ANTIBIOTIC PERMEATION IN GRAM-NEGATIVE BACTERIA AND CONTRIBUTION OF INFLAMMASOME ACTIVATION AND PYROPTOSIS IN PATHOGENESIS OF SALMONELLA SYSTEMIC INFECTION

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ANTIBIOTIC PERMEATION IN GRAM-NEGATIVE BACTERIA AND CONTRIBUTION OF INFLAMMASOME ACTIVATION AND PYROPTOSIS IN PATHOGENESIS OF SALMONELLA SYSTEMIC INFECTION

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By

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and Dr. Zhenyu Li, Professor of Biochemistry

Lexington, Kentucky

2022

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ABSTRACT OF DISSERTATION

Antibiotic resistance is one of the major global issues in the field of public health and medicine. Good antibiotic candidates need to be selectively toxic, inhibit cellular target, and effectively penetrate and accumulate in bacterial cells. The last factor is a formidable barrier in the development of antimicrobials effective in Gram-negative bacteria, due to the presence of two layers of cell envelope. The first half of my thesis focuses on understanding the permeation of small molecules through this formidable cell envelope, distribution inside the cell of Gram-negative bacteria, and design of novel methods to make small molecules effectively cross the cell envelope. The second half of my thesis focuses more on the crosstalk between Gram-negative bacteria and host immune system during systemic infection and sepsis. More specifically we studied the contribution of inflammasome activation and pyroptosis during pathogenesis of *Salmonella* systemic infection.

In the first project, I studied the accumulation and distribution behavior of fluoroquinolone class of antibiotics inside Gram-negative bacteria using *E. coli* as a model. Although several studies have been focused regarding the correlation between compound’s cellular accumulation and their effectiveness against Gram-negative bacteria but no correlation between accumulation of antibiotics and their efficacy has been observed. In this study, we measured the concentration of nine fluoroquinolones accumulated in the subcellular compartments of *E. coli*. Good correlation between the MIC and the cytoplasmic accumulation, but not whole cell accumulation, was observed using a pair of isogenic wild type and drug-efflux deficient strains. Our results supported the explanation that the efficacy cannot be determined by the whole cell accumulation alone. Accumulation in the target region as well as the intrinsic potency determines the effectiveness of an antimicrobial compound.

In the second project, I explored whether conjugation of biotin to small molecules can increase the permeation of small molecules through the Gram-negative cell envelope. We used a fluorescent molecule pair, Atto565 and Atto565-biotin as model compounds and studied their permeation behavior in *E. coli*. Our results indicated that biotinylation helped the molecule Atto565 to cross the outer membrane of *E. coli* through OmpC porin.

Moreover, in the third project, I studied how the inflammasome activation and pyroptosis play a role in pathogenesis of *Salmonella* systemic infection. We found that *Salmonella* systemic infection causes severe inflammation as indicated by very high plasma concentration of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α. Furthermore, it also caused disseminated intravascular coagulation (DIC) as indicated by increased prothrombin (PT) time and plasma thrombin-antithrombin (TAT) levels. Deficiency of caspase 1 protected the mice from *Salmonella* induced inflammation, coagulation and death during acute systemic infection. Similarly, deficiency of NAIP and GSDMD significantly reduced the *Salmonella* induced inflammation in vivo. Use of flagellin and *Salmonella* pathogenicity island 1 (SPI1) region knockout strains of *Salmonella* induced significantly less cytokine release in the plasma, however, could not protect from the coagulation and lethality.

*In vitro* studies showed that deficiency of Caspase 1, NAIP and GSDMD also protects the bone marrow derived macrophage’s (BMDM’s) death upon *Salmonella*...
infection during early phase of infection. *In vitro*, inflammasome activation and BMDM death was also completely abolished when flagellin or SPI1 deficient strains of *Salmonella* were used during early but not late phase of infection. During late phase of infection, caspase 11 and NLRP3 dependent pyroptosis had major contribution. These results indicate that during acute *Salmonella* systemic infection under flagellin and SPI1 expressing conditions, severe inflammation occurs mainly through NAIP/Caspase 1/GSDMD axis. However, coagulation could also be induced also by factors other than flagellin and SPI1 that contributes to lethality. Flagellin and SPI1 independent coagulation was protected by NLRP3 and caspase 11 deficiency.

KEYWORDS: *Antibiotic-permeation, Trojan-horse strategy, Salmonella, Inflammasome, Pyroptosis, Disseminated intravascular coagulation (DIC)*
DEDICATION

To my parents

For their unconditional love, support and trust which always motivated me to do something meaningful in life
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CHAPTER 1. INTRODUCTION

1.1 Antibiotics and antibiotic resistance

Antibiotic resistance has been a serious global problem of the current era. According to interagency coordination group report to the secretary general of United Nations in April 2019, about 700 thousands deaths occur every year globally by multidrug resistant infections and this figure could reach to 10 million deaths per year by the year 2050 if no significant action is taken soon \(^1\). Few major problems for the development of antibiotics include rapid emergence of resistance, low interest of industries in antibiotics production because of limited time for future production and profit as well as lack of better understanding about the science behind working of antibiotics. Most of the antibiotics that are used currently were developed during the golden era of antibiotics ranging from 1940s to 1960s \(^2,^3\). Discovery of antibiotics was a great milestone in the field of public health and medicine as these are continuously being used since then to save millions of lives. Antibiotics are critical in hospital practices including any kinds of surgery to avoid secondary infections. However, the antibiotics which were of great use in these processes in the past are gradually becoming less effective due to the development of resistant bacteria \(^4,^5\). Large number of bacteria are evolving ranging from single drug resistant to multi drug resistant to some that are almost untreatable. The problem is even worse in hospitals where nosocomial secondary infections are being life threatening. Recently a group of bacteria are referred as ESKAPE pathogens that are more common in these nosocomial multidrug resistant infections. ESKAPE refers to *Enterococcus faecium*, \(^\ldots\)
Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp. \(^6,^7\)

Figure 1.1 Timeline of antibiotic discovery. \(^3\)
(Reprinted from Biochemical Pharmacology, Volume 134, Kim Lewis, New approaches to antimicrobial discovery, 87-89, 2017, with permission from Elsevier)

1.2 Types and characteristics of antibiotics

Antibiotics work by targeting different sites in bacteria. Antibiotics are classified according to their targets as shown in Table 1.1.
**Table 1.1. Types of antibiotics**

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Working mechanism</th>
<th>Class of Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cell wall biosynthesis inhibition</td>
<td>Penicillins, Cephalosporins, Carbapenems, Monobactams, Cycloserines, Fosfomycins, Glycopeptides, Lipoglycopeptides</td>
</tr>
<tr>
<td>2.</td>
<td>Protein Synthesis Inhibition</td>
<td>Aminoglycosides, Tetracyclines, Oxazolidinones, Macrolides, Thiopeptides, Chloramphenicol, Fusidic acid, Clindamycin</td>
</tr>
<tr>
<td>3.</td>
<td>DNA replication and repair inhibition</td>
<td>Rifamycins, Ansamycins, Actinomycins, Tiacumycins, Fluoroquinolones, Aminocoumarins</td>
</tr>
<tr>
<td>4.</td>
<td>Folic acid metabolism inhibition</td>
<td>Sulfonamides, Trimethoprim</td>
</tr>
<tr>
<td>5.</td>
<td>Membrane disruption</td>
<td>Lipopeptides, Polymyxins</td>
</tr>
</tbody>
</table>

To become a good antibiotic candidate one compound should have selective toxicity towards bacteria, good inhibition of bacterial target as well as effective penetration and accumulation inside the bacterial cells. Selective toxicity refers to the phenomena that antibiotics should have the capacity to inhibit the growth or kill only bacterial cells but not the eukaryotic cells. For example, in case of penicillins, they inhibit the cell wall synthesis in bacteria, however, since there is no cell wall in animal cells, animal cell growth is not inhibited. Similarly, another important feature of good antibiotic is that it should be effective at low concentrations. When given to higher organisms by various means the antibiotics should be absorbed, assimilated and distributed inside the body which will cause
decrease in the concentration by multiple folds than the given concentration. Hence, antibiotics should be able to inhibit the bacterial growth at low concentrations. Also, at higher concentrations they can be toxic and have several side effects to higher animals. Another most important feature of good antibiotic is the ability to penetrate and accumulate inside the bacterial cell. Although the antibiotic might have capacity to bind and inhibit the target inside the bacterial cell, they should first enter and accumulate inside the cell so that they can reach the site where target is located. However, this is one of the formidable barriers in case of Gram-negative organisms because of stronger and double envelope barrier.

1.3 Mechanism of antibiotic resistance

As a theory of evolution bacteria are also evolving themselves against the antibiotics with time. Prolonged exposure of bacteria against non-lethal dose of antibiotics gradually evolves the bacteria to develop way to resist the drug. Resistance developed by the bacteria can be either intrinsic or acquired. Intrinsic resistance refers to the inherent resistance shown by bacteria because of lack of receptor or target while acquired resistance is shown by some population of bacteria in a group of previously sensitive bacteria. Acquired resistance can be because of the acquisition of resistant genes either by outside source or self-mutations. Antibiotic resistance genes may be present either in chromosomes, plasmid or transposons. Several mechanisms have been identified from which bacteria develop the resistance for antibiotics. These mechanisms can be classified mainly into following four groups:
a) Decreased uptake of antibiotics

One of the major ways to develop resistance by bacteria is that they change their permeability of their cell envelope due to which the uptake of antibiotics is reduced. Because of this, the available antibiotic to inhibit their target inside the bacterial cell is not enough, hence causing resistance.

b) Increased efflux of antibiotics

Apart from decreased permeability another way bacterium develop resistance is because of the efflux pumps. Even though the permeability might have not been changed, the antibiotics entered inside are effluxed out by the different types of efflux pumps present in the membrane e.g., AcrAB-TolC pump in *E. coli*.

c) Target modification

Different antibiotics have different targets located at different regions of the bacterial cells. For example, beta lactams interact with penicillin binding proteins in cell wall whereas fluoroquinolones interact with DNA gyrase and topoisomerase IV located in cytoplasm. These targets could mutate to eliminate binding with the antibiotics.

d) Enzymatic inactivation of antibiotics

Another most common way by which bacteria develop resistance is the presence of enzymes that can degrade or modify the antibiotics applied. For example, penicillinase can hydrolyze the penicillin and carbapenemase can hydrolyze the carbapenems so that these antibiotics are no longer effective. In some cases, the antibiotics can be modified which impedes the target binding ability of
the antibiotics. The modification might be phosphorylation, acetylation, adenylation, hydroxylation, etc of the antibiotics ⁹.

Figure 1.2. Mechanisms of antibiotic resistance ⁵
(Reprinted from Bioorganic and medicinal chemistry letters, Volume 27, Issue 18, Concepcion Gonzalez-Bello, Antibiotic adjuvants- a strategy to unlock bacterial resistance to antibiotics, 4221-4228, 2017. Copyright @authors, publised by Elsevier under creative commons attribution-non commercial-no derivative license)

1.4 Major problem in Gram-negative bacteria

Gram-negative bacteria are getting more attention than Gram-positive bacteria since there are less effective drug against Gram-negative bacteria. Several drug inactivating enzymes are evolving including extended spectrum β-lactamases, carbapenemases and recently found New-Delhi metallo-β-lactamases that confer β-lactam drug resistance to Gram-negative bacteria. Similarly, these Gram-negative bacteria are more common in causing nosocomial infections as there are many which live as commensals in human gut
Another interesting thing about Gram-negative bacteria is that because of the capsule-like layer in the outer membrane it hides most of the surface antigens causing a camouflage-like phenomena during recognition by human immune system. Also, presence of lipopolysaccharides (LPS) on the outer leaflet of the outer membrane makes them more detrimental as LPS is a potent trigger of inflammation and septic shock. Gram-negative bacteria contain two layers of cell envelope, one outer membrane with LPS and phospholipids and another inner lipid bilayer, also referred to as plasma membrane as shown in Figure 1.3. The space in between these two membranes is called the periplasmic space. The outer membrane is a barrier for penetration of a lot of molecules. Whatever fraction of the applied drug can pass through this barrier some of them are effluxed back from the periplasm by the multi-drug resistance efflux pumps. As an additional barrier, inner lipid bilayer blocks the entry of highly polar compounds. Thus in combination of these barriers the penetration of antibiotics inside a Gram-negative bacteria is extremely hard. The outer membrane is highly charged mostly anionic at neutral pH. Cations like Mg$^{2+}$ interact between these anionic groups and maintain the stability of the outer membrane. In addition to its role as a protection barrier for environmental toxic chemicals, outer membrane also harbors outer membrane protein channels called porins that are essential for the uptake of nutrients. Study shows that generally antibiotics are effective at very high concentrations compared to compounds used against eukaryotic target despite having similar binding constants. This difference was attributed to the low penetration of compounds to bacterial cells. Except for membrane disrupting antimicrobials, antibiotics need to rely on the porins in the outer membrane and/or free...
diffusion to enter the cells. Hence, one of a great demand for now is the development of those antimicrobials that effectively accumulate in Gram-negative bacteria \(^{13}\).

**Figure 1.3. Structure of Gram-negative cell envelope.\(^ {14}\)**

(Reprinted from ACS infectious diseases, Volume 6, Issue 9, Ankit Pandeya, Isoiza Ojo, Olaniyi Alegun, Yinan Wei, Periplasmic targets for the development of effective antimicrobials against gram-negative bacteria, 2337-2354, 2020, with permission. Copyright @ American Chemical Society)

1.5 **Permeability rules in Gram-negative bacteria**

One of the major hurdles in the development of new antibiotics that are effective against Gram-negative bacteria is the permeability barrier of these organisms due to the presence of double layer cell envelope. In general, it is believed that the smaller compounds penetrate the outer membrane through porins and larger compounds by simple diffusion through the membrane. Since the porins favors hydrophilic compounds for
transport small hydrophilic compounds are most like transported through porins. The inner plasma membrane however favors the entry of hydrophobic compounds than hydrophilic by diffusion through the lipid bilayer \(^15\). Because of these barriers various studies have been done to find out the rules that can govern the permeability of compounds through these membranes. Some common structure features have been reported for compounds that enters Gram-negative bacteria \(^15, 16\). In case of mammalian penetration there is a rule called Lipinsky’s rule of 5 which says in order to be an effective penetrator a compound should have following properties: a. \(\leq 5\) hydrogen bond donors, b. \(\leq 5\) hydrogen bond acceptors, c. molecular mass <500 Da, d. octanol-water partition coefficient (clogP) < 5 \(^17\). However, these rules do not necessarily apply to compounds for penetration into Gram-negative organisms \(^15, 18-20\). Moser et. al., in 2008 reported some of the observations they found for the drugs effective against Gram-negative organisms. They observed that most of the drugs were less than 600Da and they were much more polar than other drugs. However there are antibiotics that meet these criteria but still inactive against Gram-negative bacteria \(^19\). In another study by Silver et al. in 2016, they observed that the antimicrobials that are active against Gram-negative bacteria are smaller in size and less polar than those active against Gram-positive bacteria. Among the ones active against Gram-negative organisms those enter through passive diffusion are less polar compared to the ones entering through active transport \(^20\). Richter et. al. did the computational studies with the same goal. Based on their observations they made the rule that antibiotics that are most likely to accumulate inside the Gram-negative bacteria have amine group, are amphiphilic and rigid, and have low globularity. They applied these rules to change a
Gram-positive only effective antibiotic, deoxyribomycin, into an antibiotic that is active against a wide range of multi-drug resistant bacteria including Gram-negative ones. Acosta-Gutierrez et. al. used a different approach by understanding the structures of porins to see how they allow molecules to pass through. They studied the constricted region of porins and studied the electric field, electrostatic interactions and size to understand the mechanisms of molecules entry through them. They highlight the importance of positively charged group for better penetration, which is consistent with the mechanisms of the rules proposed by Richter et al.

1.6 Quinolones and Fluoroquinolones

The quinolones and fluoroquinolones are an extremely successful class of broad-spectrum antibiotics which are used to treat a wide variety of infections. They are the only major synthetic class of antibiotics which are currently used globally. Discovered in an attempt to optimize the efficacy of nalidixic acid this class of antibiotics have a core bicyclic structure related to compound 4-quinolone. The first member of quinolones, nalidixic acid, was isolated as a byproduct of chloroquine synthesis in 1962. Upon further modification, it was developed into a series of compounds making the first generation of quinolones. Later, in early 1980s, the second generation quinolones were developed and referred to as fluoroquinolones containing fluorine atom at the C-6 position and a major ring substituent at the C-7 position, typically piperazine or methyl piperazine. These fluoroquinolones were much more effective because of their high penetration against Gram-positive organisms. Ciprofloxacin and norfloxacin from this second
generation fluoroquinolones class are few of the commonly used antibiotics for broad spectrum infections since their discovery. Development of levofloxacin, moxifloxacin and trovafloxacin constitute the third and fourth generation fluoroquinolones which has even higher broad-spectrum activity. Generally, fluoroquinolones work by interacting with enzymes DNA gyrase and topoisomerase IV. These enzymes are responsible for introducing supercoils in DNA during replication. Whenever the fluoroquinolones interact with these enzyme-DNA complex their ligase activity is disrupted and release DNA with single or double strands break ultimately leading to the cell death. In this class of antibiotics the position 7 is believed to be the one that interacts with the DNA gyrase and topoisomerase so the substituent group present in the position 7 greatly affects the potency, spectrum and pharmacokinetics. Similarly, the C3 carboxylic acid and C4 ketone are responsible for binding with the cleaved DNA, thus forming a complex of DNA and gyrase bridged by drug.

![Figure 1.4. General chemical structure of fluoroquinolone](image)
1.7 Trojan-horse strategy

Because of this formidable double layered cell envelope in Gram-negative bacteria, scientists are constantly working to design new techniques through which the small molecule inhibitors can be easily penetrated through this cell envelope. One of these techniques which have been explored recently is the “Trojan-horse” technique. The name is derived from the ancient Greek mythology referring the war between Greece and city of Troy. The Greek soldier penetrated the defense wall of Troy by hiding inside a large wooden horse, which was sent as the emblem of Trojans. Similar to this ancient strategy, currently an antibiotic molecule is conjugated with a nutrient molecule needed for bacterial survival. Hence, upon up taking the nutrient molecules through their dedicated transporters, bacteria can also uptake the antibiotic molecule which alone cannot breach the cell envelope of Gram-negative bacteria. One of the best examples of this strategy is the development of sideromycins, in which an antibiotic molecule is conjugated with the siderophore, a molecule secreted by bacteria to chelate the iron for its uptake.

Siderophores are naturally synthesized molecules by bacteria, which in iron deprived conditions are responsible to capture and chelate the extracellular iron and then are uptaken through their dedicated transporters. Upon reaching to the intracellular sites the iron is then released and is used by the bacteria for certain metabolic processes. Even in nature bacteria use these siderophores conjugated with molecules that have antibacterial properties to inhibit the other bacterial population. One of such examples is albomycin, which has one iron carrying siderophore moiety and another thionucleoside moiety which has antibacterial properties. Bacteria uptake these molecules when they are in need of iron.
but eventually that move becomes suicidal as the thionucleoside moiety targets protein synthesis.\textsuperscript{30}

Conjugation of these siderophores with antibiotic molecules for their effective delivery started as early as late 70s, 80s to early 90s.\textsuperscript{28, 33, 34} This “Trojan-horse” strategy has since been most studied in conjugation of siderophores with β-lactam antibiotics but not limited to them. It is relatively easier to design these for beta-lactams as they have to cross only one membrane in the Gram-negative cell envelope. Typically the design of siderophore conjugated antibiotics contain one siderophore moiety, one antibiotic moiety and a linker in between them.\textsuperscript{30} Sometimes the linker could be cleavable after reaching to the intracellular target region such as the use of reducible disulfide bonded linker.\textsuperscript{32} Apart from beta-lactams, recently several fluoroquinolones have also been explored using this technique.\textsuperscript{35, 36}

Several siderophore conjugated antibiotics are currently under development. One siderophore conjugated monocarbam called MC-1 was being investigated by Pfizer, which has shown promising activity against multi drug resistant \textit{Pseudomonas} and \textit{Enterobacteriaceae} species. Another compound called BAL30072, which is a monosulfactam conjugated siderophore developed by Basilea Pharmaceutica also showed promising activity against \textit{Pseudomonas} and \textit{Acinetobacter} species.\textsuperscript{26} So far, the most promising candidate clinically is a siderophore antibiotic conjugate called Cefiderocol (S-649266), which is in phase 3 clinical trial.\textsuperscript{37} This catechol cephalosporin conjugate has showed a promising antibacterial activity against a variety of Gram-negative pathogens including \textit{Pseudomonas aeruginosa}, \textit{Burkholderia cepacia}, \textit{Acinetobacter baumannii}, \textit{Pseudomonas aeruginosa}, \textit{Burkholderia cepacia}, \textit{Acinetobacter baumannii},
Enterobacteriaceae and even the strains producing carbapenemases like Klebsiella pneumoniae carbapenemase and New Delhi metallo-β-lactamases (NDM)-1. The “Trojan-horse” strategy has also been explored for effective drug delivery in mammalian cells. Just like siderophores in case of antibacterial design, vitamin conjugation has also been explored to enhance drug penetration, mostly in mammalian cells, such as cancer cells. For mammalian cells drug delivery conjugation of drug with some vitamins are being utilized. Conjugation of drugs with vitamins have several advantages because vitamins are needed for several metabolic processes, hence, cells must either synthesize or uptake these vitamins from external environment through their transporters. Anticancer drugs conjugation with Biotin (vitamin B7) has been shown to be effective in mammalian cancer cells drug delivery. Sodium dependent multivitamin transporters (SMVT) are overexpressed in several different types of cancer cells, and it has been shown that biotin conjugation enhanced the uptake of anticancer molecule through these transporters. Biotin conjugates are being studied because they can be both target-specific to cancer cells and can enhance the accumulation of drug through overexpressed biotin transporters.

