Characterization of Cell death responses induced by *Salmonella enetrica* Typhimurium Effector protein SteA

Celina Meena
(MS16014)

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Supervisor- Dr. Arunika Mukhopadhaya

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Certificate of Examination

This is to certify that the dissertation titled "Characterization of Cell death responses induced by *Salmonella enterica* Typhimurium effector protein SteA" submitted by Ms. Celina Meena, (Registration number MS16014) 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 recommends that the report is accepted.



Dr. Samarjit Bhattacharyya Dr. Kausik Chattopadhyay Dr. Arunika Mukhopadhaya (Supervisor)

Dated- May 9th, 2021

Declaration

The work presented in the dissertation has been carried out by me under the guidance of Dr. Arunika Mukhopadhaya 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 effect is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Celina Meena

Dated- May 9th, 2021

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 Mukhopadhaya

(Supervisor)

Acknowledgment

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List of figures

Notations

Abbreviation	Full form
SPI	*Salmonella* pathogenicity island
SteA	*Salmonella* translocates effector A
T3SS	Type III secretion system
FITC	Fluorescein Isothiocyante
PI	Propidium Iodide
WT	Wild type strain of *Salmonella typhimurium* SL1344
Δ*steA*	*steA*-deletion mutant of Wild Type bacteria
SCF complex	Skp, Cullin, F-box containing complex
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
LPM	Low Phosphate low-magnesium minimal media
Bid	BH3-interacting domain death agonist
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
RIP	Ribosome-inactivating proteins
TRAIL	TNF-related apoptosis-inducing ligand
NFκB	Nuclear factor kappa light chain enhancer of activated B cells
Apaf-1	Apoptotic protease activating factor-1

IκB	NF-kappa-B inhibitor alpha
SMAC	Second mitochondria-derived activator of caspases
API-5	Apoptosis inhibitor-5
p53	Tumor protein
PIDD	p53 induced protein with a death domain
CARD	Caspase activation and recruitment domain
RAIDD	RIP-associated ICH1-homologous protein with death domain
NADH	Nicotinamide adenine dinucleotide
NEDD-8	Neural precursor cell expressed developmentally down-regulated 8

List of Tables

Table of contents

Abstract

Salmonella enterica serovar Typhimurium is a pathogenic gram-negative bacterium responsible for causing self-limiting gastroenteritis through contaminated food and water. *Salmonella* uses its secretion system to invade in epithelial cells of humans and disseminate them into the body by living inside a vacuole known as *Salmonella*-containing vacuole. Type III secretion system translocates the effector protein, encoded within *Salmonella* pathogenicity island-1 (SPI-1) and SPI-2 where SPI-1 induced effector protein are responsible for causing invasion and SPI-2 effector proteins are involved in the intracellular replication and dissemination of *Salmonella typhimurium* inside the host cell. Similarly, SteA is an effector protein induced by T3SS encoded by both SPI-1 and SPI-2. It is an essential molecule that helps in the upregulation of the expression of genes that regulate ECM organization and reported as it interacts with Cullin-1, a component of SCF E3 ubiquitin ligases and responsible for the virulence of *Salmonella* Typhimurium. This project aimed to observe the effect of SteA effector protein on cell cytotoxicity in SPI-2 induced conditions and characterizing the type of cell death SteA promoting in macrophages. The experiments involved checking the cell death by LDH, which indicated that SteA could directly affect macrophages cell health. Further, various experiments were performed to check apoptosis markers to characterize the type of cell death occurring in macrophages due to SteA. The data revealed that SteA could affect the cell cycle progression.

Chapter-1

Introduction

1.1 Basic theory

1.1.1 *Salmonella enterica* serovar Typhimurium

1.1.2 Type III secretion system (T3SS)

1.1.3 *Salmonella* pathogenicity islands (SPIs)

1.1.4 SteA- *Salmonella* translocation effector A

1.2 Cell death

Introduction

Types of cell death

1.2.1 Apoptosis

1.2.2 Signalling pathway of apoptosis

1.2.3 Caspases

1.1 Basic Theory

1.1.1 *Salmonella enterica* serovar Typhimurium

Salmonella enterica is a motile, gram-negative, facultative bacterium capable of causing enteric diseases, commonly found in eggs and dairy products, including unhygienic food. It enters our body through contaminated food and water, and some of its serotypes are harmful to the gastrointestinal tract as they cause self-limiting gastroenteritis. Approximately around 93.8 million cases of non-typhoid *Salmonella* gastroenteritis occur each year which leads to 155,000 deaths [1]. Serovars are distinct variations within a species. *Salmonella* consists of approximately 2,500 serovars, from which two serovars- Enteriditis and Typhimurium are responsible for causing gastroenteritis in humans and many other animals. In immunosuppressed hosts, including both young and older individuals, it is responsible for causing severe systemic infection [2]. *Salmonella enterica Typhimurium* invades into intestinal epithelial cells with the help of their secretion system known as Type III secretion system (T3SS). These T3SS encodes within *Salmonella* pathogenicity island-1 (SPI-1) and SPI-2 to translocate effectors across the host cell plasma membrane. *Salmonella* gets internalized in a vacuole known as *Salmonella*-containing vacuole, which helps in the transportation of bacteria inside the host eukaryotic cells. SPI-2 encoded T3SS gets activated inside the SCV. The effector proteins aid in the transportation of SCV into the host cell cytoplasm. These SPI-2 effector proteins are also involved in the intracellular replication and dissemination of *Salmonella* inside the host cell.

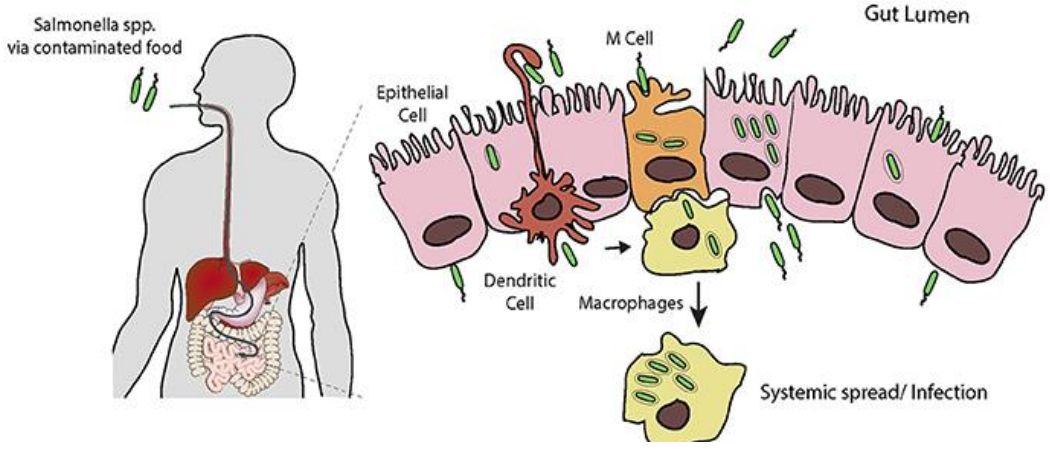


Figure 1- *Salmonella* invasion into the cells. Source- (Peter J. Hume et al 2017)

1.1.2 Type III secretion system (T3SS)

There are seven types of secretion systems present classified as Type I to Type IV (T1SS-T6SS) and the Chaperone-usher system. Among which T3SS is needle-like apparatus present in several gram-negative pathogenic bacteria [3]. T3SS utilizes its conserved genes and assembles a structure like a nano-syringe that can transverse through cell membrane for delivery of proteins from bacterium to host. This nano-syringe is composed of 20 subunits responsible for causing signal transducers, leading to actin cytoskeleton remodulation. The structure of T3SS contains three parts (i) basal body, (ii) extracellular needle, and (iii) a translocon [4]. These are the crucial parts of T3SS responsible for its functioning, but some other elements, such as chaperons that assert the secretion of effector proteins and an ATPase, present at the basal body are the additional factors that help in its functioning [5].

Salmonella typhimurium, a gram-negative and pathogenic bacterium, can invade into a host cell where it survives, replicate and disseminate. The delivery of effector proteins into host cells depends on the Type III secretion system that helps make this complex crucial for *Salmonella* pathogenicity. SPI-1 encoded T3SS gets activated extracellularly inside the gut lumen, where an anaerobic environment, high osmolarity, and basic pH helps in the induction of SPI-1 [6]. T3SS1-dependent invasion is a process with several steps. It utilizes conserved genes to assemble a structure of injectosome that helps to transports effector proteins, enables the bacterial invasion, and capable enough to induce inflammatory responses [7]. Similarly, SPI-2 encodes for T3SS that transport effector proteins responsible for the intracellular survival, multiplication, and movement of SCV across the membrane of the endocytic vacuole. SCV is an endocytic vacuole in which *Salmonella* enters and survives while invaded into the host cells [8]. T3SS-2 is expressed under SPI-2 inducing conditions like low phosphate and low pH ion post-infection. The function of effector protein is not only limited to the internalization of bacteria, but they are also crucial for the intracellular survival of *Salmonella* inside the vacuole and modulate host proteins [9-14].

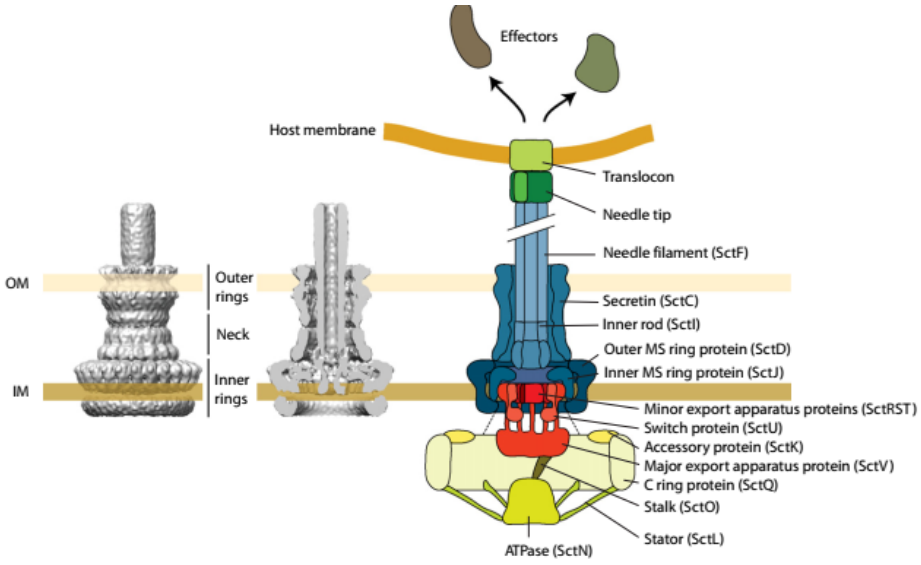


Figure 2 - Overview of injectosome and its components. Source- (Diepold and Wagner 2014)

1.1.3 *Salmonella* Pathogenicity Islands (SPIs)

Salmonella is a pathogenic bacterium whose virulence depends on many factors. One of these factors is the presence of *Salmonella* pathogenicity islands (SPIs) and their involvement in the invasion and replication process [15]. SPIs are encoded horizontally, which remain conserved in almost all serovars of *Salmonella*, but there also exist serovars that contain unique SPIs as well. Other than SPIs, *Salmonella* contains virulence plasmids but talking about the *Salmonella enterica* Typhimurium, i.e., our bacteria of interest doesn't contain such type of virulence plasmids. SPIs are horizontally acquired stretch of DNA that helps encode various virulence genes of the bacteria. Till now, in total, 21 different SPIs have been discovered, but all of them are not present in all types of serovars. S. Typhimurium contains 11 different types of SPIs that include SPI- 1 to 6 and 9,11,12,13,14,16 [16]. SPI-1 and SPI-2 encode for T3SS, a needle-like structure that facilitates the entry of bacteria into the host eukaryotic cell with the release of various effector proteins. After the invasion of bacteria into host cells, it causes the formation of *Salmonella*-containing vacuole (SCV), and then other effector proteins came into play for the replication and survival of bacteria.

