A study on the phagosomal survival mechanisms of *Salmonella* enterica Serovar Typhi in macrophages: focus on type III secretion system 2 (T3SS-2)-independent mechanisms

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By

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This is to certify that the thesis entitled "**A study on the phagosomal survival mechanisms of *Salmonella* enterica Serovar Typhi in macrophages: focus on type III secretion system 2 (T3SS-2)-independent mechanisms**" submitted by Smt. Swarnali Chakraborty who got her name registered on **02.03.2022 Index No: 76/22/LifeSc./27 Registration No: SLSBT1107622** for the award of Ph.D. (Science) Degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Dr. Santasabuj Das** and that neither this thesis nor any part of it has been submitted for either any degree/ diploma or any other academic award anywhere before.

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DECLARATION

I do, hereby declare that the thesis entitled "**A study on the phagosomal survival mechanisms of *Salmonella* enterica Serovar Typhi in macrophages: focus on type III secretion system 2 (T3SS-2)-independent mechanisms**" submitted for the award of Ph.D. (Science) in Life Science and Biotechnology, is the completion of work carried put under the supervision of Dr. **Santasabuj Das, Scientist G** at the division of Clinical Medicine, ICMR- National Institute of Cholera and Enteric Diseases, Kolkata. Neither this thesis nor any part of it has been submitted for either any equivalent degree/ diploma or any other academic award anywhere before.

Date –10/03/2025

Place – Kolkata

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.....

(Signature of the candidate)

SWARNALI CHAKRABORTY

I Dedicate this thesis to
Maa, Babai, Didibhai and Anoy.

The people who made this journey
easier, swifter, and bearable

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Swarnali Chakraborty

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Abbreviations

µg	microgram
µL	microliter
µM	micromolar
M	Molar
APCs	Antigen Presenting Cells
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CFU	Colony-forming units
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DTT	Dithiothreitol
e.g.	Exempli Gratia / For Example
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate g Gram
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescence Protein
HRP	Horseradish peroxidase
IκB	International Klein Blue
IL	Interleukin
in vitro	latin for within glass
in vivo	latin for within living
iNOS	Inducible Nitric Oxide Synthase
IP	Intra Peritoneal

IPTG	Isopropyl β-D-Thiogalcopyranoside
Kb	Kilo base
kDa	Kilodalton
kg	Kilogram
L	Liter
LA	Luria Bertani Agar
LB	Luria Bertani Broth
LD50	Lethal Dose 50%
LMP	Lysosomal Membrane Permeabilization
LPS	Lipopolysaccharide
MDR	Multi Drug Resistance
MFI	Mean Fluorescence Intensity
mg	milligram
MHC	Major Histocompatibility Complex
mL	milliliter
MLNs	Mesenteric lymph nodes
MOI	Multiplicity of Infection
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
OMP	Outer Membrane Protein
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate-buffered saline
pBSK	pBluescriptSK
pH	Negative logarithmic measure of hydrogen ion concentration
PP	Peyer's Patches
PVDF	Polyvinylidenedifluoride
qPCR/qRT-PCR	Quantitate/ Quantitate real-time polymerase chain reaction
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species

RPMI	Roswell Park Memorial Institute Medium
RT-PCR	Real-time polymerase chain reaction
SCV	*Salmonella* Containing Vacuole
SD	Standard Deviation
SDM	Site-Directed Mutagenesis
SDS	Sodium Dodecyl Sulfate
Sec	Second
SPI	*Salmonella* Pathogenicity Island
STm	Salmonetlla Typhimurium
STy	*Salmonella* Typhi
T3SS/ TTSS	Type 3 Secretion System
TBS	Tris-Buffered Saline
TCR	T cell Receptor
TEMED	Tetra methyl ethylene diamine
Th1	Type 1 T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TT	Tetanus Toxoid
Vi	Vi capsular Polysaccharide viz namely
WHO	World Health Organization

Symbols

%	Percentage
°C	Degree Celsius
±	plus-minus
α	Alpha
β	Beta
γ	Gamma
Δ	Delta
K	Kappa

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Chapter-1

General introduction and review of literature

1.1 Background: -

Intracellular pathogens spend a substantial part of their life cycle within host cells and have evolved specialized virulence strategies to thrive in this environment. Despite the host's robust defence mechanisms, including innate microbial recognition, autophagy, and cell-mediated immunity, these pathogens have adapted to exploit the intracellular milieu. The variation between intracellular and extracellular pathogens can be blurry. Instead, it's more relevant to consider whether a pathogen is highly specialized to thrive inside host cells, relying on this intracellular environment to drive its infectious process. Intracellular pathogens can be categorized into two groups: those that reside within membrane-bound vacuoles, such as phagosomal vacuoles, and those that proliferate in the host cell cytoplasm. Upon host cell entry, phagosomes rapidly become a hostile environment, triggering acidification and fusion with lysosomes, which can lead to microbial killing (Flannagan, Cosio, et al. 2009). To evade this fate, intracellular bacteria employ strategies such as vacuolar manipulation or escape into the cytosol, allowing them to survive and propagate within the host cell. Among them, *Salmonella* is a professional intracellular bacteria that divides in a specialized compartment, termed *Salmonella* Containing Vacuoles (SCV), and exploits the host endo-lysosomal pathway (Eswarappa, Negi et al. 2010).

1.2. Eukaryotic endo-lysosomal pathway: -

The Endocytic Pathway constitutes the uptake mechanism of cells and is made of different organelles known as endosomes. Endosomes traffic from Early endosomes to Late endosomes and finally to Lysosomes (Gruenberg and van der Goot 2006). (Figure 1)

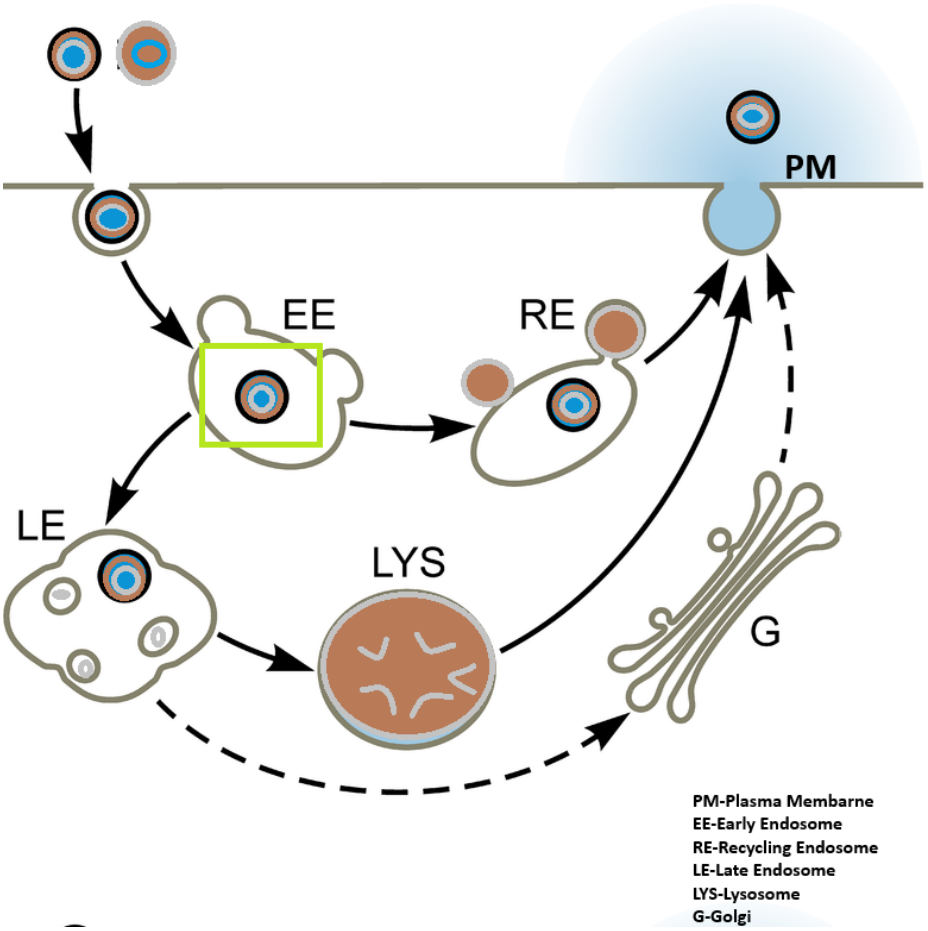

Figure 1: - Eukaryotic endo-lysosomal pathway (Burks, Legenzov et al. 2015)

Early Endosomes

Early endosomes are the first sorting station in the endocytic pathway. They receive incoming vesicles from the plasma membrane and sort their contents for recycling, degradation, or transport to other compartments. Some lipids and proteins are recycled back to the plasma membrane through recycling endosomes or the trans-Golgi network (TGN). Other molecules are collected in multivesicular endosomes and transported to late endosomes for degradation.

Multivesicular Endosomes

Multivesicular endosomes form through inward membrane invaginations. They accumulate intraluminal vesicles, which contain molecules for degradation. Multivesicular bodies (MVBs) transport these vesicles to late endosomes.

Late Endosomes: A Crucial Sorting Hub

Late endosomes serve as the second key sorting station in the endocytic pathway, playing a pivotal role in directing the fate of internalized molecules. Within late endosomes, molecules are sorted and routed to different destinations. Some proteins and lipids are recycled back to the Trans Golgi Network (TGN), while others are transported to lysosomes for degradation. Late endosomes can associate directly with the plasma membrane in specialized cell types.

Lysosomes

Lysosomes are membrane-bound organelles responsible for cellular digestion and recycling. They were first discovered by Christian René de Duve in 1955. Lysosomes contain various hydrolytic enzymes that break down complex molecules into their constituent parts, which are then recycled through anabolic pathways(Sachdeva and Sundaramurthy 2020).

Key Functions of Lysosomes

1. **Degradation and Recycling**: Lysosomes digest and recycle cellular waste, foreign substances, and damaged organelles.

2. **Cellular Defence**: Lysosomes play a crucial role in defending against pathogens by degrading bacterial and viral components.

Biogenesis of Lysosomes

Lysosomal biogenesis is a complex, highly regulated process involving the coordinated action of various genes, proteins, and cellular pathways. It begins with transcriptional regulation, where the transcription factor EB (TFEB) plays a key role in regulating the expression of genes involved in

lysosomal biogenesis, while the mechanistic target of rapamycin complex 1 (mTORC1) controls TFEB activity. Lysosomal proteins, such as lysosomal-associated membrane proteins (LAMPs) and soluble lysosomal enzymes, are synthesized in the endoplasmic reticulum (ER) and transported to the Golgi apparatus for modification and processing. The modified proteins are then transported to the lysosome, where they are assembled into a functional lysosome. This process is regulated by nutrient availability, energy status, and growth factors, and is critical for maintaining cellular homeostasis, with impaired lysosomal biogenesis implicated in various diseases, including lysosomal storage diseases, neurodegenerative diseases, and cancer.

Regulation of Lysosomal Function

Lysosomal regulation is a multifaceted process that ensures proper lysosomal function and maintenance. pH regulation is critical, as lysosomes maintain an acidic pH (around 4.5-5.0) through the V-ATPase complex, which is essential for the optimal activity of lysosomal enzymes. Calcium regulation also plays a vital role, as calcium ions modulate enzyme activity, membrane fusion, and lysosomal exocytosis, with calcium channels and pumps controlling calcium influx and efflux. Furthermore, the cytoskeleton, comprising microtubules, microfilaments, and intermediate filaments, regulates lysosomal movement and fusion, ensuring efficient digestion and recycling of cellular components, with motor proteins like dynein and kinesin facilitating lysosomal transport along microtubules. These regulatory mechanisms collectively maintain lysosomal homeostasis, enabling cells to adapt to changing conditions and maintain cellular integrity. Furthermore, post-translational modifications, such as phosphorylation and ubiquitination, also regulate lysosomal enzyme activity and lysosomal membrane protein stability, ensuring proper lysosomal function and cellular homeostasis.

Diseases Associated with Lysosomal Dysfunction

1. **Lysosomal Storage Diseases**: Genetic disorders that result in the accumulation of toxic substances within lysosomes, leading to cellular damage and disease.

2. **Neurodegenerative Diseases**: Lysosomal malfunction can cause neurodegenerative diseases, like Alzheimer's and Parkinson's.

1.3. Intra-cellular bacteria that exploit host endo-lysosomal pathway: -

1.3.1. Entry within cell: -

Intracellular pathogens utilize various entry routes into host cells, primarily via uptake by phagocytes, such as macrophages, which serve as their primary intracellular survival and replication niche(Thi, Lambertz et al. 2012).

To optimize survival, pathogens often bypass opsonophagocytosis via the Fcγ receptor (FcγR) and utilize alternative phagocytic receptors, such as complement receptor 3(Underhill and Ozinsky 2002). Pathogens have also evolved to induce their uptake by non-phagocytic cells, including epithelial and endothelial cells, using mechanisms like the 'zipper' and 'trigger' mechanisms (Groves, Dart et al. 2008). (Figure2) (Ribet and Cossart 2015)

The trigger mechanism causes dramatic extensions of cells or membrane 'ruffles,' resembling macropinosome formation (Groves, Dart et al. 2008), and is employed by pathogens like *Salmonella* and *Shigella* to enter epithelial cells via a type III secretion system (T3SS), which delivers effector proteins into the host cell, subverting host F-actin polymerization (Galan and Collmer 1999, Cossart and Sansonetti 2004).

Zipper-type phagocytosis involves a sequential and continuous interaction between host cell receptors and bacterial surfaces. This process is facilitated by bacterial surface ligands binding to host cell receptors, mimicking conventional phagocytosis. As a result, pseudopodia engulf the bacteria, forming a phagosome. Unlike the trigger mechanism, zipper-type phagocytosis does not require extensive

reorganization of the cortical actin cytoskeleton. Instead, it is driven by signaling events initiated at the cell surface (Galan and Collmer 1999). A well-documented example of a zippering entry is the invasion of non-phagocytic cells by Listeria monocytogenes, a Gram-positive foodborne pathogen(Pizarro-Cerda, Kuhbacher et al. 2012).

Microbial pathogens mainly employ two distinct entry processes, zipper and trigger mechanisms, to invade host cells. Despite their differences, both mechanisms involve the manipulation of host pathways that regulate the cortical actin cytoskeleton, specifically targeting Rho GTPases and PI3Ks. This manipulation can occur directly or indirectly through interactions with cell surface signaling receptors(da Silva, Cruz et al. 2012).

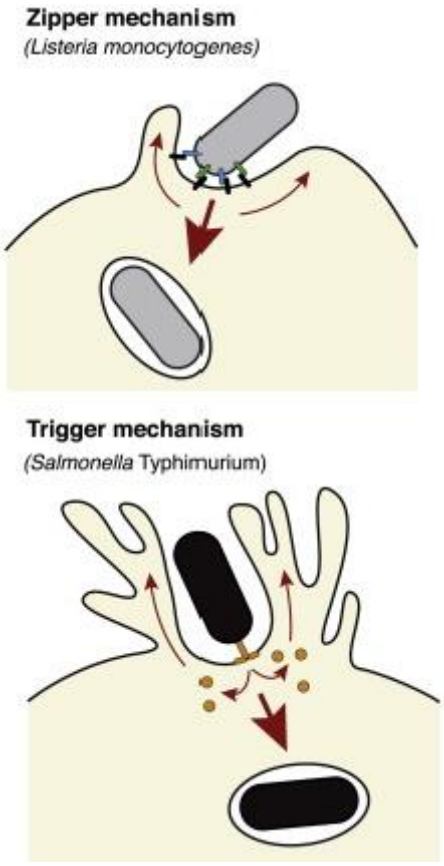

Figure 2: - Different types of bacterial entry within the cell

1.3.2. Vacuolar life after entry: -

Despite the challenges, most intracellular pathogens thrive in the phagocytic vacuole, utilizing it as a niche for survival and replication. This membrane-bound compartment provides microbes with enhanced protection against immune recognition (Kumar and Valdivia 2009). However, maintaining a vacuolar lifestyle requires reprogramming endosomal traffic to evade lysosomal destruction and remodeling the vacuole to acquire essential nutrients (Kinchen and Ravichandran 2008). Key regulators, such as Rab GTPases, play a crucial role in endocytic traffic and membrane transport regulation, enabling pathogens to exploit the host's cellular machinery for their survival and proliferation(Stein, Pilli, et al. 2012). The localization of Rab5 to early phagosomes is crucial, as it orchestrates the recruitment of effector proteins, promoting maturation and downstream recycling. This process involves Rab conversion, where Rab5 is replaced by Rab7, regulating the transition to the late-endosome stage (Flannagan, Cosio et al. 2009). The accumulation of PtdIns(3)P, a product of the Rab5 effector Vps34, on the early endosome facilitates this conversion (Fairn and Grinstein 2012) (Cain and Ridley 2009). Ultimately, Rab7 recruits the vacuolar proton pump ATPase (V-ATPase), leading to phagosome acidification and fusion with lysosomes. Pathogens exploit the complexity of host signalling pathways, particularly the PI3K and small GTPases, to disrupt phagosome maturation and create a conducive environment for microbial proliferation (Fairn and Grinstein 2012) (Cain and Ridley 2009). The PI3K isoform regulates the recruitment of PtdIns(3)P-binding proteins, which execute critical membrane trafficking steps (Fairn and Grinstein 2012). Pathogens target these pathways, leveraging the multitude of effectors and intricate mechanisms to intervene and transform the phagocytic compartment into a hospitable habitat.

1.4. Different survival strategy of intracellular bacteria by altering the host’s endo-lysosomal pathway: -

1.4.1. Delay of inhibition in phagosome maturation: -

Microbes employ a key strategy to evade lysosomal killing by disrupting the endosome maturation pathway. Specifically, pathogens such as *Mycobacterium tuberculosis* and *Salmonella* manipulate phosphoinositide metabolism, altering the levels of the crucial signaling lipid PtdIns(3)P. This modification enables the microbes to subvert endocytic trafficking and create a hospitable environment for survival and replication.

Mycobacterium tuberculosis (M. tuberculosis) employs multiple mechanisms to evade lysosomal killing by interfering with the endosome maturation pathway. The bacterium alters phosphoinositide metabolism, specifically reducing levels of PtdIns (3)P, a key signaling lipid, in the Mycobacterium-containing vacuole (MCV). *M. tuberculosis* utilizes two redundant mechanisms to deplete PtdIns(3)P: (1) inhibition of Vps34 PI3K membrane recruitment and activation by its cell envelope glycolipid lipoarabinomannan (LAM) (Fratti, Chua et al. 2003) (Vergne, Chua et al. 2003) and (2) secretion of effector proteins with PtdIns phosphatase activity, including SapM and MptpB, which dephosphorylate and remove phagosomal PtdIns(3)P (Vergne, Chua et al. 2005), (Beresford, Patel et al. 2007). *M. Tuberculosis* manipulates endosomal trafficking by depleting PtdIns(3)P, which interferes with the recruitment of EEA1, a crucial effector for early-endosome fusion (Fratti, Backer et al. 2001). To compensate, the mycobacterial cell envelope glycolipid PIM stimulates early-endosomal fusion, ensuring the exchange of essential cargo (Vergne, Fratti et al. 2004). The depletion of PtdIns(3)P also prevents Rab7 activation and V-ATPase recruitment, arresting the vacuole in a non-acidic early phagosome state. Additional mycobacterial products, such as PtpA and a Rab7-deactivating factor, further prevent lysosomal fusion by inhibiting V-ATPase recruitment and disrupting Rab7 interactions (Wong, Bach, et al. 2011) (Sun, Deghmane et al. 2007).

Salmonella, a Gram-negative enteric pathogen, actively increases PtdIns(3)P levels in its vacuole (Salmonella-containing vacuole, SCV) through the action of its T3SS effector SopB (Steele-Mortimer 2008) (Marcus, Wenk et al. 2001). This increase in PtdIns (3)P recruits PtdIns (3)P-binding proteins, such as sorting nexin-1 (SNX1), contributing to SCV remodeling (Hernandez, Hueffer et al. 2004).

The SCV transiently accumulates markers, including EEA1, Rab5, Rab7, and LAMP1, before fusing with lysosomes. However, extensive communication with the endosomal and trans-Golgi network leads to the formation of large, modified vacuoles that impair the accumulation of bactericidal factors, allowing *Salmonella* to persist intracellularly (Mukherjee, Parashuraman et al. 2001). These processes help to escape the lysosomal degradation pathway and the bacteria get nutrition from the vesicles of Golgi traveling via microtubules.

1.4.2. Escaping the endocytic pathway: -

L. pneumophila, the causative agent of Legionnaires' disease, evades lysosomal killing by connecting its vacuole to the endoplasmic reticulum (ER) by targeting host cell GTPases, forming a distinct compartment called the 'Legionella-containing vacuole' (LCV)(Newton, Ang et al. 2010). The bacterium utilizes effectors delivered by its Dot/Icm type IV secretion system (T4SS) to target GTPases, including Rab1, Sar1, and Arf1, and divert the phagosome into the ER network(Nagai, Kagan et al. 2002, Kagan, Stein et al. 2004), with over 50 identified Dot/Icm effectors targeting GTPases, PtdIns metabolism, and apoptotic pathways(Bruggemann, Cazalet et al. 2006). The key Effectors involved in this process are RalF, SidM/DrrA (GEF activity), and LepB (GAP activity) and the role of these effectors are GEF/GAP pair modulation, GDI displacement, and AMPylation of Rab1 switch II region (Nagai, Kagan et al. 2002, Machner and Isberg 2006, Murata, Delprato et al. 2006, Ingmundson, Delprato et al. 2007). Legionella effectors SidM/DrrA and SidD engage in antagonistic interactions, with SidM/DrrA AMPylating Rab1 and SidD functioning as a de-AMPylating enzyme (Muller, Peters et al. 2010, Tan and Luo 2011). To create an ER-like structure for intracellular growth, Legionella exploits host PtdIns lipid signaling, anchoring effectors like SidM/DrrA, SidC, and SdcA to the LCV membrane via PtdIns (4)P binding(Machner and Isberg 2006, Weber, Ragaz et al. 2006). Key Effectors are SidM/DrrA (Rab1 AMPylation and PtdIns(4)P binding), SidD (de-AMPylation),

SidC and SdcA (PtdIns(4)P binding and ER marker recruitment), and cross-talk between Rab and PtdIns signaling on the LCV membrane helps in the bacterial survival (Ragaz, Pietsch et al. 2008, Brombacher, Urwyler et al. 2009, Zhu, Hu et al. 2010).

Chlamydia, an obligate intracellular pathogen, diverts its vacuole from the endocytic pathway to survive, colonizing epithelia and causing genital, ocular, and respiratory infections. The Life-Cycle Stages include Elementary bodies (metabolically inactive) which transform into reticulate bodies (metabolically active) upon uptake, proliferating in a large endosome-derived vacuole called an 'inclusion' or Chlamydia-containing vacuole (CCV)(Fields and Hackstadt 2002). CCV divergences from the endocytic pathway, associates with Golgi apparatus, and causes dynein-dependent transport to the perinuclear region, which coalesce with exocytic Golgi-to-plasma membrane pathway to recruit Rab GTPases (excluding Rab5 and Rab7)(Rzomp, Scholtes et al. 2003). CCV acquires sphingomyelin, ceramide, cholesterol, and other cargo lipids from Golgi and multivesicular body (MVB) compartments for inclusion membrane growth, stability, and nutrient support (Beatty 2006) (Carabeo, Mead et al. 2003, Elwell, Jiang et al. 2011) (Moore, Fischer et al. 2008, Heuer, Rejman Lipinski et al. 2009). Chlamydia's effector proteins, including CPAF (serine protease/proteasome-like) and Inc proteins (T3SS effectors), support CCV biogenesis and stabilization (Valdivia 2008, Jorgensen, Bednar et al. 2011). Inc proteins, such as CT229, Cpn0585, CT147, IncA, and IncG, have diverse functions, including binding to Rab GTPases (Rab4, Rab1, Rab10, and Rab11), modulating transport, tethering endosomes, promoting homotypic inclusion fusion, inhibiting endocytic SNARE-mediated membrane fusion, and exerting antiapoptotic effects. The key Inc Proteins are CT229 (Rab4 binding)(Rzomp, Moorhead et al. 2006), Cpn0585 (Rab1, Rab10, and Rab11 binding)(Cortes, Rzomp et al. 2007), CT147 (EEA1 mimic)(Ronzone and Paumet 2013), IncA (SNARE-like domains), IncG (antiapoptotic effect) (Ronzone and Paumet 2013) (Verbeke, Welter-Stahl et al. 2006), and IncB, Inc101, Inc222, and Inc850 (Src family kinase binding)(Mital, Miller et al. 2010).

1.4.3. Surviving in phagolysosome: -

Coxiella burnetii, the causative agent of Q-fever, is a γ-Proteobacteria that targets macrophages and replicates in an acidic parasitophorous vacuole (PV) resembling a large lysosome (Voth and Heinzen 2007). PV formation follows the default endosomal maturation-phagolysosomal fusion pathway, with sequential recruitment of Rab5 and Rab7 GTPases, V-type H1 ATPase, lysosomal markers (LAMP1, LAMP2, LAMP3), and lysosomal enzymes (acid phosphatase, cathepsin D) (Ghigo, Colombo et al. 2012). *Coxiella* resists PV's microbicidal conditions and acidic pH (pH 5.0), using the latter as a signal to become metabolically active. It delays the transit to the lysosomal stage, allowing time for activation of the metabolically dormant infective form and manipulating the expanding PV (Ghigo, Colombo et al. 2012) (Hackstadt and Williams 1981). The parasitophorous vacuole (PV) interacts with autophagosomes, which are marked by LC3 and Rab24, and the early secretory pathway, regulated by Rab1b GTPase (Romano, Gutierrez et al. 2007) (Gutierrez, Vazquez et al. 2005) (Campoy, Zoppino et al. 2011). These interactions enable the PV to acquire membrane components and nutrients, facilitating its expansion. The PV's interaction with autophagosomes may play a crucial role in Coxiella's survival. This interaction may recruit anti-apoptotic Bcl-2 through Beclin 1, a regulator of autophagy localized to the PV (Vazquez and Colombo 2010). This recruitment can potentially protect Coxiella from host cell apoptosis. *Coxiella* deploys its effectors using a Type IV secretion system (T4SS) that shares homology with the *Legionella* Dot/Icm secretion apparatus (Voth and Heinzen 2009).

1.4.4. Escaping from vacuole to cytosol: -

A subset of intracellular pathogens, including *Shigella, Listeria monocytogenes, Rickettsia*, and *Burkholderia pseudomallei*, employ a bolder strategy to escape destruction by breaking free from the phagosome and multiplying in the cytosol. *Shigella* enters host cells via the 'trigger' mechanism using a T3SS apparatus, then disrupts the phagosome using pore-forming effectors (Cossart and Sansonetti 2004) (Senerovic, Tsunoda et al. 2012). *L. monocytogenes* causes listeriosis, a severe invasive foodborne infection, and escapes the phagosome using listeriolysin O (LLO) and phospholipase C (PLC) effectors

(Bielecki, Youngman et al. 1990, Vazquez-Boland, Kocks et al. 1992, Smith, Marquis et al. 1995, Schnupf and Portnoy 2007, Lin, Xing et al. 2024) (Marquis and Hager 2000). *Rickettsia* is an obligate intracellular α-proteobacterium that causes vector-borne spotted fevers via endothelial colonization (Walker 2007). *Burkholderia pseudomallei* causes melioidosis, an invasive infection endemic in tropical and subtropical areas, and escapes the phagosome using type III secretion system (T3SS) effectors (Stevens and Galyov 2004). Cytosolic pathogens, including *Burkholderia, Rickettsia,* and *Mycobacterium marinum*, employ various strategies to enter non-phagocytic cells, such as the trigger mechanism and interaction with host cell receptors. To thrive in the cytosol, pathogens must evade autophagy, innate immune recognition via cytosolic NOD-like receptors (NLRs), and CD8+ T cell-mediated cytotoxic immunity (Franchi, Warner et al. 2009) (Portnoy, Auerbuch et al. 2002, Wong and Pamer 2003). Highly adapted cytosolic pathogens have evolved a "runaway" escape strategy, leveraging actin-mediated motility to navigate through the cytosol and spread to adjacent cells (Stevens, Galyov et al. 2006). By hijacking the host cell's actin cytoskeletal machinery, these pathogens can propel themselves rapidly and evade immune detection. In contrast, pathogens that adopt a vacuolar lifestyle appear to be more effective at evading immune recognition and promoting long-term persistence (Stevens, Galyov et al. 2006). This strategy is widespread among intracellular pathogens, suggesting its advantages in facilitating survival and replication within host cells.

To avoid vacuole acidification and destruction, cytosolic pathogens like *Listeria monocytogenes* rapidly escape from the phagosome within 30 minutes of internalization (Henry, Shaughnessy et al. 2006). Escape is triggered by environmental cues in the vacuole, such as a drop in pH, and facilitated by secreted membrane-damaging toxins and enzymes (Cossart and Mengaud 1989). *Listeria* employs listeriolysin O (LLO), a cholesterol-dependent cytolysin (CDC), and phospholipases PlcA and PlcB to disrupt the vacuole membrane. LLO is optimally active at acidic conditions (pH 5.5), confining its pore-forming activity to the late-endosomal compartment, and preventing host cell destruction (Bielecki, Youngman et al. 1990, Smith, Marquis et al. 1995) (Vazquez-Boland, Kocks et al. 1992,

Schnupf and Portnoy 2007). PlcA and PlcB cooperate with LLO to rapidly achieve vacuole membrane disruption, with PlcB playing a key role in disrupting the double-membrane vacuole formed during cell-to-cell spread (Marquis, Doshi et al. 1995, Marquis and Hager 2000).

While *Listeria's* escape mechanism is well-studied, other cytosolic pathogens employ distinct strategies. *Shigella flexneri* uses T3SS effectors IpaB and IpaC to form a pore in the target eukaryotic membrane, promoting entry and potentially rupturing the *Shigella*-containing vacuole (High, Mounier et al. 1992). IpaB exhibits hemolytic activity, and both IpaB and IpaC contribute to the lysis of the double-membrane vacuole during cell-to-cell spread (High, Mounier et al. 1992, Page, Ohayon et al. 1999). The escape of *Rickettsia* spp. , likely involves a membrane-damaging mechanism, utilizing hemolysin C and phospholipases (Whitworth, Popov et al. 2005). In the case of *Burkholderia*, the escape mechanism is unclear but dependent on its T3SS, as mutants lacking this secretion system fail to escape from phagosomes (Renesto, Dehoux et al. 2003, Burtnick, Brett et al. 2008).

1.4.5 By evading autophagy: -

Autophagy is an ancient, universally conserved process that targets and eliminates harmful cytoplasmic entities, including misfolded protein aggregates, damaged organelles, and invasive microorganisms(Mizushima and Komatsu 2011). This process involves the sequestration of these entities within double-membrane vesicles called autophagosomes, which subsequently fuse with lysosomes for degradation. In the case of bacterial infections, recognition by the autophagic machinery involves the activation of cytosolic pattern recognition receptors and/or the binding of ubiquitinated bacterial proteins to autophagy receptors. This binding process enables the recruitment of microbial cargo to autophagosomes via the phosphatidylethanolamine-LC3 conjugate (Ogawa, Mimuro et al. 2011, Jo, Yuk et al. 2013) (Mostowy, Bonazzi et al. 2010). To evade autophagic degradation, bacterial pathogens rely on actin-based motility to escape the autophagic machinery. Moreover, some pathogens have evolved mechanisms to directly interfere with and disrupt the autophagic process.

Listeria monocytogenes employ multiple strategies to avoid autophagy, including the phospholipases PlcA and PlcB, which help evade destruction in autophagosomes (Birmingham, Canadien et al. 2007). The actin-polymerizing protein ActA plays a key role in autophagy avoidance by disguising the bacterium with host cell proteins from the actin cytoskeletal machinery(Yoshikawa, Ogawa et al. 2009). The surface protein InlK binds to the cytoplasmic ribonucleoprotein organelle major vault protein (MVP), contributing to autophagy avoidance (Dortet, Mostowy et al. 2011). In contrast, *Shigella* triggers autophagy through recognition of the actin-polymerizing protein IcsA, involving recruitment of septins, which form cage-like structures around the bacteria (Ogawa, Yoshimori et al. 2005, Ogawa and Sasakawa 2011). *Shigella* may counteract septin-promoted autophagy via the IcsB virulence effector (Ogawa, Yoshimori et al. 2005, Mostowy and Cossart 2011) and suppress autophagocytic activity through the GAP activity of VirA (Dong, Zhu et al. 2012).

Certain pathogens, including *Listeria, Staphylococcus aureus, Brucella*, *Legionella*, and *Coxiella*, have evolved strategies to exploit autophagy for their benefit. *Listeria* dampens LLO activity to avoid cellular membrane damage(Cemma and Brumell 2012), colonizing the autophagosome and converting it into a spacious Listeria-containing phagosome (SLAP) for persistence(Birmingham, Canadien et al. 2008). S. aureus invades cells, subverts the autophagy pathway, and replicates within autophagosome-like vacuoles (Schnaith, Kashkar et al. 2007). *Brucella, Legionella,* and *Coxiella* recruit autophagocytic markers to their intracellular vacuoles, persisting in compartments with autophagolysosome characteristics(Starr, Child et al. 2012) (Joshi and Swanson 2011).

Among these intracellular pathogens, that exploit host phagolysosomal pathway to promote their survival, *Salmonella* is one of the important pathogens to study and explore.

1.5. Discovery of *Salmonella*: -

Salmonella, one of the most potential foodborne pathogens, has a history dating back to the late 19th century. The bacteria were first identified in 1885 by Daniel Elmer Salmon, an American veterinarian,

and Theobald Smith, a German-American microbiologist. In the 1880s, Salmon and Smith were investigating a swine disease outbreak in the United States. They isolated the bacteria from infected pigs and named it Eberth's *bacillus* , later renamed *Salmonella* choleraesuis in honor of Salmon(1978). In 1885, Salmon and Smith published their findings. After that, in 1890, *Salmonella* outbreaks occurred in poultry and livestock. After 1950, vaccines were developed for poultry.(Patrick, Adcock et al. 2004). *Salmonella*'s history highlights the importance of microbiological research, public health vigilance, and continued efforts to combat foodborne illnesses. Understanding its evolution informs strategies to mitigate risks and protect global health.

Nowadays, a total of around 200 million to 1 billion cases of *Salmonella* infections are documented worldwide per year. Among them, 93 million cases are recorded for gastroenteritis, and deaths are counted (155,000 deaths annually). Out of the deaths, the majority (approximately 85%) are due to the intake of contaminated food(Lamichhane, Mawad et al. 2024).

1.6. Classification of *Salmonella:* -

Salmonella can be classified in several ways: taxonomy, serological, phylogenetic, and clinical classification. Based on Taxonomic classification, *Salmonella* belongs to Kingdom Bacetria, Phylum Proteobacteria, Class Gammaproteobacteria, Order Enterobacteriales, Family Enterobacteriaceae, and Genus *Salmonella*.

Serological classification is also termed as Kauffmann-White Scheme. this classification is based on the presence of surface antigens of *Salmonella*. There are mainly O antigen, H antigen, and Vi antigen present in *Salmonella* (Popoff, Bockemuhl et al. 2001) and there are more than 2500 serotypes of *Salmonella* identified(Issenhuth-Jeanjean, Roggentin et al. 2014).

According to phylogenetic classification, *Salmonella* is currently classified into two species: *Salmonella bongori* (not much related to human infection) and *Salmonella enterica. Salmonella*

enterica is further divided into subspecies such as enterica (l) ,salamae (ll) , airzonae (lll) , diarizonae (llla) , houtenae (lV) (Achtman, Wain et al. 2012).

The clinical classification divides between Typhoidal *Salmonella* and non-Typhoidal *Salmonella*. Typhoidal *Salmonella* includes *Salmonella* Typhi (Sty) and *Salmonella* Paratyphi which cause typhoid fever. Non-typhoidal *Salmonella* causes gastroenteritis, and bacterrmia. Examples of non-typhoidal *Salmonella* include *Salmonella* enteritidis, *Salmonella* Typhimurium (STm), *Salmonella* Newport, *Salmonella* Javiana, etc(Hurley, McCusker et al. 2014). *Salmonella* classification is listed in Figure 1.3.

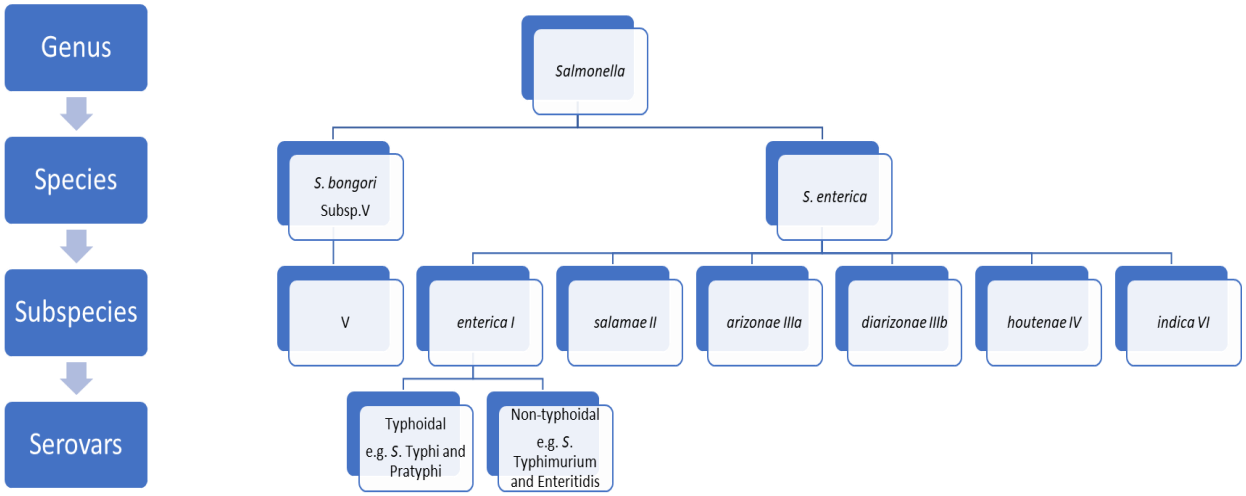

Figure 1.3: - Classification of *Salmonella*

1.7. Disease caused by STy: -

The bacterium STy causes life-threatening infection Typhoid fever. This fever typically spreads by consumption of contaminated food or water. After ingestion, the bacteria start to multiply and go into the bloodstream. Urbanization, civilization, and quick climate change increase the actual burden of the occurrence of the disease. Another cause is antibiotic resistance which makes typhoid spread easily.

Epidemiology, risk factors, and disease burden

WHO African, Eastern Mediterranean, South-East Asia, and Western Pacific Regions have become a prone area for Typhoid fever where Typhoid has become a public health problem. The proper doses of antibiotics and improvement in living conditions help to reduce typhoid fever morbidity and mortality in developing countries. People from the areas who cannot access pure water and face inadequate sanitation are at higher risk of Typhoid fever, especially children.

Symptoms

STy is a human-restricted pathogen. The bacteria reside in the bloodstream and intestinal tract of the infected person. According to WHO, the main symptoms include prolonged high fever, fatigue, headache, nausea, abdominal pain, and constipation or diarrhea. Some patients carry a rash. In severe cases, it may lead to even death. A blood test is the confirmatory test for typhoid fever.

Diagnosis

Diagnosis of typhoid fever typically involves a combination of clinical evaluation, laboratory tests, and imaging studies. Clinical evaluation involves assessing symptoms such as fever, headache, abdominal pain, and diarrhea. Laboratory tests include blood cultures, which are the gold standard for diagnosis, as well as serological tests such as the Widal test and the Typhidot test. The Widal test detects antibodies against *Salmonella* Typhi, while the Typhidot test detects antibodies against the O and H antigens of *Salmonella* Typhi. Imaging studies such as ultrasound and CT scans may be used to detect complications such as intestinal perforation or hemorrhage. Molecular diagnostic tests such as PCR (polymerase chain reaction) may also be used to detect the presence of *Salmonella* Typhi DNA in blood or stool samples. A diagnosis of typhoid fever is typically confirmed by a combination of positive blood cultures and clinical symptoms.

Treatment

Typhoid vaccines are commercially available, Ty21a (oral) and Vi polysaccharide (parenteral). The World Health Organization has added a Vi tetanus toxoid (Vi-TT) conjugate vaccine, Typbar-TCV.

The last one is the recommended vaccine for all age groups. (Milligan, Paul et al. 2018) .Antibiotics also play a role in Typhoid eradication.

A person having no symptoms of Typhoid but still carries the bacteria, can spread it through the shedding of bacteria in their faeces.

The other precautions are as follows-

Taking prescribed antibiotics for as long as the doctor has consulted.

Wash hands with soap and water after using the bathroom and avoid preparing or serving food for other people. This will lower the chance of passing the infection on to someone else.

Even if there are no symptoms, a further final blood test is required to conclude typhoid a typhoid-free person.

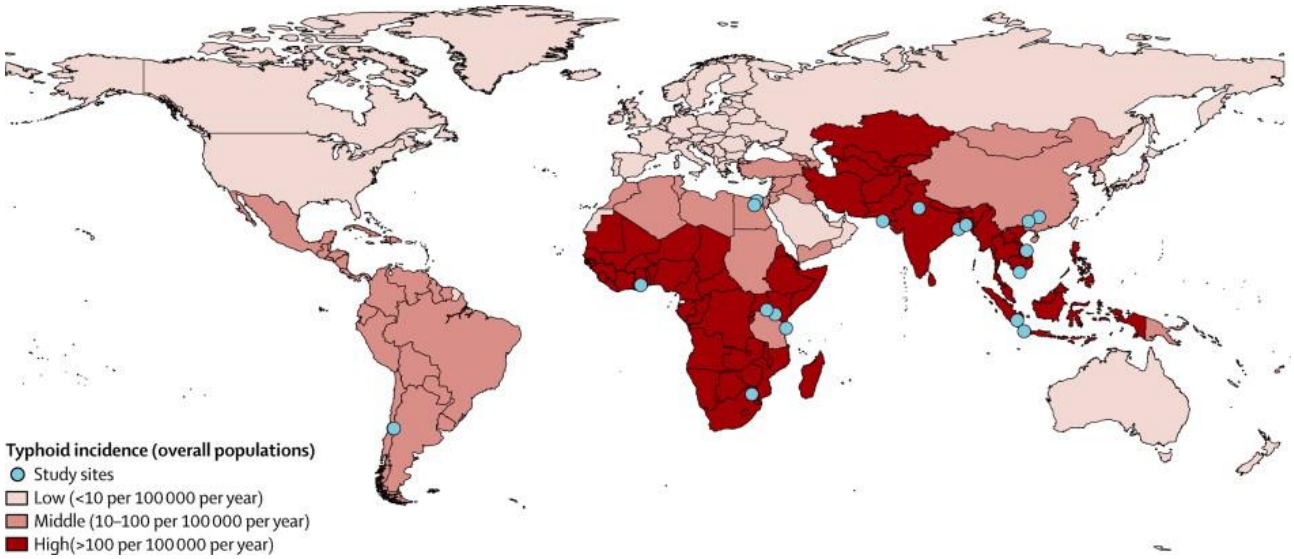

Figure 1.4: - Typhoid incidence worldwide [Mogasale, V., Maskery, B., Ochiai, R. L., Lee, J. S., Mogasale, V. V., Ramani, E., ... & Wierzba, T. F. (2014). The burden of typhoid fever in low-income and middle-income countries: a systematic, literature-based update with risk-factor adjustment. The Lancet Global Health, 2(10), e570-e580.]

1.8. Pathogenesis of *Salmonella:* -

The pathogenesis of *Salmonella* is intimately linked to the presence of specific pathogenicity islands (SPIs), which are distinct genetic regions that encode virulence factors essential for colonization, invasion, and survival within the host. *Salmonella* Typhi and Paratyphi possess multiple SPIs, including SPI-1, SPI-2, and SPI-3, which encode type III secretion systems (T3SSs), allowing the bacteria to inject effector proteins into host cells, modulating cellular processes and immune responses. SPI-1, in particular, is crucial for the initial stages of infection, encoding the invasion-associated genes that facilitate entry into intestinal epithelial cells. SPI-2, on the other hand, is involved in the survival and replication of *Salmonella* within host cells, encoding genes that enable the bacteria to evade the host's immune system. The presence and expression of these SPIs are critical for the pathogenesis of *Salmonella,* enabling the bacteria to establish a successful infection.

Salmonella pathogenicity islands: -

Pathogenicity Islands (PAIs) are distinct classes of genomic islands acquired by microorganisms through horizontal gene transfer (Juhas, van der Meer et al. 2009). These islands, typically 10-200 kilobases in size, carry virulence genes and are characteristic of pathogenic bacteria, distinguishing them from non-pathogenic strains(Hacker, Bender et al. 1990). PAIs exhibit distinct features, including varying G+C content and codon usage, and are often situated near tRNA genes, associated with mobile genetic elements, and flanked by direct repeats (Juhas, van der Meer et al. 2009). Notably, *Salmonella* Pathogenicity Islands (SPIs) are crucial for virulence in *Salmonella*, with 17 SPIs recognized to date (Riquelme, Varas et al. 2016). A list of important effectors present in that particular SPI is mentioned in Table 1.1.

1.8.1. *Salmonella* Pathogenicity Island 1 (SPI-1):

Salmonella species possess the capability to invade non-phagocytic host cells, a process facilitated by genes identified in STm (Galan and Curtiss 1989). These genes are clustered within a 40-kilobase

region at centisome 63, designated as *Salmonella* Pathogenicity Island 1 (SPI-1) (Mills, Bajaj et al. 1995). SPI-1 encodes a type III secretion system (T3SS), a complex macromolecule machine that translocates virulence proteins into host cells, earning it the nickname "injectisome" or "molecular needle" (Cornelis 2006). T3SS is a common feature among Gram-negative bacteria, including *Salmonella*, Yersinia, and E. coli, enabling them to exert virulence functions (Ghosh 2004). Notably, *Salmonella* mutants lacking a functional SPI-1 T3SS are unable to invade epithelial cells (Watson, Paulin et al. 1995). Notably, SPI-1 T3SS mutants exhibit impaired epithelial cell invasion in tissue culture, underscoring the critical role of this secretion system in *Salmonella* pathogenicity (Watson, Paulin et al. 1995).

The invasion of epithelial cells by *Salmonella* necessitates the coordinated action of at least five translocated proteins, with the complexity of invasion increasing in animal tissues (Haraga, West et al. 2008). SPI-1 orchestrates the expression of two subsets of effector proteins, one facilitating invasion through cytoskeletal modulation and the other contributing to enteropathogenesis and intestinal epithelial inflammation. The central transcription factor HilA, a member of the OmpR/ToxR family, drives SPI1 expression in response to environmental cues such as osmolality, antimicrobial peptides, and pH, which are perceived by two-component regulatory systems including BarA/SirA (Ahmer, van Reeuwijk et al. 1999), OmpR/EnvZ (Lucas and Lee 2001), PhoBR (Lucas, Lostroh et al. 2000), and PhoPQ (Pegues, Hantman et al. 1995). Furthermore, HilD, HilC, and RtsA form a feed-forward loop amplifying SPI1 expression, while nucleoid proteins HU and Fis, and transcriptional regulators InvF and SicA, also play critical roles in regulating SPI1 expression and function (Baxter, Fahlen et al. 2003, Ellermeier, Ellermeier et al. 2005) (Gerlach and Hensel 2007).

1.8.2. *Salmonella* Pathogenicity Island 2 (SPI-2):

Salmonella's survival within macrophages and establishment of systemic infection rely on a second type III secretion system (T3SS) encoded on *Salmonella* pathogenicity island 2 (SPI-2), a 40 Kb

genomic region comprising two distinct regions (Kuhle and Hensel 2004). The larger region, exclusive to *S. enterica*, encodes the T3SS essential for systemic pathogenesis, while the smaller region, found in *S. bongori*, encodes the tetrathionate reductase involved in anaerobic respiration. SPI2-translocated proteins facilitate *Salmonella*'s escape from intracellular killing by inducing phagosome tubulation, a dynamic process dependent on microtubule motor recruitment, membrane lipid alteration, and GTPase activation (Ramsden, Mota et al. 2007). Seventeen effectors, mostly encoded outside the SPI2 locus, are translocated across the *Salmonella*-containing vacuole (SCV) membrane, with three effectors, SpiC, SseF, and SseG, encoded within SPI2 (Uchiya, Barbieri et al. 1999, Kuhle and Hensel 2004).

The expression of *Salmonella* Pathogenicity Island 2 (SPI2) genes is orchestrated by the SsrAB two-component system, a global regulatory framework essential for SPI2 regulon expression within host cells (Gerlach and Hensel 2007). Additionally, the expression of SPI2 genes is influenced by other key global regulatory systems, including the EnvZ/OmpR and PhoPQ two-component systems, as well as the transcriptional regulators SlyA and Fis, which collectively modulate the expression levels of SPI2 genes to facilitate *Salmonella*'s survival and replication within host cells.

1.8.3. *Salmonella* Pathogenicity Island 3 (SPI-3):

The *Salmonella* Pathogenicity Island 3 (SPI3) locus, spanning 17 kilobases, harbors the MgtCB operon, a crucial virulence determinant comprising the magnesium transport system, MisL, and MarT, which enables *Salmonella*'s survival within the intra-phagosomal environment under nutrient-deprived conditions(Amavisit, Lightfoot et al. 2003). Notably, MisL, an autotransporter protein, exhibits adhesin properties and is essential for long-term intestinal persistence, as evidenced by animal studies (Dorsey, Laarakker et al. 2005). Furthermore, SPI3 exhibits significant sequential variation among different serovars, yet remains conserved in STy and. STm, highlighting its importance in *Salmonella* pathogenicity.

1.8.4. *Salmonella* Pathogenicity Island 4 (SPI-4):

The 27 Kb *Salmonella* Pathogenicity Island 4 (SPI4) comprises six genes (siiA-F), with SiiC, SiiD, and SiiF encoding a type I secretion system that exports the massive 600 kDa protein SiiE, facilitating bovine intestinal colonization (Gerlach, Jackel et al. 2007). Despite unclear molecular functions, SPI4's role in *Salmonella* virulence involves promoting gastrointestinal inflammation in murine colitis models and mediating adhesion to epithelial cells (Kiss, Morgan et al. 2007). Additionally, SPI4 contributes to intra-macrophage survival, similar to SPI2 (De Keersmaecker, Marchal et al. 2005), and exhibits remarkable conservation across diverse *Salmonella* serovars (Edwards, Olsen et al. 2002).

1.8.5. *Salmonella* Pathogenicity Island 5 (SPI-5):

The 7.6 Kb *Salmonella* Pathogenicity Island 5 (SPI5) locus encodes effector proteins for both SPI-1 and SPI-2 type III secretion systems (T3SS), including PipA and PipB, which play critical roles in systemic infection and lipid raft accumulation, respectively(Knodler, Celli et al. 2002) . Although PipB is dispensable for intracellular survival and systemic virulence in mice, SPI5-deficient *Salmonella* exhibits significant attenuation in cattle infection models, highlighting its importance in enteropathogenicity (Wood, Jones et al. 1998).

1.8.6. *Salmonella* Pathogenicity Island 6 (SPI-6):

The 59 Kb *Salmonella* Pathogenicity Island 6 (SPI6) locus, also known as the *Salmonella* centisome 7 genomic island (SCI), is a genomic region present in STy and STm, encoding genes such as saf (fimbriae) and pagN (invasion protein) (Folkesson, Lofdahl et al. 2002). Microarray analysis revealed SPI6 conservation among *S. enterica* subspecies I serovars, with partial homology detected in subspecies IIIb, IV, and VII, as well as sequential homology with *P. aeruginosa* and *Y. pestis* genomes(Hensel 2004). Deletion of SPI6 diminished bacterial invasiveness in tissue-cultured cells, although it did not impact systemic pathogenesis.

1.8.7. *Salmonella* Pathogenicity Island 7 (SPI-7) and *Salmonella* Pathogenicity Island 8 (SPI-8):

The *Salmonella* Pathogenicity Islands 7 and 8 (SPI7 and SPI8) span approximately 133 Kb and 6.8 Kb, respectively, with SPI7, also known as the major Pathogenicity Island, being specific to STy, *S.* Dublin, and *S.* Paratyphi(Pickard, Wain et al. 2003). SPI7 encodes the Vi antigen, a pil gene cluster, and putative virulence factors, including a type IV fimbrial adhesin, and exhibits sequential homology with *Xanthomonas axonopodis* and *Pseudomonas aeruginosa.* In contrast, SPI8, identified in STy, encodes putative virulence factors with unknown functions, highlighting the complexity and diversity of *Salmonella* pathogenicity islands.

1.8.8. *Salmonella* Pathogenicity Island 9 (SPI-9):

The 16,281 basepairs *Salmonella* Pathogenicity Island 9 (SPI9) locus in STy harbors four open reading frames (ORFs) that encode a type I secretion system (T1SS), including a large RTX-like protein with repeated Ig domains, which facilitates virulence factor secretion (Velasquez, Hidalgo et al. 2016). Notably, SPI9 is functional in STy, promoting adhesion under high osmolarity conditions, although it does not contribute to biofilm formation, highlighting its specific role in *Salmonella* pathogenicity.

1.8.9. *Salmonella* Pathogenicity Island 10 (SPI-10):

The 32.8 Kb *Salmonella* Pathogenicity Island 10 (SPI10) locus, inserted at the tRNA leuX gene, exhibits hypervariability and serves as a hotspot for the insertion of diverse mobile genetic elements(Bishop, Baker et al. 2005). Notably, SPI10 harbors the sef and pef gene clusters, encoding fimbrial adhesins in *S. Enteritidis*, whereas STy and *S.* Paratyphi A strains contain a cryptic bacteriophage within this locus, STm exhibits a distinct gene content, underscoring the dynamic nature of this genomic region.

1.8.10. *Salmonella* Pathogenicity Island 11 (SPI-11) and *Salmonella* Pathogenicity Island 12 (SPI-12):

.*Salmonella* Pathogenicity Islands 11 and 12 (SPI11 and SPI12) were identified in *Salmonella choleraesuis*, exhibiting characteristic properties of pathogenicity islands (PAIs), including association

with bacteriophage genomes and tRNA genes(Chiu, Tang et al. 2005). Notably, SPI11 displays a low G+C content of 41.32%, and while the proteins encoded by these islands contribute to *Salmonella* virulence, their exact roles remain unclear, necessitating further characterization to elucidate their mechanisms of action.

1.8.11. *Salmonella* Pathogenicity Island 13 (SPI-13) and *Salmonella* Pathogenicity Island 14 (SPI-14):

Salmonella Pathogenicity Islands 13 and 14 (SPI13 and SPI14) were initially identified in *S. Gallinarum*, the causative agent of fowl typhoid, and are avian-adapted (Shah, Lee et al. 2005). SPI13, located near the tRNA pheV gene, comprises 18 open reading frames (ORFs), whereas SPI14, lacking association with a tRNA gene, consists of 6 ORFs. Interestingly, these islands are absent in STy and *S.* Paratyphi A but present in STm and *S. Enteritidis,* suggesting a potential role in host specificity, although the functions of the encoded proteins remain to be elucidated through further molecular characterization.

1.8.12. *Salmonella* Pathogenicity Island 15 (SPI-15), *Salmonella* Pathogenicity Island 16 (SPI-16) and *Salmonella* Pathogenicity Island 17 (SPI-17):

Salmonella Pathogenicity Islands 15, 16, and 17 (SPI15, SPI16, and SPI17) were identified in STy using a bioinformatics approach, revealing associations with tRNA genes (Vernikos and Parkhill 2006). While SPI16 and SPI17 encode genes responsible for lipopolysaccharide (LPS) modification, SPI15, uniquely present in STy isolate CT18, encodes effector proteins with unclear functions. Notably, SPI16 and SPI17 are conserved across STy and most *S. enterica* genome sequences, highlighting their potential role in *Salmonella* pathogenicity.

Table 1.1: - Important effectors encoded by SPIs and their functions

SPI	SIZE in KB	Important genes for Virulence	Function of the SPI
SPI 1	40	SipC & SipA	Helps in membrane ruffling at the time of *Salmonella* entry by interacting with the actin cytoskeleton
		SopE, SopE2 and SopB	Promotes membrane ruffling. It takes part in the *Salmonella* invasion by directly activating Rac1 and Cdc42 by acting as GDP/GTP exchange factors (GEFs) and inducing membrane ruffling.
		SopA and SopD	Intestinal secretory and inflammatory

			responses are modulated by the recruitment of immune cells and secretion of fluid in the intestinal lumen
		SptP	It reverses the cytoskeletal rearrangements induced by SopE/E2 and SopB by acting as GTPase activating protein (GAP) and acts on Rac1 and Cdc42
SPI2	40	SpiC	Block fusion of the SCV with lysosomes
		Sif A	*Salmonella* containing vacuole membrane integrity
		SseJ	Cytoskeleton rearrangements

		SsPH2	Cytoskeleton rearrangements
		Ttr genes	Tetrathionate respiration and outgrowth in the intestine.
		SseF and SseG	It is helpful to maintain the juxtanuclear position of the SCV in HeLa cells
		SpvB	*Salmonella* virulence protein that is secreted into the macrophage cytoplasm,
SPI3	17	MgtCB	Enables Mg++ in a low magnesium

			environment to promote bacterial survival inside macrophages.
SPI4	27	SiiABCDF	*Salmonella* uptake in epithelial cells. Helps in membrane ruffling.
SPI5	7	PipABCD, SopB	Shows inflammatory responses
SPI6	59	PagN, safABCD	Establishment of the bacteria in the gut of the host during infection
SPI7	146	ViaB	Encodes the virulent genes Vi and IVB opreron
SPI8	6.8	STY3273, STY3292	Related to bacterial fitness in gut
SPI9	16	SpvA, SpvB, SpvC, SpvD	Helps in the invasion of epithelial cells
SPI10	32.8	SefB, sefC, SefR, PrpZ	Helps in STy intra-macrophage survival

SPI11	15	InvA, InvB, InvC, RtsA	Intra-macrophage survival
SPI12	6.3	RpoS, TviA, SpvA, SpvB	Helps in vivo adaptability
SPI13	19	SitA, SitB, SpvD	Virulent gene
SPI14	9	MgtC, TviA, SseD	Helps in the invasion into epithelial cells
SPI15	6.5	SseB, SseC, SpvA	Increases bacterial ability to invade host tissues, and helps the bacteria to survive within immune cells.
SPI16	4.5	SseK, SseJ, SseI	Helps in the persistence of the bacteria in the intestine
SPI17	5.1	SifA, SifB, SifC	Helps in bacterial survival within cells by manipulating SCV.

1.9. *Salmonella* secretion systems: -

Salmonella utilizes a complex secretion system to deliver effector proteins into host cells, facilitating infection and survival. To date, four protein secretion systems have been discovered in *Salmonella*, including the type I secretion system (T1SS), the type III secretion system (T3SS), the type IV

secretion system (T4SS) and the type VI secretion system (T6SS). The effectors secreted from these secretion systems are reported to play essential roles during infection in the animal host cells(Bao, Wang et al. 2020).

1.9.1. Type I secretion system –

T1SS was first reported in 1985 in Escherichia coli which is responsible for delivering many molecules, such as adenylate cyclases, lipases, proteases, surface layer proteins, and toxins, into the extracellular space (Lenders, Weidtkamp-Peters et al. 2015)During colonization into the host cells, *Salmonella* requires many virulence factors for attachment to host surfaces. Some of them are fimbrial adhesins like type 1 fimbriae (Fim)(Lockman and Curtiss 1992), plasmid-encoded fimbriae (Pef) (Baumler, Tsolis et al. 1996), long polar fimbriae (Lpf) (Baumler and Heffron 1995), thin aggregative fimbriae (Agf) (Grund and Weber 1988), T1SS-secreted adhesins(Barlag and Hensel 2015) (Gerlach and Hensel 2007), etc. The α-hemolysin (HlyA) secretion machinery from *E. coli* is one of the best-studied T1SSs, which is a member of an ATP-binding cassette (ABC) transporter (Holland, Schmitt et al. 2005))In *Salmonella*, BapA and SiiE are two T1SS-secreted non-fimbrial adhesins have been documented (Barlag and Hensel 2015) (Wagner, Barlag et al. 2014). SiiE is the largest adhesion protein, which helps Salmonella to attach by its N-terminal moiety to the microvilli of the apical side of polarized epithelial cells by its (Wagner, Polke et al. 2011, Wagner, Barlag et al. 2014, Barlag and Hensel 2015) close contact with polarized epithelial cells helps the bacteria to trigger the remodeling of SPI-1 mediated host cell cytoskeleton, enabling the uptake of the bacteria (Wagner, Barlag et al. 2014). It was found that SiiE is not required in non-polarized cells for adhesion (Gerlach, Jackel et al. 2007). T1SS-dependent intestinal colonization was found in a bovine infection model infected by STm but no significant role was found in chicken or pigs (Morgan, Campbell et al. 2004, Morgan, Bowen et al. 2007). It is reported that SiiA binds to peptidoglycan in vitro in a pH-dependent manner by the periplasmic domain of SiiA and the knockdown mutation in Arg162 affects the binding affinity with peptidoglycan and invasion into the polarized epithelial cells (Kirchweger, Weiler et al. 2019). BapA

is another adhesin that is reported to take part in *S. enteritidis* biofilm formation and invasion within murine cells (Latasa, Roux et al. 2005).In STy, SPI-9-derived T1SS putative effector protein STY2875 (orthologous to BapA) was stated to be responsible for bacterial adherence in epithelial cells. Unlike BapA, it does not take part in biofilm formation(Velasquez, Hidalgo et al. 2016). The report says Salmonella siiD/siiE/siiF get upregulated under low pH conditions (Lawley, Chan et al. 2006). Another observation was the T1SS gene increment on SPI-9 in STy under low pH (10.1099/mic.0.000319.SPI-4-). T1SS helps in SPI-1-T3SS-mediated breaching of epithelial barriers. These two are virulence loci that play complementary roles by helping the interactions of STm with host intestinal epithelial cells (Main-Hester, Colpitts et al. 2008). The main role of TISS is to help the SPI-1-T3SS to accomplish the invasion into host cells (Main-Hester, Colpitts et al. 2008).

1.9.2. The type 3 secretion system (T3SS)-

The type 3 secretion system (T3SS) is a complex molecular machine used by certain bacteria to inject effector proteins into eukaryotic host cells. These effector proteins can manipulate host cell processes, allowing the bacteria to colonize, survive, and proliferate within the host.

The T3SS is like a molecular syringe, consisting of:

- Needle complex: A hollow, needle-like structure that spans the bacterial inner and outer membranes.
- Basal body: A ring-like structure that anchors the needle complex to the bacterial membrane.
- Effector proteins: Proteins that are injected into the host cell to modulate its processes.
- Chaperones: Proteins that assist in the secretion and translocation of effector proteins(Notti and Stebbins 2016, Deng, Marshall et al. 2017) (Green and Mecsas 2016). The T3SS structures resemble the structures of flagella and the structures are made of a homologous set of over 20 proteins. The overall structure contains five main parts which are

- a hollow cylindrical structure of outer and inner membrane joining
- a needle that acts as a basal body,
- a needle tip complex termed translocon that forms pores at host cell membrane during infection
- a cytoplasmic ring (C-ring),
- an ATPase complex combining an apparatus required for export located to the inner membrane (Deng, Marshall et al. 2017)

T3SS is used by certain pathogenic bacteria, such as:

- *Yersinia* (plague)
- *Salmonella* (food poisoning)
- *Shigella* (dysentery)
- *Pseudomonas aeruginosa* (opportunistic infections)

The T3SS plays a crucial role in bacterial pathogenesis, enabling bacteria to:

- Inhibit phagocytosis
- Modulate host cell signalling
- Induce inflammation
- Suppress immune responses

Understanding the T3SS is essential for developing strategies to combat bacterial infections and designing novel antimicrobial therapies.