1.8 Inflammasomes and pyroptosis

Inflammasomes are multiprotein complexes present in the cytosol as a part of innate immune system which sense pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) and upon assembly cause inflammatory responses. Generally, inflammasomes consist of a sensor and an adaptor which recruits the procaspase 1 eventually causing the self-cleavage and activation of caspase 1. Upon
activation, caspase 1 can cause cleavage of pro-interleukin (IL)-1β and -18, to form active IL-1β and IL-18, which are then secreted from the cells. In addition to the activation of IL-1β and -18, caspase 1 also causes the cleavage of Gasdermin D (GSDMD) to generate its N-terminal fragment, which then goes to the plasma membrane to form pores and subsequent pyroptosis. The receptors in the inflammasome complex belong to the family of pattern recognition receptors (PRRs), which could be NLRs (nucleotide binding domain, leucine rich repeats containing proteins or NOD like receptors) or ALRs (absent in melanoma 2 or AIM2 like receptors). These receptors when sense the pathogen or danger associated molecules can oligomerize, assemble and recruit caspase 1 to activate it and cause downstream events.

Pyroptosis is a form of pro-inflammatory programmed cell death. The term “pyroptosis” was first coined by Cookson and Brennan in 2001, to describe “the screaming, alarm-ringing pro-inflammatory death of a potentially dangerous cell in an organism.” The term is constructed from the Greek roots “pyro” for fire or fever and “ptosis (to-sis)” for falling. Although discovered relatively recently, pyroptosis has been observed to occur in different types of cells ranging from the immune system, digestive system, central nervous system, reproductive system, to cardiovascular system. Pyroptosis is provoked by stimuli including infectious signal such as virulent factors and structures from pathogens, and non-infectious messages including DNA fragments, reactive oxygen species (ROS), monosodium urate, calcium pyrophosphate dehydrate crystals, and uric acid crystals, which normally signals cells in stress.
Pyroptosis has initially been defined as a process of “caspase 1-dependent programmed cell death” \(^{51}\). However, later other caspases have been reported to lead to pyroptosis as well. Intracellular lipopolysaccharide (LPS) from Gram-negative bacteria activates caspase-11 (in mice) and caspase-4/5 (in human), leading to pyroptosis \(^{52-57}\). More recently, caspase-8 has been shown to be activated by a bacterial virulent effector YopJ and flagellin, and subsequently cleaved of both gasdermin D (GSDMD) and gasdermin E (GSDME) in murine macrophages, resulting in pyroptosis \(^{58,59}\). Such a mechanism is absent in human macrophages. In cancer chemotherapy, caspase-3 can cleave GSDME to induce pyroptosis of certain GSDME-expressing cancer cells \(^{60}\). Due to the involvement of multiple caspases, more recently pyroptosis is defined as “Gasdermin-Mediated Programmed Necrotic Cell Death”, as gasdermin activation and pore formation in the cell membrane is a feature shared by both canonical and non-canonical pyroptosis \(^{61}\).

1.8.1 Mechanism of inflammasome activation and pyroptosis

The mechanism of pyroptosis has been the topic of several excellent reviews \(^{51,61-63}\) (Figure 1.5). Mammalian cells have sensors and receptors on their surface to detect environmental cues. The pattern recognition receptors (PRRs) can detect damage-associated molecular patterns (DAMPs) such as stress signals, uric acid crystals, oxidized lipoproteins, or pathogen-associated molecular patterns (PAMPs) such as flagellin, type 3 secretion system (T3SS) structure proteins, LPS, and nucleic acids \(^{64}\). Upon stimulation by extracellular signals, the PRRs initiate a cascade of responses inside the cells, including
the activation of caspases and transcriptional effectors such as NF-κB (nuclear factor-κB), induction of the expression and activation of pro-inflammatory cytokines, and secretion of these cytokines to escalate immune response. In parallel, certain bacteria such as *Salmonella typhimurium*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Burkholderia pseudomallei*, and *Yersinia enterocolitica*, can directly deliver effector proteins into the cytoplasm of host cells through the T3SS\(^{65,66}\). Another route of entry of pathogenic signal molecules to the cytoplasm is endocytosis\(^{67}\). Together with host-derived cytoplasmic signal molecules, they interact with intracellular receptor NAIPs (NLR family apoptosis inhibitory proteins) and NLRs (Nod-like receptors) to trigger the activation and assembly of multiple protein complexes called inflammasomes\(^{68,69}\).
Several types of inflammasomes have been found to be involved in pyroptosis, including NLRC4 (NOD-like receptor family, CARD domain containing 4), NLRP3 (NOD-like receptor family, pyrin domain containing 3), NLRP1, NLRP6, NLRP9, AIM2, and PYRIN. The NLRP3 inflammasome is unique in that it can be activated by the internal stress signals and a large number of metabolites, responding to both DAMPs and PAMPs. It is also involved in infections as well as several inflammatory disorders.
Activation of the NLRP3 in macrophages is a two-step process involving priming, through receptors including TLRs, IL-1R, tumor necrosis factor receptor, and NOD2, leading to activation of the NF-kB (nuclear factor kB pathway, and assembly and activation of the inflammasome. In certain cell types (such as monocyte), priming alone can activate NLRP3 inflammasome. How single NLRP3 detect a variety of intracellular stimuli remains elusive Direct binding to the stimuli seemed unlikely, since a variety of stimuli with drastically different structure can activate the NLRP3 inflammasome. It has been suggested that NLPR3 could sense a common “cellular event” induced by its stimuli. While certain stimuli share a common feature such as the stimulation of K⁺ efflux or Ca²⁺ signaling, an event that is shared by all stimuli is yet to be determined.

The NLRC4 inflammasome is mainly triggered by PAMPs from various microbial pathogens. Oligomerization and assembly of NLRC4 inflammasome are triggered by PAMP binding to NAIPs. While a mouse carries seven NAIPs (NAIP1-7), a human only has one NAIP protein. Mouse NAIPs recognize and bind to specific ligands. For example, NAIP5 and NAIP6 detect flagellin, and NAIP1 and NAIP2 recognize T3SS needle and rod proteins, respectively. Human NAIP binds with both flagellin and T3SS proteins. Binding of the PAMP signal leads to conformational changes in the corresponding NAIP, exposing a previously hidden surface, which promotes the interaction with NLRC4 and activation of the NAIP/NLRC4 inflammasome. NLRC4 contains an N-terminal CARD domain, which can recruit pro-caspase-1 and assemble inflammasome.
Mice carry three NLRP1 paralogs (NLRP1a-c), while human only carry one. Among them NLRP1b is the most well characterized and is activated by bacterial and parasitic toxins and virulent factors. Activation of the NLRP1b inflammasome is initiated by the autoproteolysis within the Function to Find Domain (FIIND) domain, which splits NLRP1b into a N-terminal and a C-terminal domain that remain noncovalently bound. Several currently identified activators have been shown to initiate the ubiquitination and proteasomal degradation of the N-terminal domain, releasing the C-terminal domain to initiate inflammasome assembly. This is consistent with the observation that NLRP1b activation can be blocked by proteasome inhibition in murine macrophages. Thus, N-terminal degradation is likely a common mechanism of NLRP1b inflammasome activation.

Other well characterized inflammasomes involved in pyroptosis include the AIM2 and Pyrin inflammasomes. AIM2 directly binds to cytosolic double stranded DNA at least 70 bp in length in a sequence-independent manner. DNA binding triggers inflammasome assembly. The Pyrin inflammasome indirectly senses inactivating modifications of host Rho GTPases by bacterial toxins. Rho-modifying proteins from pathogens induce pyrin-dependent activation of pyroptosis in macrophages. In the resting state pyrin is phosphorylated at two sites and remains bound with its endogenous inhibitor. RhoA plays an important role in maintaining pyrin inhibited by facilitating phosphorylation through recruitment of specific kinases. Thus, bacterial toxins that inactivate RhoA lead to pyrin activation through disrupting the phosphorylation of pyrin and subsequently binding with its inhibitor.
As a direct consequence of inflammasome activation, pro-caspases are recruited and activated. Caspases that have been identified to be involved in pyroptosis include 1, 3, 4, 5, 8, and 11. Activated caspases digest the pro-inflammatory cytokines IL-1β and IL-18 to generate mature cytokines, as well as digest a critical substrate GSDMD, which initiates pore-formation in the cell membrane. GSDMD-mediated pore-formation is a key feature of pyroptosis. Activated caspases cleave GSDMD in the loop connecting the N-terminal and C-terminal domains, which lifts the autoinhibition of the C-terminal domain on the N-terminal domain. The N-terminal domain then migrates and inserts into the plasma membrane, forming pores with a functional diameter of 1.1-2.4 nm\textsuperscript{101-103}. Pore formation and cytokine release initiate a cascade of downstream consequences.

1.8.2 Consequence of pyroptosis at the cellular and subcellular level

Since its discovery in early 2000, pyroptosis has been observed to occur in many different cell types. The wide occurrence of this programed cell death process indicates its involvement in various disorders and disease conditions. Pyroptosis first occurs at the cellular level, upon detection of pathogen invasion or cell stress/danger signals. Pyroptotic cells then release a large amount of pro-inflammatory cytokines and other mediators, triggering inflammatory responses from neighboring cells, similar as sparks that eventually lead to the burning of the entire forest. Distinct features of pyroptosis include the dependence on caspase activation and cleavage of a key substrate GSDMD, pore-formation in the plasma membrane, rapid cell swelling and lysis, and release of proinflammatory
cytokines and microvesicles. Among them, pore formation is the first step of the physical changes of the pyroptotic cells. Pores formed in the plasma membrane lead to the release of pro-inflammatory cytokines, as well as cellular content and disruption of the ionic gradients and osmostasis, and eventually cell lysis. Activation of caspases leads to the proteolytic digestion of many downstream substrates and their resultant biological processes. Release of the inflammatory cellular contents, and the cascade of reactions lead to tissue damage, and sometimes organ failure and host death.

Two direct pro-inflammatory cytokines generated in pyroptosis are IL-1β and IL-18. IL-1β and IL-18 lack secretion signals and their mechanism of release has been attributed to the pore-formation in the plasma membrane. Similarly, a role in protein secretion has been previously proposed for caspase-1, which is likely the result of pore-formation. Both cytokines play crucial roles in the pathogenesis of a range of inflammatory and autoimmune diseases. IL-1β binds to IL-1 receptors on the surface of immune cells and triggers various processes including fever, vasodilation, hematopoiesis, leukocyte tissue migration, antibody synthesis, and expression of cytokines and chemokines. IL-18 plays an important role in immune responses through inducing IFNγ production, activating T cells, macrophages, and natural killer (NK) cells, and plays a role in angiogenesis.

DNA damage also occurs during pyroptosis. While the mechanism of DNA damage during apoptosis is well understood and the formation of characteristic DNA fragment patterns during apoptosis is well documented, the mechanism of DNA damage
in pyroptosis remains elusive. DNA cleavage during pyroptosis depends on the caspase 1-activation of a yet to be identified endonuclease, but nuclear integrity is maintained \(^{101}\).

Activation of caspases is an important step in pyroptosis. While some substrates that are processed by activated caspases have been linked to pyroptosis, including IL-18, IL-1β, and GSDMD, other less documented substrates may also play important roles in inflammation and tissue damage. Through a proteomic study, 41 proteins were discovered that are cleaved by caspase-1 directly, including chaperones, cytoskeletal and translation machinery proteins, cytokines, and interestingly, several proteins along the glycolysis pathway \(^{115}\). It was then confirmed that digestion by caspase-1 reduced the catalytic activity of these metabolic enzymes \textit{in vitro}, and caspase-1 activation led to a significant digestion of these proteins in cells including macrophages. Thus, it is expected that a disruption of energy production would occur upon caspase-1 activation, which may contribute to cell death. Another substrate of caspase-1 is silent information regulator factor 2 related enzyme 1 (SIRT1), a histone deacetylase that promotes insulin secretion by β cells \(^{116,117}\).

A high-fat diet has been shown to trigger NLRP3 inflammasome activation in mouse model, which leads to caspase-1 mediated cleavage and reduction of SIRT1 function, and subsequent reduces insulin sensitivity in peripheral tissues \(^{118}\). This connection is confirmed by observations that mice lacking caspase-1 or NLRP3 are protected from high-fat diet–induced insulin resistance, metabolic dysfunction, and obesity \(^{119}\). Caspase-1 activation bridges two cellular processes, metabolic dysfunction and inflammation, highlighting the intricated cellular response to stimulations.
1.8.3 Pyroptosis is a double-edged sword

While the pyroptosis is a host response that is evolved to protect against infection and cell stress, overactivation of pyroptosis can be detrimental and lead to tissue damage and even host death. Inflammation is initiated by harmful stimuli, as a means of self-protection by the cells. The production, activation, and release of cytokines, transcriptions factors, and proteases collectively promote the elimination of the harmful stimuli and protect the host. Thus when under careful control, inflammation is a critical defense mechanism beneficial for the survival of the organism \(^{120}\). However, many executing molecules released as a result of inflammation could be toxic to the host cells as well, acting as double-edged sword. When overactivated, this beneficial mechanism could cause damage to tissues and could also evolve into chronic inflammatory disease. IL-1β has been shown to trigger generation of several cytokines \textit{in vivo} \(^{121}\). Therefore, although IL-1β and IL-18 are the only known proinflammatory cytokines generated directly from inflammasome activation, inflammasome activation \textit{in vivo} may lead to generation of multiple proinflammatory cytokines indirectly, including TNF-α and IL-6, resulting in cytokine storm and tissue damage.

Recently, our lab and another research group reported that pyroptosis can trigger disseminated intravascular coagulation (DIC) \(^{104, 122, 123}\), an acquired syndrome characterized by widespread intravascular activation of coagulation resulting in formation of thrombi throughout the vasculature. Tissue factor released from pyroptotic monocytes and macrophages was found to be the initiator of coagulation cascade during sepsis. DIC
can be caused by both infectious insults (such as COVID-19 and sepsis) and non-infectious insults (such as trauma and deep vein thrombosis)\textsuperscript{124, 125}. DIC often leads to bleeding due to consumption of coagulation factors and platelets, and formation of microvascular thrombi inevitably results in multiple organ dysfunction.

1.8.4 Inflammasome activation and pyroptosis during \textit{Salmonella} infection

\textit{Salmonella} is one of the Gram-negative bacterial pathogens which is prevalent in developing and under developing countries as a food borne pathogen. It is responsible for severe typhoid to self-limiting gastroenteritis. \textit{Salmonella enterica} serovars Typhi and Paratyphi are mainly responsible for typhoid fever whereas serovar Typhimurium generally causes self-limiting gastroenteritis and in severe cases can causes systemic infection and sepsis.\textsuperscript{126, 127} To study the role of innate immune system and inflammasomes during \textit{Salmonella} infection, several mouse models with \textit{Salmonella} infection have been established. In mice \textit{Salmonella} infection can cause systemic infection and lethality, whereas it can also be chronic in some resistant mouse strains.\textsuperscript{128}

\textit{Salmonella} infection has been shown to activate the NLRC4 inflammasomes causing the caspase 1 activation in macrophages.\textsuperscript{129} Activation of caspase 1 causes maturation of proinflammatory cytokines like Interleukin-1β and Interleukin 18 and pyroptotic cell death. The caspase 1 dependent pyroptosis during \textit{Salmonella} infection has been shown to act as a defense mechanism by the host.\textsuperscript{48} NLRC4 activation during \textit{Salmonella} infection has been attributed to its flagellin.\textsuperscript{130, 131} Apart from flagellin, type
three secretion system (T3SS) components of *Salmonella* like PrgJ and PrgI can also cause NLRC4 inflammasome activation. Type three secretion system can also deliver flagellin to activate the NLRC4 inflammasome. *Salmonella* contains two types of three secretion system, one from Salmonella pathogenicity island 1 (SPI1) and another from Salmonella pathogenicity island 2 (SPI2). SPI1 is responsible for early phase of host invasion and SPI2 is important for survival inside the host cells. It is believed that *Salmonella* downregulates the expression of flagellin and SPI1 during late systemic phase of infection. During this phase, NLRC4 inflammasome is activated by a SPI2 type three secretion system needle protein called SsaG in human macrophages. SPI2 rod protein has not been shown to activate the NLRC4 inflammasome. Besides NLRC4 inflammasome, *Salmonella* can also activate the NLRP3 inflammasome. However, *Salmonella* activates NLRP3 when both SPI1 and flagellin are absent through SPI2, but what molecule through SPI2 is responsible for NLRP3 activation remains elusive. Being a Gram-negative bacteria, *Salmonella* also contains LPS which is known to activate the caspase 4/5/11 non-canonical inflammasome.

Inflammasome components and pyroptosis play a protective role during the early phase of gastrointestinal *Salmonella* infection. Inflammasome activation and pyroptosis protect against intestinal infection and help to inhibit systemic dissemination of the bacteria. Both caspase 1 and caspase 11 have been shown to have protective role in intestinal restriction of bacteria during *Salmonella* infection. In addition, caspase 8 activation downstream of NLRC4 inflammasome in intestinal epithelial cells also caused infected cell expulsion and restricted the systemic dissemination of *Salmonella*. 

26
Although caspase 1 deficiency has been attributed to increased lethality during oral *Salmonella* infection,\textsuperscript{142,143} its role during systemic phase of infection is not very clear. IL-18 produced from caspase 1 activation has been found to be responsible for host defense against systemic *Salmonella* infection.\textsuperscript{143} However, septic shock induced by high dose of growth attenuated *Salmonella* was protected by caspase 1 and IL-18 deficiency.\textsuperscript{143} Also, since the expression of flagellin and SPI1 downregulated during systemic phase of infection, it has been shown that NLRC4 plays a minor role during systemic infection.\textsuperscript{48,142} However, if the host is infected by a *Salmonella* which constantly expresses flagellin through plasmid with SPI2 promoter, NLRC4 inflammasome had a protective role in host defense.\textsuperscript{48} The non-canonical caspase 11 activation and pyroptosis was observed to have detrimental role by allowing the intracellular bacteria to release and spread in the system during systemic *Salmonella* infection when there is absence of caspase 1 and defective neutrophil mediated clearance.\textsuperscript{138}
CHAPTER 2. DISTRIBUTION OF FLOROQUINOLONES IN TWO AQUEOUS COMPARTMENTS OF E. COLI

Chapter 2, in full is a reprint of the article published in Biochemistry and Biophysics Reports, 2020, vol. 24, 100849. Ankit Pandeya, Olaniyi Alegun, Yuguang Cai, Yinan Wei. https://doi.org/10.1016/j.bbrep.2020.100849. Out of nine compounds studied Dr. Olaniyi Alegun performed the studies of three: moxifloxacin, marbofloxacin and enrofloxacin. Dr. Yuguang Cai performed the microscopy presented in figure 2.5. But for the completion of the story, I am keeping these data in this chapter.

