ΔsteA S. Typhimurium strain**.

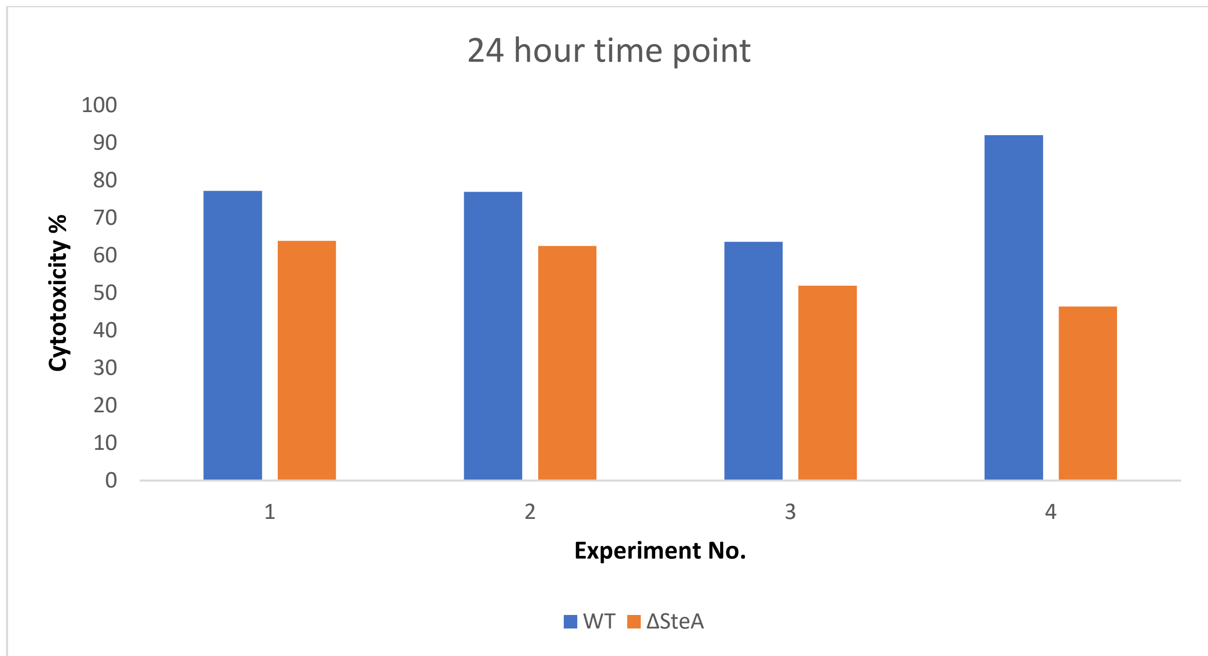


Figure 7 Bar graph represents cell cytotoxicity induced in presence and absence of SteA. Data represents **% cytotoxicity** on **Y** axis and experiment numbers on **X** axis. Cytotoxicity was determined using LDH release at 24 hours post infection **wild type and ∆steA S. Typhimurium strain**