There are two T3SS gene clusters, T3SS-1 and T3SS-2, on SPI-1 and SPI-2, respectively are present in *S. enterica.* It is reported that the effectors of the T3SS-1 cluster are required for the invasion of

intestinal epithelial cells and M cells in the gut lumen in the early phase and the other one, which is T3SS-2 effectors, ensure intracellular survival and replication within phagocytes such as macrophages at the later stages within the host (Hensel, Shea et al. 1998, Anderson and Kendall 2017).

One of the most critical and extensively studied aspects of *Salmonella* infection is its ability to invade non-phagocytic cells. Upon contacting epithelial cells, Salmonella triggers rapid and extensive reorganization of the actin cytoskeleton at the entry site. This reorganization leads to membrane ruffling, macropinocytosis, and ultimately, bacterial internalization(Galan 1999). Research has consistently shown that five Type III secretion system (T3SS) effectors play a crucial role in directly or indirectly regulating actin cytoskeleton dynamics, facilitating *Salmonella*'s invasion process.

Salmonella effector proteins, injected into host cells through the Type III Secretion System (T3SS-1), modulate various host cell processes to facilitate bacterial internalization and colonization. SipA and SipC directly bind to and polymerize F-actin, while SopE, SopE2, and SopB indirectly modulate the actin network by activating host Rho GTPases (Zhou, Mooseker et al. 1999, Bayer-Santos, Durkin et al. 2016); (Friebel, Ilchmann et al. 2001, Patel and Galan 2005) (van der Heijden and Finlay 2012). Additionally, SptP reverses membrane ruffling by downregulating Cdc42 and Rac-1 (Fu and Galan 1999). *Salmonella* infection also triggers intestinal inflammation, which is modulated by T3SS-1 through the delivery of SipA (Lee, Silva et al. 2000); (Stecher, Robbiani et al. 2007)

Salmonella Type III Secretion System (T3SS) effectors modulate host immune responses to facilitate bacterial survival and colonization. SopE, SopB, and SopE2 activate Rho GTPases, leading to NF-κB activation and pro-inflammatory cytokine release (van der Heijden and Finlay 2012, Ilyas, Tsai et al. 2017). SipB increases IL-1β and IL-18 production by activating caspase-1(Agbor and McCormick 2011);). Conversely, effectors like SptP, AvrA, SseL, SspH1, and SpvC antagonize immune responses by inhibiting MAPK, JNK, and NF-κB activation, promoting intracellular bacterial survival (Agbor and McCormick 2011) (Lee, Silva et al. 2000, Guiney 2005) (Mazurkiewicz, Thomas et al. 2008).

Following internalization, *Salmonella* forms SCVs to facilitate intracellular survival and growth. Effectors SopB, SopD, and SipC mediate initial SCV formation and maturation, while SptP enhances bacterial replication within SCVs (van der Heijden and Finlay 2012) (Bakowski, Cirulis et al. 2007, Humphreys, Hume et al. 2009);. SCVs then migrate to the perinuclear region along microtubules (Agbor and McCormick 2011) .T3SS-2 effector SifA induces SCV tubulation, forming *Salmonella*-induced filaments (Sifs), and regulates bacterial localization and replication by binding Rab7 (Garcia-del Portillo, Zwick et al. 1993, Bakowski, Cirulis et al. 2007) ; Other effectors, including SopD2, PipB, PipB2, SseG, SseJ, SseF, play crucial roles in SCV tubulation, maintenance, and Sif formation (van der Heijden and Finlay 2012). After phagocytosis by macrophages, *Salmonella* employs its Type III Secretion System-2 (T3SS-2) to evade immune responses. T3SS-2 prevents the co-localization of NADPH oxidase on *Salmonella*(Agbor and McCormick 2011)-containing vacuoles (SCVs), protecting the bacteria from reactive oxygen species (ROS) (Vazquez-Torres, Xu et al. 2000). Additionally, T3SS-2 mutants show increased co-localization of inducible nitric oxide synthase (iNOS) with SCVs, indicating a role for T3SS-2 in evading reactive nitrogen species (RNS)(Chakravortty, Hansen-Wester et al. 2002). *Salmonella* can also induce macrophage cell death through different mechanisms, including the interaction of T3SS-1 effector SipB with caspase-1, and the release of pro-inflammatory cytokines by T3SS-2(Hueffer and Galan 2004) (Kuhle and Hensel 2004);(Monack, Detweiler et al. 2001). A list of effectors of T3SS and their function are enlisted in Table-1.2 (Agbor and McCormick 2011, Bao, Wang et al. 2020)

1.9.3. Type IV secretion system (T4SS)

The *Salmonella* T4SS is a multi-protein complex that spans the bacterial inner and outer membranes. It transports different types of substrates including DNA or DNA-protein complexes into plant or animal cells ((Backert and Meyer 2006, Galan and Waksman 2018). It consists of several components, including the **Inner membrane complex**: Complex is composed of several proteins, including SsaL, SsaM, and SsaN, which form a ring-like structure, **Outer membrane complex**: This complex is

composed of proteins such as SsaB, SsaC, and SsaD, which form a pore-like structure, **Pilus**: The pilus is a long, thin structure composed of SsaG protein that protrudes from the bacterial surface, **Effector proteins**: These are proteins that are secreted through the T4SS and injected into host cells (Kubori, Matsushima et al. 1998) (Lostroh and Lee 2001, Marlovits, Kubori et al. 2004)

T4SSs are of 3 types. The first one takes part in conjugation by transferring the DNA from one cell to recipient cells in a contact-dependent manner. The second type confirms DNA uptake or release. The third type transfers proteins and other substances into eukaryotic cells and is mostly present in pathogenic bacteria (Backert and Meyer 2006, Alvarez-Martinez and Christie 2009, Christie 2016). There are reports stating the role of T4SS in many diseases progressions like gastric ulcers, brucellosis, or Legionnaire diseases, and the causative agents are *Helicobacter pylori*, *Brucella suis*, and *Legionella pneumophila*, respectively (Backert and Meyer 2006, Galan and Waksman 2018) Some pathogens use T4SS effectors to promote their intracellular survival and replication (Sexton and Vogel 2002). According to the studies, few *Salmonella enterica* serotypes have a T4SS cluster. As discovered, *S. enterica* serotype Enteritidis phage type 34, *S. enterica* serotype Montevideo isolates, *S. enterica* Serovar STm strain ST1660/06, and most *S. enterica* Serovar Heidelberg contain a T4SS on different plasmids (Chen, Wang et al. 2009, Li, Cheng et al. 2012) (Delgado-Suarez, Selem-Mojica et al. 2018). A published study stated the role of VirB/D4 T4SS plasmids in *Salmonella enterica* serovar Heidelberg Virulence. the bacteria with the plasmid penetrated and survived in epithelial cells and macrophages better than those without the plasmid. After uptake, the VirB/D4 T4SS encoding genes are up-regulated in a time-dependent manner. To study the mechanism of survival, several host anti-microbial genes were analyzed. The strain containing the T4SS downregulated several host's innate immune response genes which may take part in the increased entry and survival within macrophages and epithelial cells. There was a downregulation of several of the antimicrobial peptide (AMP) pathway genes. The down-regulated genes were those encoding bacterial/permeability-increasing protein (BPI), cationic AMP

(CAMP), Ctsg, lipocalin 2 (LCn2), lactotransferin (LTF), LYZz and Prtn3, etc. (Gokulan, Khare et al. 2013)

1.9.4. Type 6 Secretion System (T6SS) :-

T6SS is a contact-dependent nanomachine found in 25% of Gram-negative bacteria which delivers toxins directly into eukaryotic or prokaryotic cells(Boyer, Fichant et al. 2009). It was first discovered functionally in 2006 by Pukatzki et al. in Vibrio cholera ((Pukatzki, Ma et al. 2006, Ho, Dong et al. 2014) It is structurally similar to the inverted T4 contractile bacteriophage tail(Cascales and Cambillau 2012).**T6SS** Consists of 13 core components (TssA-TssM) and It has three main types: type i, type ii, and type iii **(Cianfanelli, Monlezun et al. 2016)** . The basic structure includes

1. **Inner membrane complex**: This complex is composed of several proteins, including TssL, TssM, and TssN, which form a ring-like structure (Coulthurst 2019).

2. **Outer membrane complex**: This complex is composed of proteins such as TssB, TssC, and TssD, which form a pore-like structure(Liu, Guo et al. 2013).

3. **Hcp proteins**: These are proteins that form a tube-like structure that protrudes from the bacterial surface (Shneider, Buth et al. 2013)

4. **VgrG proteins**: These are proteins that form a spike-like structure that protrudes from the bacterial surface (Lopez, Ly et al. 2020).

Salmonella contains five phylogenetically distinct T6SSs which are encoded by SPI-6, SPI-19, SPI-20, SPI-21, and SPI-22. (Blondel, Jimenez et al. 2009, Fookes, Schroeder et al. 2011). T6SS acts as a weapon against commensal bacteria. STm uses SPI-6 T6SS to kill the commensal bacterium *Klebsiella oxytoca*, facilitating its colonization (Sana, Flaugnatti, et al. 2016).T6SS is identified as an

important virulence-related factor in *Salmonella* enterica, contributing to its pathogenesis ((Haneda, Ishii et al. 2009, Liu, Guo et al. 2013). It was found that T6SS having SPI-6 is responsible for the virulence of STm in the murine model of infection. (Liu, Guo et al. 2013). SPI-6 T6SS is involved in the virulence of various *Salmonella* serotypes, including STy, Dublin, and Gallinarum (Wang, Luo et al. 2011) (Blondel, Yang et al. 2010, Pezoa, Blondel et al. 2014). Global regulators histone-like nucleoid structuring protein (H-NS) and the ferric uptake regulator (Fur) repress T6SS gene expression during *Salmonella* infection, indicating precise control of T6SS by *Salmonella* for adaptation to different environments. (Brunet, Khodr et al. 2015, Wang, Yang et al. 2019). *Salmonella* strains with T6SSs encoding on SPI-19 are mostly isolated from warm-blooded hosts, suggesting a potential host adaptation-related function (Bao, Zhao et al. 2019).

Table 1.2 List of effectors of T3SS and their function

Name of the Effector	T3SS1 or T3SS2	Function	Host Target
AvrA	1	Regulates Salmonella-induced inflammation	IκBα, β-catenin
SipA	1	Mediates *Salmonella* invasion of epithelial cells by inducing actin bundling and polymerization, promotes formation and maintenance of SCV, induces neutrophil transmigration, and also processes and activates caspase-3	Actin
SipB	1	Translocon	Cholesterol

SipC	1	Promotes bacteria entry (invasion) by mediating actin bundling, a component of the translocon	Actin
SipD	1	Component of the translocon	
SopA	1	Ubiquitin E3 ligase promotes bacteria escape from SCV and is required for invasion, promotes neutrophil transmigration	
SopB	1	Phosphatidyl inositol phosphatase, promotes bacteria entry, macropinocytosis, regulates SCV localization and formation, and promotes fluid secretion during infection, Akt activation	inositol phosphates, phosphoinositides
SptP	1	Contains GTPase activating Protein (GAP) and tyrosine activities, reverses *Salmonella*-induced pro-inflammatory responses	Rac-1 and Cdc42
SpiC	2	Regulates Spi-2 T3SS	Hook3 and TassC
SseF	2	Regulation and maintenance of SCV	Microtubules
SseG	2	Regulates SCV positioning	Microtubules
SopD	½	Promotes bacteria invasion and fluid secretion/accumulation	

SopE	2	Mediates bacteria invasion by promoting membrane ruffling and actin cytoskeletal rearrangements disrupt tight junctions	Rac-1 and Cdc42
SopE2	2	Membrane ruffling and disruption of tight junctions	Cdc42
SopD2	2	Sifs formation and intracellular replication of bacteria in mouse macrophages	
SifA	2	Required for Sif formation and SCV membrane integrity, SCV maintenance, C-terminal has GTPase activity	SKIP, Rab7
PipB2	2	Promotes Sifs extension	Kinesin-1
PipB	2	homologous to PipB2 localizes to SCV and Sif, and is required for cecal colonization in chicks and induction of secretory and inflammatory responses in bovine-ligated ileal loops	
SlrP	2	ubiquitin ligase for thioredoxin targets the human chaperone ERDj3	theorodexin' ERDj3
SspH1	½	E3 ubiquitin ligase	
SspH2	2	E3 ubiquitin ligase	

SseJ	2	Antagonizes SifA mediated stability of SCV, negatively regulates Sifs	Cholesterol
SseL	2	Helps to attenuate *Salmonella* virulence, has de-ubiquitinating activity, and is a cysteine protease	
SteB	½	Putative piconate reductase, required for *Salmonella* biofilm formation	
SteC	2	Kinase that promotes F-actin meshwork formation	F-actin
SpvB	2	Depolymerizes actin filaments in vitro	Actin
SpvC	2	Phosphothreonine lyase necessary for complete virulence in mice	
SseK1	2	Inhibit NF-κB signaling, manipulate the host cell microtubule network	TRADD, TBCB
SseK3	2	Inhibit NF-κB signaling	TNFR1, TRAILR, TRADD
GogB	½	Inhibit proinflammatory response	Skp1, FBXO22

1.10. *Salmonella* invasion into Host cells: -

Salmonella internalization into host cells occurs through two distinct processes: phagocytic uptake by professional phagocytes, such as macrophages, and active invasion using the type III secretion system (T3SS), T3SS1 (Haraga, Ohlson et al. 2008, Kumar, Kawai et al. 2011). Phagocytosis involves multiple receptors that recognize pathogen-associated molecular patterns, including lipopolysaccharide (LPS) and flagellin, leading to phagosome maturation, signaling, and gene expression (Kumar, Kawai et al. 2011, McCoy 2016). Pattern-recognition receptors, such as Toll-like receptors (TLRs), play a crucial role in recognizing these molecular patterns and activating immune response (Akira, Uematsu et al. 2006, Kumar, Kawai et al. 2011). Furthermore, studies have shown that *Salmonella* can manipulate the host cell's signaling pathways to facilitate its survival and replication within the host cell (Haraga, Ohlson et al. 2008, Azimi, Zamirnasta et al. 2020). Overall, understanding the mechanisms of *Salmonella* internalization is essential for developing effective strategies to combat *Salmonella* infections. *Salmonella* transmission occurs through the fecal-oral route, infecting the distal small intestine. The bacteria use T3SS/SPI-1 to enter specialized M cells, then translocate into Peyer's patches, where they're engulfed by phagocytic cells (Kurtz, Goggins et al. 2017). *Salmonella* penetrates the intestinal epithelium using virulence factors, disseminating systemically to colonize the liver and spleen. To invade the non-phagocytic cells, *Salmonella* uses the trigger mechanism(Groves, Dart et al. 2008). *Salmonella*'s genome contains *Salmonella* pathogenicity islands (SPIs) encoding virulence factors. SPI-1 and SPI-2 encode type III secretion systems (T3SS), a molecular syringe injecting virulence factors into host cells (Galan 1996, Galan and Wolf-Watz 2006). Environmental conditions induce SPI-1 T3SS and effector protein expression(Bajaj, Lucas et al. 1996). T3SS assembles at the epithelial surface, releasing effector molecules into the host cytoplasm (Kubori, Matsushima et al. 1998, Schlumberger, Muller et al. 2005). Real-time imaging experiments show that effector proteins SipA and SopE are translocated within 80-200 seconds(Schlumberger, Muller et al. 2005). This "regulated translocation" requires a translocation pore (translocon) composed

of bacterial proteins SipC and SipB. Cellular cholesterol is necessary for effector protein translocation and may trigger translocon assembly (Hayward, Cain et al. 2005).

The *Salmonella* Pathogenicity Island 1 (SPI-1) Type III Secretion System (T3SS) mediates the secretion of at least 13 proteins, including AvrA, SipA, SipB, SipC, SipD, SlrP, SopA, SopB/SigD, SopD, SopE, SopE2, SptP, and SspH1(Galan 1996). Of these, six proteins have been shown to regulate actin cytoskeleton dynamics, either directly or indirectly, by modulating the polymerization and depolymerization of actin filaments. Many of these proteins have homologs or orthologues in other pathogens, such as *Shigella* or *Yersinia* species, highlighting the conserved nature of these virulence mechanisms.

SipC, a component of the bacterial translocon, contains two membrane-spanning domains, with its N-terminus and C-terminus extending into the host cytoplasm(Hayward and Koronakis 1999). The C-terminal cytoplasmic domain of SipC nucleates the assembly of actin filaments, leading to rapid filament growth from the barbed ends. SipC's efficiency in filament nucleation is similar to that of the eukaryotic nucleating factor, the Arp2/3 complex. The SipC C-terminus also modulates translocon assembly and/or function, and its trimeric structure suggests it may mediate oligomerization. Recent work by Chang et al. (2005) shows that actin nucleating and effector translocation functions are dissociable. SipA, a secreted effector, promotes actin filament polymerization by reducing monomer concentration, enhancing filament-bundling activity, and potentiating SipC's nucleating activity (Zhou, Mooseker et al. 1999) (McGhie, Hayward et al. 2001). SipA binds to assembled filaments, prevents depolymerization by ADF/cofilin, and protects filaments from fragmentation induced by gelsolin(McGhie, Hayward et al. 2004). Structural studies reveal SipA acts as a 'molecular staple' using extended 'arm' domains to tether actin monomers(Lilic, Galkin et al. 2003). SipA and SipC combined activities promote actin filament formation and stability, enhancing bacterial internalization efficiency(Zhou, Mooseker et al. 1999). SipC and SipA stimulate actin polymerization, forming an unbranched actin filament (Hayward and Koronakis 1999). However, phagocytic apparatus formation

requires highly branched and interconnected actin networks, mediated by Rho family GTPases, primarily Rac and Cdc42 (Jaffe and Hall 2005). These GTPases function as molecular switches, cycling between GTP-bound and GDP-bound states, facilitated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Activated Rac and Cdc42 stimulate downstream effector proteins, driving actin cytoskeleton assembly, leading to membrane ruffles, lamellipodia, and filopodia formation. *Salmonella* employs a direct activation mechanism of Rho family GTPases through effector proteins SopE and SopE2, which are bacterially encoded guanine nucleotide exchange factors (GEFs) targeting Rac and Cdc42(Hardt, Chen et al. 1998); (Stender, Friebel et al. 2000). The activation of Rac and Cdc42 are required for the *salmonella* internalization(Chen, Hobbie et al. 1996). Despite lacking sequence similarity to eukaryotic GEFs, SopE and SopE2 catalyze nucleotide exchange using similar mechanisms (Buchwald, Friebel et al. 2002) (Schlumberger, Friebel et al. 2003). SopE activates both Rac and Cdc42, while SopE2 selectively activates Cdc42(Friebel, Ilchmann et al. 2001). *Salmonella* triggers actin rearrangements using secreted effector protein SopB/SigD (Norris, Wilson et al. 1998) (Zhou, Chen et al. 2001). SopB/SigD induces membrane ruffling independently of SopE and SopE2, and its transient expression in host cells causes local membrane ruffles(Zhou, Chen et al. 2001). Although SopB/SigD requires Rho family GTPase activity for actin reorganization, it lacks GEF activity and instead contains a domain related to eukaryotic phosphatidylinositol phosphatases. SopB/SigD acts on various phosphoinositide species, including PIP (3,4,5) P3, PI (3,4) P2, and PI (3,5) P2 (Norris, Wilson et al. 1998, Marcus, Wenk et al. 2001). SopB/SigD's catalytic activity suggests it activates Rho GTPases indirectly through phosphoinositide hydrolysis. Patel and Galán (2006) showed that SopB promotes RhoG activation through SGEF, an endogenous RhoG GEF. RhoG activates Rac by recruiting the ELMO/DOCK180 complex(Katoh and Negishi 2003). However, *Salmonella*-induced RhoG activation doesn't activate Rac (Patel and Galan 2006) Depletion of RhoG or SGEF via RNAi reduced membrane ruffling in *Salmonella*-infected cells, but RhoG's necessity for efficient bacterial entry remains unclear.

Collectively, these findings indicate that *Salmonella* employs SopE, SopE2, and SopB to synergistically stimulate multiple Rho GTPase family members, facilitating rapid and efficient bacterial internalization. The formation of branched actin filament networks, triggered by Rho family GTPase activation, requires the heptameric Arp2/3 complex, which initiates new filament assembly, introduces branch points, and cross-links filaments (Goley and Welch 2006). The Arp2/3 complex is necessary for efficient bacterial internalization(Criss and Casanova 2003) (Unsworth, Way et al. 2004) However, incorporation of the Arp2/3 complex into growing filaments requires upstream regulatory molecules, including WASP, N-WASP, Scar/WAVE1, cortactin, and IQGAP1 (Takenawa and Suetsugu 2007). WASP and N-WASP are activated directly by Cdc42 binding, whereas WAVE proteins are activated indirectly by Rac. WAVE2, a ubiquitously expressed isoform, exists as part of a multimeric protein complex containing Abi1, Nap1, HSPC300, and a Rac-binding subunit, PIR121 (Stradal and Scita 2006). The WAVE2 complex is recruited to sites of Rac activation via PIR121, promoting actin assembly at or near the membrane surface. Recent evidence shows that the knockdown of endogenous WAVE2 or Abi1 substantially inhibits STm internalization (Shi, Scita et al. 2005). This suggests WAVE2 couples SopE-mediated Rac activation to the assembly of branched actin networks, providing the driving force for *Salmonella* entry into host cells.

Focal adhesions are complex protein assemblies mediating the linkage between integrins and the actin cytoskeleton, transmitting integrin-dependent signals to the cell interior(Zamir and Geiger 2001). Focal adhesion proteins coordinate actin cytoskeleton dynamics in response to integrin-mediated adhesion. Many bacterial pathogens subvert focal adhesion proteins, such as Y. pseudotuberculosis, which interacts with β1 integrins but prevents focal adhesion complex assembly by injecting bacterial effectors, including the tyrosine phosphatase YopH. Reports stated that focal adhesion complexes play a crucial role in STm entry into host cells(Shi and Casanova 2006). Infection of cultured epithelial cells induces the assembly of focal adhesion-like structures at the apical plasma membrane. FAK and p130Cas are necessary for bacterial internalization. Surprisingly, FAK autophosphorylation isn't

induced by *Salmonella* infection, and the tyrosine kinase domain is dispensable for infection. Instead, the C-terminal proline-rich domain, mediating FAK-p130Cas interaction, is required (Shi and Casanova 2006).

Bacterial internalization involves engulfment and plasma membrane annealing to enclose bacteria in a sealed phagosome. Phagosome closure requires, in part, SopB/SigD's inositol phosphatase activity. Research showed that PtdIns (4,5) P2 accumulates at *Salmonella*-induced membrane ruffling sites but depletes from nascent phagosomes as invagination progresses(Terebiznik, Vieira et al. 2002). After bacterial entry, the host cell cytoskeleton returns to its basal state, mediated by the dual-function secreted effector SptP. SptP contains two catalytic modules: an N-terminal RhoGAP domain and a C-terminal tyrosine phosphatase domain(Kaniga, Uralil et al. 1996). SptP's GAP domain mimics eukaryotic RhoGAPs in structure and mechanism(Stebbins and Galan 2000). Introducing purified SptP into host cells reduces actin assembly (Fu and Galan 1998), likely due to inactivating Rac and Cdc42 (Fu and Galan 1999). SopE/SopE2 and SptP have opposing functions, yet are simultaneously injected into host cells. Experiments by Kubori and Galán showed that while SopE and SptP are injected in equimolar amounts, SopE is degraded within 30 minutes of infection via ubiquitin/proteasome-mediated mechanism. In contrast, SptP persists for several hours (Kubori and Galan 2003). This differential stability is due to the N-terminal translocation domain of each protein.

1.11. Intracellular survival of *salmonella* within cells: -

Following entry into host cells, *Salmonella* employs a range of effectors to reverse actin cytoskeleton rearrangements, which are essential for bacterial entry, and to suppress inflammation. The tyrosine phosphatase SptP plays a crucial role in reversing MAPK-mediated inflammation and IL-8 secretion (Murli, Watson et al. 2001, Lin, Le et al. 2003), and persists during the late stages of infection to dephosphorylate the AAA+ ATPase valosin-containing protein (VCP), which is vital for intracellular replication and endosomal tubule formation(Humphreys, Hume et al. 2009). Furthermore, the SPI-1

effector AvrA and the SPI-1 and SPI-2 E3 ubiquitin ligase effector SspH1 contribute to the downregulation of *Salmonella*-induced IL-8 production by epithelial cells, with SspH1 binding to and ubiquitylating the serine/threonine protein kinase N1 (PKN1), thereby inhibiting NF-κB activity (Haraga and Miller 2003, Haraga and Miller 2006, Keszei, Tang et al. 2014). The acetyltransferase activity of AvrA, for instance, can suppress inflammation triggered by apoptosis of macrophages and enhance bacterial intracellular survival(Wu, Jones et al. 2012). Other effectors, including AvrA (Jones, Wu et al. 2008, Du and Galan 2009), the phosphothreonine lyase SpvC (Haneda, Ishii et al. 2012), and the anti-inflammatory effector GogB (Pilar, Reid-Yu et al. 2012), target the MAPK and NF-κB pathways to downregulate inflammation. Specifically, SpvC reduces inflammation by inhibiting the production of IL-8 and tumor necrosis factor (TNF) (Mazurkiewicz, Thomas et al. 2008), while GogB blocks ubiquitylation and degradation of inhibitor of NF-κB (IκB), leading to inhibition of NF-κB pro-inflammatory signaling (Pilar, Reid-Yu et al. 2012). Furthermore, the SPI-1 and SPI-2 effector SteA elicits changes in the expression of several genes involved in the extracellular matrix organization, cell proliferation, and serine/threonine kinase signaling pathways, while repressing genes involved in immune processes, cell death, cell adhesion, and cell migration (Cardenal-Munoz, Gutierrez et al. 2014).

The maturation of the SCV involves transient interactions with early endosomes, resulting in the recruitment of early endosomal markers, including the transferrin receptor, early endosomal antigen 1, and the small GTPase RAB5 (Steele-Mortimer, Meresse et al. 1999), (Smith, Cirulis et al. 2005)]. As the SCV matures, these markers are replaced with late endosomal and lysosomal markers, such as lysosome-associated membrane proteins (LAMPs), RAB7, RAB-interacting lysosomal protein (RILP), vacuolar ATPase, and cholesterol (Steele-Mortimer, Meresse et al. 1999), (Garcia-del Portillo and Finlay 1995, Meresse, Steele-Mortimer et al. 1999, Brumell, Tang et al. 2001). However, the presence of certain lysosomal markers, such as mannose-6-phosphate receptor (MPR) and lysobisphosphatidic acid, can vary depending on the cell type (Garcia-del Portillo and Finlay 1995). It

was found that virulent STm inhibits phagosome/lysosome fusion and preferentially divides in unfused phagosomes 10.1128/iai.59.7.2232-2238.1991. Strikingly, evidence stated that STm containing vacuoles acquires lysosomal molecules and lysosomal acid phosphatases, but is essentially devoid of lysosomal markers (Meresse, Steele-Mortimer et al. 1999). Despite this complexity, SPI-2 T3SS effector proteins play a crucial role in modifying the endosomal membrane, with many effectors localizing to the SCV, including SifA and SseJ. The SPI-2 effector SseJ plays a crucial role in modifying the composition of cholesterol and phospholipids in the SCV membrane by activating its glycerophospholipid: cholesterol acyltransferase activity, which ultimately increases the accumulation of cholesterol esters within lipid droplets in the cytoplasm(Christen, Coye et al. 2009) (Nawabi, Catron et al. 2008, LaRock, Brzovic et al. 2012)

This alteration in lipid composition has the potential to dramatically change the repertoire of proteins that associate with the SCV, such as the recruitment of SifA, which links the SCV to the microtubular network (Boucrot, Henry et al. 2005). Other SPI-2 effectors, including SseL, a deubiquitinase, may prevent changes in lipid composition on the SCV (Arena, Auweter et al. 2011). Additionally, effectors like SspH2, a ubiquitin ligase, may function in removing host proteins through ubiquitylation. The specific enzymatic activities of SPI-2 effectors may include manipulation of small GTPases, removal of proteins from the SCV, inhibition of protein trafficking, and deubiquitylation, resulting in a vacuole with unique protein and lipid content. *Salmonella* T3SS2 effectors play a crucial role in positioning the *Salmonella*-containing vacuole (SCV) and forming *Salmonella*-induced filaments (Sifs) in epithelial cells (Kuhle and Hensel 2002) (Salcedo and Holden 2003). Specifically, SseF and SseG are required for maintaining the SCV at the microtubule-organizing center (MTOC) and promoting intracellular replication(Kuhle and Hensel 2002) (Salcedo and Holden 2003). SifA is essential for Sif formation, a process linked to SCV membrane integrity, as mutants lacking SifA are released into the cytosol(Beuzon, Salcedo et al. 2002) (Brumell, Tang et al. 2002).Additionally, PipB2 and SseJ cooperate with SifA to facilitate Sif formation through interactions with mammalian proteins,

including the kinesin light chain, which recruits the kinesin-1 motor complex to the SCV surface(Henry, Couillault et al. 2006), driving the extension of Sif tubules towards the periphery of the host cell (Knodler and Steele-Mortimer 2005). Recent studies have shed light on the complex interactions between SifA, the mammalian protein SKIP, and small GTPases, which play a crucial role in regulating host cell membrane dynamics. Specifically, SKIP interacts directly with rab9, a GTPase involved in the lysosome and late endosome function and positioning, and SifA may displace rab9 from this complex (Barbero, Bittova et al. 2002), (Ganley, Carroll et al. 2004), (Jackson, Nawabi et al. 2008). Moreover, SifA can bind directly to rab7 and acts as an exchange factor (GEF) for RhoA, a small GTPase that promotes host membrane tubulation when activated (Harrison, Brumell et al. 2004), (Lossi, Rolhion et al. 2008, Ohlson, Huang et al. 2008). The T3SS2 effector SseJ also plays a crucial role in regulating host cell membrane dynamics, as mutants lacking SseJ exhibit increased cholesterol accumulation and decreased intracellular replication (Ruiz-Albert, Yu et al. 2002),(Nawabi, Catron et al. 2008). Interestingly, high levels of cholesterol can sequester rab9, causing defects in membrane trafficking (Ganley and Pfeffer 2006), suggesting a possible link between SseJ and rab9. Three T3SS2 effectors, SspH1, SspH2, and SseL, interfere with host cell ubiquitin pathways, thereby modulating various cellular processes(Quezada, Hicks et al. 2009). SspH1 and SspH2 are ubiquitin E3 ligases that share homology with similar proteins found in other pathogenic bacteria, such as *Shigella* and Yersinia (Miao, Scherer et al. 1999, Quezada, Hicks et al. 2009). While their exact functions remain unknown, SspH2 has been shown to colocalize with actin around the SCV and target tight junctions in polarized epithelial cells (Miao, Scherer et al. 1999) (Quezada, Hicks et al. 2009). In contrast, SseL is a deubiquitinase that modulates NF-κB activation downstream of IκBα kinases, although its exact impact on the pathway remains unclear (Coombes, Lowden et al. 2007)., (Rytkonen, Poh et al. 2007). ; (Le Negrate, Faustin et al. 2008).

The SPI-2 type III secretion system (T3SS) and its effectors play a crucial role in promoting the replication of STm in bone marrow-derived macrophages and virulence in inbred mice (Figueira,

Watson et al. 2013). While individual SPI-2 effectors have overlapping and partially redundant functions, strains lacking specific effectors exhibit attenuated virulence (Beuzon, Meresse et al. 2000). Notably, the SifA deletion mutant has the most pronounced virulence defect, and SseJ and SopD2 promote the release of bacteria into the cytosol in this mutant (Schroeder, Henry et al. 2010), (Ohlson, Fluhr et al. 2005). It is known that T3SS II effector protein SifA of *Salmonella* hampers the retrograde trafficking of mannose-6-phosphate receptors (MPRs) from late endosomes and lysosomes to the trans-Golgi network in macrophages. So, the lysosomes are formed with a reduced potency (McGourty, Thurston et al. 2012). Another effector protein SseL of STm, is a deubiquitinase that modulates autophagy receptor p62 thus hampers LC3 recruitment and inhibits autophagy to promote its survival in macrophages(Mesquita, Thomas et al. 2012). The presence of cytosolic *salmonella*e triggers host stress response pathways, leading to rapid bacterial clearance in macrophages. Reports from STm state that, effector protein SOPE2 accumulates glycolytic intermediate which they use as a carbon source, helping bacterial survival (Roy Chowdhury, Sah et al. 2022). SseF and SseG form a tethering complex with ACBD3, a protein associated with the Golgi network. This close proximity to the Golgi network facilitates interactions with dense vesicular tubular compartments, enabling the acquisition of membrane and nutrients (Yu, Liu et al. 2016), (Figueira, Watson et al. 2013), (Deiwick, Salcedo et al. 2006).SteA contributes to bacterial growth in epithelial cells and macrophages. It is involved in the partitioning process of the Salmonella-containing vacuole (SCV), and its mutation leads to the congregation of bacteria in tight clusters within enlarged vacuoles (McQuate, Young et al. 2017), (Domingues, Holden et al. 2014), (Domingues, Ismail et al. 2016). SpvC is a phosphothreonine lyase that dephosphorylates ERK, p38, and JNK mitogen-activated protein kinases (MAPKs), thereby inhibiting the transcription of proinflammatory cytokines. A strain lacking SpvC showed attenuated systemic virulence in mice (Mazurkiewicz, Thomas et al. 2008). GtgA, GogA, and PipA effectors cleave the DNA-binding loop within the Rel-homology domain of NF-κB transcription factors p65 and RelB, suppressing proinflammatory immune responses. A *Salmonella* Typhimurium (STm) strain

lacking these effectors showed an increased ability to stimulate NF-κB and increased virulence in an animal model of infection (Sun, Kamanova et al. 2016).

Rab32 contributes to host defence against *Salmonella* by delivering antimicrobial-peptide-containing lysosomal-related organelles (LROs) to the SCV. STm targets Rab32 using SopD2 and GtgE, a cysteine protease that cleaves Rab29, Rab32, and Rab38. Deletion of both SopD2 and GtgE severely inhibits replication in murine macrophages and attenuates virulence in the STm mouse model (Spano and Galan 2012), (Spano, Gao et al. 2016), (Xu, Kozlov et al. 2016).SPI-2 effectors, including SpvB, SseJ, SseF, SseG, SseI, SopD2, SteA, and PipB2 confer virulence defects when deleted individually (Beuzon, Meresse et al. 2000, Ruiz-Albert, Yu et al. 2002, Freeman, Ohl et al. 2003, Jiang, Rossanese et al. 2004, Deiwick, Salcedo et al. 2006) (Knodler, Vallance et al. 2003, Browne, Hasegawa et al. 2008, McLaughlin, Govoni et al. 2009) (Geddes, Worley et al. 2005).

1.12. Difference in the pathogenicity of STy and STm: -

STy belongs to Typhoidal *Salmonella* and STm belongs to the non-Typhoidal *Salmonella* group. Notwithstanding their genetic similarity, with over 90% shared genes, typhoidal and non-typhoidal *Salmonella* exhibit distinct differences in disease manifestation, potentially attributed to hundreds of unique genes encoded by each serovar (Sabbagh, Forest et al. 2010). Both types possess two type 3 secretion systems (T3SSs) on *Salmonella* pathogenicity islands (SPI) 1 and 2, crucial for translocating effector proteins into mammalian cells. However, research indicates that the SPI-2 T3SS is dispensable for intracellular survival and replication of typhoidal *Salmonella* in human macrophages (Sabbagh, Lepage et al. 2012), (Forest, Ferraro et al. 2010) and infection in humanized mouse models (Karlinsey, Stepien et al. 2019). Moreover, many T3SS effector genes in STy and Paratyphi A are either absent or pseudogenes, suggesting that the functions of T3SS-1 and -2, and their effectors, may be altered or absent in these serovars(Gal-Mor, Boyle et al. 2014).

Salmonella Pathogenicity Islands (SPIs) harbor serovar-specific genes that significantly influence virulence. SPIs are large, horizontally acquired genetic cassettes that often encode virulence genes, with SPI-7, -15, -17, and -18 being unique to typhoidal serovars, while SPI-14 is exclusive to non-typhoidal isolates. The STm and STy genomes contain 11 common SPIs (SPIs-1 to 6, 9, 11, 12, 13 and 16). SPIs-8 and 10 were initially found in STy. There is only one, i.e. SPI 14 is specific to STm and four SPIs are specific to STy (SPIs-7, 15, 17, and, 18) (Sabbagh, Forest et al. 2010). Notably, SPI-7 in STy encodes the Vi antigen, a capsular polysaccharide that enhances virulence by preventing complement binding (Wilson, Winter et al. 2011) and masking lipopolysaccharide (LPS) from TLR-4 recognition (Wilson, Raffatellu et al. 2008). The Vi capsule also promotes phagocytosis by human macrophages while evading phagocytosis by human neutrophils (Zhang, Lepenies et al. 2022). Furthermore, TviA, the transcriptional activator of Vi production, suppresses the expression of flagellin and T3SS-1, allowing STy to evade TLR-5 and NAIP-mediated inflammatory responses (Winter, Winter et al. 2015). Collectively, these findings underscore the crucial role of Vi capsule production in conferring a "stealth" phenotype to STy, enabling it to disseminate systemically without triggering robust inflammatory immune responses.

A distinctive characteristic of typhoidal serovars is the extensive presence of pseudogenes in their genomes, indicative of genome degradation. Approximately 5% of the genome in sequenced typhoidal isolates comprises pseudogenes, resulting from frameshift mutations or premature stop codons (Sabbagh, Forest et al. 2010). These pseudogenes encompass a wide range of functions, including metabolic genes (e.g., narW, wcaK), transporters (e.g., fhuA, mglA), chemotaxis genes (e.g., trg), and cell-wall related genes (e.g., dacD) (McClelland, Sanderson et al. 2004),(Holt, Thomson et al. 2009)]. The loss of specific operons, such as the ydiQRSTD operon in STy, may have contributed to the transition of STy from a gastrointestinal to an extraintestinal pathogen (Bronner, Faber et al. 2018). Notably, multiple virulence genes in STm are pseudogenes in STy and/or *S.* Paratyphi A, including

several SPI-1 and SPI-2 T3SS effectors (i.e., sopA, sseI, steB, sopE2, sseK2, and sopD2) (McClelland, Sanderson et al. 2004).

The pseudogenization of specific genes, such as sseI, in non-typhoidal *Salmonella* belonging to the ST313 lineage, contributes to their ability to cause systemic bacteremia in humans (Carden, Okoro et al. 2015). Similarly, in STy and *S.* Paratyphi A, pseudogenes include genes involved in gut colonization, such as misL, siiE, and T6SS genes sciI and sciS (Sabbagh, Forest et al. 2010). The loss of these genes is likely not necessary for systemic disease in humans, as they promote intestinal persistence, a hallmark of non-typhoidal serovars (Sabbagh, Forest et al. 2010). Gene loss has been shown to fundamentally alter bacterial pathogen behavior, enhancing virulence phenotypes in some cases (Maurelli, Fernandez et al. 1998, Moore, Reckseidler-Zenteno et al. 2004) (Maurelli 2007, Hottes, Freddolino et al. 2013). In STy, the loss of the fepE gene has been reported to enhance immune evasion mediated by the Vi capsule (Crawford, Wangdi et al. 2013). The Type 3 Secretion System-1 (T3SS-1), encoded in the *Salmonella* Pathogenicity Island-1 (SPI-1) locus, facilitates *Salmonella* invasion of non-phagocytic cells, including epithelial cells, in the gastrointestinal tract. However, the regulation of T3SS-1-dependent invasion differs between typhoidal and non-typhoidal *Salmonella*. For instance, TviA, a transcriptional regulator exclusive to STy, positively regulates Vi capsule production while repressing T3SS-1 expression (Winter, Winter et al. 2009). Additionally, STy uniquely up-regulates T3SS-1 and invades epithelial cells in response to bile, present in the gallbladder, whereas STm does not (Johnson, Ravenhall et al. 2018). This bile-mediated response may contribute to the gallbladder being a site of chronic STy infections in humans (Basnyat and Baker 2015). Beyond differences in T3SS regulation, the effectors encoded on these virulence loci also exhibit distinct profiles between non-typhoidal and typhoidal *Salmonella*. Notably, many T3SS-1 effectors contributing to intestinal inflammation in STm are pseudogenes in STy and *S.* Paratyphi A, suggesting genetic degradation as a possible explanation for the lack of acute intestinal inflammation in typhoidal serovars. For instance, six T3SS-1 effectors (SipA, SopA, SopB, SopD, SopE, and SopE2) induce

neutrophil infiltration and fluid accumulation during STm infection, but SopA and SopE2 are pseudogenized in STy (Zhang, Santos et al. 2002, Valenzuela, Hidalgo et al. 2015). Alternatively, typhoidal serovars may encode unique genes to dampen inflammatory responses, such as the T3SS-1 dependent effector StoD in STy, which functions as an E3/4 ubiquitin ligase to degrade host targets and potentially modulate immune responses (McDowell, Byrne et al. 2019).

Following adherence to epithelial cells, T3SS-1 in both STy and STm injects effector proteins into the host cell cytoplasm to facilitate internalization. Both serovars induce T3SS-1-dependent ruffling of epithelial cell surfaces, indicating a conserved "trigger" mechanism of invasion mediated by cytoskeletal rearrangement. Injected effectors SipA and SipC directly nucleate actin (McGhie, Hayward et al. 2001), while SopE and SopE2 initiate actin polymerization through host GTPases Cdc42 and Rac1 (Friebel, Ilchmann et al. 2001). Notably, SipA, SipC, and SopE are conserved in typhoidal serovars, whereas SopE2 is pseudogenized (Valenzuela, Hidalgo et al. 2015). In contrast to STm, which deploys SptP to antagonize SopE-mediated cytoskeletal rearrangements (Fu and Galan 1999), STy's SptP is non-functional due to a point mutation (Johnson, Byrne et al. 2017)leaving the mechanism of post-invasion cytoskeletal resolution unclear.

While T3SS-1-dependent invasion facilitates entry into non-phagocytic cells, macrophages naturally phagocytose pathogens, rendering SPI-1-mediated invasion unnecessary for *Salmonella* internalization (van der Velden, Lindgren et al. 2000). Intriguingly, macrophages, which typically eliminate phagocytosed pathogens, serve as a primary replicative niche for *Salmonella* during systemic infection (Jiang, Wang et al. 2021). To replicate within macrophages, *Salmonella* employs multiple molecular mechanisms. T3SS-2 effector proteins are essential for STm intra-macrophage replication within the *Salmonella*-containing vacuole (SCV) (Figueira, Watson et al. 2013). However, T3SS-2 is not strictly required for STy intracellular survival and/or replication in THP-1 macrophages (Sabbagh, Lepage et al. 2012),(Forest, Ferraro et al. 2010). The underlying molecular mechanisms driving this discrepancy between non-typhoidal and typhoidal serovars remain unclear. Research on *Salmonella*

survival and replication within macrophages has primarily focused on STm (Jennings, Thurston et al. 2017). Studies have shown that T3SS-2-dependent effectors, such as SseF and SseG, play a crucial role in intracellular trafficking of the *Salmonella*-containing vacuole (SCV) (Yu, Liu et al. 2016), and these effectors are conserved in typhoidal *Salmonella*. In STm, multiple effectors, including SifA, PipB2, SopD2, and SseJ, contribute to the growth and elongation of the SCV membrane, forming *Salmonella*-induced filaments (Sifs) (Knuff and Finlay 2017). However, these Sif-inducing effectors are not universally conserved; SseJ and SopD2 are pseudogenes in typhoidal *Salmonella* (Holt, Thomson et al. 2009),(Trombert, Berrocal et al. 2010). The absence of GtgE, which cooperates with SopD2 in STm, is also notable in typhoidal serovars (Spano and Galan 2012). GtgE degrades Rab GTPases, regulating membrane trafficking and potentially delivering antimicrobial factors to the SCV (Spano 2014). Previous studies have demonstrated that the presence of Rab32 in mouse macrophages leads to the death of STy, potentially explaining why typhoidal *Salmonella* cannot infect mice (Spano and Galan 2012). Interestingly, the trans-expression of GtgE from STm enables STy to overcome host restriction by removing Rab32 from the SCV surface and replicating in murine macrophages (Spano and Galan 2012). Although typhoidal *Salmonella* lacks GtgE, a recent study suggests that STy employs its SPI-1 T3SS to evade Rab32-mediated killing in human macrophages (Baldassarre, Solano-Collado et al. 2021). The molecular mechanisms underlying STy's ability to overcome Rab-mediated killing in human macrophages, but not in murine macrophages, remain unclear. Intracellular *Salmonella* in human macrophages exhibits heterogeneous fates, including host-killed, non-replicating, persisting, and actively replicating bacteria (Helaine, Cheverton et al. 2014). The transcriptomic phenotype of macrophages with host-killed bacteria and bystander cells is characterized by an M1 polarization state, whereas macrophages allowing intracellular replication and persistence exhibit a transcriptomic profile dominated by M2 polarization genes. The state of individual macrophages influences the ability of intracellular *Salmonella* to replicate, and manipulating macrophage activation states can alter the outcome of *Salmonella* infection. Ongoing research aims to determine whether these bacterial

populations and/or macrophage polarization states differ between typhoidal and non-typhoidal *Salmonella* infections. Elucidating the molecular mechanisms underlying each state and characterizing the role of each population in maintaining persistent infection may reveal additional serovar-specific differences during macrophage infection. The difference in the presence of effector molecules between STy and STm is listed in Table 1.3.(Saliba, Li et al. 2016)

Table 1.3**. List of SPI-1 and SPI-2 effectors from STm and STy**

Effectors **SPI1 T3SS**	**LT2**	**CT18**	**% identity**	**% homology**
AvrA	STM2865	A[e]		-
SipA (SspA)	STM2882	STY3005	97	98
SipB (SspB)	STM2885	STY3008	99	99
SipC (SspC)	STM2884	STY3007	99	99
SipD (SspD)	STM2883	STY3006	87	93
SopA	STM2066	(STY2275) [b]	-	-
SopB (SigD)	STM1091	STY1121	98	98
SopE	A	STY4609	-	-
SopE2	STM1855	(STY1987)	-	-
SptP	STM2878	STY3001	94	96
SopD	STM2945	STY3073	98	99
SteA	STM1583	STY1482	88	93
SteB	STM1629	A	-	-
SPI2 T3SS				
GogB	STM2584	A	-	-

PipB	STM1088	STY1117	97	98
PipB2	STM2780	STY2897	91	96
SifA	STM1224	STY1264	93	95
SifB	STM1602	STY1462	98	99
SopD2	STM0972	(STY0971)	-	-
SpiC (SsaB)	STM1393	STY1727	100	100
SseF	STM1404	STY1716	96	96
SseG	STM1405	STY1715	98	99
SseI	STM1051	A	-	-
SseJ	STM1631	(STY1439a)	-	-
SseK1	STM4157	A	-	-
SseK2	STM2137	A	-	-
SseK3	A	A	-	-
SseL	STM2287	STY2517	93	97
SspH2	STM2241	STY2467	99	99
SteC	STM1698	STY1353	91	95
SpvB	pSLT039	A	-	-
SpvC	pSLT038	A	-	-
Translocated by both SPI1 and SPI2 T3SS				
SlrP	STM0800	(STY0833)	-	-
SspH1	A	A	-	-

[b]ORFs in parentheses and yellow shade are pseudogenes.

[e]A, absent from chromosome

1.13. Role of bacterial serine-threonine kinases in bacterial pathogenesis: -

The discovery of serine/threonine-protein-kinases in bacteria has unearthed a complex regulatory paradigm. Over the past three decades, technological advancements have facilitated the characterization of numerous kinases and phosphorylation substrates. Research has revealed that serine/threonine phosphorylation plays a pivotal role in modulating various bacterial processes, including, Cell growth and division, Antibiotic resistance, Virulence and pathogenesis, Metabolic regulation, Chromosomal dynamics, and cellular differentiation. These findings have significantly expanded our understanding of bacterial signaling, underscoring the intricacies of microbial regulation.

The M. tuberculosis serine/threonine-protein-kinase (STPK) PknG, initially identified as the first non-membrane STPK secreted into the host cytosol (Walburger, Koul et al. 2004), plays a crucial role in preventing phagolysosome fusion, thereby enabling bacterial survival within the host. Subsequent investigations have elucidated the mechanistic details of PknG's virulence modulation, revealing that the SecA2 secretion system is essential for PknG secretion, alongside the phosphatase SapM. Upon secretion, PknG and SapM collectively block the exchange and recruitment of the late endosomal marker Rab7 into the phagosomal membrane (Zulauf, Sullivan et al. 2018), ultimately inhibiting phagolysosome fusion and facilitating *M. Tuberculosis* pathogenesis. A recent study (Pradhan, Shrivastva et al. 2018) has provided novel insights into the mechanisms underlying PknG's virulence modulation, revealing that PknG interacts directly with the host endosomal marker Rab7L1 within the Golgi complex. Through this interaction, PknG prevents the conversion of Rab7L1-GDP into its GTP-bound isoform, thereby blocking the recruitment of Rab7 and lysosomal markers into the phagosome membrane (Pradhan, Shrivastva et al. 2018). As a consequence, PknG prevents phagosome maturation and phagolysosome fusion, ultimately enabling *M. tuberculosis* to evade host cellular defenses and establish a replicative niche within the host. *M. tuberculosis* has been shown to occasionally escape the phagosome and enter the cytosol, where it induces autophagy in a PknG-dependent manner (Ge, Lei et al. 2022). Specifically, PknG competitively binds with the AKT protein kinase, blocking its

activation and resulting in the downregulation of mTOR signaling, which in turn leads to autophagosome formation that recaptures cytosolic *M. Tuberculosis* (Ge, Lei et al. 2022). Furthermore, PknG binds to the endosomal marker Rab14 and its activator protein TBC14, interfering with autophagosome maturation (Ge, Lei et al. 2022). This complex interplay enables M. tuberculosis to re-establish a replicative niche within the macrophage, facilitating its survival and persistence within the host. PknG plays a crucial role in maintaining intracellular redox balance and metabolic adaptation in *M. Tuberculosis*, particularly under hypoxia and oxidative stress conditions (Wolff, de la Pena et al. 2015) (Khan, Bhaskar et al. 2017). Specifically, PknG associates with the ribosomal protein L13 and the nudix hydrolase RenU to facilitate NADH hydrolysis, thereby mitigating oxidative stress (Wolff, de la Pena et al. 2015). Additionally, PknG mediates persistence and drug tolerance by phosphorylating GarA in response to hypoxia, enabling *M. Tuberculosis* to adapt to adverse environmental conditions and evade host immune responses (Khan, Bhaskar et al. 2017). PknG has been found to possess an unusual role in proteasomal degradation, in addition to its protein-kinase activity (Wang, Ge et al. 2021). The N-terminal lobe of PknG's protein-kinase domain contains a β-grasp fold similar to ubiquitin-like proteins, enabling it to mimic both E1 and E3 ubiquitin enzymes (Wang, Ge et al. 2021). PknG interacts with the host E2 ubiquitin-conjugating enzyme UbcH7, transferring activated ubiquitin and subsequently functioning as a ubiquitin ligase E3 to target host proteins, including TRAF2 and TAK1, for degradation(Wang, Ge et al. 2021). This ubiquitination results in the downregulation of the NF-κB pathway, highlighting PknG's multifaceted role in manipulating host cellular processes to facilitate *M. tuberculosis* survival and persistence (Wang, Ge et al. 2021).

Legionella pneumophilia, an intracellular bacterium, utilizes its Dot/Icm Type IV secretion system to secrete approximately 300 virulence effectors, including four serine/threonine-protein-kinases (STPKs): LegK1, LegK2, LegK4, and LegK7, which play critical roles in manipulating host cellular processes (Segal, Feldman et al. 2005). LegK1 activates the NF-κB signal cascade by phosphorylating IκB and p100, leading to proteasomal degradation and subsequent inflammatory cytokine response

(Ge, Xu et al. 2009). LegK2 interacts with the ARP2/3 complex, phosphorylating ARPC1B/APR3 subunits, which impacts global cytoskeleton remodeling and prevents phagolysosome fusion (Michard, Sperandio et al. 2015). LegK7 modulates host signaling by phosphorylating MOB1A, leading to the proteasomal degradation of YAP1 and TAZ, resulting in differential gene expression that facilitates bacterial survival within macrophages (Lee and Machner 2018). Finally, LegK4 interferes with the host translation machinery by phosphorylating the Hsp70 chaperone, affecting its ATPase activity and reducing protein folding efficiency, thereby impacting protein synthesis (Moss, Taylor et al. 2019).

The STPK CstK of *Coxiella burnetii* is secreted into the host cytosol via a Type IV secretion system, where it interacts with TBC1D5 to regulate phagosome maturation and bacterial survival (Martinez, Huc-Brandt et al. 2020). Similarly, the STPKs PpkA and SiaB of Pseudomonas aeruginosa are essential for infection, with PpkA mediating the phosphorylation of Fha1 to facilitate the secretion of virulence factors through a Type VI secretion system (Mougous, Gifford et al. 2007), and SiaB, in conjunction with phosphatase SiaA, controlling the phosphorylation of SiaC to modulate biofilm formation through the synthesis of cyclic-di-GMP (Mougous, Gifford et al. 2007, Poh, Lin et al. 2020). The serine/threonine-protein-kinase (STPK) StkP of *Streptococcus pneumoniae* plays a crucial role in modulating the intracellular survival of the pathogen. StkP phosphorylates the response regulator ComE, which influences global transcriptional regulation under acidic stress conditions (Pinas, Reinoso-Vizcaino et al. 2018). This phosphorylation event favors resistance to cell wall-damaging antibiotics, thereby promoting *S. pneumoniae* survival and persistence within the host (Pinas, Reinoso-Vizcaino et al. 2018).

STm STPK SteC causes major alterations to the microtubule, intermediate filament, and actin networks. The kinase activity of the STPK SteC, which is secreted by the SPI-2 T3SS, induces a meshwork of F-actin that is formed around SCVs and bacterial microcolonies. SteC promotes actin cytoskeleton reorganization by activating a signaling pathway involving the MAPKs MEK and ERK,

myosin light chain kinase, and myosin IIB. Specifically, SteC phosphorylates MEK directly on Ser200, a previously unknown phosphorylation site. Ser-200 phosphorylation is predicted to displace a negative regulatory helix causing autophosphorylation on the known MEK activatory residues, Ser-218 and Ser-222. Both SteC-null and kinase-deficient mutant strains display enhanced replication in infected cells, suggesting that the effects of SteC on the actin cytoskeleton limit bacterial growth. (Odendall, Rolhion et al. 2012)

OspG, a serine/threonine protein kinase (STPK) produced by *Shigella*, has been found to subvert the host's innate immune system by suppressing the NF-κB signaling pathway (Kim, Lenzen et al. 2005).OspG achieves this immune evasion by binding to ubiquitinylated E2 enzymes, such as UbcH7 and UbcH5, without phosphorylating them. This sequestration of E2 enzymes leads to a decrease in the degradation of IκBα, thereby blocking the activation of the NF-κB pathway. Notably, the immune-modulating activity of OspG appears to be dependent on its kinase activity, highlighting the complex mechanisms employed by *Shigella* to manipulate host innate immunity.

Previously this lab had employed a bioinformatics approach to identify a putative eukaryotic-like serine/threonine kinase (eSTK) in *Salmonella* Typhi. A BLAST search revealed two candidate kinases, T4519 (STY4822) and T4520 (STY4823), with significant sequence homology to eukaryotic protein kinases(Theeya, Ta et al. 2015). Notably, T4519 exhibited 58% query coverage and 23% identity to eukaryotic protein kinases, with the conservation of 11 critical amino acid residues defining the eukaryotic protein kinase superfamily. T4519's sequence was further analyzed using similarity searches against known crystal structures in the Protein Data Bank (PDB). The results showed a striking resemblance to *M. tuberculosis* PknB, a well-characterized eSTK. The conservation of critical residues, including the RD sequence and the activation loop, suggests that T4519 may possess similar catalytic activity. Furthermore, the presence of an STK active-site signature motif, {[LIVMFYC]-x-[HY]-x-D-[LIVMFY]-K-(2)-N-LIVMFYCT}, supports T4519's classification as an eSTK. T4519 is encoded within the prpZ gene cluster, which is exclusively present in *S.* Typhi and absent from other

Salmonella serovars. This cluster also includes a second eSTK (prkY) and a serine/threonine phosphatase 2C gene (prpZ). Deletion of this gene cluster has been shown to compromise *S.* Typhi's viability within host cells. Recent studies have demonstrated that T4519 is induced by reactive oxygen species (ROS) within the phagosome, followed by translocation into the cell cytosol, where it modulates innate immune responses. The discovery of T4519, a novel eSTK in *S*. Typhi, provides new insights into the molecular mechanisms underlying *Salmonella* pathogenesis. Further research is warranted to elucidate the precise functions of T4519 and its role in modulating the factors host cells to promote bacterial survival.

1.14. Knowledge Gap: -

Despite being closely related, STy and STm exhibit distinct differences in their pathogenesis, virulence mechanisms, and host interactions. However, there is a significant knowledge gap regarding the specific mechanisms underlying these differences.

Key areas of uncertainty:

1. **T3SS-2 effector functions**: While STm T3SS-2 effectors have been well-characterized, the functions of STy T3SS-2 effectors remain largely unknown.

2. **Macrophage interactions**: The dynamics of STy-macrophage interactions, including the role of Rab GTPases and macrophage polarization states, require further investigation.

3. **Virulence gene regulation**: The regulation of virulence genes in STy, particularly those not involved in T3SS-1 and T3SS-2, is not well understood.

4. **Host adaptation and specificity**: The mechanisms underlying STy's host specificity and adaptation to the human host, compared to STm's broader host range, remain unclear.

5. **T3SS-2 effector independent survival mechanism:-** As previously reported, T3SS-2 is not required for STy intracellular survival in human macrophages and in mice (Sabbagh, Lepage et al.

2012), (Forest, Ferraro et al. 2010),(Karlinsey, Stepien et al. 2019). However, how it shows T3SS-2 independent survival and which effectors are involved in it was not investigated.

Previously it was reported that a gene of prpZ cluster, T4519, exclusively present in Typhoidal *Salmonella* helps in the bacterial survival inside human macrophages (Theeya, Ta et al. 2015). T4519 works T3SS-II independently and works through SPI 10. However, how it causes less survival was not investigated previously. Addressing these knowledge gaps is essential for developing effective vaccines and therapies. Understanding the unique mechanisms of STy pathogenesis will inform the development of targeted interventions. It will also help in Improving diagnostics and surveillance. Elucidating the differences between STy and STm will enhance diagnostic accuracy and inform public health strategies.

Future research directions:

1. Comparative genomics and transcriptomics
2. Functional characterization of T3SS-2 effectors
3. Investigation of macrophage interactions and polarization states
4. Analysis of virulence gene regulation and host adaptation mechanisms

By addressing these knowledge gaps, we can gain a deeper understanding of STy pathogenesis which will be different from STm, and develop effective strategies for prevention, diagnosis, and treatment of typhoid fever.

To overcome these knowledge gaps, my three objectives are: -

1. **Objective 1 - To study the maturation and traffic of STy-containing phagosomes.**
2. **Objective 2: - To study lysosomal biogenesis, turnover, and membrane integrity in STy infected Macrophages**
3. **Objective 3: - To study the redox biology of STy infected macrophages**

Chapter 2

Rationality of the objectives

Rationale of objective 1: To study the maturation and traffic of STy-containing phagosomes.

The objective of studying the maturation and trafficking of STy -containing phagosomes is crucial for understanding the pathogenesis and survival mechanisms of this intracellular pathogen. Here's a detailed description of the rationality behind this objective, supported by relevant references:

Intracellular Survival and Replication

STy, the causative agent of typhoid fever, survives and replicates within host cells, particularly macrophages and monocytes. Understanding how STy-containing phagosomes mature and traffic within host cells can provide insights into their intracellular lifestyle.

Phagosome Maturation and Trafficking

Phagosome maturation involves a series of fusion and fission events with endosomes and lysosomes, leading to the formation of phagolysosomes (Desjardins, Huber et al. 1994). However, STy can manipulate this process to avoid degradation and create a replicative niche (Rajashekar, Liebl et al. 2008). Studying the maturation and trafficking of STy-containing phagosomes can reveal how the bacterium interacts with host cell machinery.

Host-Pathogen Interactions

The maturation and trafficking of STy-containing phagosomes involve complex interactions between host cell proteins and bacterial effectors. For example, STy's type III secretion system (T3SS) and type IV secretion system (T4SS) inject effectors into host cells, modulating phagosome dynamics (Galan and Waksman 2018) (Backert and Meyer 2006). Investigating these interactions can uncover novel targets for therapeutic intervention.

Implications for Vaccine Development and Disease Control

Understanding the intracellular life cycle of STy is essential for developing effective vaccines and therapies. By studying the maturation and trafficking of STy-containing phagosomes, researchers can:

1. Identify novel antigenic targets for vaccine development
2. Develop strategies to disrupt STy's intracellular survival mechanisms
3. Enhance our understanding of typhoid fever pathogenesis and transmission

Studying the maturation and trafficking of STy-containing phagosomes is crucial for understanding the pathogenesis and survival mechanisms of this intracellular pathogen. Investigating these processes can reveal novel targets for therapeutic intervention, enhance our understanding of typhoid fever, and inform the development of effective vaccines and therapies.

Rationalate of objective 2: To study lysosomal biogenesis, turnover, and membrane integrity in STy infected Macrophages

Intracellular bacteria have evolved various strategies to manipulate host cell processes, including lysosomal biogenesis, turnover, and membrane integrity, to promote their survival and replication within the host cell. Some examples are

- **Inhibiting Lysosomal Biogenesis**: it was reported that *Coxiella burnetii* inhibits nuclear translocation of TFEB which is the master transcription factor for lysosomal biogenesis to avoid lysosomal killing (Killips et al. 2024).
- **Modulating Lipid Metabolism**: *Mycobacterium tuberculosis* can alter lipid metabolism in the host cell, leading to changes in the composition and fluidity of lysosomal membranes (Gago et al.2018).

  For every intracellular bacterial survival, the lysosomal pathway is the most important pathway that can be altered by the pathogen to facilitate its survival. The mechanisms of intracellular survival of *S.* Typhi are no exception. Reports are less finding the effectors of *S.* Typhi manipulating lysosomal pathways.

  Lysosomal dysfunction in STy-infected macrophages can lead to

1. Impaired antigen presentation and immune response.

2. Altered cellular homeostasis and nutrient availability.

3. Enhanced bacterial survival and replication.

Understanding lysosomal biogenesis, turnover, and membrane integrity in STy-infected macrophages can help in the development of novel therapeutic strategies targeting lysosomal function.

Rationalate of the objective 3: - To study the redox biology of STy infected macrophages

Intracellular bacteria can alter the redox balance in host macrophages, influencing bacterial survival, replication, and immune evasion. Investigating the redox biology of STy-infected macrophages can reveal critical insights into the pathogenesis of typhoid fever.

Reactive Oxygen Species (ROS) and Nitrogen Species (RNS) in Infection

ROS and RNS play pivotal roles in host defense against pathogens, but excessive production can lead to oxidative stress and tissue damage (Di Meo, Reed et al. 2016). STy infection modulates ROS and RNS levels to create a favorable intracellular environment (Rhen 2019). It was found that , *S.* Typhimurium infection increases ROS production but the bacteria enables several mechanisms like the degradation of Reactive Oxygen Species (ROS) before they act on target molecules by increasing Superoxide dismutases (SodA and SodCI) ,Catalase (KatE), Thiol peroxidase (Tpx) (Rhen 2019).But the effector molecules that directly operates ROS are unknown. Furthermore, studies regarding redox biology changes due to *S.* Typhi infection in macrophages are very inadequate.