2.1 Introduction

Antibiotic resistance is becoming a serious global problem of the current era. According to an interagency coordination group report to the Secretary-General of the United Nations in April 2019, approximately 700,000 deaths occur every year globally due to multidrug resistant infections and this figure could reach 10 million deaths per year by 2050.1 Most of the antibiotics that are used currently were developed during the golden era of antibiotics in the 1940s to 1960s and are gradually becoming less effective due to the development of bacterial resistance.2-5 A clinically useful antimicrobial needs to be selectively toxic towards bacteria, with excellent potency, and effectively penetrating to the target site inside the bacteria cell. The latter is a major barrier in the development of drugs for Gram-negative bacteria due to their double-membrane cell envelope structure. The outer membrane contains lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet. LPS forms a hydrophilic barrier on the surface of the bacteria. The outer
membrane contains protein channels called porins, which allow the passage of selected compounds.\textsuperscript{144-148} The inner membrane is a phospholipid bilayer, which is a hydrophobic barrier.\textsuperscript{3} In addition, Gram-negative bacteria have an array of drug efflux pumps that remove toxic compounds from the cells.\textsuperscript{149} Because of these obstacles many antimicrobials effective against Gram-positive bacteria do not work for Gram-negative bacteria.\textsuperscript{3, 150, 151}

The importance of cellular accumulation to any pharmaceuticals is apparent. Several studies have been conducted to determine the correlation between antibiotic accumulation in bacteria and its MIC.\textsuperscript{152-157} Bazile et al. determined the cellular accumulation of 11 fluoroquinolones and their DNA gyrase inhibition activity.\textsuperscript{156} No correlation was found between MIC and accumulation, but the correlation between the MIC and the minimal effective dose improved on considering accumulation as a factor. In addition, they observed that the hydrophobicity of compounds correlated positively with accumulation in \textit{S. aureus} and negatively with accumulation in \textit{E. coli} and \textit{P. aeruginosa}.\textsuperscript{156} Inspired by this pioneering work, Piddock and her group conducted a series of studies on the accumulation of compounds in bacteria. They found that the highly hydrophobic rifampicin accumulated much less in \textit{E. coli} compared to \textit{S. aureus} and speculated that the presence of the outer membrane in \textit{E. coli} was the major reason for the reduced accumulation, while efflux also played a minor role.\textsuperscript{157} In the study of fluoroquinolones accumulation in \textit{Mycobacterium tuberculosis},\textsuperscript{154} no correlation between MIC and accumulated concentration of fluoroquinolones was found. Instead, a clear negative correlation between the molecule size and accumulation, and positive correlation between hydrophobicity and accumulation were observed.\textsuperscript{154}
accumulation in *S. aureus*, no correlation of accumulation with hydrophobicity, but positive correlation with molecular weight, was observed. However, the steady state concentration of accumulated compounds did not correlate with their effectiveness as revealed by MIC.¹⁵²

In the study of accumulation of 10 fluoroquinolones in *Streptococcus pneumoniae*, hydrophobicity and molecular weight of the compounds were observed to correlate negatively with the steady state accumulation concentration of fluoroquinolones. Surprisingly, MIC of the fluoroquinolones was found to correlate negatively with steady state concentration with more effective antibiotics accumulating less.¹⁵³

In a recent study, Iyer et al. determined accumulation of fluoroquinolones and a collection of other DNA ligase inhibitors in *E. coli* using mass spectroscopy and observed that there was no correlation between accumulation of fluoroquinolones versus their effectiveness in inhibiting bacteria cell growth.¹⁵⁵ No correlation was observed between accumulation concentration and the ratio of IC₅₀ to MIC of the DNA ligases. It was suggested that the lack of correlation could be because the accumulation data represented the accumulation of drugs in entire cell rather than the amount available for target inhibition.¹⁵⁵ When we first started this project, no publications were available to our knowledge that studied the accumulation of antibiotics in subcellular fractions of Gram-negative bacteria. A very recent report described fractionation and quantification of the antibiotic accumulation using mass spectroscopy.¹⁵⁸ The accumulation of four antibiotics from different classes were investigated. But the drastic differences between the structure and mechanism of the drugs made comparison among them difficult.
To further explore the penetration barrier presented by Gram-negative bacteria, in this study we examined the accumulation of nine fluoroquinolones in subcellular compartments of an *E. coli* strain and its isogenic *tolC* knock out strain. The potency of the compounds as a growth inhibitor was determined using the minimum inhibitory concentration (MIC) assay. Relative distribution of fluoroquinolones in the periplasm and cytoplasm, and their correlation with antimicrobial activity were studied to reveal new insight in our understanding about how antimicrobials work.

### 2.2 Materials and methods

#### 2.2.1 Drug accumulation assay

Two strains of *E. coli*, both obtained from the Yale Coli Genetic Stock Center, were used in the measurement of accumulation, *BW25113* and *BW25113 ΔtolC* (referred to below as WT and ΔtolC). Bacteria were cultured in LB broth to mid-log phase and harvested by centrifugation at 3,000 g for 15 mins at room temperature. All following operations were conducted at room temperature unless otherwise noted. The cell pellet was resuspended in a NaPi-Mg buffer (50 mM sodium phosphate buffer, 0.25% MgCl$_2$, pH 7.0) to a final concentration of optical density at 600 nm (OD600) of 6.4. The bacterial density was approximately 6.4×10$^9$ colony-forming units per ml. For drug treatment, the indicated fluoroquinolone was added to the bacteria suspension to a final drug concentration of 2.0 µg/ml. The mixture was incubated for 15 mins with constant shaking at 250 rpm. After incubation, 700 µl of the drug-treated bacterial cells was layered carefully on top of 700 µl
of silicon oil in a microcentrifuge tube. The sample was centrifuged at 13,000g for 1 minute. The supernatant was discarded, and the inner wall of the centrifuge tubes cleaned carefully using a paper rod made out of Kimwipe. For the “whole cell” samples, 1.0 ml of glycine-HCl buffer (0.1 M glycine-HCl, pH 3.0) was added directly to lyse the cell as described\(^{159}\). The pellet was resuspended and incubated overnight. The next morning, cell debris and membrane were removed through centrifugation at 15,000g for 15 minutes. The supernatant was collected and diluted 1:1 using the glycine-HCl buffer before fluorescence emission spectra were measured. For the fractionated samples, the periplasm and cytoplasm were obtained as described below, and 1.0 ml of glycine-HCl buffer was added into each sample followed by mixing and overnight incubation. In the next morning, all samples were centrifuged after a brief vertex, and the supernatant was collected and diluted before measurement as described above. The peak intensity at the corresponding excitation and emission wavelength was used to determine the concentration of each fluoroquinolone according to its standard calibration curve (see below). Figure 2.1 is a flow chart of the procedure. All measures were done in triplicate and the data were presented as the average ± standard deviation.

To evaluate the effect of wash, cells were incubation with drugs and centrifuged through silicon oil as described above. The cell pellet was quickly resuspended in 300 µl of 70 mM Tris buffer (pH 8.0) and centrifuged again at 13,000g for 1 min. The supernatant was collected as the “wash solution”. Glycine-HCl buffer (1.0 ml) was added and the concentration of fluoroquinolone in the wash solution was determined similarly as in the other samples.
2.2.2 Separation of periplasm and cytoplasm

An osmotic shock method was used to extract the periplasm with modifications to minimize the incubation time. Briefly, cell pellet was resuspended in 100 µl of periplasm preparation buffer (200 mM Tris-Cl, 1 mM EDTA, 20% sucrose, 1 mg/ml lysozyme, pH 8.0) and incubated for 5 minutes. Next, 200 µl of ice-cold deionized water was added and the sample was mixed by tapping. The mixture was incubated on ice for 2 minutes and then
centrifuged at 13,000g for 1 min. The supernatant was collected, which contains the periplasmic component. The pellet contains the cytoplasm and cell membranes.

2.2.3 Monitoring potential leakage during the osmotic shock procedure

To monitor the leakage of cytosolic protein, BW25113 transformed with plasmid pBAD-sfGFP (a gift from Ryan Mehl, Addgene plasmid # 85482) was cultured to OD600 0.5 and induced using 0.2% arabinose for 0.5 h. Cell pellet was collected by centrifugation through silicon oil and subjected to the osmotic shock procedure exactly as described above. After the supernatant containing the periplasm was collected, the spheroplast was resuspended in 300 µl tris buffer, lysed through sonication and centrifuged to collect the solution component (cytoplasm). In parallel, a duplicate cell pellet was resuspended in 300 µl tris buffer, sonicated and centrifuged to collect the supernatant (whole cell). The sfGFP concentration in all three samples were determined using the intrinsic fluorescence of sfGFP at excitation and emission wavelengths of 495 nm/510 nm.

To monitor the leakage of ATP from the cytoplasm, the periplasm, cytoplasm, and whole cell samples were prepared from BW25113 cell pellet as described above for the drug accumulation assay, and the ATP concentration was determined following well established protocols. Briefly, cell pellet was collected by centrifugation through silicon oil and subjected to the osmotic shock procedure exactly as described above. After the supernatant containing the periplasm was collected, the spheroplast was resuspended in 450 µl ice cold 0.4 M perchloric acid and vortexed for 10 s. The mixture was incubated on ice
for 15 min and spun down at 13,000 × g for 5 min. To neutralize the acid, 200 µl of the supernatant was transferred to a fresh tube and mixed with 100 µl of a solution containing 0.72 M KOH and 0.16 M KHCO₃. The neutralized mixture was then centrifuged at 13,000 × g for 5 min and the supernatant was transferred to a fresh tube for use in the ATP assay. Similarly, 150 µl of ice cold 1.2 M perchloric acid was added into 300 µl of the periplasm sample (to a final concentration of 0.4 M perchloric acid) and vortexed for 10 s, followed by the same procedure to prepare samples for ATP analysis. In parallel, a duplicate cell pellet was resuspended in 450 µl ice cold 0.4 M perchloric acid and processed exactly as described for the spheroplast sample. ATP concentration in the samples were determined using a luciferase – based assay (Staybrite™ Highly stable ATP bio luminescence kit, BioVision Incorporated.). The ATP level was determined by measuring luminescence levels using BioTek Synergy HT multimode plate reader.

### 2.2.4 Preparation of calibration curves

Cell culture was prepared similarly as described above except that NaPi-Mg buffer, instead of the fluoroquinolone solution, was used in the “drug treatment” step. Cells were aliquoted and pelleted through silicon oil similarly and the whole cell, periplasm, and cytoplasm extracts were obtained separately as described above. These solutions were used as the background to obtain the respective calibration curves. For each fluoroquinolone used in this study, standard solutions were prepared by spiking the extracts with known concentration of each compound. All samples were vortexed and centrifuged for 15 minutes
at 15,000g and the supernatant was used to measure the fluorescence after 1:1 dilution using the glycine-HCl buffer.

The emission spectra of the samples were obtained to determine the peak intensity. Graph was plotted with the emission peak intensity in Y-axis and the concentration of drug in X-axis. Every sample was done in triplicate and plotted as the average with error bars representing the standard deviation. Standard calibration curves for all compounds used in this study can be found in Figure 2.2. Excitation/emission wavelength (nm) of compounds used in this study: ciprofloxacin (Cipro, 280/447), norfloxacin (Nor, 278/444), enrofloxacin (Enro, 278/445), levofloxacin (Levo, 293/502), lomefloxacin (Lome, 286/450), ofloxacin (Oflo, 293/501), fleroxacin (Flero, 286/453), marbofloxacin (Marbo, 298/510), moxifloxacin (Moxi, 296/509).

2.2.5 Quantification of accumulated drug

For each sample, the measured fluorescence intensity was converted into sample concentration using the respective calibration curve. The product of the sample concentration and sample volume yield the mass of the compounds associated in the whole cell pellet (Mt), periplasm (Mp) or cytoplasm (Mc) from 0.7 ml of OD600 6.4 bacteria, or ~4.5x10⁹ cells. As described above, total volume of the periplasm, cytoplasm, and whole cell samples are approximately 1.3, 1.0, and 1.0 ml, respectively.

Similarly, the mass of the compounds in the wash solution (Mw) was determined from multiplying the wash solution concentration and the wash sample volume (1.3 ml).
To calculate the total concentration of each compound in the cell \((C_t)\), since the actual cell volume of the pellet was 16.1 µl (see below):

\[
C_t = \frac{Mt}{16.1}
\]

Concentration of drug in the periplasm \((C_p)\) is determined assuming the periplasm volume to be 10% of the total cell volume:

\[
C_p = \frac{Mp}{1.6}
\]

Similarly, the total volume of the cytoplasm was approximately 13.8 µl, thus the concentration in the cytoplasm \((C_c)\):

\[
C_c = \frac{Mc}{13.8}
\]

### 2.2.6 Volume of cellular compartments

The reported volume of the periplasmic space of Gram-negative bacteria ranges from 7% to 40%. A seminal study by Stock et al. reported that the periplasmic space in *E. coli* and *S. Typhimurium* constitute of approximately 20-40% of the total cell volume.

The volume was determined experimentally through exploiting the selective permeability of the inner and outer membranes. More recent studies used electron microscopy to measure the gap between the outer membrane and inner membrane, and the reported thickness of the periplasmic space ranged from 10 nm to 33 nm.
recently, Pilizota et. al. estimated the periplasmic space to be approximately 16% of the total cell volume based on fluorescence imaging of *E. coli* cells expressing fluorescent proteins.\textsuperscript{167} Prochnow et al. took the cellular dimensions from different published sources and calculated the volume of the periplasm of *E. coli* to be \textasciitilde7% of the total cell volume.\textsuperscript{158} Based on these discussions, in our study we assumed the periplasmic volume to be 10% of the total cell volume.

The total volume of *E. coli* in 1 ml of OD600 1.0 culture has been reported to be approximately 3.6 µl.\textsuperscript{170} This value was used as the cellular volume for the calculation of drug concentration in “whole cell” samples. The cell density in the drug treatment mixture is OD600 6.4. Each pellet contained cells from 700 µl of OD600 6.4 culture, which yielded a total cell volume of $3.6 \times 6.4 \times 0.7 = 16.1$ µl. Assuming 10% of the total volume to be the periplasm, volume of the periplasm was 1.6 µl. Cell membranes has been estimated to account for 4% of the overall volume, and thus the cytoplasm was 13.8 µl.\textsuperscript{158}

### 2.2.7 Determination of MIC

BW25113 wild type and *tolC* knockout strains were cultured overnight in LB broth at 37°C with shaking at 250 rpm. The overnight culture was diluted into fresh LB broth at a final concentration of $5 \times 10^5$ CFU/ml. The diluted cells were then aliquoted into a 48-well plate, the indicated drug was added with 2-fold dilutions to create a series of concentrations. Plates were then incubated at 37°C with shaking at 160 rpm overnight. The concentration of drug at which no visible growth was observed was reported as the MIC.
2.3 Results and discussion

2.3.1 Calibration curves

Calibration curves were made as described in materials and methods. Figure 2.2 shows the calibration curves used for the quantification of accumulated fluoroquinolones.
Enrofloxacin WT

Enrofloxacin ΔtolC

Levofloxacin WT

Levofloxacin ΔtolC

\[ y = 680.73x + 10.947 \]
\[ R^2 = 0.9993 \]
Periplasm

\[ y = 481.91x + 35.3 \]
\[ R^2 = 0.9936 \]
Spheroplast

\[ y = 505.06x + 36.239 \]
\[ R^2 = 0.9969 \]
Whole cell

\[ y = 639.09x + 10.28 \]
\[ R^2 = 0.9987 \]
Wash

\[ y = 540.01x + 12.767 \]
\[ R^2 = 0.9998 \]
Periplasm

\[ y = 457.96x + 23.344 \]
\[ R^2 = 0.9938 \]
Spheroplast

\[ y = 432.85x + 29.783 \]
\[ R^2 = 0.9985 \]
Whole cell

\[ y = 638.05x + 8.4238 \]
\[ R^2 = 0.9997 \]
Wash

\[ y = 262.18x + 3.082 \]
\[ R^2 = 0.9998 \]
Periplasm

\[ y = 241.03x + 6.9628 \]
\[ R^2 = 0.9996 \]
Spheroplast

\[ y = 227.46x + 8.6558 \]
\[ R^2 = 0.9998 \]
Whole cell

\[ y = 248.35x + 0.8719 \]
\[ R^2 = 0.9999 \]
Wash

\[ y = 260.55x + 2.9224 \]
\[ R^2 = 0.9998 \]
Periplasm

\[ y = 231.89x + 8.7998 \]
\[ R^2 = 0.9991 \]
Spheroplast

\[ y = 210.53x + 10.32 \]
\[ R^2 = 0.9975 \]
Whole cell

\[ y = 247.51x + 0.8738 \]
\[ R^2 = 1 \]
Wash
Lomefloxacin WT

**Periplasm**
- \( y = 238.16x + 11.213 \)
- \( R^2 = 0.9999 \)

**Spheroplast**
- \( y = 214.07x + 20.533 \)
- \( R^2 = 0.9987 \)

**Whole cell**
- \( y = 244.83x + 6.3696 \)
- \( R^2 = 0.9994 \)

**Wash**
- \( y = 224.36x + 10.867 \)
- \( R^2 = 0.9969 \)

Ofloxacin WT

**Periplasm**
- \( y = 314.83x + 2.3074 \)
- \( R^2 = 1 \)

**Spheroplast**
- \( y = 282.95x + 7.4298 \)
- \( R^2 = 1 \)

**Whole cell**
- \( y = 306.92x + 1.0016 \)
- \( R^2 = 0.9998 \)

**Wash**
- \( y = 301.86x + 3.0978 \)
- \( R^2 = 0.9997 \)

Lomefloxacin \( \Delta tolC \):

**Periplasm**
- \( y = 200.56x + 18.733 \)
- \( R^2 = 0.9959 \)

**Spheroplast**
- \( y = 198.31x + 23.021 \)
- \( R^2 = 0.9989 \)

**Whole cell**
- \( y = 218.8x + 8.6174 \)
- \( R^2 = 0.9989 \)

**Wash**
- \( y = 218.8x + 8.6174 \)
- \( R^2 = 0.9989 \)

Ofloxacin \( \Delta tolC \):

**Periplasm**
- \( y = 273.12x + 7.1964 \)
- \( R^2 = 0.9996 \)

**Spheroplast**
- \( y = 263.86x + 10.133 \)
- \( R^2 = 1 \)

**Whole cell**
- \( y = 305.16x + 0.9686 \)
- \( R^2 = 0.9998 \)

**Wash**
- \( y = 305.16x + 0.9686 \)
- \( R^2 = 0.9998 \)
2.3.2 Cellular accumulation of fluoroquinolones

We analyzed nine fluoroquinolone family compounds that are commercially available and have high fluorescence intensity. Accumulation studies were performed as described in Materials and Methods. After incubation with fluoroquinolones and cell fractionation, fluorescence emission spectra were collected for each sample. The intensity at the corresponding wavelength was used to determine compounds concentration using the calibration curves. Calculated mass $M_p$, $M_c$, and $M_t$ were shown in Figure 2.3 and Table 2.1.
Figure 2.3. Accumulation of fluoroquinolones (FQs) in the periplasm ($M_p$), cytoplasm ($M_c$), and whole cell ($M_t$) in $BW25113$ (WT) and $BW25113\Delta tolC$ ($\Delta tolC$) cells.

Gram negative bacteria have four subcellular compartments, the outer membrane (OM), the inner membrane (IM), the periplasm, and the cytoplasm. It is impossible to separately the IM and OM quantitatively in a timely manner, thus our original plan was to fractionate cells into three components, the periplasm, the cytoplasm, and the membrane. However, in practice we realized that fractionating the membrane component while keeping the original compound distribution was technically impossible. To separate the membrane component, cells need to be disrupted vigorously to burst the cells and fragment the cell membrane, followed by prolonged ultra-centrifugation to collect the membrane vesicles as a pellet, as described in the recent publication by Prochnow et al.\textsuperscript{158} The entire process takes longer than an hour. During the process, drug redistribution among lysed cell fragments and the soluble component would certainly occur. Thus, the value determined may not
faithfully reflect the actual amount of compound in each component. In this study we will focus on the quantification of accumulation in the two aqueous compartments, the periplasm and cytoplasm.

2.3.3 All compounds accumulated more in BW25113ΔtolC than in BW25113

As expected, for all compounds, the accumulation levels in the wild-type strain are lower than that in the tolC knockout strain. This is additional evidence validating the experimental method and measurements in this study. The ratio of difference varies drastically among compounds used in this study, potentially reflecting the difference in efflux efficiency of the AcrAB-TolC system for different fluoroquinolones. Ciprofloxacin, norfloxacin, and enrofloxacin are the top three accumulated drugs in the whole-cell and the cytoplasm, while enrofloxacin, levofloxacin, and marbofloxacin are the top three accumulators in the periplasm. The accumulation in the whole cell should equal to the sum of accumulations in the cytoplasm, periplasm and the membranes. The difference between the combined accumulation measured in the aqueous compartments and the whole cell accumulation was calculated: \( \Delta = (M_p + M_c) - M_t \) (Table 2.1). Overall, the \( \Delta \) values are reasonably small, validating the accuracy of the measurements. The accumulation in the cell membrane, which was not measured in this study, could also contribute to a negative value to the difference.
2.3.4 Intracellular concentration of fluoroquinolones

To further evaluate the concentration of fluoroquinolones in the periplasm, cytoplasm, and the whole cell, we divided the mass of the accumulated compounds by the volume of each compartment. The calculated concentration can be found in Table 2.1. Interestingly, we found the periplasmic concentrations of the compounds (C_{p}) were much higher than the concentrations in the cytoplasm (C_{c}) and the whole cell (C_{t}).

Table 2.1. Accumulation of fluoroquinolones (FQs) in two strains, BW25113 (WT) and BW25113\textDelta{tolc} (\Delta C). M_{t}, M_{p}, and M_{c} are mass of accumulated compounds in the whole cell, periplasm, and cytoplasm, respectively. The difference (\Delta) between the sum of the measured periplasmic and cytoplasmic accumulation to that of the whole cell accumulation was calculated. C_{p}, C_{c} and C_{t} refer to the concentration of drug in the periplasm, cytoplasm, and whole cell respectively.