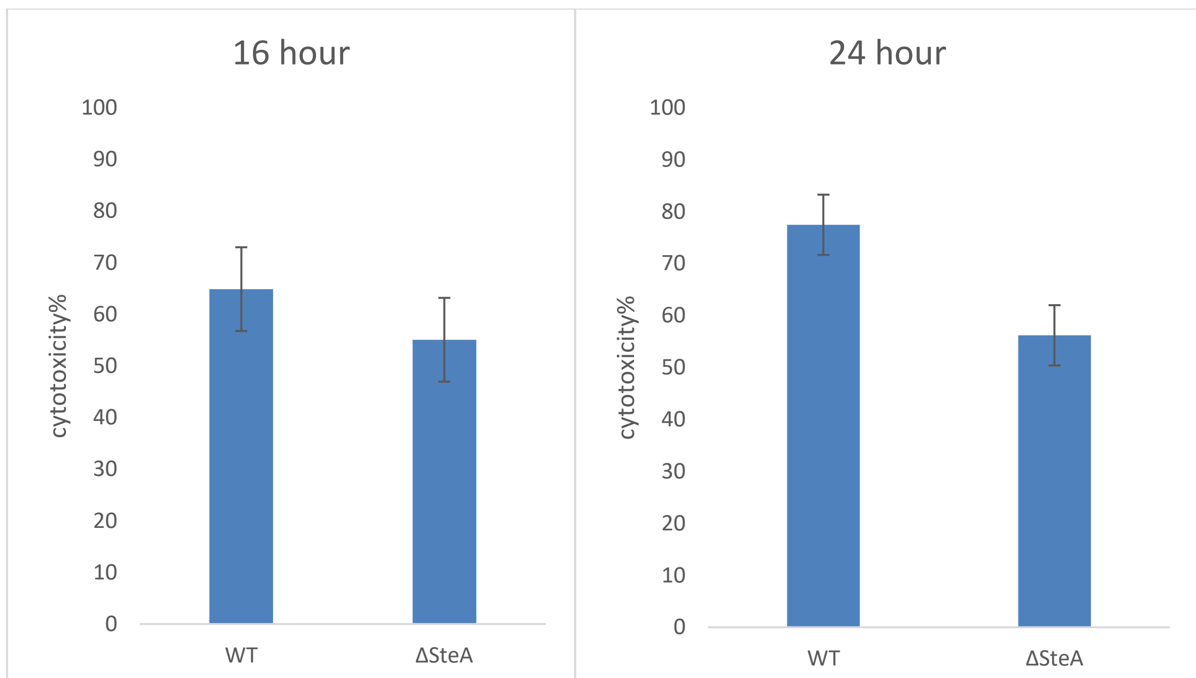
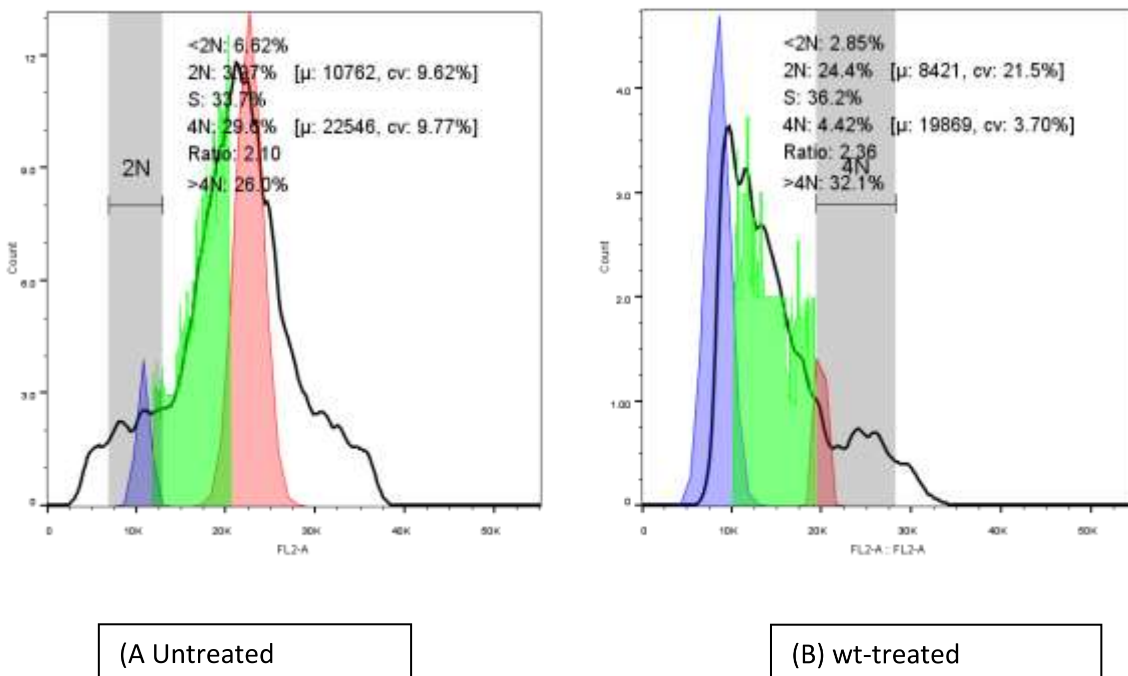


Figure 8 Bar graph represents cell cytotoxicity induced in presence and absence of SteA. Data represents **% cytotoxicity** on **Y** axis and experiment numbers on **X** axis. Cytotoxicity was determined using LDH release at 16 h and 24 h post infection **wild type and ∆steA S. Typhimurium strain**