Altered redox balance in STy-infected macrophages can affect:

1. Immune response and cytokine production

2. Antimicrobial activity and phagocytosis
3. Cellular signaling pathways and gene expression
4. Mitochondrial function and energy metabolism

Studying redox biology in STy-infected macrophages can uncover:

1. Activation of redox-sensitive transcription factors (e.g., NF-κB, Nrf2).
2. Reveal novel therapeutic targets to modulate redox balance.
3. Enhance our understanding of typhoid fever pathogenesis and transmission.
4. Identify potential biomarkers for disease diagnosis and monitoring.

Studying the redox biology of STy-infected macrophages provides valuable insights into the complex interactions between host and pathogen. Understanding these mechanisms can reveal novel therapeutic targets, enhance our understanding of typhoid fever pathogenesis, and inform the development of antioxidant-based adjunct therapies.

Chapter 3

Materials and Methods

3.1. Cells and reagents.

Cell culture reagents and THP-1 cell line were bought from Invitrogen and the American Type Culture Collection (ATCC) respectively. RPMI 1640 and FBS were bought from GIBCO. Culture media such as Luria broth, and Luria agar for bacterial growth were procured from BD Difco. RNA was isolated using TRISOL from Sigma. cDNA synthesis reagents from Fermentas and SYBR green master mix from ABI were purchased. TNF-α and IL-6 ELISA kits were purchased from R&D systems (DY206-05 for IL-6 and DY210-05 for TNF- α). All cell culture plasticware used were from Nunc, Antibodies against IκBα (9242S), p65(8242S), histone H4(13919S), β- actin(3700S), mTOR (2983S), galectin-1(12936S) were purchased from Cell Signalling, cystatin B (PA5110828) and cystatin C (PA5110779) were bought from Thermofisher, Cathepsin B antibody (ab214428) and cathepsin B substrate Magic red (ab270772) were from ABCAM, horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies bought from Pierce, horseradish peroxidase (HRP)-conjugated anti-rabbit secondary (7074P2) antibodies procured from CST. Other reagents such as MG-132 \, SN-50 and Ca-074, triton X-100, and Hoechst were bought from Sigma. Reagents for confocal microscopy such as Lamp1 antibody, Rab7 antibody, lysotracker, Dextran Alexa-Fluor 546, fluorochrome tagged secondary antibodies (Alexa-Fluor conjugated anti-rabbit, Alexa-Fluor conjugated anti-mouse, Alexa-Fluor conjugated anti-rabbit), Lysosensor yellow blue DND-160 were taken from Invitrogen. Cystatin B Knock out plasmids and transfection media were taken from Santacruz. Streptomycin, Ampicillin, Kanamycin antibiotics and other chemicals used were procured from Sigma. Annexin V and Pi and DCFDA (for ROS) were purchased from Thermo.

Table 3.1- List of Reagents and catalog no.

Reagent Name	Catalog No	Manufacturer
THP-1 cell line	TIB-202	ATCC
RPMI 1640	31800022	GIBCO
FBS	26140079	GIBCO
Luria broth	241420	BD Difco
Luria agar	211829	BD Difco
TRISOL	T9424	Sigma

cDNA kit	1708891	Bio-Rad
SYBR green Master Mix	4368708	ABI
TNF-α	DTA00D	R&D systems
IL-6 ELISA kits	D6050B	R&D systems
IκBα	9242S	Cell Signalling Technology
p65	8242S	Cell Signalling Technology
Histone H4	13919S	Cell Signalling Technology
β-actin	3700S	Cell Signalling Technology
m-TOR	2983S	Cell Signalling Technology
Galectin-1	12936S	Cell Signalling Technology
TLR9	13674S	Cell Signalling Technology
HRP-conjugated anti-rabbit IgG	7074P2	Cell Signalling Technology
Cystatin B	PA5110828	Thermofisher Scientific
Cystatin C	PA5110779	Thermofisher Scientific
Cathepsin B	ab214428	ABCAM
Cathepsin B substrate Magic red	ab270772	ABCAM
TLR2	ab213676	ABCAM
TLR4	ab13556	ABCAM
MG-132	474790	Sigma
SN-50	SML1471	Sigma
Ca-074	C5732	Sigma
triton X-100	T8787	Sigma
Monensin	M5273	Sigma
Hoechst	94403	Sigma
Lamp1	MA1-164	Invitrogen
Rab7	PA5-52369	Invitrogen
lysotracker	L7528	Invitrogen
Dextran Alexa-Fluor 546	D22911	Invitrogen
Alexa-Fluor-conjugated anti-rabbit 594 IgG	A-21123	Invitrogen
Alexa Fluor conjugated anti-rabbit 633 IgG	A21070	Invitrogen
H2DCFDA	D399	Invitrogen
LysoSensor™ Yellow/Blue DND-160	DCF D399	Invitrogen
siRNAs for p65	L7545	Ambion
Annexin V	V13242	Invitrogen

3.2.Bacterial strains, plasmids and primers

Bacterial strains used in the study were *Salmonella* Typhi Ty2, Ty2 ΔT4519, αSDM-T4519 Ty2, and *Salmonella* Typhimurium LT2. The GFP, expression plasmid pAKGFP1(14076) was acquired from Addgene. For cystatin B knockout, si-RNA and transfection media were bought from Santacruz. Cystatin B siRNA (h)(sc-4443), siRNA Dilution Buffer (sc-29527) siRNA Transfection Reagent (sc-

29528), siRNA Transfection Medium (sc-36868), siRNA Reagent System (sc-45064) Control siRNA-A (sc-37007) were purchased from Santacruz .A list of primers was added in Table 3.2.

Table 3.2- List of Primers used in this study.

Primer Name	**5'<-----Sequence----->3'**
RAB7 FP.	TGGGACACAGCAGGACAGGAAC
RAB7 RP.	TGTTGGGGGCAGTCACATCAAA
LiMP2 FP.	GGAAACCGAAACCGAGTCC
LIMP2 RP.	CAACTGCAAGGAGGGAGGAG
Cystatin B FP.	CTGTGTTTAAGGCCGTGTCA
Cystatin B RP.	AGGTCAGCTCATCATGCTTG
Cystatin C FP.	GCGGCGTGCACTGGACTTTG
Cystatin C RP.	GCCGCCTGCTGCCTTCTCTG
β-Actin FP.	ACAATGTGGCCGAGGACTTT
β-Actin RP.	GCACGAAGGCTCATCATTCA
NOVA1 FP.	TTGCCATCTTCCCCAACTAC
NOVA1 RP.	TTACAGCCTTCACAGTAGCAC
YB-1 FP.	AAGTGATGGAGGGTGCTGAC
YB-1 RP.	TTCTTCATTGCCGTCCTCTC
RAB 12 FP.	GGGAGGTTATAGACACTGGTGC
RAB 12 RP.	AACTGCTCCCCATGTGCAAG
CTSH FP.	CAAGGGGATCATGGGTGAAG
CTSH RP.	TCAAAGGCAAAGCTCACAGG
RAB10 FP.	CGATGCCTTCAATACCACCT
RAB10 RP.	GCCACTTGCTGATGTTCTCA

RAB34 FP.	CACCCTCATTCCTCCAGAGC
RAB34 RP.	TAGGGGGCATCCAGTCTTTG
RAB5A FP.	CAGAGGAGCACAAGCAGCCATAG
RAB5A RP.	GTCGGCCTTGTTTCCCGATA
TFEB FP.	CTAACAGATGCTGAGAGCAGAG
TFEB RP.	CCAGCGCACGTCCTTAG
LAMP2 FP	CAGTGGCACCCACCATACACAC
LAMP2 RP.	CCCATGGTAGCCAGCAGACAAG
LAMP1 FP.	ACCACCGTCCTGCTCTTCCA
LAMPI RP.	TCCTCCGCGTTGCACTTGTA
CTSD FP.	CCCATTCCCGAGGTGCTCAA
CTSD RP.	TTGGAGGAGCCCGTGTCGAA
CTSB FP.	TTCTCCTGCTTCAGCCTTCC
CTSB RP.	TCACGCCTGTAATCCCAACA

3.3.Cell culture: -

Media used for the human acute monocytic leukemia cell line THP-1 was RPMI 1640 (Gibco) supplemented with 10% Fetal Bovine Serum (FBS). Cells were differentiated into macrophages by adding 100 nM phorbol 12-myristate 13-acetate (PMA) for 24 h, followed by maintaining the cells in the culture media in the absence of PMA for 24 h before the experiment. For PBMC, RPMI 1640 (Gibco) supplemented with 10% male Human serum was used as media. For HT-29, DMEM media (Gibco) with 10% FBS was used.

3.4.PBMC isolation: -

3.4.1. Materials: -

- Whole Human Blood

- 1X PBS
- Histopaque
- Centrifugation Machine
- RBC lysis Buffer
- 24 wells plate
- RPMI Medium
- Male Human Serum
- Haemocytometer
- Recombinant human Macrophage Colony Stimulating Factor [rh-MCSF (R& D)].

3.4.2. Procedure: -

Peripheral blood mononuclear cells were isolated from whole human blood collected from Apollo Glenicals Hospital. Human blood was diluted in a 1:1 ratio with 1X PBS and then loaded onto Histopaque at a ratio of 1 volume Histopaque and 3 volume blood. Then centrifugation was done at 550 g for 30 minutes at acceleration and deceleration of 1 and 1. Different layers of blood cells were found. From them, a white layer found that contained monocytes was aspirated carefully and washed with 1X PBS through centrifugation. After washing with PBS, the pellet was resuspended with RBC lysis buffer (11814389001 Roche). It was used to eliminate RBC contamination from PBMCs. After isolation, cells were counted using a Haemocytometer and seeded at 2* 10^6 cells per well in a 24-well plate in RPMI medium supplemented with 10 % male human serum. After 24 hours of cell seeding, media is replaced with fresh media containing 50 µM rh-MCSF (R& D). This process was repeated thrice on alternative days. At day 7, around 10% of monocytes were converted into macrophages.

3.5.Gentamycin protection Assay: -

3.5.1. Materials: -

- THP-1 cells
- PMA
- 24 well plates
- RPMI media
- FBS
- PBMC
- rh-MCSF (R& D).
- Centrifugation Machine
- 1X PBS
- 5% CO2 Incubator
- Gentamycin Solution
- LA plates

3.5.2. Procedure: -

Gentamicin protection assay was done with both THP-1- and MoM-derived macrophages. THP-1 macrophages were infected at a multiplicity of infection (MOI) of 50 for Ty2 and LT2 and 100 for Ty2Δt4519. The corresponding MOI for MoM cells were 20 and 40, respectively. Prior to infection, bacterial cultures were opsonized with complete RPMI1640. Cells were synchronized with the opsonized bacteria by centrifugation at 400 × g for 5 min, followed by incubation for 30 min at 37°C (5% CO2). Extracellular bacteria were removed by washing the cells 3 times with 1× phosphate-buffered saline (PBS) and the infected cells were cultured in a complete RPMI medium, containing 100 µg/ml of gentamicin (Gibco) for 1 h, followed by 15 µg/ml of gentamicin till the end of the experiment. Cells were lysed with 0.25% Triton X-100, dissolved in 1xPBS. Intracellular CFU were counted after culturing the bacteria overnight on LA plates at 37 º C.

3.6.Gene expression analysis: -

For the analysis of host mRNA expression, cells were seeded at a density of 5 x 10 cells / well in 24-well plates and infected with bacteria as above or treated with the recombinant proteins.

3.6.1. Total RNA extraction

3.6.1.1 Materials:

- TRIZOL Reagent (InvitrogenTM, CA).
- Chloroform.
- Isopropanol.
- 75% Ethanol.
- RNAse-free water (DEPC-treated).

3.6.1.2 Procedure:

RNA Was isolated by TRIZOL reagent (Invitrogen, USA) essentially following the manufacturer's instructions. Briefly, cells grown in a 24-well tissue culture plate were harvested with 200ul of TRIZOL reagent. Samples were incubated for 5 min at room temperature (RT) to permit complete dissociation of nucleoprotein complexes. 40ul of chloroform was added to each sample and the tubes were vigorously shaken by vortexing for 15 seconds. After incubation at RT for 2-3 minutes, cell samples were centrifuged at 14,000 g for 15 min at 4 °C. Clear aqueous phase at the uppermost layer was transferred to a fresh tube and RNA was precipitated by mixing with 100 % of isopropyl alcohol. Samples were incubated at RT for 10 minutes and centrifuged at 14,000 g for 10 minutes at 4 C. Precipitated RNA was washed with 200ul of 75% ethanol and collected by centrifuging at 14,000 g for 5 minutes at 4 °C. RNA pellet was air dried and dissolved in 50ul of RNase free water by gently tapping the tubes followed by incubation at 58°C for 10 min Quantification of RNA was done by spectrophotometric reading at 260 nm and purity was checked by OD at 260/280 nm using Uy-Vis Spectrophotometer (UV1700, Shimadzu Corporation, Kyoto, Japan).

3.6.2 cDNA synthesis:

3.6.2.1 Materials:

- Isolated total RNA.
- superscript Il cDNA Synthesis Kit (Invitrogen Life Technologies).
- SX RT buffers.
- RNase H inhibitor.
- MuLV Reverse Transcriptase (RT) Enzyme.
- Random hexamers (InvitrogenM, CA).
- DEPC-treated water.
- dNTP mix (InvitrogenTM, CA).

3.6.2.2 Procedure:

In vitro reverse transcription was performed using Superscript II cDNA Synthesis Kit (Invitrogen Life Technologies) following the manufacturer's instructions. RNA samples were then treated with RNase-free DNasel (NEB). Each 20 ul PCR reaction mix contains 1 ug (for host mRNA expression), 0.25 nM each of dATP, dCTP, dGTP, dTTP, 300 ng random hexamers or oligo dT, and nuclease-free DEPC water. RNA was denatured at 65 C for 5 minutes and the mixture of 1unit MuLV RT enzyme, 5X RT buffer, and RNase Inhibitor was added to the RNA mix on ice. cDNA was generated by incubating the mixture at 25 °C for 5 minutes, 42 °C for 50 hours, and 72 °C for 10 minutes.

3.6.3 Quantitative Real-time PCR

3.6.3.1 Materials:

- SX SYBR GreenTM mastermixTM (Applied Biosystems, USA).
- Forward and reverse primers (Tabel 2).

- Nuclease-free water.

3.6.3.2 Procedure:

Real-time quantitative PCR was performed using ABI7300 (Applied Biosystems, USA). Relative quantitation was done by the comparative CT method. PCR was performed with SYBR Green TM mastermixTM (Applied Biosystems, USA), where SYBR green was the Asorescent reporter. The internal control gene GAPDH was amplified simultaneously in separate reaction tubes. To eliminate primer dimers, the fluorescent signal was collected at a temperature of 82 C, where the primer dimmers melted, but the PCR products were still in their double-stranded form, thereby emitting fluorescence. The reaction conditions were set as follows: initial heating at 95 °C for 5 min, followed by 40 cycles of reactions at 95 °C (30 seconds) and 62 °C (1 minute). Relative quantitation was done by the comparative C method (Chakraborty et al., 2009). The levels of expression of the genes of interest were normalized against β-actin (Cytokines and chemokines) using the formula $2^{-\Delta\Delta CT}$ where $-\Delta\Delta C_T = \Delta C_T$ (sample) - ΔC_T (calibrator) and ΔC_T is the C_T of the target gene subtracted from the Cr of the housekeeping gene (β-actin). The calibrator used in our experiments was the untreated or uninfected THP-1, RAW 264.7, and human primary macrophages.

3.7.RNA Interference: -

3.7.1. Materials: -

- THP-1 Cells
- RPMI medium
- Scrambled siRNA
- gene-specific siRNA (TLR2, 4 and 9 siRNA, p65 siRNA
- siRNA transfection medium

- 5 % CO2 Incubator

3.7.2. Procedure: -

PMA-differentiated THP-1 cells were transfected with the control (Scrambled siRNA) or gene-specific siRNA (TLR2, 4 and 9 siRNA, p65 siRNA) using siPort as per manufacturer instructions (Invitrogen). Briefly, the siRNA complex and siRNA transfection reagent were diluted in the siRNA transfection medium and incubated at room temperature for 30 minutes. Cells were overlaid with the transfection mixture for 5 hours at 37 °C in the presence of 5% CO2, followed by culture in a complete RPMI1640 medium. Gene knockdown was confirmed by immunoblots 72 hours post-transfection. Cystatin B knockdown was done in the THP-1 cell line by Santacruz siRNA cystatin B (sc-44431), siRNA Transfection Medium (sc-36868), siRNA Transfection Reagent (sc-29528) by following manufacturer's protocol. Briefly, THP-1 cells were seeded and differentiated with PMA for 24 hours. Cystatin B siRNA and Transfection medium were mixed to make solution A and transfection medium and transfection reagent were mixed to make solution B. Solution A &B were mixed and cells were incubated with this mixture for 6 hours. The next day, this solution was replaced by a complete medium and kept for 24 hours. The next day, again the media was replaced with fresh media and kept for 48 hours. After 48 hours, control siRNA (sc-37007) and Cystatin B siRNA-treated cells were lysed by RIPA buffer, and western blotting was done using cystatin B antibody.

3.8. Purification of recombinant protein T4519:

3.8.1 Materials:

- Isopropyl B-D-1-thiogalactopyranoside (IPTG).
- Guanidium hydrochloride.
- Urea.
- B121 (DE3).

- Ni-NTA Agarose beads.
- Renaturation buffer.
- Anti-HIS monoclonal antibody.

3.8.2. Procedure:

T4519 *E. coli* BL21 (DE3) bacterial cultures were grown till the OD600 reached 0.6 and all protein expressions were induced with 1 mM IPTG for 5 hours at 37 °C. To obtain the purified proteins, pellets were resuspended in the denaturation buffer [(0.1 M phosphate buffer, 8 M Urea, 10 mM Tris-Hcl, and 100 mM imidazole (pH 8.0)] and incubated for 1 hour at room temperature, followed by centrifugation at 16,000 g for 30 minutes. Supernatants were collected and passed through Ni-NTA columns (Qiagen). The columns were washed with the wash buffer [0.1 M phosphate buffer, 8 M Urea, 10 mM Tris-Hcl, and 30 mM imidazole (pH 6.8)] and the bound proteins were eluted using the elution buffer [0.1 M phosphate buffer, 8 M Urea, 10 mM Tris-Hcl and 30 mM imidazole (pH 5.2)]. Denatured proteins were refolded by dialysis with gradient phosphate-urea buffer [50 mM Glycine, 5 mM EDTA, 10% glycerol, 10% glucose, 250 mM NaCl, 5 mM HEPES, 0.5 mM PMSF, 0.1 mM DTT, 1 M Na2HPO4 and 1 M NaHPO, (pH 7.2)]. Purity and size of the proteins were confirmed by SDS-PAGE and protein concentrations were quantified by Bradford Assay. Western blotting was done using anti-His antibody.

3.9. Vacuolar pH Measurement: -

For vacuolar PH measurement, the following buffers were made of different pH to obtain a standard curve by following protocol (Ma, Ouyang et al. 2017).

Recipes for preparing pH calibration curve buffers are mentioned in Table 3.3

	1 M KCl	5 M NaCl	72 mM Monensin	0.5 M HEPES	0.5 M MES	1 N NaOH*	1 N HCl*	H_2O
Buffer of pH 7.5	6.25 mL	0.25 mL	6.9 µL	**2.5 mL**	–	*	*	to 50 mL
Buffer of pH 7.0	6.25 mL	0.25 mL	6.9 µL	**2.5 mL**	–	*	*	to 50 mL
Buffer of pH 6.5	6.25 mL	0.25 mL	6.9 µL	–	2.5 mL	*	*	to 50 mL
Buffer of pH 6.0	6.25 mL	0.25 mL	6.9 µL	–	2.5 mL	*	*	to 50 mL
Buffer of pH 5.5	6.25 mL	0.25 mL	6.9 µL	–	2.5 mL	*	*	to 50 mL
Buffer of pH 5.0	6.25 mL	0.25 mL	6.9 µL	–	2.5 mL	*	*	to 50 mL
Buffer of pH 4.5	6.25 mL	0.25 mL	6.9 µL	–	2.5 mL	*	*	to 50 mL
Buffer of pH 4.0	6.25 mL	0.25 mL	6.9 µL	–	2.5 mL	*	*	to 50 mL
Buffer of pH 3.5	6.25 mL	0.25 mL	6.9 µL	–	2.5 mL	*	*	to 50 mL
Final concentration	125 mM KCl	25 mM NaCl	10 µM Monensin	25 mM HEPES	25 mM MES	–	–	–

Each buffer solution is adjusted to the appropriate final pH using 1 N NaOH or 1 N HCl.

In living cells, the fluorescent dye Lysosensor Yellow-blue DND 160 (Thermo) produces yellow fluorescence in acidic environments, such as lysosomes, whereas it produces blue fluorescence in neutral environments.

3.9.1. For standard curve preparation: -

3.9.1.1. Confocal microscopy-based method

3.9.1.1.1. Loading of LysoSensor™ dye

Two days before the experiment, THP-1 cells were seeded along with PMA into a 35 mm glass bottom dish at a density of 1×10^6. The next day one complete media change was given. On the day of the experiment, the LysoSensor™ Yellow/Blue DND-160 stock solution (1 mM) was diluted to the final

working concentration of 1 μM, in a normal cell culture medium. The cells were incubated at 37°C in 1 mL of pre-warmed, normal cell culture medium containing LysoSensor™ Yellow/Blue DND-160 for 3 mins. The cells were rinsed with 1X PBS and left in 1 mL of 1X PBS.

3.9.1.1.2. Imaging

- The excitation filter settings appropriate for imaging of LysoSensor™ Yellow/Blue DND-160 are 329(blue) and 384 (yellow) and emission 440(blue) and 540(yellow)
- The cells were incubated for 2 mins with the most alkaline of the pH calibration curve buffers (e.g., pH 7.5) for imaging.
- The cells were rinsed once with 1X PBS.
- The cells were incubated for 2 min in 1 mL of the next pH calibration curve buffer for imaging.
- The steps were repeated to proceed from the most alkaline (pH 7.5) to the most acidic (pH 3.5).
- Fluorescence intensity values were recorded for emissions at both wavelengths (e.g., 440 and 540 nm).

3.9.1.2. Fluorimeter based method: -

3.9.1.2.1. Loading of LysoSensor™ dye

Two days before the experiment, THP-1 cells were seeded along with PMA into a 24-well plate at a density of 5×10^5. The next day one complete media change was given. On the day of the experiment, the LysoSensor™ Yellow/Blue DND-160 stock solution (1 mM) was diluted to the final working concentration of 1 μM, in a normal cell culture medium. The cells were incubated at 37°C in 1 mL of pre-warmed, normal cell culture medium containing LysoSensor™ Yellow/Blue DND-160 for 3 mins. The cells were rinsed with 1X PBS and left in 1 mL of 1X PBS.

3.9.1.2.2. Reading of fluorimeter

The fluorimeter was set at excitation and emission settings appropriate for reading LysoSensor™ Yellow/Blue DND-160 fluorescence. Multiple wells of cells were used for each pH calibration curve buffer (e.g., three wells per buffer), and each well of cells was rinsed once with its respective pH calibration curve buffer. Each well of cells was incubated for 5 mins in 500 µL of its respective pH calibration curve buffer for reading.

3.9.1.3. Microplate reading results analysis and plotting of data-

For each pH calibration curve buffer, the fluorescence intensity measurement data were exported based on the microplate readings and for emissions at both wavelengths (e.g., 440 and 540 nm) to Microsoft Excel. The fluorescence intensity ratio of the intensity of emission at shorter wavelength (e.g., 440 nm): and intensity of emission at longer wavelength (e.g., 540 nm) for each pH calibration curve buffer was calculated. The average fluorescence intensity ratio for each pH calibration curve buffer was plotted and calculated to generate a pH calibration curve for use in determining pH values based on experimental data.

3.9.2 Measurement of intraorganellar pH: -

After infection with *Salmonella* by following Gentamycin survival assay, the infected and uninfected wells were incubated with Lysosensor dye for 5 mins. Cells were rinsed with 1x PBS and then reading was taken in a fluorimeter following the previous protocol. The fluorescent intensity ratio was plotted in the equation of the standard curve (here the obtained equation is y=0.75395x+ 2.71824) to obtain the intravacuolar pH of the infected cells.

3.10. Confocal Lesser Scanning Microscopy: -

3.10.1. For lysotracker staining: -

3.10.1.1. Materials: -

- 24 well plate (Nunc)
- 18 mm coverslip (Bluestar)
- Lysotracker Red (Thermo)
- Hoechst (Sigma)
- 1X PBS
- Zeiss LSM 710 Confocal Microscope

3.10.1.2. Procedure: -

THP-1 cells were seeded in a density of 5*10^5 cells per well in a 24-well plate onto Rat tail collagen (Sigma) coated coverslip along with 100 nM PMA. TDM cells were infected with GFP-tagged bacteria following the Gentamycin protection assay. Then the cells were incubated in a lysotracker (100nm) containing complete media for 1hr at 37º C at dark. After that, cells were washed once in 1XPBS and then the cells were incubated in 200µl PBS solution containing Hoechst stain (10µg/ml) for 30 mins at RT in the dark. Then the coverslips were mounted on the slide and viewed under ZEISS LSM 710 confocal microscope. Quantification in confocal microscopy was done by taking 50 cells from each experiment from random fields. Punctas were counted manually for each cell individually and plotted in graphs. All the images were taken in Axio observer LSM 710 ZEISS confocal microscopy, 63X /1.4 oil immersion lens, scale bar 5 µm.

3.10.2. For antibody Staining: -

3.10.2.1. Materials: -

- 24 well plate (Nunc)
- 18 mm coverslip (Bluestar)
- Primary antibody
- Fluorochrome conjugated Secondary antibody

- 4%PFA (Ph 7.2-7.4)
- 0.1% Triton X-100 containing PBS
- 1%BSA containing 0.1% Triton X -100 PBS
- 1% BSA
- Hoechst (Sigma)
- 1X PBS
- Zeiss LSM 710 Confocal Microscope

3.10.2.2. Procedure: -

For confocal microscopy staining, coverslips were coated in 24 well plates with rat tail collagen for 6 hours at 37º C. Then it was dried and the cell was seeded onto it. On the day of infection, each well in a 24-well plate contained 2*10^5 PBMC-derived macrophage cells. THP-1 cells were seeded at a density of 5*10^5 cells per well in a 24-well plate. For antibody staining, after infection with GFP-tagged bacteria, cells were permeabilized with 4% PFA in PBS for 15 mins at 37 degrees. Then the cells were permeabilized in 0.1% Triton X-100 containing PBS for 10 mins followed by blocking in 1%BSA containing 0.1% Triton X -100 PBS for 30 mins. The primary antibody was diluted following the manufacturer's protocol in 1% BSA in PBS and incubated overnight at 4 degrees. Fluorochrome conjugated secondary antibody was diluted following the manufacturer's protocol in 1% BSA in PBS and incubated for 1 hr at dark. For nucleus staining, Hoechst stain is diluted (Working concentration 10 µg/ml)in PBS and incubated for 30 mins at dark. Then the coverslips were mounted on the slide and viewed in ZEISS LSM 710 confocal microscope. Quantification in confocal microscopy was done by taking 50 cells from each experiment from random fields. Punctas were counted manually and the co-relation coefficient was measured by ZEISS software for each cell individually and plotted in graphs. All the images were taken in Axio observer LSM 710 ZEISS confocal microscopy, 63X /1.4 oil immersion lens, scale bar 5 µm.

3.10.3. For Alexa-Fluor Dextran staining: -

3.10.3.1. Materials: -

- 24 well plate (Nunc)
- 18 mm coverslip (Bluestar)
- Dextran conjugated Alexa-Fluor (Thermo)
- Hoechst (Sigma)
- 1X PBS
- Zeiss LSM 710 Confocal Microscope

3.10.3.2. Procedure: -

For confocal microscopy staining, coverslips were coated in 24 well plates with rat tail collagen for 6 hours at 37º C. Then it was dried and the cell was seeded onto it. On the day of infection, each well in a 24-well plate contained 2*10^5 PBMC-derived macrophage cells. THP-1 cells were seeded at a density of 5*10^5 cells per well in a 24-well plate. For dextran Alexa-Fluor staining (10µM), cells were incubated with Dextran Alexa-Fluor in complete media overnight so that dextran could reach all the vacuoles of the cell including the lysosomes. in acidic vacuoles, it will give red fluorescence. Then the cells were infected with GFP-tagged bacteria following Gentamycin survival assay. For nucleus staining, Hoechst stain is diluted (Working concentration 10 µg/ml) in PBS and incubated for 30 mins at dark. Then the coverslips were mounted on the slide and viewed in ZEISS LSM 710 confocal microscope. Quantification in confocal microscopy was done by taking 50 cells from each experiment from random fields. Puncta were counted manually and for each cell individually and plotted in graphs. All the images were taken in Axio observer LSM 710 ZEISS confocal microscopy, 63X /1.4 oil immersion lens, scale bar 5 µm.

3.10.4. For Magic Red staining: -

3.10.4.1. Materials: -

- 24 well plate (Nunc)
- 18 mm coverslip (Bluestar)
- Cathepsin B substrate Magic Red (Abcam)
- Hoechst (Sigma)
- 1X PBS
- Zeiss LSM 710 Confocal Microscope

3.10.4.2. Procedure: -

For confocal microscopy staining, coverslips were coated in 24 well plates with rat tail collagen for 6 hours at 37º C. Then it was dried and the cell was seeded onto it. On the day of infection, each well in a 24-well plate contained 2*10^5 PBMC-derived macrophage cells. THP-1 cells were seeded at a density of 5*10^5 cells per well in a 24-well plate. Then the cells were infected with GFP-tagged bacteria following Gentamycin survival assay. For Magic Red staining after infection, cells were incubated in Magic Red-containing media for 1hr at 37ºC. For nucleus staining, Hoechst stain is diluted (Working concentration 10 µg/ml) in PBS and incubated for 30 mins at dark. Then the coverslips were mounted on slide and viewed in a ZEISS LSM 710 confocal microscope. Quantification in confocal microscopy was done by taking 50 cells from each experiment from random fields. All the images were taken in Axio observer LSM 710 ZEISS confocal microscopy, 63X /1.4 oil immersion lens, scale bar 5 µm.

3.11. Flow cytometer: -

3.11.1. Lysotracker staining: -

3.11.1.1. Materials: -

- THP-1 Cells

- rt4519
- RPMI medium
- FBS
- 1XPBS
- Lysotracker Red
- FACS Tube
- FACS Aria II flow-cytometer
- 24 well plates

3.11.1.2. Procedure: -

THP-1-derived macrophage cells were incubated with rt4519(30µg) in complete RPMI for 3 hours at 37°C. Then the cells were incubated with lysotracker (100nm) containing complete media for 1hr at 37º C. After that, cells were washed once with 1XPBS and then the cells were scraped in 200µl PBS followed by transfer to the FACS tube. For FACS, a PE channel was used (Ex 565 nm and emits at 573 nm) in BD FACS Aria II flow-cytometer to obtain the required result.

3.11.2. Measurement of ROS: -

3.11.2.1. Materials: -

- MoM cells
- Ty2
- Ty2ΔT4519

- 1XPBS
- CM-H_2DCFDA
- RPMI medium
- FBS
- 24 well plates
- FACS Tube
- FACS Aria II flow-cytometer

3.11.2.2. Procedure: -

To measure ROS generation MoM cells were seeded at the density of 2 x 10^5 cells / well in 24-well plates and infected with wild-type and mutant bacteria. At the indicated time points, cells were twice washed with PBS and incubated with PBS containing10 µM of CM-H_2DCFDA (Invitrogen) at room temperature for 45 minutes. The dye was removed by washing with PBS and the cells were resuspended in a complete RPMI 1640 medium to determine ROS levels by flow cytometer (FACS Arya II, BD), using excitation 488 nm (FITC channel) after gating on the basis of the basal level of fluorescence intensity for unstained cells.

3.11.3. Measurement of Apoptosis: -

3.11.3.1. Annexin V-PI staining protocol: -

3.11.3.1.1. Materials: -

- 12 x 75 mm round-bottom tubes
- 1X PBS

- Annexin V FITC Apoptosis Detection kit (Thermo Cat. Nos., 88-8005-74)
- 10X Binding buffer
- RPMI medium
- FBS
- 24 well plates
- FACS Tube
- FACS Aria II flow-cytometer
- Centrifugation Machine

3.11.3.1.2. Procedure: -

MoM cells were infected following the Gentamycin protection assay. 1X binding buffer was prepared by mixing 1 part of 10X binding buffer with 9 parts of distilled water. The cells were harvested by scraping after the Gentamycin protection assay. Cells were washed once in 1X PBS, then once in 1X binding buffer. Cells were resuspended in 1X Binding Buffer at 2.5 x 106 cells/mL.10 µL of fluorochrome-conjugated Annexin V was added to 200 µL of the cell suspension. Cells were incubated for 10-15 minutes at room temperature in the dark. 2 mL 1X binding buffer was added and centrifuged at 400-600 x g for 5 minutes at room temperature. The supernatant was discarded. The cells were resuspended in 200 µL of 1X binding buffer. 5 µL of Propidium Iodide Staining Solution was added and incubated for 5 minutes on ice or at room temperature. Cells were analyzed by flow cytometry in FITC and PE channels.

3.12 ELISA:

3.12.1 Material:

- Flat bottom 96 well plate (Nunc).
- Wash buffer (1X PBS +0.05% Tween-20).
- 2N H_2SO_4 (stop solution).
- ELISA Microplate Reader.
- ELISA Kit

3.12.2 Procedure:

Levels of cytokines were checked in culture supernatants of *Salmonella* infected PBMC derived cells. To quantify the cytokines, ELISA was performed using an ELISA kit (eBioscience) as per the manufacturer's instructions. ELISA plates were coated with the capture antibody in I X coating buffer (100 µl/well) and incubated overnight at 4 °C. After aspiration, wells were washed with wash buffer (1X PBS + 0.05% Tween-20) five times and blocked with 200µl/ well IX assay diluent (provided with the kit) for 1 h at RT. After aspiration, wells were again washed 5 times with the wash buffer and incubated with 100 µl/well of the standard (different dilutions) and test samples (serum diluted 1:10). The Plates were incubated at room temperature for 2 h. Test samples (serum diluted 1:10) were added directly to each well. the plate was sealed and incubated at RT for 2 hr. After aspiration, wells were further washed and avidin-HRP (100 µl/well) diluted in IX assay diluent was added to each well and incubated at room temperature for 30 min. After aspiration, wells were washed 5 times and 100 µl/well of substrate solution was added and incubated at room temperature for 15 min. Once the color started developing, 50 µl of stop solution was added to each well to stop the reaction, and the reading was taken at 450 nm in an ELISA reader. The standard graph was prepared according to data for different standard dilutions and the quantitative level of secreted cytokines (in pg/ml) in test samples was calculated accordingly.

3.13. Cytoplasmic and lysosomal fractions isolation protocol

3.13.1. Materials: -

- Homogenization Buffer
- Ice
- Centrifugation machine
- 1XPBS
- MSH Buffer
- 25 G needle
- Trypan Blue
- Haemocytometer
- Triton-X 100

3.13.2. Procedure: -

Cytosolic extracts were isolated as described (Oberle et al.,2010)**.** Briefly, infected cells were lysed in homogenization buffer (50 µg Digitonin, 250mM sucrose, 20 Mm HEPES, 10 mM KCL, 1.5 mM $MgCl_2$, 1 mM EDTA, 1mM EGTA, and 1mM PMSF) and homogenize (100 µl) on ice by shaking for 30 minutes. Lysates were centrifuged at 14000 rpm for 5 minutes to obtain cytosolic proteins.

Preparation of lysosomal membrane fractions (LF) and cytosolic fractions (CF) was done as before Colletti et al.,2012)**.** Briefly, cells were washed with chilled 1X PBS, gently scrapped and centrifuged at 600g for 5 minutes at 4 º C. Cells were incubated with twice the volume of MSH buffer (200 mM mannitol, 70 mM sucrose, 20 mM HEPES (pH 7.5), 1mM EDTA, 100 µM PMSF) for 45 minutes on ice followed by lysis using a 25 G needle until 75% of the cells were trypan blue positive. Cells were

centrifuged for 5 minutes at 350 g to pellet cellular debris and nuclei. The supernatant was centrifuged to 100000 X g for 1 hr to obtain the cytosol. The pellets found here contain lysosomal fraction. To obtain the crude membrane/ lysosomal fraction, (containing all other organelles except nuclei), the pellets were resuspended in MSH buffer containing 1% Triton-X.

3.14. Western Blotting: -

3.14.1 Materials:

- Protein lysates
- Resolving SDS-polyacrylamide gel (10 ml)

Composition of SDS-PAGE gel 10 (10ml)	10%
40% Acrylamide (ml)	2.5
Resolving buffer (pH-8.8) (ml)	2.5
10% APS (ul)	100
TEMED (ul)	8
Sterile Water	4.9

	Composition	Amount
• Stacking 5% gel (10 ml).		
	40% Acrylamide	0.5 ml

	Stacking buffer (pH-6.8)	0.5 ml
	10% APS	40 µl
	10% TEMED	4 µl
	Sterile water	2.95 ml
• 1 X Tri-glycine electrophoresis buffer (1 litre).		
	25 mM Tris Base	3.02 g
	250 mM Glycine	18.8 g
	SDS	1g
• 1 X Transfer buffer (1 litre)		
	25 mM Tris Base	3.3 g
	192 mM Glycine	14.41 g
	Methanol	200 ml
• 1X Tris Buffered Saline (TBS) 1 Litre		
	Tris (25 mM)	3.028 g
	NaCl (0.15 M)	8.775 g

• 4X SDS Gel-loading buffer		
	200mM Tris-Cl (pH 6.8)	
	8% (w/v) SDS (electrophoresis grade)	
	0.4 (w/v) bromophenol blue	
	40% (v/v) glycerol	
	400mM B-mercaptoethanol (added just before use)	

- PVDF membrane.
- 3% BSA or 5% Skimmed milk.
- HRP-conjugated secondary antibody.
- Supersignal West Pico plus chemiluminescent substrate (Thermo)

3.14.2. Procedure:

Post-experimental cells were lysed with RIPA lysis buffer (G BIOSCIENCES) with an added protease inhibitor cocktail. Equal amounts of total proteins in the cell lysates were separated in SDS-PAGE and transferred to a PVDF membrane (Millipore). Blots were incubated overnight at 4 °C with primary

antibodies diluted in blocking buffer [TBS containing 3% (w/v) BSA]. After washing (3 times) with IX TBS containing 0.1% tween-20 (TBS-T) blots were incubated with HRP-conjugated secondary antibodies (1:20000) dissolved in TBS-T containing 3% (w/v) BSA for 1 hour at room temperature. Blots were developed with Supersignal West Pico plus chemiluminescent substrate (Thermo). Signal intensity was measured by Bio-Rad Image-Lab Software.

3.15. Co-immunoprecipitation

3.15.1. Materials: -

- HEK293TLR2 cells
- rT4519 or rT4519-SDM
- 1X PBS
- 1% paraformaldehyde (PFA)
- Non-iodent P-40 lysis buffer
- Centrifugation Machine
- Bradford
- protein A/G agarose beads
- primary antibody

3.15.2. Procedure: -

HEK293TLR2 cells (10^8) were seeded and treated either with 3 µg of rT4519 or rT4519-SDM proteins for 24 hrs. Cells were washed in ice-cold PBS, cross-linked with 1% paraformaldehyde (PFA) for 10 minutes, and twice washed with ice-cold PBS to remove PFA. Cells were lysed with Non-iodent P-40

lysis buffer containing protease inhibitors, lysates were cleared by centrifugation at 14,000 rpm for 20 minutes at 4 °C and the supernatants were collected in fresh tubes. After the determination of the protein concentration by Bradford protein assay, a primary antibody was added (1:50 dilution) to the cell lysates (containing 600 µg of total protein in each tube), and the mixtures were kept overnight on a rocker at 4 °C. 50 µl of protein A/G agarose slurry (50%) (Santa Cruz) equilibrated with the lysis buffer was added to each cell lysate and incubated for 4 hours on a rocker at 4 °C. Protein A/G agarose beads were collected by pulse centrifugation (14,000 rpm for 5 seconds). Supernatants were removed and the beads were washed three times with 500 µl of cold PBS. Agarose beads were resuspended in 1X sample loading buffer and boiled at 94 °C for 10 minutes to dissociate the bound proteins. Proteins were analyzed by SDS-PAGE, transferred to a PVDF membrane, and probed with primary monoclonal anti-His or TLR2 antibody.

3.16. Homology modelling

T4519 sequence from *Salmonella* enterica subsp. enteric serovar Typhi str. Ty2 was subjected to HHPRED (Soding et al.,2005) and PSIPRED v3.0 (http://bioinf.cs.ucl.ac.uk/psipred/) (Buchan et al, 2010, Sadowski et al.,2009) server for prediction of secondary and tertiary structure. Fold prediction analyses and psiBLAST (Astlchul et al.,1997) against protein data bank (PDB) sequences suggest a strong structural similarity between T4519 and the kinase family. The crystal structure of PknB from Mycobacterium tuberculosis (PDB ID 1O6Y), which possesses maximum sequence identity (29%) with T4519was used as a template to model the three-dimensional (3D) structure of T4519. The model was generated using Modeller and further refined with repetitive loop modelling (Fiser et al.,2000)**.** Finally, the potential energy of the model was minimized using the steepest descent followed by the conjugate gradient method in Discovery Studio 2.5. 3D models were filtered based on the DOPE score and validated using PROCHECK and verify_3D (Laskowski et al., 1996 Luthy et al., 1992) structure validation tools. To check the stability of the T4519 atomic model a short duration (5ns) molecular dynamic simulation in GROMACS 4.**5** (Pronk et al, 2013) was performed.

3.17. In-silico protein-protein interaction study: -

To check the interaction pattern of T4519 with TLR2, first, we retrieved the TLR2 crystal structure from PDB (PDB ID 2Z7X), which is a TLR1-TLR2 heterodimer (Jin et al., 2007) and then docked each other using ClusPro 2.0 protein-protein docking server (Comeau et al.,2004) with all the default parameters. The docked complexes were refined further in the FireDock server (http://bioinfo3d.cs.tau.ac.il/FireDock/) and the global energies of the complexes were measured.

3.18. Statistical analysis: -

Statistical analysis was performed by using GraphPad Prism. Data are expressed as mean ± standard deviations of at least three independent experiments. All the graphs were plotted and the statistical analysis was done by unpaired Students t-test in GraphPad Prism Version 8.0.1 with $P < 0.05$ considered as significant. If a p-value is less than 0.05, it is flagged with one star (*). If a p-value is less than 0.01, it is flagged with 2 stars (**). If a p-value is less than 0.001, it is flagged with three stars (***).

Chapter 4

Results

Objective 1 - To study the maturation and traffic of STy**-containing phagosomes.**

Results: -

1. STy strain Ty2 infected THP-1 derived macrophage cells (TDM) reduce Lamp1+ vesicles at 24 hours PI by STy serine-threonine kinase T4519.-

It is a well-known fact that STy resides within a specialized compartment called *Salmonella* containing vacuole (SCV) which initially acquires the markers of early endosome and gradually accumulates late endosome markers and finally fuses with lysosomes as a protective mechanism of host cells to eliminate the bacteria (Krieger, Liebl et al. 2014). The bacteria itself tries to enable various manipulations to get rid of this protective mechanism of host cells. To inhibit the SCV maturation is certainly one of them. To monitor the SCV maturation, we stained the cells with late endosomal marker Rab7 and lysosomal marker LAMP1. TDM cells were infected with wild-type STy Ty2 strain with 50 MOI followed by gentamycin protection assay. Cells were fixed and stained with Rab7 and LAMP1 primary antibody followed by fluorochrome-conjugated secondary antibody. After 24 hours of infection, the intensity of Rab 7 was increased and the intensity of LAMP1 was markedly decreased (Fig 1A) in TDM cells. Reduction in the LAMP1 intensity is a measure of the reduction in lysosomal number. There were previous reports from our lab that proved that a serine-threonine kinase T4519 present in STy is required for bacterial survival inside THP-1 macrophages (Theeya, Ta et al. 2015). As lysosomal reduction can support the bacteria to survive within SCV, we wanted to check whether this lysosomal reduction is somehow related to T4519 or not. TDMs were infected with Ty2ΔT4519 at 100 MOI followed by Lamp1 and Rab7 immunofluorescence staining. Double the MOI of the mutant bacteria equalled its number same as the wild-type bacteria after 24 hrs of infection (Theeya, Ta et al. 2015). However, in Ty2 ΔT4519 infected cells, no reduction of the LAMP1 intensity was found after 24 hrs P.I. indicating that the mutant form was unable to reduce lysosomes in macrophages (Fig 1B,1C). No significant change in Rab7 fluorescence intensity was found between Ty2 and Ty2ΔT4519 infected cells suggesting no T4519-mediated change in late endosomal maturation. This

experiment proved that, T4519 present in STy was responsible for the lysosomal membrane protein LAMP1 intensity reduction.

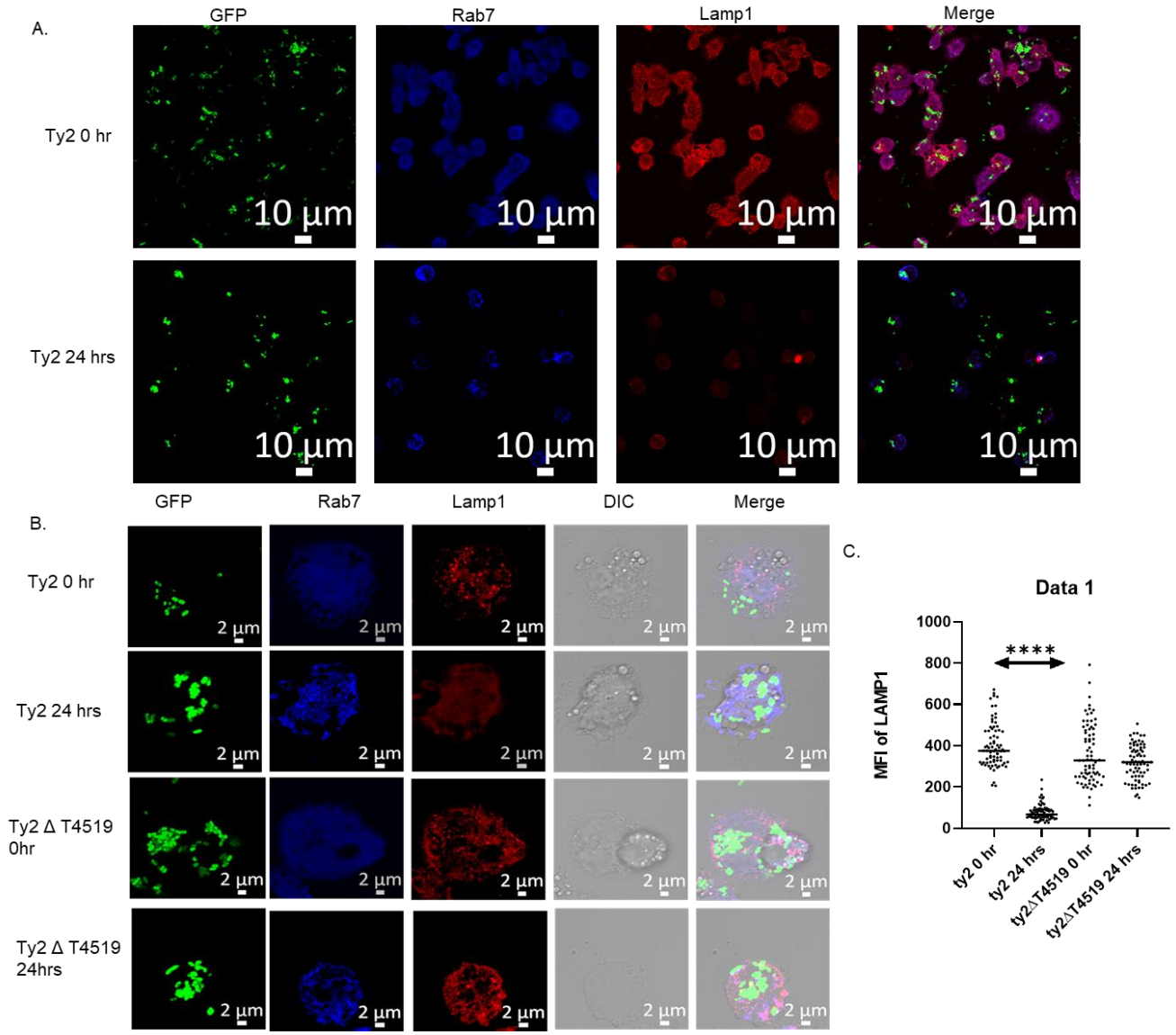

Figure1:- ***Salmonella*** **Typhi Ty2 infected THP-1 macrophage cells reduce Lamp1+ vesicles at 24 hours PI by Typhi serine threonine kinase T4519.**THP1-derived macrophage cells were infected with wild-type Ty2 and Δ T4519 Ty2 and stained with Rab7 and LAMP1 antibodies. A, B .Rab7 (blue) and LAMP1 (red) antibody staining of THP-1 derived macrophages visualized in a confocal microscope. C. Quantification of mean fluorescence intensity (MFI) of LAMP1 in individual cells. Images taken in Zeiss LSM710 confocal microscopy. n=50 cells. A representative image from all experiments was given.

2. T4519 present in STy increases vacuolar pH in infected human macrophages from acidic to neutral –

Intracellular pathogen *Salmonella* survives and replicates inside a modified phagosome, known as *Salmonella*-containing vacuole (SCV) within macrophages. We investigated if the lysosomal reduction by T4519, as described above contributed to a change in the vacuolar pH of STy infected macrophages. Manipulation of phagosomal acidification and phagolysosome formation are common survival mechanisms of different intracellular pathogens. Lysosensor yellow-blue DND from Thermofisher was used to determine the value of vacuolar pH. This dye gives yellow fluorescence in acidic pH and blue fluorescence in neutral pH (Fig 2A). The emission ratio of 440 and 540 was measured in a fluorimeter and plotted in a graph to make the standard curve. Cells were exposed to buffers of different pH starting from pH 7 to pH 4 for 3 mins in each buffer and then the emission ratio was taken in a fluorimeter. The emission ratio was plotted against each of the corresponding pH to obtain the standard curve. We measured the pH of the acidic organelles of macrophage cells after infection with the *STm* strain Lt2 and wild type and Ty2 ΔT4519 strains by staining the cells with lysosensor yellow blue DND (Ma, Ouyang et al. 2017)and the same emission ratio was measured. After measuring the ratio, the value was plotted in the standard curve to get the actual pH value of the vacuole. After 24 hours, it was found that the Ty2 infected cells showed much higher pH than Ty2 ΔT4519 infected cells. The pH starts rising for both the infected cells as early as 3h, but the vacuolar pH of the Ty2 infected cells rose beyond 7.0 after 24h of infection, whereas after Ty2ΔT4519 infection it stopped at pH 5.5 (Fig 2B,2C). As lysosomes were the main acidic vacuoles of the cell and T4519 reduced the lysosomal membrane proteins, we can presume the overall lysosomes were getting reduced. As a result, the overall number of acidic vacuoles in the cells was decreased enabling the vacuolar pH rise in Ty2 infected cells.

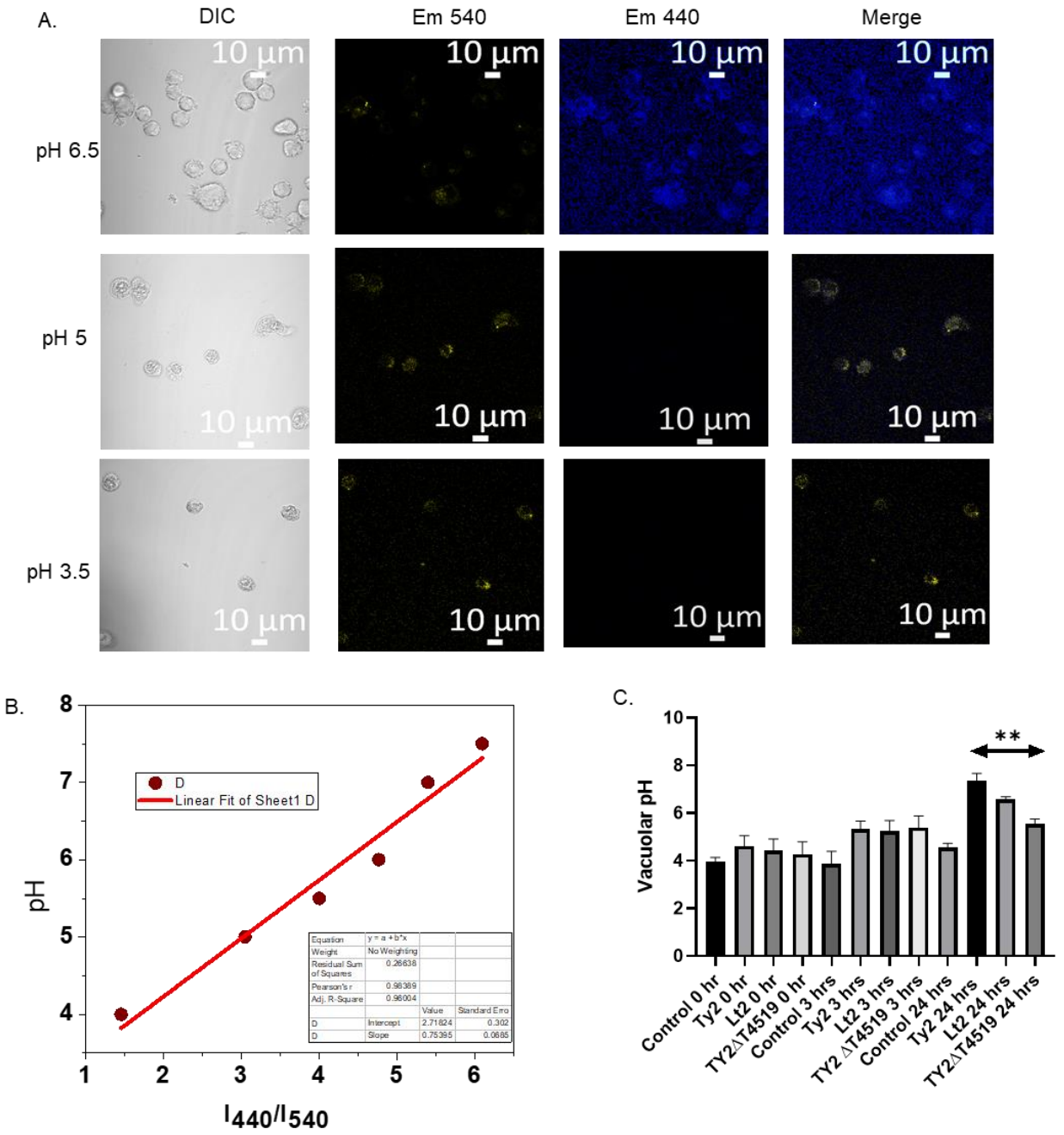

Figure 2: **-T4519 present in Salmonella Typhi increases vacuolar pH in infected human macrophages from acidic to neutral** –THP-1 derived macrophages were stained with lysosensor yellow blue DND dye and exposed to different pH-containing buffers from 3 to 8.

A. Representative image of cells stained with lysosensor yellow-blue DND was given. The images were captured by LSM 710 confocal microscopy and processed using ZEISS ZEN BLUE software. B-C. An excitation and emission ratio of 440/540 was taken in a fluorimeter. The values were plotted to make the standard curve. C. THP-1 derived macrophage cells were infected and then incubated in lysosensor Yellow-Blue DND dye and an excitation and emission ratio of 440/540 was taken in a fluorimeter. The value was plotted in the standard curve to obtain the correct pH. graphical representation of different pH was given.

3. T4519 reduces lysosomes after 24 hours of infection.

LAMP1 may accumulate in the late endosomes. As we did not find any reduction in late endosome marker Rab7, we considered the LAMP1 reduction by T4519 was due to the decrease in the lysosome. To further confirm this, TDM cells were infected with wild-type STy Ty2 strain, and acidic vacuoles were stained by lysotracker red dye. Lysotracker red dye preferably accumulates in highly acidic vacuole lysosomes and stains those vacuoles. A drastic reduction in the number of lysosomes (red signal) was found after 24 hours post-infection which again supported our previous findings. As expected, Ty2ΔT4519 infected cells showed proper punctated lysotracker staining of lysosomes (Fig 3A, 3B). The disappearance of lysosomal puncta might happen due to the lysosomal membrane permeabilization (LMP) or reduced lysosomal biogenesis. The above experiments suggested that T4519 did not interfere with the endosomal maturation pathway, but reduced the intracellular lysosomes after 24 hrs of infection.

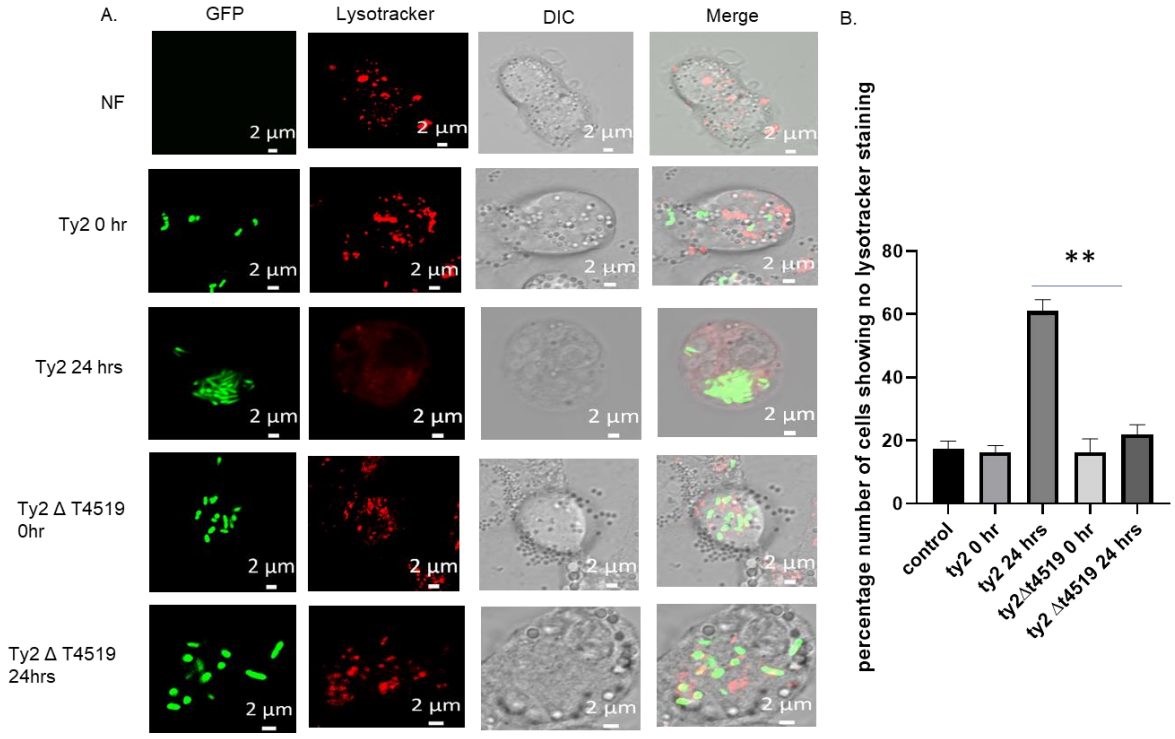

Figure 3 -**T4519 reduces lysotracker staining after 24 hours of infection.** TDM cells were infected with wild-type Ty2 and Δ T4519 Ty2 and stained with Lysotracker red. A, B. Lysotracker staining of TDM cells visualized in a confocal microscope. B. Quantification of the percentage of cells showing lysotracker staining. A representative image from all experiments was given. Images taken in Zeiss LSM710 confocal microscopy. n=50 cells

Objective 2: - To study lysosomal biogenesis, turnover, and membrane integrity in STy infected Macrophages

1. STy does not affect Lysosomal biogenesis

In our earlier experiments, we found that wild-type STy caused lysosomal reduction which was found to be controlled by STy protein T4519. The lysosomal reduction could be due to lysosomal rupture (LMP) or lysosomal biogenesis impairment. So, we measured the expression of the master regulator of lysosomal genes, Transcription Factor EB (TFEB). We found no change in the mRNA expression of TFEB at different time points after infecting the TDM cells with Ty2 (Fig 4A). This proved that the lysosomal biogenesis was not influenced by Ty2. Several other lysosomal gene expressions were measured, among them, Lamp1 m-RNA was reduced significantly and Limp1, Rab5, Rab7, and Rab 34 increased both after 6 hrs and 18 hrs post infection (Fig 4B). The unchanged expression level of TFEB confirmed no change in lysosomal biogenesis.

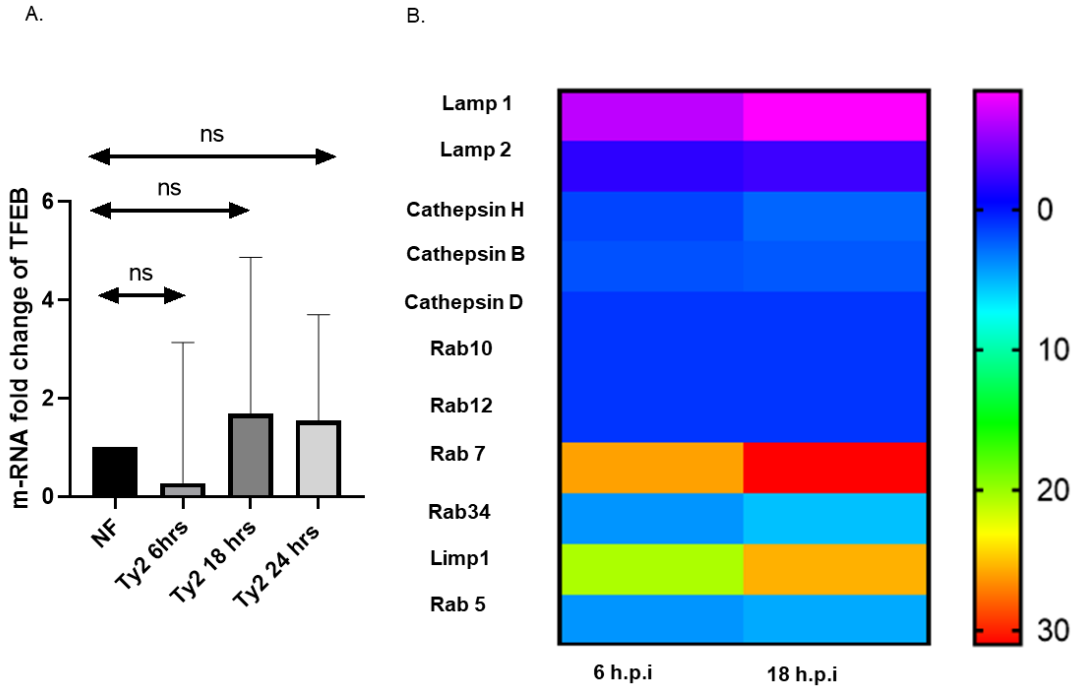

Figure 4- ***Salmonella* Typhi does not affect Lysosomal biogenesis**. A. m-rna fold change of TFEB measured by rt-PCR. B. A. m-RNA expression change of different lysosomal gene measured by rt-PCR. Each color denoted the amount of fold change. β-actin was used as a housekeeping gene. Error bars represent SD. Significance was calculated using a two-tailed unpaired T-test. Statistical analysis was done by using GraphPad Prism 8.