<table>
<thead>
<tr>
<th>FQs / strain</th>
<th>M_{p} (ng)</th>
<th>M_{c} (ng)</th>
<th>M_{t} (ng)</th>
<th>\Delta (ng)</th>
<th>C_{p} (\mu g/ml)</th>
<th>C_{c} (\mu g/ml)</th>
<th>C_{t} (\mu g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin ΔC</td>
<td>81.4 ± 3.1</td>
<td>118.0 ± 1.1</td>
<td>183.0 ± 18.8</td>
<td>16.5</td>
<td>50.9 ± 1.9</td>
<td>8.6 ± 0.1</td>
<td>11.4 ± 1.2</td>
</tr>
<tr>
<td>Ciprofloxacin WT</td>
<td>54.2 ± 1.0</td>
<td>70.2 ± 5.3</td>
<td>123.1 ± 6.8</td>
<td>1.4</td>
<td>33.9 ± 0.6</td>
<td>5.1 ± 0.4</td>
<td>7.6 ± 0.4</td>
</tr>
<tr>
<td>Norfloxacin ΔC</td>
<td>67.9 ± 4.1</td>
<td>111.4 ± 0.9</td>
<td>180.8 ± 1.9</td>
<td>-1.4</td>
<td>42.5 ± 2.6</td>
<td>8.1 ± 0.1</td>
<td>11.2 ± 0.1</td>
</tr>
<tr>
<td>Norfloxacin WT</td>
<td>54.4 ± 1.6</td>
<td>93.0 ± 0.5</td>
<td>147.7 ± 4.7</td>
<td>-0.3</td>
<td>34.0 ± 1.0</td>
<td>6.7 ± 0.1</td>
<td>9.2 ± 0.3</td>
</tr>
<tr>
<td>Enrofloxacin ΔC</td>
<td>128.6 ± 0.3</td>
<td>82.5 ± 5.8</td>
<td>187.4 ± 16.6</td>
<td>23.7</td>
<td>80.4 ± 0.2</td>
<td>6.0 ± 0.4</td>
<td>11.6 ± 1.0</td>
</tr>
<tr>
<td>Enrofloxacin WT</td>
<td>22.8 ± 0.9</td>
<td>13.5 ± 1.0</td>
<td>42.9 ± 3.7</td>
<td>-6.6</td>
<td>14.2 ± 0.5</td>
<td>1.0 ± 0.1</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Levofoxacin ΔC</td>
<td>72.2 ± 1.6</td>
<td>17.9 ± 2.2</td>
<td>117.5 ± 10.4</td>
<td>-27.4</td>
<td>45.1 ± 1.0</td>
<td>1.3 ± 0.2</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td>Levofoxacin WT</td>
<td>38.8 ± 1.9</td>
<td>8.7 ± 0.5</td>
<td>52.7 ± 2.4</td>
<td>-5.2</td>
<td>24.2 ± 1.2</td>
<td>0.6 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>Lomefloxacin ΔC</td>
<td>91.1 ± 2.4</td>
<td>47.4 ± 5.0</td>
<td>149.3 ± 11.1</td>
<td>-10.7</td>
<td>56.9 ± 1.5</td>
<td>3.4 ± 0.3</td>
<td>9.3 ± 0.7</td>
</tr>
<tr>
<td>Lomefloxacin WT</td>
<td>33.5 ± 3.1</td>
<td>9.5 ± 6.5</td>
<td>37.0 ± 6.4</td>
<td>6.0</td>
<td>20.9 ± 1.9</td>
<td>0.7 ± 0.4</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>Ofloxacin ΔC</td>
<td>70.4 ± 6.2</td>
<td>23.7 ± 3.1</td>
<td>96.5 ± 4.6</td>
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<td>44.0 ± 3.9</td>
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<td>Ofloxacin WT</td>
<td>28.8 ± 2.1</td>
<td>3.0 ± 2.3</td>
<td>35.3 ± 3.2</td>
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<td>18.0 ± 1.3</td>
<td>0.2 ± 0.2</td>
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<td>Fleroxacin ΔC</td>
<td>100.3 ± 3.5</td>
<td>49.0 ± 3.4</td>
<td>146.4 ± 5.1</td>
<td>3.0</td>
<td>62.7 ± 2.2</td>
<td>3.6 ± 0.2</td>
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<tr>
<td>Fleroxacin WT</td>
<td>58.4 ± 1.6</td>
<td>20.2 ± 1.7</td>
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<td>36.5 ± 1.0</td>
<td>1.5 ± 0.1</td>
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Table 2.1. continued. Accumulation of fluoroquinolones (FQs) in two strains, BW25113 (WT) and BW25113Δtolc (ΔC). Mᵣ, Mₑ, and Mᵣₑ are mass of accumulated compounds in the whole cell, periplasm, and cytoplasm, respectively. The difference (Δ) between the sum of the measured periplasmic and cytoplasmic accumulation to that of the whole cell accumulation was calculated. Cₑ, Cₑₑ and Cₑᵣ refer to the concentration of drug in the periplasm, cytoplasm, and whole cell respectively.

<table>
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<tr>
<th></th>
<th>Marbofloxacin</th>
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<tr>
<td></td>
<td>ΔC</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>76.7 ± 4.8</td>
<td>34.0 ± 4.7</td>
<td>100.5 ± 3.8</td>
<td>10.2</td>
<td>47.9 ± 3.0</td>
<td>2.5 ± 0.3</td>
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<tr>
<td></td>
<td>39.6 ± 1.1</td>
<td>14.5 ± 3.5</td>
<td>46.7 ± 5.6</td>
<td>7.4</td>
<td>24.7 ± 0.7</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Marbofloxacin</td>
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<tr>
<td></td>
<td>WT</td>
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<tr>
<td></td>
<td>68.1 ± 2.6</td>
<td>47.8 ± 0.5</td>
<td>124.2 ± 0.9</td>
<td>-8.3</td>
<td>42.5 ± 1.6</td>
<td>3.5 ± 0.1</td>
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<td></td>
<td>Moxifloxacin</td>
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<tr>
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<tr>
<td></td>
<td>13.9 ± 0.6</td>
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<tr>
<td></td>
<td>WT</td>
<td></td>
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</tbody>
</table>
| 2.3.5  Quantification of compounds loss during washing

While the logic of washing the cell pellet to remove compounds loosely attached to the exterior of the bacteria is apparent, there is also concern of losing compound, especially from the periplasm, during the washing step. To avoid the wash step, a method was developed to centrifuge cells through a silicon oil layer. We adopted the silicon oil method in this study, but the very high drug concentration measured in the periplasm made us reconsider the procedure. Inefficient separation of exterior compounds is likely to lead to a larger false positive impact for the periplasmic accumulation, as these compounds could be washed off during the osmotic shock procedure and contribute to the measured periplasmic accumulation.

To evaluate the potential impact of a wash step to the measurement, we quantified compounds that were released from the drug-treated cell pellet during a quick wash step
after centrifugation of the bacteria through the silicon oil layer. Figure 2.4 showed the $M_w$ values for all fluoroquinolones in the two strains, as well as the respective $M_t$ for comparison. Even after centrifugation through the silicon oil layer, the washing process still led to a significant reduction of the associated compounds for all fluoroquinolones. The level of reduction varies among the compounds. Compounds “washed off” could either be loosely associated to the exterior of the cell surface or flushed out from the cell. Interestingly, for every compound, the wild type (WT) strain BW25113 (diagonal lines) lost a higher percentage of associated drug during the wash step than the efflux- deficient strain (dotted), clearly indicating the involvement of active efflux and thus loss of compound from the inside of the bacteria during the quick wash procedure (Figure 2.4B).

The accumulation data of the periplasm and whole cell accumulation, after wash correction, were shown in Table 2.2. Since the cytoplasmic accumulation data were collected from samples after the removal of the periplasm, there is no need to apply wash correction to the cytoplasmic accumulation data. Even with the wash correction, the periplasmic concentrations remain to be higher than both the cytoplasmic and external media concentrations.
Figure 2.4. Loss of compounds during wash. A. Measured M_w values. The corresponding M_t was also shown for comparison. B. Relative M_w to M_t for BW25113 (diagonal lines) and BW25113∆tolC (dotted).
Table 2.2. Accumulation of fluoroquinolones (FQs) in two strains, *BW25113* (WT) and *BW25113Δtolc* (ΔC) after correction for wash. M<sub>w</sub> and M<sub>pw</sub> are accumulation numbers in the whole cell and periplasm and cytoplasm, respectively. C<sub>pw</sub> and C<sub>tw</sub> refer to the concentration of drug in the periplasm and whole cell respectively.

<table>
<thead>
<tr>
<th>FQs / strain</th>
<th>M&lt;sub&gt;w&lt;/sub&gt; (ng)</th>
<th>M&lt;sub&gt;pw&lt;/sub&gt; (ng)</th>
<th>M&lt;sub&gt;tw&lt;/sub&gt; (ng)</th>
<th>C&lt;sub&gt;pw&lt;/sub&gt; (µg/mL)</th>
<th>C&lt;sub&gt;tw&lt;/sub&gt; (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin ΔC</td>
<td>40.4 ± 7.3</td>
<td>41.0 ± 7.9</td>
<td>142.6 ± 20.1</td>
<td>25.6 ± 5.0</td>
<td>8.9 ± 1.2</td>
</tr>
<tr>
<td>Ciprofloxacin WT</td>
<td>37.0 ± 5.6</td>
<td>17.3 ± 5.7</td>
<td>86.1 ± 8.8</td>
<td>10.9 ± 3.5</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>Norfloxacin ΔC</td>
<td>32.4 ± 1.1</td>
<td>35.6 ± 4.3</td>
<td>148.4 ± 2.2</td>
<td>22.3 ± 2.7</td>
<td>9.2 ± 0.1</td>
</tr>
<tr>
<td>Norfloxacin WT</td>
<td>32.6 ± 1.4</td>
<td>21.8 ± 2.1</td>
<td>115.0 ± 4.9</td>
<td>13.6 ± 1.3</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>Enrofloxacin ΔC</td>
<td>37.9 ± 3.9</td>
<td>90.7 ± 3.9</td>
<td>149.5 ± 17.0</td>
<td>56.7 ± 2.4</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td>Enrofloxacin WT</td>
<td>20.2 ± 3.4</td>
<td>2.7 ± 3.5</td>
<td>22.8 ± 5.0</td>
<td>1.7 ± 2.2</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Levofloxacin ΔC</td>
<td>42.6 ± 0.6</td>
<td>29.6 ± 1.7</td>
<td>74.9 ± 10.5</td>
<td>18.5 ± 1.1</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>Levofloxacin WT</td>
<td>36.2 ± 3.1</td>
<td>2.6 ± 3.7</td>
<td>16.5 ± 3.9</td>
<td>1.6 ± 2.3</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Lomefloxacin ΔC</td>
<td>21.0 ± 5.5</td>
<td>70.1 ± 6.0</td>
<td>128.0 ± 12.4</td>
<td>43.8 ± 3.7</td>
<td>8.0 ± 0.8</td>
</tr>
<tr>
<td>Lomefloxacin WT</td>
<td>21.6 ± 5.9</td>
<td>11.9 ± 6.6</td>
<td>15.4 ± 8.7</td>
<td>7.4 ± 4.2</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Ofloxacin ΔC</td>
<td>40.1 ± 5.0</td>
<td>30.3 ± 8.0</td>
<td>56.4 ± 6.8</td>
<td>19.0 ± 5.0</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Ofloxacin WT</td>
<td>25.7 ± 0.7</td>
<td>3.2 ± 2.2</td>
<td>9.7 ± 3.2</td>
<td>2.0 ± 1.4</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Fleroxacin ΔC</td>
<td>65.4 ± 6.4</td>
<td>34.9 ± 7.3</td>
<td>81.0 ± 8.1</td>
<td>21.8 ± 4.5</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>Fleroxacin WT</td>
<td>55.8 ± 2.0</td>
<td>2.6 ± 2.5</td>
<td>17.6 ± 5.0</td>
<td>1.7 ± 1.6</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Marbofloxacin ΔC</td>
<td>22.3 ± 3.5</td>
<td>54.4 ± 5.9</td>
<td>78.1 ± 5.2</td>
<td>34.0 ± 3.7</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>Marbofloxacin WT</td>
<td>12.0 ± 1.6</td>
<td>27.6 ± 1.9</td>
<td>34.8 ± 5.8</td>
<td>17.2 ± 1.2</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>Moxifloxacin ΔC</td>
<td>37.7 ± 4.3</td>
<td>30.3 ± 5.0</td>
<td>86.5 ± 4.3</td>
<td>19.0 ± 3.1</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>Moxifloxacin WT</td>
<td>10.1 ± 2.1</td>
<td>3.9 ± 2.2</td>
<td>17.4 ± 2.3</td>
<td>2.4 ± 1.4</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>
2.3.6 Accumulated fluoroquinolones were at higher concentrations than the external media

In the efflux deficient strain ΔtolC, the concentrations of compound accumulated in the cells were always higher than the external concentration of compound used in the study (2.0 µg/ml). The observation of higher drug concentration collected inside the cell than the external concentration has been reported in several previous studies, however, whole cell accumulations were measured in these cases. We found that the accumulated concentrations in the periplasm were higher than concentrations both the in the cytoplasm and the external media. Two potential artifacts could lead to evaluated number of the measured Cp: first, nonspecific binding of compounds to the cell surface, which was later detached from the membrane during the osmotic shock step; second, leakage during the osmotic shock step, which may lead to the release of compounds from the cytoplasm to the osmotic shock solution. To minimized potential effect of the first possibility, we evaluated the effect of washing as described above. Even with the wash correction, which likely leads to an underestimate on the measured Cpw due to loss of compound from the periplasm during wash, the periplasmic concentrations for most cases remained higher than 2.0 µg/ml. To address the second possible source of error, we used a very quick osmotic shock procedure (less than 10 mins) during our study. To examine the integrity of the inner membrane during the process, we conducted two assays. First, we used BW25113 expressing sfGFP. As described in materials and methods, the cells were subjected to identical treatment as cells used in the drug accumulation assay to obtain the periplasmic,
cytoplasm, and whole cell extractions. Measurement of sfGFP fluorescence in the three samples revealed that the signal in the periplasmic sample was less than 2% of the whole cell signal (Figure 2.5). We acknowledge that sfGFP is much larger than the fluoroquinolones, however the lack of sfGFP leakage suggests that the procedure did not lead to a large-scale membrane disruption. To further confirm that the high fluoroquinolone concentration in the periplasm was not a result of leakage from the cytoplasm, we fractionated BW25113 as described and measured the ATP concentration in the periplasm, cytoplasm, and the whole cell samples. Since ATP is only present in the cytoplasm, the detection of ATP in the periplasm would indicate that leakage occurred during the osmotic shock procedure. We found that the relative ATP concentration in the periplasm was ~6% of the concentration determined for the whole cell sample, indicating that the leakage of small molecules such as ATP from the cytoplasm was minimal during the osmotic shock procedure (Figure 2.6).
Figure 2.5. Cell viability assay.

A. Fluorescence images of the cells after staining with the bacteria live/dead staining kit. Left panel, BW25113ΔtolC cells without drug treatment. Right panel, BW25113ΔtolC cells incubated with 2.0 μg/mL ciprofloxacin for 15 min. Green indicates live bacteria, while red indicates dead bacteria. B. EtBr accumulation assay of BW25113 (black smooth line), BW25113ΔtolC (grey smooth line), BW25113 treated with ciprofloxacin (black dotted line) and BW25113ΔtolC treated with ciprofloxacin (grey dotted line). Accumulation in dead BW25113 (blue line) and BW25113ΔtolC (red line) pre-treated with isopropanol were also shown as controls.
Figure 2.6. Evaluation of potential leakage of the inner membrane during the osmotic shock procedure.
A. Fluorescence emission spectra of the whole cell (black), cytoplasm (grey), and periplasm (red) samples prepared from BW25113 expressing sfGFP. The relative fluorescence of the cytoplasm over the whole cell samples, and periplasm over the whole cell samples were also shown. B. The relative ATP concentration in the cytoplasm and periplasm versus in the whole cell.

2.3.7 Correlation between MIC ratio versus accumulation ratio in two isogenic strains

MIC of all fluoroquinolones was determined for both BW25113 and BW25113ΔtolC (Table 2.3). Compounds with higher MIC are less effective against a certain bacterium. While higher accumulation inside the cell makes a compound a better antibiotic, accumulation alone never correlated well with the effectiveness of an antibiotic as reported
in previous studies.\textsuperscript{152-157} Although the efficacy of an antibiotic against a bacterium depends on several factors, if other parameters remain consistent except for accumulation, we expect that the accumulation at the target site to correlate directly with the efficacy of the antibiotic.

We measured the accumulation of the nine fluoroquinolones in a pair of isogenic strains, \textit{BW25113} and \textit{BW25113\textDelta tolC}. The only difference between the two strains is the deficiency of efflux in the knockout strain, thus we expect the MIC ratio between the two strains to correlate negatively with the cytosolic accumulation ratio of these compounds.

As expected, the MIC ratio correlated very well with the ratio of cytoplasmic accumulation $C_c$ with a Pearson’s correlation coefficient of 0.94 (Figure 2.7). However, no correlation was observed when the MIC ratio was plotted against the ratio of the whole cell accumulation $C_t$.

\textbf{Table 2.3. MIC of fluoroquinolones in two E. coli strains, BW25113 (WT) and BW25113 \textDelta tolC.}

<table>
<thead>
<tr>
<th>Fluoroquinolones</th>
<th>MIC (µg/ml)</th>
<th>$R_{\text{MIC}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>$\Delta \text{tolC}$</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.016</td>
<td>0.004</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0.064</td>
<td>0.016</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0.032</td>
<td>0.002</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.032</td>
<td>0.008</td>
</tr>
<tr>
<td>Lomefloxacin</td>
<td>0.125</td>
<td>0.016</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0.125</td>
<td>0.008</td>
</tr>
<tr>
<td>Fleroxacin</td>
<td>0.064</td>
<td>0.016</td>
</tr>
<tr>
<td>Marbofloxacin</td>
<td>0.032</td>
<td>0.008</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.064</td>
<td>0.008</td>
</tr>
</tbody>
</table>
2.3.8 Effect of hydrophobicity in partition across the inner membrane

As mentioned in the introduction, the correlation between hydrophobicity of a compound with its accumulation has been investigated by several studies.\textsuperscript{152-154, 156, 157} There is no consistent conclusion about how hydrophobicity affects the overall accumulation in Gram-negative bacteria. Here, the cytoplasmic and periplasmic accumulation data offered us an opportunity to evaluate the role of the inner membrane as a hydrophobic barrier. The ratio of the cytoplasmic to periplasmic accumulation (\(C_c/C_p\)) were plotted against the hydrophobicity of the compounds (Figure 2.8). Higher hydrophobicity, as represented by the clogD values, did not correlate with a higher percentage of compounds accumulated into the cytoplasm.
Figure 2.8. $C_c/C_p$ plotted against clogD. clogD values were calculated using Chemaxon, MarvinSketch version 18.10 at pH 7.4, with electrolyte concentration of 0.1 mol/dm$^3$ Cl$^-$, 0.1 mol/dm$^3$ Na$^+$/K$^+$. 

2.4 Discussion

The main purpose of this study was to characterize antimicrobials accumulation and distribution inside the periplasm and cytoplasm of Gram-negative bacterial cell and examine the impact of this distribution on the efficacy of the drug. The separation of the whole cell accumulation into the periplasmic and cytoplasmic components offered us the unique opportunity to make several interesting observations.
First, we observed that inside the Gram-negative bacterium *E. coli*, the accumulated fluoroquinolone concentration was very different in different subcellular compartments. For all nine compounds in both strains, we observed a much higher concentration of fluoroquinolones accumulated in the periplasm compared to the cytoplasm. In most case the periplasmic fluoroquinolone concentration is even higher than the concentration in the exterior media. Prochnow et al. also observed that the concentration of ciprofloxacin in the periplasm of *E. coli* was higher than the external concentration used in the accumulation assay.\textsuperscript{158} While no active uptake has been identified so far for fluoroquinolones, the negative inside Donnan potential across the outer membrane has been considered to be a major contributing factor that lead to the accumulation of these compounds inside the Gram-negative bacteria.\textsuperscript{163, 177} Another possibility is the binding of fluoroquinolones to yet unknown periplasmic components, which may have reduced the concentration of free drug in the periplasm to be lower than the exterior concentration.

Another interesting observation is the lack of correlation between the hydrophobicity of the compounds and their ability to accumulate in the cytoplasm. The inner membrane is clearly not just a hydrophobic barrier in this case. Other factors that could potentially affect the \( C_v/C_p \) ratio are the structure of the compounds, and presence of binding partners in the cytoplasm and/or periplasm. Binding to a partner will switch a molecule from the free state to a bound state, which may affect the equilibrium across the inner membrane.