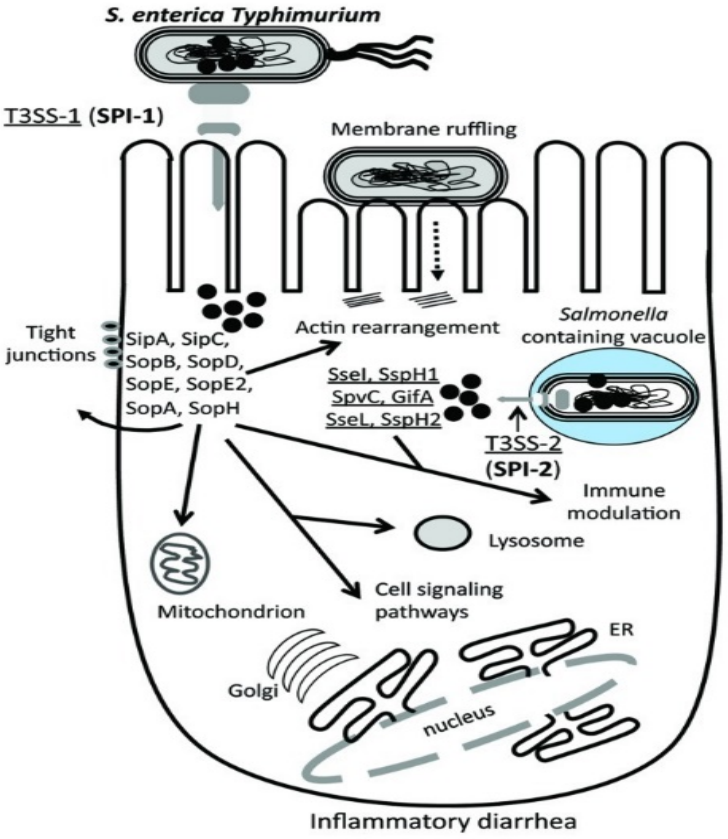


Figure 3 - Schematic of role of different effector proteins functions in *Salmonella* Spp. Source- (E Fidelma Boyd, Megan R Carpenter, Nityananda Chowdhury; 2012)

1.1.3.1 *Salmonella* pathogenicity Island-1 (SPI-1)

SPI-1 is found in S. Typhimurium responsible for causing invasion. It is approximately 40 Kb in size and located at 63 centisomes of the bacteria. SPI-1 encodes for T3SS that translocates the effector protein across the plasma membrane into the host cell cytoplasm. SPI-1 effectors proteins are crucial for invasion and also for inducing inflammation of intestinal epithelium [7]. Anaerobic environment, high osmolarity, and basic pH are the major contributor to causing the SPI-1 condition that encodes for effector proteins including SopB, SteA, SopE, SptP, SipA, SipC, SpvD and facilitate the entry of S. Typhimurium into the host cell. These effector proteins play a crucial role in bacterium's virulence by performing a different function like molecule SopB recruits RAB5 and annexin A2 that are important to function as a platform for actin rearrangements [9]. Similarly, another molecule, SipA, and SipC binds directly with actin and involved in bacterial internalization. SipA is known for increasing the actin-bundling and inhibits the actin

depolymerization, whereas SipC is necessary as it bundles and nucleates actin upon translocon insertion for invasion [12,13,17,18]. This way, many molecules come together and perform different functions, and some of them also have overlapping tasks for ensuring the invasion of S. Typhimurium into the host eukaryotic cells. SPI-1, along with invasion, helps induce inflammation, which indirectly helps bacteria provide a growth advantage over other bacteria present in the gut lumen. This is achieved by effector proteins that contribute to intestinal inflammation and are induced by the synthesis of pro-inflammatory cytokines, including IL-8 through MAPK and NF-κB pathways [19]. After the entry of *Salmonella* into host cells, it resides in a vacuole as *Salmonella*-containing vacuole, effector proteins SopB, SptP, and SopE are necessary for SCV maturation. SCV maturation is transient interactions with early endosomes and recruits early endosomal markers, which are slowly replaced with late endosomal and lysosomal markers [20].

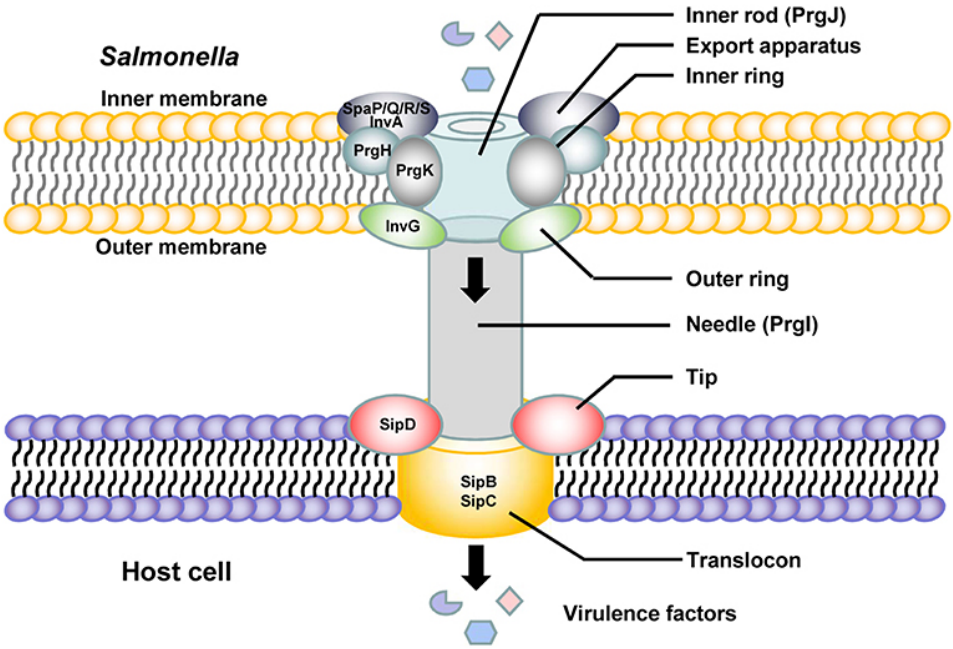


Figure 4 - Schematic diagram of the SPI-1 encoded T3SS needle apparatus in contact with host cell. Source- (Lixin Lou, Peng Zhang et al. 2019)

1.1.3.2 *Salmonella* pathogenicity Island-2 (SPI-2)

SPI-2 effector proteins are known to be responsible for making the SCV membrane and its positioning in the infected host cell [21]. After SPI-1 completes its function in the invasion of bacteria into the infected cells, the role of SPI-2 starts to promote the survival of bacteria and helps in its replication and dissemination. This aim is achieved by secreting the effector proteins encoded by SPI-2 induced T3SS. From the effectors proteins that get secreted, some proteins are most essential for virulence that includes sifA, spvB, sseF, sseJ, and steA [22]. Environmental changes occur when bacteria get invaded into vacuole known as SCV, environment changes from alkaline pH to acidic pH of the SCV, and phosphate ion concentration reduces. Many effector proteins induced by SPI-2 conditions play different roles in achieving their aim. Like RAB-7 interacts with a molecule known as Rab7-interacting lysosomal protein (RILP) and motor protein dynein, these three molecule form complex that is responsible for the centripetal movement of the SCV at an earlier stage [23]. Another protein secreted by SPI-2, SseJ, binds with Rhoa to activate its glycerophospholipid cholesterol acyltransferase activity and modifies the lipid content of endosomal tubules and SCV, and also affect the proteins associated with SCV [24]. SseF and SseG are vital effector proteins for promoting microtubule bundling of endosomal vesicles. SifA is reported to connect the SCV with the microtubular network [25] and break the complex formed by the Rab7-RILP-dynein motor complex by binding with Rab7 and increases the peripheral movement of the endosomal tubules. A complex of SifA-SKIP (SKI-interacting protein) is used to seize Rab9 and block the trafficking of mannose-6-phosphate receptors. It is one reason by which SCV can avoid the degradation of lysosomes [26]. SteC helps to alter the position of SCV within the cell, whereas PipB2 contributes to an endosomal tabulation by recruiting kinesin to the endosomal tubules and SCV [27]. SCV moves to peripheral regions in the later stage of infection and ready to disseminate and infect other host cells [28]. SPI-2 effector protein, SteA, and other effector proteins like SifA, SseF, SseJ, PipB2, SpvB, SopD2, and SseG reported controlling the dynamics of SCV [29-31].