2. T4519 causes lysosomal membrane permeabilization in THP-1-derived macrophage cells and primary human macrophages.

As the lysosomal reduction was not caused by lysosomal biogenesis impairment, we wanted to check whether the lysosomes were ruptured. LMP was studied by the appearance of diffused Dextran Alexa Fluor staining after Ty2 infection as dextran conjugated Alexa-Fluor would give red fluorescence puncta only in the lysosomes, which was observed at 0 hr post-infection by wild-type Ty2. Strikingly, at 24 hrs post infection diffused staining of Dextran Alexa fluor was observed indicating lysosomal leakage (Fig. 5A, 5B). LMP caused by STy infection was also confirmed by Galectin 1 staining. Galectin is a carbohydrate-binding protein that accumulates within the inner lysosomal membrane if the lysosome is ruptured. In intact lysosomes, endogenous galectin remains in the cytoplasm and shows diffused staining. Ty2 infection was done in TDM cells and stained with galectin 1 antibody. LMP was observed by the appearance of prominent Galectin 1 puncta at 24 hours post-infection. At 0 hr, no puncta was found showing no LMP (Fig 5C,5D). To study the role of T4519 in Ty2-induced LMP, THP1-derived human macrophage cells were infected with Ty2 ΔT4519 strain, stained with galectin 1 antibody, and analyzed by confocal microscopy. Even after 24 hours post-infection, no galectin 1 puncta was observed indicating no LMP (Fig 5C,5D) in the mutant bacteria-infected cells. The role of T4519 in LMP was further elucidated by studying the staining pattern of dextran Alexa fluor in THP-1-derived human macrophage cells infected with Ty2 ΔT4519 strain at 24 hours. No diffused staining of Dextran Alexa fluor was found even after 24 hours post-infection indicating no lysosomal leakage (Fig. 5A, 5B) in Ty2 ΔT4519 infected cells. We isolated Peripheral blood mononuclear cells (PBMC) from whole human blood to see if the Ty2 bacteria can cause LMP in PBMC also. PBMCs were converted in macrophages by 7 days of treatment with Macrophage Colony Stimulating Factor. Monocyte-derived macrophage (MoM) cell line was incubated with Dextran Alexa-fluor overnight. Then cells were infected with wild-type Ty2 at 20 MOI and with Ty2 ΔT4519 at 40 MOI followed by gentamycin protection assay. Then cells were visualized in confocal microscopy. Just like THP1, the

puncta indicating intact lysosomes got diffused after 24 hours in PBMC but in Ty2 ΔT4519 infected cells, the punctas remain intact like 0 hr infected cells (Fig 6 A-B). From this, it can be stated that Ty2 component T4519 can induce LMP in PBMC-derived macrophages also.

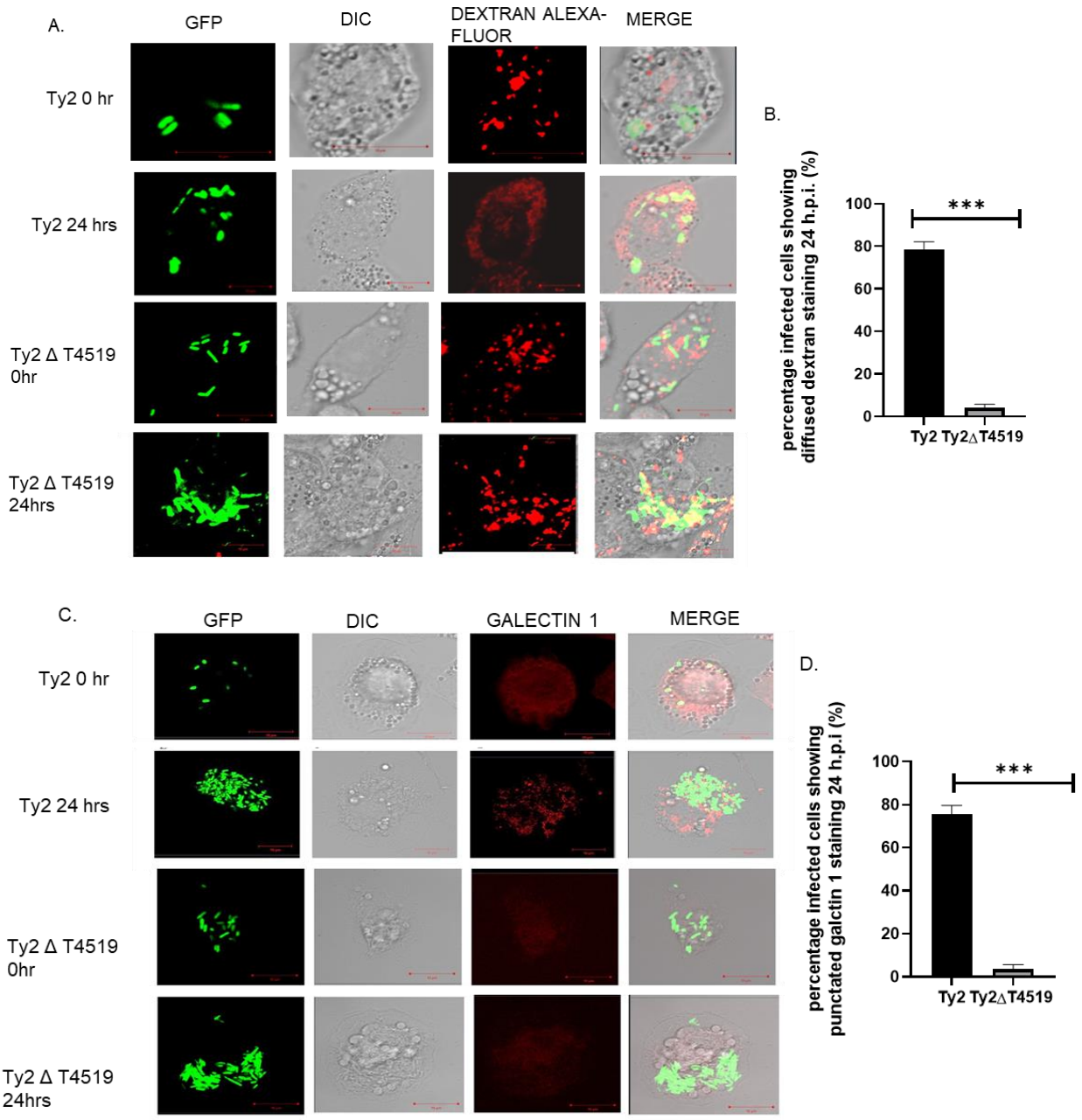

Figure 5-**T4519 causes lysosomal membrane permeabilization in THP-1-derived macrophage cells**. A-B. Representative confocal images showing LMP caused by Ty2 by Dextran AlexaFluor staining. TDM cells, treated with Dextran Alexa-fluor were infected and were analyzed by confocal microscopy. B. Quantification of the percentage of cells showing diffused Dextran Alexa-fluor staining. N=50 Cells C-D.THP-1-derived human macrophage cell lines were infected and stained with Galectin 1 antibody and were analyzed by confocal microscopy at 0 hr and 24 hrs post-infection. D. Quantification of the percentage of cells showing Galectin-1

puncta. N=50 Cells. A representative image from all experiments was given. Images taken in 63X oil-immersion lens of Zeiss LSM710 confocal microscopy. Error bars represent SD. Significance was calculated using two two-tailed unpaired T tests. Statistical analysis was done by using GraphPad Prism 8.

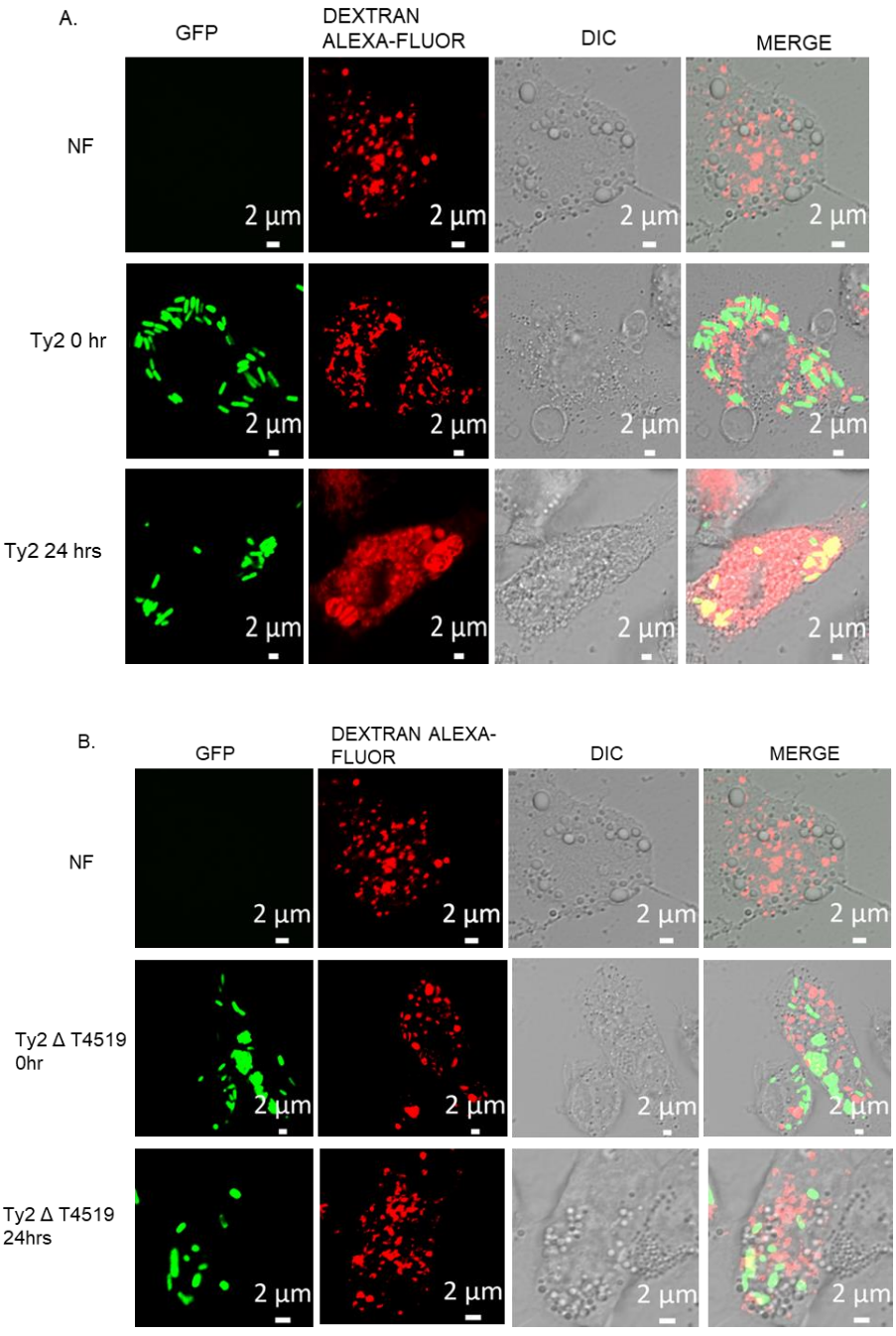

Figure 6-**T4519 causes lysosomal membrane permeabilization in primary human macrophages**. A. Representative confocal images showing LMP caused by Ty2 by Dextran Alexa-fluor staining. MoM cells, treated with Dextran Alexa-fluor were infected and were analyzed by confocal microscopy. B. MoM cells were infected with ΔT4519 Ty2 and stained with Galectin 1 antibody and were analyzed by confocal microscopy at 0 hr and 24 hrs post-infection. A representative image from all experiments was given. Images taken in 63X oil-immersion lens of Zeiss LSM710 confocal microscopy.

3. T4519-mediated LMP releases active cathepsin B in the cytosol

Previously it was found that, wild type Ty2 bacteria can cause lysosomal membrane permeabilization but the mutant bacteria ΔT4519Ty2 was unable to cause LMP. Cathepsin B release in the cytosol is a consequence of unrepaired LMP. Cathepsin B release was studied by staining cells with cathepsin B antibody. THP1-derived human macrophage cell line was infected with wild-type STy Ty2 strain with 50 MOI and Ty2 ΔT4519 strain with 100 MOI followed by gentamycin protection assay. Cells were fixed and stained with cathepsin B primary antibody followed by fluorochrome-conjugated secondary antibody. After 24 hours of infection of wild type bacteria, cytosolic diffuse cathepsin B was found which was not found in Ty2 ΔT4519 infected cells (Fig 7A,7B). Punctated lysosomes containing cathepsin B were found even after 24 hours in Ty2 ΔT4519 infected macrophages. Sometimes minor LMP is repaired by ESCRT pathway or by lysophagy (Radulovic, Schink et al. 2018). The cytosolic cathepsin B can also be inactivated by cystatins present in the cytosol which are the main inhibitors of cathepsins and by the neutral pH of the cytosol (Cavallo-Medved, Moin et al. 2011). Cathepsin B substrate Magic Red (ab270772) is cleaved by active cathepsin B and gives red fluorescence. To see if the released cathepsin B was active or not, TDM cells were stained with magic red after infecting cells with wild-type Ty2 and visualized under a microscope. After 24 hours of infection, red fluorescence was found in cytosol indicating active cathepsin B in a diffused pattern (Fig 7C). At 0 hr of infection and in control cells this diffused pattern of magic red staining was not found. It showed that the released cathepsin B due to LMP remained active in the cytosol after 24 hours of infection.

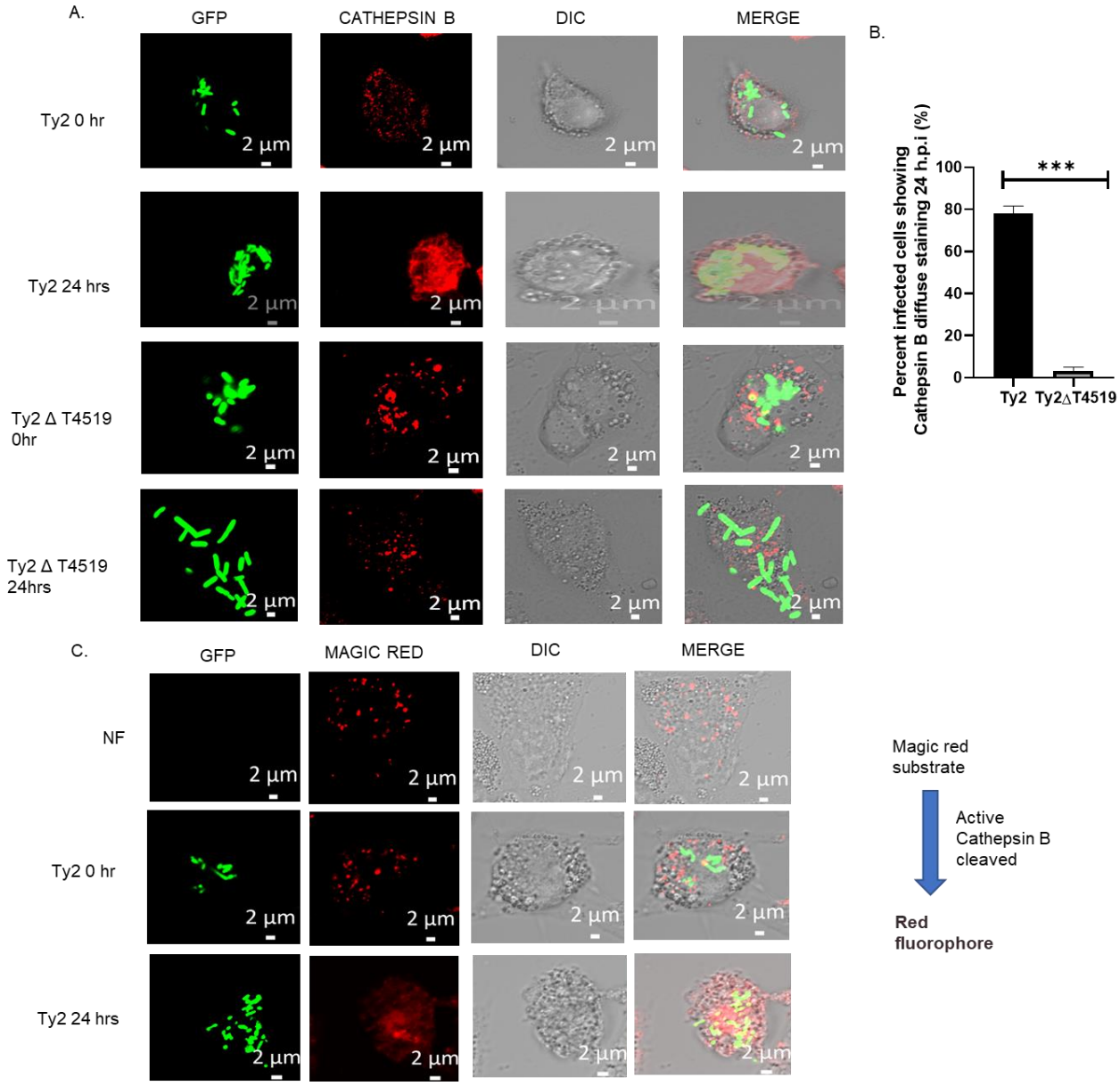

Fig 7 **T4519 mediated LMP releases active cathepsin B in the cytosol**. A-B.TDM cells were infected with wild-type Ty2 and Δ T4519 Ty2 and stained with Cathepsin B antibody. B. Quantification of the percentage of cells showing diffused Cathepsin B staining. N=50 Cells. C. TDM cells were infected with wild-type Ty2 and Δ T4519 Ty2 and stained with Cathepsin B substrate Magic Red. A representative image from all experiments was given. Images taken in 63X oil-immersion lens of Zeiss LSM710 confocal microscopy. Error bars represent SD. Significance was calculated using a tailed unpaired T test. Statistical analysis was done by using GraphPad Prism 8.

4. Apoptosis occurs as a consequence of cathepsin B release in the cytosol after LMP: -

There are several reports and well-established pathways saying cytosolic cathepsin B triggers apoptosis or programmed cell death (Xie, Zhao et al. 2023). So here we wanted to investigate whether

this cytosolic cathepsin B can trigger apoptosis or not. TDM cells were infected with wild-type Ty2 at 50 MOI and with Ty2 ΔT4519 at 100 MOI followed by a gentamycin protection assay. Then cells were stained with annexin V and PI and a number of annexin-positive cells were quantified in FACS. Early apoptotic cells are Annexin V-positive and PI-negative (Annexin V-FITC+/PI−), whereas late (end-stage) apoptotic cells are Annexin V/PI-double-positive (Annexin V-FITC+/PI+). Total annexin v positive cells indicating both early and late apoptosis were counted in FACS. Cells infected with wild-type bacteria showed 10-20% apoptosis whereas Ty2 ΔT4519 bacteria showed almost 3-5 % apoptosis and control cells showed around 1-3 % apoptosis after 24 hours of infection (Fig 8A-B).

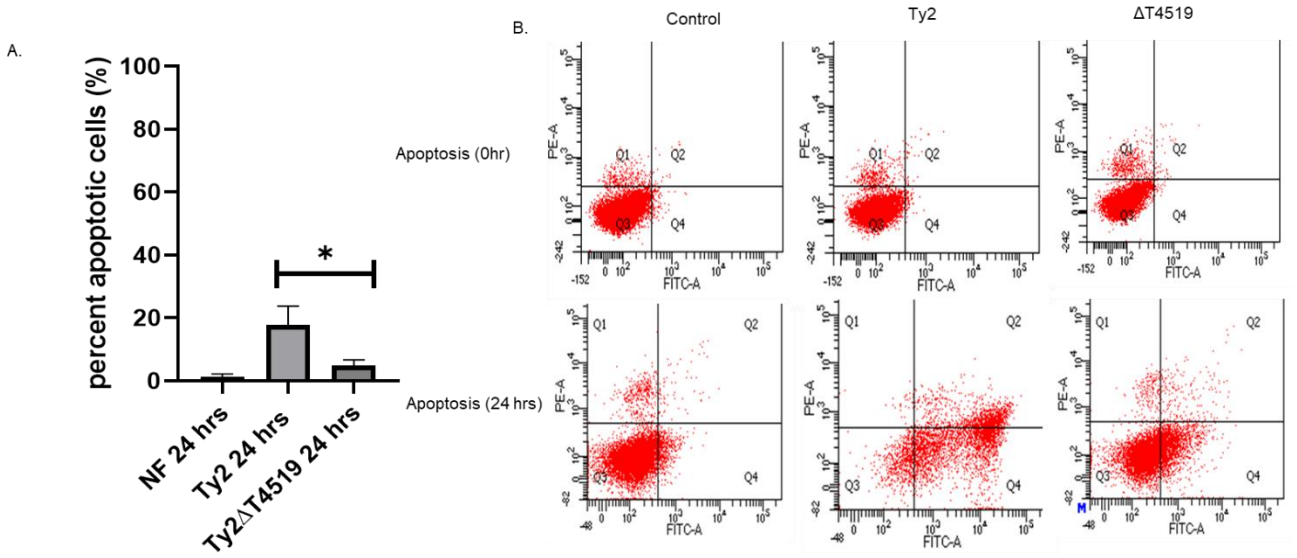

Fig 8 - **Apoptosis occurs as a consequence of cathepsin B release in the cytosol after LMP**. TDM cells were infected and stained with Annexin V and PI at indicated time points. FACS analysis was done after staining. A. Percentage of apoptotic cells. B. FACS analysis of infected cells. Error bars represent SD. Significance was calculated using a tailed unpaired T test. Statistical analysis was done by using GraphPad Prism 8.

5. T4519 protein induces LMP: -

It was previously reported that T4519 is a secreted serine-threonine kinase present in STy and the protein gets secreted out in the cytosol (Theeya, Ta et al. 2015). Whether the purified protein itself was enough for lysosomal rupture or not was our next question. To investigate this, TDM cells were incubated with Dextran Alexa-fluor overnight. Purified protein rT4519 (0.3 mg/ml) was added to cell media and incubated with TDM cells for 3 hrs and LMP was studied in confocal microscopy. rT4519

treated cells showed a diffused pattern of dextran staining. The cells containing denatured protein (rT4519+ 8M urea) and protein isolation buffer alone showed punctate staining of intact lysosomes (Fig 9A-B). We measured the number of punctae as intact lysosomes and found a significantly lesser number of Dextran punctas in purified protein-treated cells (Fig 9B). Cells were incubated with purified protein and stained with Lysotracker Red followed by FACS to measure the intensity of lysotracker. Protein-treated cells showed significantly reduced MFI (Mean Fluorescence Intensity) compared with buffer-treated cells (Fig 9C). To check if the protein released in the culture supernatant of the infected cells was responsible for LMP, TDM cells were infected for 24 hrs with wild-type Ty2, and the media was taken from the infected well and incubated with fresh TDM cells for 3 hrs. Interestingly, the supernatant of the infected cells caused diffused dextran staining of the cells suggesting LMP, which the mutant bacteria-infected cells did not (Fig 9D-E)

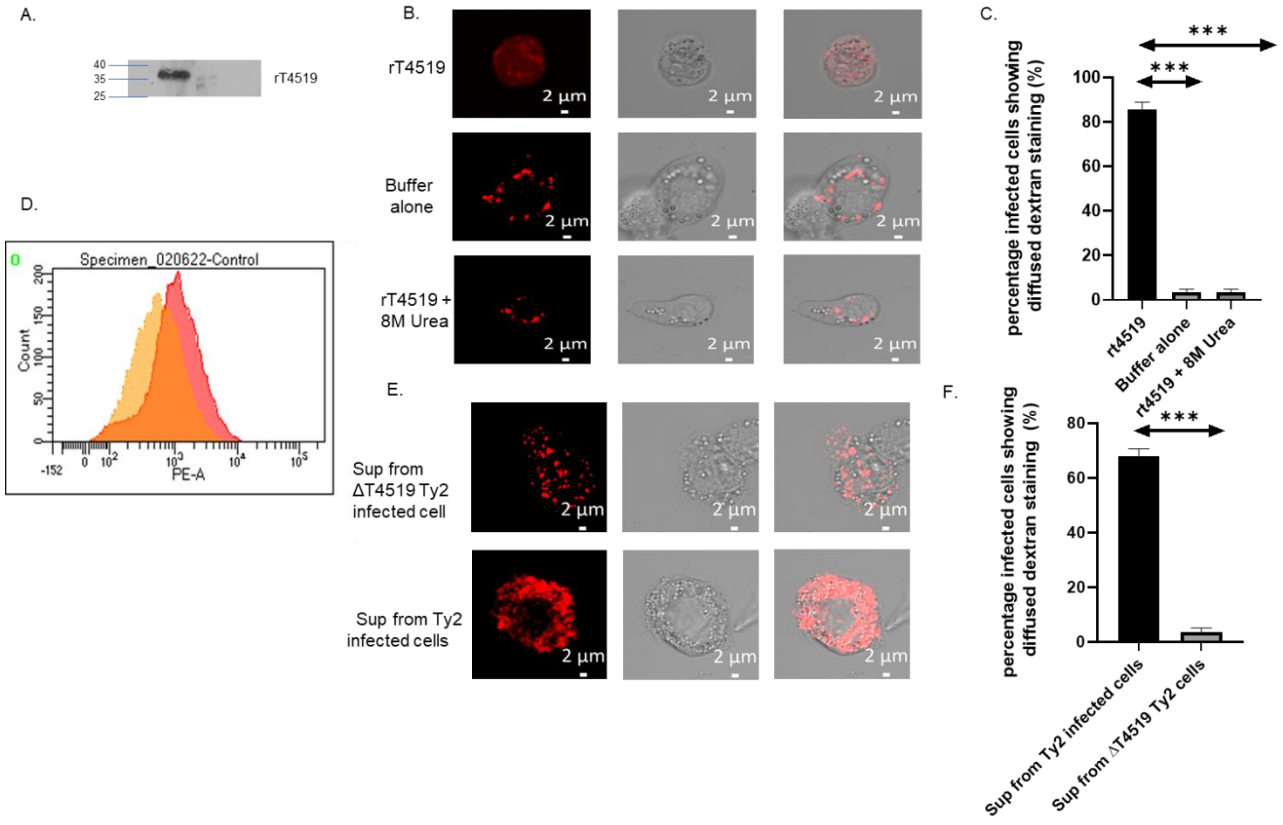

Figure 9: - **rT5619 can directly cause lysosomal membrane permeabilization**. A. Western blot of purified protein rT4519. B- Cells are incubated with Dextran Alexa–fluor to stain lysosomes and viewed in a confocal microscope. Upper panel- purified protein rT4519 treated THP1 cells, middle panel- only buffer treated THP1 cells, lower panel- denatured rT4519 (urea containing) treated THP1 cells. C. Quantification of the percentage of cells showing diffused dextran staining in a confocal microscope. N=50 cells. D. Quantification of loss of lysotracker by FACS after treating the cells with purified rT4519 protein. Red- Non-infected cells with buffer stained with lysotracker, yellow- Non-infected cells with rT4519 stained with lysotracker. E-F. Supernatants from infected cells are incubated with non-infected TDM cells for 3hrs. The non-infected TDM cells contained Dextran Alexa-fluor overnight. F. Quantification of the percentage of cells showing diffused dextran staining in a confocal microscope. N=50 cells. A representative image from all experiments was given. Images taken in 63X oil-immersion lens of Zeiss LSM710 confocal microscopy. Error bars represent SD. Significance was calculated using a tailed unpaired T test. Statistical analysis was done by using GraphPad Prism 8.

6. T4519 promoted delayed IκB-α degradation followed by p65 nuclear translocation independent of proteasomal degradation of IκB-α in human MoM cells.

We had earlier reported that a eukaryote-type serine/threonine kinase (T4519) of STy promoted bacterial survival in macrophages, derived from human myelomonocytic cell line THP-1 (THP-1 derived macrophages or TDM). We further demonstrated that T4519 is a secreted kinase that degrades

IκB-α and induces nuclear translocation of NF-κB p65 in TDM cells (Theeya, Ta et al. 2015). Here, we investigated the detailed mechanisms underlying IκB-α degradation and NF-κB nuclear translocation by T4519 and its role, if any in STy T4519-mediated lysosomal rupture and bacterial survival within human primary macrophage cells. LMP by T4519 is a phenomenon that happened in MoM cells also (Fig 5). To this end, we generated macrophages from the peripheral blood monocytes (monocyte-derived macrophages or MoM) by the treatment of M-CSF (Macrophage Colony-Stimulating Factor) and infected them with wild-type STy Ty2 strain (Ty2) or its isogenic deletion mutant lacking T4519 (Ty2ΔT4519). Similar to the TDM infection, both the wild-type and mutant bacteria were equally efficient in infecting MoM cells. However, intracellular bacterial counts were reduced after the cells were cultured for 24h, as opposed to increased numbers in TDM, suggesting that human primary macrophage cells were more efficient in limiting the intracellular growth of STy. However, the reduction was only modest for the Ty2 strain as compared with the markedly reduced count of Ty2ΔT4519 (Fig 10A). This result indicated that T4519 contributed to the intracellular survival of STy Ty2 in human primary macrophages. We further standardized that the numbers of live intracellular bacteria recovered 24h post-infection were equalized for the wild-type and mutant STy strains when the MoI for the latter was made double that of the wild-type bacteria (Fig 10B). To prove that our results were determined by the presence or absence of T4519 rather than the number of bacteria, we infected the MoM cells with twice as many Ty2Δt4519 as the Ty2 strain for our subsequent experiments.

To investigate if the canonical pathway was involved in NF-κB activation by T4519, we first examined IκB-α degradation in the infected cell lysates by western blots. The results showed biphasic IκB-α degradation in Ty2-infected cells, while Ty2Δt4519 only induced the early IκB-α degradation (Fig 10C-D). Next, the involvement of the proteasome in IκB-α degradation was studied by pre-treatment of the cells with proteasome inhibitor (MG132), followed by infection with Ty2 or Ty2ΔT4519.

Intriguingly, we found that T4519-independent early degradation of IκB-α, but not the late degradation induced by T4519 was proteasome-mediated (Figure 10E-F)

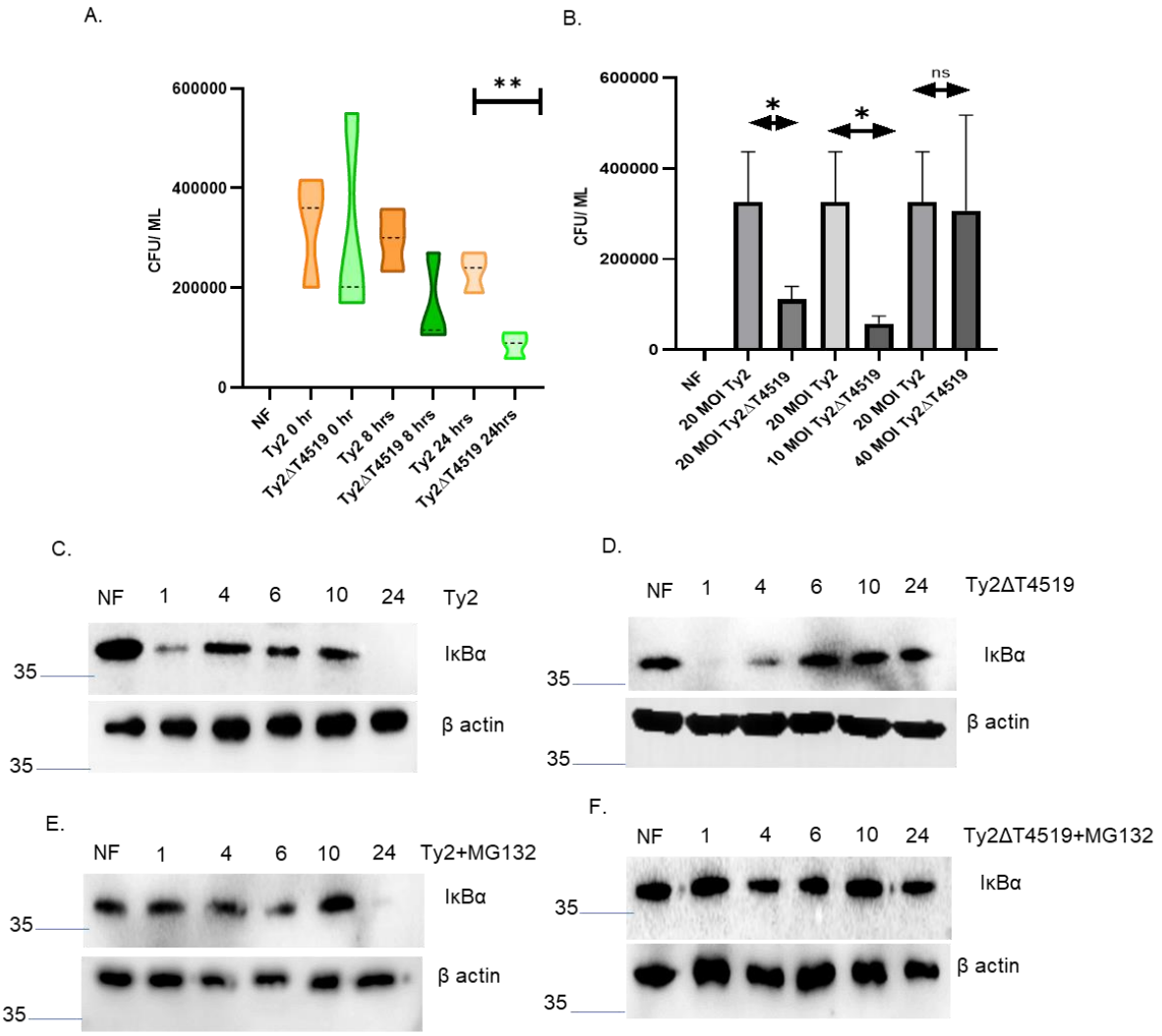

Figure 10**. T4519 promoted delayed IκBα degradation independent of proteasomal degradation of IκBα in human MoM cells.** A. Intracellular bacterial CFU counts in MoM cells. PBMC-derived macrophages were infected with wild-type *S.* Typhi or Ty2ΔT4519 for 30 mins followed by gentamycin protection assay as described under Materials and Methods. CFU determined T4519 mediated survival. Intracellular CFU were counted after 24 hrs of infection following cell lysis and the lysates were platted on LA plates, which were incubated overnight at 37° C. B. CFU was calculated at different MOI, 24 hrs after infection in MoM cells. Significance was calculated using a tailed unpaired T test. Statistical analysis was done by using GraphPad Prism 8. NS stands for not significant. C-F.NF stands for no Infection. MoM was treated with a proteasome inhibitor (MG132, 20 μM) 3 hours prior to infection with Ty2 or Ty2ΔT4519. Western blots of IκB-α. The numbers denoted hours after infection. The experiments were repeated three times and one representative blot in each case is shown here. β-actin was used as a loading control for all blots.

The late IκB-α degradation by T4519 also led to NF-κB nuclear translocation was proved by staining of the STy-infected MoM cells with p65 antibodies. P65 was predominantly present in the nucleus

after 24 hours of Ty2 infection as opposed to largely cytosolic localization in the uninfected and Ty2ΔT4519-infected cells. This resulted in a significantly higher co-localization coefficient of the red and blue fluorescence, used for the staining of p65 and the cell nucleus, respectively in Ty2-infected cells compared with the uninfected and mutant bacteria-infected cells (Fig 11 A-C). However, p65 nuclear translocation at the early time point was observed after both Ty2 and Ty2Δt4519 infection (Fig 12 A-C). In corroboration with the above findings, p65 nuclear translocation after 1h, but not after 24h of infection was proteasome-dependent (Fig 11).

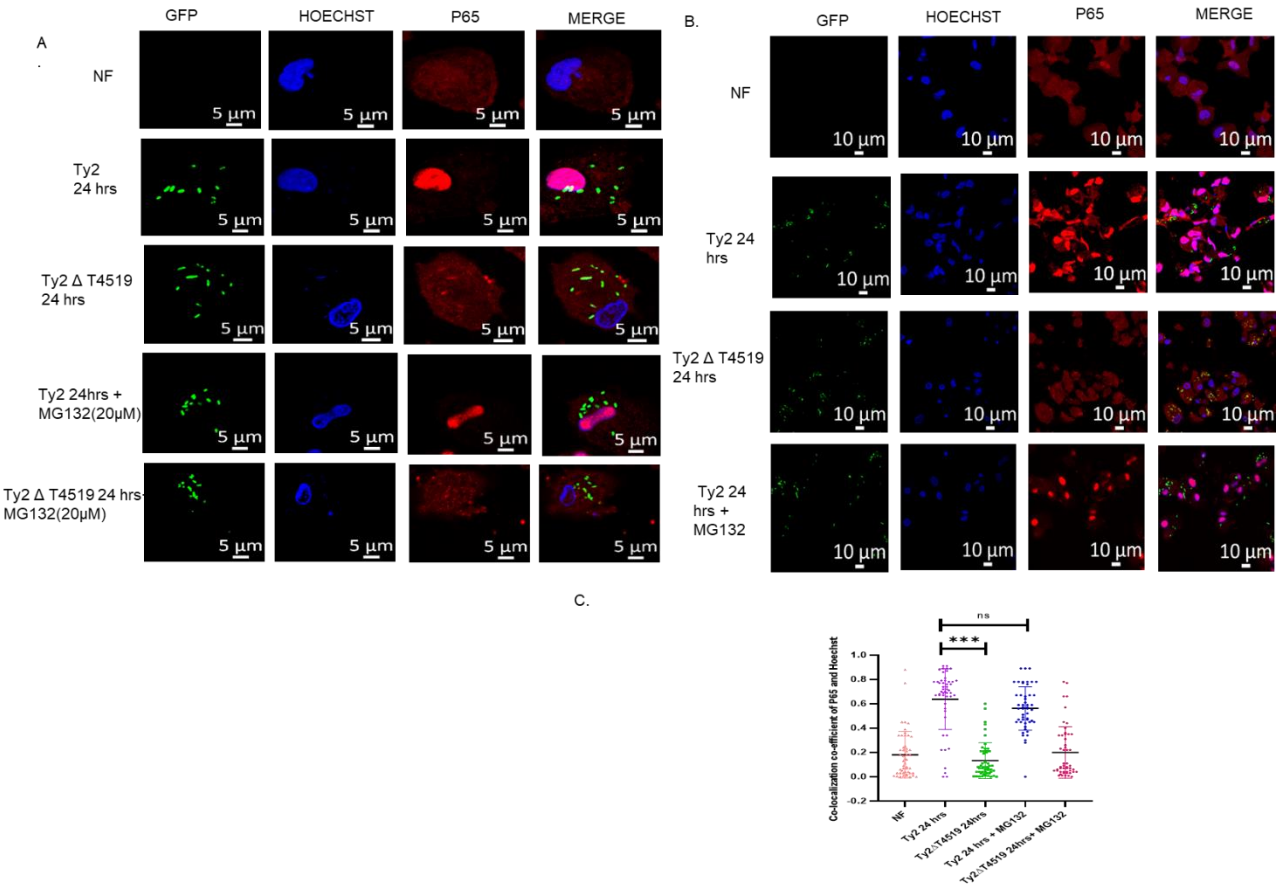

Figure 11. T4519 promoted delayed IκBα degradation followed by p65 nuclear translocation independent of proteasomal degradation of IκBα in human MoM cells. A-C. P65 staining viewed under the confocal microscope showed P65 nuclear translocation in GFP-tagged Ty2-infected cells. Alexa-fluor conjugated secondary antibody (Alexa-fluor 594 anti-rabbit) was used for p65 staining (red) and the nucleus was stained by Hoechst (blue). Images were taken in 63X oil immersion lens of LSM 710 confocal microscope, Scale bar 5μm. Representative images from three independent experiments were shown. Error bars represent SD. Significance was calculated using a two-tailed unpaired T-test. Statistical analysis was done by using GraphPad Prism 8. C. Quantification of fluorescence colocalization of Hoechst (blue) and p65 (red), was done by taking random fields, from which in a total of 50 cells, blue and red co-localization was measured by using LSM 710 Zeiss Zen Blue software of confocal microscope. D.D

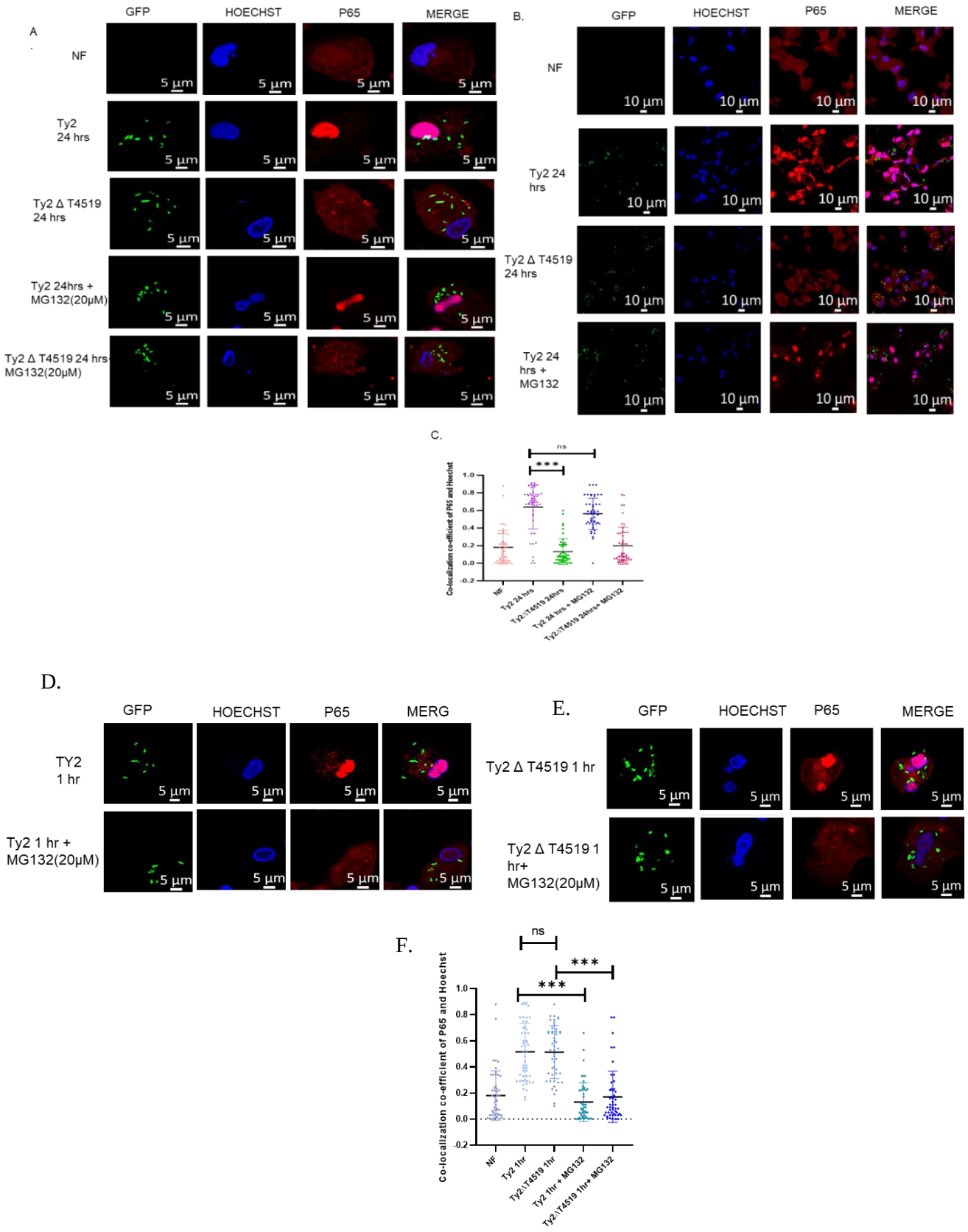

Figure 12- Delayed p65 nuclear translocation is T4519 dependent and early p65 nuclear translocation is proteasome-dependent in human MoM cells. PBMC-derived macrophages were infected with wild-type *S.* Typhi or Ty2ΔT4519 for 30 mins followed by gentamycin protection assay as described under Materials and

Methods. A-F–p65 confocal microscopy staining after infection with GFP-tagged bacteria. MoM cells were incubated with indicated inhibitors followed by infection. Alexa-fluor conjugated secondary antibody (Alexa-fluor 594 anti-rabbit) was used for p65 staining and the nucleus was stained by Hoechst (blue). Here NF stands for no Infection. The above experiments were repeated three times and the values from those three experiments were plotted. Error bars represent SD. Significance was calculated using a tailed unpaired T test. Statistical analysis was done by using GraphPad Prism 8. NS stands for not significant. A representative image from each experiment was given. C,F. – Quantification of fluorescence colocalization of Hoechst (blue) and p65 (red), done by taking random fields, from which in a total of 50 cells, blue and red co-localization was measured by using LSM 710 Zeiss Zen Blue software of confocal microscope.

7. Delayed, but not early IκB-α degradation and p65 nuclear translocation in STy-infected human MoM cells are cathepsin B dependent

Published studies suggested that cathepsin B can induce proteasome-independent IκB-α degradation and consequent NF-κB activation (de Mingo, de Gregorio et al. 2016) (Li, Wu et al. 2016). Given that T4519-regulated IκB-α degradation was proteasome-independent, we investigated if this was mediated by cathepsin B. To this end, MoM cells were treated with cathepsin B inhibitor, followed by STy infection. Probing the cell lysates for IκB-α by western blots showed complete blockade of the degradation after 24h of Ty2 infection in the inhibitor-treated cells, but early IκB-α degradation was spared (Fig 13A-C). Accordingly, nuclear translocation of p65 at 24 hrs, but not at 1 hr post-infection (p.i.) was completely abrogated by cathepsin B inhibitor pre-treatment of the Ty2-infected cells (Fig 13D-G). Together the above results suggested that T4519 stimulated cathepsin B to promote IκB-α cleavage, followed by p65 nuclear translocation in human MoM cells only at the later time point of infection. This would require active cathepsin B in the cytosol which is normally absent but may be released from the damaged lysosomes. However, minor damages are successfully repaired, while the enzymatic activity of the released catB is suppressed by the default cytosolic catB inhibitor, cystatins.

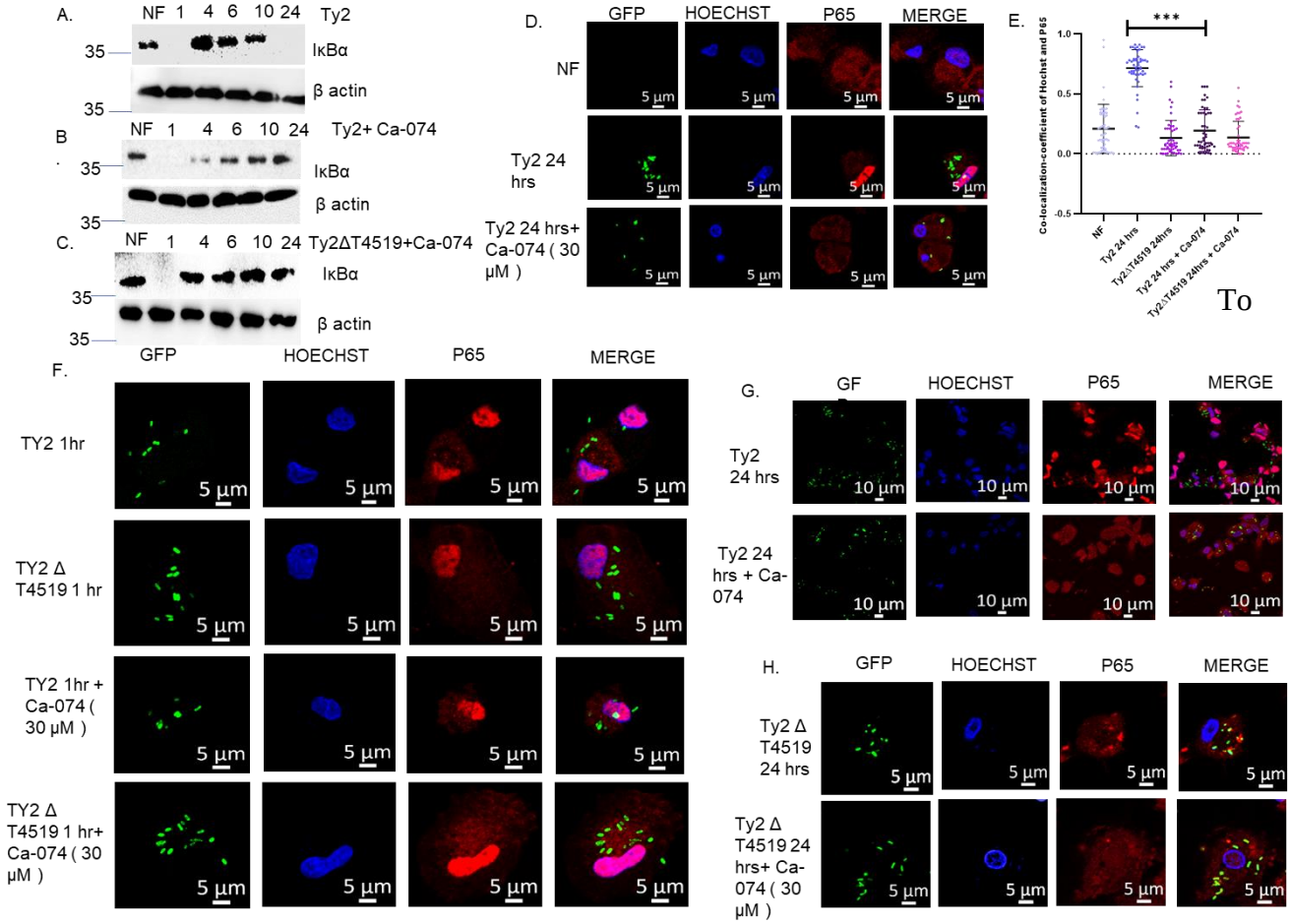

Fig 13. Delayed, but not early IκB-α degradation and p65 nuclear translocation in *S.* Typhi-infected human MoM cells are cathepsin B dependent. MoM was treated with cathepsin B inhibitor (Ca-074 30 μM) 3 hours prior to infection. A to C. Western blot of IκBα in the infected cell lysates, showing Cathepsin B mediated IκBα degradation by T4519 only at the later time point (24h). β-actin was used as a loading control. The experiments were repeated three times and one representative blot from each experiment is shown here. D-H. P65 staining viewed under a confocal microscope shows cathepsin B-mediated P65 nuclear translocation in Ty2-infected cells. Alexa-fluor 594 anti-rabbit was used for p65 staining (red) and the nucleus was stained by Hoechst (blue). Images were taken in 63X oil immersion lens of LSM 710 confocal microscope, Scale bar 5μm. Representative images from three independent experiments are shown. E. Quantification of fluorescence colocalization of Hoechst (blue) and p65 (red) was done. Error bars represent SD. Significance was calculated using two-tailed unpaired T-tests. Statistical analysis was done by using GraphPad Prism 8.

Next, we investigated if active catB was present in the cytosol of Ty2-infected macrophages, we first stained the infected MoM cells with cathepsin B antibody at different time points. The results showed diffuse Cathepsin B staining of the cytosol after both the wild-type and mutant bacterial infections

during the 8-12 hrs post-infection period (Fig 14A). This was corroborated by cathepsin B western blots of the cytosolic extracts from the above cells (Fig 14B). These results indicated early lysosomal damage of the infected cells. Further to investigate if the cytosolic catB was enzymatically active, cells were stained with Magic Red, a cathepsin B substrate. We observed a speckled Magic Red staining pattern for both wild-type and mutant bacterial infections in the early hours (8 h p.i.), representing exclusively lysosomal active catB (Fig 14C-D). Together these results suggested the presence of inactive catB in the cytosol of the infected cells. However, at 12h p.i., strong cytosolic Magic Red staining, suggestive of enzymatic activity was observed in the Ty2-, but not in the Ty2ΔT4519-infected cells (Fig 14C-E).

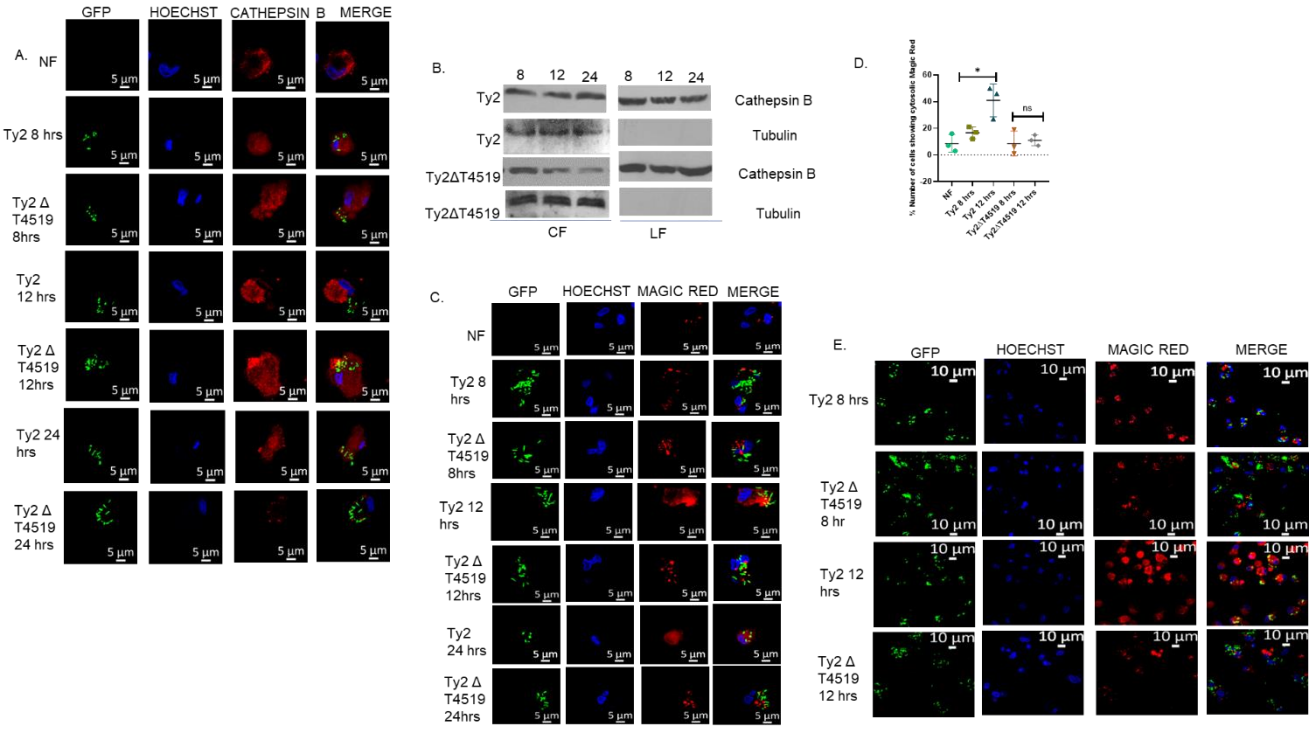

Fig 14- **T4519 in *S*. Typhi activates cytosolic Cathepsin B in human MoM cells.** A. Cathepsin B antibody staining in infected MoM cells. B. Infected MoM cells were lysed and cytoplasmic fraction (CF) and lysosomal fraction (LF) were isolated. Western blot was done by cathepsin B antibody at 8 hrs, 12 hrs, and 24 hrs PI respectively. Tubulin was used as a loading control. C-E. Ty2 or Ty2ΔT4519 Infected MoM cells were infected with GFP-expressing bacteria and stained with Cathepsin B substrate, Magic Red, excitation 592nm (red). The nucleus is stained with Hoechst (blue). Representative confocal microscopic images from three independent experiments are given. D. Quantification of magic red stained cells showing cytoplasmic staining. Manual

counting was done by randomly selecting 50 cells from different fields, Error bars represent SD. Significance was calculated using two tailed unpaired T test. Statistical analysis was done by using GraphPad Prism 8.

8. T4519-induced cathepsin B activation, IκB-α degradation, and p65 nuclear translocation are cystatin B-dependent

To address the underlying mechanisms behind T4519-dependent cytosolic cathepsin B activation, we checked for mRNA expression levels of cystatin B and C, which are default cellular inhibitors of cytosolic cathepsin B (Haves-Zburof, Paperna et al. 2011). We observed a progressive reduction of cystatin B mRNA expression levels in the infected cells, reaching nearly 16-fold lower after 12h of Ty2 infection compared with the uninfected cells. However, minimal changes in the cystatin B levels were noticed following infection with the mutant bacteria (Figure 15A). Cystatin B protein expression in the cells matched mRNA expression levels and showed reduction after Ty2, but not after Ty2ΔT4519 infection (Figure 15B). Pre-treatment of the cells with cathepsin B inhibitor had no effects on cystatin B expression, suggesting that it was not under the control of cathepsin B (Figure 15B). In contrast, cystatin C expression remained unaltered after infection with Ty2 or Ty2ΔT4519 (Fig 15C-D).

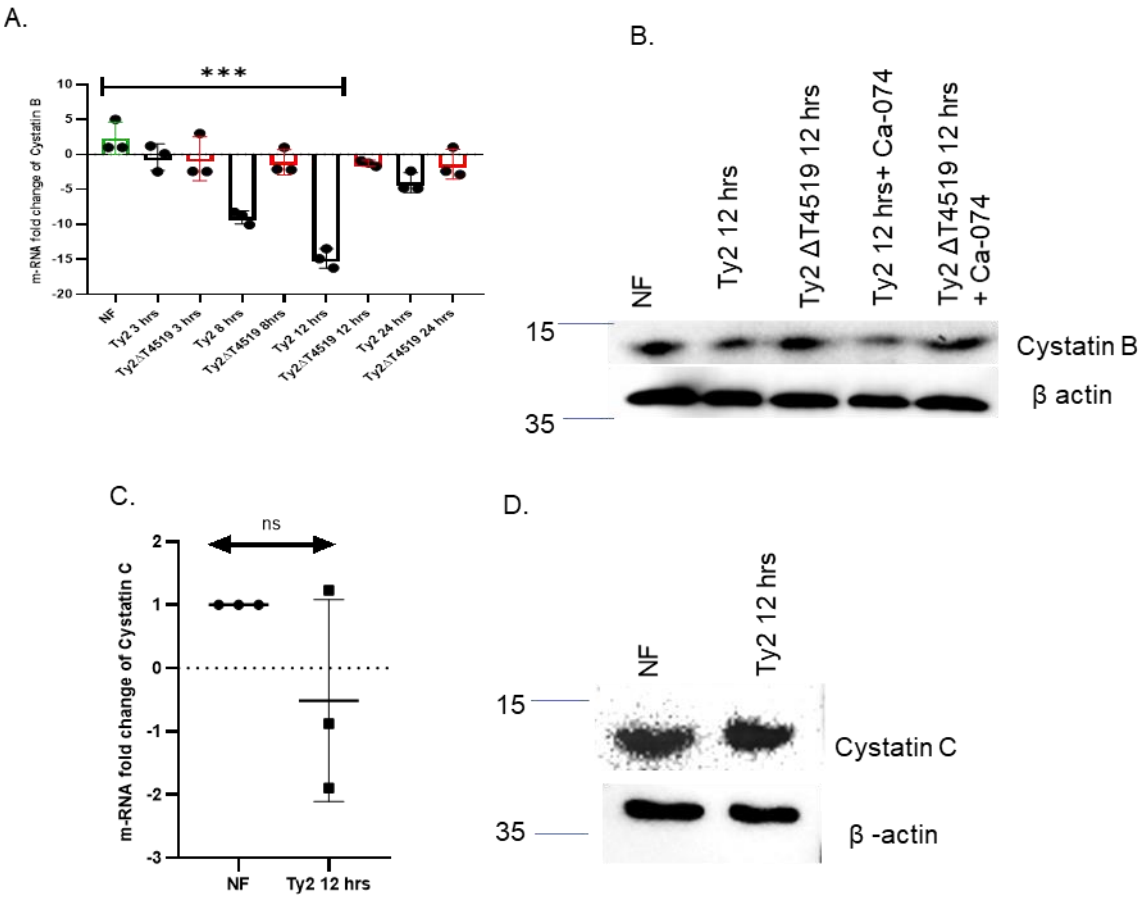

Fig 15. Cathepsin B activation is cystatin B dependent and early events are independent
A. m-RNA fold changes of cystatin B measured by RT-qPCR. B. Western blot of cystatin B in MoM cell lysates. β-actin was used as a loading control. C. m-RNA fold change of cystatin C was measured by RT-PCR. β-actin was used as the control gene. D. The western blot of cystatin C. The time points taken was 12 hrs PI. β- actin was used as loading control for all the blots.

Given the reciprocal relations between the cystatin B and active cytosolic cathepsin B levels in the cells, we investigated if Ty2 infection regulated cathepsin B activation in the cytosol by altering cystatin B levels. To this end, we checked for cathepsin B activity by staining the cells with magic red after silencing cystatin B with siRNA (Fig 16A). The results showed marked cytosolic cathepsin B

activity in both Ty2 and Ty2Δt4519 strain-infected cells after silencing of cystatin B (Fig 16B). This suggested that T4519 inhibited cystatin B expression, leading to the activation of cytosolic cathepsin B.

Next, we investigated if T4519-dependent suppression of cystatin B expression could result in IκB-α degradation and NF-κB nuclear translocation through cathepsin B activation. To this end, cystatin B gene-silenced TDM cells were pre-treated with or without cathepsin B inhibitor followed by STy infection. In agreement with the above results, IκB-α was degraded in the parental Ty2-infected as well as in mutant bacteria-infected cystatin B silenced cells. However, inhibition of cathepsin B in these cells completely restored IκB-α levels, suggesting that cystatin B downregulation activated cathepsin B that was responsible for IκB-α degradation (Fig 16C). That cystatin B downregulation also activated NF-κB in a cathepsin B-dependent way was proved by confocal microscopy that showed nuclear translocation of NF-κB in the Ty2-infected as well as cystatin B gene-silenced cells, which had active cytosolic cathepsin B but not in the cathepsin B inhibitor pre-treated cells (Fig 16D-E). Consequently, the co-localization coefficient of p65 and the nucleus was significantly higher in the cells having active cathepsin B in the cytosol. These results suggested that T4519 downregulated cystatin B expression levels to activate the cathepsin B-NF-κB axis in the MoM cells. However, this phenomenon was a rather late event (beyond 12 hrs of infection), while cystatin B expression remained unaltered in the early hours of infection.