To understand the structure features that promote a compound’s ability to penetrate the Gram-negative cellular envelope, several studies were performed recently to investigate
the accumulation of compounds in Gram-negative bacteria. O’shea and Moser observed that antimicrobials effective for Gram-negative bacteria were normally small (less than 600 Da) and much more polar in general than other pharmaceuticals. Silver et al. compared the antimicrobials effective against Gram-negative bacteria with the ones effective against Gram-positive bacteria and reported that the former group are smaller in size and less polar. Among the ones active against Gram-negative bacteria, those enter through passive diffusion are less polar compared to the those entering through active transport. Richter et al. measured accumulation of a large group of compounds to distill principles favoring accumulation in Gram-negative bacteria. They concluded that good accumulators tend to have amine functional groups, be amphiphilic and rigid, and with low globularity. More importantly, these rules were applied to convert deoxynybomycin from a Gram-positive-only antibiotic into an antibiotic that is active against a wide range of multi-drug resistant bacteria including Gram-negative ones. Acosta-Gutierrez et. al. focused on porins to study the constricted region of the channel, the electrostatic interactions, and size limit to understand the mechanisms of penetration through these channels. They concluded that positively charged groups are important to promote penetration, which is consistent with the mechanisms of the rules proposed by Richter et al. In all these studies, whole cell accumulation was measured. We argue that quantification of the accumulation at different subcellular compartment will lead to valuable insight into the mechanism of effective penetration. Collecting data on the accumulation of a large group of compounds in sub-cellular compartments is a cumbersome and labor-intensive, yet necessary effort. Here we reported the preliminary result on a group
of nine fluoroquinolones. Additional studies on diverse sets of compounds would be critically necessary to fully understand the structure features that favor the accumulation of antimicrobials in bacterial cells.
3.1 Introduction

The ever-growing antibiotic resistance in pathogens has become a serious global health threat and warrants a need for development of new therapeutics. Among drug-resistant superbugs, gram-negative bacteria cause more serious concerns because of the dwindling pool of effective therapeutics. Their double-layered cell envelope is difficult for antibiotics to penetrate. The asymmetrical outer membrane consists of lipopolysaccharides (LPS) and phospholipids in the outer and inner leaflet, respectively. Together with the phospholipid inner membrane, they make a tough barrier against both hydrophilic and hydrophobic antibiotics. Another major hurdle for antibiotics to cross the cellular envelope is the multiple efflux pumps that effectively transport many antibiotics out of cells. Hence, there is an ongoing hunt for novel approaches to breach the barrier to increase the penetration and accumulation efficiency of antibiotics in gram-negative bacteria.
Several different approaches are being studied to address this penetration issue. Researchers are trying to establish the profiles that describe compound with good penetration behavior through the gram-negative cell envelope.\textsuperscript{18, 151, 180} Retrospective study using the active antimicrobials as well as screening and computational algorithms have been successful in discovering some features shared by good penetrators. However, these rules are not holistic.\textsuperscript{181} Apart from designing a potent inhibitor with optimal penetration features, an alternative approach that has been explored to address the impermeability issue is the “Trojan horse” technique. In this technique, the antibiotic is conjugated to a nutrient molecule and the uptake is driven by dedicated transporters of those nutrients present in the bacterial membrane.\textsuperscript{26} The iron uptake system is such a system that has been the subject of study for several decades.\textsuperscript{27-30, 182} Siderophores are iron chelators released by bacteria to capture iron in the surrounding environment. Uptake of chelated iron is facilitated by the siderophore transporters. Antibacterial conjugated siderophores (sideromycins) occur in nature, for example albomycin, and are released by bacteria when they compete with other species for growing resources.\textsuperscript{183} Mimicking nature, siderophore conjugates have been created for a couple of compounds to enhance their cellular accumulation through siderophore transporters. In some cases, the conjugates were cleaved once inside the cell to release the free drug.\textsuperscript{27, 31, 32} Antibacterials made through this technique are showing promising results, especially the β-lactam-siderophore conjugates.\textsuperscript{31} So far, the most promising candidate clinically is a siderophore antibiotic conjugate called Cefiderocol (S-649266), which is in phase 3 clinical trial.\textsuperscript{37} This catechol cephalosporin conjugate has showed a promising antibacterial activity against a variety of Gram-negative pathogens.
including *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Enterobacteriaceae* and even the strains producing carbapenemases like *Klebsiella pneumoniae* carbapenemase and New Delhi metallo-β-lactamases (NDM)-1.31, 38

Vitamin conjugation has also been explored to enhance drug penetration, mostly in mammalian cells, such as cancer cells. Since vitamins are critical for various metabolic reactions essential for survival, cells must either synthesize or uptake these vitamins from external environment through their transporters. Biotin (vitamin B7) mediated enhanced uptake has been shown to be effective in mammalian cell drug delivery, especially in cancer cells.39-41 Sodium dependent multivitamin transporters (SMVT) are overexpressed in several different types of cancer cells, and it has been shown that biotin conjugation enhanced the uptake of anticancer molecule through these transporters.42, 43 Biotin conjugates are being studied because they can be both target-specific to cancer cells and can enhance the accumulation of drug through overexpressed biotin transporters.44

However, in case of gram-negative bacteria, approaches involving biotinylation to enhance the uptake of small molecules have not been explored. Bacteria also require biotin for several metabolic processes, mainly the carboxylation and decarboxylation reactions. For this, bacteria either synthesize the biotin or uptake it whenever available through the biotin transporters.184 In *E. coli*, there is only one major essential metabolic reaction carried out by Acetyl CoA carboxylase complex that needs biotin as a cofactor, although requirement in propionate metabolism have been reported in certain strains.185, 186 Acetyl CoA carboxylase acts in the conversion of acetyl CoA to malonyl CoA as in the fatty-acid biosynthesis process. The synthesis of biotin is such an energy expensive process that *E.
coli prefers to uptake biotin from the environment whenever available.\textsuperscript{185} Biotin uptake in prokaryotes is believed to mostly occur through ATP dependent energy coupling factor (ECF) transporters, consisting of substrate specific S unit, transmembrane T unit, and ATPase containing A unit. In biotin transport, proteins BioY, BioN and BioM represent the S, T and A unit, respectively. However, in some bacteria the substrate specific BioY alone has been shown to transport biotin.\textsuperscript{187} Recently it has been identified that in \textit{E. coli}, biotin transport is mediated by a different protein called YgiM (BioP) through a yet not completely understood mechanism.\textsuperscript{184}

Walker et. al.\textsuperscript{188} have shown that biotinylation facilitated the uptake of very large peptides up to 31 amino acids long that otherwise could not breach the membrane barrier of gram-negative bacteria. It was interesting to see that bacteria like \textit{E. coli}, \textit{Salmonella enterica} Typhimurium and \textit{Pseudomonas aeruginosa} were able to uptake these large peptides through their biotin transporters.\textsuperscript{188} Another example of exploiting nutrient uptake pathways for small molecules translocation was shown by Dumont et. al. where they demonstrated that the maltose porin LamB can transport and translocate the malto-triose conjugated fluorophores into the periplasm and cytoplasm of \textit{E. coli}.\textsuperscript{189} Recently, one study by Zhao et. al.\textsuperscript{190} focused on vitamin B12 mediated enhanced uptake where they showed ampicillin upon conjugation with vitamin B12 had about 500 folds improved efficacy than the parent ampicillin. They demonstrated that this increased efficacy is due to the enhanced uptake through vitamin B12 transporters.\textsuperscript{190}

However, besides biotinylation mediated peptide uptake, to our knowledge there are no such studies that focused on the biotinylation mediated uptake of small molecules.
in gram-negative bacteria. Thus, we tried to probe how biotinylation will affect the penetration and accumulation of small molecules using florescent compound Atto565 and *E. coli* as a gram-negative model.

### 3.2 Materials and methods

#### 3.2.1 Bacterial strains and chemicals

Atto565, Atto565-biotin, Atto565-NHS and all the amino acids used were obtained from Sigma-Aldrich (St. Louis, MO). Bacterial strain BW25113 was obtained from Yale E. coli Genetic Stock Centre, BL21(DE3) strain was obtained from New England Biolabs (Ipswich, MA) and MG1655WT, ΔOmpF, ΔOmpC and ΔOmpF&C were kindly provided as a gift by Dr. Chang-Ro Lee (Department of Biological Sciences and Bioinformatics, Myongji University, Yongin, South Korea).

#### 3.2.2 Compound accumulation assay

Overnight bacterial culture was diluted 50 folds in fresh and sterile LB and cultured at 37 degree Celsius with constant shaking at 250 rpm until they reach mid-log phase (OD ~0.6). After that, the cells were harvested through centrifugation at 3000×g for 15 minutes, washed once using the NaPi-Mg buffer (50 mM sodium phosphate buffer with 5 mM MgCl₂, pH 7.4) and then resuspended in the same buffer to a final concentration of OD₆₀₀nm 8.0. From this cell suspension, 2 mL was taken in a glass vial and 250 µL of compound (10X) was added and final volume was adjusted to 2.5 mL using the buffer to make the final concentration of compound 2 µM and final OD₆₀₀nm 6.4. The mixture was then
incubated for 30 minutes with constant shaking at 250rpm at 28 degree Celsius. After the incubation, 700 µL of suspension was taken and carefully layered on top of 700 µL silicone oil (AR20: Sigma High temperature=9:1)\textsuperscript{151} in a microcentrifuge tube. It was then centrifuged for 1 minute at 13,000g and the supernatant composed of upper aqueous layer and lower oil layer was carefully removed using micropipette. Kim wipes were then used to wipe the inner wall of the tube and the pellet was resuspended in 1 mL of the lysis buffer (50mM Tris pH 8.0, 0.5% Triton-X100, 0.5% glycerol, 100 µg/mL lysozyme, 10 µg/mL DNase). Next, samples were subjected to 6 cycles of freeze and thaw, followed by centrifugation for 15 minutes at 15,000×g to remove the cell debris. The supernatant was collected to measure the florescence. For both Atto565 and Atto565-biotin, excitation wavelength used was 565 nm and the emission was monitored from 575 nm to 620 nm, and the emission maxima as 585 nm were recorded. All the measurements were done in triplicate and the data were presented as average ± standard deviation.

3.2.3 Fractionation of periplasm and cytoplasm

Periplasm and cytoplasm were separated according to the osmotic shock method we described previously.\textsuperscript{191} Briefly, the pellet obtained after incubation with compounds and centrifugation was resuspended in 100 µL periplasm preparation buffer (200 mM Tris-Cl, 1 mM EDTA, 20% sucrose, 1 mg/mL lysozyme, pH 8.0). The resuspension was then incubated at room temperature for 5 minutes, followed by the addition of 200 µL ice cold deionized water. The mixture was mixed properly by tapping or pipetting and was incubated on ice for 2 minutes. Samples were then centrifuged for 1 minute at 13,000×g.
The supernatant contained the periplasmic components, and the pellet contained the spheroplast. Spheroplast was further lysed according to the procedure as mentioned above to release the cytoplasmic components.

### 3.2.4 Expression of streptavidin and biotin-streptavidin binding

Streptavidin was expressed using the pET30b vector. pET30b-OmpAT7Sav (Addgene #138589) was used to express streptavidin in the periplasm, and pET30b-T7Sav (Addgene #138588) was used to express streptavidin in the cytoplasm of BL21(DE3) cells. Biotin is essential for cell growth as it is a cofactor for the enzyme Acetyl CoA carboxylase that converts acetyl CoA into Malonyl CoA needed for fatty acid biosynthesis. Expression of streptavidin inside the cell is toxic as it will bind to the biotin and deprive biotin for fatty acid biosynthesis. To circumvent this detrimental effect, streptavidin was expressed in conjunction with MatC transporter and MatB enzyme from the plasmid pCKmatBC (Addgene #138587), which transport malonate from the media (MatC) and convert it into malonyl CoA (MatB). Through this strategy, the need for biotin in the conversion of acetyl CoA to malonyl CoA was bypassed.

The expression of streptavidin was carried out by inducing the mid log phase cells with 0.5 mM IPTG for 45 minutes and then accumulation experiment was done as described in section 2.2. Binding to streptavidin quenches the fluorescence of biotinylated Atto565. To dissociate Atto565-biotin from streptavidin, a very high concentration (3 mM) of free biotin was added into the cell lysate and samples were subjected to a heat treatment of 78 degree Celsius for 10 minutes. Next the samples were cooled down, centrifuged for
15 minutes at 15,000×g, and then the supernatant was collected for fluorescence measurement. High temperature helped to break the biotin/streptavidin interaction, and the presence of high concentration of free biotin helped to saturate the binding site of streptavidin to free Atto565-biotin.

### 3.2.5 Cloning and expression of OmpC

The OmpC gene was amplified using PCR from the genomic DNA of *E. coli* MG1655WT strain and it was cloned into plasmid pACYCT2 (Addgene #45799)\textsuperscript{193} using the “Fast cloning” method.\textsuperscript{194} The OmpC encoding plasmid, pACYCT2-OmpC, was then transformed into the *E. coli* MG1655\textDeltaompC strain. Fresh transformants were picked and cultured overnight in LB with 25 µg/ml chloramphenicol. This overnight culture was used to inoculate a fresh LB/Chloramphenicol media. OmpC expression was induced when the culture grew to the mid log phase with 1 mM IPTG for 30 mins at 28 degree Celsius. Cells were then harvested and subjected to accumulation experiment as described in section 2.2. For the visualization of OmpC expression, 1 mL of OD 8.0 cells were lysed through sonication (50% amplitude, 1 min, 5 secs on/off) and centrifuged for 15 minutes at 15,000×g to get the membrane fraction. Supernatant was discarded and the membrane pellet was resuspended in 50 µL NaPi-Mg buffer, 75 µL 10% SDS and 25 µL of 6X SDS loading dye. The mixture was incubated for 1 hour at room temperature for protein extraction. After that, the samples were boiled for 10 minutes and visualized through SDS-PAGE in 15% polyacrylamide gel.
3.2.6 Site directed mutagenesis and knockouts

Site directed mutagenesis was done using the QuikChange Site Directed Mutagenesis Kit (Agilent, Santa Clara, CA) following the manufacturer’s protocols. Primers for the introduction of D18V, W72K and D171T mutations are shown in Table 3.1. LamB knockout strain was created using the Quick & easy E. coli gene deletion kit (Gene Bridges GmbH, Heidelberg, Germany) following the manufacturer’s protocol. The target gene on the MG1655 chromosome was replaced with an FRT-flanked kanamycin resistance marker cassette. Colony PCR was performed to confirm that the target gene has been replaced correctly. All constructs were confirmed through sequencing.

Table 3.1. List of primers used

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>D18V</td>
<td>5'-CGGTAAAGTAGTGCGCTGCAC-3'</td>
<td>5'-GTGCAAGCCGACTAATTACCG -3'</td>
</tr>
<tr>
<td>W72K</td>
<td>5'-CGAAAACAACTCCAAGACCCGTGAGCC -3'</td>
<td>5'-GCCACACGGGCTTTGGAGTTTAC -3'</td>
</tr>
<tr>
<td>D171T</td>
<td>5'-CTAACAAACGGGACTACCCGACTGC -3'</td>
<td>5'-GACGCAGTGCGGACCGCTTACAG -3'</td>
</tr>
</tbody>
</table>

3.2.7 Preparation of amino acids labelled Atto565

For the preparation of amino acids labelled Atto565, we used the Atto565-NHS ester and used it to react with amino groups of amino acids using the same principle of protein labeling by NHS ester of a compound. Labeling was done using 20 µM Atto565-NHS and 50 mM amino acids in a 200 mM Na-Pi buffer, pH 8.0 for 2 hours at room temperature. The resulting labeled solution was then used for the accumulation experiment.
Seven different amino acids (Glycine, Arginine, Lysine, Aspartic acid, Glutamic acid, Asparagine, Glutamine) were used including representatives of acidic, basic, and neutral amino acids.

3.2.8 Density functional theory (DFT) calculations

Density functional theory (DFT) calculations were carried out at the \( \omega B97XD/6-31G(d,p) \) level of theory to optimize molecular geometries, and charges were explicitly determined using the Charge Model 5. The Polarizable Continuum Model (PCM) with the integral equation formalism variant (IEFPCM) was used to simulate water solvent reaction field. The multi-wfn\textsuperscript{195} package was used for the generation of Hirshfield charge distributions\textsuperscript{196} for the DFT level energy optimized structures. All visualizations were carried out using Visual Molecular Dynamics (VMD) package version 1.9.3\textsuperscript{197} with colors red and blue representing the atomic charge range of -0.5 and +0.5 (a.u.) respectively.

3.2.9 Statistical Analysis

All the data shown were represented as mean±S.D. Significance was analyzed by either One-way or Two-way analysis of variance (ANOVA). Specific details about the asterisks presented above the bar or group of bars and significance measurements are presented in the figure legends.
3.3 Results

3.3.1 Atto565-biotin accumulates higher in *E. coli* than the Atto565

To determine whether biotinylation of compounds would enhance their accumulation in gram-negative bacterial cells, we first measured the accumulation of a fluorescent compound, Atto565, and its biotinylated form, Atto565-biotin, in *E. coli*. The cells were incubated with the compound and then harvested. After cell lysis and centrifugation, fluorescence signal in the supernatant was monitored. We observed that Atto565-biotin accumulated at a significantly higher level compared to the parent compound Atto565 (Figure 3.1). To determine if the higher accumulation is because of the uptake through biotin transporter, we did a competitive experiment by adding an excess of free biotin (4 mM) in the presence of Atto565-biotin (2 µM) or Atto565 (2 µM) during the incubation. If the uptake was through biotin transporters, the presence of a high concentration of free biotin would compete and impair the uptake of Atto565-biotin but not Atto565. However, we did not see a competitive effect from the 2000-fold excess of free biotin (Figure 3.1). The accumulation of Atto565-biotin was very similar with or without the presence of free biotin in the medium, indicating that biotin transporters are not involved in the enhanced uptake of Atto565-biotin.
3.3.2 Biotinylation enhances the penetration of Atto565 across the OM

Next, we measured the subcellular distribution of the compounds in the periplasm and cytoplasm to determine whether biotinylation promotes uptake of Atto565 through breaching the OM or the IM. We separated the periplasm from the cytoplasm and measure the fluorescence intensities in each sample. We found that the compounds were mostly located in the periplasmic fraction and a small portion of the compounds was in the cytoplasmic fraction (Fig 3.2).
To verify that Atto565 entered the cells, we measured accumulation of the fluorescent compound in *E. coli* strains expressing streptavidin. We expressed streptavidin in the periplasm or cytoplasm using plasmids pET30b_OmpAT7Sav and pET30b_T7Sav, respectively, as described in materials and method. We expect that if biotinylation really enhanced cellular accumulation of Atto565, a higher accumulation would be observed in strains expressing streptavidin. As a control, we also examined the impact of streptavidin expression on the accumulation of Atto565. We found that when streptavidin was expressed in the periplasm, the accumulation of Atto565-biotin increased but the accumulation of Atto565 remained unchanged (Figure 3.3). When free biotin was present in large excess, the accumulation of Atto565-biotin decreased. This clearly indicated that
Atto565-biotin entered the periplasm. In contrast, when streptavidin was expressed in the cytoplasm of the cell, the accumulation of Atto565-biotin did not increase (Figure 3.3).

**Figure 3.3. Accumulation of Atto565 and Atto565-biotin in *E. coli* expressing streptavidin either in the periplasm or cytoplasm.**
A) Schematics showing plasmid-expressed streptavidin binds to Atto565-biotin. B) Image of cell pellets showing redder color (higher accumulation) of Atto565-biotin in cells expressing periplasmic streptavidin and decrease in the accumulation in the presence of free biotin. C) Quantitative analyses of accumulation of Atto565 and Atto565-biotin in cells expressing streptavidin in the presence or absence of free biotin. Data were presented as mean±S.D. (Two-way analysis of variance; ****p<0.0001, *p<0.05). Asterisks above the Atto565 accumulation bar represent significance when compared them with Atto565 accumulation in WT strain, whereas asterisk above the Atto565-biotin accumulation bar represent the significance when compared with Atto565-biotin accumulation in WT strain.
3.3.3 Biotinylation does not affect efflux

Another possible factor that may lead to the observed higher accumulation of Atto565-biotin than Atto565 is efflux. If biotinylation makes a compound a bad substrate for efflux pumps, then higher accumulation would also be observed. We tested the efflux efficiency of both compounds by doing the accumulation experiment in wild type as well as in a deficient strain lacking major efflux systems. We used BW25113 and its isogenic ΔacrB and ΔtolC strains. Deletion of the acrB gene did not have a significant effect on accumulation of both compounds. A higher accumulation for both compounds were observed in the ΔtolC strain than in the WT strain, indicating that they both are substrates of efflux involving TolC (Figure 3.4). The ratio of accumulation of Atto565 between the WT strain and ΔtolC strain was very similar to the ratio of accumulation of Atto565-biotin between the WT and ΔtolC strain, suggesting that the extent of efflux was similar for both compounds. Thus, higher accumulation of Atto565-biotin is not because it is a bad substrate for TolC-involved efflux. Also, since we know efflux pumps can pump out substrates from either periplasm, inner membrane or cytoplasm\textsuperscript{198}, the observation that both of these compounds were substrate of efflux pumps further verified that the compounds entered the cells, not just attached to the cell surface.
Figure 3.4. Accumulation of Atto565 and Atto565-biotin in wild type versus efflux knock out BW25113 strains.

Data were presented as mean±S.D. (Two-way analysis of variance; ****p<0.0001, **p<0.01, *p<0.05). Asterisks above the Atto565 accumulation bar represent significance when compared them with Atto565 accumulation in WT strain, whereas asterisk above the Atto565-biotin accumulation bar represent the significance when compared with Atto565-biotin accumulation in WT strain.