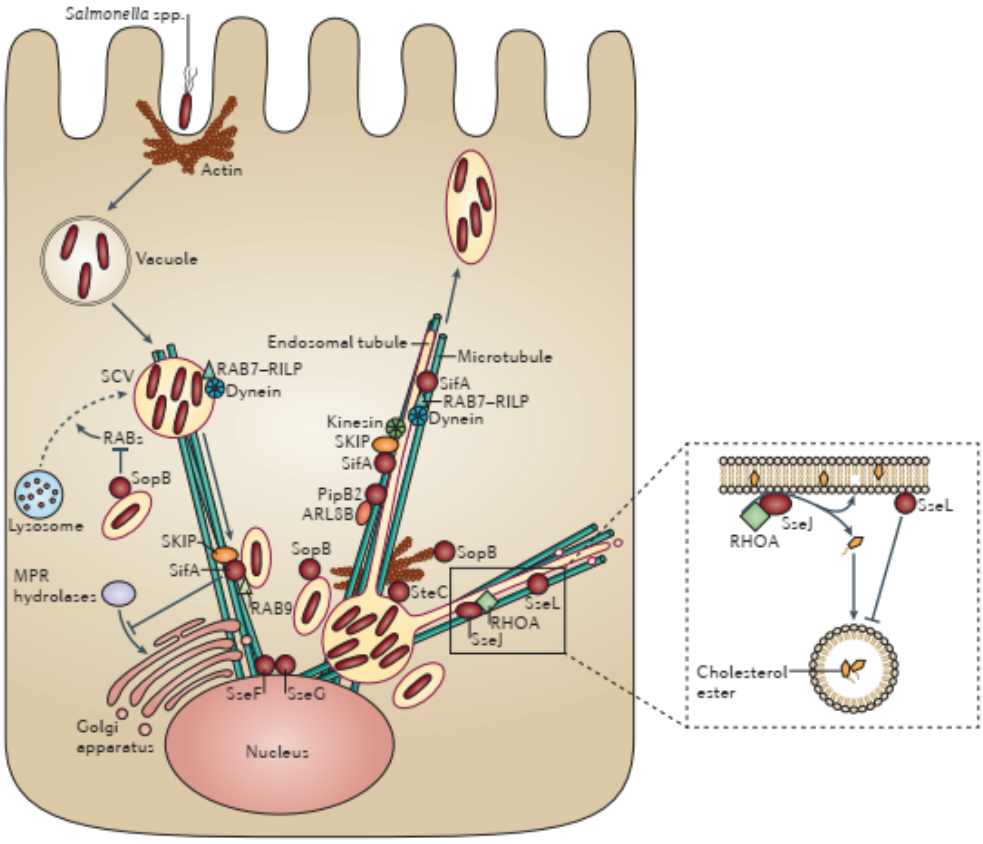


Figure 5 - Schematic diagram of SPI-2 mediated *Salmonella* Spp. Modulation in host membrane. Source- (Doris L LaRock et al 2015)

1.1.4 SteA- *Salmonella* translocates effector A

SPI-1 and SPI-2 are both responsible for secreting effector protein SteA that carry out different functions depending on the source of the secretion system. It upregulates the expression of genes that regulate extracellular matrix (ECM) organization and cell proliferation [32]. In our laboratory, one of the senior shown that SteA is an essential effector molecule responsible for the virulence of S. Typhimurium as it binds with Cullin-1, an integral component of SCF E3 ubiquitin ligases which is a complex of proteins necessary for the regulation of apoptosis, cell proliferation and degradation of many cellular proteins [33]. After binding with cullin-1, it modulates immune responses in host cells in SPI-1 mediated conditions [34].

Cullin-1 is an essential component in the SCF E3 complex as it helps in the activation of the ubiquitin activity of the complex by interacting with another component named NEDD-8. In normal conditions, Cullin-1 is attached with NEDD-8, which brings the complex in close

proximity with the ubiquitinating enzymes; this whole process is known as Neddylation. Taking the case in which *Salmonella* interferes with the function of this complex when the host machinery gets affected by the entry of bacteria, the function of cullin-1 also gets compromised. SteA, which is secreted by bacteria's secretion system, interacts with cullin-1, and there is no space for NEDD-8 to bind to Cullin-1 left; this causes the degradation of IκB, an inhibitor of NF-κB, and hence modulate the immune responses in host cell [35].

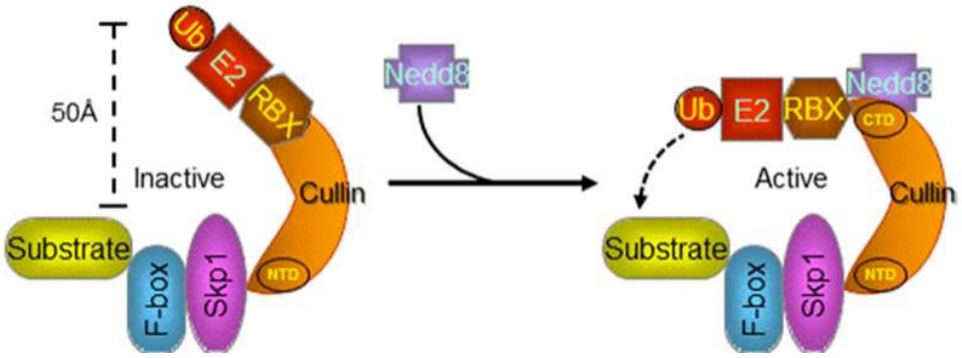


Figure 6 - Diagrammatic representation of Neddylation process by SCF E3 ubiquitin ligases complex. Source- (Malaria Parasite Metabolic Pathways)

1.2 Cell death

Cell death is an event that takes place in our body and indicates cells ceasing to carry out their functions. Our body maintains the constant number of cells for normal functioning, so, in appropriate condition, cells that are not functioning normally or old cells get removed. Cells get removed in situations including when they are not needed, like during certain stages of development, or the effect of radiation and toxins causes cells damaged or infected by viruses or bacteria. Cell kills themselves when there is a problem with the proliferation or the DNA repairing pathway. Cell killing takes place in distinct ways, including programmed cell death or activating the immune system with the substances from the dead cell.

Cell death is divided into various type based on their morphological and chemical changes take place that are i) Apoptosis ii) Necrosis iii) Pyroptosis iv) Autophagy etc., we will briefly talk about these type of cell death and will discuss in detail about apoptosis.

I) **Necrosis**- First, necrosis is a type of unprogrammed death of cells that occurs when a cell dies because of a lack of blood supply or a toxin. The bursting or release of content present in cells undergoing necrosis causes harmful effects on neighbouring cells and trigger inflammation [36-38]. In necrosis, different cytoplasmic changes occur that include increase eosinophilia, granular or vacuolated cytoplasm, swollen mitochondria, and some nuclear changes. This type of cell death occurs due to several factors, including injury, cancer, toxins, inflammation, infection, and can also arise due to lack of care at the wound site. It is accompanied by the release of enzymes stored by lysosomes capable of digesting the cell components or the whole cell itself. Necrotic cells are internalized by the macropinocytotic mechanism, which means only part of the cell is taken by phagocytes [39].

II) **Pyroptosis**- It is a form of necrotic and programmed cell death induced by inflammatory Caspases. It is also known as Caspase-1 dependent programmed cell death, a recently identified pathway of host cell death [40-43]. This pathway gets induced or stimulated by various microbial infections, including *Salmonella*, *Legionella*, and *Francisella*, and non-infectious stimuli like host factors that get produced during myocardial infarction [44].

III) **Autophagy**- In this type of cell death, the degradation of cellular components within the dying cell occurs in autophagic vacuoles [45]. The morphological Characteristics of autophagy are degradation of cytoplasmic contents, chromatin condensation, and vacuolization [46]. The autophagic pathway starts with sequestrating of cytoplasmic material in autophagosomes. These autophagosomes fuse with lysosomes which depends on the microtubules [47]. Cells that are undergoing autophagy can be phagocytosed by neighbouring cells.

1.2.1 Apoptosis-

Apoptosis is a programmed cell death, in biology it is a mechanism that allows cell to destruct themselves when they stimulated by agent and it is crucial for both homeostasis and normal development of multicellular organisms. Several morphological and biochemical changes take place during this process given below (Table-1). Apoptosis gets activated by stimuli that includes cell stressors, like lack of nutrients or hypoxia, and by agents that are responsible for causing damage of DNA or other cell structure.

Morphological Changes	Biochemical changes
• Cell shrinkage	• Chromosomal DNA cleaved into fragments
• Organelle reduction	• Change in the plasma membrane (exposure of PS on the outer surface of membrane)
• Mitochondrial leakage	• Loss of electrical potential across the inner membrane of mitochondria
• Chromatin condensation	• Relocation of cytochrome C from mitochondria to cytosol
• Nuclear fragmentation	
• Membrane blebbing and changes	

Table 1 - Morphological and Biochemical changes in Apoptosis

Apoptosis is different from other cell death process known as necrosis as it is a morphologically distinct form of programmed cell death. Necrosis and apoptosis can be distinguishing morphologically as they show different characters shown below (Table2).

Apoptosis	Necrosis
• Chromatin condensation	• Nuclear swelling
• Cell shrinkage	• Cell swelling
• Membrane remain intact	• Membrane is broken

• Cells are phagocytosed, no tissue reaction	• Cell lyses, eliciting an inflammatory reaction
• Ladder-like DNA fragmentation	• DNA fragmentation is random or smeared

Table 2 - Difference in morphological characters of Apoptosis Vs Necrosis

1.2.2 Signalling pathway of apoptosis

The two most widely studied or characterized apoptotic pathways are the Intrinsic and Extrinsic pathways. The extrinsic pathway is mediated by the death receptor, which includes Tumor necrosis factor (TNF) receptors, Fas receptors, and TNF- related apoptosis-inducing ligand (TRAIL) receptors. The signalling pathway starts with a death ligand binding with a death receptor; for example, TNF-α binds with TNFR1. The TNFR family consists of approximately 29 transmembrane receptor proteins, and these proteins share a similar cysteine-rich extracellular domain and contain a cytoplasmic domain known as the death domain (DD). This domain is used to pass signals from the cell surface to the intracellular pathways. This signalling pathway results in inflammation, proliferation, and apoptosis, depends upon the adaptor proteins [48,49].

It has been identified that the binding of ligand TNF-α with receptor TNFR1 is responsible for activating the NFκB pathway. This pathway gets activated via the recruitment of RIP, essential for TRAIL-induced IκB activation by receptor TNFR, leading to cell survival or apoptosis, depending upon the biological context. Recruitment of the death domain eventually activates caspase-8 and caspase-10. caspase-8 and 10 are initiator caspases whose function is to either initiate apoptosis or cleave executioner caspases and activate them, or can also activate another signalling pathway known as the intrinsic pathway through cleavage of BID, which induces cell death [48,49].

Intrinsic pathway initiates by exogenous or endogenous stimuli like oxidative stress or DNA damage and helps in eliminating damaged cells. The B-cell lymphoma 2 (Bcl-2) is the critical regulator for initiating the Intrinsic pathway.

The mitochondrial pathway is influenced by Bcl family members, including anti-apoptotic regulatory protein Bcl-2 and pro-apoptotic regulatory protein Bax [54,55]. Bax molecule causes permeabilization of the outer membrane of mitochondria which eventually leads to cytochrome c release. Cytochrome c binds to APaf-1 and caspase-9 molecule and causes the formation of apoptosome complex. Whereas the function of Bcl- is to inhibit the efflux of cytochrome c while Bak, Bax, and Bid, all pro-apoptotic proteins, stimulate its release from mitochondria. During the process of apoptotic cell death, various caspases like caspase-2, caspase-9, caspase-8, and caspase-10 are involved in initiating the process. Other Caspases are also involved in activating different molecules that eventually leads to apoptosis. caspase-3 and caspase-7 start the DNA degradation and inhibit DNA repair, and caspase-6 helps in the disintegration of cytoskeleton and lamina [50,51].