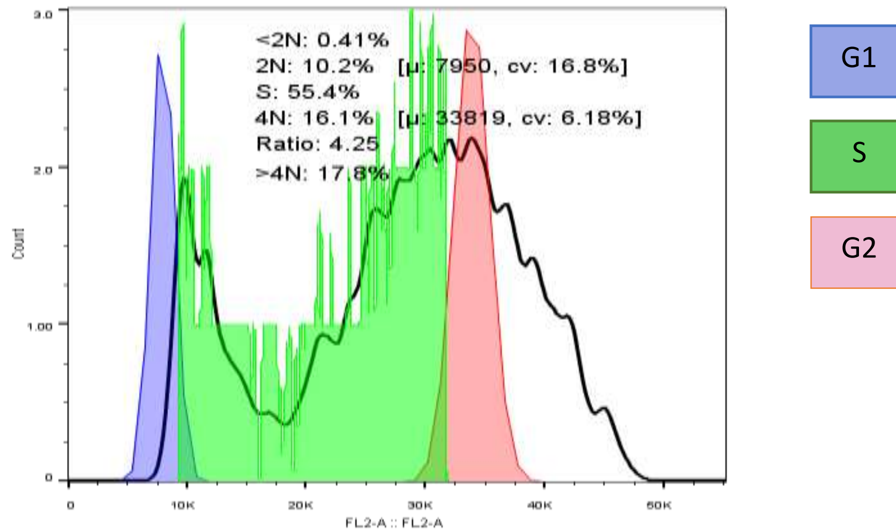
SteA affects cell cycle progression

After observing that there is a decrease in cell cytotoxicity in absence of SteA, we suspected that this could be due to inactivity of Cullin-1 upon interaction with SteA. As literature suggested that Cullin-1 functioning is essential for cell cycle progression, therefore, we proceeded to check the cell cycle in cells infected with wt and ∆steA. Towards this, we fixed the cells and stained the DNA with PI (Propidium Iodide, which is a DNA staining dye) and analysed the cell cycle status by flow cytometer and FlowJo. This study still needs to be repeated and completed but we have preliminary results that suggested that in case of wt infection, most of the cells are in the G1 phase of the cell cycle showing an arrest in the cell cycle progression after the G1 phase, whereas, in case of ∆steA infection, the G1 and G2 peaks are clearly visible with most of cells in S phase of cell cycle, suggesting cell cycle progression without any arrest (Figure 9). This indicated that cell cycle is getting affected in wt infection because of the presence of SteA.



(A Untreated

(B) wt-treated



G1

S

G2

(C) cells treated with ∆steA

Figure 9. Cell cycle analysis showing more population of cells in the G1 phase following infection with wild type *S.* Typhimurium suggesting cell cycle arrest. The above represented data shows **flow cytometric analysis of cell cycle** where cells are in different phases of cell cycle. the blue coloured peak represents cells in G1 phase of cell cycle, the cells in the S phase are shown as green coloured peak and G2 phase shown as pink coloured peak. Untreated cells (A) Wild type treated cells (B) and ∆steA treated cells (C).

Discussion

According to WHO, every year there are 550 million people falling ill and about 220 million children under the age of 5 years getting infected from *S.* Typhimurium*.* In the quest of decoding the mechanisms enteric bacterial infection machinery, we tried to explore the unexplored areas about the pathogenesis of S. Typhimurium. We explored the function of an SPI-2 mediated effector protein SteA towards influence on cell viability during the pathogenesis of *S.* Typhimurium. Our study suggested that absence of this effector molecule from the S. Typhimurium in SPI-2 environment resulted in decreased cytotoxicity in comparison to the wild type strain of the bacteria. As the infection persists, difference in cytotoxicity is increased, indicating that SteA is an essential effector molecule in pathogenesis. SteA can directly affects cell health and increases cytotoxicity in RAW 264.7 macrophages. Following this observation, we explored how SteA cytotoxicity. Towards, it we recalled the work done by Dr. Aakanksha Gulati in the lab, where she showed that SteA interacts with Cullin-1 to suppress host's innate immune response. Cullin-1 is an essential SCF E3 ligase component (47). Further, Cullin-1 has been reported to influence cell cycle and thus regulate cell proliferation. Considering that, we then went explored the possibility that interaction of SteA could affect cell cycle In our attempt to probe this we got a

preliminary idea that most of the cells in case of wild type bacterial infection were in G1phase of cell cycle while very few in other phases whereas in ∆steA, most of the cells were in S phase and G2 phase and very few in G1 phase suggesting progression of cell cycle. Which indicates that there is a cell cycle arrest mainly in the G1 phase when SteA is present and there is progression of the cell cycle in absence of SteA. This result corroborates with our idea that SteA might affect the cell cycle progression, cell proliferation, hence cytotoxicity by interacting with Cullin-1, as the literature suggested Cullin-1 compromised cells should have higher G1 population of cells (43). Further, work is needed to prove the hypothesis generated from our preliminary data. We now have indication to proceed research on this aspect to check whether the interaction of SteA with Cullin-1 is affecting cell proliferation and thereby increasing cell cytotoxicity. Further we can check the Caspase activity (ubiquitinating enzyme deployed by cell mechanisms to kill itself through various fashions) to see if the cells are entering programmed cell death in response to cell cycle arrest.

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