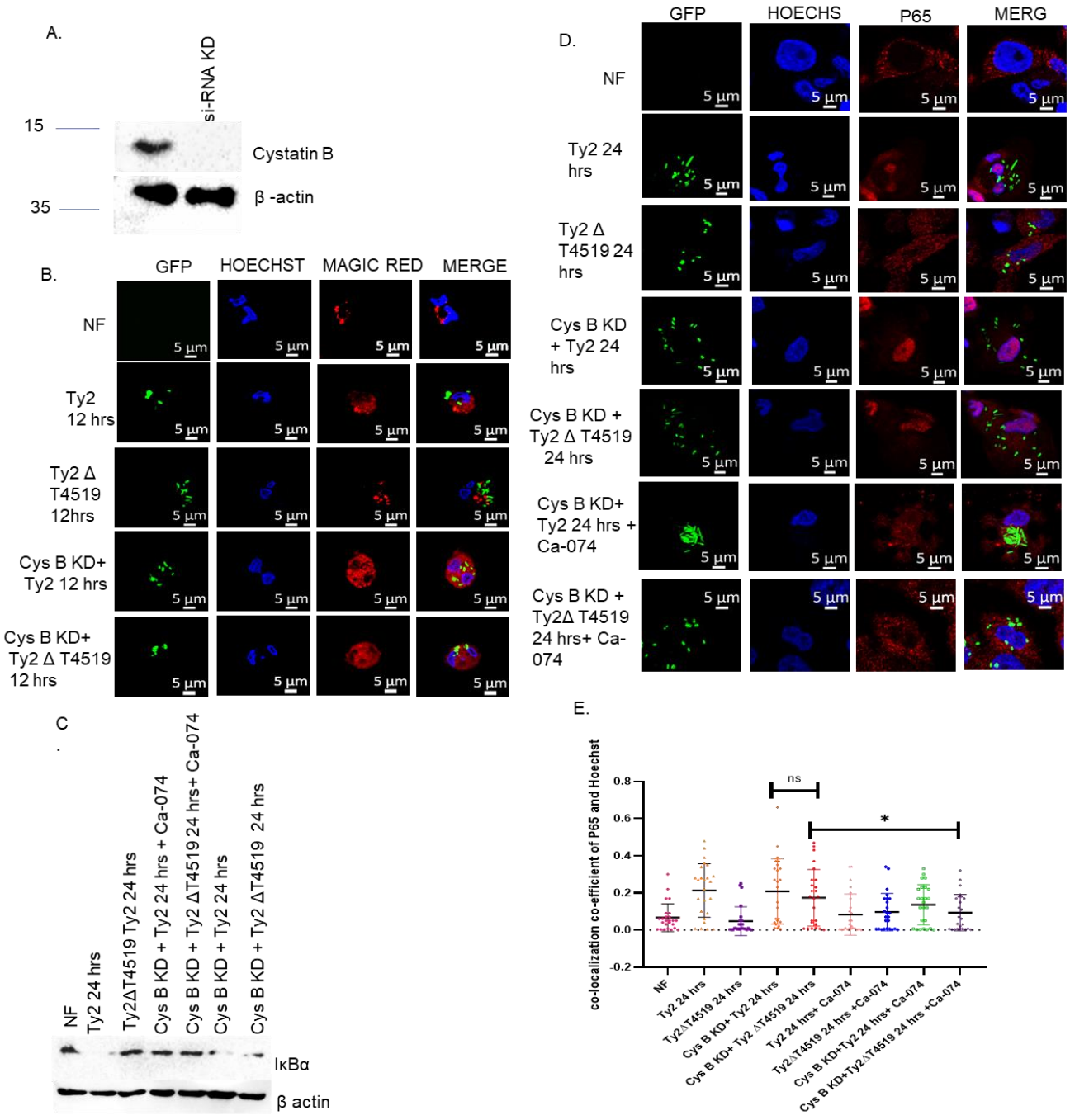

Fig 16. **Cathepsin B activation as well as the delayed events (IKB-α degradation and nuclear translocation) are cystatin B dependent and early events are independent**. A. WB showing expression of cystatin B in Scramble Control and knockout cells. B. Experiments were done as under B- E above after silencing of Cystatin B expression by siRNA in the TDM cells. Scrambled siRNA was used as a control. Representative confocal microscopic images from three independent experiments are shown. The microscopic images were taken using LSM 710 confocal microscope with 63X oil immersion objective lens and processed in ZEISS ZEN BLUE software. Error bars represent SD. Significance was calculated using two two-tailed unpaired T test. Statistical analysis was done by using GraphPad Prism 8. B. TDM cells were infected with GFP-tagged bacteria followed by Magic red staining. C. Western blots of IκB-α in Cystatin B KD TDM cells. β-actin was used as a loading control. One representative blot from three independent experiments is shown D. Above cells were stained with p65 antibody, followed by Alexa-fluor conjugated secondary antibody (Alexa-fluor 594, anti-rabbit). The nucleus was stained by Hoechst (blue), Cells were viewed under the confocal

microscope as described above. E. Quantification of fluorescence colocalization of Hoechst (blue) and p65 (red), done by randomly selecting 25 cells from different fields and using Zeiss Zen Blue software of LSM 710 confocal microscope.

9. Cystatin B-Cathepsin B-NF-κB axis induces LMP

As we have found T4519 caused both LMP and Cathepsin B dependent NF-κB activation in MoM cells, we wanted to check whether T4519 used the same Cystatin B-Cathepsin B- NF-κB pathway to finally promote LMP or not. To check this, pre-treatment of the 24h Ty2-infected cells with cathepsin B and p65 nuclear translocation inhibitors retained the diffuse galectin 1 staining pattern, while proteasome inhibitor pre-treated, infected cells still showed the punctate stains (Fig 17A-C). As expected, none of the inhibitors produced any change in the staining pattern of Ty2ΔT4519- infected cells (Fig 17D).

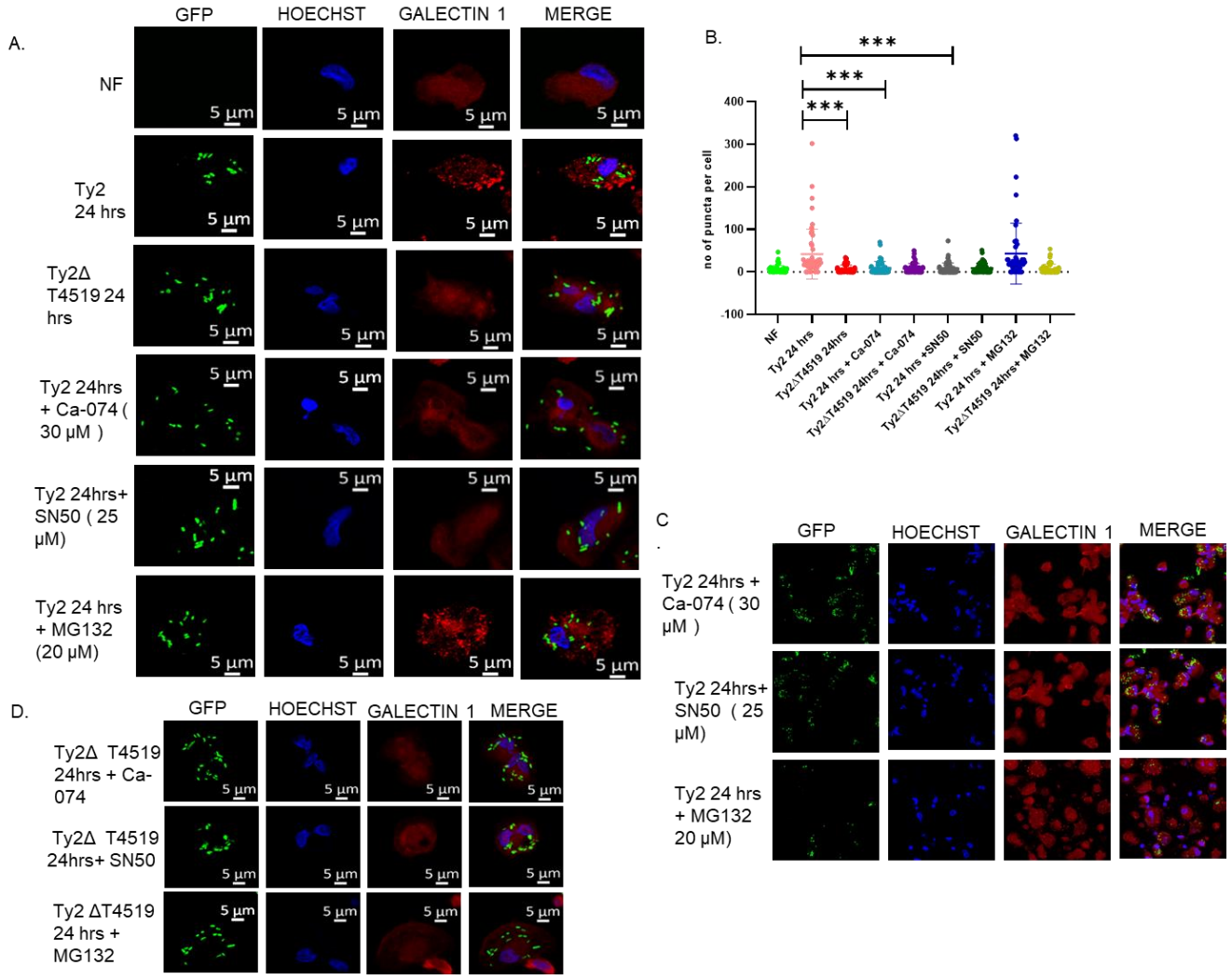

Fig 17**. Cystatin B-Cathepsin B-NF-κB axis induces LMP.** MoM cells were pre-treated with different inhibitors, followed by infection with Ty2 or Ty2ΔT4519. A-D. After 24 hrs, cells were stained with Galectin 1 antibody (Red), followed by Alexa-fluor conjugated secondary antibody, while the nucleus was stained with Hoechst (blue). The images were captured by LSM 710 confocal microscopy in 63X oil immersion lens and processed using ZEISS ZEN BLUE software. A. Representative images from three independent experiments were given. B. Quantification of Galectin-1 puncta per cell, counted manually under the microscope by randomly selecting cells from each field. N=50 cells. The images were taken in LSM 710 confocal microscopy and processed in ZEISS ZEN BLUE software. Error bars represent SD. Significance was calculated using two-tailed unpaired t test. Statistical analysis was done by using GraphPad Prism 8

Parallel staining with dextran Alexa-fluor that stains the acidic vacuoles of the cells corroborated with the above findings, showing lysosomal puncta, except in cells infected with Ty2 for 24h, which showed diffuse cytosolic staining that was abrogated by pre-treatment with cathepsin B or p65 nuclear translocation inhibitors (Fig 18A-E). Lysotracker red staining showed decreased numbers of lysosomal puncta in Ty2 infected parental, but not p65 knockdown TDM cells at 24 hrs p.i. (Fig 18F).

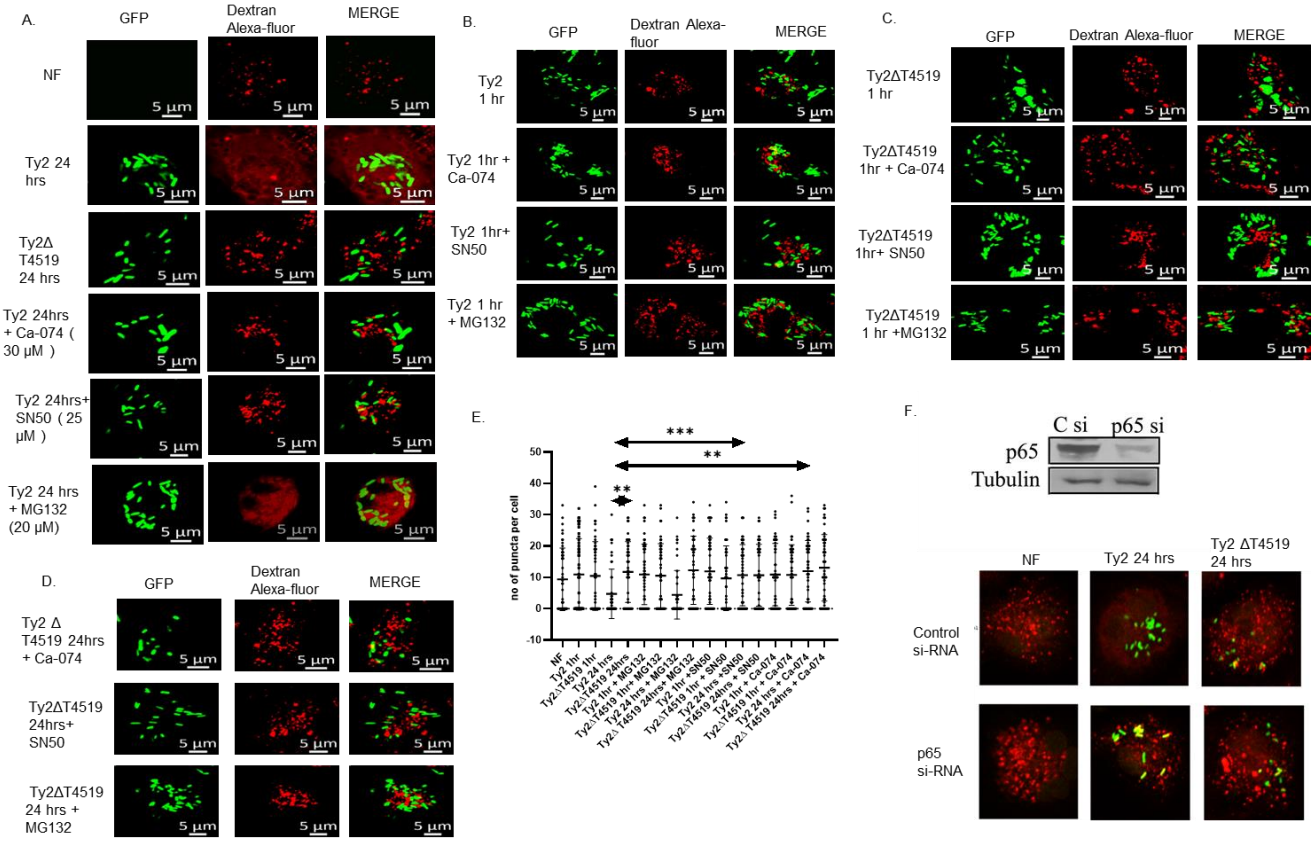

Fig 18**. T4519 induces LMP through Cat B-NF-kB**. A-E- Cells were incubated with Dextran Alexa-fluor-containing media overnight and then infected with GFP-tagged bacteria. E- Quantification of dextran puncta. N=50 Cells F. Lysotracker red staining after infecting p65 si-RNA KD TDM cells. TDM cells were treated with p65 si-RNA to knock down p65 followed by infection. KD was known in Western blot.

As expected, the un-infected and 1 hr infected cells treated with all the inhibitors showed diffused galectin-1 staining (Fig 19A-C), These results together indicated that cathepsin B-dependent, but proteasome-independent NF-κB activation mediated LMP, which was induced by T4519 after 24h of Ty2 infection.

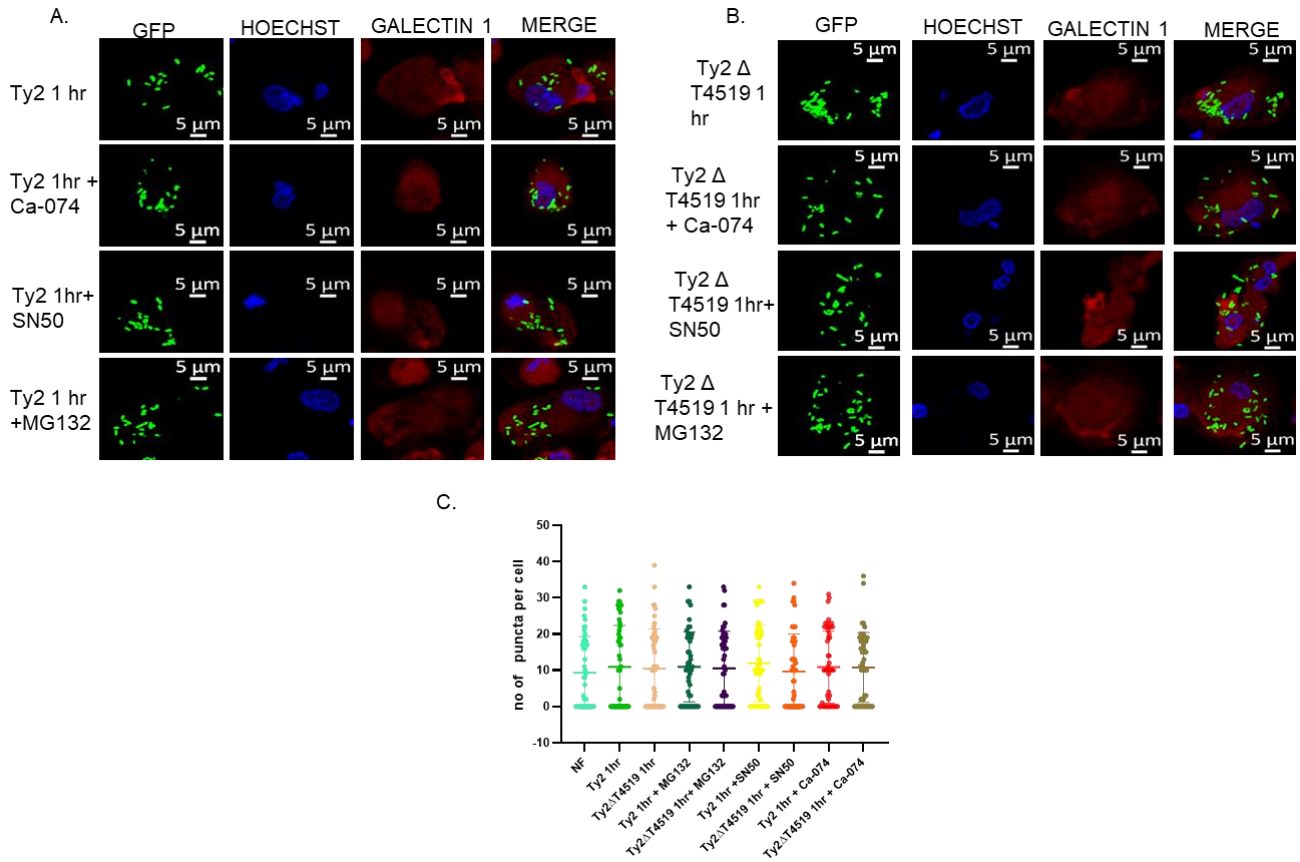

Fig 19**. Cat B-NF-kB induces LMP at later time point of infection proved by Galecyin-1 puncta assay**. A-C- PBMC derived macrophage cells were infected and stained with Galectin 1 antibody followed by Alexa-fluor conjugated secondary antibody and nucleus was stained by Hoechst (blue). C. Quantification of Galectin-1 puncta was done manually by taking cells from random fields, under LSM 710 ZEISS confocal microscope. N= 50 cells. A representative image from all experiments was given. Error bars represent SD. Significance was calculated using two tailed unpaired t-test. Statistical analysis was done by using GraphPad Prism 8

Our earlier experiments showed that T4519 activated cytosolic cathepsin B by downregulating cystatin B expression, thereby inducing IκB-α cleavage and nuclear translocation of NF-κB. We investigated if T4519-elicited LMP, which was regulated by the cathepsin B-NF-κB axis also depended on cystatin

B expression. To address this issue, we infected the TDM cells with the wild-type or mutant Ty2 strain for 24hrs after silencing cystatin B expression. Staining for galectin-1 showed prominent puncta in the cystatin B knockdown cells after Ty2ΔT4519 infection that was identical to the Ty2-infected cells, suggesting LMP induction. However, inhibition of cathepsin B abolished LMP in all the cells (Fig 20A-D).

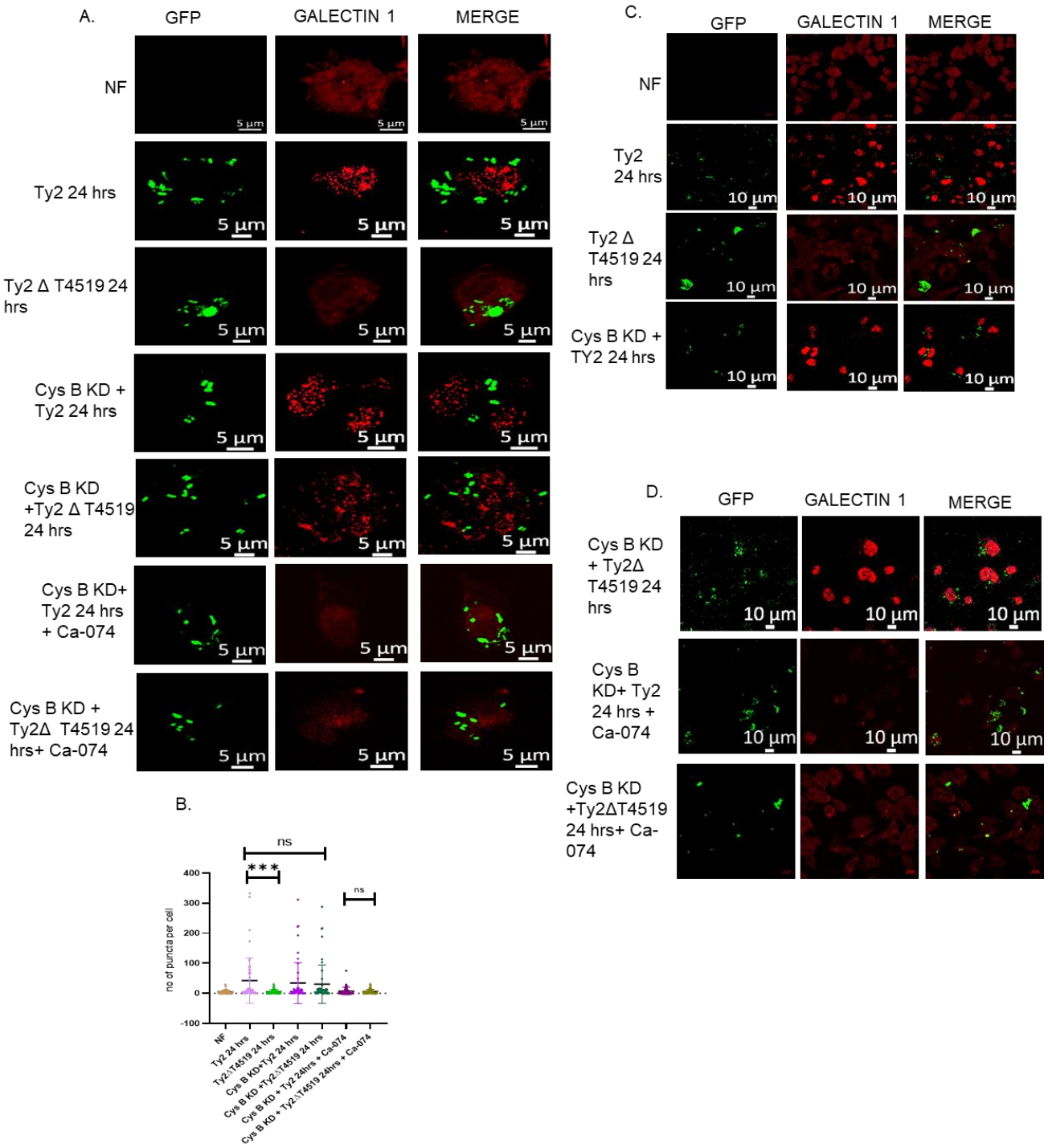

Fig 20. **Cys B-Cat B-NF-kB induces LMP** A-D. Cystatin B KD was done by si-RNA A-D. Parental and cystatin B gene silenced TDM cells were infected with Ty2 or Ty2ΔT4519 strain. 24 hours later, cells were stained with galectin 1 antibody. Representative images from one out of three independent experiments, captured by LSM 710 confocal microscope and processed with ZEISS ZEN BLUE software are shown. B. Quantification of Galectin-1 puncta (red) counted under confocal microscope as described above. Statistical analysis were done in Graph Pad-Prism 8 by unpaired Students' T-test.

Similar experiments followed by dextran Alexa-fluor staining of the cells corroborated with the above findings, with cystatin B knockdown resulting in diffuse cytosolic staining pattern after Ty2ΔT4519 infection that was similar to Ty2 infection of both the parental and cystatin B knockdown cells and suggested LMP (Fig 21A-B). Together these results underscored that cystatin B downregulation by T4519 was indeed responsible for the induction of LMP after Ty2 infection, which was mediated by the cathepsin B-NF-κB axis (Fig 20A).

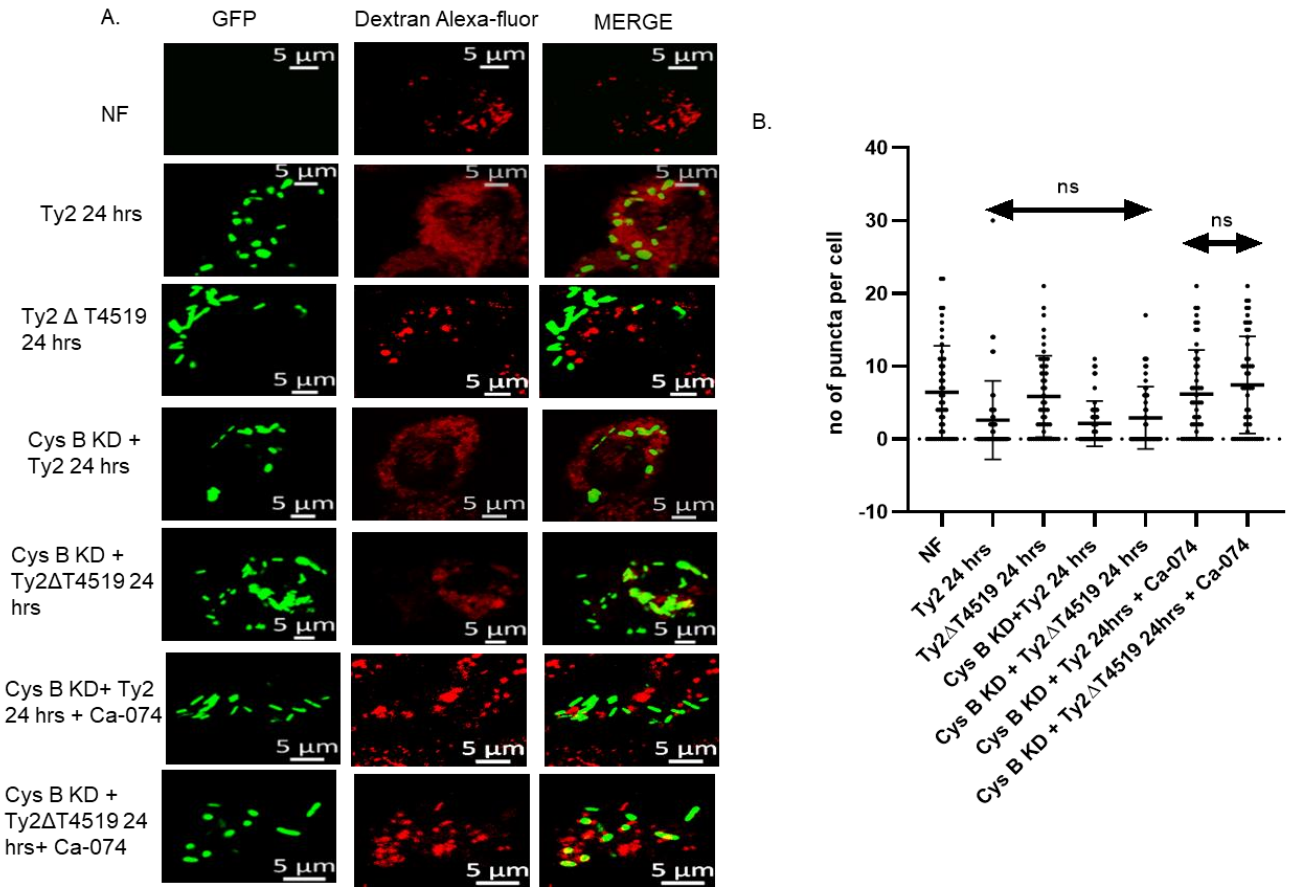

Fig 21**. Cys B-Cat B-NF-kB induces LMP** A-B. Cystatin B KD was done by si-RNA. A.THP-1 derived macrophages were incubated with Dextran Alexa-fluor containing media overnight and then infected with bacteria. B. Quantification of dextran puncta was done manually by taking cells from random fields, N= 50 cells. Representative images from all these experiments were given. Confocal images were taken in LSM 710 confocal microscope and statistical analysis were done in Graph Pad-Prism 8 by unpaired Students' T-test

10. T4519 functions as TLR2 ligand to regulate the TLR2-Cys b-Cat B-NF-κB-LMP pathway

Our results thus far have shown that T4519 promotes bacterial survival and replication within macrophages and activates NF-κB. Since TLRs constitute the major family of microbial recognition receptors in macrophages, we investigated if T4519 functions as a TLR ligand to promote STy survival in MoM cells. To this end, TLR expression in TDM cells was silenced with si-RNAs, followed by infection with Ty2 for 24 hrs, and intracellular CFUs were counted. The results showed significantly reduced recovery of live bacteria from the cells silenced for TLR2, but not after other TLR expressions, suggesting that TLR2 played a critical role in Ty2 survival. (Fig 22A). This was supported by nearly equal recovery of the wild-type and mutant strains when TLR2 was blocked by antibodies, suggesting that TLR2-mediated survival of STy in MoM cells was dependent on T4519 expression (Fig 22B).

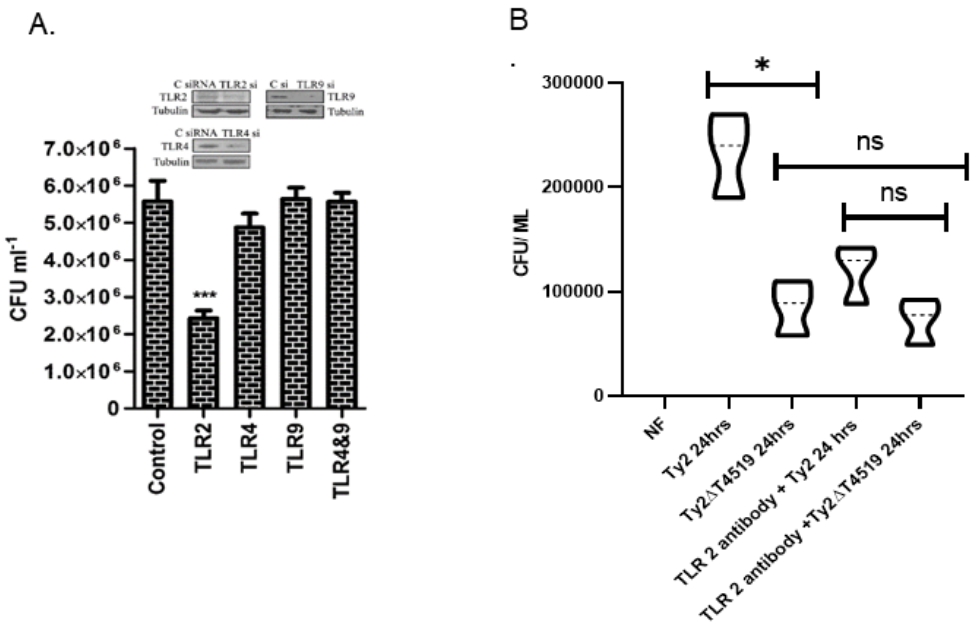

Fig 22. **TLR2 played a critical role for Ty2 survival** A. CFU of wild type bacteria after infecting TDM cells at 24 hours. TDM cells were treated with different si-RNA of that particular TLR to knock down. Knock down was tested by western blot. Tubulin was used as loading control. B. CFU of wild type and mutant bacteria at 24 hrs PI after lysing MoM cells. Error bars, means, standard deviations were done for three independent experiments, *** by students' T Test. All statistical analysis were done in Graph Pad-Prism 8.

To address if TLR2 transduced signals through Cystatin B-Cathepsin B-NF-kB--LMP pathway, cystatin B m-RNA expression was checked in the untreated and TLR2 antibody pre-treated MoM cells, infected with Ty2. Cystatin B downregulation was abolished in the presence of TLR-2 antibody, suggesting that cystatin B downregulation by T4519 was TLR2-mediated (Fig 23A). To probe the underlying mechanism of cystatin B downregulation, we checked for the regulation of gene silencer elements by TLR2 signals. A literature search revealed that out of several cystatin B gene silencers, TLR2 promoted the expression of three splicing factors, namely NOVA-1, SRSF3, and YB-1 through its interaction with the Wnt signaling pathway (Tredicine, Camponeschi et al. 2022)We studied the temporal profile of NOVA-1 and YB-1 m-RNA expression in Ty2-infected cells that showed a 3 to 7-fold increase of YB-1 m-RNA at 12 hrs P.I., but no changes in NOVA-1 expression. (Fig 23B-C). However, YB-1 expression dropped to a level similar to the non-infected cells if the cells were treated with TLR2 antibody before the infection (Fig 23C). These results suggested that T4519-induced cystatin B downregulation could be mediated by the induction of YB-1 downstream of TLR2 activation. Further, the blocking of TLR2 by antibodies resulted in the absence of active cytosolic cathepsin B (Fig 23D) as well as the abrogation of p65 nuclear translocation (Fig 23E) in the Ty2-infected cells. We studied by confocal microscopy if lysosomal damage induced by T4519 was regulated by TLR2 signals. We observed significantly reduced staining of the lysosomes when T4519 was expressed in the parental, but not in TLR2 knockdown cells, suggesting the involvement of TLR2 signals in lysosomal damage (Fig 23 F). This was further corroborated by the absence of Galectin-1 puncta in the cells blocked for TLR-2 signals, suggesting the absence of LMP after Ty2 infection (Fig 23G).

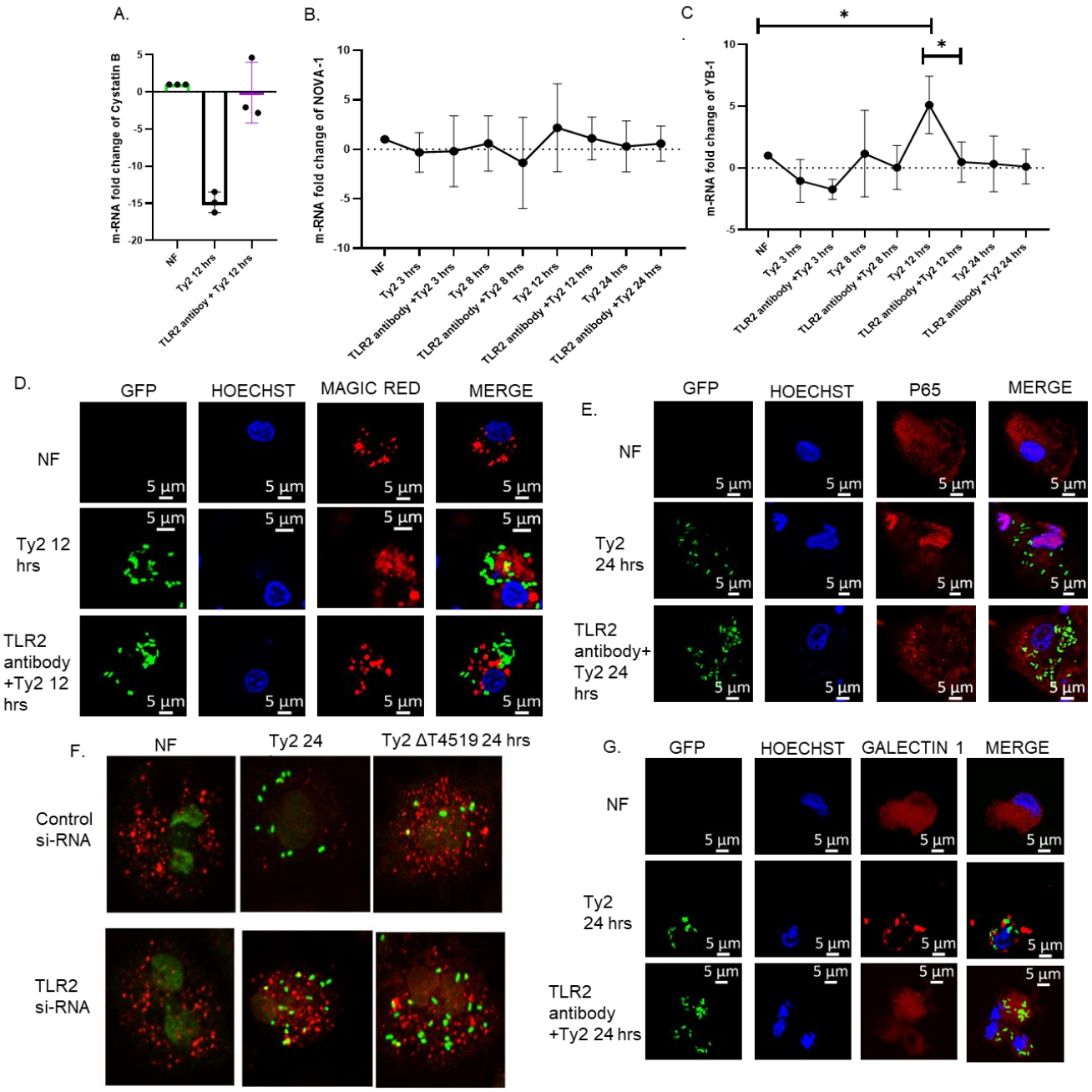

Fig 23. **TLR2 controls T4519 mediated Cystatin B-Cathepsin B-NF-kB-LMP axis.** MoM cells were incubated with TLR2 antibody for 5 hours followed by infection. A. m-rna expression change of cystatin B measured by rt-PCR after TLR2 antibody blocking. B-C. m-RNA expression was measured by RT-PCR after infecting the MoM cells with Ty2. B. m-RNA fold change of NOVA-1, C. m-RNA fold change of YB-1. D. Magic red staining in confocal microscope after infection by GFP tagged bacteria in TLR2 antibody treated MoM cells. E. p65 nuclear translocation study in confocal microscope after infection by GFP tagged bacteria in TLR2 antibody treated MoM cells. F. Lysotracker red staining after 24 hrs of infection in TDM cells. TDM cells were treated with TLR2 si-RNA to knock down TLR-2 before infection with GFP tagged bacteria. G. Galectin-1 antibody staining in confocal microscopy after infecting the cells with GFP tagged bacteria. A representative image from all experiments was shown. Confocal images were taken in Zeiss 63 X oil-immersion LSM 710 confocal microscope. Error bars represent SD. Significance was calculated using two tailed unpaired T test. Statistical analysis was done by using GraphPad Prism 8.

To study the interactions between T4519 and TLR2 in further detail, a 3D homology model of T4519 was constructed using the crystal structure of PknB (PDB ID: 1O6Y), a ser/thr kinase of Mycobacterium tuberculosis, bearing significant sequence homology to T4519 as the template (Theeya, Ta et al. 2015). The model structure conformed to the conserved internal architecture of a protein kinase(Kornev and Taylor 2010). The 3D model structure of T4519 is comprised of 6 anti-parallel, β sheets at the N terminal domain and 9 α helices at the C terminal end. ATP and substrate binding regions were present in between N and C terminal domains. (Fig 24A). The overall acceptable quality and compatibility of the 3D atomic model were suggested by the Ramachandran plot (90.6% of the residues in the allowed regions), 'Verify_3D' (score 81.58), and 'Errat' (score 90.661) (24B-C). After 5ns simulation, the resulting root mean square deviation (RMSD) plot showed that the 3D model was stable in aqueous medium at constant temperature and pressure (Fig 24D).

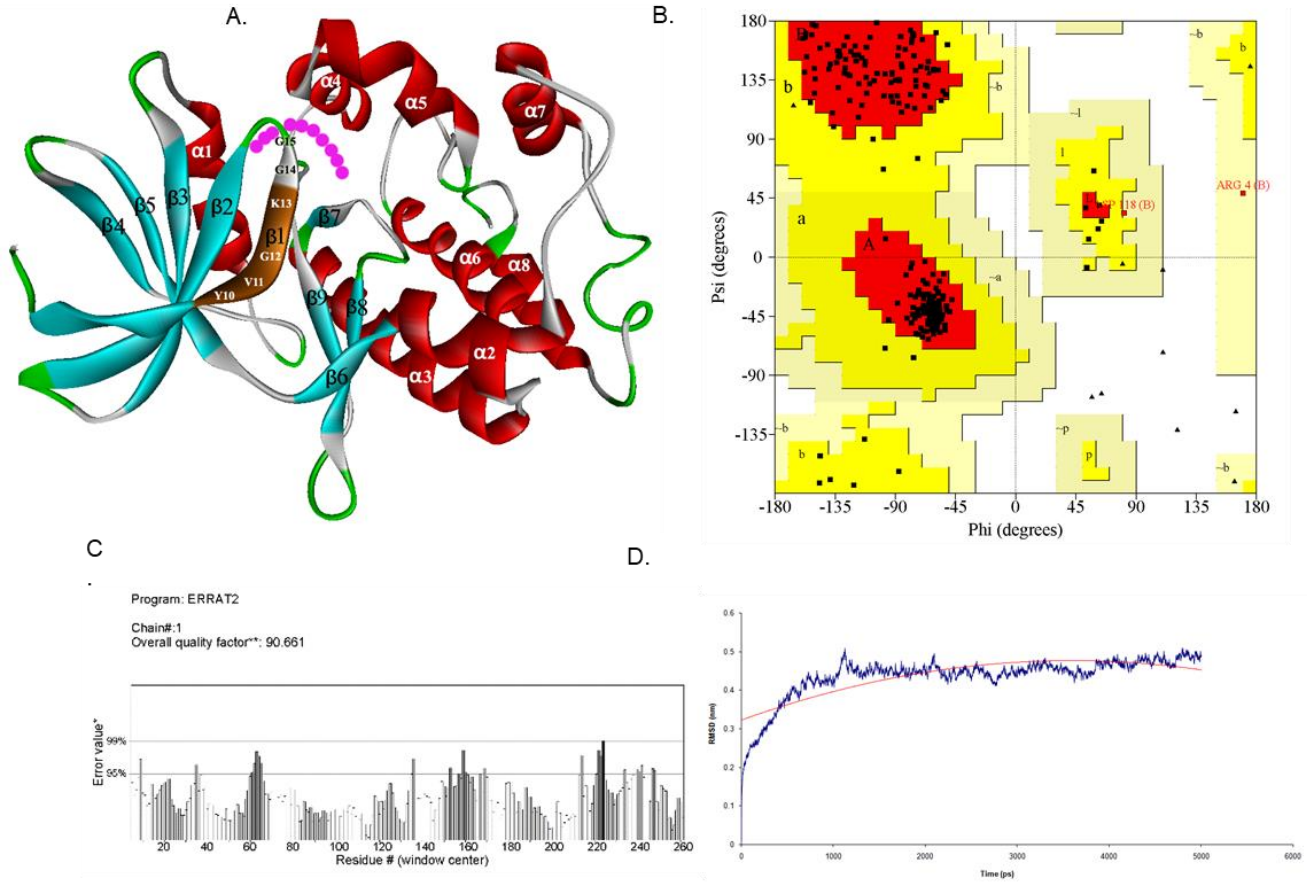

Fig 24. **T4519 acts as a TLR2 ligand. A**. Homology model of T4519 sowing substrate binding pocket (pink motifs) and the residues from glycine-rich loop (marked in brown and labeled in white) involved in H-bond

interaction with TLR2. B. Ramachandran plot of the 3D model structure of T4519. Ramachandran plot for the t4519 model structure shows 90.6% of the residues are in the allowed regions whereas 8.6% and 0.9% residues are observed in the additional and generously allowed regions, respectively C. ERRAT plot shows the statistics of non-bonded interactions between different atoms. The overall quality of the modeled protein structure is indicated by the score of 90.661 (Low resolution structures produce values around 91). D. 5 nanoseconds molecular dynamics simulation plot of T4519 showing relative deviation of the protein backbone atoms during simulation. Polynomial curve (Red) shows that the protein is quite stable after 2 ns as it fluctuates within the range of 0.4-0.5 nm

As per Mi Sun Jin.et. al. (2007), the tri-acylated lipopeptide induces the formation of an "m" shaped heterodimer of TLR1 and TLR2, but it was uncertain how relatively large protein ligands interact with the TLR2/1 heterodimer. Subsequently, there were efforts where researchers had identified the critical region of TLR2 which interacted with other bacterial proteins like PPE18 and LT-IIb-B5(Nair, Ramaswamy et al. 2009) (Liang, Hosur et al. 2009)which lacked acyl chains. In their work, they used different protein-protein docking programs to predict the most favorable binding site of TLR2 and afterward, they validated their result by site-directed mutagenesis.

Here we used a similar approach to find out the most favorable site of the kinase, binding to TLR2. We used ClusPro 2.0 (http://cluspro.bu.edu) protein-protein docking server for our prediction. The resulting 120 complexes were analyzed to get the most favorable binding pose of T4519 with TLR2. In the majority of these complexes (79 out of 120 conformations), the N terminal domain of the kinase bound to the border of the convex surface of the central domain of TLR2, which was similar to other TLR2 ligand proteins (Nair, Ramaswamy et al. 2009) (Liang, Hosur et al. 2009)Further analysis of these 79 conformations revealed that 62 of the interactions involved glycine-rich loop (12-GKGG-15) (Fig. 25A-B) of the kinase, which is known to be involved in the interaction with other proteins (Terasawa, Yoshimatsu et al. 2006) .Finally, to select the most favorable of the 62 interactions, we used three parameters, H-bond count, global energy value, and electrostatic charge complementarity. The final selected model consisted of 30 H-bond interactions with TLR2, involving the residues from

the lateral convex surface of the central domain and with a global energy value of -131.23, which was a measure of binding free energy of the complex. Interestingly we found that the hydrophobic core between the N and C terminal domain with the positively charged lining residues (K13,R90,H91,K125,R166,K207) and a deep cleft of negatively charged central residues (D123, D144, E77, E78) interacted mainly with the two loop regions (290-300 and 323-330) from the convex surface of TLR2 central domain that consists of positively charged hinge region (ARG321 at the tip of the loop), surrounded by negatively charged residues (D233, D235, D263, E264, D327) (Fig 25C-D). Moreover, these two loops from TLR2 provided a pedestal to the kinase hydrophobic surface (Fig 25E), giving rest to the complex.

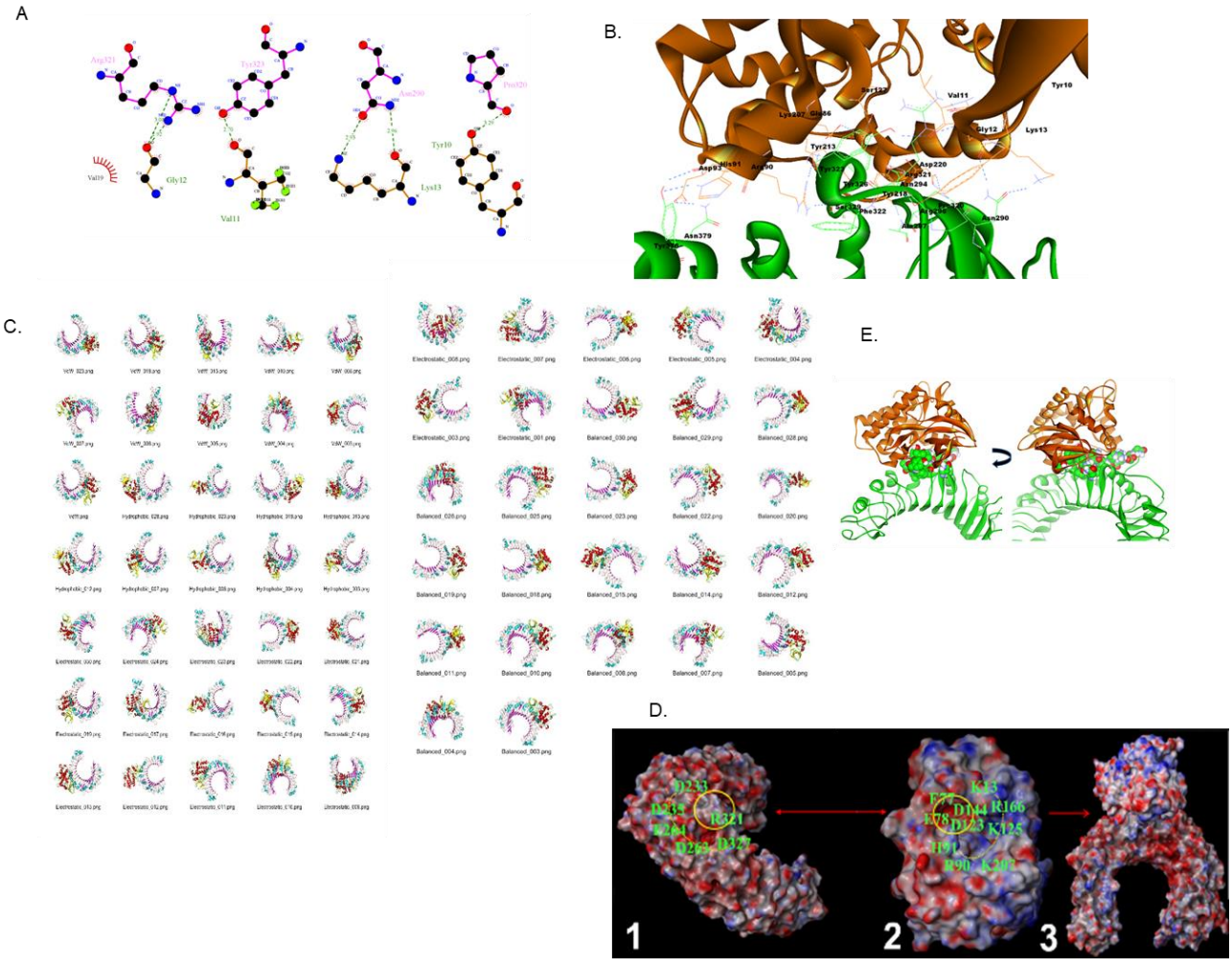

Fig 25. **T4519 acts as a TLR2 ligand.** A. H-bond interaction between glycine-rich loop of T4519 (brown) and TLR2 (pink). Hydrogen bonds are indicated by dashed green lines, with bond lengths shown in Å. B. Overall H-bond (blue) interactions between T4519 (brown) and TLR2 (green). C. 62 interactions between TLR2 and T4519 produced by ClusPro 2.0 docking server. Figure represents favored interactions, which include 11

Vanderwalls, 9 Hydrophobic, 22 Electrostatic and 20 balanced interactions. D. Electrostatic surface representation of TLR2(1), T4519(2) and the complex (3). Yellow circles represent positively charged hinge region of TLR2 (surface marked in blue) and negatively charged groove of T4519 (surface marked in red) and the corresponding amino acids are labeled. Yellow dotted lines represent the distribution of the corresponding charged residues. E. The cartoon representation of t4519 and TLR2 complex. The image showing two loops from TLR2 (in CPK) positioned between N and C terminal hydrophobic region of t4519.

To confirm that the glycine-rich loop (12-GKGG-15) of T4519 binds to TLR2, we generated mutant T4519 by site-directed mutagenesis of the glycine G12/A, G14/A) and lysine (K13/V) residues and purified the recombinant T4519 and rSDM-T4519 proteins. TLR2 immunoprecipitated from HEK293 cells that expressed TLR2/6 and co-receptor molecules and were stimulated with rT4519 or rSDM-T4519 showed significantly fewer interactions with the latter (Fig 26A). Finally, to study if TLR2-T4519 interaction was functionally relevant for intracellular survival of STy, MoM cells were infected with Ty2, Ty2ΔT4519, and Ty2 expressing the mutant T4519 gene (αSDM-T4519 Ty2). CFUs recovered after 24h were equal for both the mutant strains, but significantly less than the wild type bacteria. However, survival of all the above three strains was similar in TLR2 gene-silenced TDM cells, suggesting that the glycine-rich loop of T4519 was required to transduce survival signals through TLR2 (26B-C).

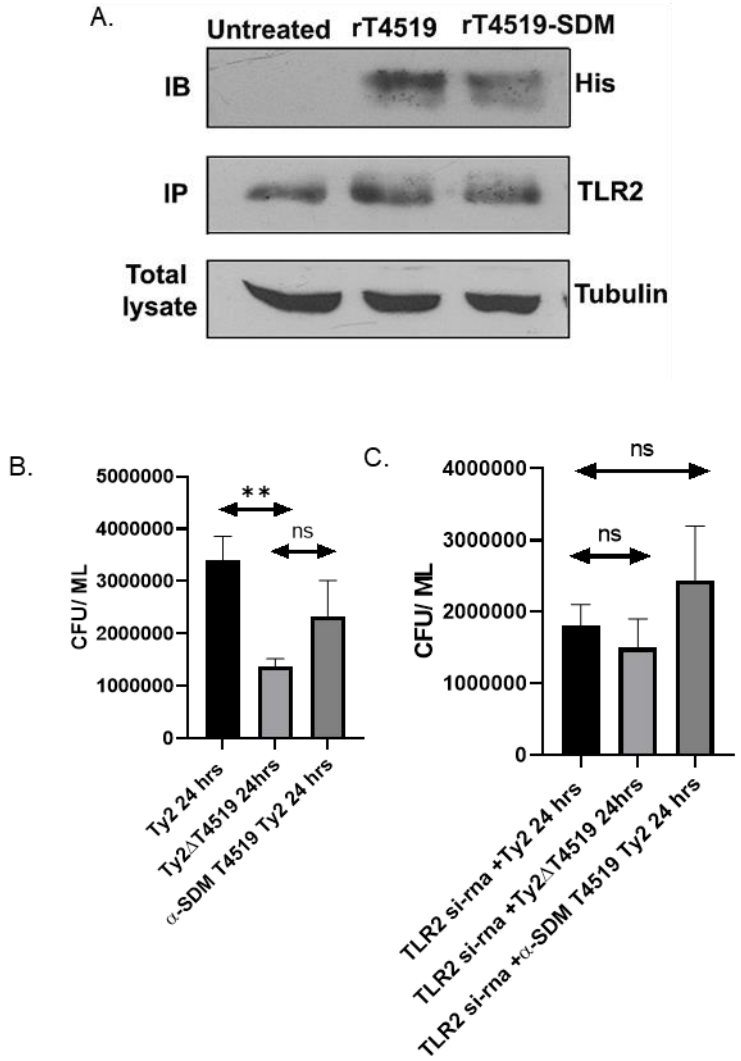

Fig 26. Glycine-rich loop (12-GKGG-15) of T4519 binds to TLR2 A. Stable expression TLR2/6 HEK293 cells were transiently transfected with TLR accessory proteins. Cells were stimulated with rT4519, rT4519-SDM (G12A/K13V/G14A) for 24 hrs and immunoprecipitation were done. B, C. Cells were infected with the indicated bacterial strains. CFU counts of intracellular bacteria recovered from the lysed TDM cells after 24 hrs were plotted.

Objective 3: - To study the redox biology of STy infected macrophages

1. STy increased intracellular ROS in human macrophages

ROS is an important modulator in host-pathogen response. When host macrophages encounter any foreign particles, they modulate the ROS levels to evoke the immune response. So, we measured the ROS levels in STy-infected macrophages. We infected the MoM cells with wild-type STy Ty2 bacteria and measured the intracellular ROS by Flow cytometer after staining the cells with DCFDA. Ty2 showed a significant increase in ROS production compared with control cells at 24 hrs PI. (Fig 27)

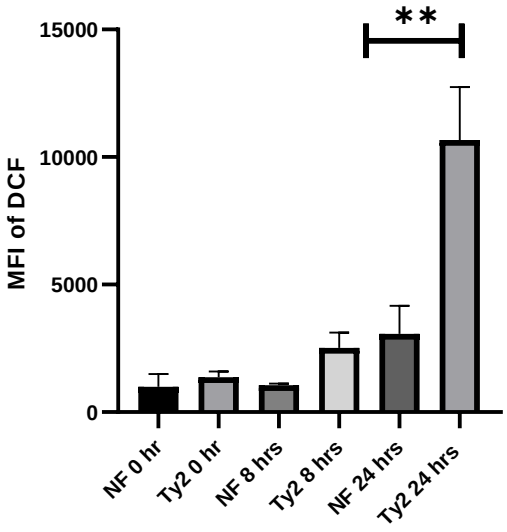

Fig 27. ***S*. Typhi increased intracellular ROS in human macrophages.** MoM cells were infected followed by staining with PBS containing 10 µM of CM-H_2DCFDA (Invitrogen) and FACS was done in FITC filter. NF stands for No Infection. Error bars represent SD. Significance was calculated using two tailed unpaired T test. Statistical analysis was done by using GraphPad Prism 8

2. ROS inhibition compromised the survival of the bacteria in MoM cells.

As Ty2 increased the bacterial survival in MoM cells, we wanted to check whether this increased ROS took any part in the bacterial survival or not. ROS was scavenged by NAC (1mM) for 3hrs. We infected MoM cells with wild-type STy and bacterial CFU was measured at 0 hr and 24 hrs PI after following the Gentamycin protection assay. Surprisingly the bacterial survival was hampered after ROS scavenging (Fig 28) which depicted the role of ROS in Ty2 survival.

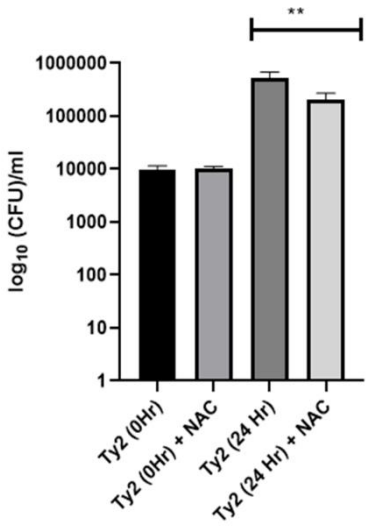

Fig 28. **ROS inhibition compromised the survival of the bacteria in MoM cells**. MoM cells were treated with NAC (1 mM) for 3 hrs. CFU of wild type bacteria at 0 hr and 24 hrs PI after lysing MoM cells. Error bars, means, standard deviations were done for three independent experiments, *** by students' T Test. All statistical analysis was done in Graph Pad-Prism 8.

3. T4519 activated ROS through TLR2-Cys B-Cat B-NF-κB- pathway and promotes LMP

Previous studies suggested T4519 helped in the survival of the bacteria (Theeya, Ta et al. 2015) and here we found ROS-mediated survival of the bacteria. So, we wanted to test, if T4519 promoted the ROS induction or not. We measured intracellular ROS in Ty2ΔT4519 bacteria-infected MoM cells and compared it with the ROS generation of wild-type bacteria-infected MoM cells. We found a decreased level of ROS production in the mutant bacteria-infected cells (Fig 29A-B). So, T4519 was the cause of increased intracellular ROS in MoM cells. ROS is known to cause LMP (Boya and Kroemer 2008) and was induced by T4519 in MoM cells. NF-Kb activation is another cause of ROS induction(Morgan and Liu 2011). We investigated if ROS production underlies LMP induction. Scavenging ROS 12 hrs post-infection completely inhibited LMP in Ty2-infected cells, but the effects were not observed when ROS was scavenged after 24 hrs (Fig 29C). Taken together, these findings

revealed ROS-mediated LMP by T4519 downstream cystatin B suppression, which may lead to the activation of the cathepsin B- NF-κB axis.

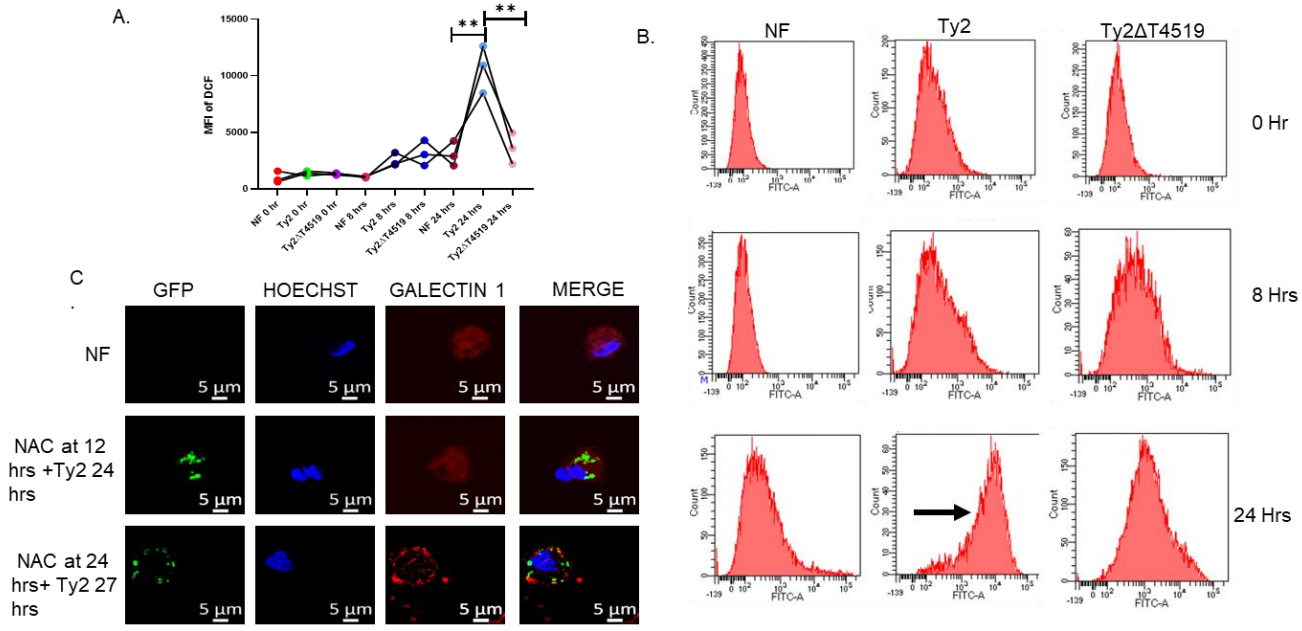

Fig 29. **T4519 activated ROS through TLR2-Cys B-Cat B-NF-κB- pathway and promotes LMP** .MoM cells were infected followed by staining with PBS containing 10 µM of CM-H_2DCFDA (Invitrogen) and FACS was done in FITC filter A. Measurement of ROS generation by flow cytometer (FACS Arya II, BD) in FITC channel in MoM cells after infection.NF stands for No Infection. B. Representative histogram images of FACS.The shift of MFI (Mean Fluorescent Intensity) of FITC peak was shown by an arrow. F. MoM cells, were infected and treated with NAC (1mM for 3hrs), followed by galectin-1 antibody staining like previously. Error bars represent SD. Significance was calculated using two tailed unpaired t test. Statistical analysis was done by using GraphPad Prism 8

4. NF-κB activation by cys B-cat B axis regulated pro-inflammatory response such as cytokines and ROS production

Previous studies indicated that intracellular bacteria may induce pro- or anti-inflammatory cytokines release from the infected macrophage cells. A recently published article reported replicating STy to preferentially choose pro-inflammatory M1 macrophages(Schade, Butler et al. 2024)**.** We investigated if Ty2 and Ty2ΔT4519 differentially stimulated pro-inflammatory cytokines in MoM cells. In contrast to rapid induction of cytokines by STm (Pietila, Veckman et al. 2005)**.** STy strains minimally induced TNF-α after 8h of infection. However, Ty2, but not Ty2ΔT4519 significantly augmented pro-inflammatory response such as TNF-α and ROS production after 24h (Fig 30A-B). This suggested that

T4519 contributed to late cytokine and ROS induction in STy-infected human macrophages. Next, we investigated if the pro-inflammatory mediator release might be regulated by cys B-cat B-IkB-α-NF-κB pathways, activated by T4519. We observed failure of cytokine surge and ROS production when cathepsin B activity was inhibited in Ty2-infected cells (Fig 30B-D) or p65 nuclear translocation was abrogated by SN-50 (Fig 30B-D).

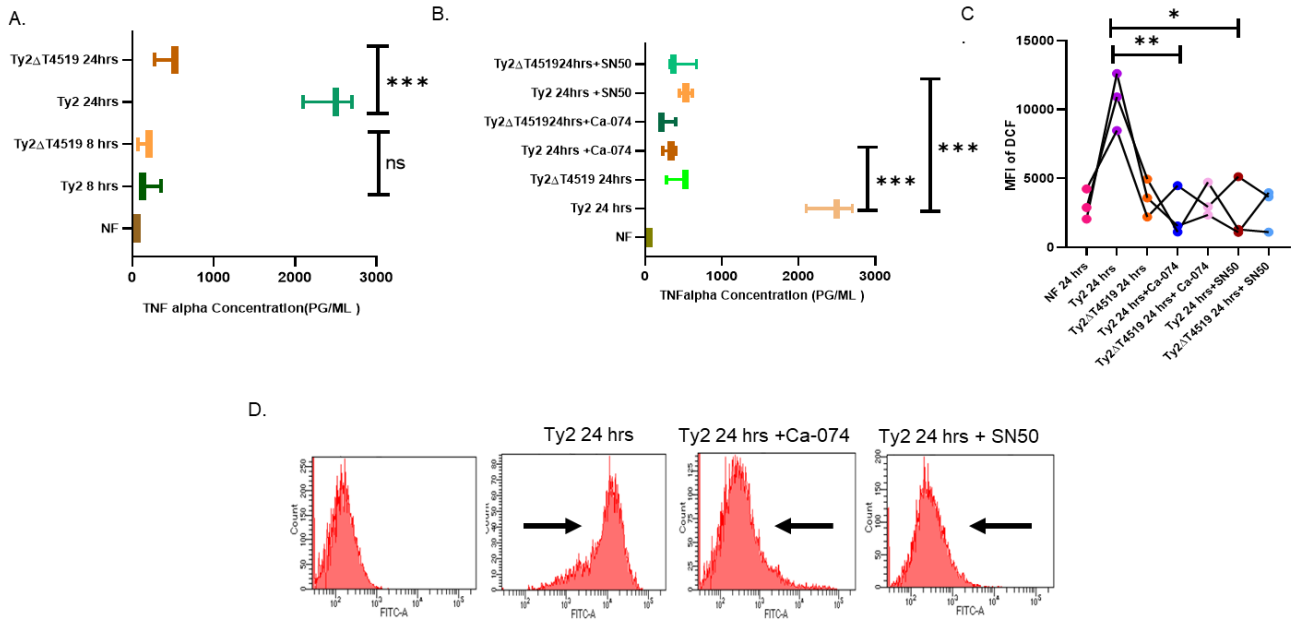

Fig 30. **NF-κB activation by cys B-cat B axis regulated pro-inflammatory response such as cytokines and ROS production**. MoM were incubated with indicated inhibitors followed by infection. The above experiments were repeated three times and the values from those three experiments were plotted. Error bars represent SD. Significance was calculated using two tailed unpaired t test. Statistical analysis was done by using GraphPad Prism 8. NF stands for not significant. A, B. ELISA to quantitate TNF-α concentration in MOM culture. C-D. Measurement of Ros generation by flow cytometer (FACS Arya II, BD) in FITC channel in MoM cells.