3.3.4 OmpC contributes to the elevated accumulation of Atto565-biotin

Generally, compounds cross the outer membrane through porins, lipid mediated diffusion, or self-promoted uptake.\textsuperscript{147} We performed the accumulation assay using MG1655 and various isogenic porin knockout strains. We found that the accumulation of Atto565-biotin was greatly reduced in MG1655\textDeltaompC where the reduction of accumulation of parent compound was minimal (Figure 3.5). This reduction was not observed in MG1655\textDeltaompF, which showed similar accumulation as the wild-type strain. Also, MG1655\textDeltaompF\textDeltaompC double knockout strain showed similar accumulation as the
MG1655ΔompC single knock out strain. Another major porin apart from OmpF and OmpC is the sugar porin LamB, which is regarded mostly as the pathway for sugar transport including maltose and maltodextrins. We also tested the accumulation in the strain lacking this sugar porin LamB. As expected, the accumulation was similar as the wild-type strain, ruling out the involvement of LamB in higher accumulation of Atto565 after biotinylation. This indicated that OmpC serves as a port of entry for Atto565-biotin. Biotinylation makes the Atto565 a better passenger through OmpC.

**Figure 3.5. Accumulation of Atto565 and Atto565-biotin in wild type versus porin knock out strains.**

Data were presented as mean±S.D. (Two-way analysis of variance; ****p<0.0001, *p<0.05). Asterisks above the Atto565 accumulation bar represent significance when compared them with Atto565 accumulation in WT strain, whereas asterisk above the Atto565-biotin accumulation bar represent the significance when compared with Atto565-biotin accumulation in WT strain.
To further verify the role of OmpC, we complemented the \textit{BW25113}\textit{∆ompC} strain with plasmid pACYC2-OmpC, which encodes the \textit{ompC} gene under the control of the \textit{tac} promoter and IPTG inducible \textit{lac} operator. Upon induction of OmpC expression from the plasmid, accumulation of Atto565-biotin was partial restored in the \textit{BW25113}\textit{∆ompC} strain (Figure 3.6).

Both OmpC and OmpF are major porins in \textit{E. coli} and favor cationic molecules, but OmpC has a higher preference to cations than OmpF.\textsuperscript{199-201} The sequences of the two proteins are highly homologous, with 59.5\% identity and 72.1\% similarity. A few different ionizable amino acid residues lining the pore lumen have been proposed to be involved in making interactions with substrates during their passage through the channel.\textsuperscript{201} We replaced these residues in OmpC with their corresponding residues in OmpF and tested the impact of these mutations (D18V, W72K and D171T) on the accumulation of Atto565 and Atto565-biotin (Figure 3.6A). We found that the W72K mutation reduced the accumulation the most. When the tryptophan was converted into a lysine, expression of the mutant did not restore accumulation to the \textit{ompC} knock out strain. In contrast, the D18V mutation did not seem to affect passage of Atto565-biotin. Expression of the D171T mutant partially restored accumulation, at a level in between the levels in cells expressing W72K (close to the \textit{ompC} knockout strain) and D18V (similar to the \textit{ompC} knockout strain supplemented with plasmid encoded OmpC). We examined the expression levels of these mutants and confirmed that the mutants expressed at a similar level as the wild type OmpC (Figure 3.6B). The observed differences in accumulation are not due to variation in protein expression.
Figure 3.6. Accumulation of Atto565 and Atto565-biotin upon plasmid complementation of wild type OmpC as well as OmpC with different single mutations. Data were represented as mean±S.D. (Two-way analysis of variance; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Asterisks above the Atto565 accumulation bar represent significance when compared with Atto565 accumulation in WT strain, whereas asterisk above the Atto565-biotin accumulation bar represent the significance when compared with Atto565-biotin accumulation in WT strain.
3.3.5 Effect of labels other than biotin in accumulation of Atto565

The structure of Atto565 contains one ionizable amine group and two carboxylic acid groups, so at neutral pH the molecule will have a net negative charge. In Atto565-biotin, one of the carboxylic acid groups is altered to form an amide bond to connect with biotin, thereby eliminating one negative charge. We speculated that the elimination of one negative charge could make it a more favorable passenger through the OmpC channel as OmpC prefers cations.\(^{200,201}\) To elucidate the role of eliminating a negative charge, we reacted Atto565-NHS ester with various neutral (glycine, glutamine, and asparagine), positively charged (lysine and arginine), and negatively charged amino acids (aspartic acid and glutamic acid) (Figure 3.7).
Figure 3.7. Chemical structures of Atto565, Atto565-biotin, Atto565-NHS ester and the Atto565 labelled with various amino acids (Glycine, Lysine, Arginine, Aspartic acid, Glutamic acid, Asparagine, Glutamine) represented by -R group.

Accumulation of Atto565-amino acid derivatives are shown in Figure 3.8. Florescence was also measured for each of the labelled compound to control for the potential effect of the labeling on fluorescence intensity (Figure 3.9). When the fluorescent intensities were normalized using the calibration curves of each compound, it was observed that in general all Atto565 conjugates accumulated less than the parent compound Atto565. Among the conjugate compounds, negatively charged amino acids (glutamic acid and aspartic acid) reduced accumulation further than neutral and positively charged amino acids. This is consistent with the know property of OmpC for preferring positive charges.
Figure 3.8. Accumulation of different amino acids labelled Atto565 in wild type (A) and ΔOmpC (B) strains.

Data were presented as mean±S.D. (One-way analysis of variance; ****p<0.0001). Asterisks above the bars represent the significance compared with the accumulation of Atto565.
Figure 3.9. A) Florescence intensities of Atto565 and its conjugates showed that conjugation did not significantly affect the florescence quantum yield. B) Representative florescence intensities of Atto565 and its conjugates are linear with the concentration range used.

3.3.6 Molecular charge distributions and geometries

To further probe the potential impact of biotinylation on Atto565, we examined the Hirshfield charge distributions and energy optimized geometries as determined by DFT calculations at the ωB97XD/6-31G(d,p) level of theory. All structures showed similar charge distribution along the π-conjugated backbone, with the extra positive charge
delocalized between the amides. The DFT-optimized molecular geometries, as shown in Figure 3.10, reveal no real structural difference of the Atto565 in either the biotinylated or non-biotinylated forms. We note, however, that these are static pictures, and that molecular dynamics in the cellular environment could demonstrate variations in conformational flexibility. Further, the alkyl chains with functional groups could be of importance for establishing non-covalent interactions with OmpC to accommodate cellular penetration.

![Figure 3.10](image)

**Figure 3.10. Lowest energy conformations of the DFT-minimized structures.** The red and blue colors represent the atomic charge range of -0.5 and +0.5 respectively.

### 3.4 Discussion

Protection from the outer membrane and the inner membrane, as well as the presence of efflux pumps make the gram-negative bacterial envelope a formidable barrier for compounds to penetrate. This is becoming a significant hurdle in the development of new antimicrobials. Small hydrophilic molecules generally cross the outer membrane
through porin pores and hydrophobic molecules pass through passive diffusion, which is also not easy because of the presence of lipopolysaccharides outside the outer membrane. In addition, the inner membrane restricts penetration of small hydrophilic molecules. Several approaches are being developed to solve this issue. Conjugation of antimicrobials with nutrient molecules to facilitate their uptake through the nutrient uptake systems is such an approach.

In this study we examined the concept of using biotin to assist efficient penetration of selected compound into Gram-negative membrane. Based on our results, we found that biotin conjugation helps in the increased penetration of florescent molecules. However, in contrary to our expectation, the increased penetration did not seem to occur through biotin-specific uptake. When we measured the subcellular distribution of the accumulated compounds, it was observed that the majority of the compounds were accumulated in the periplasmic space. To determine if biotinylation actually promoted accumulation of Atto565 inside of cells, we examined the effect of expressing streptavidin. Our result clearly showed that expression of streptavidin in the periplasm specifically increased accumulation of Atto565-biotin, which was repressed in the presence of excess free biotin. This observation indicates that Atto565-biotin did enter the periplasm and was trapped by streptavidin. However, upon cytoplasmic expression of streptavidin the accumulation of Atto565-biotin did not increase significantly. Biotinylation of Atto565 seemed to promote penetration across the outer membrane but not the inner membrane. Further experiment using efflux knockout strains further confirmed that the compound did enter the cells, as non-specific cell surface attachment would have similar effect in different efflux knockout
strains. We used \textit{acrB} and \textit{tolC} gene knockout strains to evaluate the role of efflux in the accumulation of these compounds. AcrABToIC system is the resistance nodulation division (RND) superfamily type efflux pump which is a major contributor of the drug efflux in \textit{E. coli}. AcrB is the inner membrane pump, AcrA is the periplasmic adapter and TolC is the outer membrane channel in this system.\textsuperscript{202} However, TolC can also be coupled with several other efflux pump systems in \textit{E. coli}.\textsuperscript{203} We observed higher accumulation of both compounds in \textit{tolC} deficient strain, indicating that the efflux system involving TolC could pump out Atto565 and Atto565-biotin. In contrast, deletion of the \textit{acrB} gene did not have such an effect, indicating that TolC worked with another inner membrane protein/adaptor protein pair to efflux Atto565 and Atto565-Biotin.

If not through biotin-specific transporters, how did biotinylation improve accumulation? We found that the pathway of entry involves OmpC, but not a closely related porin OmpF. OmpC deficient strain accumulated less Atto565-biotin, while complementation with plasmid-encoded OmpC partially restored accumulation. Although the restoration was not 100\%, the increase was significant. Also, single residue mutations introduced to replace ionizable amino acids in the pore lining of OmpC affected Atto565 accumulation, further confirming the role of OmpC as a route of entry. This evidence supported that the Atto565-biotin permeated \textit{E. coli} outer membrane better than Atto565, and that this enhanced penetration is at least partially through the OmpC porin. This enhanced penetration through the outer membrane could be of great importance as the outer membrane is a critical barrier of defense for Gram-negative bacteria. There are several antibiotics which are effective against gram-positive bacteria but not against gram-negative
bacteria, because of this extra outer membrane and active efflux. So, development of methodologies that can help the otherwise impermeable molecules to pass through the outer membrane is beneficial to design antibiotics effective against Gram-negatives. Subcellular distribution studies and the streptavidin expressing experiments revealed that the accumulation of both compounds was not very significant in the cytoplasm and biotinylation only increased the cytoplasmic accumulation slightly. Biotinylation did not seemed to promote Atto565 permeability across the inner membrane.

OmpC is considered a non-specific porin present in the outer membrane of *E. coli*, with an hourglass shape pore lumen. The constriction region is formed by the extracellular L3 loop with the narrowest diameter in the region about 5.5 – 6 Å, slightly narrower than the constriction region of OmpF. While it has been generally accepted that these porins work as channels for small molecules of 600 Daltons or less, Ruggiu et. al. have demonstrated that even the molecules larger than the molecular weight of 600 Daltons can pass through these porins depending on their three-dimensional conformation, projection area, and dipole moment.

There could be several reasons of how and why biotinylation helps in the enhanced penetration through OmpC pore. One reason could be that biotinylation changes the molecular conformation and/or charge distribution in such a way that favors the passage through the OmpC pore. Further, attachment of biotin through an alkyl linker neutralizes the negative charge in the carboxylic acid group, increasing the net positive charge in the molecule, making it more favorable to pass through OmpC, which prefers cationic substrates. Our DFT calculations reveal regions of charge in the biotin tail, which may
contribute to interactions with the ionizable residues in the pore lumen of OmpC and facilitate the molecule to slip through the pore.

OmpF and OmpC are highly similar non-specific porins. Kojima and Nikaido highlighted the major difference in the pore lumen of OmpF and OmpC. There are 10 major ionizable residues which differ between OmpF and OmpC, namely D18, V29, N67, E68, W72, D135, D171, L173, R246 and K317. We picked three out of them, which were D18, W72 and D171 at three very different locations in the pore lumen. We found that when we mutate W72 into Lys and D171 into Thr, the corresponding amino acids present in OmpF, the accumulation of Atto565-biotin was reduced, suggesting that these ionizable residues might be involved in interactions with Atto565-biotin.

Recently, Prajapati et al. studied the translocation pathways of two fluoroquinolones, ciprofloxacin and norfloxacin, through OmpC, using molecular dynamics simulation with metadynamics and string method. They observed the presence of interactions including salt bridges, hydrogen bonds, π-stacking, and hydrophobic contacts. It was observed that these fluoroquinolones orient differently when they are present at the beginning of the pore versus when they are in the constriction region depending upon the interactions involved. Also, even a small difference in structure between ciprofloxacin and enrofloxacin had a significant change in how they orient and with what residues in the pore they interact. They also observed that because of these differences in interactions, enrofloxacin translocation is associated with a higher energy barrier, which is in line with the experimental observation of slower diffusion of enrofloxacin than ciprofloxacin. Relating this study to our observation, we might assume
a significant difference in the interactions and orientations during the translocation of Atto565 versus Atto565-biotin. However, further studies involving the molecular dynamics simulation is required to shed light on the exact mechanism.

3.5 Concluding remarks

In this study, we examined the effect of biotinylation on the accumulation of a fluorescent compounds, Atto565, in *E. coli*. We found that biotinylation indeed promote accumulation of Atto565, but not through the specific biotin-specific uptake system as we had expected. We found a two-fold increase of accumulation in the periplasm upon biotinylation. Several antibacterial targets have been explored in the periplasmic space of gram-negative bacteria and because of their location, antibacterial molecules need to cross only the outer membrane compared to both outer and inner membrane for cytoplasmic targets\textsuperscript{14}. Hence, development of strategies that allow for the better penetration of molecules through the outer membrane are helpful in designing new antibacterial therapeutics. On a positive note, Sauvageot et al. reported that in the absence of photo illumination, biotinylation reduced the MIC of an Iridium (III) dipyridylamine complex by 8-fold in *Pseudomonas aeruginosa*.\textsuperscript{207} No accumulation data was reported, thus it was not clear if the enhancement was due to accumulation. Further experimental, and molecular dynamics simulations would be necessary to understand the detail mechanism of this enhanced penetration of molecules through biotin conjugation.
CHAPTER 4. CONTRIBUTION OF INFLAMMASOME ACTIVATION AND PYROPTOSIS IN PATHOGENESIS OF *SALMONELLA* SYSTEMIC INFECTION

4.1 Introduction

Systemic bacterial infection leading to sepsis is one of the devastating consequences of bacterial infection. Sepsis often leads to critical situations such as cytokine storm as well as DIC, which are extremely infamous for bad prognosis including death.\textsuperscript{208-210} *Salmonella* is one of the enteric bacteria that enters into the body through oral route because of contaminated food and water, and then can cross the intestinal epithelial barrier to reach the systemic sites.\textsuperscript{128} Among several serovars of *Salmonella*, typhoid-causing *Salmonella* Typhi leads to more lethal infection whereas non-typhoidal strains like *Salmonella* Typhimurium often cause self-limiting gastroenteritis in humans.\textsuperscript{211} Several mouse models have been established to study the *Salmonella* Typhimurium infection where it causes lethal or chronic infection depending upon the mouse strain.\textsuperscript{211, 212}

Upon infection, *Salmonella* can cause the inflammasome activation and pyroptosis in several different cell types ranging from epithelial cells in the intestinal barrier to immune cells in the system.\textsuperscript{111, 128, 139, 141} *Salmonella* has several virulence factors that can activate the inflammasome and cause pyroptosis. Flagellin is one of the potent molecules present in *Salmonella* that can activate NLRC4 inflammasome.\textsuperscript{131} Apart from this, *Salmonella* encode two different kinds of type three secretion system, one from SPI1 and one from SPI2.\textsuperscript{213} SPI1 type three secretion system is believed to be responsible during host invasion and its components also activate NLRC4 inflammasome while SPI2 type three secretion system is essential during intracellular survival of *Salmonella*.\textsuperscript{134, 214}

Inflammasome activation and pyroptosis can sometimes be a double-edged sword during infection. It is essential to disrupt the replicative niche for intracellular bacteria,
while overactivation of inflammasome and pyroptosis can lead to severe sepsis, septic shock, coagulation, tissue, and organ damage, and even death. Cytokine storm as a result of excessive inflammation is one of the major complications in severe sepsis which leads to lethality. Apart from cytokine storm, recently it was identified that disseminated intravascular coagulation (DIC) is another complication of uncontrolled inflammasome activation and pyroptosis that can lead to lethality.\textsuperscript{104}

The role of inflammasome in \textit{Salmonella} infection has been studied by several groups but most of them focused on the gastrointestinal infection model, which is the major route of \textit{Salmonella} infection.\textsuperscript{139-143} Extensive study has been performed to find out the role of inflammasome activation and pyroptosis during the intestinal infection and bacterial clearance. Inflammasome components and pyroptosis are shown to have protective roles during the early phase of gastrointestinal \textit{Salmonella} infection. Inflammasome activation and pyroptosis during intestinal infection helped to inhibit systemic dissemination of \textit{Salmonella}. Caspase 1 and caspase 11 have been shown to have protective role in intestinal restriction of bacteria during \textit{Salmonella} infection.\textsuperscript{139, 140} NLRC4 inflammasome and caspase 8 activation in intestinal epithelial cells also caused infected cell expulsion and restricted the systemic dissemination of Salmonella.\textsuperscript{141} Caspase 1 deficiency has been also attributed to increased lethality during oral \textit{Salmonella} infection.\textsuperscript{142, 143} However, during the systemic phase of infection the role of inflammasome disruption have been found both protective and detrimental. Interleukin-18 produced from caspase 1 activation has been found to be responsible for host defense against systemic \textit{Salmonella} infection. However, septic shock induced by high dose of attenuated \textit{Salmonella} was protected by caspase 1
and IL-18 deficiency. Also, considering the observations that Salmonella downregulates the expression of flagellin and SPI1 genes during systemic phase of infection, NLRC4 inflammasome has been shown to have a minor role during systemic infection.\textsuperscript{48, 142} However, if the host is infected by Salmonella which constantly expresses flagellin, NLRC4 inflammasome had a protective role in the host defense and bacterial clearance.\textsuperscript{48}

Also, non-canonical caspase 11 activation and pyroptosis were observed to have detrimental role by allowing the intracellular bacteria to release and spread in the system during systemic Salmonella infection when there is an absence of caspase 1 and defective neutrophil mediated clearance.\textsuperscript{138}

Although extensive study has been done about inflammasome activation during Salmonella infection model, the relative contribution of the inflammasomes in pathogenesis remains unclear. The inflammasome involved might depend on several factors such as the phase of infection, severity of infection, upregulation or downregulation of these virulence factors during different phase and site of infection. We aimed to study the role of the inflammasome activation and pyroptosis in pathogenesis of severe Salmonella systemic infection. Under severe systemic infection, patient with compromised immune system might not be able to clear the bacteria, but severe uncontrolled activation of inflammasome and pyroptosis worsen the prognosis. We characterized the role of inflammasome activation in pathogenesis of Salmonella infection when the bacteria are already colonizing the system by using different strains of bacteria and different inflammasome components knockout mouse model. We aim to identify the mechanism of
lethality during severe sepsis and also the role of inflammasome in different phases of infection by using in vivo and in vitro mice models.

4.2 Materials and methods

4.2.1 Mice

C57BL/6J (WT), Naip-/-, Caspase 1-/-, Caspase 11-/-, Caspase 1&11-/-, Tlr5-/-, Gsdmd-/- and NLRP3-/- mice were housed in the University of Kentucky Animal Care Facility as well as Texas A&M University, College of Pharmacy, following institutional and National Institutes of Health guidelines after approval by the Institutional Animal Care and Use Committee. Male mice at 8-14 weeks were used in all experiments.

4.2.2 Bacteria

Salmonella enterica serovar Typhimurium (ATCC 14028) and its mutant ∆fliCfljB was obtained from Dr. Edward Miao at Duke University. FliC ON, a strain in genomic flagellin knockout background harboring the plasmid which can constitutively express flagellin through SPI2 promoter was also obtained from Dr. Miao. ∆SPI1 was obtained from Dr. James M Slauch at the University of Illinois. Using the obtained ∆SPI1 strain, we created ∆SPI1∆fliCfljB knockout using the Quick and easy gene deletion kit (Gene bridges GmbH, Heidelberg, Germany) following the manufacturer’s protocol. The target gene fliC was first replaced with FRT-flanked kanamycin resistance cassette which was then removed and again inserted in place of fljB. Colony PCR was performed to confirm the gene replacement and later confirmed through sequencing. All the bacterial strains were
grown in LB with appropriate antibiotics for selection and used for infection after counting the number using plate-based colony counting method.

4.2.3 BMDMs isolation and culture

BMDMs were isolated and cultured as described in published protocols. Briefly, mouse leg bones were isolated and bone marrow was flushed out and cultured in BMDM medium (RPMI-1640 supplemented with 15% L929-cell conditioned medium, 10% FBS, 1% HEPES, 1% L-Glutamine and 1% Penicillin/Streptomycin) for 5-6 days. Then the BMDMs were harvested and seeded in 100 µL in 96 well plate or 1 mL in 12 well plate at a density of 1x10^6 cells/mL.