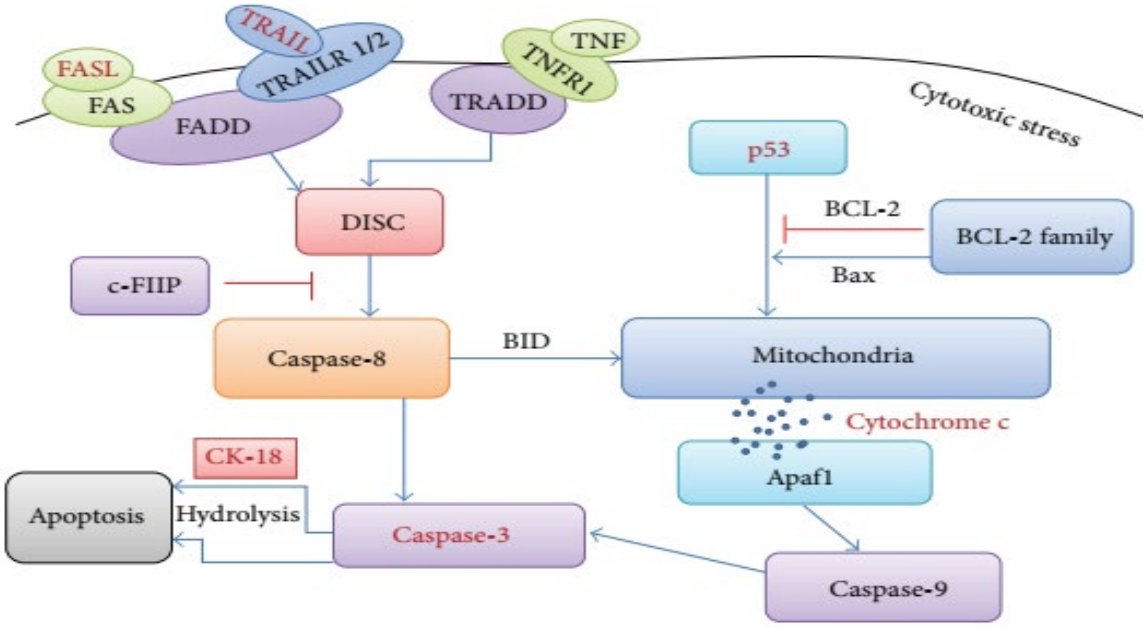


Figure 7 - Diagrammatic representation of Apoptosis Pathway. Source- (Kongning Li et al. 201, Biomed Research International).

Mitochondria are also known for releasing proteins called SMACs (second mitochondria-derived activator of caspases), leading to an increase in the permeability of the mitochondrial membrane. SMAC proteins bind with IAPs that inhibit apoptosis by deactivating them and allow apoptosis to proceed. Therefore, Mitochondrial permeability is regulated indirectly by various degradation enzymes.

1.2.3 Caspases

Caspases are aspartate-specific cysteine proteases, evolutionarily conserved necessary for the initiation and execution of apoptosis [52,53]. They play an essential role in apoptosis, and mammalian caspases are divided into three categories commonly known as Initiator caspases, executioner caspases, and inflammatory caspases. Initiator caspase includes caspase-2,8,9 and 10, while caspases 3, 6, and 7 falls in the executioner caspase category, and inflammatory caspase has 1,4,5,11 and 12 caspases [54]. The function of initiator caspases is to initiate apoptosis, while executioner caspases help in mass proteolysis that eventually leads to apoptosis. Caspases get activated when they get cleaved and converted from procaspase to active caspase in response to granzyme B, apoptosome stimuli, and death receptors. Initiator caspases are responsible for cleaving inactive pro-forms of effector or executioner caspases, thereby activating them.

In our laboratory, Mass spectrometry was performed after GST pull-down, which showed the interaction of SteA with proteins like API-5 involved in apoptosis and other proteins involved in endocytosis. p53, tumor suppressor gene stops the replication of damaged or mutated DNA. When DNA damage or cell cycle arrest occurs, p53 gets activated and leads to initiation of signaling cascade, as shown below in figure 7. Apoptosis inhibitor-5 (API-5) is seen to inhibit Caspase-2 that gets activated by the p53-indued death domain.

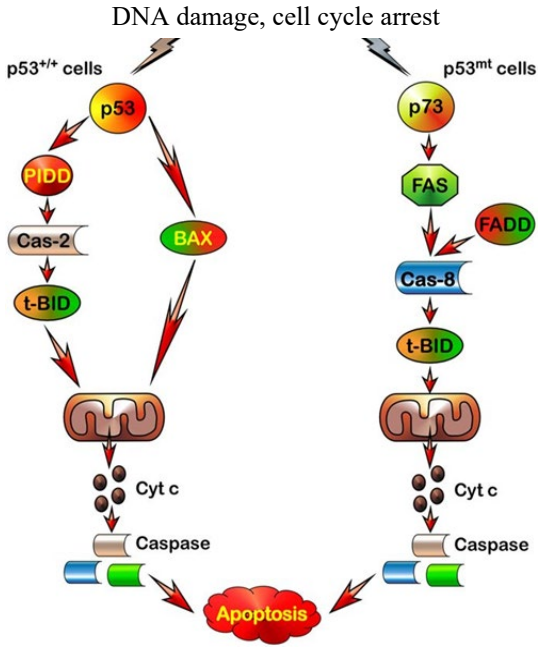


Figure 8 - Diagrammatic representation of p53-induced death domain (PIDD) to induce apoptosis. Source- (Ray, P., Guha, D., Chakraborty, J. et al .2016)

Activation of caspase-2 takes place by p53-induced protein with a death domain (PIDD1), that is cloned as death-domain containing protein get induced by p53. It is a core of a molecule activating caspase-2, named as PIDDosome [55]. PIDD1 undergo autoprocessing to generate fragments that are PIDD-C and PIDD-CC. Caspase activation and recruitment domain (CARD) of RAIDD interact with proCaspase-2 [55]. PIDDosome complex formed by the interaction of PIDD-CC and RAIDD molecule via Death domain. Pro-caspase-2 binds with the RAIDD molecule and promotes proximity-induced dimerization, and auto-cleavage of caspase-2 leads to its activation (figure-9) [55].

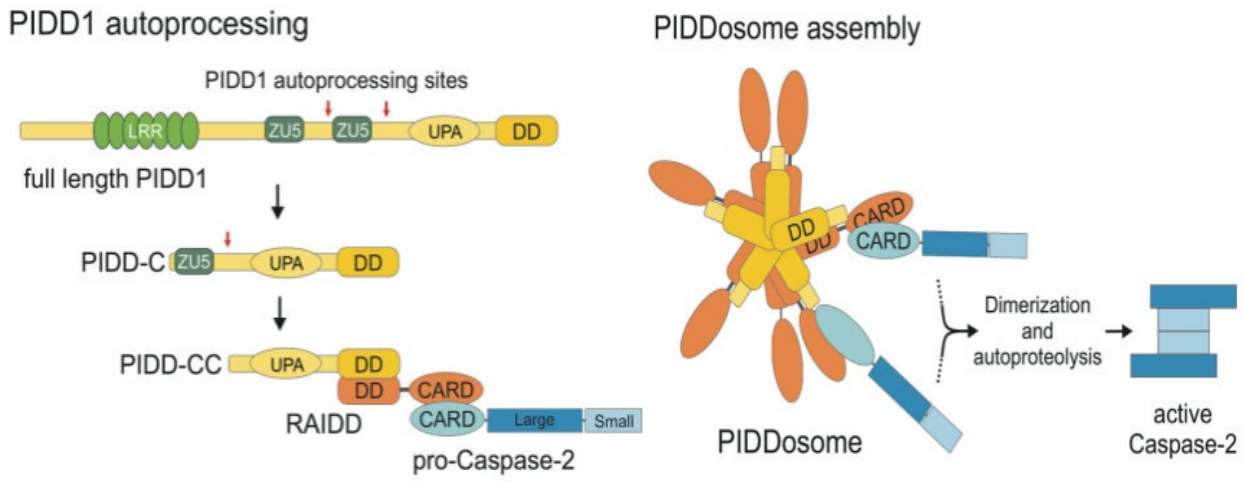


Figure 9 - PIDD1 autoprocessing and caspase-2 activation. Source- (Valentina C. Sladky et al 2020)

Chapter- 2

Experimental Materials and Methods

2.1 Materials

2.2 Methods

2.1 Materials:

Bacterial Strains-

Salmonella enterica serotype Typhimurium of strain SL1344 and mutant of effector protein SteA derived from the same strain were used in this study. Mutant *steA* was constructed using one step inactivation method of Datsenko and Warner, in which the *steA* gene was replaced with a Kanamycin cassette.

Bacterial Culture-

S. Typhimurium wild type and SteA mutant were cultured in Luria Bertani medium (HIMEDIA). For generating different SPI conditions, different cultures were used and the effect of SteA effector protein in that particular condition was checked.

SPI-1 inducing condition-

1. μaer-ST (non-aerated stationary phase bacteria) were used. *S.* Typhimurium strains were grown overnight at 37°C in LB–0.3 M NaCl medium under static conditions.
2. Aer-LL (aerated late-log phase bacteria with OD_{600} = 3.0): Bacteria were grown for 16 h at 37°C with shaking and then sub-cultured (1:33) in Luria Bertani broth for 3 h.

SPI-2 inducing condition-

SteA effector protein's role was observed by inducing SPI-2 condition. This condition was achieved by growing the bacterial strains on LB culture, and after developing bacterial culture, this culture was resuspended with dilution 1:100 in Low phosphate, low magnesium minimal media (LPM) culture at a pH of 5.8 and incubated overnight at 37°C with shaking.

Low Phosphate, Low-magnesium minimal media (LPM) consists of-

1.80mM 2-(N-morpholino) ethanesulfonic acid (pH 5.8)
2. 5 M KCl
3. 7.5 mM (NH4)2SO4
4. 0.5 mM K2SO4
5. 0.1% Casamino acids
6. 38 mM Glycerol
7. 337.5 µM K2HPO4-KH2PO4 (pH 7.4)
8. 8 µM MgCl2

From the above-given components, Casamino acids and MES were filter-sterilized with a 0.22 µm filter (Millipore millex)

Antibiotics used-

S. Typhimurium wild type and SteA mutant culture were prepared using antibiotics given below.

1. Streptomycin antibiotic from HIMEDIA with a concentration of 100 mg/ml was prepared for both WT and Δ*steA* cultures.

2. Kanamycin antibiotic from HIMEDIA with a concentration of 100 mg/ml was prepared for both WT and Δ*steA* culture.

3. Gentamicin antibiotic from HIMEDIA with a concentration of 100 mg/ml was prepared for gentamicin protection assay.

Cell Culture-

RAW 264.7 murine macrophages cell line (ATCC) were used in our study that was maintained in RPMI (Roswell Park Memorial Institute) media supplemented with 10% FBS (Fetal bovine serum) and incubated in 5% CO_2 at 37 °C.

Measurement of bacterial growth-

The OD_{600} readings for bacterial culture of WT and Δ*steA* were taken using Jenway Genova plus spectrophotometer.

Flow cytometry and post-acquisition analysis-

Experiments of flow cytometry were performed on FACS Aria flow cytometer, and FITC conjugated Annexin V Apoptosis Detection Kit was used for fluorescent staining. Post-acquisition analysis was performed on software FlowJo V10, and spectral compensation was done by using the 'compensation' tab available on the software itself. In Annexin-PI experiment, we used Staurosporine as a positive control as it is a protein-kinase inhibitor used to induce apoptosis in cells.