In contrast, TNF-α and ROS were also induced in the mutant bacteria infected cells when cystatin B expression was silenced (Fig 31A-C), indicating a crucial role for cystatin B downregulation-dependent cathepsin B activation, leading to IκB- α cleavage and NF-κB nuclear translocation in the regulation of TNF-α and ROS production after 24 hrs of Ty2 infection.

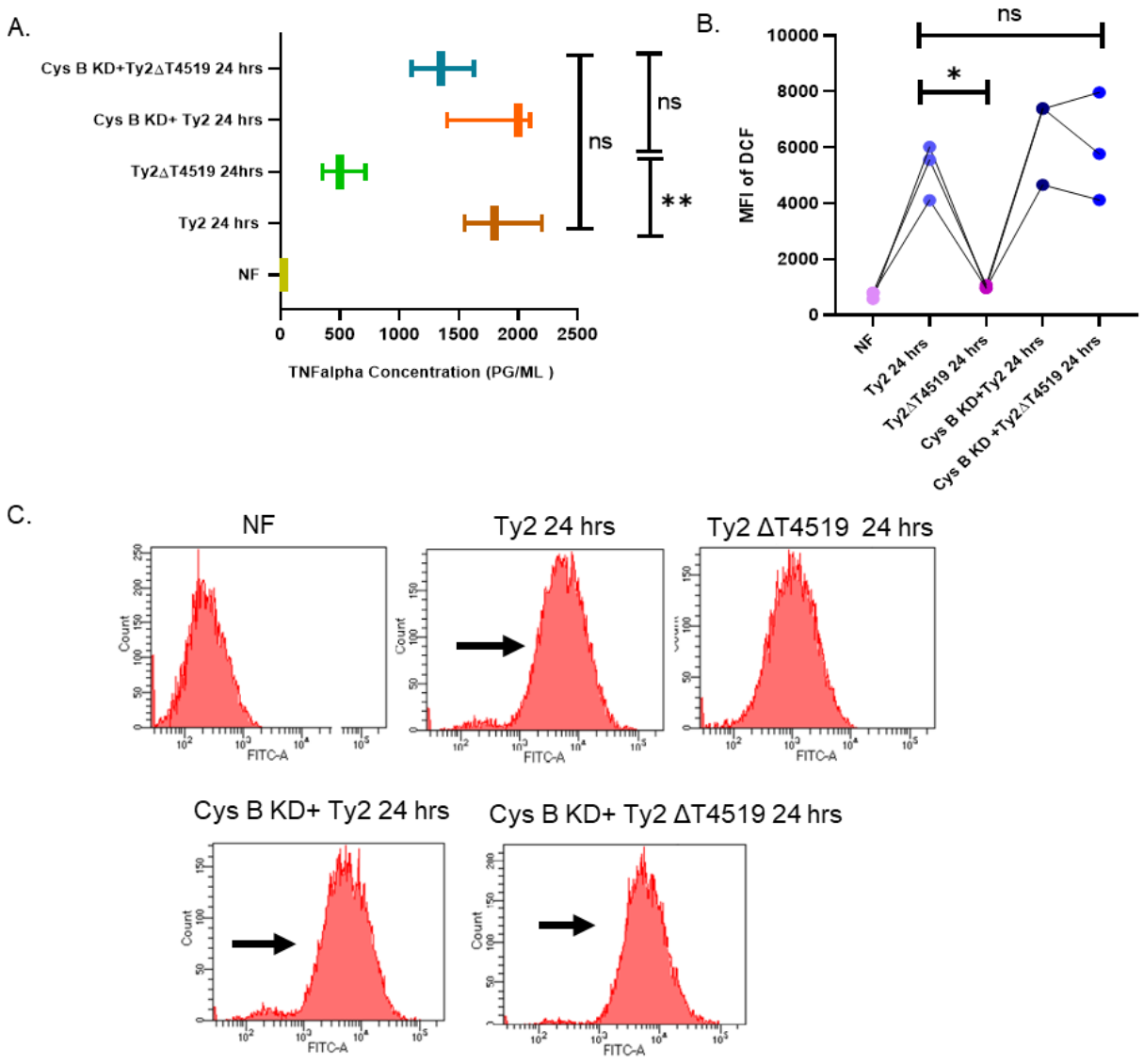

Fig 31. **NF-κB activation by cys B-cat B axis regulated pro-inflammatory response such as cytokines and ROS production**. MoM were incubated with indicated inhibitors followed by infection. The above experiments were repeated three times and the values from those three experiments were plotted. Error bars represent SD. Significance was calculated using two tailed unpaired t test. Statistical analysis was done by using GraphPad Prism 8. NF stands for not significant A. ELISA to quantitate TNF-α concentration in TDM culture P**= 0.0036 compared between Ty2 24 hrs and ΔT4519Ty2 24 hrs B-C. FACS to quantitate ROS generation in TDM culture, F. Here, P**= 0.0018 compared between Ty2 24 hrs and ΔT4519Ty2 24 hrs.

5. T4519 activated TLR2-Cystatin B-Cathepsin B-NF-κB-ROS pathway promotes bacterial survival in MoM cells.

Since T4519 promoted survival of STy Ty2 in the MoM cells (Fig 10A), we investigated if the regulation of cystatin B-cathepsin B-NF-κB axis by T4519 was responsible for enhanced bacterial survival. Early on (0h time point after infection), PBMCs pre-treated with different inhibitor molecules did not change intracellular bacterial counts compared with the inhibitor-untreated cells, indicating that phagocytosis was unaffected by the inhibitor treatment (Fig 32A). In contrast, significantly fewer intracellular bacteria were recovered from the cells, which were pre-treated with the cathepsin B or NF-κB nuclear translocation inhibitors 24h post-infection of Ty2. However, no changes in the intracellular CFU counts were found after pre-treatment with proteasome inhibitor, suggesting that cathepsin B-dependent, but proteasome-independent IκB-α degradation and NF-κB nuclear translocation augmented survival of STy in the MoM cells (Fig 32B). The mutant bacteria showed significantly reduced CFU counts than Ty2, both in the presence and absence of inhibitors (Fig 32B), suggesting the role of T4519 in activating the cathepsin B-NF-κB axis (Fig 32B). Ros scavenging after 10 hours inhibited survival promotion by T4519 at 24 hrs PI, depicting ROS-mediated survival of the bacteria (Fig 32B). Further, silencing of the Cystatin B gene equalized the intracellular survival of Ty2 and Ty2ΔT4519, whereas prior inhibition of cathepsin B in Cystatin B gene-silenced cells significantly reduced the survival of both strains (Fig 32C). Together the above results underscored the role of T4519 in the survival of STy in human macrophage cells through the engagement of TLR2 by cystatin B downregulation, leading to the activation of cathepsin B-NF-κB axis and finally ROS generation, independent of proteasomal degradation of IκB-α.

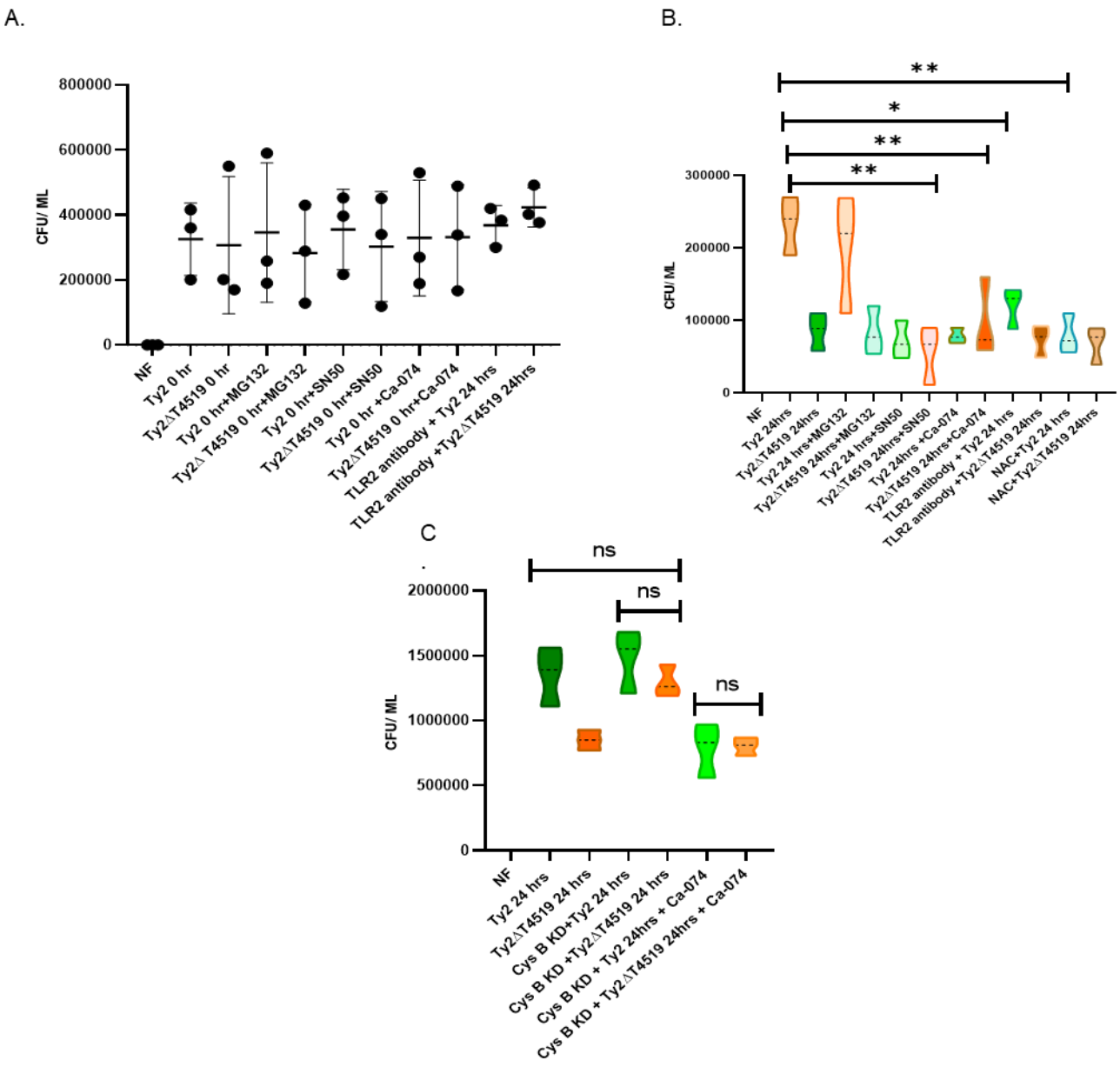

Fig 32**. T4519 promotes intracellular survival through TLR2- cysB- Cat B- NFκB- ROS pathway**. A-C. PBMC derived macrophages were infected with wild type *S.* Typhi or Ty2ΔT4519 for 30 minutes followed by gentamycin protection assay as described under Materials and Methods. Here NF stands for no Infection The above experiments were repeated three times and the values from those three experiments were plotted. Ns stands for not significant. CFU determined T4519 mediated survival in MoM cells. A. Bacterial CFU at 0 hr time point after infection B. Intracellular bacterial CFU were counted after 24 hours of the infection following cell lysis and plating the lysate on LA plates, which were incubated overnight in at 37° C. C. Intracellular bacterial CFU determined after lysing the TDM cells. Cystatin B KD was done in TDM cells by cystatin B si-RNA pretreatment.

Chapter 5

General Discussion

Discussion: -

After phagocytosis in macrophages, *Salmonella* resides in a modified phagosome-like compartment termed as *Salmonella-containing* vacuole (SCV). This SCV gradually acquires markers of early endosome and then late endosome and finally, it fuses with host cell lysosome. Lysosomal hydrolases efficiently kill the bacteria. To evade lysosomal hydrolases, bacteria employ several mechanisms. It is known that T3SS II effector protein SifA of *Salmonella* Typhimurium hampers the retrograde trafficking of mannose-6-phosphate receptors (MPRs) from late endosomes and lysosomes to the trans-Golgi network in macrophages. So, the lysosomes are formed with a reduced potency(McGourty, Thurston et al. 2012). In our case, we found reduced Lamp1 and reduced Lysotracker staining after 24 hrs of Ty2 infection (Fig 1) denoting a reduction in the lysosome in human macrophages. The lysosomal reduction was caused by STy protein T4519 which also decreased phagosomal acidification. (Fig 2). To investigate the mechanism of T4519-mediated lysosomal reduction, we have encountered lysosomal membrane rupture by STy protein T4519. Released dextran all over the cytoplasm (Fig 5, 6) clearly showed the damage to the lysosomes. As previous reports postulated about the deficiency of the lysosomal hydrolases and/or problems in the phagosomal acidification, our study first discovered the lysosomal damage caused by STy protein T4519. Lysosomal hydrolases are powerful bactericidal agents and hence, LMP leading to loss of lysosomal enzymes has remained an important immune evasion mechanism for bacterial pathogens. *B. anthracis* lethal toxin induces LMP through activation of NLR signaling, leading to apoptosis (Averette, Pratt et al. 2009). In our case also, the LMP releases active cathepsin B in the cytosol followed by apoptosis (Fig 7). The purified protein rT4519 is also able to cause LMP. (Fig 9)

NF-κB is a key molecule modulated by several intracellular bacteria for manipulating host cell immune response. Most intracellular pathogens inhibit NF-κB signaling, either by acting upstream of IKKs or by directly targeting the core signalling components of the pathway. This prevents the elicitation of pro-inflammatory response, favouring bacterial survival and growth. Many prokaryotic ser/thr kinases

were found to play a critical role in regulating this response, although an opposite function, leading to the activation of NF-κB and induction of pro-inflammatory cytokines and ROS was also observed for some eSTKs. *L. pneumophilia* LegK1 stimulates canonical NF-κB activation through direct phosphorylates and degradation of IκB-α (Ge, Xu et al. 2009)**.** In addition, it also activates the non-canonical NF-κB pathway by targeting another IκB family inhibitor, p100, and processing it to generate p52 (NF-κB2). LegK1 functionally replaces host IKKs by targeting the same motif in the IκB family of proteins, but unlike IKKs, this function was independent of the upstream adaptor molecules (TRAF2, TRAF6) and kinases, (TAK1, NIK and MEKK3) (Ge, Xu et al. 2009)**.** NF-κB activation by LegK1 was reported to delay macrophage apoptosis, allowing more time for *Legionella* to replicate. NF-κB activation through TLR-2 by Mycobacterium protein EsxL contributes to TNF-α and IL-6 pro-inflammatory cytokine production in murine macrophages. However, whether this protein had any role in bacterial survival was not investigated(Pattanaik, Ganguli et al. 2021). Studies have shown that STm flagellin FliC and FljB were able to activate NF-κB(Simon and Samuel 2007) and *Salmonella* induced Map Kinase activation can lead to NF-κB activation (Hobbie, Chen et al. 1997) whereas, NF-κB activation by *Salmonella* in epithelial cells was mediated by calcium signaling (Gewirtz, Rao et al. 2000). Virulence protein SrfA of STm dissociated IL-1R associated kinase (IRAK-1) to promote its phosphorylation and NF-κB activation in macrophages. This subsequently led to inflammatory cytokine production such as IL-8, IL-1β, and TNF-α. When SrfA is deleted, no effect is found regarding bacterial replication but NF-κB activation was hampered (Lei, Wang, et al. 2016). The temporal dynamics of NF-κB response to *Bordetella pertussis* filamentous haemagglutinin (FHA) includes an early pathway activation, leading to pro-inflammatory cytokines production and inhibition of NF-κB upon longer exposure to FHA (Abramson, Kedem et al. 2008)**.** In contrast, obligate intracellular bacterial pathogen, *Rickettsia rickettsii* prolongs macrophage survival by inducing biphasic NF-κB activation in cultured human endothelial cells (ECs) – an early, transient phase at 3h and a late, sustained response from 18 to 24 h(Sporn, Sahni et al. 1997)**.** The early phase activation

requires both IKKα and IKKβ, while the late response was IKKβ- and proteasome-independent and perhaps required a rickettsial or a host cell protease-mediated IκB-α degradation (Clifton, Rydkina et al. 2005) (Clifton, Goss et al. 1998, Haenssler and Isberg 2011).

Here we also report biphasic NF-κB activation in STy-infected MoM cells, where the early phase was proteasome-dependent, but independent of T4519 (Fig 10). In contrast, late NF-κB activation was induced by T4519 and mediated by proteasome-independent IκB degradation. A recent study suggested that multiple SPI-2 effectors of STm cooperate to inhibit NF-κB activation and their absence in STy, especially the lack of GogA and GtgA results in p65 phosphorylation in the infected Thp-1 macrophages Deletion of these effectors cause less virulence in mice (Stepien, Singletary et al. 2024). However, it is well established that NF-κB p65 gets phosphorylated at multiple sites, often by different upstream kinases after its release from the IkB complex. However, the above study did not identify the phosphorylation site(s) or the kinases involved and whether NF-κB phosphorylation followed proteasome-dependent or independent IκB-α degradation. Other reported studies identified deubiquitylation of IκB-α by STm T3SS-2 effectors, AvrA and SseL being responsible for the inhibition of NF-κB activation(Le Negrate, Faustin et al. 2008),(Yin, Liu et al. 2020).

In search of the lysosomal and endosomal genes that could be the main survival targets of the bacteria to reside inside vacuoles of human macrophages, we found cathepsin B gene upregulation in STy infected cells (fig). Cathepsin B was implicated in the proteasome-independent, alternate pathway of NF-κB activation by several studies(de Mingo, de Gregorio et al. 2016) (Ni, Wu et al. 2015)Another report suggested that cathepsin B caused prolonged activation of NF-κB in fibroblasts as inhibition of cathepsin B by ca-074 hampered IκBα degradation (Li, Wu et al. 2016). Following hypoxia/ischemia, NF-κB activation and pro-inflammatory cytokine and ROS induction in microglia/macrophages were mediated by Cathepsin B-dependent autophagic degradation of IκB-α that was unaffected by proteasome inhibitor. That IκB is a substrate of Cathepsin B was proved by the digestion of recombinant IκB-α in an *in vitro* assay (Ni, Wu et al. 2015). We also observed proteasome-

independent, but Cathepsin B-dependent IκB-α degradation (Fig 13) and pro-inflammatory cytokine induction (Fig 30) in STy-infected MoM cells. Cathepsin B inhibition also decreased p65 nuclear translocation (Fig 13) and reduced the survival rate of bacteria to become like ΔT4519 infection (Fig 32). T4519 was controlling cathepsin B expression to activate it so that NF-κB activation could happen. A previous study suggested that Cathepsin B promotes hepatic inflammation through NF-κB activation by proteolytic cleavage of Sirt1, a nuclear deacetylase that inhibits NF-κB transactivation capacity by deacetylating lysine310 residue(de Mingo, de Gregorio et al. 2016)**.** However, Sirt1 played no role in Cathepsin B-dependent NF-κB activation in STy-infected cells in our study.

We detected in the cytosol of MoM cells infected with both Ty2 and Ty2ΔT4519 enzymatically inactive Cathepsin B (Fig 14), which was subsequently activated only in the Ty2-infected cells. This correlated with T4519-dependent downregulation of the default Cathepsin B inhibitor, Cystatin B expression (Fig 15). As cystatins were the main cytosolic inhibitors of cathepsins, precisely cystatin B and cystatin C inhibit cathepsin B, and expression levels of both cystatins are measured (Fig 15). Cystatin B was found to be downregulated by T4519 to activate cytosolic cathepsin B (Fig 15) Infection of human oral epithelial cells with *P. gingivalis* or stimulation with bacterial LPS resulted in a dose-dependent increase of Cathepsin B and reduction in Cystatin C expression levels. However, LPS effects were delayed compared with the whole bacteria, suggesting the involvement of additional bacterial molecules(Elkaim, Werner et al. 2008)**.** In contrast, T4519 was solely responsible for Cystatin B downregulation (Fig15) and the activation of Cathepsin B-NF-κB axis, promoting survival of STy in MoM cells (Fig 32). Several reports have already discovered the antimicrobial and anti-inflammatory roles of several cystatins or derivatives of cystatins (Pikula, Smuzynska et al. 2017) (Blancas-Luciano, Becker-Fauser et al. 2022) (Naito, Sasaki et al. 1995) but controversial results were also found in which cystatin C downregulation improved the intracellular killing of *Mycobacterium tuberculosis* inside macrophages(Pires, Calado et al. 2021). In our case, cystatin B KD cells, infected with Ty2ΔT4519 showed a similar kind of NF-κB activation (Fig 16) compared with

wild-type bacteria and showed an increased rate of bacterial survival in human macrophages (Fig 32). Downregulation of cystatin B by T4519 in STy caused cytosolic cathepsin B activation followed by NF-κB activation which contributed the bacterial survival in human macrophages. Other studies reported decreased interactions of Cathepsin B with Cystatin B and Cystatin C in HIV-1 infected MoM cells, leading to increased Cathepsin B activity(Rodriguez-Franco, Cantres-Rosario et al. 2012). However, we found no changes in Cystatin C expression after Ty2 infection (Fig 15 C-D), while we did not check Cathepsin B interaction with the inhibitor cystatins. Cystatins also exert direct antimicrobial functions, such as those purified from chicken egg white and A20 peptide, mimicking the N-terminus of human cystatin C act against several pathogenic bacteria, including *E. coli*, Acinetobacter and MRSA (Moreau, Gautron et al. 2022) (Ferguson, Komenda et al. 2015). However, the antibacterial functions of cystatin B against STy in human macrophages were primarily mediated through the inhibition of Cathepsin B. This corroborates with the studies that reported increased resistance of mice and macrophages, lacking Cathepsin B against *Francisella novicida* (Qi, Man et al. 2016). Also, macrophages lacking cathepsin B do not support productive intracellular replication of *L. pneumophila* harboring wild-type RpsL, which induces cell death via LMP (Zhu, Tao et al. 2015). Cathepsin B functions as a negative regulator of lysosomal biogenesis and autophagy through mTor-mediated cleavage of lysosomal Ca channel TRPML1, suppressing TEFB (Qi, Man et al. 2016).

Recent studies suggested that intracellular persistence and growth of STy is promoted by NF-κB activation that prevents death of infected macrophages (Stepien, Singletary et al. 2024). This observation corroborates with another recent report that documented M1 macrophages to house the replicating STy, since NF-κB polarizes macrophages to the M1 phenotype and remains the major transcription factor in these cells (Schade, Butler et al. 2024). However, NF-κB, at the same time is a key molecule that orchestrates the antibacterial immune response of macrophages. It is currently unknown if STy could directly exploit NF-κB signaling pathways to boost its intracellular survival. We found here that STy induced LMP through the Cystatin B-Cathepsin B-NF-κB pathways in a

T4519-dependent way, leading to reduced numbers of acidic vesicles in the infected cells and an increase in intracellular pH (Fig 17,18,19,20,21). Reports reveal sphingosine-mediated LMP is cathepsin B dependent. Here TNF-α induction causes a change in the ceramide concentration which is responsible for LMP. The process is dependent on cathepsin B. (Werneburg, Guicciardi et al. 2002, Ullio, Casas et al. 2012). In our study, the downregulation of cystatin B by T4519 causes Cathepsin B activation in the cytoplasm which activates NF-KB to cause LMP**.**

Toll-like receptors recognize pathogen-associated molecular patterns to promote innate immunity. In general, TLR2 plays a more important role in protection against Gram-positive bacteria, while TLR4 for the Gram-negative species. However, several bacterial pathogens have evolved mechanisms to exploit TLR signals to augment their virulence. Yersinia spp. LcrV induces TLR2-mediated immunosuppression and a point mutation in the TLR2-binding epitope of LcrV abrogates interactions and decreases virulence (Sing, Reithmeier-Rost et al. 2005)**.** *S.* aureus prolongs its own survival within the phagosomes of macrophages by inhibiting the generation of superoxides other than NO through TLR2-dependent JNK activation (Watanabe, Ichiki et al. 2007) **.** While TLR2 plays a critical role in the early-stage macrophage defense against Mycobacterium tuberculosis by suppressing proliferation, by direct bactericidal effects or induction of apoptosis, it may help Mtb in the later stages to escape recognition by inhibiting MHC II expression or inducing IL10 and IL-4 using the bacterial 19 kDa glycolipoprotein (p19), which is both cell wall-associated and secreted and a candidate virulence factor (Yoshida, Inagawa et al. 2009)**.** We show here that T4519 signals through TLR2 to suppress cystatin B expression, allowing enzymatically active cytosolic cathepsin B to induce LMP by the activation of NF-κB- pathway (Fig 22, 23). On the other hand, *T. denticola* antimicrobial peptide in the gingival epithelial cells. TLR2 knockout mice have decreased pathology and increased survival compared with the wild-type mice from sepsis caused by *Burkholderia pseudomellei*, suggesting that TLR2 mediates morbidity and mortality in murine models of this pathogen (Wiersinga, Wieland et al. 2007). On the other hand, penumolysin selectively induces the loss of lysosomal acids (LLA)/ LMP, leaking smaller

molecules (up to 40 kDa) into the cytosol. This is mediated through multiple TLR activation, especially TLR2, and TLR4, but independent of the pore-forming ability of pneumolysin and does not induce cell death (Bewley, Naughton et al. 2014). In contrast, T4519 exclusively used TLR2 to induce LMP, leading to a marked loss of lysosomal staining and increased bacterial survival (Fig 22,23, 32). TLR-mediated augmentation of phagosomal persistence was also reported for STm, where TLR2, 4, and 9 together were found to provide cues to the bacteria to sense the phagosome and regulate virulence genes required for intracellular survival and replication (Arpaia, Godec et al. 2011). *Brucella suis* LPS, a ligand for TLR4 may inhibit phagosome-lysosome fusion (Porte, Naroeni et al. 2003).

ROS production by macrophage helps to clear bacterial infection, but excessive ROS generation, especially within lysosomes can trigger LMP through multiple mechanisms, including lysosomal free iron-dependent H_2O_2 and hydroxyl radical generation, membrane phospholipase A_2 degradation, etc(Boya and Kroemer 2008). ROS and NF-κB have reciprocal regulation where one can induce the other (Morgan and Liu 2011). NF-κB induces ROS by activating NADPH oxidase, NOX2, and upregulation of iNOS (Morgan and Liu 2011). We observed a surge in ROS production in Ty2-infected macrophages by a novel, T4519-activated Cathepsin B-NF-κB pathway (Fig 29) and ROS-mediated LMP (Fig 29), promoting phagosomal persistence of STy (Fig 32). To the best of our knowledge, this is the first study that demonstrated a direct role of NF-κB in promoting phagosomal survival of intracellular bacteria and inducing LMP.

In conclusion, we report here a novel mechanism of cytosolic cathepsin B-mediated NF-κB activation by STy that promotes bacterial survival within human macrophages. T4519, a bacterial eSTK indirectly activated cathepsin B by downregulating the expression of its inhibitor molecule, cystatin B in a TLR2-dependent way. This led to a surge of ROS production by NF-κB, leading to ROS-induced LMP.

There are a few limitations of the study. We did not directly demonstrate T4519 and TLR2 interaction in the infected macrophages. Instead, we showed this interaction by co-immunoprecipitation

experiment in TLR2 over-expressed 293 cells and purified T4519 protein. However, this is the first study in which we are going to state a novel survival strategy of STy by rupturing lysosomes which involves several other important host molecules like cystatin B, Cathepsin B, NF-κB, and Mtor. More precisely, this study also identifies the factor, T4519 a serine-threonine kinase of STy which is responsible for operating the entire pathway.

Chapter 6

L form *Salmonella* Typhi can infect and survive inside murine and human macrophages

6. Introduction: -

Antibiotics are widely used to treat different kinds of bacterial infections. Ampicillin belongs to the first line of antibiotics. While administered inside the body of a patient ampicillin increases in concentration slowly over time. So, the bacterial population is exposed to an increasing concentration gradient of ampicillin. In this study, we exposed wild-type ampicillin-sensitive STy (Ty2) strain to an increasing ampicillin concentration over a period of 14 days. Due to this exposure, the Ty2 strain exhibits different properties of L form *Salmonella*. In this study, the pathogenesis of these L forms of *Salmonella* has also been studied. L form has been found to infect murine and human macrophage cell lines more efficiently than wild-type Ty2. However, in the mice model, they show infection with lesser efficiency than WT when administered orally. These results show that L form *Salmonella* can efficiently infect, survive, and replicate inside macrophage cells but its efficiency fails to cross the gut immune barrier decreases as compared to WT. Around 11 to 12 million cases of typhoid fever have been reported worldwide. Multi-drug resistance strains of STy have been reported for several decades from different parts of the globe. STy was initially treated with Ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole (Rowe, Ward et al. 1997). The appearance of multidrug resistance to these three first lines of antibiotics at around late 1980 to 1990s led to ciprofloxacin as the drug of choice to treat typhoid fever. In 1991 STy strain resistant to ciprofloxacin appeared and resulted in the 1997 outbreak (Threlfall, Ward et al. 1999). After that, macrolides, third-generation cephalosporin, and carbapenems have been used extensively against STy. In 2018 and 2019, extreme drug-resistant STy strains which are resistant to macrolide, fluoroquinolones, and third generation of cephalosporin emerged at Hyderabad (Qamar, Yousafzai et al. 2018, Yousafzai, Qamar et al. 2019), India and Pakistan respectively. The World Health Organization (WHO) has reported a significant number of infections with extensively drug-resistant (XDR) STy strains in Pakistan and travel-associated cases in several countries, including China, the USA, Australia, Denmark, and Canada, posing a global health threat.

However, recent studies in India have revealed a promising trend: the reemergence of ampicillin-sensitive STy strains. Several investigations have reported a decrease in the minimum inhibitory concentration (MIC) of ampicillin for various Indian STy strains (Krishnan, Stalin et al. 2009). A notable study by Harish et al. (2011) found that ampicillin-resistant strains in Chennai decreased from 52% to 23% between 2002 and 2009. These findings suggest that ampicillin-sensitive STy strains may be reemerging, potentially making ampicillin a viable treatment option for typhoid fever. The emergence of antibiotic-resistant strains was initially thought to be plasmid-borne. Genome sequencing of the Ct18 strain and other ampicillin-resistant STy strains revealed the presence of the Salmonella-associated transferable R plasmid, pHCM1 (Parkhill, Dougan et al. 2001). This plasmid confers resistance to multiple first-line antibiotics. Ampicillin resistance can also be conferred temporarily through the formation of L-form bacteria. L-form bacteria overcome the effects of ampicillin by shutting down cell wall production. First discovered in 1935, L-form bacteria have been the subject of several studies. However, most of these investigations were conducted before the molecular biology era, resulting in a limited understanding of L-form biology. Recent work by Errington and his group has significantly contributed to our understanding of L-form transition in B. subtilis (Kawai, Mercier et al. 2015), (Kawai, Mercier et al. 2019). Their research has shown that knocking out cell wall machinery (MurE operon) is insufficient to induce stable L-form growth. In addition to MurE knocking out a mutation in ispA or any glycolytic enzymes or any enzymes participating in the Respiratory chain is absolutely necessary for the L form to survive (Kawai, Mercier et al. 2019).

Salmonella has also been reported to undergo L form transition in the presence of cell wall targeting drugs. A recent study with STm showed that L form can readily be induced by using ciprothoxanone (Yang, Li et al. 2020). These L forms of STm had also been shown to be resistant to third-generation cephalosporin drugs (Yang, Li et al. 2020). L form *Salmonella* has also been responsible for the long-term effect of attenuated bacterial vaccines indicating that L forms are non-pathogenic in nature (Kita,

Nishikawa et al. 1992).On the contrary, in the 1970s several works proved that L form *Salmonella* indeed can cause diseases. However, most of these studies were either not in English or not available in the databases. So complete knowledge about the pathogenesis of L form *Salmonella* remains obscured.

In this study, we induce the formation of STy L form by treating an ampicillin-sensitive strain (Ty2) with an increasing concentration of ampicillin over a period of 14 days. This exposure of a sensitive STy strain to an increasing concentration gradient of ampicillin would mimic the real scenario of antibiotic treatment of typhoid patients. We characterize the L form *Salmonella* and study whether it can infect murine and human macrophage cell lines. Finally, we tested whether these L forms of STy can show infection in mice models. This study, for the first time, explores the pathogenesis of ampicillin-induced L form STy in great detail.

6.1 Rationality of the Study: -

The study investigates the ability of L-form STy to infect and survive within macrophages. This research has significant implications for understanding STy's pathogenesis and developing effective treatments.

Relevance to STy Survival in Macrophages-

L-form bacteria lack a cell wall, which helps them evade antibiotic treatments and immune responses. This is also a survival mechanism of STy to potentially sustain within macrophages as reservoirs. The study sheds light on L-form STy being cell wall-less, infects macrophages, potentially exploiting host cell processes. From this study, researchers can identify mechanisms enabling L-form STy to survive within macrophages, informing novel therapeutic strategies. This study can reveal insights into the complex interactions between STy and host immune cells, particularly macrophages.

6.2. Materials and Method:-

6.2.1. Cells and reagents.

Cell lines and cell culture reagents were procured from the American Type Culture Collection (ATCC) and Invitrogen, respectively. Bacterial culture media were purchased from BD Difco.

6.2.2. Bacterial culture.

E. coli DH5α, transformed with pAKGFP1 (Kan^r) was grown in LB broth containing 60 μg/mL of kanamycin at 37°C. Ty2 and CT18 strains were grown in LB broth in the presence of 50 μg/mL of streptomycin and 100 μg/mL of ampicillin, respectively, at 37°C. STy L-form (A200) was grown in LB medium in the presence of 50 μg/mL of streptomycin and 200 μg/mL of ampicillin.

The GFP-Ty2 and GFP-A200 strains were generated by introducing the pAKGFP1 (Kanr) plasmid into Ty2 and A200 by electroporation (5 pulses of 2,500 V each). Engineered bacterial strains, expressing green fluorescent protein (GFP) were grown in LB medium in the presence of streptomycin (50 μg/mL) and kanamycin (60 μg/mL) at 37°C. For GFP-A200, ampicillin (200 μg/mL) was also added to the culture medium in addition to the other antibiotics.

6.2.3. Generation of L form of STy

Ampicillin-sensitive strain (Ty2) was exposed to increasing concentrations of ampicillin (0.05 ug/ml to 200 ug/ml) over a period of 14 days. Bacteria was allowed to grow in each ampicillin concentration for two days before transferring into media containing higher ampicillin concentration.

6.2.4. In vitro growth assay

About 105 cells were inoculated into 100 mL LB medium containing the appropriate antibiotic(s) (50 μg/mL streptomycin for Ty2, 50 μg/mL streptomycin, and 200 μg/mL ampicillin for A200, 100 μg/mL ampicillin for CT18) and cultured at 37°C. For culture at low pH, 0.1 M HCl was added to the LB medium until the pH dropped to 5.0. Growth curves were obtained by plotting optical densities

of the bacterial cultures, taken at 600-nm wavelength (OD600) after different time intervals from the start of the culture.

6.2.5.LPS extraction and quantification

LPS was purified using hot phenol and quantified using the phenol-sulfuric acid method, as described previously (34). Briefly, bacteria were cultured for 18 h in LB medium at 37°C. The harvested cells (~1010 cells) were resuspended in distilled water and lysed by sonication (7 watts, 10-s pulse, and 10-s interval for 20 min). The cell lysates produced were treated with DNase I (10 mg/mL) and RNase A (5 mg/mL), followed by proteinase K (20 mg/mL) at 60°C for 1 h. The lysate was then incubated with phenol at 65 to 70°C with vigorous shaking for 1 h, and the solution was allowed to cool down to 10°C before being centrifuged at 10,000 rpm for 10 min to separate the organic and aqueous phases. Finally, the aqueous phase was mixed with double the volume of ethanol, and the mixture was incubated overnight at −20°C for complete precipitation of LPS.

Purified LPS was quantified using the phenol-sulfuric acid method as described earlier (35). Briefly, LPS was incubated with 50% phenol at room temperature for 10 min. Then, 500 µL of concentrated sulfuric acid was added to the mixture, and the absorbance of the resulting yellow-colored solution was measured. The LPS concentration was finally determined using a standard curve, generated with commercially available LPS (Sigma).

6.2.6. Peptidoglycan estimation

Peptidoglycan was measured according to the standard protocol (36). Parental Ty2 and A200 were cultured in LB medium with or without ampicillin at pH 7.0 and pH 5.0 for 7 h at 37°C. About 106 bacterial cells from each culture were incubated with 50 µL of 1 M NaOH at 37°C for 30 min, followed by the addition of 1 mL of concentrated sulfuric acid, and kept in a boiling water bath for 3 min. The mixture was cooled down rapidly on ice for 1 min and treated with 10 µL of 0.16 M copper sulfate and

20 µL of 0.09 M p-hydroxy biphenyl at 30°C for 30 min. Peptidoglycan was estimated by measuring the absorbance at 570 nm using a spectrophotometer.

6.2.7. Cell culture

The human acute monocytic leukemia cell line, THP-1, was maintained in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin (50 µg/mL penicillin and 50 µg/mL streptomycin). Cells were maintained at a density of 5 × 105 to 1.0 × 106 cells/mL. To differentiate THP-1 cells into macrophages, they were cultured in the above-described medium in the presence of 100 nM phorbol 12-myristate 13-acetate (PMA) for 24 h, followed by a recovery phase culture of 24 h in the absence of PMA. Before every experiment, cells were cultured overnight in RPMI 1640 containing 10% FBS but no antibiotics. Mouse macrophage cell line RAW264.7 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and penicillin-streptomycin. Cells were sub-cultured when 90% confluence was attained.

6.2.8. Characterization of L form

The growth curves of WT and A200 were obtained by plotting OD at different time intervals. LPS was purified by the hot phenol method and was quantified by the phenol-sulfuric acid method. Antibiotic susceptibility was quantified using disk diffusion assay.

6.2.9. Gentamycin Protection Assay

THP-1 cells seeded in 24-well plates (5×10^5 cells/well) were differentiated with PMA (100 nM, 24 h). RAW 264.7 Cells seeded in 24–well plates (5* 10^5 cells/well). Cells were infected at a multiplicity of infection (MOI) of 50 with bacterial cultures opsonized with RPMI 1640 medium containing 10% FBS for THP1 and with DMEM medium containing 10% FBS for RAW 267.4 and were synchronized with the bacteria by centrifugation at 400 × *g* for 5 min. Infection was continued for 30 min at 37°C in the presence of 5% CO_2. Extracellular bacteria were removed by repeated washing with 1× phosphate-

buffered saline (PBS), and the cells were cultured in complete RPMI medium for THP1 and with complete DMEM MEDIUM for RAW 267.4 containing 100 μg/ml of gentamicin for 1 h, followed by 15 μg/ml of gentamicin until the time of the experiment. Cells were lysed with 1× PBS containing 0.25% Triton X-100, and intracellular CFU counts were carried out after the bacteria recovered from the cells were grown overnight on a Luria agar plate containing suitable antibiotics.

6.2.10. Fluorescent confocal microscopy

THP1 and RAW cells were seeded in a 24-well plate at a density of 5×10^5 per well on collagen-coated coverslips and were infected with the bacteria. One day before infection with bacteria, Dextran conjugated Alexa Fluor 594 (5mg/ml) (thermofisher D22913) was added to the antibiotic-free fresh media and was incubated to/with the cells. Dextran is taken up by the cells and in the acidic vacuole, due to the fluorophore conjugated with it, it stains red in a pH-dependent manner. After 3 times washing with 1× PBS, coverslips were mounted on clean glass slides with fluorophore mounting media (sigma). Stained cells were imaged under a Zeiss LSM 710 confocal microscope, and bacteria were counted manually.

6.2.11. In vivo experiments

All animal experiments were carried out following the protocols approved by the Institutional Animal Ethics Committee of the National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India (Project license # PRO/103/May 2014-September 2017, Date 11.03.1999). NICED adheres to the animal handling protocols issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forest and DASGUPTA ET AL. 13 of 17 Climate Change, Government of India. (Registration no. 68/GOReBi/ S/99/CPCSEA. Validity from 11.03.2015 to 10.03.2019).

An iron overload mouse model was used for oral STy infection. Briefly, 6- to 8-week-old female BALB/c mice were injected intraperitoneally with desferrioxamine (Novartis, Switzerland; 0.025 mg

g−1 of mice body weight) and Fe3+ (0.32 mg g−1 of mice body weight) 4 hr before bacterial infection. Gastric acid of mice was neutralized with 5% sodium bicarbonate 20 min before oral administration of bacterial cultures grown till OD600 ~ 1.0. To study systemic infection, 6- to 8- week-old female BALB/c mice were infected orally with a sublethal dose (5 × 105 CFU) of STy strain Ty2 and L form of Ty2, and the liver, spleen, and gall bladder, MLN and payer's patches were collected 2 days, 4 days and 8 days post-infection respectively. Single-cell suspensions prepared from the liver, spleen, MLN, gall bladder, and peyer; 's patches were lysed with 0.25% Triton X-100 (Sigma- Aldrich, USA), and plated on LA containing suitable antibiotics from control mice, wild-type TY2 infected mice and L form of Ty2 infected mice separately and colonies were counted and plotted.

An intraperitonial infection mouse model was used without iron. 6- to 8- week-old BALB/c mice were infected intraperitoneally with a sublethal dose (5 × 10^4 CFU) of STy strain Ty2 and L form of Ty2, and the liver, spleen, and gall bladder were collected 2 days, 4 days and 8 days post-infection respectively. Single-cell suspensions prepared from the liver, spleen, and gall bladder, were lysed with 0.25% Triton X-100 (Sigma- Aldrich, USA) and plated on LA containing suitable antibiotics from control mice, wild type TY2 infected mice and L form of Ty2 infected mice separately and colonies were counted and plotted.

6.2.12.Statistical analysis

All graphs were plotted on Graph Pad Prism, version 8 and a T-Test was performed.

6.3. Results:-

6.3.1. Induction and characterization of STy L form

Wild-type Ty2 was subjected to increasing concentrations of Ampicillin over 14 days. On the 14th day, the bacterial strain obtained (A200) was able to grow in the presence of 200 ug/ml Ampicillin. A200 strain was then visualized under a Scanning electron microscope. Fig. 1A and B reveal a gross morphological change of A200 as compared to the WT strain. A200 strain had become more elongated

with a higher cytoplasmic volume as compared to WT. DAPI staining reveals the presence of more than one nucleoid region in the A200 strain (Fig. 1C) indicating compromised cell division. Furthermore, a comparison of the growth curves of the A200 and WT strains (Fig. 1D) indicates A200 strain had significantly reduced growth as compared to Wt.

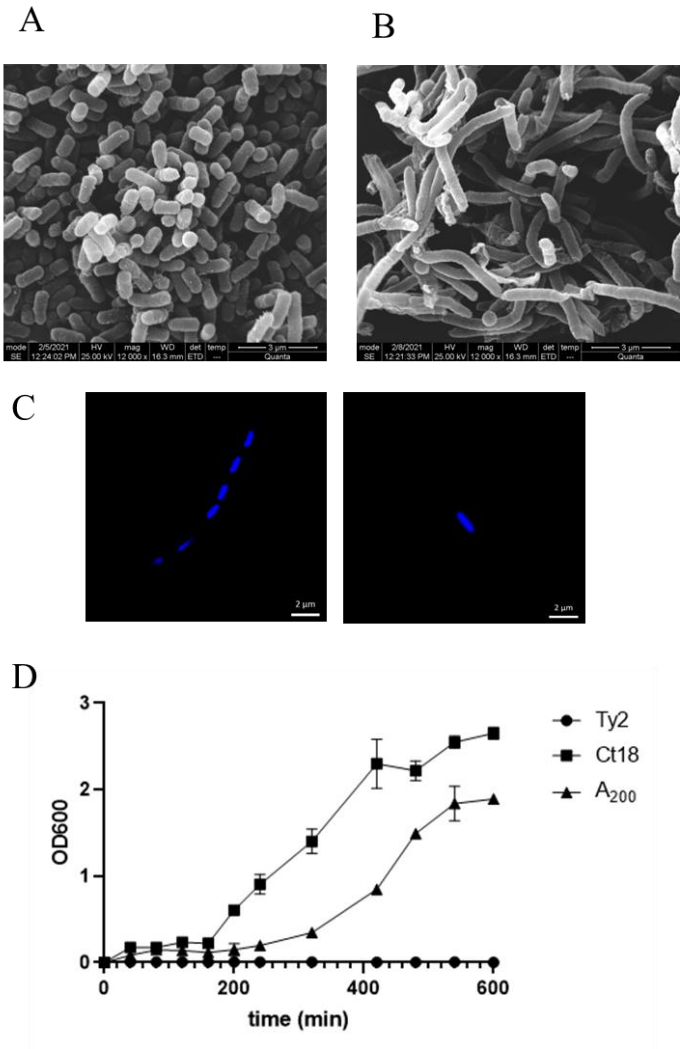

Figure 1: **General characterization of A200:** (A) Electron microscopy image of overnight grown WT Ty2. (B) Electron microscopy image of overnight grown A200. (C) DAPI staining of overnight grown WT Ty2 culture (Left). (B) DAPI staining of overnight grown A200 culture (Right). (D) Growth curve comparison of Ampicillin sensitive (Ty2), Ampicillin resistant (Ct18) and A200 L form. Data represents the mean of three independent experiments with SD value indicated by the error bar.

However, when A200 was allowed to grow in the absence of Ampicillin, it resumed the growth rate (Fig. 2A). A200 transformed with GFP containing plasmid enabled us to directly visualize its morphological changes during growth under different conditions. Fig. 2B reveals that when grown in the presence of Ampicillin, the majority of the cell population remained elongated. However, when

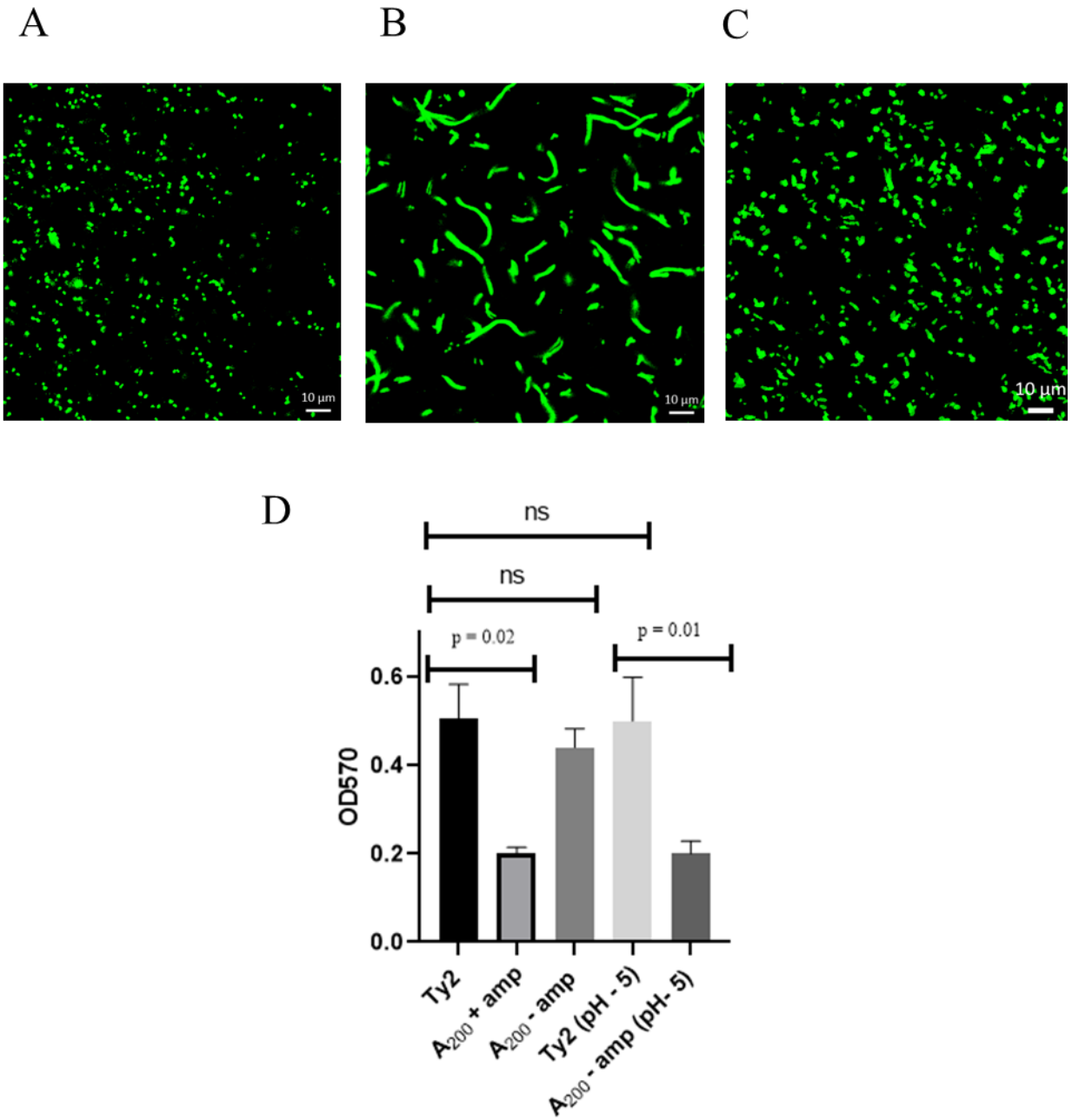

Figure 2 **General Characterization of A200 L form A**) Confocal microscopy of GFP containing Ty2 cells grown overnight in the absence of Ampicillin. (B) Confocal microscopy of GFP containing A200 cells grown overnight in the presence of Ampicillin. (C) GFP containing A200cells grown overnight in the absence of Ampicillin. (D) Quantification of total peptidoglycan in Ty2 and A200 strains. Peptidoglycan was measured from ~ 10^{10} cells grown as mentioned in materials and methods. Data shown as mean of three independent experiments with SD value shown by error bar

Ampicillin was removed, and most of the cells had reverted back into WT morphology (Fig. 2C). Time kinetics also revealed the same result (Fig 2D). When grown in the absence of Ampicillin, within 4 hrs majority of the bacterial cells assumed the WT morphology with the concomitant resumption of the growth rate. All the above-mentioned observations strongly suggested that A200 might display the characteristics of L-form bacteria.

Previous studies showed that in the L form of STy LPS production was significantly reduced as compared to WT (Stepanova, Gorelov et al. 1983). Fig. 3A shows that like L form, A200 also showed a drastic reduction in LPS formation. L forms are also shown to be more resistant to several antibiotics as compared to WT. When the antibiotic susceptibility of A200 was tested compared to WT A200 was found to be more resistant to several antibiotics tested as compared to WT further suggesting that it's the L form *Salmonella* (Fig. 3B).

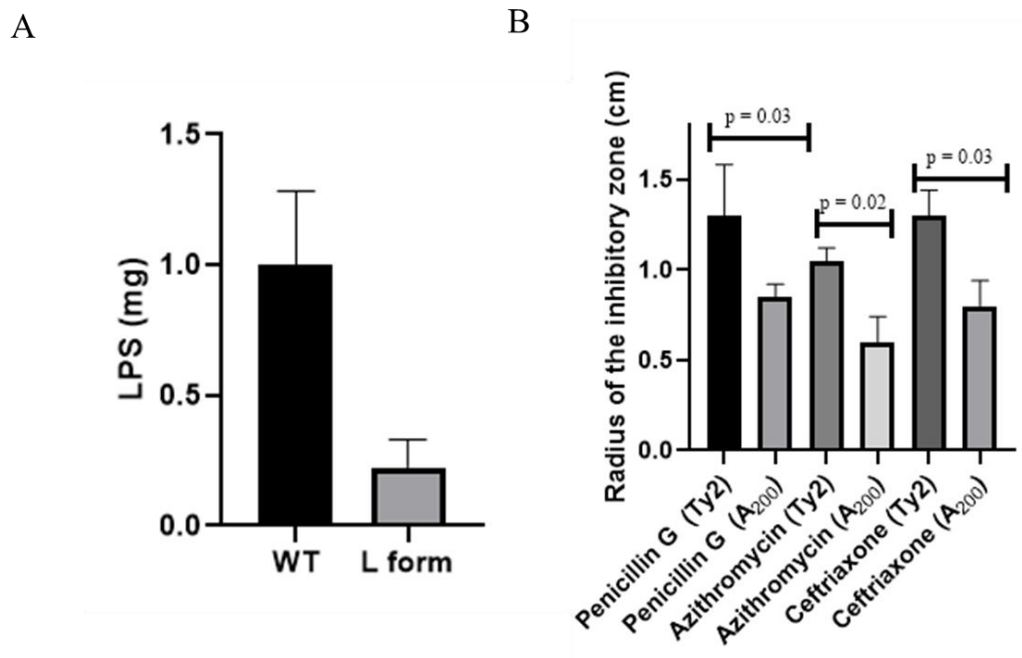

Figure 3 -**LPS synthesis and antibiotic resistance of A200 L- form.** (A)Quantification of LPS purified from 1010 Ty2 and A200 L bacterial cells by phenol- sulphuricacid method. Data represents the mean of three independent experiments with SD value indicated by the error bar (B) Radius of the inhibitory zones as observed in disk diffusion assay with antibiotic disc. Data represents the mean of three independent experiments with SD value indicated by the error bar. Statistical significance was measured by two tailed paired t test.

6.3.2. Infection of murine RAW cells by L form STy

The mouse RAW cell line was infected with 10 MOI of WT and GFP-A200 following the gentamycin assay. At 0 hr both WT and A200 showed a significant number of colonies indicating that A200 despite its altered morphology was able to infect RAW cells (Fig. 4A). At 24 hrs, the number of colonies of both WT and A200 significantly increased indicating that A200 can survive and multiply in RAW cells (Fig 4A). However, at both 0 hr and 24 hrs number of colonies of A200 was significantly higher than that of the WT strain indicating that A200 is more competent than WT in infecting the RAW cells. To get further insight into the infection caused by A200 in RAW cells, confocal microscopy was employed. Fig. 4B reveals at 0 hr few A200 bacteria were able to gain entry into the RAW cells. Surprisingly A200 colocalises with acidic vacuoles of RAW cells (stained with Dextran Alexa fluor 594) (Fig. 4B, lower panel). At 24 hrs (Fig. 4B), the number of A200 inside the cells had significantly increased as compared to 0 hr and most of the A200 cells showed co-localization with acidic vacuoles. These results indicate that A200 despite its altered behaviour was able to infect RAW macrophage cell lines.

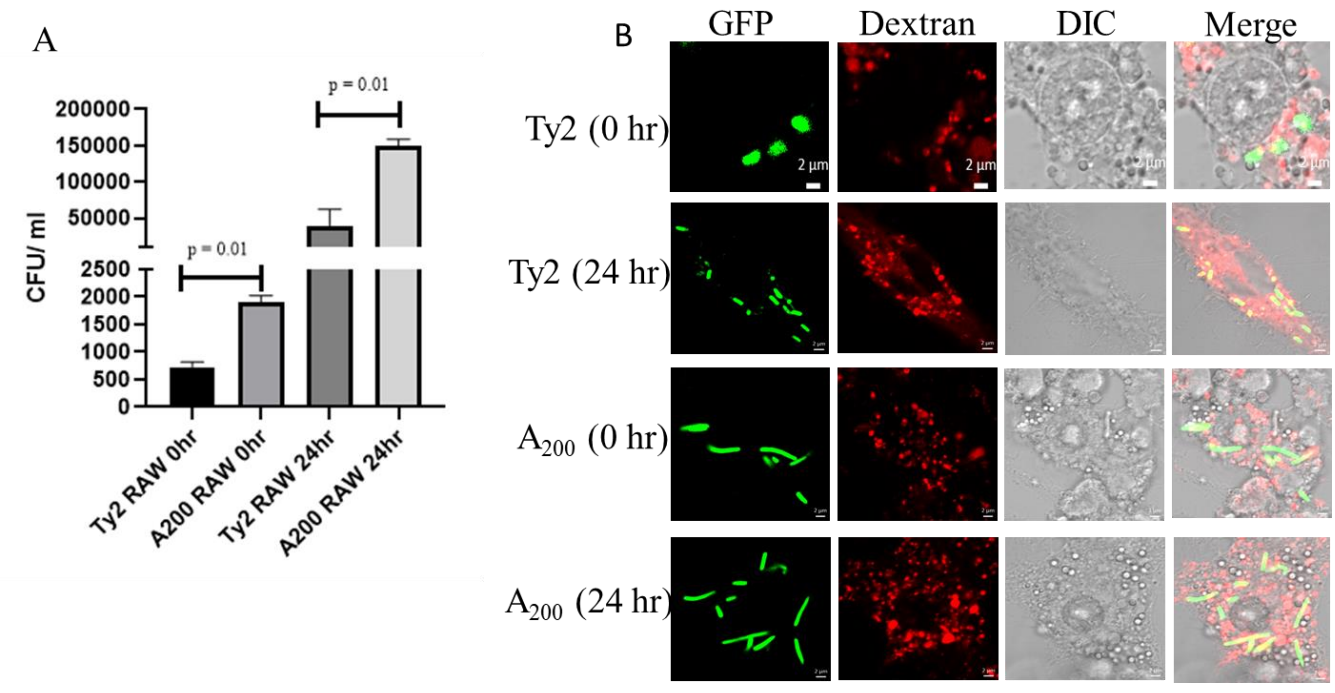

Figure 4. **Infection of murine RAW cell line with Ty2 and A200 L-form**(A) Raw cells were infected with WT Ty2 and A200 L-form following standard gentamycin assay. Bacteria were isolated from cells at 0 hr and 24 hrs and were platted on Luria Bertani agar containing 50 ug/ ml streptomycin. CFU was calculated and plotted. (B). Infection of murine RAW cell line with GFP- Ty2 and GFP- A200 L-form: Representative confocal microscopy images showing intracellular Ty2 (upper two panels) andA200 (lower two panels) bacteria at 0hr and 24 hrs post infection. RAW cells were stained with dextran and were infected with 50 MOI GFP- Ty2 and GFP- A200. After 0 hr and 24 hrs post infection, cells were mounted and visualized under fluorescence confocal microscope (63X/ 1.4 NA). Scale bar- 2 µm

6.3.3. Infection of human THP-1 cells by L form STy

Next, the ability of A200 cells to infect human macrophage cell line THP-1 was monitored. THP-1 cell line was infected with 10 MOI of both WT and A200 strains and the gentamycin assay was performed. At 0 hr both WT and A200 showed a significant number of colonies indicating that A200 can infect THp-1 (Fig. 5A) cells more efficiently than WT. After, 24 hrs, both WT and A200 showed an enhancement in numbers indicating that A200, like WT, can survive and multiply in human THP-1 cells. Confocal microscopy with GFP-A200 showed that at 0 hr few A200 cells colocalize with acidic vacuoles of Thp-1 as evidenced by the merging of red Alexa fluor and GFP (Fig 5B). At 24 hrs, the number of A200 cells was increased with the majority of the bacteria within acidic vacuoles. These results strongly support the fact that A200 can infect mammalian Thp-1 cells (Fig 5).

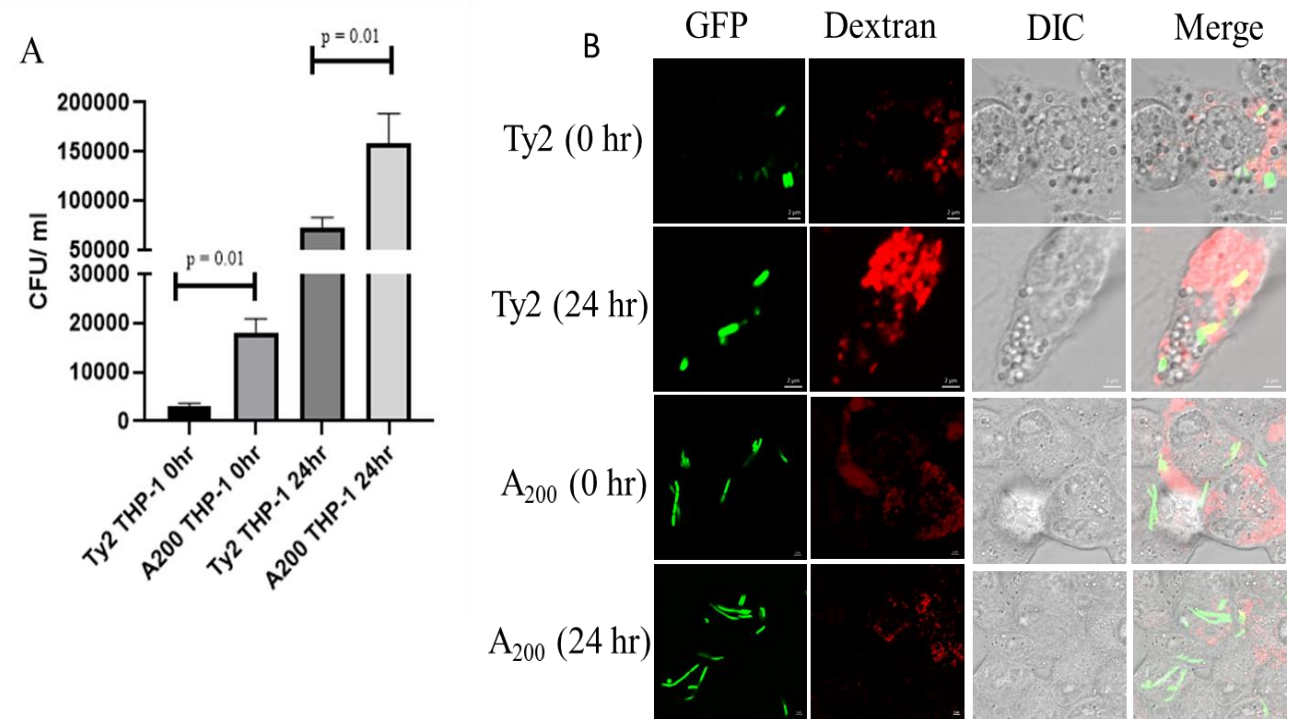

Figure 5: **Infection of THP-1 cell line with Ty2 and A200 L-form** (A) THP-1 cells were infected with WT Ty2 and A200 L- form following standard gentamycin assay. Bacteria were isolated from cells at 0 hr and 24 hrs and were platted on Luria Bertani agar containing 50 ug/ ml streptomycin. CFU was calculated and plotted. Data represents the mean of three independent experiments with SD value indicated by the error bar. Statistical significance was measured by two tailed paired t test. (B) Infection of THP-1 cell line with GFP- Ty2 and GFP- A200 L-form: Representative confocal microscopy images showing intracellular Ty2 (upper two panels) and A200 (lower two panel)bacteria at 0hr and 24 hrs post infection. THP-1 cells were stained with dextran and were infected with 50 MOI GFP- Ty2 and GFP- A200. After 0 hr and 24 hrs post infection, cells were mounted and visualized under fluorescence confocal microscope (63X/ 1.4 NA). Scale bar- 2 µm

6.3.4. Infection of mice by L form STy (Orally)

The ability of A200 to infect the mouse macrophage cell line prompted us to question whether it is able to show infection in mice. Mice were infected orally with a sub-lethal dose of WT and A200 strains and bacterial load was measured in the liver, spleen, Payer's patch, MLN, and gall bladder after 2, 4, and 8 days post-infection (dpi). Fig 6 reveals that a significant number of bacteria were isolated from all the organs with a drastic increase in A200 number at 8 dpi indicating that A200 can infect, survive, and multiply in mice. Surprisingly, in this experiment, the number of bacteria isolated for WT is significantly higher than that of A200 indicating that A200 are less competent in infecting mice (orally) than the WT.