4.2.4 In vitro infection

For in vitro infection assays, isolated BMDMs in BMDM medium were plated in either 96 well plate or 12 well plate at a density of 1x10^6 cells/mL. The BMDMs were then allowed to attach to the plates by incubating at 37 °C and 5% CO₂ few hours to overnight. After they are properly attached, the medium was changed to Opti-MEM low serum medium for infection. For infection designated strains of bacteria resuspended in sterile PBS were added to the cells. Required number of bacteria for 25 MOI infection was added to the cells, briefly centrifuged at 500 g for 5 mins to allow the bacteria to reach the macrophages and incubated at 37 °C and 5% CO₂. For early phase infection, supernatant and lysate samples were collected 90 mins post infection. For long-term infection, after 90
mins of infection gentamicin was added to a concentration of 100 µg/mL and further incubated to a total of 18 hours.

4.2.5 LDH release assay

To determine cell viability, the lactate dehydrogenase (LDH) CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Cat#G1780) was used. To 50 µL of supernatant samples, 50 µl of CytoTox 96 Reagent was added in each well of 96-well plate and incubated for 10 minutes at room temperature or until a sufficient colorimetric change was visible. The reaction was stopped using the stop solution, and the absorbance was read at 490 nm. Percentage LDH release was calculated using the maximum LDH release prepared using lysis buffer.

4.2.6 Western blots

For western blot, the collected cell supernatants were subjected to TCA precipitation of proteins whereas cell lysates were made using SDS loading dye and sonication. These samples were then used to perform western blots for caspase 1 and IL-1β. Both pro-caspase-1 and caspase-1-p20 were determined using anti-caspase-1(p20) (Adipogen, Cat#AG-20B-0042) at 1:1000 dilution. Pro-IL-1β and IL-1β (p17) was detected using anti-IL-1β (GeneTex Cat#GTX74034) at 1:1000 dilution. Blots were imaged using BIO-RAD ChemiDoc MP imaging system as well as Azure imaging system.
4.2.7 In vivo inflammation study

For the in vivo studies C57BL6 and the corresponding gene deficient mice Naip\(^{-/-}\), Caspase 1\(^{-/-}\), Caspase 11\(^{-/-}\), Tlr5\(^{-/-}\) and Gsdmd\(^{-/-}\) (8-14 weeks old) were intraperitoneally injected with \(1 \times 10^8\) CFU Salmonella in 0.2 mL sterile PBS. Blood samples were collected before or at various time (90 mins, 4 h, and 8 h) following injection. The blood samples were collected via retro-orbital bleeding in an EDTA tube and were centrifuged at 10,000 g for 1 minute at room temperature to obtain plasma. IL-1\(\beta\), IL-6, and TNF\(\alpha\) were measured using ELISA kit (Invitrogen) following manufacturer’s instruction.

4.2.8 DIC

For the determination of DIC, mice were infected with \(1 \times 10^8\) CFU bacteria through intraperitoneal injection in 0.2 mL sterile saline. After 7.5-8 hours of infection, blood was collected, and Prothrombin time (PT) and plasma thrombin-antithrombin complex (TAT) concentrations were measured as the indicators of DIC.

4.2.9 Prothrombin time (PT)

To measure Prothrombin time (PT), mice were subjected to tribromoethanol (avertin) anesthesia and blood was collected by cardiac puncture method. Blood was collected by a 23-gauge needle in a syringe containing 3.8% sodium citrate (final citrate to blood ratio 1:7). Plasma was obtained after centrifugation at 1500 g for 15 minutes at 4 \(^\circ\)C. PT was determined with Thromboplatin-D (Pacific Hemostasis, Cat#100357) in a manual
setting according to manufacturer’s instruction, using CHRONO-LOG #367 plastic cuvette.

4.2.10 Plasma TAT concentration

Using the plasma obtained while determining the PT, plasma TAT concentrations were determined using a mouse TAT ELISA kit (Abcam, Cat#ab137994) at 1:50 dilution according to manufacturer’s instruction.

4.2.11 Survival assays

For the survival assays, different genotypes of mice were infected with different strains of Salmonella through intraperitoneal injection of 5x10^6 CFU bacteria in 0.2 mL sterile saline. The mice were then left with ad libitum food and water and observed over time to monitor their activity and health deterioration symptoms.

4.2.12 Statistical analysis

All the data are presented in mean±SEM. Depending upon the case, either two-way ANOVA with Holm-Sidak multiple comparisons or one-way ANOVA with Holm-Sidak multiple comparisons were used to determine the significance. p < 0.05 was considered statistically significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. All statistical analyses were conducted on biological replicates in GraphPad Prism 6.
4.3 Results

4.3.1 Deficiency of caspase 1 protects mice mortality from systemic *Salmonella* infection

We studied the role of caspase 1, a major inflammation initiator downstream of inflammasome activation, during pathogenesis of severe systemic *Salmonella* infection. Systemic infection was induced in C57BL/6J mice by intraperitoneal injection of 5x10^6 CFU of *Salmonella* in 0.2 mL sterile saline. It was observed that the deficiency of caspase 1 significantly prolonged survival [Figure 4.1], suggesting a protective role of inflammasome during the systemic *Salmonella* infection. In contrast, deficiency of caspase 11 only slightly delayed mortality.

Our findings seem to be contradictory to the previous studies, which show that caspase 1 is essential for bacterial clearance and to protect the host from *Salmonella* infection. In these studies, caspase 1 protects against *Salmonella* infection either by the gastrointestinal route of infection or when the mice were challenged with a very low dose of bacteria. In contrast, we challenged the mice with high-dose bacteria by i.p, which resembles severe sepsis. Our data suggest that although the inflammasomes play a protective role during early *Salmonella* infection, they could become detrimental when infection progresses. Next, we investigated the mechanism behind the protection from caspase 1 deficiency.
Figure 4.1. Caspase 1 deficiency prolonged the C57BL/6J mice survival upon systemic infection by *Salmonella* Typhimurium.

5x10⁶ CFU bacteria were intraperitoneally injected in 0.2 mL sterile saline to cause infection and mice were then monitored over time. 10-14 weeks old, 9-11 mice were used for the experiment.

4.3.2 Deficiency of intracellular flagellin receptor Naip but not extracellular receptor TLR5 protects from mice death

After we observed the elongated survival for mice with caspase 1 deficiency, we wanted to test whether deficiency of inflammasome sensors, such as Naip or membrane receptor TLR5 show similar effect. *Salmonella* contains very potent virulence factors like flagellin and T3SS components, causing activation of the NLRC4 inflammasome and caspase 1. Naip 5&6 are the intracellular receptor for flagellin, Naip 1 detects the needle protein PrgI and Naip 2 detects the inner rod protein PrgJ from *Salmonella.*²¹⁸ We used mice deficient in all NAIP 1-6 to further investigate the role of inflammasome in
Salmonella infection. Consistent with a protective role of caspase-1 deficiency, the Naip deficient mice were less susceptible than WT mice to Salmonella infection [Figure 4.2]. It is known that flagellin can also activate its extracellular receptor TLR5. Deficiency of TLR5 did not protect against Salmonella systemic infection [Figure 4.2].

![Graphs showing survival rates of Naip and TLR5 deficient mice after Salmonella infection.](image)

**Figure 4.2.** Naip but not TLR5 deficiency prolonged the C57BL/6J mice survival upon systemic infection by Salmonella Typhimurium. 5x10^6 CFU bacteria were intraperitoneally injected in 0.2 mL sterile saline to cause infection and mice were then monitored over time. 10-14 weeks old, 5-11 mice were used for the experiment.

### 4.3.3 Inhibition of pyroptosis protects from Salmonella induced mice mortality

Since we observed the protective effect from deficiency of inflammasome components, we evaluated whether the downstream pyroptosis has any role in the pathogenesis. Since Gasdermin D (GSDMD) is one of the major gasdermins responsible for pyroptosis during bacterial infection, we tested the role of GSDMD during the pathogenesis and observed that the deficiency of GSDMD also prolonged survival upon systemic infection by Salmonella, indicating that pyroptosis indeed contributes to pathogenesis by systemic Salmonella infection. [Figure 4.3]
Figure 4.3. Loss of pyroptosis by GSDMD deficiency prolonged the C57BL/6J mice survival upon systemic infection by *Salmonella Typhimurium*. 5x10^6 CFU bacteria were intraperitoneally injected in 0.2 mL sterile saline to cause infection and mice were then monitored over time. 10-14 weeks old, 9-11 mice were used for the experiment.

4.3.4 *Salmonella* causes severe inflammation in vivo in a Naip, Caspase 1 and GSDMD dependent manner

One of the major causes of mouse mortality upon bacterial infection is the occurrence of septic shock due to the release of a large amount of pro-inflammatory cytokines, causing cytokine storm. Inflammasome activation and subsequent pyroptosis lead to maturation and release of pro-inflammatory cytokines from different immune cells in the body, contributing to this process. To further evaluate the role of inflammasome activation and pyroptosis in *Salmonella* pathogenesis, we examined the role of inflammasome and pyroptosis in *Salmonella*-induced inflammation.
We measured plasma level of the proinflammatory cytokines; IL-1β, IL-6 and TNF-α. These proinflammatory cytokines are not only used to evaluate the severeness of inflammation, but also play important roles in the pathogenesis of sepsis. The plasma level of TNF-α reached peak in 90 minutes post infection, whereas the level of IL-1β and IL-6 kept on increasing to reach almost a plateau in 4-8 hours post infection.

The release of these proinflammatory cytokines were dependent upon the activation of caspase 1 because the plasma levels of these cytokines were almost abolished in the mice deficient in caspase 1. [Figure 4.4A] There was almost negligible amount of IL-1β detected in plasma of caspase 1 deficient mice, whereas there was some amount of TNF-α and IL-6, but they were significantly less compared to plasma level in WT mice. Similarly, the plasma levels of these pro-inflammatory cytokines were significantly less in Naip deficient mice. [Figure 4.4B] These data indicate that the NLRC4 inflammasome plays an important role in Salmonella-induced inflammation. We also studied the extent of inflammation in GSDMD deficient mice and found that the plasma concentrations of these cytokines reduced as compared to WT mice, but not as dramatic as in Caspase 1−/− or Naip−/− mice. [Figure 4.4A] Overall, these results indicated that, upon Salmonella infection, Naip is responsible for detection and activation of the NLRC4 inflammasome followed by recruitment of caspase 1, which is then involved in cytokines maturation and release.
Figure 4.4. Salmonella causes systemic inflammation through Naip, Caspase 1 and GSDMD dependent manner.
Mice were intraperitoneally infected with $1 \times 10^8$ CFU bacteria, and blood was collected over a period of time through retroorbital bleeding. Levels of IL-1β, TNF-α and IL-6 were measured in plasma by ELISA.

4.3.5 Deficiency of Caspase 11 and TLR5 did not protect against inflammation in vivo

Gram-negative bacteria can also activate the caspase 11 non-canonical inflammasome through LPS and cause TLR5 mediated signaling through flagellin. We tested the role of caspase 11 in inflammation in vivo during Salmonella systemic infection. Plasma concentrations of IL-1β, TNF-α, and IL-6 in caspase 11 deficient mice were very similar to WT mice, suggesting that caspase 11 plays a minimal role in inflammation upon Salmonella infection. [Figure 4.5A] Similarly, plasma concentrations of these cytokines in
TLR5 deficient mice were similar to wild-type mice, suggesting that TLR5 plays a minimal role in inflammation upon *Salmonella* infection. [Figure 4.5B]

**Figure 4.5. Salmonella induced systemic inflammation is independent of TLR5 and Caspase 11.**

Mice were intraperitoneally infected with $1 \times 10^8$ CFU bacteria, and blood was collected over a period of time through retroorbital bleeding. Levels of IL-1β, TNF-α and IL-6 were measured in plasma by ELISA.

### 4.3.6 Salmonella causes DIC upon systemic infection through a caspase 1 dependent manner

Occurrence of DIC is one of the major hallmarks of severe sepsis. During DIC, the coagulation cascade is activated uncontrollably causing blood clot in various smaller blood capillaries inside the body. Because of this, the blood flow is restricted to the vital organs causing the organ damage and failure. And at the same time, in case of any injury, there is
a chance of excessive bleeding because of lack of clotting factors due to their unregulated consumption. We evaluated the occurrence of DIC during systemic *Salmonella* infection. Recently, our lab and Yang et. al. have shown that inflammasome activation and pyroptosis play a major role in DIC during sepsis. Since we observed inflammasome activation and pyroptosis are important during *Salmonella* infection, we investigated whether they contributes to DIC during the *Salmonella* pathogenesis. [Figure 4.6] Prothrombin time (PT) was significantly prolonged 8 hours after *Salmonella* inoculation. Similarly, the plasma level of thrombin-antithrombin (TAT) complex was also significantly increased upon *Salmonella* infection. These two indicators of DIC were significantly less in caspase 1 deficient mice compared to the WT, indicating a role of caspase 1 in the process. Similarly, the mice with GSDMD deficiency also had shorter PT time and lesser plasma TAT level compared to the WT mice in response to *Salmonella* challenge, indicating that pyroptosis plays an important role in this process. The mice deficient in caspase 11 did not show shorter PT time and less TAT showing no significant role of caspase 11 in the DIC caused by *Salmonella* infection. These results also correlate with the mice survival data upon *Salmonella* infection showing the important role of caspase 1 and pyroptosis while not much role of caspase 11 in pathogenesis of *Salmonella* infection. Surprisingly, the mice deficient in Naip were not as protected from DIC, although the PT time and TAT levels were lesser compared to the WT. These data suggest a possible role of another inflammasome activation which also activates caspase 1.
4.3.7 *Salmonella* causes rapid and robust inflammasome activation and pyroptosis in BMDMs in vitro

Since one of the major immune cells that plays a role during bacterial infection and undergoes pyroptosis is macrophage, we tested *in vitro* inflammasome activation and pyroptosis in macrophages to see if they undergo pyroptosis upon *Salmonella* infection. LDH release from cells is a standard assay to evaluate cell viability. We observed that the macrophages undergo cell death when infected with *Salmonella*. [Figure 4.7] *Salmonella* at 25 MOI infection caused rapid cell death with more than 50% cell death occurring within 90 minutes of infection.

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**Figure 4.6. **Salmonella causes DIC in mice upon systemic infection. Measurement of prothrombin time (A) and plasma TAT concentration (B) in different genotypes of mice upon infection by Salmonella at a dose of 1x10^8 CFU for 7.5-8 hours.
Figure 4.7. *Salmonella* causes rapid and robust inflammasome activation and pyroptosis in BMDMs.  
A. LDH release in supernatant of BMDMs after infection by different strains of *Salmonella* at 25 MOI for 90 minutes. B. Time dependent LDH release in supernatant of BMDMs after infection by different strains of *Salmonella* at 25 MOI. C. Western blot for caspase 1 and IL-1β in supernatants after infection by *Salmonella* strains at 25 MOI for different time points.

This rapid inflammasome activation and cell death induced by *Salmonella* was mediated mainly through flagellin, since LDH release was almost abolished upon infection with flagellin deficient strain of *Salmonella*. Also, Western blot results showed that there was no caspase 1 and IL-1β cleavage in the samples infected with flagellin deficient strain. In contrast, caspase 1 and IL-1β cleavage induced by a mutant strain that constitutively expresses flagellin referred as FliC ON was elevated than that caused by wild-type strain.
4.3.8 Salmonella caused rapid inflammasome activation and pyroptosis of BMDMs through the Naip/Caspase 1/Gsdmd axis

As expected, we observed that the macrophages isolated from caspase 1 deficient mice did not undergo lytic cell death as indicated by lack of LDH release in the supernatant. Similarly, it was also observed that the macrophages deficient in Naip and Gsdmd also did not undergo lytic cell death. [Figure 4.8A-B] These data demonstrate that the macrophages undergo pyroptosis upon Salmonella infection through the Naip/Caspase-1/Gsdmd axis.

Western blot result also showed that upon Salmonella infection, the caspase 1 and IL-1β were activated into their mature form in NLRP3 deficient macrophages but not in Naip deficient macrophages. [Figure 4.8C] These data indicate that the rapid inflammasome activation and pyroptosis during Salmonella infection is mediated by Naip/NLRC4 inflammasome. Cell death was not protected by caspase 11 deficiency.
Figure 4.8. *Salmonella* causes rapid inflammasome activation and pyroptosis in BMDMs through Naip/Caspase 1/GSDMD dependent manner.

A-B. LDH release determined in the supernatant of different genotypes of BMDMs after infection by 25 MOI *Salmonella* for 90 minutes. C. Western blot for caspase 1 and IL-1β in supernatant of different genotypes BMDMs after infection by 25 MOI *Salmonella* for 90 minutes.

4.3.9 Rapid inflammasome activation and pyroptosis caused by *Salmonella* are also SPI1 dependent

Apart from flagellin, *Salmonella* also has other molecules that can activate the Naip/NLRC4 inflammasome, such as the type three secretion system components rod protein PrgJ and needle protein PrgI. Flagellin can be secreted into the cytoplasm of host cells from *Salmonella* containing vacuole using type three secretion system. *Salmonella* has two major type three secretion system, one encoded in SPI1 and another
encoded in SPI2. SPI1 type three secretion system is mainly responsible during the early phase of infection whereas SPI2 type three secretion system helps to maintain bacterial survival inside the host cells for chronic infection. We used SPI1 deficient *Salmonella*, which does not contain the SPI1 coded type three secretion system to investigate its role in inflammasome activation and pyroptosis. SPI1 deficient strain did not induce rapid cell death in macrophages *in vitro*. [Figure 4.9A] SPI1 deficient strains did not cause activation of caspase 1 and IL-1β as shown by Western blot. [Figure 4.9B] These data are consistent with the previous findings that during early phase of infection, the inflammasome activation and pyroptosis mediated by flagellin also require functional T3SS encoded by SPI1.130, 131, 133

Figure 4.9. Salmonella induced rapid inflammasome activation and pyroptosis are SPI1 dependent.
A. LDH release determined in the supernatant of BMDMs after infection by different strains of *Salmonella* at 25 MOI for 90 minutes. B. Western blot for caspase 1 and IL-1β in supernatant and cell lysate samples of BMDMs after infection by different strains of *Salmonella* at 25 MOI for 90 minutes.
4.3.10 Deficiency of flagellin or SPI1 alone was not sufficient to prevent inflammation in vivo

To investigate the contribution of flagellin to in vivo inflammation during Salmonella infection, we compared plasma concentrations of the proinflammatory cytokines in mice challenged with WT or flagellin deficient strains. Plasma concentrations of IL-1β in the mice challenged with the flagellin deficient strain was reduced to about half of those in the mice challenged with wild type mice. [Figure 4.10] There was only a little difference in the TNF-α levels and no significant changes in the IL-6 levels. This indicates that flagellin is only one of the virulence factors causing inflammation. Next, we used a strain lacking the whole SPI1 gene cluster and evaluated the extent of inflammation in mice upon infection by this strain. It was observed that the deficiency of SPI1 genes was not able to completely abolish the inflammation in vivo. [Figure 4.10]

Since deficiency of just flagellin or just SPI1 was not able to abolish systemic inflammation in vivo, we constructed the Salmonella strain without SPI1 and flagellin both (∆SPI1∆fliCfljB). When mice were infected with this strain the severity of inflammation indicated by the amount of cytokine levels detected in the plasma was drastically reduced compared to the mice infected by either WT or SPI1 or flagellin only deficient strains. [Figure 4.10]
Figure 4.10. Deficiency of both flagellin and SPI1 reduces *Salmonella* induced systemic inflammation.
Mice were injected intraperitoneally with $1 \times 10^8$ CFU bacteria and blood was collected through retroorbital bleeding and plasma concentrations of IL-1β, IL-6 and TNF-α are determined through ELISA.

4.3.11 Deficiency of SPI1 and flagellin both was not sufficient to protect against mouse mortality from *Salmonella* systemic infection

After we observed that the deficiency of both flagellin and SPI1 genes reduce the inflammation in vivo, we evaluated the mice survival upon infection by these strains to see if they are less virulent. Surprisingly, although the inflammation was reduced, the removal of SPI1 and flagellin both from *Salmonella* was not sufficient to protect the mice mortality. [Figure 4.11] These data suggest that systemic inflammation may not be the major cause of mice mortality during systemic *Salmonella* infection.
4.3.12 Deficiency of SPI1 and flagellin alone or both did not protect DIC during Salmonella systemic infection

Since we observed that although the inflammation was reduced significantly, and that there was not much protection from the mortality upon infection by flagellin and SPI1 knockout strain, another obvious contributing factor to lethality could be DIC. We, then, tested the occurrence of DIC upon infection by these knockout strains and it was observed that the extent DIC indicated by PT time and plasma TAT concentration was not much different compared with infection by WT strain. [Figure 4.12] These results indicate that there must be some factors other than flagellin and SPI1 that can induce DIC and cause lethality.

Figure 4.11. Survival of mice upon infection by different strains of bacteria. Mice were injected intraperitoneally with different strains of bacteria at a dose of 5x10^6 CFU in 0.2 mL sterile saline and monitored over time.
Figure 4.12. DIC induced by different strains of Salmonella.
Prothrombin time (A) and Plasma TAT concentration (B) after infection of mice by $1 \times 10^8$ CFU bacteria intraperitoneally for 7.5-8 hours.