2.2 Methods:

Invasion assay (Gentamicin protection assay) -

1. Bacterial stocks of S. Typhimurium SL1344 of wild type and Δ*steA* mutant were streaked on LB-agar plates containing the appropriate antibiotics and incubated overnight at 37°C.
2. Bacterial primary culture of WT and Δ*steA* were prepared in LB media (5 ml) by putting antibiotics mentioned above according to their concentration and incubated overnight at 37°C and 5% CO_2.
3. Cultured prepared of bacteria in LB media was taken in 1.5 ml of MCT and centrifuged at 10,000 rpm for 5 min, supernatant was discarded and pellet resuspended in LPM culture in dilution of 1:100 for inducing SPI-2 conditions, then the culture was incubated overnight. For example, 5 ml of LPM culture was prepared by taking 500 µl of LB culture, centrifuged and then pellet was resuspended in 500 µl of LPM media. In the same way SPI-1 condition can also be induced by using the media mentioned in the experimental materials section.

4. RAW 264.7 murine macrophages cells (0.5 million/well) were plated in 24-well plate for 10-12 hours before the infection at 37 ℃ with 5% CO_2.
5. After overnight incubation, OD_{600} readings were taken by setting LPM as a blank for the LPM culture prepared of both bacterial strains in a Jenway Genova Plus spectrophotometer. Then according to their values, the amount of culture needed to mix in PBS is calculated. The calculated culture was taken in 1.5 ml MCT and centrifuged at 10,000 rpm for 5 min and the pellet was resuspended in 900µl of 1XPBS.
6. OD_{600} of resuspended sample is taken by setting 1XPBS as a blank, and OD_{600} was made equal for both *Salmonella* bacterial strains of WT and Δ*steA* till the second decimal place and were equalize to approximately 1. As OD_{600} equal to 1 contains $2.5x10^8$ cells, the OD of both samples set to 1 added in each well is calculated by taking MOI (multiplicity of infection) according to the experiment requirement.
7. Bacteria is added in each well with RPMI without 10% FBS and then the plate was centrifuged at 200 g for 2 min, 4 °C so that bacteria can settle down and incubated for 30 min at 37 °C.
8. After the incubation period of 30 min, RPMI media containing bacteria was removed and RPMI media containing 100 µg/ml gentamicin (300 µl/well) was added and again incubated for 1 hour at 37°C.
9. Gentamicin containing RPMI media was removed after 1 hour of incubation and fresh RPMI media containing 20 µg/ml gentamicin was added in each well (500 µl/well) and incubated at 37°C and 5% CO_2.
10. To check the equal invasion of WT and Δ*steA* bacteria in RAW264.7 cells, cells present in 24 well plate after treating with bacteria and 100 µg/ml of gentamicin were first washed with 1XPBS twice (500µl) and then harvested in 450 µl of 1XPBS and then 50 µl of Triton was added for lysis. The plate was incubated for 30 min at 37°C.
11. After the lysis of cells, the media was collected in 1.5 ml MCT and diluted to 10^3 for plating on LB-agar plates containing appropriate antibiotics i.e., Streptomycin in WT plate and Streptomycin and kanamycin both for Δ*steA* plate. These Plates were incubated overnight in incubator and next day number of colonies (bacterial CFU count) in WT and Δ*steA* was counted and the invasion is compared.

Lactate dehydrogenase assay (LDH assay)-

Lactate is an enzyme involved in energy production in all cells, and it helps in catalyzing the conversion of lactate to pyruvate, which is a useful process in cellular respiration. Lactate is a reliable marker for checking cell death as when the tissue gets damaged, it gets released in the surrounding, and quantified to check cell damage. For checking the cell cytotoxicity, RPMI media in which cells treated with bacterial strain of WT and Δ*steA* were incubated overnight is collected at time point according to our experiment requirement and centrifuged at 3500 rpm for 5 min. As negative control, we took untreated cells and cells treated with Triton-X as positive control. After centrifugation 50 µl sample was put in 96-well plate in triplets, followed by 50 µl of substrate i.e., yellow coloured tetrazolium salt was added in each well-containing sample and incubated for 10-15 min at room temperature till the color changes from yellow to red colour of formazan salt. The change in colour is due to the NADH produced by lactate during the conversion of pyruvate into lactate, reducing tetrazolium and converting it into formazan salt. For stopping the reaction, stop solution was added and then optical density of samples were taken at 490 nm using spectrophotometer and cell cytotoxicity was quantified using the formula given below.

$$\% \text{ cell cytotoxicity} = [(\text{test- negative control}) \div (\text{positive control- negative control})] \times 100$$

Statistical Analysis-

Data of cell cytotoxicity and other experiments was analyzed in Microsoft excel, where calculated the standard deviation and standard error of our data using the formula mentioned below.

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

n = number of observations

Annexin-FITC/PI assay-

1. 12-hour post infection of bacteria strain WT and Δ*steA*, cells were harvested from wells and collected in 1.5 ml MCT. The sample was centrifuged at 1800 rpm for 5 min at 4 °C.
2. The pellet obtained was washed in 500 µl of 1X PBS twice. Then the pellet was resuspended in 350 µl of 1X Binding buffer prepared from the stock of 10X binding buffer.
3. For flow cytometry analysis, 100 µl of sample was added in FACS tubes and then added 2.5 µl of FITC and PI each. Samples were prepared according to table 3 given below.
4. Incubating time of 15 minutes in the dark was given to the samples for staining of FITC and PI.
5. After staining, 300 µl of 1X binding buffer was added in each tube and analyzed in Aria flow cytometer. Spectral compensation was performed in Flow Jo V10 using function compensation.

	Unstained	Single Stained FITC	Single Stained PI	Double Stained FITC+PI
WT infected				✓
Δ*steA* infected				✓
Staurosporine		✓	✓	✓
Untreated	✓			✓

Table 3 - Flow cytometry based apoptosis detection-table of samples prepared

Cell cycle arrest-

1. The infection of bacteria by taking MOI 20 was done on RAW264.7 cells at a time point of 12 hours to check cell cycle arrest.
2. Post 12 hour of infection, cells were harvested from the 12-well plate containing 1 million/well, and the media was put in 1.5 ml MCT, pellet down by centrifuging at 3500 rpm for 5 min at 4 °C.
3. The pellet got after centrifugation was washed in 500 µl 1XPBS twice. After washing, cells were fixed and permeabilized by chilled 70% ethanol and incubated for 20-30 min on ice.
4. The fixed cells were centrifuged again at 3500 rpm for 5 min, 4 °C and washed with 500 µl of 1X PBS twice.
5. The pellet obtained after centrifugation was resuspended in 100 µl of 1X PBS, then 5 µl RNase A and 2.5 µl of propidium iodide, DNA staining dye (stock concentration- 50 µg/ml) staining dye was added in each sample.
6. The Staining dye was incubated for 15-30 min and after staining 400 µl of 1X PBS was added.
7. The sample prepared for cell cycle was analyzed in Flow cytometer aria and done cell cycle analysis.

Western Blotting (checking the involvement of Caspase-3) –

Caspase-3 antibody	Santa cruz Anti-Rabbit antibody
GAPDH	Santa cruz Anti-mouse antibody

1. Cell lysate of the samples was prepared at the time point according to the experiment requirement after the bacterial infection of WT and Δ*steA*. The Bradford of the prepared sample was performed by adding 99 µl of Bradford and 1µl of sample in 96 well plate and incubated in dark for 10-15 min to quantify the amount of protein present by using spectrophotometer at 595 nm.
2. Sample preparation- 5 µl of loading dye was added in the sample and heated at 95 °C for 15-20 min. 12.5% SDS-PAGE gel was run and by transfer, proteins were transferred to the PVDF membrane.

3. The blot we got after transfer was put in blocking solution (5% BSA in 1X TBST) for at least 1 hour. After 1 hour, primary antibody of Caspase-3 and GAPDH (as mentioned in above table) was added in 1:1000 dilution in blots and incubated overnight at 4 °C.
4. After overnight incubation, primary antibody was removed and the blot was washed 4-5 times with 1X TBST for 15 min each by putting on rocker. After washing, secondary antibody (anti-rabbit and anti-mouse for Caspase-3 and GAPDH respectively) was added in 1:5000 dilutions in 1X TBST or blocking solution and incubated for 1 hour at room temperature on rocker.
5. Washing of blot was done with 1X TBST after incubation for at least 4-5 times for 15 min each.
6. Blot was developed using ECL substrate and Image Quant LAS4000 Imager.

RT-PCR for checking the gene expression level of *Bcl-2* and *Bax*-

Primers	Sequence
Mouse-*Bcl-2*-Forward primer	GTCGCTACCGTCGTGACTTC
Mouse-*Bcl-2*-Reverse primer	CAGACATGCACCTACCCAGC
Mouse-*Bax*-Forward primer	CCGGCGAATTGGAGATGAACT
Mouse-*Bax*- Reverse primer	CCAGCCCATGATGGTTCTGAT

1. Infection on RAW264.7 murine macrophages was performed by gentamicin protection assay by taking the MOI=20 and time point according to the experiment requirement for the infection.
2. Post Infection, invasion of bacteria in cells was checked by counting the number of colonies and after getting equal invasion, RNA of cells is isolated by using the protocol mentioned below.
3. For RNA isolation, first wash the cells with 500 µl of 1X PBS twice. Then add 1ml of trizol in each well and harvest the cells in trizol, put the sample in 1.5 ml MCT.
4. Homogenize the cells with 1 ml syringe after harvesting and put it at room temperature for 5 min.

5. After 5 min, 200 µl of chloroform per 1ml of trizol is added and mix it by shaking. Then spin the sample at 12000 g for 15 min at 4°C.
6. Collect aqueous phase solution (upper) and transfer it to new 1.5 ml MCT and add 500 µl of 100% isopropanol, mix it by shaking and keep at room temperature for at least 10 min at 4 °C. After 10 min, spin at 12000 g for 10 min at 4 °C
7. Discard the supernatant and wash the pellet with 500µl volume of 75% ethanol twice at 7500 g for 5 min at 4 °C.
8. After washing, discard the supernatant and air dry or at 55°C heating block.
9. Elute in 30µl of RNase free water and check concentration by using Nanodrop at 260/280.
10. cDNA was prepared by using verso cDNA kit from Thermofisher scientific.
11. qRT-PCR was performed using SyBr probe and the kit from Thermofisher, data was analyzed in Microsoft excel.

Chapter-3
Results and Discussions

3.1 Background and Hypothesis

3.2 Results

3.3 Discussion and conclusion

3.4 Future outlook

3.1 Background and hypothesis:

Cullin-1, an essential component of SCF E3 ubiquitin ligases is seen to be interacting with *Salmonella* effector protein SteA. In absence of bacteria, the role of cullin-1 is to attach with NEDD-8 and help the complex to bring it in close proximity with the ubiquitinating enzymes, this whole process of ubiquitin activity of SCF E3 complex is known as Neddylation. In case when SteA binds with cullin-1 instead of NEDD-8, the function got disrupted and IκB does not get degraded. The inhibition of degradation of IκB causes NF-κB to remain it active state and hence play essential role for modulating immune response in host cell as shown in figure below.