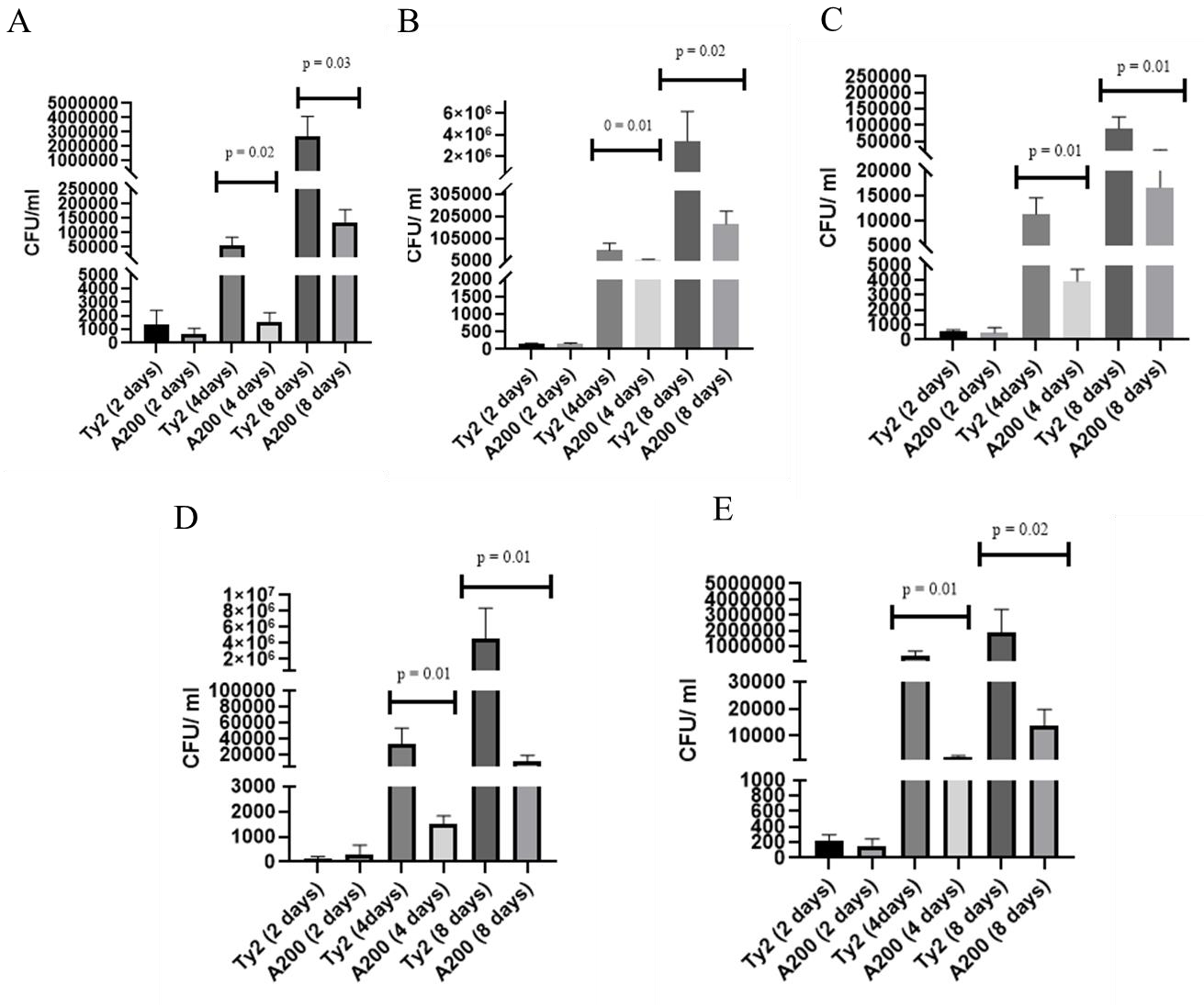

Figure 6: **Infection of mice model by A200 L form (oral):** Mice (n = 5) were infected orallywith A200 L- form. Bacteria was isolated from different organs, (A) liver , (B) Spleen , (C) Gall bladder, (D) MLN , (E) Payers' patch after 2, 4, and 8 dpi and platted on Leuria Bertani agar plates containing 50 ug/ ml strptomycin. CFU was calculated and plotted. Statistical significance was measured by two tailed paired t test.

6.3.4. Infection of mice by L form STy (Intraperitoneally)

Mice were infected intraperitoneally (i.p.) with a sublethal dose of either the parental Ty2 or A200 strains. The bacterial load in visceral organs, including the liver, spleen, and gallbladder, was assessed at 2, 4, and 8 days post-infection (dpi). At 2 dpi, similar numbers of both A200 and Ty2 bacteria were recovered from the visceral organs, indicating that A200 was equally competent in infecting live animals (Fig. 7). The bacterial load increased for both strains at 4 dpi, demonstrating their ability to

survive and replicate in vivo. However, by 8 dpi, there was a significant reduction in bacterial load in the visceral organs, suggesting clearance by the host immune system. Notably, A200 exhibited a higher visceral load than the parental strain at this time point, implying that A200 was more efficient in evading the host immune response. A200 isolates from the liver and spleen after 8 days of both peritoneal and oral infection retained their ability to grow in the presence of ampicillin (200 µg/mL), indicating that A200 did not revert to the parental Ty2 strain.

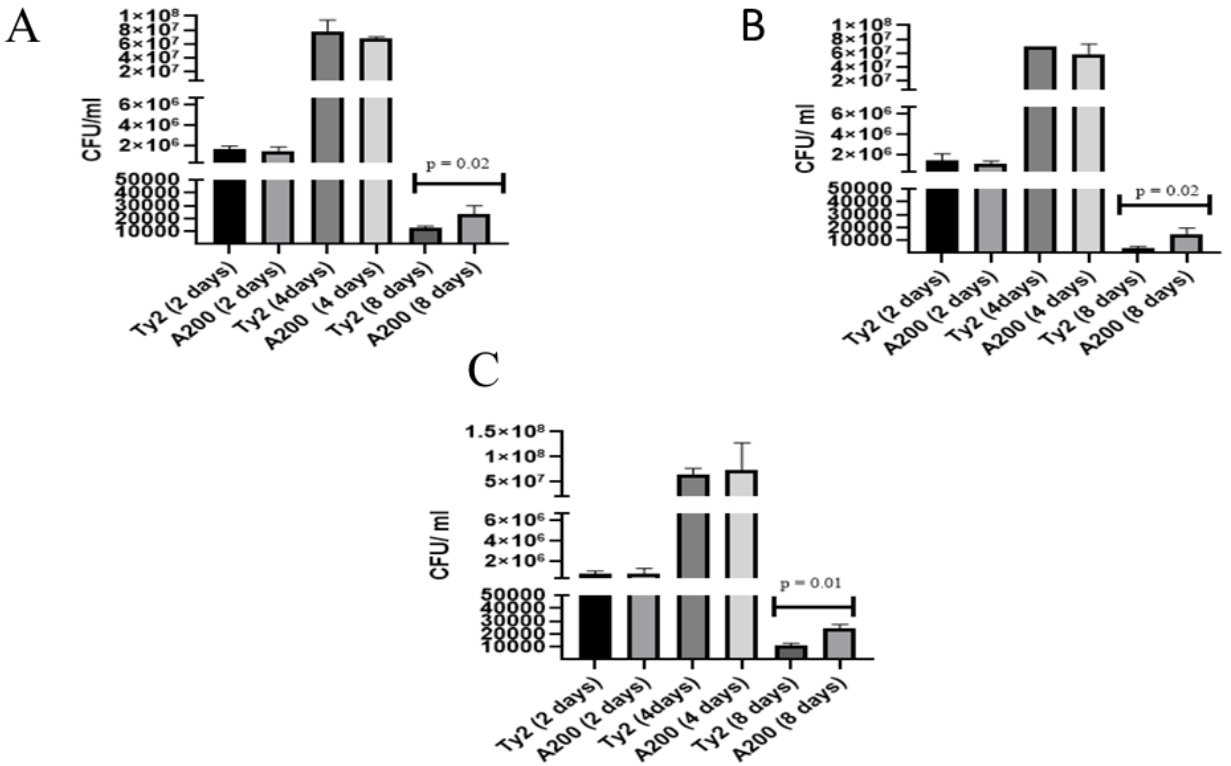

Figure 7: **Infection of mice model by A200 L form (IP)**: Mice (n = 5) were infected intraperitoneally with A200 L- form. Bacteria was isolated from different organs, (A) liver, (B) spleen, (C) Gall bladder after 2, 4, and 8 dpi and platted on Luria Bertani agar plates containing 50 ug/ ml streptomycin. CFU was calculated and plotted. Statistical significance was measured by two tailed paired t test.

6.4. Discussion

Bacterial diseases are treated using a wide variety of antibiotics which either kill the bacteria or slow their growth. However, the antibiotic concentration in vivo increases with time. So, the bacterial population is subjected to an increasing gradient of antibiotics. In this study, we aimed to investigate

the effect of this increasing gradient of antibiotic concentration on STy. Ampicillin is a well-known cell wall-targeting compound that kills bacteria by inhibiting transpeptidase. When ampicillin-sensitive STy was subjected to increasing concentration of ampicillin over 14 days, it changed its morphology, shut down its cell wall and LPS synthesis, and was able to grow in the presence of 200 ug/ml Ampicillin. However, the same thing was not observed when Ty2 was allowed to grow in the presence of 200 ug/ml Ampicillin directly. In this case, no growth was observed (data not shown). The increasing concentration gradient of Ampicillin caused Ty2 to display the following characteristics of L-form bacteria: I) cell wall and LPS production was drastically reduced, II) it became more elongated with higher cytoplasmic volume with more than one nucleoid per cell, III) the growth was highly compromised. However, with the removal of Ampicillin, most of the altered behavior was reverted to that of the WT indicating that these changes are mostly reversible.

In this study, L form STy was also shown to infect murine as well as human macrophage cell lines. Gentamycin assay and confocal microscopy revealed that the A200 strain can enter and multiply inside THP-1 and RAW cell lines. Surprisingly, its competence to infection was more as compared to WT. A200 was also shown to cause infection in mice albeit with a reduced efficiency as compared to WT. Bacteria were isolated from all the organs tested after oral infection in mice indicating successful colonization in the gut. At 8 dpi, the number of A200 increased manifold suggesting that A200 can survive as well can replicate inside the organs. These results indicate that A200 despite an altered morphology can successfully colonize and replicate inside the gut. It should be mentioned here that at 2 and 4 dpi A200 from different organs form mixed colonies with the majority being smaller ones indicating slow slow-growing L form. However, at 8 dpi most of the colonies were bigger indicating the reversion of A200 into WT (data not shown).

Previous studies with L form *Salmonella* do not deal with the pathogenesis of L form. In this study, for the first time, the pathogenesis of *Salmonella* L form was explored in great detail. This study reveals that subjecting *Salmonella* with an increasing concentration gradient of ampicillin does not kill the

entire bacterial population. Rather it causes *Salmonella* to be resistant without any notable genetic mutations. In this resistant state, it will be able to infect macrophage cells and multiply with efficiency. However, in the mice model the infection efficiency was lesser as compared to WT. This indicates that the L form may be unsuccessful in crossing the gut immune barrier. The underlying reason for this observation is still under investigation.

Chapter 8

References

Abramson, T., H. Kedem and D. A. Relman (2008). "Modulation of the NF-kappaB pathway by Bordetella pertussis filamentous hemagglutinin." PLoS One **3**(11): e3825.
Achtman, M., J. Wain, F. X. Weill, S. Nair, Z. Zhou, V. Sangal, M. G. Krauland, J. L. Hale, H. Harbottle, A. Uesbeck, G. Dougan, L. H. Harrison, S. Brisse and S. E. M. S. Group (2012). "Multilocus sequence typing as a replacement for serotyping in Salmonella enterica." PLoS Pathog **8**(6): e1002776.
Agbor, T. A. and B. A. McCormick (2011). "Salmonella effectors: important players modulating host cell function during infection." Cell Microbiol **13**(12): 1858-1869.
Ahmer, B. M., J. van Reeuwijk, P. R. Watson, T. S. Wallis and F. Heffron (1999). "Salmonella SirA is a global regulator of genes mediating enteropathogenesis." Mol Microbiol **31**(3): 971-982.
Akira, S., S. Uematsu and O. Takeuchi (2006). "Pathogen recognition and innate immunity." Cell **124**(4): 783-801.
Alvarez-Martinez, C. E. and P. J. Christie (2009). "Biological diversity of prokaryotic type IV secretion systems." Microbiol Mol Biol Rev **73**(4): 775-808.
Amavisit, P., D. Lightfoot, G. F. Browning and P. F. Markham (2003). "Variation between pathogenic serovars within Salmonella pathogenicity islands." J Bacteriol **185**(12): 3624-3635.
Anderson, C. J. and M. M. Kendall (2017). "Salmonella enterica Serovar Typhimurium Strategies for Host Adaptation." Front Microbiol **8**: 1983.
Arena, E. T., S. D. Auweter, L. C. Antunes, A. W. Vogl, J. Han, J. A. Guttman, M. A. Croxen, A. Menendez, S. D. Covey, C. H. Borchers and B. B. Finlay (2011). "The deubiquitinase activity of the Salmonella pathogenicity island 2 effector, SseL, prevents accumulation of cellular lipid droplets." Infect Immun **79**(11): 4392-4400.
Arpaia, N., J. Godec, L. Lau, K. E. Sivick, L. M. McLaughlin, M. B. Jones, T. Dracheva, S. N. Peterson, D. M. Monack and G. M. Barton (2011). "TLR signaling is required for Salmonella typhimurium virulence." Cell **144**(5): 675-688.
Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997 Sep 1;25(17):3389-402. doi: 10.1093/nar/25.17.3389. PMID: 9254694; PMCID: PMC146917.
Averette, K. M., M. R. Pratt, Y. Yang, S. Bassilian, J. P. Whitelegge, J. A. Loo, T. W. Muir and K. A. Bradley (2009). "Anthrax lethal toxin induced lysosomal membrane permeabilization and cytosolic cathepsin release is Nlrp1b/Nalp1b-dependent." PLoS One **4**(11): e7913.
Azimi, T., M. Zamirnasta, M. A. Sani, M. M. Soltan Dallal and A. Nasser (2020). "Molecular Mechanisms of Salmonella Effector Proteins: A Comprehensive Review." Infect Drug Resist **13**: 11-26.
Backert, S. and T. F. Meyer (2006). "Type IV secretion systems and their effectors in bacterial pathogenesis." Curr Opin Microbiol **9**(2): 207-217.
Bajaj, V., R. L. Lucas, C. Hwang and C. A. Lee (1996). "Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression." Mol Microbiol **22**(4): 703-714.
Bakowski, M. A., J. T. Cirulis, N. F. Brown, B. B. Finlay and J. H. Brumell (2007). "SopD acts cooperatively with SopB during Salmonella enterica serovar Typhimurium invasion." Cell Microbiol **9**(12): 2839-2855.
Baldassarre, M., V. Solano-Collado, A. Balci, R. A. Colamarino, I. M. Dambuza, D. M. Reid, H. M. Wilson, G. D. Brown, S. Mukhopadhyay, G. Dougan and S. Spano (2021). "The Rab32/BLOC-3-dependent pathway mediates host defense against different pathogens in human macrophages." Sci Adv **7**(3).
Bao, H., S. Wang, J. H. Zhao and S. L. Liu (2020). "Salmonella secretion systems: Differential roles in pathogen-host interactions." Microbiol Res **241**: 126591.
Bao, H., J. H. Zhao, S. Zhu, S. Wang, J. Zhang, X. Y. Wang, B. Hua, C. Liu, H. Liu and S. L. Liu (2019). "Genetic diversity and evolutionary features of type VI secretion systems in Salmonella." Future Microbiol **14**: 139-154.
Barbero, P., L. Bittova and S. R. Pfeffer (2002). "Visualization of Rab9-mediated vesicle transport from endosomes to the trans-Golgi in living cells." J Cell Biol **156**(3): 511-518.
Barlag, B. and M. Hensel (2015). "The giant adhesin SiiE of Salmonella enterica." Molecules **20**(1): 1134-1150.
Basnyat, B. and S. Baker (2015). "Typhoid carriage in the gallbladder." Lancet **386**(9998): 1074.
Baumler, A. J. and F. Heffron (1995). "Identification and sequence analysis of lpfABCDE, a putative fimbrial operon of Salmonella typhimurium." J Bacteriol **177**(8): 2087-2097.

Baumler, A. J., R. M. Tsolis, F. A. Bowe, J. G. Kusters, S. Hoffmann and F. Heffron (1996). "The pef fimbrial operon of Salmonella typhimurium mediates adhesion to murine small intestine and is necessary for fluid accumulation in the infant mouse." Infect Immun **64**(1): 61-68.
Baxter, M. A., T. F. Fahlen, R. L. Wilson and B. D. Jones (2003). "HilE interacts with HilD and negatively regulates hilA transcription and expression of the Salmonella enterica serovar Typhimurium invasive phenotype." Infect Immun **71**(3): 1295-1305.
Bayer-Santos, E., C. H. Durkin, L. A. Rigano, A. Kupz, E. Alix, O. Cerny, E. Jennings, M. Liu, A. S. Ryan, N. Lapaque, S. H. E. Kaufmann and D. W. Holden (2016). "The Salmonella Effector SteD Mediates MARCH8-Dependent Ubiquitination of MHC II Molecules and Inhibits T Cell Activation." Cell Host Microbe **20**(5): 584-595.
Beatty, W. L. (2006). "Trafficking from CD63-positive late endocytic multivesicular bodies is essential for intracellular development of Chlamydia trachomatis." J Cell Sci **119**(Pt 2): 350-359.
Beresford, N., S. Patel, J. Armstrong, B. Szoor, A. P. Fordham-Skelton and L. Tabernero (2007). "MptpB, a virulence factor from Mycobacterium tuberculosis, exhibits triple-specificity phosphatase activity." Biochem J **406**(1): 13-18.
Beuzon, C. R., S. Meresse, K. E. Unsworth, J. Ruiz-Albert, S. Garvis, S. R. Waterman, T. A. Ryder, E. Boucrot and D. W. Holden (2000). "Salmonella maintains the integrity of its intracellular vacuole through the action of SifA." EMBO J **19**(13): 3235-3249.
Beuzon, C. R., S. P. Salcedo and D. W. Holden (2002). "Growth and killing of a Salmonella enterica serovar Typhimurium sifA mutant strain in the cytosol of different host cell lines." Microbiology (Reading) **148**(Pt 9): 2705-2715.
Bewley, M. A., M. Naughton, J. Preston, A. Mitchell, A. Holmes, H. M. Marriott, R. C. Read, T. J. Mitchell, M. K. Whyte and D. H. Dockrell (2014). "Pneumolysin activates macrophage lysosomal membrane permeabilization and executes apoptosis by distinct mechanisms without membrane pore formation." mBio **5**(5): e01710-01714.
Bielecki, J., P. Youngman, P. Connelly and D. A. Portnoy (1990). "Bacillus subtilis expressing a haemolysin gene from Listeria monocytogenes can grow in mammalian cells." Nature **345**(6271): 175-176.
Birmingham, C. L., V. Canadien, E. Gouin, E. B. Troy, T. Yoshimori, P. Cossart, D. E. Higgins and J. H. Brumell (2007). "Listeria monocytogenes evades killing by autophagy during colonization of host cells." Autophagy **3**(5): 442-451.
Birmingham, C. L., V. Canadien, N. A. Kaniuk, B. E. Steinberg, D. E. Higgins and J. H. Brumell (2008). "Listeriolysin O allows Listeria monocytogenes replication in macrophage vacuoles." Nature **451**(7176): 350-354.
Bishop, A. L., S. Baker, S. Jenks, M. Fookes, P. O. Gaora, D. Pickard, M. Anjum, J. Farrar, T. T. Hien, A. Ivens and G. Dougan (2005). "Analysis of the hypervariable region of the Salmonella enterica genome associated with tRNA(leuX)." J Bacteriol **187**(7): 2469-2482.
Blancas-Luciano, B. E., I. Becker-Fauser, J. Zamora-Chimal, J. Delgado-Dominguez, A. Ruiz-Remigio, E. R. Leyva-Huerta, J. Portilla-Robertson and A. M. Fernandez-Presas (2022). "Antimicrobial and anti-inflammatory activity of Cystatin C on human gingival fibroblast incubated with Porphyromonas gingivalis." PeerJ **10**: e14232.
Blondel, C. J., J. C. Jimenez, I. Contreras and C. A. Santiviago (2009). "Comparative genomic analysis uncovers 3 novel loci encoding type six secretion systems differentially distributed in Salmonella serotypes." BMC Genomics **10**: 354.
Blondel, C. J., H. J. Yang, B. Castro, S. Chiang, C. S. Toro, M. Zaldivar, I. Contreras, H. L. Andrews-Polymenis and C. A. Santiviago (2010). "Contribution of the type VI secretion system encoded in SPI-19 to chicken colonization by Salmonella enterica serotypes Gallinarum and Enteritidis." PLoS One **5**(7): e11724.
Boucrot, E., T. Henry, J. P. Borg, J. P. Gorvel and S. Meresse (2005). "The intracellular fate of Salmonella depends on the recruitment of kinesin." Science **308**(5725): 1174-1178.
Boya, P. and G. Kroemer (2008). "Lysosomal membrane permeabilization in cell death." Oncogene **27**(50): 6434-6451.
Boyer, F., G. Fichant, J. Berthod, Y. Vandenbrouck and I. Attree (2009). "Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources?" BMC Genomics **10**: 104.

Brombacher, E., S. Urwyler, C. Ragaz, S. S. Weber, K. Kami, M. Overduin and H. Hilbi (2009). "Rab1 guanine nucleotide exchange factor SidM is a major phosphatidylinositol 4-phosphate-binding effector protein of Legionella pneumophila." J Biol Chem **284**(8): 4846-4856.
Bronner, D. N., F. Faber, E. E. Olsan, M. X. Byndloss, N. A. Sayed, G. Xu, W. Yoo, D. Kim, S. Ryu, C. B. Lebrilla and A. J. Baumler (2018). "Genetic Ablation of Butyrate Utilization Attenuates Gastrointestinal Salmonella Disease." Cell Host Microbe **23**(2): 266-273 e264.
Browne, S. H., P. Hasegawa, S. Okamoto, J. Fierer and D. G. Guiney (2008). "Identification of Salmonella SPI-2 secretion system components required for SpvB-mediated cytotoxicity in macrophages and virulence in mice." FEMS Immunol Med Microbiol **52**(2): 194-201.
Bruggemann, H., C. Cazalet and C. Buchrieser (2006). "Adaptation of Legionella pneumophila to the host environment: role of protein secretion, effectors and eukaryotic-like proteins." Curr Opin Microbiol **9**(1): 86-94.
Brumell, J. H., P. Tang, S. D. Mills and B. B. Finlay (2001). "Characterization of Salmonella-induced filaments (Sifs) reveals a delayed interaction between Salmonella-containing vacuoles and late endocytic compartments." Traffic **2**(9): 643-653.
Brumell, J. H., P. Tang, M. L. Zaharik and B. B. Finlay (2002). "Disruption of the Salmonella-containing vacuole leads to increased replication of Salmonella enterica serovar typhimurium in the cytosol of epithelial cells." Infect Immun **70**(6): 3264-3270.
Brunet, Y. R., A. Khodr, L. Logger, L. Aussel, T. Mignot, S. Rimsky and E. Cascales (2015). "H-NS Silencing of the Salmonella Pathogenicity Island 6-Encoded Type VI Secretion System Limits Salmonella enterica Serovar Typhimurium Interbacterial Killing." Infect Immun **83**(7): 2738-2750.
Buchwald, G., A. Friebel, J. E. Galan, W. D. Hardt, A. Wittinghofer and K. Scheffzek (2002). "Structural basis for the reversible activation of a Rho protein by the bacterial toxin SopE." EMBO J **21**(13): 3286-3295.
Burks, S. R., E. A. Legenzov, E. W. Martin, C. Li, W. Lu and J. P. Kao (2015). "Co-encapsulating the fusogenic peptide INF7 and molecular imaging probes in liposomes increases intracellular signal and probe retention." PLoS One **10**(3): e0120982.
Burtnick, M. N., P. J. Brett, V. Nair, J. M. Warawa, D. E. Woods and F. C. Gherardini (2008). "Burkholderia pseudomallei type III secretion system mutants exhibit delayed vacuolar escape phenotypes in RAW 264.7 murine macrophages." Infect Immun **76**(7): 2991-3000.
Cain, R. J. and A. J. Ridley (2009). "Phosphoinositide 3-kinases in cell migration." Biol Cell **101**(1): 13-29.
Campoy, E. M., F. C. Zoppino and M. I. Colombo (2011). "The early secretory pathway contributes to the growth of the Coxiella-replicative niche." Infect Immun **79**(1): 402-413.
Carabeo, R. A., D. J. Mead and T. Hackstadt (2003). "Golgi-dependent transport of cholesterol to the Chlamydia trachomatis inclusion." Proc Natl Acad Sci U S A **100**(11): 6771-6776.
Carden, S., C. Okoro, G. Dougan and D. Monack (2015). "Non-typhoidal Salmonella Typhimurium ST313 isolates that cause bacteremia in humans stimulate less inflammasome activation than ST19 isolates associated with gastroenteritis." Pathog Dis **73**(4).
Cardenal-Munoz, E., G. Gutierrez and F. Ramos-Morales (2014). "Global impact of Salmonella type III secretion effector SteA on host cells." Biochem Biophys Res Commun **449**(4): 419-424.
Cascales, E. and C. Cambillau (2012). "Structural biology of type VI secretion systems." Philos Trans R Soc Lond B Biol Sci **367**(1592): 1102-1111.
Cavallo-Medved, D., K. Moin and B. Sloane (2011). "Cathepsin B: Basis Sequence: Mouse." AFCS Nat Mol Pages **2011**.
Cemma, M. and J. H. Brumell (2012). "Interactions of pathogenic bacteria with autophagy systems." Curr Biol **22**(13): R540-545.
Chakravortty, D., I. Hansen-Wester and M. Hensel (2002). "Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates." J Exp Med **195**(9): 1155-1166.
Chen, C. L., C. Y. Wang, C. Chu, L. H. Su and C. H. Chiu (2009). "Functional and molecular characterization of pSE34 encoding a type IV secretion system in Salmonella enterica serotype Enteritidis phage type 34." FEMS Immunol Med Microbiol **57**(3): 274-283.
Chen, L. M., S. Hobbie and J. E. Galan (1996). "Requirement of CDC42 for Salmonella-induced cytoskeletal and nuclear responses." Science **274**(5295): 2115-2118.
Chiu, C. H., P. Tang, C. Chu, S. Hu, Q. Bao, J. Yu, Y. Y. Chou, H. S. Wang and Y. S. Lee (2005). "The genome sequence of Salmonella enterica serovar Choleraesuis, a highly invasive and resistant zoonotic pathogen." Nucleic Acids Res **33**(5): 1690-1698.

Christen, M., L. H. Coye, J. S. Hontz, D. L. LaRock, R. A. Pfuetzner, Megha and S. I. Miller (2009). "Activation of a bacterial virulence protein by the GTPase RhoA." Sci Signal **2**(95): ra71.
Christie, P. J. (2016). "The Mosaic Type IV Secretion Systems." EcoSal Plus **7**(1).
Cianfanelli, F. R., L. Monlezun and S. J. Coulthurst (2016). "Aim, Load, Fire: The Type VI Secretion System, a Bacterial Nanoweapon." Trends Microbiol **24**(1): 51-62.
Clifton, D. R., R. A. Goss, S. K. Sahni, D. van Antwerp, R. B. Baggs, V. J. Marder, D. J. Silverman and L. A. Sporn (1998). "NF-kappa B-dependent inhibition of apoptosis is essential for host cellsurvival during Rickettsia rickettsii infection." Proc Natl Acad Sci U S A **95**(8): 4646-4651.
Clifton, D. R., E. Rydkina, R. S. Freeman and S. K. Sahni (2005). "NF-kappaB activation during Rickettsia rickettsii infection of endothelial cells involves the activation of catalytic IkappaB kinases IKKalpha and IKKbeta and phosphorylation-proteolysis of the inhibitor protein IkappaBalpha." Infect Immun **73**(1): 155-165.
Colletti GA, Miedel MT, Quinn J, Andharia N, Weisz OA, Kiselyov K. Loss of lysosomal ion channel transient receptor potential channel mucolipin-1 (TRPML1) leads to cathepsin B-dependent apoptosis. J Biol Chem. 2012 Mar 9;287(11):8082-91. doi: 10.1074/jbc.M111.285536. Epub 2012 Jan 18. PMID: 22262857; PMCID: PMC3318733.
Comeau SR, Gatchell DW, Vajda S, Camacho CJ. ClusPro: a fully automated algorithm for protein-protein docking. Nucleic Acids Res. 2004 Jul 1;32(Web Server issue):W96-9. doi: 10.1093/nar/gkh354. PMID: 15215358; PMCID: PMC441492.
Coombes, B. K., M. J. Lowden, J. L. Bishop, M. E. Wickham, N. F. Brown, N. Duong, S. Osborne, O. Gal-Mor and B. B. Finlay (2007). "SseL is a salmonella-specific translocated effector integrated into the SsrB-controlled salmonella pathogenicity island 2 type III secretion system." Infect Immun **75**(2): 574-580.
Cornelis, G. R. (2006). "The type III secretion injectisome." Nat Rev Microbiol **4**(11): 811-825.
Cortes, C., K. A. Rzomp, A. Tvinnereim, M. A. Scidmore and B. Wizel (2007). "Chlamydia pneumoniae inclusion membrane protein Cpn0585 interacts with multiple Rab GTPases." Infect Immun **75**(12): 5586-5596.
Cossart, P. and J. Mengaud (1989). "Listeria monocytogenes. A model system for the molecular study of intracellular parasitism." Mol Biol Med **6**(5): 463-474.
Cossart, P. and P. J. Sansonetti (2004). "Bacterial invasion: the paradigms of enteroinvasive pathogens." Science **304**(5668): 242-248.
Coulthurst, S. (2019). "The Type VI secretion system: a versatile bacterial weapon." Microbiology (Reading) **165**(5): 503-515.
Crawford, R. W., T. Wangdi, A. M. Spees, M. N. Xavier, R. M. Tsolis and A. J. Baumler (2013). "Loss of very-long O-antigen chains optimizes capsule-mediated immune evasion by Salmonella enterica serovar Typhi." mBio **4**(4).
Criss, A. K. and J. E. Casanova (2003). "Coordinate regulation of Salmonella enterica serovar Typhimurium invasion of epithelial cells by the Arp2/3 complex and Rho GTPases." Infect Immun **71**(5): 2885-2891.
da Silva, C. V., L. Cruz, S. Araujo Nda, M. B. Angeloni, B. B. Fonseca, O. Gomes Ade, R. Carvalho Fdos, A. L. Goncalves and F. Barbosa Bde (2012). "A glance at Listeria and Salmonella cell invasion: different strategies to promote host actin polymerization." Int J Med Microbiol **302**(1): 19-32.
De Keersmaecker, S. C., K. Marchal, T. L. Verhoeven, K. Engelen, J. Vanderleyden and C. S. Detweiler (2005). "Microarray analysis and motif detection reveal new targets of the Salmonella enterica serovar Typhimurium HilA regulatory protein, including hilA itself." J Bacteriol **187**(13): 4381-4391.
de Mingo, A., E. de Gregorio, A. Moles, N. Tarrats, A. Tutusaus, A. Colell, J. C. Fernandez-Checa, A. Morales and M. Mari (2016). "Cysteine cathepsins control hepatic NF-kappaB-dependent inflammation via sirtuin-1 regulation." Cell Death Dis **7**(11): e2464.
Deiwick, J., S. P. Salcedo, E. Boucrot, S. M. Gilliland, T. Henry, N. Petermann, S. R. Waterman, J. P. Gorvel, D. W. Holden and S. Meresse (2006). "The translocated Salmonella effector proteins SseF and SseG interact and are required to establish an intracellular replication niche." Infect Immun **74**(12): 6965-6972.
Delgado-Suarez, E. J., N. Selem-Mojica, R. Ortiz-Lopez, W. A. Gebreyes, M. W. Allard, F. Barona-Gomez and M. S. Rubio-Lozano (2018). "Whole genome sequencing reveals widespread distribution of typhoidal toxin genes and VirB/D4 plasmids in bovine-associated nontyphoidal Salmonella." Sci Rep **8**(1): 9864.
Deng, W., N. C. Marshall, J. L. Rowland, J. M. McCoy, L. J. Worrall, A. S. Santos, N. C. J. Strynadka and B. B. Finlay (2017). "Assembly, structure, function and regulation of type III secretion systems." Nat Rev Microbiol **15**(6): 323-337.
Desjardins, M., L. A. Huber, R. G. Parton and G. Griffiths (1994). "Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus." J Cell Biol **124**(5): 677-688.

Di Meo, S., T. T. Reed, P. Venditti and V. M. Victor (2016). "Role of ROS and RNS Sources in Physiological and Pathological Conditions." Oxid Med Cell Longev **2016**: 1245049.
Domingues, L., D. W. Holden and L. J. Mota (2014). "The Salmonella effector SteA contributes to the control of membrane dynamics of Salmonella-containing vacuoles." Infect Immun **82**(7): 2923-2934.
Domingues, L., A. Ismail, N. Charro, I. Rodriguez-Escudero, D. W. Holden, M. Molina, V. J. Cid and L. J. Mota (2016). "The Salmonella effector SteA binds phosphatidylinositol 4-phosphate for subcellular targeting within host cells." Cell Microbiol **18**(7): 949-969.
Dong, N., Y. Zhu, Q. Lu, L. Hu, Y. Zheng and F. Shao (2012). "Structurally distinct bacterial TBC-like GAPs link Arf GTPase to Rab1 inactivation to counteract host defenses." Cell **150**(5): 1029-1041.
Dorsey, C. W., M. C. Laarakker, A. D. Humphries, E. H. Weening and A. J. Baumler (2005). "Salmonella enterica serotype Typhimurium MisL is an intestinal colonization factor that binds fibronectin." Mol Microbiol **57**(1): 196-211.
Dortet, L., S. Mostowy, A. Samba-Louaka, E. Gouin, M. A. Nahori, E. A. Wiemer, O. Dussurget and P. Cossart (2011). "Recruitment of the major vault protein by InlK: a Listeria monocytogenes strategy to avoid autophagy." PLoS Pathog **7**(8): e1002168.
Du, F. and J. E. Galan (2009). "Selective inhibition of type III secretion activated signaling by the Salmonella effector AvrA." PLoS Pathog **5**(9): e1000595.
Edwards, R. A., G. J. Olsen and S. R. Maloy (2002). "Comparative genomics of closely related salmonellae." Trends Microbiol **10**(2): 94-99.
Elkaim, R., S. Werner, L. Kocgozlu and H. Tenenbaum (2008). "P. gingivalis regulates the expression of Cathepsin B and Cystatin C." J Dent Res **87**(10): 932-936.
Ellermeier, C. D., J. R. Ellermeier and J. M. Slauch (2005). "HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in Salmonella enterica serovar Typhimurium." Mol Microbiol **57**(3): 691-705.
Elwell, C. A., S. Jiang, J. H. Kim, A. Lee, T. Wittmann, K. Hanada, P. Melancon and J. N. Engel (2011). "Chlamydia trachomatis co-opts GBF1 and CERT to acquire host sphingomyelin for distinct roles during intracellular development." PLoS Pathog **7**(9): e1002198.
Eswarappa, S. M., V. D. Negi, S. Chakraborty, B. K. Chandrasekhar Sagar and D. Chakravortty (2010). "Division of the Salmonella-containing vacuole and depletion of acidic lysosomes in Salmonella-infected host cells are novel strategies of Salmonella enterica to avoid lysosomes." Infect Immun **78**(1): 68-79.
Fairn, G. D. and S. Grinstein (2012). "How nascent phagosomes mature to become phagolysosomes." Trends Immunol **33**(8): 397-405.
Ferguson, T. W., P. Komenda and N. Tangri (2015). "Cystatin C as a biomarker for estimating glomerular filtration rate." Curr Opin Nephrol Hypertens **24**(3): 295-300.
Fields, K. A. and T. Hackstadt (2002). "The chlamydial inclusion: escape from the endocytic pathway." Annu Rev Cell Dev Biol **18**: 221-245.
Figueira, R., K. G. Watson, D. W. Holden and S. Helaine (2013). "Identification of salmonella pathogenicity island-2 type III secretion system effectors involved in intramacrophage replication of S. enterica serovar typhimurium: implications for rational vaccine design." mBio **4**(2): e00065.
Fiser A, Do RK, Sali A. Modeling of loops in protein structures. Protein Sci. 2000 Sep;9(9):1753-73. doi: 10.1110/ps.9.9.1753. PMID: 11045621; PMCID: PMC2144714.
Flannagan, R. S., G. Cosio and S. Grinstein (2009). "Antimicrobial mechanisms of phagocytes and bacterial evasion strategies." Nat Rev Microbiol **7**(5): 355-366.
Folkesson, A., S. Lofdahl and S. Normark (2002). "The Salmonella enterica subspecies I specific centisome 7 genomic island encodes novel protein families present in bacteria living in close contact with eukaryotic cells." Res Microbiol **153**(8): 537-545.
Fookes, M., G. N. Schroeder, G. C. Langridge, C. J. Blondel, C. Mammina, T. R. Connor, H. Seth-Smith, G. S. Vernikos, K. S. Robinson, M. Sanders, N. K. Petty, R. A. Kingsley, A. J. Baumler, S. P. Nuccio, I. Contreras, C. A. Santiviago, D. Maskell, P. Barrow, T. Humphrey, A. Nastasi, M. Roberts, G. Frankel, J. Parkhill, G. Dougan and N. R. Thomson (2011). "Salmonella bongori provides insights into the evolution of the Salmonellae." PLoS Pathog **7**(8): e1002191.
Forest, C. G., E. Ferraro, S. C. Sabbagh and F. Daigle (2010). "Intracellular survival of Salmonella enterica serovar Typhi in human macrophages is independent of Salmonella pathogenicity island (SPI)-2." Microbiology (Reading) **156**(Pt 12): 3689-3698.

Franchi, L., N. Warner, K. Viani and G. Nunez (2009). "Function of Nod-like receptors in microbial recognition and host defense." Immunol Rev **227**(1): 106-128.
Fratti, R. A., J. M. Backer, J. Gruenberg, S. Corvera and V. Deretic (2001). "Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest." J Cell Biol **154**(3): 631-644.
Fratti, R. A., J. Chua, I. Vergne and V. Deretic (2003). "Mycobacterium tuberculosis glycosylated phosphatidylinositol causes phagosome maturation arrest." Proc Natl Acad Sci U S A **100**(9): 5437-5442.
Freeman, J. A., M. E. Ohl and S. I. Miller (2003). "The Salmonella enterica serovar typhimurium translocated effectors SseJ and SifB are targeted to the Salmonella-containing vacuole." Infect Immun **71**(1): 418-427.
Friebel, A., H. Ilchmann, M. Aepfelbacher, K. Ehrbar, W. Machleidt and W. D. Hardt (2001). "SopE and SopE2 from Salmonella typhimurium activate different sets of RhoGTPases of the host cell." J Biol Chem **276**(36): 34035-34040.
Fu, Y. and J. E. Galan (1998). "The Salmonella typhimurium tyrosine phosphatase SptP is translocated into host cells and disrupts the actin cytoskeleton." Mol Microbiol **27**(2): 359-368.
Fu, Y. and J. E. Galan (1999). "A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion." Nature **401**(6750): 293-297.
Gago, G., Diacovich, L., & Gramajo, H. (2018). Lipid metabolism and its implication in mycobacteria–host interaction. Current opinion in microbiology, 41, 36-42.
Gal-Mor, O., E. C. Boyle and G. A. Grassl (2014). "Same species, different diseases: how and why typhoidal and non-typhoidal Salmonella enterica serovars differ." Front Microbiol **5**: 391.
Galan, J. E. (1996). "Molecular and cellular bases of Salmonella entry into host cells." Curr Top Microbiol Immunol **209**: 43-60.
Galan, J. E. (1999). "Interaction of Salmonella with host cells through the centisome 63 type III secretion system." Curr Opin Microbiol **2**(1): 46-50.
Galan, J. E. and A. Collmer (1999). "Type III secretion machines: bacterial devices for protein delivery into host cells." Science **284**(5418): 1322-1328.
Galan, J. E. and R. Curtiss, 3rd (1989). "Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells." Proc Natl Acad Sci U S A **86**(16): 6383-6387.
Galan, J. E. and G. Waksman (2018). "Protein-Injection Machines in Bacteria." Cell **172**(6): 1306-1318.
Galan, J. E. and H. Wolf-Watz (2006). "Protein delivery into eukaryotic cells by type III secretion machines." Nature **444**(7119): 567-573.
Ganley, I. G., K. Carroll, L. Bittova and S. Pfeffer (2004). "Rab9 GTPase regulates late endosome size and requires effector interaction for its stability." Mol Biol Cell **15**(12): 5420-5430.
Ganley, I. G. and S. R. Pfeffer (2006). "Cholesterol accumulation sequesters Rab9 and disrupts late endosome function in NPC1-deficient cells." J Biol Chem **281**(26): 17890-17899.
Garcia-del Portillo, F. and B. B. Finlay (1995). "Targeting of Salmonella typhimurium to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors." J Cell Biol **129**(1): 81-97.
Garcia-del Portillo, F., M. B. Zwick, K. Y. Leung and B. B. Finlay (1993). "Salmonella induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells." Proc Natl Acad Sci U S A **90**(22): 10544-10548.
Ge, J., H. Xu, T. Li, Y. Zhou, Z. Zhang, S. Li, L. Liu and F. Shao (2009). "A Legionella type IV effector activates the NF-kappaB pathway by phosphorylating the IkappaB family of inhibitors." Proc Natl Acad Sci U S A **106**(33): 13725-13730.
Ge, P., Z. Lei, Y. Yu, Z. Lu, L. Qiang, Q. Chai, Y. Zhang, D. Zhao, B. Li, Y. Pang, C. H. Liu and J. Wang (2022). "M. tuberculosis PknG manipulates host autophagy flux to promote pathogen intracellular survival." Autophagy **18**(3): 576-594.
Geddes, K., M. Worley, G. Niemann and F. Heffron (2005). "Identification of new secreted effectors in Salmonella enterica serovar Typhimurium." Infect Immun **73**(10): 6260-6271.
Gerlach, R. G. and M. Hensel (2007). "Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens." Int J Med Microbiol **297**(6): 401-415.
Gerlach, R. G. and M. Hensel (2007). "Salmonella pathogenicity islands in host specificity, host pathogen-interactions and antibiotics resistance of Salmonella enterica." Berl Munch Tierarztl Wochenschr **120**(7-8): 317-327.

Gerlach, R. G., D. Jackel, B. Stecher, C. Wagner, A. Lupas, W. D. Hardt and M. Hensel (2007). "Salmonella Pathogenicity Island 4 encodes a giant non-fimbrial adhesin and the cognate type 1 secretion system." Cell Microbiol **9**(7): 1834-1850.
Gewirtz, A. T., A. S. Rao, P. O. Simon, Jr., D. Merlin, D. Carnes, J. L. Madara and A. S. Neish (2000). "Salmonella typhimurium induces epithelial IL-8 expression via Ca(2+)-mediated activation of the NF-kappaB pathway." J Clin Invest **105**(1): 79-92.
Ghigo, E., M. I. Colombo and R. A. Heinzen (2012). "The Coxiella burnetii parasitophorous vacuole." Adv Exp Med Biol **984**: 141-169.
Ghosh, P. (2004). "Process of protein transport by the type III secretion system." Microbiol Mol Biol Rev **68**(4): 771-795.
Gokulan, K., S. Khare, A. W. Rooney, J. Han, A. M. Lynne and S. L. Foley (2013). "Impact of plasmids, including those encodingVirB4/D4 type IV secretion systems, on Salmonella enterica serovar Heidelberg virulence in macrophages and epithelial cells." PLoS One **8**(10): e77866.
Goley, E. D. and M. D. Welch (2006). "The ARP2/3 complex: an actin nucleator comes of age." Nat Rev Mol Cell Biol **7**(10): 713-726.
Green, E. R. and J. Mecsas (2016). "Bacterial Secretion Systems: An Overview." Microbiol Spectr **4**(1).
Groves, E., A. E. Dart, V. Covarelli and E. Caron (2008). "Molecular mechanisms of phagocytic uptake in mammalian cells." Cell Mol Life Sci **65**(13): 1957-1976.
Gruenberg, J. and F. G. van der Goot (2006). "Mechanisms of pathogen entry through the endosomal compartments." Nat Rev Mol Cell Biol **7**(7): 495-504.
Grund, S. and A. Weber (1988). "A new type of fimbriae on Salmonella typhimurium." Zentralbl Veterinarmed B **35**(10): 779-782.
Guiney, D. G. (2005). "The role of host cell death in Salmonella infections." Curr Top Microbiol Immunol **289**: 131-150.
Gutierrez, M. G., C. L. Vazquez, D. B. Munafo, F. C. Zoppino, W. Beron, M. Rabinovitch and M. I. Colombo (2005). "Autophagy induction favours the generation and maturation of the Coxiella-replicative vacuoles." Cell Microbiol **7**(7): 981-993.
Hacker, J., L. Bender, M. Ott, J. Wingender, B. Lund, R. Marre and W. Goebel (1990). "Deletions of chromosomal regions coding for fimbriae and hemolysins occur in vitro and in vivo in various extraintestinal Escherichia coli isolates." Microb Pathog **8**(3): 213-225.
Hackstadt, T. and J. C. Williams (1981). "Stability of the adenosine 5'-triphosphate pool in Coxiella burnetii: influence of pH and substrate." J Bacteriol **148**(2): 419-425.
Haenssler, E. and R. R. Isberg (2011). "Control of host cell phosphorylation by legionella pneumophila." Front Microbiol **2**: 64.
Haneda, T., Y. Ishii, H. Danbara and N. Okada (2009). "Genome-wide identification of novel genomic islands that contribute to Salmonella virulence in mouse systemic infection." FEMS Microbiol Lett **297**(2): 241-249.
Haneda, T., Y. Ishii, H. Shimizu, K. Ohshima, N. Iida, H. Danbara and N. Okada (2012). "Salmonella type III effector SpvC, a phosphothreonine lyase, contributes to reduction in inflammatory response during intestinal phase of infection." Cell Microbiol **14**(4): 485-499.
Haraga, A. and S. I. Miller (2003). "A Salmonella enterica serovar typhimurium translocated leucine-rich repeat effector protein inhibits NF-kappa B-dependent gene expression." Infect Immun **71**(7): 4052-4058.
Haraga, A. and S. I. Miller (2006). "A Salmonella type III secretion effector interacts with the mammalian serine/threonine protein kinase PKN1." Cell Microbiol **8**(5): 837-846.
Haraga, A., M. B. Ohlson and S. I. Miller (2008). "Salmonellae interplay with host cells." Nat Rev Microbiol **6**(1): 53-66.
Haraga, A., T. E. West, M. J. Brittnacher, S. J. Skerrett and S. I. Miller (2008). "Burkholderia thailandensis as a model system for the study of the virulence-associated type III secretion system of Burkholderia pseudomallei." Infect Immun **76**(11): 5402-5411.
Hardt, W. D., L. M. Chen, K. E. Schuebel, X. R. Bustelo and J. E. Galan (1998). "S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells." Cell **93**(5): 815-826.
Harrison, R. E., J. H. Brumell, A. Khandani, C. Bucci, C. C. Scott, X. Jiang, B. B. Finlay and S. Grinstein (2004). "Salmonella impairs RILP recruitment to Rab7 during maturation of invasion vacuoles." Mol Biol Cell **15**(7): 3146-3154.

Haves-Zburof, D., T. Paperna, A. Gour-Lavie, I. Mandel, L. Glass-Marmor and A. Miller (2011). "Cathepsins and their endogenous inhibitors cystatins: expression and modulation in multiple sclerosis." J Cell Mol Med **15**(11): 2421-2429.
Hayward, R. D., R. J. Cain, E. J. McGhie, N. Phillips, M. J. Garner and V. Koronakis (2005). "Cholesterol binding by the bacterial type III translocon is essential for virulence effector delivery into mammalian cells." Mol Microbiol **56**(3): 590-603.
Hayward, R. D. and V. Koronakis (1999). "Direct nucleation and bundling of actin by the SipC protein of invasive Salmonella." EMBO J **18**(18): 4926-4934.
Helaine, S., A. M. Cheverton, K. G. Watson, L. M. Faure, S. A. Matthews and D. W. Holden (2014). "Internalization of Salmonella by macrophages induces formation of nonreplicating persisters." Science **343**(6167): 204-208.
Henry, R., L. Shaughnessy, M. J. Loessner, C. Alberti-Segui, D. E. Higgins and J. A. Swanson (2006). "Cytolysin-dependent delay of vacuole maturation in macrophages infected with Listeria monocytogenes." Cell Microbiol **8**(1): 107-119.
Henry, T., C. Couillault, P. Rockenfeller, E. Boucrot, A. Dumont, N. Schroeder, A. Hermant, L. A. Knodler, P. Lecine, O. Steele-Mortimer, J. P. Borg, J. P. Gorvel and S. Meresse (2006). "The Salmonella effector protein PipB2 is a linker for kinesin-1." Proc Natl Acad Sci U S A **103**(36): 13497-13502.
Hensel, M. (2004). "Evolution of pathogenicity islands of Salmonella enterica." Int J Med Microbiol **294**(2-3): 95-102.
Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang and D. W. Holden (1998). "Genes encoding putative effector proteins of the type III secretion system of Salmonella pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages." Mol Microbiol **30**(1): 163-174.
Hernandez, L. D., K. Hueffer, M. R. Wenk and J. E. Galan (2004). "Salmonella modulates vesicular traffic by altering phosphoinositide metabolism." Science **304**(5678): 1805-1807.
Heuer, D., A. Rejman Lipinski, N. Machuy, A. Karlas, A. Wehrens, F. Siedler, V. Brinkmann and T. F. Meyer (2009). "Chlamydia causes fragmentation of the Golgi compartment to ensure reproduction." Nature **457**(7230): 731-735.
High, N., J. Mounier, M. C. Prevost and P. J. Sansonetti (1992). "IpaB of Shigella flexneri causes entry into epithelial cells and escape from the phagocytic vacuole." EMBO J **11**(5): 1991-1999.
Ho, B. T., T. G. Dong and J. J. Mekalanos (2014). "A view to a kill: the bacterial type VI secretion system." Cell Host Microbe **15**(1): 9-21.
Hobbie, S., L. M. Chen, R. J. Davis and J. E. Galan (1997). "Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by Salmonella typhimurium in cultured intestinal epithelial cells." J Immunol **159**(11): 5550-5559.
Holland, I. B., L. Schmitt and J. Young (2005). "Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway (review)." Mol Membr Biol **22**(1-2): 29-39.
Holt, K. E., N. R. Thomson, J. Wain, G. C. Langridge, R. Hasan, Z. A. Bhutta, M. A. Quail, H. Norbertczak, D. Walker, M. Simmonds, B. White, N. Bason, K. Mungall, G. Dougan and J. Parkhill (2009). "Pseudogene accumulation in the evolutionary histories of Salmonella enterica serovars Paratyphi A and Typhi." BMC Genomics **10**: 36.
Hottes, A. K., P. L. Freddolino, A. Khare, Z. N. Donnell, J. C. Liu and S. Tavazoie (2013). "Bacterial adaptation through loss of function." PLoS Genet **9**(7): e1003617.
Hueffer, K. and J. E. Galan (2004). "Salmonella-induced macrophage death: multiple mechanisms, different outcomes." Cell Microbiol **6**(11): 1019-1025.
Humphreys, D., P. J. Hume and V. Koronakis (2009). "The Salmonella effector SptP dephosphorylates host AAA+ ATPase VCP to promote development of its intracellular replicative niche." Cell Host Microbe **5**(3): 225-233.
Hurley, D., M. P. McCusker, S. Fanning and M. Martins (2014). "Salmonella-host interactions - modulation of the host innate immune system." Front Immunol **5**: 481.
Ilyas, B., C. N. Tsai and B. K. Coombes (2017). "Evolution of Salmonella-Host Cell Interactions through a Dynamic Bacterial Genome." Front Cell Infect Microbiol **7**: 428.
Ingmundson, A., A. Delprato, D. G. Lambright and C. R. Roy (2007). "Legionella pneumophila proteins that regulate Rab1 membrane cycling." Nature **450**(7168): 365-369.

Issenhuth-Jeanjean, S., P. Roggentin, M. Mikoleit, M. Guibourdenche, E. de Pinna, S. Nair, P. I. Fields and F. X. Weill (2014). "Supplement 2008-2010 (no. 48) to the White-Kauffmann-Le Minor scheme." Res Microbiol **165**(7): 526-530.
Jackson, L. K., P. Nawabi, C. Hentea, E. A. Roark and K. Haldar (2008). "The Salmonella virulence protein SifA is a G protein antagonist." Proc Natl Acad Sci U S A **105**(37): 14141-14146.
Jaffe, A. B. and A. Hall (2005). "Rho GTPases: biochemistry and biology." Annu Rev Cell Dev Biol **21**: 247-269.
Jennings, E., T. L. M. Thurston and D. W. Holden (2017). "Salmonella SPI-2 Type III Secretion System Effectors: Molecular Mechanisms And Physiological Consequences." Cell Host Microbe **22**(2): 217-231.
Jiang, L., P. Wang, X. Song, H. Zhang, S. Ma, J. Wang, W. Li, R. Lv, X. Liu, S. Ma, J. Yan, H. Zhou, D. Huang, Z. Cheng, C. Yang, L. Feng and L. Wang (2021). "Salmonella Typhimurium reprograms macrophage metabolism via T3SS effector SopE2 to promote intracellular replication and virulence." Nat Commun **12**(1): 879.
Jiang, X., O. W. Rossanese, N. F. Brown, S. Kujat-Choy, J. E. Galan, B. B. Finlay and J. H. Brumell (2004). "The related effector proteins SopD and SopD2 from Salmonella enterica serovar Typhimurium contribute to virulence during systemic infection of mice." Mol Microbiol **54**(5): 1186-1198.
Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H, Lee JO. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell. 2007 Sep 21;130(6):1071-82. doi: 10.1016/j.cell.2007.09.008. PMID: 17889651.
Jo, E. K., J. M. Yuk, D. M. Shin and C. Sasakawa (2013). "Roles of autophagy in elimination of intracellular bacterial pathogens." Front Immunol **4**: 97.
Johnson, R., A. Byrne, C. N. Berger, E. Klemm, V. F. Crepin, G. Dougan and G. Frankel (2017). "The Type III Secretion System Effector SptP of Salmonella enterica Serovar Typhi." J Bacteriol **199**(4).
Johnson, R., M. Ravenhall, D. Pickard, G. Dougan, A. Byrne and G. Frankel (2018). "Comparison of Salmonella enterica Serovars Typhi and Typhimurium Reveals Typhoidal Serovar-Specific Responses to Bile." Infect Immun **86**(3).
Jones, R. M., H. Wu, C. Wentworth, L. Luo, L. Collier-Hyams and A. S. Neish (2008). "Salmonella AvrA Coordinates Suppression of Host Immune and Apoptotic Defenses via JNK Pathway Blockade." Cell Host Microbe **3**(4): 233-244.
Jorgensen, I., M. M. Bednar, V. Amin, B. K. Davis, J. P. Ting, D. G. McCafferty and R. H. Valdivia (2011). "The Chlamydia protease CPAF regulates host and bacterial proteins to maintain pathogen vacuole integrity and promote virulence." Cell Host Microbe **10**(1): 21-32.
Joshi, A. D. and M. S. Swanson (2011). "Secrets of a successful pathogen: legionella resistance to progression along the autophagic pathway." Front Microbiol **2**: 138.
Juhas, M., J. R. van der Meer, M. Gaillard, R. M. Harding, D. W. Hood and D. W. Crook (2009). "Genomic islands: tools of bacterial horizontal gene transfer and evolution." FEMS Microbiol Rev **33**(2): 376-393.
Kagan, J. C., M. P. Stein, M. Pypaert and C. R. Roy (2004). "Legionella subvert the functions of Rab1 and Sec22b to create a replicative organelle." J Exp Med **199**(9): 1201-1211.
Kaniga, K., J. Uralil, J. B. Bliska and J. E. Galan (1996). "A secreted protein tyrosine phosphatase with modular effector domains in the bacterial pathogen Salmonella typhimurium." Mol Microbiol **21**(3): 633-641.
Karlinsey, J. E., T. A. Stepien, M. Mayho, L. A. Singletary, L. K. Bingham-Ramos, M. A. Brehm, D. L. Greiner, L. D. Shultz, L. A. Gallagher, M. Bawn, R. A. Kingsley, S. J. Libby and F. C. Fang (2019). "Genome-wide Analysis of Salmonella enterica serovar Typhi in Humanized Mice Reveals Key Virulence Features." Cell Host Microbe **26**(3): 426-434 e426.
Katoh, H. and M. Negishi (2003). "RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo." Nature **424**(6947): 461-464.
Kawai, Y., R. Mercier, K. Mickiewicz, A. Serafini, L. P. Sorio de Carvalho and J. Errington (2019). "Crucial role for central carbon metabolism in the bacterial L-form switch and killing by beta-lactam antibiotics." Nat Microbiol **4**(10): 1716-1726.
Kawai, Y., R. Mercier, L. J. Wu, P. Dominguez-Cuevas, T. Oshima and J. Errington (2015). "Cell growth of wall-free L-form bacteria is limited by oxidative damage." Curr Biol **25**(12): 1613-1618.
Keszei, A. F., X. Tang, C. McCormick, E. Zeqiraj, J. R. Rohde, M. Tyers and F. Sicheri (2014). "Structure of an SspH1-PKN1 complex reveals the basis for host substrate recognition and mechanism of activation for a bacterial E3 ubiquitin ligase." Mol Cell Biol **34**(3): 362-373.

Khan, M. Z., A. Bhaskar, S. Upadhyay, P. Kumari, R. S. Rajmani, P. Jain, A. Singh, D. Kumar, N. S. Bhavesh and V. K. Nandicoori (2017). "Protein kinase G confers survival advantage to Mycobacterium tuberculosis during latency-like conditions." J Biol Chem **292**(39): 16093-16108.
Killips B, Heaton EJB, Augusto L, Omsland A, Gilk SD. 2024. Coxiella burnetii inhibits nuclear translocation of TFEB, the master transcription factor for lysosomal biogenesis. J Bacteriol 206:e00150-24.
Kim, D. W., G. Lenzen, A. L. Page, P. Legrain, P. J. Sansonetti and C. Parsot (2005). "The Shigella flexneri effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes." Proc Natl Acad Sci U S A **102**(39): 14046-14051.
Kinchen, J. M. and K. S. Ravichandran (2008). "Phagosome maturation: going through the acid test." Nat Rev Mol Cell Biol **9**(10): 781-795.
Kirchweger, P., S. Weiler, C. Egerer-Sieber, A. T. Blasl, S. Hoffmann, C. Schmidt, N. Sander, D. Merker, R. G. Gerlach, M. Hensel and Y. A. Muller (2019). "Structural and functional characterization of SiiA, an auxiliary protein from the SPI4-encoded type 1 secretion system from Salmonella enterica." Mol Microbiol **112**(5): 1403-1422.
Kiss, T., E. Morgan and G. Nagy (2007). "Contribution of SPI-4 genes to the virulence of Salmonella enterica." FEMS Microbiol Lett **275**(1): 153-159.
Kita, E., F. Nishikawa, N. Kamikaidou, D. Oku, K. Yasui and S. Kashiba (1992). "Mechanism of the protective immunity against murine typhoid: persistence of Salmonella L forms in the liver after immunization with live-cell vaccines." FEMS Microbiol Immunol **5**(4): 191-199.
Knodler, L. A., J. Celli, W. D. Hardt, B. A. Vallance, C. Yip and B. B. Finlay (2002). "Salmonella effectors within a single pathogenicity island are differentially expressed and translocated by separate type III secretion systems." Mol Microbiol **43**(5): 1089-1103.
Knodler, L. A. and O. Steele-Mortimer (2005). "The Salmonella effector PipB2 affects late endosome/lysosome distribution to mediate Sif extension." Mol Biol Cell **16**(9): 4108-4123.
Knodler, L. A., B. A. Vallance, M. Hensel, D. Jackel, B. B. Finlay and O. Steele-Mortimer (2003). "Salmonella type III effectors PipB and PipB2 are targeted to detergent-resistant microdomains on internal host cell membranes." Mol Microbiol **49**(3): 685-704.
Knuff, K. and B. B. Finlay (2017). "What the SIF Is Happening-The Role of Intracellular Salmonella-Induced Filaments." Front Cell Infect Microbiol **7**: 335.
Kornev, A. P. and S. S. Taylor (2010). "Defining the conserved internal architecture of a protein kinase." Biochim Biophys Acta **1804**(3): 440-444.
Krieger, V., D. Liebl, Y. Zhang, R. Rajashekar, P. Chlanda, K. Giesker, D. Chikkaballi and M. Hensel (2014). "Reorganization of the endosomal system in Salmonella-infected cells: the ultrastructure of Salmonella-induced tubular compartments." PLoS Pathog **10**(9): e1004374.
Krishnan, P., M. Stalin and S. Balasubramanian (2009). "Changing trends in antimicrobial resistance of Salmonella enterica serovar typhi and salmonella enterica serovar paratyphi A in Chennai." Indian J Pathol Microbiol **52**(4): 505-508.
Kubori, T. and J. E. Galan (2003). "Temporal regulation of salmonella virulence effector function by proteasome-dependent protein degradation." Cell **115**(3): 333-342.
Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan and S. I. Aizawa (1998). "Supramolecular structure of the Salmonella typhimurium type III protein secretion system." Science **280**(5363): 602-605.
Kuhle, V. and M. Hensel (2002). "SseF and SseG are translocated effectors of the type III secretion system of Salmonella pathogenicity island 2 that modulate aggregation of endosomal compartments." Cell Microbiol **4**(12): 813-824.
Kuhle, V. and M. Hensel (2004). "Cellular microbiology of intracellular Salmonella enterica: functions of the type III secretion system encoded by Salmonella pathogenicity island 2." Cell Mol Life Sci **61**(22): 2812-2826.
Kumar, H., T. Kawai and S. Akira (2011). "Pathogen recognition by the innate immune system." Int Rev Immunol **30**(1): 16-34.
Kumar, Y. and R. H. Valdivia (2009). "Leading a sheltered life: intracellular pathogens and maintenance of vacuolar compartments." Cell Host Microbe **5**(6): 593-601.
Kurtz, J. R., J. A. Goggins and J. B. McLachlan (2017). "Salmonella infection: Interplay between the bacteria and host immune system." Immunol Lett **190**: 42-50.
Lamichhane, B., A. M. M. Mawad, M. Saleh, W. G. Kelley, P. J. Harrington, 2nd, C. W. Lovestad, J. Amezcua, M. M. Sarhan, M. E. El Zowalaty, H. Ramadan, M. Morgan and Y. A. Helmy (2024). "Salmonellosis: An

Overview of Epidemiology, Pathogenesis, and Innovative Approaches to Mitigate the Antimicrobial Resistant Infections." Antibiotics (Basel) **13**(1).
LaRock, D. L., P. S. Brzovic, I. Levin, M. P. Blanc and S. I. Miller (2012). "A Salmonella typhimurium-translocated glycerophospholipid:cholesterol acyltransferase promotes virulence by binding to the RhoA protein switch regions." J Biol Chem **287**(35): 29654-29663.
Laskowski RA, Rullmannn JA, MacArthur MW, Kaptein R, Thornton JM. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J Biomol NMR. 1996 Dec;8(4):477-86. doi: 10.1007/BF00228148. PMID: 9008363.
Latasa, C., A. Roux, A. Toledo-Arana, J. M. Ghigo, C. Gamazo, J. R. Penades and I. Lasa (2005). "BapA, a large secreted protein required for biofilm formation and host colonization of Salmonella enterica serovar Enteritidis." Mol Microbiol **58**(5): 1322-1339.
Lawley, T. D., K. Chan, L. J. Thompson, C. C. Kim, G. R. Govoni and D. M. Monack (2006). "Genome-wide screen for Salmonella genes required for long-term systemic infection of the mouse." PLoS Pathog **2**(2): e11.
Le Negrate, G., B. Faustin, K. Welsh, M. Loeffler, M. Krajewska, P. Hasegawa, S. Mukherjee, K. Orth, S. Krajewski, A. Godzik, D. G. Guiney and J. C. Reed (2008). "Salmonella secreted factor L deubiquitinase of Salmonella typhimurium inhibits NF-kappaB, suppresses IkappaBalpha ubiquitination and modulates innate immune responses." J Immunol **180**(7): 5045-5056.
Lee, C. A., M. Silva, A. M. Siber, A. J. Kelly, E. Galyov and B. A. McCormick (2000). "A secreted Salmonella protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration." Proc Natl Acad Sci U S A **97**(22): 12283-12288.
Lee, P. C. and M. P. Machner (2018). "The Legionella Effector Kinase LegK7 Hijacks the Host Hippo Pathway to Promote Infection." Cell Host Microbe **24**(3): 429-438 e426.
Lei, L., W. Wang, C. Xia and F. Liu (2016). "Salmonella Virulence Factor SsrAB Regulated Factor Modulates Inflammatory Responses by Enhancing the Activation of NF-kappaB Signaling Pathway." J Immunol **196**(2): 792-802.
Lenders, M. H., S. Weidtkamp-Peters, D. Kleinschrodt, K. E. Jaeger, S. H. Smits and L. Schmitt (2015). "Directionality of substrate translocation of the hemolysin A Type I secretion system." Sci Rep **5**: 12470.
Li, L., C. K. Cheng, M. K. Cheung, P. T. Law, J. M. Ling, K. M. Kam, W. M. Cheung and H. S. Kwan (2012). "Draft genome sequence of Salmonella enterica serovar Typhimurium ST1660/06, a multidrug-resistant clinical strain isolated from a diarrheic patient." J Bacteriol **194**(22): 6319-6320.
Li, X., Z. Wu, J. Ni, Y. Liu, J. Meng, W. Yu, H. Nakanishi and Y. Zhou (2016). "Cathepsin B Regulates Collagen Expression by Fibroblasts via Prolonging TLR2/NF-kappaB Activation." Oxid Med Cell Longev **2016**: 7894247.
Liang, S., K. B. Hosur, S. Lu, H. F. Nawar, B. R. Weber, R. I. Tapping, T. D. Connell and G. Hajishengallis (2009). "Mapping of a microbial protein domain involved in binding and activation of the TLR2/TLR1 heterodimer." J Immunol **182**(5): 2978-2985.
Lilic, M., V. E. Galkin, A. Orlova, M. S. VanLoock, E. H. Egelman and C. E. Stebbins (2003). "Salmonella SipA polymerizes actin by stapling filaments with nonglobular protein arms." Science **301**(5641): 1918-1921.
Lin, H., J. Xing, H. Wang, S. Wang, R. Fang, X. Li, Z. Li and N. Song (2024). "Roles of Lipolytic enzymes in Mycobacterium tuberculosis pathogenesis." Front Microbiol **15**: 1329715.
Lin, S. L., T. X. Le and D. S. Cowen (2003). "SptP, a Salmonella typhimurium type III-secreted protein, inhibits the mitogen-activated protein kinase pathway by inhibiting Raf activation." Cell Microbiol **5**(4): 267-275.
Liu, J., J. T. Guo, Y. G. Li, R. N. Johnston, G. R. Liu and S. L. Liu (2013). "The type VI secretion system gene cluster of Salmonella typhimurium: required for full virulence in mice." J Basic Microbiol **53**(7): 600-607.
Lockman, H. A. and R. Curtiss, 3rd (1992). "Isolation and characterization of conditional adherent and non-type 1 fimbriated Salmonella typhimurium mutants." Mol Microbiol **6**(7): 933-945.
Lopez, J., P. M. Ly and M. F. Feldman (2020). "The Tip of the VgrG Spike Is Essential to Functional Type VI Secretion System Assembly in Acinetobacter baumannii." mBio **11**(1).
Lossi, N. S., N. Rolhion, A. I. Magee, C. Boyle and D. W. Holden (2008). "The Salmonella SPI-2 effector SseJ exhibits eukaryotic activator-dependent phospholipase A and glycerophospholipid : cholesterol acyltransferase activity." Microbiology (Reading) **154**(Pt 9): 2680-2688.
Lostroh, C. P. and C. A. Lee (2001). "The Salmonella pathogenicity island-1 type III secretion system." Microbes Infect **3**(14-15): 1281-1291.
Lucas, R. L. and C. A. Lee (2001). "Roles of hilC and hilD in regulation of hilA expression in Salmonella enterica serovar Typhimurium." J Bacteriol **183**(9): 2733-2745.

Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M. P. Spector, B. L. Wanner and C. A. Lee (2000). "Multiple factors independently regulate hilA and invasion gene expression in Salmonella enterica serovar typhimurium." J Bacteriol **182**(7): 1872-1882.
Lüthy R, Bowie JU, Eisenberg D. Assessment of protein models with three-dimensional profiles. Nature. 1992 Mar 5;356(6364):83-5. doi: 10.1038/356083a0. PMID: 1538787.
Ma, L., Q. Ouyang, G. C. Werthmann, H. M. Thompson and E. M. Morrow (2017). "Live-cell Microscopy and Fluorescence-based Measurement of Luminal pH in Intracellular Organelles." Front Cell Dev Biol **5**: 71.
Machner, M. P. and R. R. Isberg (2006). "Targeting of host Rab GTPase function by the intravacuolar pathogen Legionella pneumophila." Dev Cell **11**(1): 47-56.
Main-Hester, K. L., K. M. Colpitts, G. A. Thomas, F. C. Fang and S. J. Libby (2008). "Coordinate regulation of Salmonella pathogenicity island 1 (SPI1) and SPI4 in Salmonella enterica serovar Typhimurium." Infect Immun **76**(3): 1024-1035.
Malik-Kale, P., C. E. Jolly, S. Lathrop, S. Winfree, C. Luterbach and O. Steele-Mortimer (2011). "Salmonella - at home in the host cell." Front Microbiol **2**: 125.
Marcus, S. L., M. R. Wenk, O. Steele-Mortimer and B. B. Finlay (2001). "A synaptojanin-homologous region of Salmonella typhimurium SigD is essential for inositol phosphatase activity and Akt activation." FEBS Lett **494**(3): 201-207.
Marlovits, T. C., T. Kubori, A. Sukhan, D. R. Thomas, J. E. Galan and V. M. Unger (2004). "Structural insights into the assembly of the type III secretion needle complex." Science **306**(5698): 1040-1042.
Marquis, H., V. Doshi and D. A. Portnoy (1995). "The broad-range phospholipase C and a metalloprotease mediate listeriolysin O-independent escape of Listeria monocytogenes from a primary vacuole in human epithelial cells." Infect Immun **63**(11): 4531-4534.
Marquis, H. and E. J. Hager (2000). "pH-regulated activation and release of a bacteria-associated phospholipase C during intracellular infection by Listeria monocytogenes." Mol Microbiol **35**(2): 289-298.
Martinez, E., S. Huc-Brandt, S. Brelle, J. Allombert, F. Cantet, L. Gannoun-Zaki, M. Burette, M. Martin, F. Letourneur, M. Bonazzi and V. Molle (2020). "The secreted protein kinase CstK from Coxiella burnetii influences vacuole development and interacts with the GTPase-activating host protein TBC1D5." J Biol Chem **295**(21): 7391-7403.
Maurelli, A. T. (2007). "Black holes, antivirulence genes, and gene inactivation in the evolution of bacterial pathogens." FEMS Microbiol Lett **267**(1): 1-8.
Maurelli, A. T., R. E. Fernandez, C. A. Bloch, C. K. Rode and A. Fasano (1998). ""Black holes" and bacterial pathogenicity: a large genomic deletion that enhances the virulence of Shigella spp. and enteroinvasive Escherichia coli." Proc Natl Acad Sci U S A **95**(7): 3943-3948.
Mazurkiewicz, P., J. Thomas, J. A. Thompson, M. Liu, L. Arbibe, P. Sansonetti and D. W. Holden (2008). "SpvC is a Salmonella effector with phosphothreonine lyase activity on host mitogen-activated protein kinases." Mol Microbiol **67**(6): 1371-1383.
McClelland, M., K. E. Sanderson, S. W. Clifton, P. Latreille, S. Porwollik, A. Sabo, R. Meyer, T. Bieri, P. Ozersky, M. McLellan, C. R. Harkins, C. Wang, C. Nguyen, A. Berghoff, G. Elliott, S. Kohlberg, C. Strong, F. Du, J. Carter, C. Kremizki, D. Layman, S. Leonard, H. Sun, L. Fulton, W. Nash, T. Miner, P. Minx, K. Delehaunty, C. Fronick, V. Magrini, M. Nhan, W. Warren, L. Florea, J. Spieth and R. K. Wilson (2004). "Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of Salmonella enterica that cause typhoid." Nat Genet **36**(12): 1268-1274.
McCoy, C. E. (2016). "Preface." Methods Mol Biol **1390**: v.
McDowell, M. A., A. M. Byrne, E. Mylona, R. Johnson, A. Sagfors, V. F. Crepin, S. Lea and G. Frankel (2019). "The S. Typhi effector StoD is an E3/E4 ubiquitin ligase which binds K48- and K63-linked diubiquitin." Life Sci Alliance **2**(3).
McGhie, E. J., R. D. Hayward and V. Koronakis (2001). "Cooperation between actin-binding proteins of invasive Salmonella: SipA potentiates SipC nucleation and bundling of actin." EMBO J **20**(9): 2131-2139.
McGhie, E. J., R. D. Hayward and V. Koronakis (2004). "Control of actin turnover by a salmonella invasion protein." Mol Cell **13**(4): 497-510.
McGourty, K., T. L. Thurston, S. A. Matthews, L. Pinaud, L. J. Mota and D. W. Holden (2012). "Salmonella inhibits retrograde trafficking of mannose-6-phosphate receptors and lysosome function." Science **338**(6109): 963-967.

McLaughlin, L. M., G. R. Govoni, C. Gerke, S. Gopinath, K. Peng, G. Laidlaw, Y. H. Chien, H. W. Jeong, Z. Li, M. D. Brown, D. B. Sacks and D. Monack (2009). "The Salmonella SPI2 effector SseI mediates long-term systemic infection by modulating host cell migration." PLoS Pathog **5**(11): e1000671.
McQuate, S. E., A. M. Young, E. Silva-Herzog, E. Bunker, M. Hernandez, F. de Chaumont, X. Liu, C. S. Detweiler and A. E. Palmer (2017). "Long-term live-cell imaging reveals new roles for Salmonella effector proteins SseG and SteA." Cell Microbiol **19**(1).
Meresse, S., O. Steele-Mortimer, B. B. Finlay and J. P. Gorvel (1999). "The rab7 GTPase controls the maturation of Salmonella typhimurium-containing vacuoles in HeLa cells." EMBO J **18**(16): 4394-4403.
Mesquita, F. S., M. Thomas, M. Sachse, A. J. Santos, R. Figueira and D. W. Holden (2012). "The Salmonella deubiquitinase SseL inhibits selective autophagy of cytosolic aggregates." PLoS Pathog **8**(6): e1002743.
Miao, E. A., C. A. Scherer, R. M. Tsolis, R. A. Kingsley, L. G. Adams, A. J. Baumler and S. I. Miller (1999). "Salmonella typhimurium leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems." Mol Microbiol **34**(4): 850-864.
Michard, C., D. Sperandio, N. Bailo, J. Pizarro-Cerda, L. LeClaire, E. Chadeau-Argaud, I. Pombo-Gregoire, E. Hervet, A. Vianney, C. Gilbert, M. Faure, P. Cossart and P. Doublet (2015). "The Legionella Kinase LegK2 Targets the ARP2/3 Complex To Inhibit Actin Nucleation on Phagosomes and Allow Bacterial Evasion of the Late Endocytic Pathway." mBio **6**(3): e00354-00315.
Milligan, R., M. Paul, M. Richardson and A. Neuberger (2018). "Vaccines for preventing typhoid fever." Cochrane Database Syst Rev **5**(5): CD001261.
Mills, D. M., V. Bajaj and C. A. Lee (1995). "A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome." Mol Microbiol **15**(4): 749-759.
Mital, J., N. J. Miller, E. R. Fischer and T. Hackstadt (2010). "Specific chlamydial inclusion membrane proteins associate with active Src family kinases in microdomains that interact with the host microtubule network." Cell Microbiol **12**(9): 1235-1249.
Mizushima, N. and M. Komatsu (2011). "Autophagy: renovation of cells and tissues." Cell **147**(4): 728-741.
Monack, D. M., C. S. Detweiler and S. Falkow (2001). "Salmonella pathogenicity island 2-dependent macrophage death is mediated in part by the host cysteine protease caspase-1." Cell Microbiol **3**(12): 825-837.
Moore, E. R., E. R. Fischer, D. J. Mead and T. Hackstadt (2008). "The chlamydial inclusion preferentially intercepts basolaterally directed sphingomyelin-containing exocytic vacuoles." Traffic **9**(12): 2130-2140.
Moore, R. A., S. Reckseidler-Zenteno, H. Kim, W. Nierman, Y. Yu, A. Tuanyok, J. Warawa, D. DeShazer and D. E. Woods (2004). "Contribution of gene loss to the pathogenic evolution of Burkholderia pseudomallei and Burkholderia mallei." Infect Immun **72**(7): 4172-4187.
Moreau, T., J. Gautron, M. T. Hincke, P. Monget, S. Rehault-Godbert and N. Guyot (2022). "Antimicrobial Proteins and Peptides in Avian Eggshell: Structural Diversity and Potential Roles in Biomineralization." Front Immunol **13**: 946428.
Morgan, E., A. J. Bowen, S. C. Carnell, T. S. Wallis and M. P. Stevens (2007). "SiiE is secreted by the Salmonella enterica serovar Typhimurium pathogenicity island 4-encoded secretion system and contributes to intestinal colonization in cattle." Infect Immun **75**(3): 1524-1533.
Morgan, E., J. D. Campbell, S. C. Rowe, J. Bispham, M. P. Stevens, A. J. Bowen, P. A. Barrow, D. J. Maskell and T. S. Wallis (2004). "Identification of host-specific colonization factors of Salmonella enterica serovar Typhimurium." Mol Microbiol **54**(4): 994-1010.
Morgan, M. J. and Z. G. Liu (2011). "Crosstalk of reactive oxygen species and NF-kappaB signaling." Cell Res **21**(1): 103-115.
Moss, S. M., I. R. Taylor, D. Ruggero, J. E. Gestwicki, K. M. Shokat and S. Mukherjee (2019). "A Legionella pneumophila Kinase Phosphorylates the Hsp70 Chaperone Family to Inhibit Eukaryotic Protein Synthesis." Cell Host Microbe **25**(3): 454-462 e456.
Mostowy, S., M. Bonazzi, M. A. Hamon, T. N. Tham, A. Mallet, M. Lelek, E. Gouin, C. Demangel, R. Brosch, C. Zimmer, A. Sartori, M. Kinoshita, M. Lecuit and P. Cossart (2010). "Entrapment of intracytosolic bacteria by septin cage-like structures." Cell Host Microbe **8**(5): 433-444.
Mostowy, S. and P. Cossart (2011). "Autophagy and the cytoskeleton: new links revealed by intracellular pathogens." Autophagy **7**(7): 780-782.
Mougous, J. D., C. A. Gifford, T. L. Ramsdell and J. J. Mekalanos (2007). "Threonine phosphorylation post-translationally regulates protein secretion in Pseudomonas aeruginosa." Nat Cell Biol **9**(7): 797-803.

Mukherjee, K., S. Parashuraman, M. Raje and A. Mukhopadhyay (2001). "SopE acts as an Rab5-specific nucleotide exchange factor and recruits non-prenylated Rab5 on Salmonella-containing phagosomes to promote fusion with early endosomes." J Biol Chem **276**(26): 23607-23615.
Muller, M. P., H. Peters, J. Blumer, W. Blankenfeldt, R. S. Goody and A. Itzen (2010). "The Legionella effector protein DrrA AMPylates the membrane traffic regulator Rab1b." Science **329**(5994): 946-949.
Murata, T., A. Delprato, A. Ingmundson, D. K. Toomre, D. G. Lambright and C. R. Roy (2006). "The Legionella pneumophila effector protein DrrA is a Rab1 guanine nucleotide-exchange factor." Nat Cell Biol **8**(9): 971-977.
Murli, S., R. O. Watson and J. E. Galan (2001). "Role of tyrosine kinases and the tyrosine phosphatase SptP in the interaction of Salmonella with host cells." Cell Microbiol **3**(12): 795-810.
Nagai, H., J. C. Kagan, X. Zhu, R. A. Kahn and C. R. Roy (2002). "A bacterial guanine nucleotide exchange factor activates ARF on Legionella phagosomes." Science **295**(5555): 679-682.
Nair, S., P. A. Ramaswamy, S. Ghosh, D. C. Joshi, N. Pathak, I. Siddiqui, P. Sharma, S. E. Hasnain, S. C. Mande and S. Mukhopadhyay (2009). "The PPE18 of Mycobacterium tuberculosis interacts with TLR2 and activates IL-10 induction in macrophage." J Immunol **183**(10): 6269-6281.
Naito, Y., M. Sasaki, T. Umemoto, I. Namikawa, K. Sakae, Y. Ishihara, S. Isomura and I. Suzuki (1995). "Bactericidal effect of rat cystatin S on an oral bacterium Porphyromonas gingivalis." Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **110**(1): 71-75.
Nawabi, P., D. M. Catron and K. Haldar (2008). "Esterification of cholesterol by a type III secretion effector during intracellular Salmonella infection." Mol Microbiol **68**(1): 173-185.
Newton, H. J., D. K. Ang, I. R. van Driel and E. L. Hartland (2010). "Molecular pathogenesis of infections caused by Legionella pneumophila." Clin Microbiol Rev **23**(2): 274-298.
Nguyen, J. A. and R. M. Yates (2021). "Better Together: Current Insights Into Phagosome-Lysosome Fusion." Front Immunol **12**: 636078.
Ni, J., Z. Wu, C. Peterts, K. Yamamoto, H. Qing and H. Nakanishi (2015). "The Critical Role of Proteolytic Relay through Cathepsins B and E in the Phenotypic Change of Microglia/Macrophage." J Neurosci **35**(36): 12488-12501.
Norris, F. A., M. P. Wilson, T. S. Wallis, E. E. Galyov and P. W. Majerus (1998). "SopB, a protein required for virulence of Salmonella dublin, is an inositol phosphate phosphatase." Proc Natl Acad Sci U S A **95**(24): 14057-14059.
Notti, R. Q. and C. E. Stebbins (2016). "The Structure and Function of Type III Secretion Systems." Microbiol Spectr **4**(1).
Oberle C, Huai J, Reinheckel T, Tacke M, Rassner M, Ekert PG, Buellesbach J, Borner C. Lysosomal membrane permeabilization and cathepsin release is a Bax/Bak-dependent, amplifying event of apoptosis in fibroblasts and monocytes. Cell Death Differ. 2010 Jul;17(7):1167-78. doi: 10.1038/cdd.2009.214. Epub 2010 Jan 22. PMID: 20094062.
Odendall, C., N. Rolhion, A. Forster, J. Poh, D. J. Lamont, M. Liu, P. S. Freemont, A. D. Catling and D. W. Holden (2012). "The Salmonella kinase SteC targets the MAP kinase MEK to regulate the host actin cytoskeleton." Cell Host Microbe **12**(5): 657-668.
Ogawa, M., H. Mimuro, Y. Yoshikawa, H. Ashida and C. Sasakawa (2011). "Manipulation of autophagy by bacteria for their own benefit." Microbiol Immunol **55**(7): 459-471.
Ogawa, M. and C. Sasakawa (2011). "The role of Tecpr1 in selective autophagy as a cargo receptor." Autophagy **7**(11): 1389-1391.
Ogawa, M., T. Yoshimori, T. Suzuki, H. Sagara, N. Mizushima and C. Sasakawa (2005). "Escape of intracellular Shigella from autophagy." Science **307**(5710): 727-731.
Ohlson, M. B., K. Fluhr, C. L. Birmingham, J. H. Brumell and S. I. Miller (2005). "SseJ deacylase activity by Salmonella enterica serovar Typhimurium promotes virulence in mice." Infect Immun **73**(10): 6249-6259.
Ohlson, M. B., Z. Huang, N. M. Alto, M. P. Blanc, J. E. Dixon, J. Chai and S. I. Miller (2008). "Structure and function of Salmonella SifA indicate that its interactions with SKIP, SseJ, and RhoA family GTPases induce endosomal tubulation." Cell Host Microbe **4**(5): 434-446.
Page, A. L., H. Ohayon, P. J. Sansonetti and C. Parsot (1999). "The secreted IpaB and IpaC invasins and their cytoplasmic chaperone IpgC are required for intercellular dissemination of Shigella flexneri." Cell Microbiol **1**(2): 183-193.
Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S.

Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead and B. G. Barrell (2001). "Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18." Nature **413**(6858): 848-852.
Patel, J. C. and J. E. Galan (2005). "Manipulation of the host actin cytoskeleton by Salmonella--all in the name of entry." Curr Opin Microbiol **8**(1): 10-15.
Patel, J. C. and J. E. Galan (2006). "Differential activation and function of Rho GTPases during Salmonella-host cell interactions." J Cell Biol **175**(3): 453-463.
Patrick, M. E., P. M. Adcock, T. M. Gomez, S. F. Altekruse, B. H. Holland, R. V. Tauxe and D. L. Swerdlow (2004). "Salmonella enteritidis infections, United States, 1985-1999." Emerg Infect Dis **10**(1): 1-7.
Pattanaik, K. P., G. Ganguli, S. K. Naik and A. Sonawane (2021). "Mycobacterium tuberculosis EsxL induces TNF-alpha secretion through activation of TLR2 dependent MAPK and NF-kappaB pathways." Mol Immunol **130**: 133-141.
Pegues, D. A., M. J. Hantman, I. Behlau and S. I. Miller (1995). "PhoP/PhoQ transcriptional repression of Salmonella typhimurium invasion genes: evidence for a role in protein secretion." Mol Microbiol **17**(1): 169-181.
Pezoa, D., C. J. Blondel, C. A. Silva, H. J. Yang, H. Andrews-Polymenis, C. A. Santiviago and I. Contreras (2014). "Only one of the two type VI secretion systems encoded in the Salmonella enterica serotype Dublin genome is involved in colonization of the avian and murine hosts." Vet Res **45**(1): 2.
Pickard, D., J. Wain, S. Baker, A. Line, S. Chohan, M. Fookes, A. Barron, P. O. Gaora, J. A. Chabalgoity, N. Thanky, C. Scholes, N. Thomson, M. Quail, J. Parkhill and G. Dougan (2003). "Composition, acquisition, and distribution of the Vi exopolysaccharide-encoding Salmonella enterica pathogenicity island SPI-7." J Bacteriol **185**(17): 5055-5065.
Pietila, T. E., V. Veckman, P. Kyllonen, K. Lahteenmaki, T. K. Korhonen and I. Julkunen (2005). "Activation, cytokine production, and intracellular survival of bacteria in Salmonella-infected human monocyte-derived macrophages and dendritic cells." J Leukoc Biol **78**(4): 909-920.
Pikula, M., M. Smuzynska, A. Krzystyniak, M. Zielinski, P. Langa, M. Deptula, A. Schumacher, J. Lata, M. Cichorek, A. Grubb, P. Trzonkowski, F. Kasprzykowski and S. Rodziewicz-Motowidlo (2017). "Cystatin C peptidomimetic derivative with antimicrobial properties as a potential compound against wound infections." Bioorg Med Chem **25**(4): 1431-1439.
Pilar, A. V., S. A. Reid-Yu, C. A. Cooper, D. T. Mulder and B. K. Coombes (2012). "GogB is an anti-inflammatory effector that limits tissue damage during Salmonella infection through interaction with human FBXO22 and Skp1." PLoS Pathog **8**(6): e1002773.
Pinas, G. E., N. M. Reinoso-Vizcaino, N. Y. Yandar Barahona, P. R. Cortes, R. Duran, C. Badapanda, A. Rathore, D. R. Bichara, M. B. Cian, N. B. Olivero, D. R. Perez and J. Echenique (2018). "Crosstalk between the serine/threonine kinase StkP and the response regulator ComE controls the stress response and intracellular survival of Streptococcus pneumoniae." PLoS Pathog **14**(6): e1007118.
Pires, D., M. Calado, T. Velez, M. Mandal, M. J. Catalao, O. Neyrolles, G. Lugo-Villarino, C. Verollet, J. M. Azevedo-Pereira and E. Anes (2021). "Modulation of Cystatin C in Human Macrophages Improves Anti-Mycobacterial Immune Responses to Mycobacterium tuberculosis Infection and Coinfection With HIV." Front Immunol **12**: 742822.
Pizarro-Cerda, J., A. Kuhbacher and P. Cossart (2012). "Entry of Listeria monocytogenes in mammalian epithelial cells: an updated view." Cold Spring Harb Perspect Med **2**(11).
Poh, W. H., J. Lin, B. Colley, N. Muller, B. C. Goh, D. Schleheck, A. El Sahili, A. Marquardt, Y. Liang, S. Kjelleberg, J. Lescar, S. A. Rice and J. Klebensberger (2020). "The SiaABC threonine phosphorylation pathway controls biofilm formation in response to carbon availability in Pseudomonas aeruginosa." PLoS One **15**(11): e0241019.
Popoff, M. Y., J. Bockemuhl, F. W. Brenner and L. L. Gheesling (2001). "Supplement 2000 (no. 44) to the Kauffmann-White scheme." Res Microbiol **152**(10): 907-909.
Porte, F., A. Naroeni, S. Ouahrani-Bettache and J. P. Liautard (2003). "Role of the Brucella suis lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome fusion in murine macrophages." Infect Immun **71**(3): 1481-1490.
Portnoy, D. A., V. Auerbuch and I. J. Glomski (2002). "The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity." J Cell Biol **158**(3): 409-414.
Pradhan, G., R. Shrivastva and S. Mukhopadhyay (2018). "Mycobacterial PknG Targets the Rab7l1 Signaling Pathway To Inhibit Phagosome-Lysosome Fusion." J Immunol **201**(5): 1421-1433.

Pukatzki, S., A. T. Ma, D. Sturtevant, B. Krastins, D. Sarracino, W. C. Nelson, J. F. Heidelberg and J. J. Mekalanos (2006). "Identification of a conserved bacterial protein secretion system in Vibrio cholerae using the Dictyostelium host model system." Proc Natl Acad Sci U S A **103**(5): 1528-1533.
Pronk S, Páll S, Schulz R, Larsson P, Bjelkmar P, Apostolov R, Shirts MR, Smith JC, Kasson PM, van der Spoel D, Hess B, Lindahl E. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. Bioinformatics. 2013 Apr 1;29(7):845-54. doi: 10.1093/bioinformatics/btt055. Epub 2013 Feb 13. PMID: 23407358; PMCID: PMC3605599.
Qamar, F. N., M. T. Yousafzai, M. Khalid, A. M. Kazi, H. Lohana, S. Karim, A. Khan, A. Hotwani, S. Qureshi, F. Kabir, F. Aziz, N. M. Memon, M. H. Domki and R. Hasan (2018). "Outbreak investigation of ceftriaxone-resistant Salmonella enterica serotype Typhi and its risk factors among the general population in Hyderabad, Pakistan: a matched case-control study." Lancet Infect Dis **18**(12): 1368-1376.
Qi, X., S. M. Man, R. K. Malireddi, R. Karki, C. Lupfer, P. Gurung, G. Neale, C. S. Guy, M. Lamkanfi and T. D. Kanneganti (2016). "Cathepsin B modulates lysosomal biogenesis and host defense against Francisella novicida infection." J Exp Med **213**(10): 2081-2097.
Quezada, C. M., S. W. Hicks, J. E. Galan and C. E. Stebbins (2009). "A family of Salmonella virulence factors functions as a distinct class of autoregulated E3 ubiquitin ligases." Proc Natl Acad Sci U S A **106**(12): 4864-4869.
Radulovic, M., K. O. Schink, E. M. Wenzel, V. Nahse, A. Bongiovanni, F. Lafont and H. Stenmark (2018). "ESCRT-mediated lysosome repair precedes lysophagy and promotes cell survival." EMBO J **37**(21).
Ragaz, C., H. Pietsch, S. Urwyler, A. Tiaden, S. S. Weber and H. Hilbi (2008). "The Legionella pneumophila phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole." Cell Microbiol **10**(12): 2416-2433.
Rajashekar, R., D. Liebl, A. Seitz and M. Hensel (2008). "Dynamic remodeling of the endosomal system during formation of Salmonella-induced filaments by intracellular Salmonella enterica." Traffic **9**(12): 2100-2116.
Ramsden, A. E., L. J. Mota, S. Munter, S. L. Shorte and D. W. Holden (2007). "The SPI-2 type III secretion system restricts motility of Salmonella-containing vacuoles." Cell Microbiol **9**(10): 2517-2529.
Renesto, P., P. Dehoux, E. Gouin, L. Touqui, P. Cossart and D. Raoult (2003). "Identification and characterization of a phospholipase D-superfamily gene in rickettsiae." J Infect Dis **188**(9): 1276-1283.
Rhen, M. (2019). "Salmonella and Reactive Oxygen Species: A Love-Hate Relationship." J Innate Immun **11**(3): 216-226.
Ribet, D. and P. Cossart (2015). "How bacterial pathogens colonize their hosts and invade deeper tissues." Microbes Infect **17**(3): 173-183.
Riquelme, S., M. Varas, C. Valenzuela, P. Velozo, N. Chahin, P. Aguilera, A. Sabag, B. Labra, S. A. Alvarez, F. P. Chavez and C. A. Santiviago (2016). "Relevant Genes Linked to Virulence Are Required for Salmonella Typhimurium to Survive Intracellularly in the Social Amoeba Dictyostelium discoideum." Front Microbiol **7**: 1305.
Rodriguez-Franco, E. J., Y. M. Cantres-Rosario, M. Plaud-Valentin, R. Romeu, Y. Rodriguez, R. Skolasky, V. Melendez, C. L. Cadilla and L. M. Melendez (2012). "Dysregulation of macrophage-secreted cathepsin B contributes to HIV-1-linked neuronal apoptosis." PLoS One **7**(5): e36571.
Romano, P. S., M. G. Gutierrez, W. Beron, M. Rabinovitch and M. I. Colombo (2007). "The autophagic pathway is actively modulated by phase II Coxiella burnetii to efficiently replicate in the host cell." Cell Microbiol **9**(4): 891-909.
Ronzone, E. and F. Paumet (2013). "Two coiled-coil domains of Chlamydia trachomatis IncA affect membrane fusion events during infection." PLoS One **8**(7): e69769.
Rowe, B., L. R. Ward and E. J. Threlfall (1997). "Multidrug-resistant Salmonella typhi: a worldwide epidemic." Clin Infect Dis **24 Suppl 1**: S106-109.
Roy Chowdhury, A., S. Sah, U. Varshney and D. Chakravortty (2022). "Salmonella Typhimurium outer membrane protein A (OmpA) renders protection from nitrosative stress of macrophages by maintaining the stability of bacterial outer membrane." PLoS Pathog **18**(8): e1010708.
Ruiz-Albert, J., X. J. Yu, C. R. Beuzon, A. N. Blakey, E. E. Galyov and D. W. Holden (2002). "Complementary activities of SseJ and SifA regulate dynamics of the Salmonella typhimurium vacuolar membrane." Mol Microbiol **44**(3): 645-661.
Rytkonen, A., J. Poh, J. Garmendia, C. Boyle, A. Thompson, M. Liu, P. Freemont, J. C. Hinton and D. W. Holden (2007). "SseL, a Salmonella deubiquitinase required for macrophage killing and virulence." Proc Natl Acad Sci U S A **104**(9): 3502-3507.

Rzomp, K. A., A. R. Moorhead and M. A. Scidmore (2006). "The GTPase Rab4 interacts with Chlamydia trachomatis inclusion membrane protein CT229." Infect Immun **74**(9): 5362-5373.
Rzomp, K. A., L. D. Scholtes, B. J. Briggs, G. R. Whittaker and M. A. Scidmore (2003). "Rab GTPases are recruited to chlamydial inclusions in both a species-dependent and species-independent manner." Infect Immun **71**(10): 5855-5870.
Sabbagh, S. C., C. G. Forest, C. Lepage, J. M. Leclerc and F. Daigle (2010). "So similar, yet so different: uncovering distinctive features in the genomes of Salmonella enterica serovars Typhimurium and Typhi." FEMS Microbiol Lett **305**(1): 1-13.
Sabbagh, S. C., C. Lepage, M. McClelland and F. Daigle (2012). "Selection of Salmonella enterica serovar Typhi genes involved during interaction with human macrophages by screening of a transposon mutant library." PLoS One **7**(5): e36643.
Sachdeva, K. and V. Sundaramurthy (2020). "The Interplay of Host Lysosomes and Intracellular Pathogens." Front Cell Infect Microbiol **10**: 595502.
Sadowski MI, Jones DT. The sequence-structure relationship and protein function prediction. Curr Opin Struct Biol. 2009 Jun;19(3):357-62. doi: 10.1016/j.sbi.2009.03.008. Epub 2009 May 4. PMID: 19406632.
Salcedo, S. P. and D. W. Holden (2003). "SseG, a virulence protein that targets Salmonella to the Golgi network." EMBO J **22**(19): 5003-5014.
Saliba, A. E., L. Li, A. J. Westermann, S. Appenzeller, D. A. Stapels, L. N. Schulte, S. Helaine and J. Vogel (2016). "Single-cell RNA-seq ties macrophage polarization to growth rate of intracellular Salmonella." Nat Microbiol **2**: 16206.
Sana, T. G., N. Flaugnatti, K. A. Lugo, L. H. Lam, A. Jacobson, V. Baylot, E. Durand, L. Journet, E. Cascales and D. M. Monack (2016). "Salmonella Typhimurium utilizes a T6SS-mediated antibacterial weapon to establish in the host gut." Proc Natl Acad Sci U S A **113**(34): E5044-5051.
Schade, R., D. S. C. Butler, J. A. McKenna, B. Di Luccia, V. Shokoohi, M. Hamblin, T. H. M. Pham and D. M. Monack (2024). "Transcriptional profiling links unique human macrophage phenotypes to the growth of intracellular Salmonella enterica serovar Typhi." Sci Rep **14**(1): 12811.
Schlumberger, M. C., A. Friebel, G. Buchwald, K. Scheffzek, A. Wittinghofer and W. D. Hardt (2003). "Amino acids of the bacterial toxin SopE involved in G nucleotide exchange on Cdc42." J Biol Chem **278**(29): 27149-27159.
Schlumberger, M. C., A. J. Muller, K. Ehrbar, B. Winnen, I. Duss, B. Stecher and W. D. Hardt (2005). "Real-time imaging of type III secretion: Salmonella SipA injection into host cells." Proc Natl Acad Sci U S A **102**(35): 12548-12553.
Schnaith, A., H. Kashkar, S. A. Leggio, K. Addicks, M. Kronke and O. Krut (2007). "Staphylococcus aureus subvert autophagy for induction of caspase-independent host cell death." J Biol Chem **282**(4): 2695-2706.
Schnupf, P. and D. A. Portnoy (2007). "Listeriolysin O: a phagosome-specific lysin." Microbes Infect **9**(10): 1176-1187.
Schroeder, N., T. Henry, C. de Chastellier, W. Zhao, A. A. Guilhon, J. P. Gorvel and S. Meresse (2010). "The virulence protein SopD2 regulates membrane dynamics of Salmonella-containing vacuoles." PLoS Pathog **6**(7): e1001002.
Segal, G., M. Feldman and T. Zusman (2005). "The Icm/Dot type-IV secretion systems of Legionella pneumophila and Coxiella burnetii." FEMS Microbiol Rev **29**(1): 65-81.
Senerovic, L., S. P. Tsunoda, C. Goosmann, V. Brinkmann, A. Zychlinsky, F. Meissner and M. Kolbe (2012). "Spontaneous formation of IpaB ion channels in host cell membranes reveals how Shigella induces pyroptosis in macrophages." Cell Death Dis **3**(9): e384.
Sexton, J. A. and J. P. Vogel (2002). "Type IVB secretion by intracellular pathogens." Traffic **3**(3): 178-185.
Shah, D. H., M. J. Lee, J. H. Park, J. H. Lee, S. K. Eo, J. T. Kwon and J. S. Chae (2005). "Identification of Salmonella gallinarum virulence genes in a chicken infection model using PCR-based signature-tagged mutagenesis." Microbiology (Reading) **151**(Pt 12): 3957-3968.
Shi, J. and J. E. Casanova (2006). "Invasion of host cells by Salmonella typhimurium requires focal adhesion kinase and p130Cas." Mol Biol Cell **17**(11): 4698-4708.
Shi, J., G. Scita and J. E. Casanova (2005). "WAVE2 signaling mediates invasion of polarized epithelial cells by Salmonella typhimurium." J Biol Chem **280**(33): 29849-29855.
Shneider, M. M., S. A. Buth, B. T. Ho, M. Basler, J. J. Mekalanos and P. G. Leiman (2013). "PAAR-repeat proteins sharpen and diversify the type VI secretion system spike." Nature **500**(7462): 350-353.

Simon, R. and C. E. Samuel (2007). "Activation of NF-kappaB-dependent gene expression by Salmonella flagellins FliC and FljB." Biochem Biophys Res Commun **355**(1): 280-285.
Sing, A., D. Reithmeier-Rost, K. Granfors, J. Hill, A. Roggenkamp and J. Heesemann (2005). "A hypervariable N-terminal region of Yersinia LcrV determines Toll-like receptor 2-mediated IL-10 induction and mouse virulence." Proc Natl Acad Sci U S A **102**(44): 16049-16054.
Smith, A. C., J. T. Cirulis, J. E. Casanova, M. A. Scidmore and J. H. Brumell (2005). "Interaction of the Salmonella-containing vacuole with the endocytic recycling system." J Biol Chem **280**(26): 24634-24641.
Smith, G. A., H. Marquis, S. Jones, N. C. Johnston, D. A. Portnoy and H. Goldfine (1995). "The two distinct phospholipases C of Listeria monocytogenes have overlapping roles in escape from a vacuole and cell-to-cell spread." Infect Immun **63**(11): 4231-4237.
Söding J, Biegert A, Lupas AN. The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res. 2005 Jul 1;33(Web Server issue):W244-8. doi: 10.1093/nar/gki408. PMID: 15980461; PMCID: PMC1160169.
Spano, S. (2014). "Host restriction in Salmonella: insights from Rab GTPases." Cell Microbiol **16**(9): 1321-1328.
Spano, S. and J. E. Galan (2012). "A Rab32-dependent pathway contributes to Salmonella typhi host restriction." Science **338**(6109): 960-963.
Spano, S., X. Gao, S. Hannemann, M. Lara-Tejero and J. E. Galan (2016). "A Bacterial Pathogen Targets a Host Rab-Family GTPase Defense Pathway with a GAP." Cell Host Microbe **19**(2): 216-226.
Sporn, L. A., S. K. Sahni, N. B. Lerner, V. J. Marder, D. J. Silverman, L. C. Turpin and A. L. Schwab (1997). "Rickettsia rickettsii infection of cultured human endothelial cells induces NF-kappaB activation." Infect Immun **65**(7): 2786-2791.
Starr, T., R. Child, T. D. Wehrly, B. Hansen, S. Hwang, C. Lopez-Otin, H. W. Virgin and J. Celli (2012). "Selective subversion of autophagy complexes facilitates completion of the Brucella intracellular cycle." Cell Host Microbe **11**(1): 33-45.
Stebbins, C. E. and J. E. Galan (2000). "Modulation of host signaling by a bacterial mimic: structure of the Salmonella effector SptP bound to Rac1." Mol Cell **6**(6): 1449-1460.
Stecher, B., R. Robbiani, A. W. Walker, A. M. Westendorf, M. Barthel, M. Kremer, S. Chaffron, A. J. Macpherson, J. Buer, J. Parkhill, G. Dougan, C. von Mering and W. D. Hardt (2007). "Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota." PLoS Biol **5**(10): 2177-2189.
Steele-Mortimer, O. (2008). "The Salmonella-containing vacuole: moving with the times." Curr Opin Microbiol **11**(1): 38-45.
Steele-Mortimer, O., S. Meresse, J. P. Gorvel, B. H. Toh and B. B. Finlay (1999). "Biogenesis of Salmonella typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway." Cell Microbiol **1**(1): 33-49.
Stein, M., M. Pilli, S. Bernauer, B. H. Habermann, M. Zerial and R. C. Wade (2012). "The interaction properties of the human Rab GTPase family--comparative analysis reveals determinants of molecular binding selectivity." PLoS One **7**(4): e34870.
Stender, S., A. Friebel, S. Linder, M. Rohde, S. Mirold and W. D. Hardt (2000). "Identification of SopE2 from Salmonella typhimurium, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell." Mol Microbiol **36**(6): 1206-1221.
Stepanova, L. K., A. L. Gorelov, N. S. Sergeeva, G. A. Levina and A. Belaia Iu (1983). "[Immunoelectrophoretic analysis of the antigenic composition of Salmonella typhi in the process of L transformation and reversion]." Zh Mikrobiol Epidemiol Immunobiol(10): 29-32.
Stepien, T. A., L. A. Singletary, F. E. Guerra, J. E. Karlinsey, S. J. Libby, S. L. Jaslow, M. R. Gaggioli, K. D. Gibbs, D. C. Ko, M. A. Brehm, D. L. Greiner, L. D. Shultz and F. C. Fang (2024). "Nuclear factor kappa B-dependent persistence of Salmonella Typhi and Paratyphi in human macrophages." mBio **15**(4): e0045424.
Stevens, J. M., E. E. Galyov and M. P. Stevens (2006). "Actin-dependent movement of bacterial pathogens." Nat Rev Microbiol **4**(2): 91-101.
Stevens, M. P. and E. E. Galyov (2004). "Exploitation of host cells by Burkholderia pseudomallei." Int J Med Microbiol **293**(7-8): 549-555.
Stradal, T. E. and G. Scita (2006). "Protein complexes regulating Arp2/3-mediated actin assembly." Curr Opin Cell Biol **18**(1): 4-10.

Sun, H., J. Kamanova, M. Lara-Tejero and J. E. Galan (2016). "A Family of Salmonella Type III Secretion Effector Proteins Selectively Targets the NF-kappaB Signaling Pathway to Preserve Host Homeostasis." PLoS Pathog **12**(3): e1005484.
Sun, J., A. E. Deghmane, H. Soualhine, T. Hong, C. Bucci, A. Solodkin and Z. Hmama (2007). "Mycobacterium bovis BCG disrupts the interaction of Rab7 with RILP contributing to inhibition of phagosome maturation." J Leukoc Biol **82**(6): 1437-1445.
Takenawa, T. and S. Suetsugu (2007). "The WASP-WAVE protein network: connecting the membrane to the cytoskeleton." Nat Rev Mol Cell Biol **8**(1): 37-48.
Tan, Y. and Z. Q. Luo (2011). "Legionella pneumophila SidD is a deAMPylase that modifies Rab1." Nature **475**(7357): 506-509.
Terasawa, K., K. Yoshimatsu, S. Iemura, T. Natsume, K. Tanaka and Y. Minami (2006). "Cdc37 interacts with the glycine-rich loop of Hsp90 client kinases." Mol Cell Biol **26**(9): 3378-3389.
Terebiznik, M. R., O. V. Vieira, S. L. Marcus, A. Slade, C. M. Yip, W. S. Trimble, T. Meyer, B. B. Finlay and S. Grinstein (2002). "Elimination of host cell PtdIns(4,5)P(2) by bacterial SigD promotes membrane fission during invasion by Salmonella." Nat Cell Biol **4**(10): 766-773.
Theeya, N., A. Ta, S. Das, R. S. Mandal, O. Chakrabarti, S. Chakrabarti, A. N. Ghosh and S. Das (2015). "An inducible and secreted eukaryote-like serine/threonine kinase of Salmonella enterica serovar Typhi promotes intracellular survival and pathogenesis." Infect Immun **83**(2): 522-533.
Thi, E. P., U. Lambertz and N. E. Reiner (2012). "Sleeping with the enemy: how intracellular pathogens cope with a macrophage lifestyle." PLoS Pathog **8**(3): e1002551.
Threlfall, E. J., L. R. Ward, J. A. Skinner, H. R. Smith and S. Lacey (1999). "Ciprofloxacin-resistant Salmonella typhi and treatment failure." Lancet **353**(9164): 1590-1591.
Tredicine, M., C. Camponeschi, D. Pirolli, M. Lucchini, M. Valentini, M. C. Geloso, M. Mirabella, M. Fidaleo, B. Righino, C. Moliterni, E. Giorda, M. Rende, M. C. De Rosa, M. Foti, G. Constantin, F. Ria and G. Di Sante (2022). "A TLR/CD44 axis regulates T cell trafficking in experimental and human multiple sclerosis." iScience **25**(2): 103763.
Trombert, A. N., L. Berrocal, J. A. Fuentes and G. C. Mora (2010). "S. Typhimurium sseJ gene decreases the S. Typhi cytotoxicity toward cultured epithelial cells." BMC Microbiol **10**: 312.
Uchiya, K., M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl and E. A. Groisman (1999). "A Salmonella virulence protein that inhibits cellular trafficking." EMBO J **18**(14): 3924-3933.
Ullio, C., J. Casas, U. T. Brunk, G. Sala, G. Fabrias, R. Ghidoni, G. Bonelli, F. M. Baccino and R. Autelli (2012). "Sphingosine mediates TNFalpha-induced lysosomal membrane permeabilization and ensuing programmed cell death in hepatoma cells." J Lipid Res **53**(6): 1134-1143.
Underhill, D. M. and A. Ozinsky (2002). "Phagocytosis of microbes: complexity in action." Annu Rev Immunol **20**: 825-852.
Unsworth, K. E., M. Way, M. McNiven, L. Machesky and D. W. Holden (2004). "Analysis of the mechanisms of Salmonella-induced actin assembly during invasion of host cells and intracellular replication." Cell Microbiol **6**(11): 1041-1055.
Valdivia, R. H. (2008). "Chlamydia effector proteins and new insights into chlamydial cellular microbiology." Curr Opin Microbiol **11**(1): 53-59.
Valenzuela, L. M., A. A. Hidalgo, L. Rodriguez, I. M. Urrutia, A. P. Ortega, N. A. Villagra, D. Paredes-Sabja, I. L. Calderon, F. Gil, C. P. Saavedra, G. C. Mora and J. A. Fuentes (2015). "Pseudogenization of sopA and sopE2 is functionally linked and contributes to virulence of Salmonella enterica serovar Typhi." Infect Genet Evol **33**: 131-142.
van der Heijden, J. and B. B. Finlay (2012). "Type III effector-mediated processes in Salmonella infection." Future Microbiol **7**(6): 685-703.
van der Velden, A. W., S. W. Lindgren, M. J. Worley and F. Heffron (2000). "Salmonella pathogenicity island 1-independent induction of apoptosis in infected macrophages by Salmonella enterica serotype typhimurium." Infect Immun **68**(10): 5702-5709.
Vazquez-Boland, J. A., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud and P. Cossart (1992). "Nucleotide sequence of the lecithinase operon of Listeria monocytogenes and possible role of lecithinase in cell-to-cell spread." Infect Immun **60**(1): 219-230.
Vazquez-Torres, A., Y. Xu, J. Jones-Carson, D. W. Holden, S. M. Lucia, M. C. Dinauer, P. Mastroeni and F. C. Fang (2000). "Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase." Science **287**(5458): 1655-1658.

Vazquez, C. L. and M. I. Colombo (2010). "Coxiella burnetii modulates Beclin 1 and Bcl-2, preventing host cell apoptosis to generate a persistent bacterial infection." Cell Death Differ **17**(3): 421-438.
Velasquez, J. C., A. A. Hidalgo, N. Villagra, C. A. Santiviago, G. C. Mora and J. A. Fuentes (2016). "SPI-9 of Salmonella enterica serovar Typhi is constituted by an operon positively regulated by RpoS and contributes to adherence to epithelial cells in culture." Microbiology (Reading) **162**(8): 1367-1378.
Verbeke, P., L. Welter-Stahl, S. Ying, J. Hansen, G. Hacker, T. Darville and D. M. Ojcius (2006). "Recruitment of BAD by the Chlamydia trachomatis vacuole correlates with host-cell survival." PLoS Pathog **2**(5): e45.
Vergne, I., J. Chua and V. Deretic (2003). "Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade." J Exp Med **198**(4): 653-659.
Vergne, I., J. Chua, H. H. Lee, M. Lucas, J. Belisle and V. Deretic (2005). "Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis." Proc Natl Acad Sci U S A **102**(11): 4033-4038.
Vergne, I., R. A. Fratti, P. J. Hill, J. Chua, J. Belisle and V. Deretic (2004). "Mycobacterium tuberculosis phagosome maturation arrest: mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion." Mol Biol Cell **15**(2): 751-760.
Vernikos, G. S. and J. Parkhill (2006). "Interpolated variable order motifs for identification of horizontally acquired DNA: revisiting the Salmonella pathogenicity islands." Bioinformatics **22**(18): 2196-2203.
Voth, D. E. and R. A. Heinzen (2007). "Lounging in a lysosome: the intracellular lifestyle of Coxiella burnetii." Cell Microbiol **9**(4): 829-840.
Voth, D. E. and R. A. Heinzen (2009). "Coxiella type IV secretion and cellular microbiology." Curr Opin Microbiol **12**(1): 74-80.
Wagner, C., B. Barlag, R. G. Gerlach, J. Deiwick and M. Hensel (2014). "The Salmonella enterica giant adhesin SiiE binds to polarized epithelial cells in a lectin-like manner." Cell Microbiol **16**(6): 962-975.
Wagner, C., M. Polke, R. G. Gerlach, D. Linke, Y. D. Stierhof, H. Schwarz and M. Hensel (2011). "Functional dissection of SiiE, a giant non-fimbrial adhesin of Salmonella enterica." Cell Microbiol **13**(8): 1286-1301.
Walburger, A., A. Koul, G. Ferrari, L. Nguyen, C. Prescianotto-Baschong, K. Huygen, B. Klebl, C. Thompson, G. Bacher and J. Pieters (2004). "Protein kinase G from pathogenic mycobacteria promotes survival within macrophages." Science **304**(5678): 1800-1804.
Walker, D. H. (2007). "Rickettsiae and rickettsial infections: the current state of knowledge." Clin Infect Dis **45 Suppl 1**: S39-44.
Wang, J., P. Ge, Z. Lei, Z. Lu, L. Qiang, Q. Chai, Y. Zhang, D. Zhao, B. Li, J. Su, R. Peng, Y. Pang, Y. Shi, Y. Zhang, G. F. Gao, X. B. Qiu and C. H. Liu (2021). "Mycobacterium tuberculosis protein kinase G acts as an unusual ubiquitinating enzyme to impair host immunity." EMBO Rep **22**(6): e52175.
Wang, M., Z. Luo, H. Du, S. Xu, B. Ni, H. Zhang, X. Sheng, H. Xu and X. Huang (2011). "Molecular characterization of a functional type VI secretion system in Salmonella enterica serovar Typhi." Curr Microbiol **63**(1): 22-31.
Wang, S., D. Yang, X. Wu, Z. Yi, Y. Wang, S. Xin, D. Wang, M. Tian, T. Li, J. Qi, C. Ding and S. Yu (2019). "The Ferric Uptake Regulator Represses Type VI Secretion System Function by Binding Directly to the clpV Promoter in Salmonella enterica Serovar Typhimurium." Infect Immun **87**(10).
Watanabe, I., M. Ichiki, A. Shiratsuchi and Y. Nakanishi (2007). "TLR2-mediated survival of Staphylococcus aureus in macrophages: a novel bacterial strategy against host innate immunity." J Immunol **178**(8): 4917-4925.
Watson, P. R., S. M. Paulin, A. P. Bland, P. W. Jones and T. S. Wallis (1995). "Characterization of intestinal invasion by Salmonella typhimurium and Salmonella dublin and effect of a mutation in the invH gene." Infect Immun **63**(7): 2743-2754.
Weber, S. S., C. Ragaz, K. Reus, Y. Nyfeler and H. Hilbi (2006). "Legionella pneumophila exploits PI(4)P to anchor secreted effector proteins to the replicative vacuole." PLoS Pathog **2**(5): e46.
Werneburg, N. W., M. E. Guicciardi, S. F. Bronk and G. J. Gores (2002). "Tumor necrosis factor-alpha-associated lysosomal permeabilization is cathepsin B dependent." Am J Physiol Gastrointest Liver Physiol **283**(4): G947-956.
Whitworth, T., V. L. Popov, X. J. Yu, D. H. Walker and D. H. Bouyer (2005). "Expression of the Rickettsia prowazekii pld or tlyC gene in Salmonella enterica serovar Typhimurium mediates phagosomal escape." Infect Immun **73**(10): 6668-6673.
Wiersinga, W. J., C. W. Wieland, M. C. Dessing, N. Chantratita, A. C. Cheng, D. Limmathurotsakul, W. Chierakul, M. Leendertse, S. Florquin, A. F. de Vos, N. White, A. M. Dondorp, N. P. Day, S. J. Peacock and T. van der Poll (2007). "Toll-like receptor 2 impairs host defense in gram-negative sepsis caused by Burkholderia pseudomallei (Melioidosis)." PLoS Med **4**(7): e248.

Wilson, R. P., M. Raffatellu, D. Chessa, S. E. Winter, C. Tukel and A. J. Baumler (2008). "The Vi-capsule prevents Toll-like receptor 4 recognition of Salmonella." Cell Microbiol **10**(4): 876-890.
Wilson, R. P., S. E. Winter, A. M. Spees, M. G. Winter, J. H. Nishimori, J. F. Sanchez, S. P. Nuccio, R. W. Crawford, C. Tukel and A. J. Baumler (2011). "The Vi capsular polysaccharide prevents complement receptor 3-mediated clearance of Salmonella enterica serotype Typhi." Infect Immun **79**(2): 830-837.
Winter, S. E., M. G. Winter, V. Atluri, V. Poon, E. L. Romao, R. M. Tsolis and A. J. Baumler (2015). "The flagellar regulator TviA reduces pyroptosis by Salmonella enterica serovar Typhi." Infect Immun **83**(4): 1546-1555.
Winter, S. E., M. G. Winter, P. Thiennimitr, V. A. Gerriets, S. P. Nuccio, H. Russmann and A. J. Baumler (2009). "The TviA auxiliary protein renders the Salmonella enterica serotype Typhi RcsB regulon responsive to changes in osmolarity." Mol Microbiol **74**(1): 175-193.
Wolff, K. A., A. H. de la Pena, H. T. Nguyen, T. H. Pham, L. M. Amzel, S. B. Gabelli and L. Nguyen (2015). "A redox regulatory system critical for mycobacterial survival in macrophages and biofilm development." PLoS Pathog **11**(4): e1004839.
Wong, D., H. Bach, J. Sun, Z. Hmama and Y. Av-Gay (2011). "Mycobacterium tuberculosis protein tyrosine phosphatase (PtpA) excludes host vacuolar-H+-ATPase to inhibit phagosome acidification." Proc Natl Acad Sci U S A **108**(48): 19371-19376.
Wong, P. and E. G. Pamer (2003). "CD8 T cell responses to infectious pathogens." Annu Rev Immunol **21**: 29-70.
Wood, M. W., M. A. Jones, P. R. Watson, S. Hedges, T. S. Wallis and E. E. Galyov (1998). "Identification of a pathogenicity island required for Salmonella enteropathogenicity." Mol Microbiol **29**(3): 883-891.
Wu, H., R. M. Jones and A. S. Neish (2012). "The Salmonella effector AvrA mediates bacterial intracellular survival during infection in vivo." Cell Microbiol **14**(1): 28-39.
Xie, Z., M. Zhao, C. Yan, W. Kong, F. Lan, Narengaowa, S. Zhao, Q. Yang, Z. Bai, H. Qing and J. Ni (2023). "Cathepsin B in programmed cell death machinery: mechanisms of execution and regulatory pathways." Cell Death Dis **14**(4): 255.
Xu, C., G. Kozlov, K. Wong, K. Gehring and M. Cygler (2016). "Crystal Structure of the Salmonella Typhimurium Effector GtgE." PLoS One **11**(12): e0166643.
Yang, C., H. Li, T. Zhang, Y. Chu, J. Zuo and D. Chen (2020). "Study on antibiotic susceptibility of Salmonella typhimurium L forms to the third and forth generation cephalosporins." Sci Rep **10**(1): 3042.
Yin, C., Z. Liu, H. Xian, Y. Jiao, Y. Yuan, Y. Li, Q. Li and X. Jiao (2020). "AvrA Exerts Inhibition of NF-kappaB Pathway in Its Naive Salmonella Serotype through Suppression of p-JNK and Beclin-1 Molecules." Int J Mol Sci **21**(17).
Yoshida, A., H. Inagawa, C. Kohchi, T. Nishizawa and G. Soma (2009). "The role of toll-like receptor 2 in survival strategies of Mycobacterium tuberculosis in macrophage phagosomes." Anticancer Res **29**(3): 907-910.
Yoshikawa, Y., M. Ogawa, T. Hain, M. Yoshida, M. Fukumatsu, M. Kim, H. Mimuro, I. Nakagawa, T. Yanagawa, T. Ishii, A. Kakizuka, E. Sztul, T. Chakraborty and C. Sasakawa (2009). "Listeria monocytogenes ActA-mediated escape from autophagic recognition." Nat Cell Biol **11**(10): 1233-1240.
Yousafzai, M. T., F. N. Qamar, S. Shakoor, K. Saleem, H. Lohana, S. Karim, A. Hotwani, S. Qureshi, N. Masood, M. Rauf, J. A. Khanzada, M. Kazi and R. Hasan (2019). "Ceftriaxone-resistant Salmonella Typhi Outbreak in Hyderabad City of Sindh, Pakistan: High Time for the Introduction of Typhoid Conjugate Vaccine." Clin Infect Dis **68**(Suppl 1): S16-S21.
Yu, X. J., M. Liu and D. W. Holden (2016). "Salmonella Effectors SseF and SseG Interact with Mammalian Protein ACBD3 (GCP60) To Anchor Salmonella-Containing Vacuoles at the Golgi Network." mBio **7**(4).
Zamir, E. and B. Geiger (2001). "Components of cell-matrix adhesions." J Cell Sci **114**(Pt 20): 3577-3579.
Zhang, L. F., B. Lepenies, S. Nakamae, B. M. Young, R. L. Santos, M. Raffatellu, B. A. Cobb, H. Hiyoshi and A. J. Baumler (2022). "The Vi Capsular Polysaccharide of Salmonella Typhi Promotes Macrophage Phagocytosis by Binding the Human C-Type Lectin DC-SIGN." mBio **13**(6): e0273322.
Zhang, S., R. L. Santos, R. M. Tsolis, S. Stender, W. D. Hardt, A. J. Baumler and L. G. Adams (2002). "The Salmonella enterica serotype typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea in calves." Infect Immun **70**(7): 3843-3855.
Zhou, D., L. M. Chen, L. Hernandez, S. B. Shears and J. E. Galan (2001). "A Salmonella inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization." Mol Microbiol **39**(2): 248-259.

Zhou, D., M. S. Mooseker and J. E. Galan (1999). "An invasion-associated Salmonella protein modulates the actin-bundling activity of plastin." Proc Natl Acad Sci U S A **96**(18): 10176-10181.
Zhou, D., M. S. Mooseker and J. E. Galan (1999). "Role of the S. typhimurium actin-binding protein SipA in bacterial internalization." Science **283**(5410): 2092-2095.
Zhu, W., L. Tao, M. L. Quick, J. A. Joyce, J. M. Qu and Z. Q. Luo (2015). "Sensing cytosolic RpsL by macrophages induces lysosomal cell death and termination of bacterial infection." PLoS Pathog **11**(3): e1004704.
Zhu, Y., L. Hu, Y. Zhou, Q. Yao, L. Liu and F. Shao (2010). "Structural mechanism of host Rab1 activation by the bifunctional Legionella type IV effector SidM/DrrA." Proc Natl Acad Sci U S A **107**(10): 4699-4704.
Zulauf, K. E., J. T. Sullivan and M. Braunstein (2018). "The SecA2 pathway of Mycobacterium tuberculosis exports effectors that work in concert to arrest phagosome and autophagosome maturation." PLoS Pathog **14**(4): e1007011.

Chapter 8
Publications

Research Articles:-

- **Infection & Immunity** – (**Joint 1st authorship**) - Ganguli, D., **Chakraborty, S.,** Chakraborty, S., Pal, A., Gope, A., & Das, S. (2022). Macrophage cell lines and murine infection by Salmonella enterica serovar typhi L-form bacteria. Infection and Immunity, 90(6), e00119-22.

- **Npj-vaccines**- Chakraborty, S., Dutta, P., Pal, A., **Chakraborty, S.,** Banik, G., Halder, P., ... & Das, S. (2024). Intranasal immunization of mice with chimera of Salmonella Typhi protein elicits protective intestinal immunity. npj Vaccines, 9(1), 24.

- **Plos Pathogens**- *Salmonella* Typhi Serine Threonine Kinase T4519 Induces Lysosomal Membrane Permeabilization by Manipulating Toll-like Receptor 2-Cystatin B-Cathepsin B-NF-κB-Reactive Oxygen Species Pathway and Promotes Survival within Human Macrophages- **Swarnali Chakraborty** , Debayan Ganguli, Theeyaa Nagaraja, Animesh Gope, Sudip Dey, Ananda Pal, Rahul Shubhra Mandal, Sudipta Sekhar Das, Santasabuj Das (Paper communicated and under final revision)

- FliC adjuvant augments protection of T2544 protein subunit antigen against typhoidal *Salmonella* by increasing serum antibodies, intestinal secretory immunoglobulin A and T cell memory- Suparna Chakraborty , Pujarini Dutta , Ananda Pal , **Swarnali Chakraborty** , Sneha Mitra , Shin Ichi Miyoshi , Santasabuj Das (Paper communicated)

Chapter 9
List of conferences

List of conferences attended:-

- **Genomics Analysis & Technology Conference GATC Lite (East)** from 24th-25th August , 2023 at Vedic Village, Kolkata- **poster presentation** on "Serine-threonine kinase T4519 promotes Salmonella Typhi survival in human macrophages through the modulation of cystatin B-cathepsin B-NF-κB axis"
- **Golden Jubilee Conference of Indian Immunology Society (IMMUNOCON)** from 5th – 8th Oct, 2023 at AIIMS, New Delhi **– poster presentation** on "T4519, a serine-threonine kinase of Salmonella Typhi promotes bacterial survival in human PBMC-derived macrophage cells by activating Cathepsin B-NFκB axis dependent pro-inflammatory signaling.
- **16th Asian Conference on Diarrheal Disease and Nutrition in Kolkata, from 11th to 13th Nov,2022 (ASCODD**) – **poster presentation** on "A study on the phagosomal survival mechanisms of Salmonella enterica Serovar Typhi in macrophages".