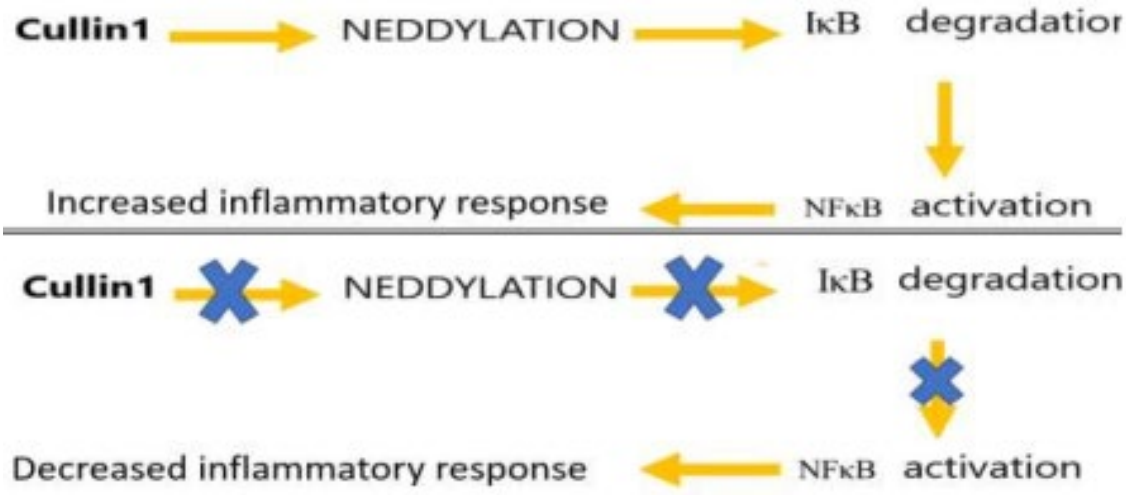


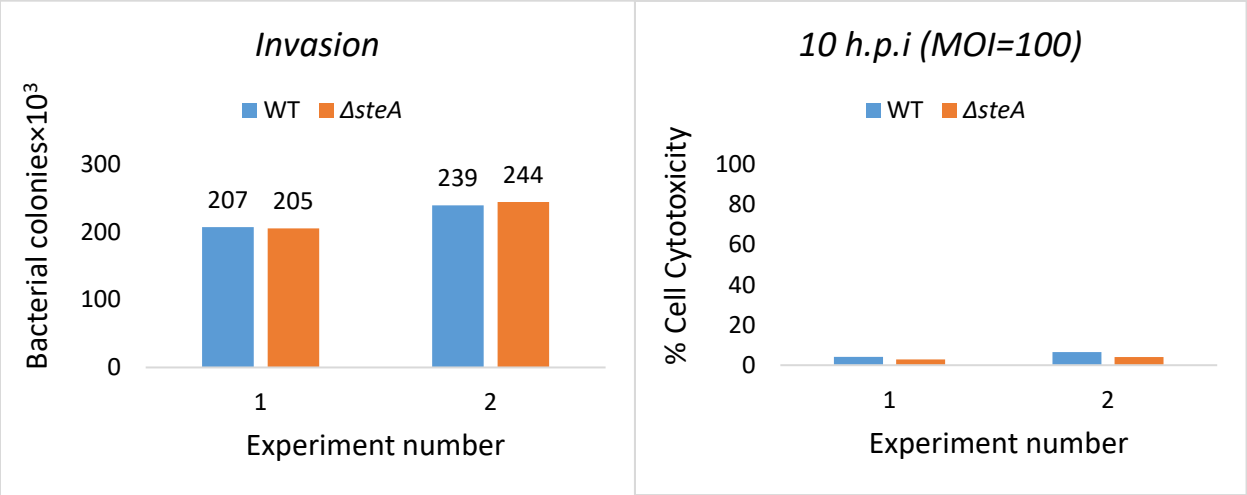
Figure 10- Schematic representation of Modulation of host's immune response upon SteA-Cullin-1 interaction. Figure courtesy- Shivansh's thesis

Secondly the aim of this project was to characterize the cell death taking place due to *Salmonella* effector protein SteA, as my seniors got the hint from mass spectrometry that SteA is seen to interact with many proteins related to Apoptosis and Endocytosis so we tried to identify the type of cell death taking place due to effector protein SteA. We have checked the apoptosis markers by using various technique as mentioned in results section. Further, we checked the role of SteA on cell cycle arrest as cullin-1 is essential for cell-cycle progression and it have been reported in an article that cullin-1 knockout causes accumulation of p27 and p21 in the nucleus and eventually leads to cell cycle arrest and DNA damage, and causes decreased level of Cyclin D1 and cyclin E [56]. Interestingly, SteA released in SPI-2 induced Condition is associated with T3SS-1 independent cytotoxicity caused in macrophages [57].

3.2 Results:

3.2.1 Cell cytotoxicity was not effected due to *Salmonella typhimurium* effector protein SteA at 10 h post infection in RAW264.7 cells

We took RAW264.7 murine macrophages cell line to understand the effect of effector protein SteA on cell health, by taking wild-type (WT) and *steA*-deleted (Δ*steA*) of *Salmonella typhimurium* strain SL1344, inducing SPI-2 conditions and observed the difference in cell cytotoxicity. First we checked the difference at 10 hpi and MOI=100. Estimation of cell cytotoxicity caused by both types of bacterial strains i.e., WT and Δ*steA* were analyzed using uninfected cells as the negative control and 100% cell cytotoxicity was shown in positive control as we have treated the cells with Triton-X 100, a strong detergent that have a capability to lyse 100% of cells. Before checking the cell death using LDH, we checked if there is an equal invasion of both bacterial strains after 2 h of infection. After getting equal invasion in each sample, we checked the cell cytotoxicity percentage and observed no difference in cell death caused by WT and Δ*steA*. Further we took different time points and different MOI to check the effect of SteA on cell death.



(a) (b)

Figure 11- Cell cytotoxicity using LDH assay at 10 h post infection Bar graphs represent (a) Invasion of bacterial strains WT and Δ*steA* 2 h.p.i on RAW264.7 cell line where Y axis represents number of colonies on LB agar plates, and observed almost equal invasion. (b) Represents % cell cytotoxicity due to 10 h.p.i by taking WT and Δ*steA* strains, LDH assay was used to determine the cytotoxicity, where no difference in cell death was observed.

3.2.2 Cell cytotoxicity was downregulated in absence of effector protein SteA at 16 and 24 h post infection

Salmonella typhimurium effector protein SteA is seen to be involved in cell death in Raw264.7 murine macrophage cell line. We took bacterial strains of Wild type and Δ*steA*, in SPI-2 inducing Condition and observed the difference in cell cytotoxicity at two different time point. For these experiments we checked the cell death by taking different MOI as shown in figure below. Before checking the cell death by LDH assay, we checked whether there is equal invasion of bacterial strains in RAW264.7 cells after 2 h post infection. After observing equal invasion in experiment, we moved further to check the cell cytotoxicity and compared the difference in both WT and Δ*steA* *Salmonella typhimurium* strains in both time points, further we repeated the experiments to get confirmation. We observed more cell death in WT as compared to Δ*steA* and it was approximately 4 % and 12 % more in WT with respect to Δ*steA* at 16 and 24 h post infection respectively by taking MOI=20.

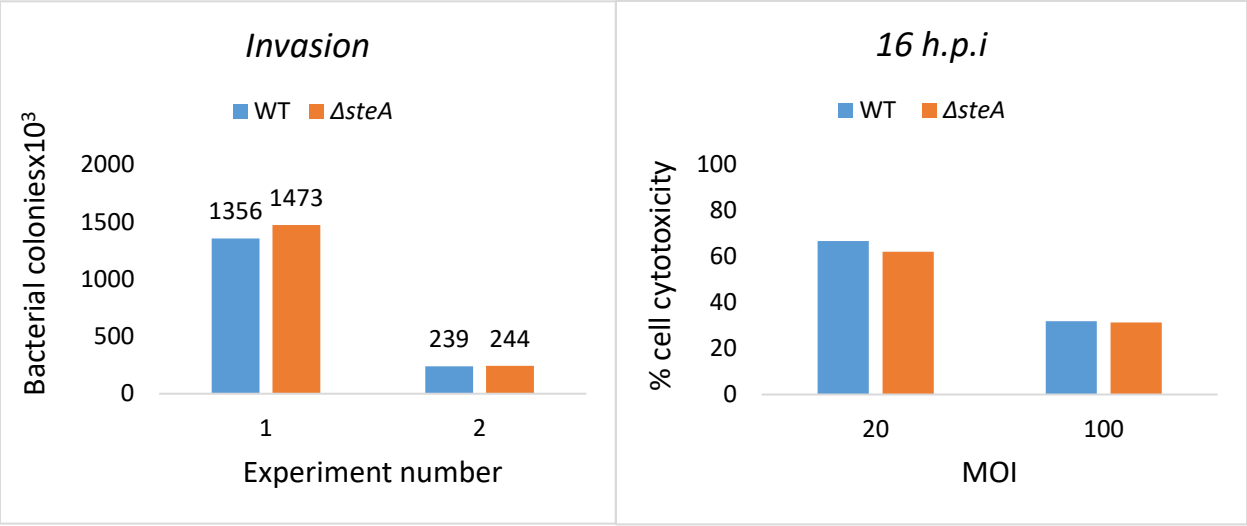
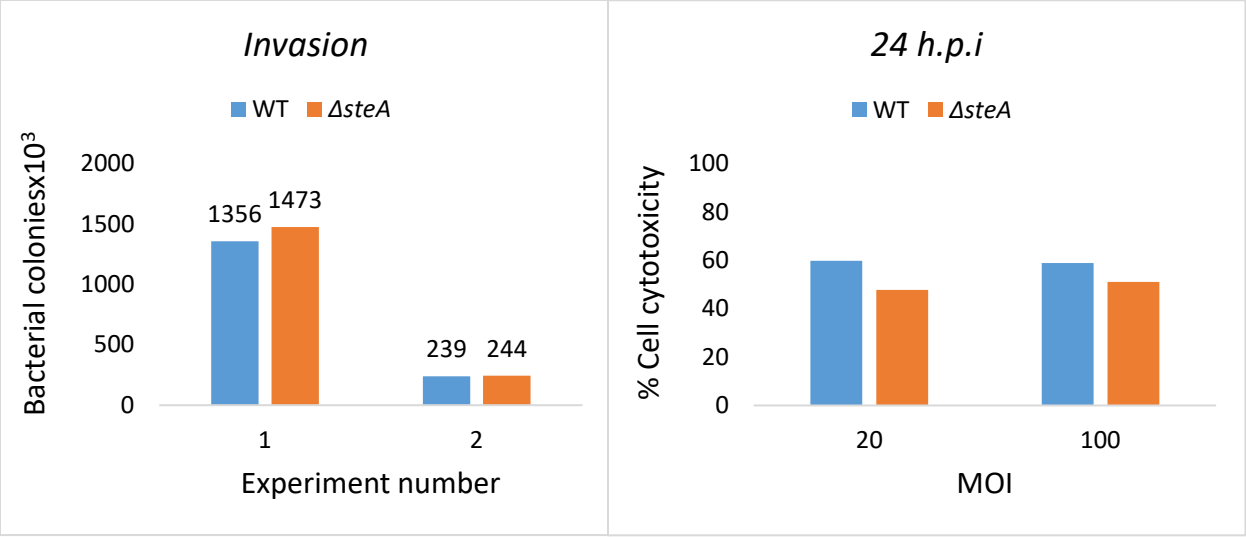


Figure 12 – Cell cytotoxicity using LDH assay at 16 h post infection Bar graphs represent (a) Invasion of bacterial strains WT and Δ*steA* 2 h.p.i on RAW264.7 cell line where Y axis represents number of colonies on LB agar plates, and observed almost equal invasion. (b) Represents % cell cytotoxicity due to 16 h.p.i by taking WT and Δ*steA* strains, in this experiment we check the cell death by taking MOI=20 and 100 as represented on the X axis of the graph. LDH assay was used to determine the cytotoxicity. And observed the difference in cell death of WT and Δ*steA*. As figure shows, MOI=20 had more difference in cell cytotoxicity



(a) (b)

Figure 13 – Cell cytotoxicity using LDH assay at 24 h post infection Bar graphs represent (a) Invasion of bacterial strains WT and Δ*steA* 2 h.p.i on RAW264.7 cell line where Y axis represents number of colonies on LB agar plates, and observed almost equal invasion. (b) Represents % cell cytotoxicity due to 24 h.p.i by taking WT and Δ*steA* strains, in this experiment we check the cell death by taking MOI=20 and 100 as represented on the X axis of the graph. LDH assay was used to determine the cytotoxicity. And observed the difference in cell death of WT and Δ*steA*. As figure shows, MOI=20 had more difference in cell cytotoxicity

As it is observable from the above graphs that SteA play role in cell death when infected in RAW264.7 cell line. We repeated the experiment for these two time points that are 16 and 24 h by taking MOI=20 as we have observed more difference in cell cytotoxicity as compared to MOI=100. We confirmed this as shown in figure below.

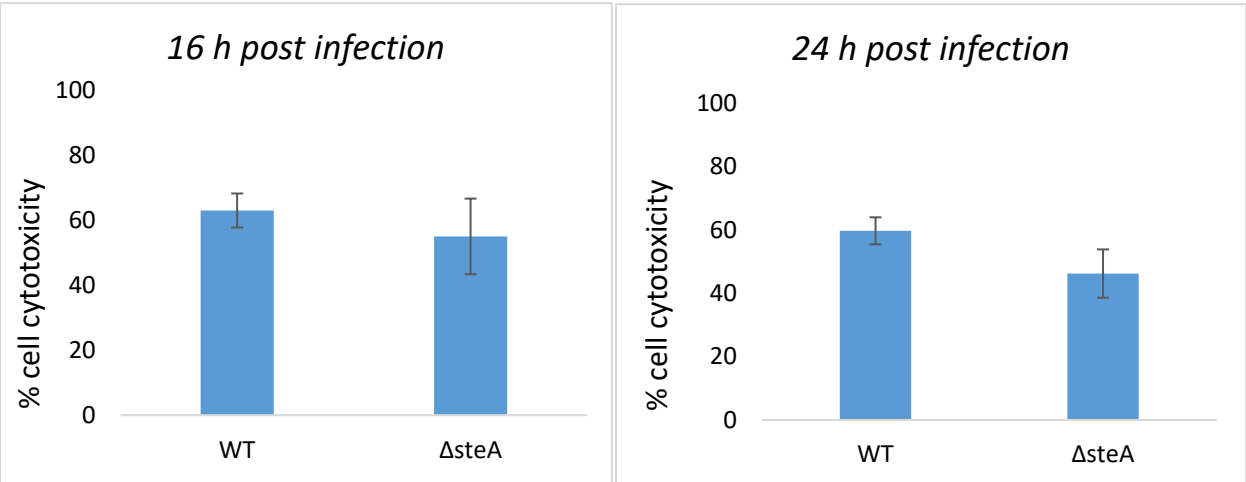


Figure 14 – **Cell cytotoxicity graphs at 16 and 24 h post infection** Bar graph represents cell death caused due to effector protein SteA, in graph Y axis represents % cell cytotoxicity and *Salmonella* bacterial strains that are WT and Δ*steA* on X axis. Cell cytotoxicity was quantified using LDH assay by taking two different time point- 16 and 24 h post infection of bacterial strains and observed induction in cell death in WT as compared to Δ*steA*.

3.2.3 Flow cytometry experiment to detect apoptosis caused due to effector protein SteA in RAW264.7 cell line

Flow cytometry-based Annexin-PI was performed for detection of apoptosis due to the effect of SteA. For this experiment, we have treated the RAW264.7 cells in SPI-2 inducing Condition with WT and Δ*steA* bacterial strains of *Salmonella typhimurium* and observed the apoptotic cells. For confirming equal invasion of bacteria in both sample of WT and Δ*steA*, infected cells from both samples were lysed and plated at 2h post-infection on LB agar plate and counted the bacterial colonies after overnight incubation.

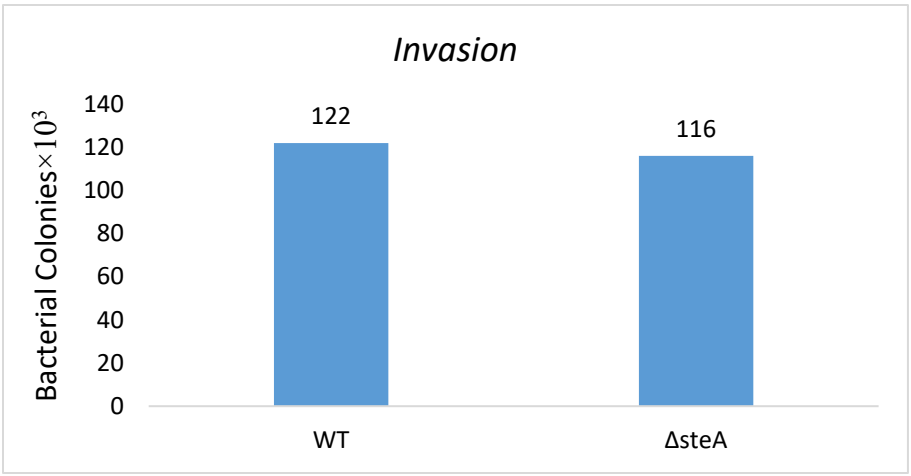
Annexin-PI is a method basically used to count the number of cells that have undergone apoptosis works on the principle of membrane flipping. As normal cells express phosphatidylserine in the inner membrane by which they are hydrophobic in nature, and when cells go through apoptosis, their inner membrane flips by which phosphatidylserine expose in the outer membrane. We took WT and Δ*steA* treated cells and stained them with Annexin-V FITC and PI stain where PI intercalates with the DNA of damaged cells and Annexin-V binds to cells showing phosphatidylserine on the outer leaflet. As shown in figure 14, the early apoptotic cell population (Q3) was almost equal in both WT and Δ*steA* treated samples. We repeated this experiment three time to get the confirmation and observed the same result, i.e. no difference in early and late apoptotic cell population in both samples.

Parameters- MOI=20

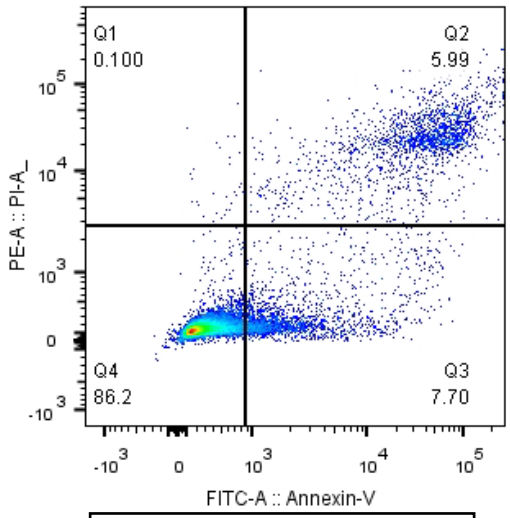
Time point- 12 h post infection

Invasion- 2 h post infection

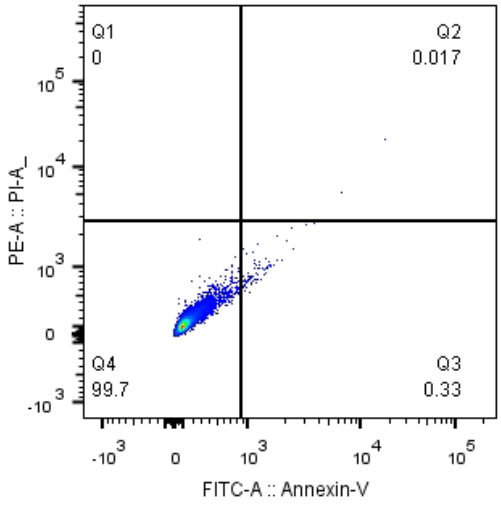
Positive control- Staurosporine (1µm)



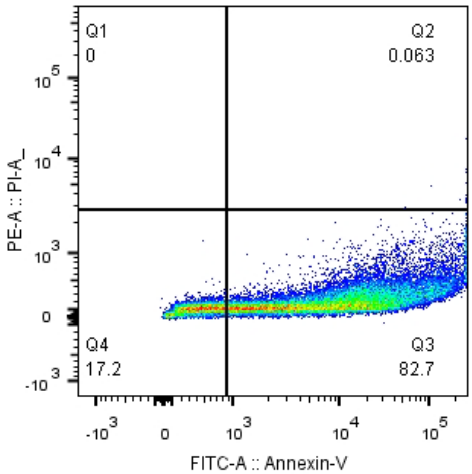
(a)



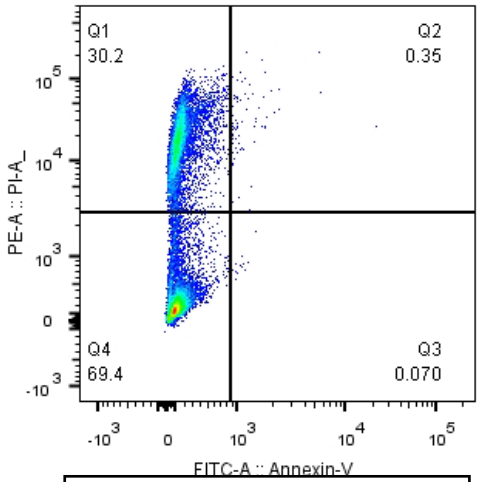
Untreated- Double stained



Untreated- Unstained



Staurosporine- FITC stained



Staurosporine- PI stained

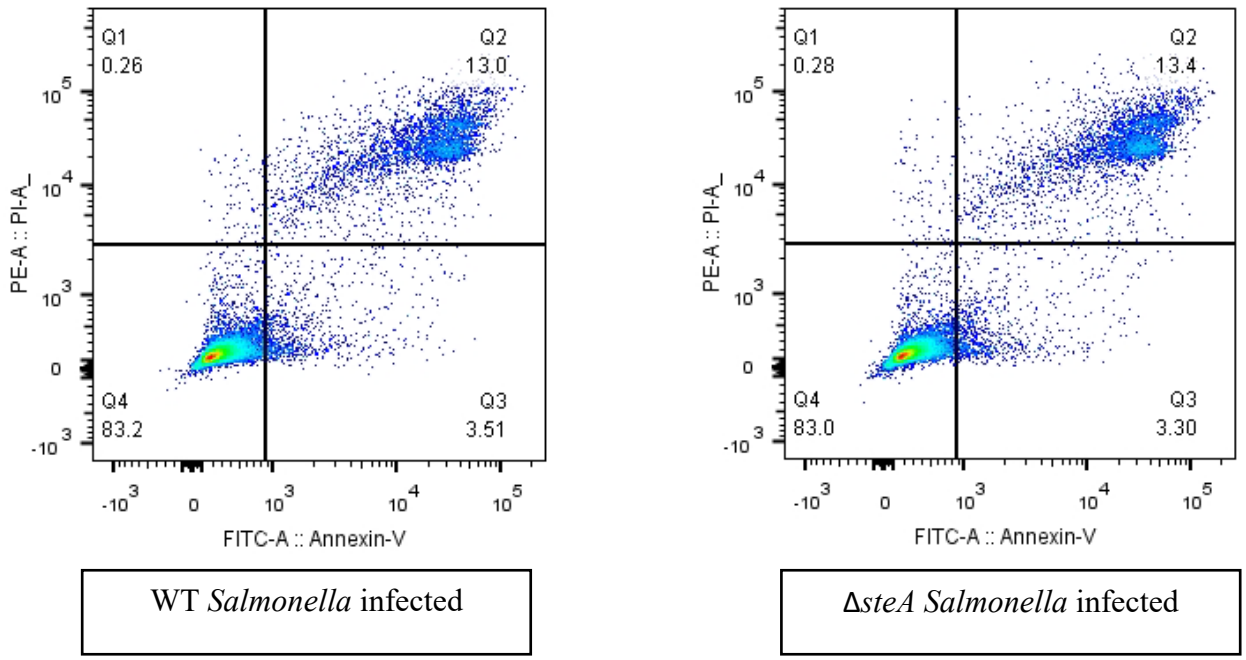
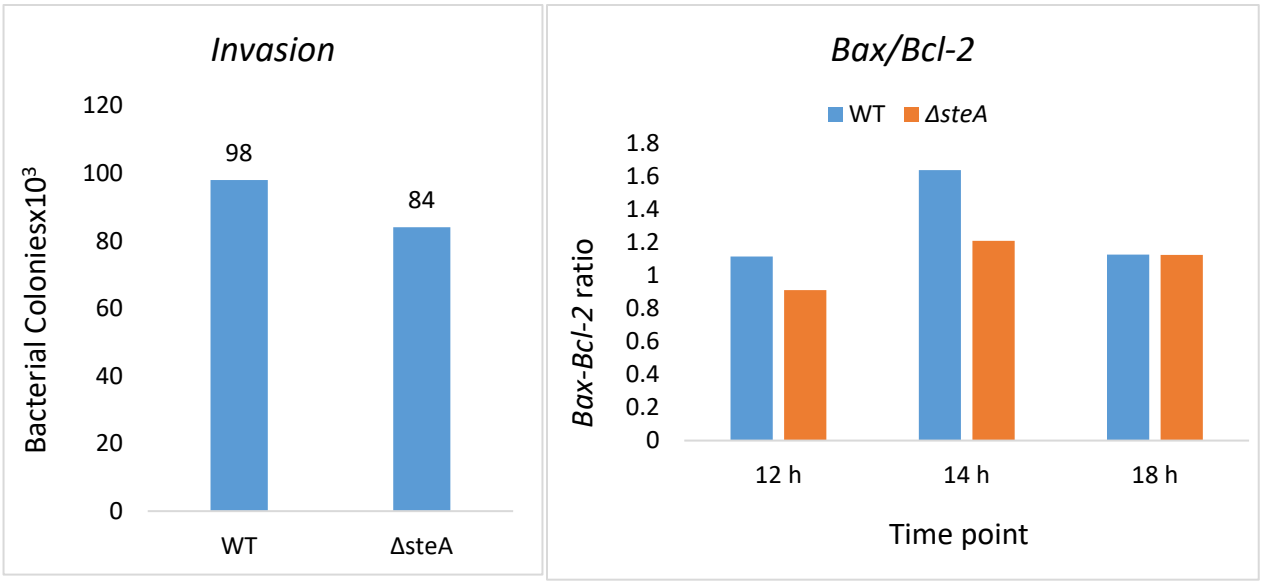


Figure 15 – **Flow cytometry based Annexin-PI data** (a) Bar graph represents equal invasion in both WT and Δ*steA* bacterial strain. The flow cytometry plots represent Annexin-V FITC on X axis and the Y axis shows PI. As it is observable from the plots that the early apoptotic cells (Q3) and late apoptotic cells (Q2) in WT and Δ*steA* are almost similar.

3.2.4 Caspase-3 is not involved in cell death due to *Salmonella* effector protein SteA in RAW264.7 cells

The Caspase cascade is an essential step for executing cell death followed by cytochrome c release. Similarly, Caspase-3 is known to be a crucial mediator of programmed cell death (Apoptosis). In this experiment, we checked the involvement of Caspase-3 in samples treated with Wild type and Δ*steA* bacterial strain in SPI-2 induced condition. Western blotting was performed and compared the level of Caspase-3 at 12 h post-infection. Uninfected RAW264.7 cells was taken as a control for conducting the experiment and analyzing the data. Before making the cell lysates of samples, we checked the invasion of bacteria in both WT and Δ*steA* by enumerating CFU after overnight incubation of plates that were plated after 2h of infection. As shown in the blots given below, we observed no difference in the level of Caspase-3 of WT and Δ*steA*. Although the housekeeping



(a) (b)

Figure 19 – qRT-PCR data at different time point showing ratio of *Bax/Bcl-2* Bar graphs represents (a) Invasion of bacterial strains WT and Δ*steA* 2 h.p.i on RAW264.7 cell line where Y axis represents number of colonies on LB agar plates, and observed almost equal invasion (b) qRT-PCR data showing the ratio of *Bax/Bcl-2* on cells treated with WT and Δ*steA* by taking different time point and observed some difference at 12 and 14 h post infection.

3.3 Discussion and conclusion:

To identify the function of *Salmonella typhimurium* effector protein SteA, we quantified the cell death that takes place in RAW264.7 cells infected with *Salmonella* WT and Δ*steA* bacterial strains by using LDH release assay. We induced SPI-2 Condition as it was already known that in SPI-1 inducing state, SteA does not play any role in cell health. Treating the cells at MOI=20 on 16 and 24 h post-infection, the difference in cell cytotoxicity of WT and Δ*steA* was observed. This project aims to identify the type of cell death taking place due to effector protein SteA in SPI-2 inducing conditions. As our senior got the hint by the mass spectrometry data that SteA shows interaction with many proteins related to Apoptosis and Endocytosis like Apoptosis inhibitor-5 (API-5). We tried to detect the apoptotic cells in both WT and Δ*steA* infected cells by using a flow cytometry-based Annexin-PI experiment. In this experiment, we didn't observe any difference in early apoptotic cells of both samples. We were not able to confirm whether apoptosis is taking place or not due to effector protein SteA.

Then we tried another method to characterize the cell death by checking the involvement of Caspase-3, which is the executioner Caspase, using western blotting. The signalling pathway of apoptosis involves the significant role of the Caspases, which includes many Caspases. Caspase-3 is found to be not involved in cell death due to *Salmonella* SteA effector protein. qRT-PCR data revealed that the *Bax/Bcl-2* ratio in cells infected with WT and Δ*steA* bacterial strain was not significantly different at 16 and 18 h of infection, but we observed some difference in this ratio at 12 and 14 h post-infection, which was more in WT sample as compared to Δ*steA*. However, this data needs to be replicated; however, Bax and Bcl-2 might be involved in the signalling pathway of cell death occurring due to effector protein SteA in SPI-2 inducing condition.

Cell cycle progression was conducted to examine the effect of SteA on cell cycle arrest as it interferes with the function of Cullin-1. As Cullin-1 helps in cell proliferation and regulates cell cycle progression, there might be a possibility that its function is getting compromised by interacting with SteA. We observed the difference in the G2 population of WT and *ΔsteA* by doing a flow cytometric analysis of the cell cycle. However, the protocol needs optimization to check the

effect of SteA on cell cycle progression, but we got the idea that cell cycle is getting affected by the effector protein SteA.

From this work we can conclude that *Salmonella* effector protein SteA plays role in cell health and causes cell death in RAW264.7 murine macrophages. Characterization of cell death was examined by checking apoptosis markers like Annexin- PI where we observed no difference in apoptotic cells in both WT and Δ*steA*. Gene expression level of *Bax* and *Bcl-2* was observed as they are crucial member of B-cell lymphoma family protein and observed some difference at 12 and 14 h post-infection of WT and *ΔsteA*. However biological replicates of this experiment need to be performed. Cell cycle analysis gave a preliminary idea that SteA is affecting cell cycle by interacting with Cullin-1.

3.4 Future outlook:

Future experiments could include the following experiments listed below:

- Biological replicates of the qRT-PCR experiment need to be performed for checking the gene expression level of *Bax* and *Bcl-2* at time points 12 and 14 h post-infection.
- Checking the involvement of p53 (tumor suppressor gene) on the signalling pathway causing cell death due to effector protein SteA.
- Checking the cell cytotoxicity of RAW264.7 cells treated with *Salmonella* bacterial strain of WT and Δ*steA* by giving treatment of tBID (truncated p15 BID) inhibitor.
- Checking the markers of other types of cell death, for example- necrosis, autophagy, etc.
- Biological replicates of cell cycle experiments need to be performed to check the effect of SteA on the cell cycle.

Salmonella typhimurium effector protein SteA can be purified, and then its effect could be studied as in general, Salmonella contains many effector proteins, so there is a probability that the two effector protein is responsible for performing similar function and altering the result that are obtained in the absence of SteA. After purification of the protein, we can check other apoptosis markers and confirm the type of cell death taking place due to SteA and further perform an experiment to analyze whether it is necessary or sufficient for causing cell cytotoxicity in macrophages.

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