4.3.13 Flagellin and SPI1 deficiency cannot protect inflammasome activation and pyroptosis upon longer infection in vitro

We observed that the deficiency of flagellin and SPI1 did not significantly improve DIC. However, upon in vitro studies the inflammasome activation and pyroptosis were observed to be completely dependent on flagellin or SPI1 during early phase of infection. This inconsistency suggests that factors other than flagellin and SPI1 also contribute to the pathogenesis of Salmonella infection. Salmonella is believed to downregulate the expression of flagellin and SPI1 genes once they start to reside in the system. Therefore, we investigated inflammasome activation in macrophages by longer infection. BMDMs were incubated with 25 MOI bacteria for 90 mins, and then added with 100 µg/mL gentamicin to kill the extracellular bacteria. Cells were continued to incubate for total to
7.5 hours and 18 hours. At 7.5 hours infection condition only little inflammasome activation was seen and cell death was not much affected. [Figure 4.13A-B] However, after 18 hours of infection, there was significant amount of LDH release as well as caspase 1 and IL-1β activation even when knockout strains of *Salmonella* were used to infect the BMDMs. [Figure 4.13C-D] These results indicate that during the longer time infection, factors other than flagellin and SPII comes into play to activate the inflammasome and cause pyroptosis.
Figure 4.13. Inflammasome activation and pyroptosis in BMDMs during long term infection.
A-B. LDH release and caspase 1 and IL-1β western blot using cell supernatant and lysate samples after 7.5 hours of infection using 25 MOI infection. C-D. LDH release and caspase 1 and IL-1β western blot using cell supernatant and lysate samples after 18 hours of infection using 25 MOI infection. In all cases, 100 µg/mL gentamicin was added 90 minutes post infection.

4.3.14 NLRP3 and Caspase 11 inflammasomes are involved during long term Salmonella infection

We then used the strains deficient in both flagellin and SPI1 to investigate the mechanism of flagellin/SPI1-independent inflammasome activation. Activation of caspase 1 and IL-1β by the ΔSPI1ΔfliCfljB strain was abolished in the NLRP3 deficient cells. In contrast, WT strain induced similar level of inflammasome activation in the NLRP3 deficient cells. [Figure 4.14] These data suggest that in the absence of flagellin and SPI1, Salmonella can activate NLRP3 inflammasome during the long term. However, in the presence of Naip/NLRC4 and their activators, the activation of NLRC4 inflammasome overshadows the NLRP3 inflammasome activation because the NLRC4 inflammasome processing is very robust and rapid while NLRP3 inflammasome activation needs time. Similarly, in case of caspase 11 deficient BMDMs, there was also no significant caspase 1 activation and IL-1β production upon infection by ΔSPI1ΔfliCfljB strain. [Figure 4.14] This suggests that the caspase 11 non-canonical inflammasome activation is also involved during the long-term infection by flagellin and SPI1 deficient strains. The activation of caspase 11 in this case could be from the LPS, which is a well-known activator of non-
canonical caspase 11 inflammasome. The activator of NLRP3 remains elusive, although a possible candidate is the caspase 11 non-canonical inflammasome activation mediated potassium efflux.

Figure 4.14. Role of NLRP3 and caspase 11 during late phase of infection.
A. LDH release in the cell supernatant after infection of different genotypes of BMDMs by 25 MOI ST WT and ΔSPI1ΔfliCfljB strains for 18h. B. Western blot for caspase 1 and IL-1β using concentrated cell supernatant samples and cell lysate samples after 18h of infection by 25 MOI ST WT and ΔSPI1ΔfliCfljB.

4.3.15 Salmonella also activates caspase 8 upon infection in vitro in BMDMs

Since we observed the IL-1β release even in caspase 1 deficient BMDM’s supernatant upon infection by wild-type Salmonella, we tried to figure out the source of the caspase 1 independent IL-1β maturation. One of the possible candidates was caspase 8 mediated IL-1β cleavage and release. We studied whether caspase 8 is activated upon
Salmonella infection and observed that indeed caspase 8 is activated upon longer time Salmonella infection [Figure 4.15A]. In the absence of this caspase 8 in caspase 1 deficient cells we did not see any IL-1β cleavage and release. However, lack of caspase 8 also affected the expression of pro-IL-1β. We also studied whether this activation of caspase 8 is downstream of any inflammasomes. However, even in the deficiency of Naip and NLRP3 inflammasome the caspase 8 activation was still there indicating the redundancy of these inflammasome in caspase 8 activation during Salmonella infection [Figure 4.15B].

**Figure 4.15. Caspase 8 activation during Salmonella infection**
A-B Western blot for caspase 8 and IL-1β using various cell lysate and concentrated supernatant samples after 18h infection of different genotypes of BMDMs by 25 MOI ST WT and ΔSPI1ΔfliCfljB.
4.3.16 Deficiency of NLRP3 and caspase 11 protects from DIC upon infection by \(\Delta SPI1\Delta fliCfljB\) strain

Since deficiency of NLRP3 and caspase 11 protected from inflammasome activation during infection by \(\Delta SPI1\Delta fliCfljB\) strain, we tested whether these deficiencies also protect from DIC \textit{in vivo} upon infection by same strain. Since the WT strain of \textit{Salmonella} was seen to majorly activate NLRC4 inflammasome and cause DIC, it was not clear what causes the DIC during infection of flagellin and SPI1 deficient strains. We observed that deficiency of NLRP3 completely protects the mice from DIC during infection by flagellin and SPI1 deficient strains. PT was not prolonged and was comparable to the uninfected controls. [Figure 4.16] This suggests that DIC is mediated by NLRP3 inflammasome activation in a condition when there is no NLRC4 inflammasome activation during \textit{Salmonella} infection, particularly during late phase of infection. Similar to the NLRP3 deficient mice, caspase 11 deficient mice also showed significant decrease in the PT as well as plasma TAT levels compared to WT mice during infection by flagellin and SPI1 \textit{Salmonella}. Overall, these results also indicate that the flagellin and SPI1 deficient strain mediated DIC is mainly through caspase 11 and NLRP3 inflammasomes.
Figure 4.16. NLRP3 deficiency protects from DIC induced by ∆SPI1∆fliCfljB strain
Prothrombin time (A) and Plasma TAT concentration (B) after infection of mice by 1x10^8 CFU Salmonella ∆SPI1∆fliCfljB bacteria intraperitoneally for 7.5-8 hours.

4.3.17 Role of Naip and Caspase 1 during gastrointestinal infection

Since there were multiple reports about protective role of intestinal epithelial inflammasome against Salmonella infection\textsuperscript{139, 141, 142}, we also tested whether they have the protective or detrimental role during gastrointestinal Salmonella infection in our setting. In contrast to the systemic infection model and consistent with the reported results, we observed no protection of death upon gastrointestinal infection with Salmonella in the absence of Naip or Caspase 1. The mice mortality rate was rather higher in the mice deficient in Naip or Caspase 1 than the WT control, although not statistically significant. [Figure 4.17] We observed only a minor difference may be because we did not pretreat the mice with oral dose of antibiotics which would kill the intestinal microbiota. The presence of intestinal microbiota might have affected the infection and partly shadowed the actual
effect. This opposite result in gastrointestinal infection model compared with systemic infection model might be because the inflammasome components are needed to recognize the bacteria and expel the intestinal epithelial cells to prevent the systemic progression of the bacteria. In the absence of these inflammasome components the bacteria can easily infect the epithelial cell lining and progress past the epithelial cell boundary to reach the systemic sites. The gastrointestinal infection therefore requires higher dose, and the mice survive longer than the systemic infection because only a subset of bacteria can progress past the intestinal epithelial boundary to cause the systemic infection. To test this model where only a small number of bacteria reach to the systemic sites, we performed the low dose bacterial intraperitoneal infection to see whether these inflammasome components have protective or detrimental role.

**Figure 4.17. Survival of mice upon gastrointestinal infection.**
Different genotypes of mice were infected with 2x10⁷ CFU of ST WT bacteria suspended in 0.2 mL sterile saline through oral gavage. Animals were left ad libitum with food and water and were observed for symptoms of health deterioration over time.
4.3.18 Inflammasome components have minor role during low dose systemic infection

We observed the detrimental role of inflammasome activation upon severe acute systemic infection, so we also wanted to evaluate the role of inflammasome activation and pyroptosis upon early systemic infection by using a very small number of bacteria. When used only 100 CFUs of *Salmonella*, it was observed that the mice mortality was not much affected by the presence or absence of inflammasome components, although the mortality rate was little higher upon deficiency of inflammasome components. [Figure 4.18] Also, upon using very small number of bacteria it takes a long time for the model animal to succumb. During this period, the virulence factors such as flagellin and SPI1 encoded components are repressed, and the bacteria cannot be cleared from the body due to lack of recognition by the inflammasome receptors. This might be the reason why we observed no difference between the mortality caused by *Salmonella* in WT and Naip deficient mice, consistent with previously studied low dose infection model.48
**Figure 4.18. Survival of mice upon low dose infection.**
Mice were infected with intraperitoneal injection of 100 CFU ST WT bacteria suspended in 0.2 mL of sterile saline. Mice were then left with ad libitum food and water and were monitored for symptoms of health deterioration over time.

4.4 Discussion

Inflammasome activation and subsequent pyroptosis is a double-edged sword of innate immune system. Its proper regulation and control support the host to fight against pathogens. However, overactivation and improper regulation can lead to severe complications. In this study, we investigated the role of inflammasome activation and pyroptosis in the pathogenesis of systemic *Salmonella* infection. We mainly studied two major complications downstream of inflammasome activation and pyroptosis, namely, DIC and systemic inflammation. In our infection model, we observed that the deficiency of inflammasome components have protective role in pathogenesis of severe systemic infection causing the significant delay in animal mortality. Our results indicated that during severe and acute systemic *Salmonella* infection, systemic inflammation mediated through inflammasome activation and pyroptosis causes cytokines storm. The release of the major pro-inflammatory cytokines like IL-1β, TNF-α and IL-6 were inflammasome activation dependent. The plasma levels of these cytokines were significantly reduced in the absence of inflammasome activation. Activation of IL-1β needs inflammasome activation but surprisingly release of the inflammasome independent cytokines like IL-6 and TNF-α in plasma also required Naip/NLRC4 inflammasome and caspase 1. [Figure 4.4A-B] This could be because the production of IL-6 in WT mice may have been triggered by the IL-1β release and its downstream signaling. However, the TNF-α release in plasma of WT
mice was even earlier than IL-1β and reached peak within 90 minutes post infection. [Figure 4.4A] This suggests the possibility of another pathway dependent on Naip/NLRC4 inflammasome and caspase 1 leading to cleavage and release of TNF-α in the blood. When we used the flagellin and SPI1 expressing wild-type Salmonella, Naip/NLRC4 inflammasome was the major inflammasome responsible for this systemic inflammation. Deficiency of this inflammasome as well as caspase 1 significantly abolished the inflammation. However, no significant difference in plasma levels of these cytokines was observed in caspase 11 and TLR5 deficient mice. [Figure 4.5A-B] LPS is the major pathogen associated molecule that can activate caspase 11. This indicates that during flagellin-expressing conditions, there is no significant role of LPS mediated signaling and flagellin is the major factor causing inflammasome activation. Also, no role of TLR5 was observed, indicating flagellin mediated extracellular signaling does not have significant role in pathogenesis of systemic Salmonella infection. TLR5 is highly expressed in intestinal epithelial cells and in cells present in mucosal surfaces. Thus, TLR5 mediated signaling in response to flagellin has been shown to be more effective in these cells.221 However, in several murine systemic immune cells there is not much expression of TLR5, which could be the reason why we did not observe any role of TLR5 during systemic inflammation.221-223

Apart from inflammation, DIC was observed during the systemic infection by Salmonella. The major hallmarks of DIC are prolonged PT and increased plasma TAT concentrations. When we measured these two parameters, both PT and plasma TAT concentrations were elevated during systemic Salmonella infection. [Figure 4.6A-B]
During sepsis, DIC is caused downstream of inflammasome activation and pyroptosis. Pyroptotic death leads to the release of micro vesicles containing tissue factor, which is responsible for activating the coagulation cascade.\textsuperscript{104} Our results were consistent with the previous findings that inhibition of pyroptosis lead to inhibition of DIC. Caspase 1 and GSDMD deficiency protected the prolongation of PT and decreased the plasma TAT concentrations compared to wild-type animals during \textit{Salmonella} systemic infection. [Figure 4.6A-B]

To understand the contribution from various virulence factors of \textit{Salmonella} in systemic inflammation and DIC, we used several mutants to cause the systemic infection. It was observed that removal of either flagellin or SPI1 genes from \textit{Salmonella} could not prevent systemic inflammation. However, removal of both significantly reduced the plasma levels of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. [Figure 4.10] Since multiple pathogen-associated molecules present in \textit{Salmonella} such as flagellin, PrgJ and PrgI can activate Naip/NLRC4 inflammasome, the effect of removal of one virulence factor could be complimented by the presence of other.\textsuperscript{82, 220, 224} Furthermore, apart from flagellin and SPI1 proteins, SPI2 type three secretion system protein SsaG\textsuperscript{136} can activate NLRC4 inflammasome and several other SPI2 dependent effectors can activate NLRP3 inflammasome.\textsuperscript{128} This could be the reason of an incomplete abrogation of systemic inflammation although reduced significantly which is consistent with the previous observation about redundancy in NLRC4 and NLRP3 activation during \textit{Salmonella} infection.\textsuperscript{137} Although we observed reduction in plasma cytokines concentration we did not see any protection from DIC and lethality upon removal of SPI1 and flagellin. [Figure 4.11,
This indicates that apart from flagellin and SPI1 other factors still contribute to DIC and lethality.

Consistent with the *in vivo* data, we observed that flagellin and SPI1 triggered rapid and robust inflammasome activation and pyroptosis *in vitro* through the Naip/NLRC4 inflammasome in murine BMDMs. [Figure 4.7] This was so robust that the macrophages underwent pyroptosis as early as 30 minutes post infection with more than half of the cell died within 90 minutes of infection.

Deficiency of flagellin and SPI1 in *Salmonella* prevented the inflammasome activation and pyroptosis *in vitro* during the early phase of infection. [Figure 4.8] However, during longer infection, *Salmonella* still caused inflammasome activation and pyroptosis even in the absence of both flagellin and SPI1. SPI1 is normally believed to be important during host invasion, whereas SPI2 is important in maintaining the intracellular survival. Different SPI2 mutants including ΔspiC, ΔsteC, ΔspvB, ΔsseK1/K2/K3, ΔsifA and ΔsifB have been previously observed to have very poor replication inside macrophages.134 Inability of SPI1 and flagellin deficiency to prevent inflammasome activation and pyroptosis during the longer infection *in vitro* is consistent with the *in vivo* results that removal of flagellin and SPI1 cannot protect *Salmonella* caused DIC and lethality. This also indicates a possible alternative virulence factor that can cause inflammasome activation and pyroptosis. To understand which inflammasome is involved during this late phase of infection, we used different genotypes of BMDMs and found that NLRP3 inflammasome and caspase 11 non-canonical inflammasome play the role during this late phase of infection. Caspase 11 can be activated by LPS present in the outer membrane of
gram-negative bacteria. Salmonella is an intracellular pathogen that resides in the vacuoles. How its LPS reaches cytosol remains elusive. Apart from caspase 11, NLRP3 inflammasome could also be activated by a Salmonella mutant lacking both flagellin and SPI1. NLRP3 inflammasome can be activated by several factors including the caspase 11 mediated GSDMD pore dependent potassium efflux. Deficiency of caspase 11 and NLRP3 abolished the caspase 1 and IL-1β activation, indicating that caspase 11 non-canonical inflammasome and NLRP3 inflammasome are activated during the late phase of infection when there are no activators of NLRC4 inflammasome. Consistent with this analysis, caspase 11 and NLRP3 deficiency also caused significant protection from DIC upon infection by ∆SPI1∆fliCfljB strain. Both the PT and plasma TAT levels were very similar to the untreated controls. Deficiency of both caspase 1 and 11 protected the pyroptosis even more than caspase 1 deficiency alone as indicated by less LDH release. This also supports the involvement of caspase 11 mediated GSDMD cleavage and pyroptosis during long-term infection. In the absence of caspase 1, a small amount of IL-1β was still released, upon infection by ST WT strain. This could be because of the involvement of caspase 8 activation which can cleave IL-1β. In fact caspase 8 was observed to be activated during Salmonella infection for longer time. There was no IL-1β maturation in RipK3−/−Caspase 1&8−/− BMDMs, suggesting the involvement of caspase 8 in caspase 1 independent IL-1β release. However, the pro-IL-1β levels were also lower in these cells because of which we could not tell whether the lack of IL-1β release is due to lack of maturation or due to less expression. Similarly, there was complete abrogation of IL-1β release in the supernatant of Caspase 1/11 deficient cells. This observation was
interesting as caspase 11 has not been previously observed to cleave IL-1β, although it has been shown to cleave IL-1α. \(^{229}\) Similarly, human counterpart caspase 4 and 5 have been shown to cleave IL-1β and IL-18 with a minor efficacy compared to caspase 1. \(^{230}\) However, caspase 11 has been shown to be essential in regulating the secretion of IL-1β by degrading Transient receptor potential channel 1 (TRPC1). \(^{231}\) TRPC1 deficiency promotes IL-1β secretion independent of caspase 1. This could be the reason why caspase 11 was observed to be important for the release of IL-1β during *Salmonella* infection.

The *in vivo* infection model we used is the acute systemic infection model and we observed some contrary results from the gastrointestinal mode of infection. When we used the gastrointestinal infection method, we observed that the inflammasome components including Naip, Caspase 1 and Caspase 8 were essential for host defense, similar to the published studies. This contrasting result is a good example of inflammasome activation and pyroptosis acting as a double-edged sword. In the gastrointestinal infection model, inflammasome activation and pyroptosis plays a significant role in destroying the replicative niche of this intracellular bacteria and also helps to prevent reaching them to the system. Inflammasome components including caspase 1, caspase 11 and NLRC4 are essential in intestinal restriction of *Salmonella* and prevent their systemic dissemination. \(^{139}\) Inflammasome components also have been shown to be essential for infected intestinal epithelial cell expulsion, preventing the bacteria to reach the systemic sites. \(^{141}\) Hence, inflammasome activation is essential in intestinal epithelial cell barrier to defend against the *Salmonella* from reaching to systemic sites. However, in the systemic infection model we used, where the bacteria are already in the system, activation of inflammasome and
pyroptosis can cause systemic inflammation and DIC, both of which are infamous to have bad prognosis. Uncontrolled activation during severe sepsis causes cytokine storm and DIC in addition to other consequences, leading to lethality. Even during systemic infection some studies have shown no role of caspase 1 during Salmonella infection. However, they used a very low dose of bacteria and studied whether they can be cleared from the body. We used comparatively high dose infection resembling the severe sepsis, which causes severe cytokine storm and DIC. When using very low dose systemic infection, the mice survival was prolonged but the role of inflammasome activation seemed to be very modest.

However, in any of the cases, we were not able to rescue the mice from mortality because we used the C57BL/6J strain, which is highly susceptible to Salmonella infection and cannot clear the bacteria from the body without antimicrobial therapies due the lack of the Natural resistance associated macrophage protein 1 (Nramp1) gene. Phagolysosomal protein Nramp1 is involved in providing resistance to the intracellular replication of multiple bacteria including Salmonella by pumping out and reducing the availability of iron inside Salmonella containing macrophages. Nramp1 expression has also been shown to be associated with increased expression of lipocalin 2 which is responsible for iron sequestering inside the macrophages. In the absence of Nramp1, Salmonella can undergo unregulated replication in various vital organs, causing eventual lethality. We did not quantify the bacterial titer inside different organs, which could give us more clues about the systemic dissemination of bacteria and the role of inflammasome activation and pyroptosis in it. Apart from pyroptotic cell death, bacterial infection can also cause several other kinds of cell death independent of inflammasome activation. In addition, we only
measured three major pro-inflammatory cytokines. There are several other cytokines that can contribute to systemic inflammation, which may contribute to mice mortality when plasma concentrations of these three cytokines were low.

Our data indicates that during systemic sepsis from *Salmonella*, depending upon the phase of infection and depending upon the presence or absence of flagellin/SP1 expression, optimum treatment strategies could be different. If it is early phase of infection, together with the antimicrobial therapies, inhibitors of NLRC4 inflammasome could be helpful. However, in late phase of infection when there is downregulation of NLRC4 activators, it seems inhibition of caspase 11 and NLRP3 inflammasome could be beneficial for the patient’s prognosis.

Several discrepancies have been observed between different cell types as well different organisms in case of inflammasome activation during *Salmonella* infection. In murine intestinal epithelial cells, Naip/NLRC4 and caspase 1 dependent inflammasome activation seems to play a major role in restriction of Salmonella from systemic sites. However, in human intestinal epithelial, Naip/NLRC4 and caspase 1 have been found to be dispensable. Instead, caspase 4/5 play a major role in inflammasome dependent responses. Although LPS is one of the activators of these inflammasomes, activators during SPI1 dependent activation of these caspases remains elusive. In contrast, human macrophages responses are similar to the murine macrophages. Because of these kinds of differences more studies are needed to translate the mice studies into clinically useful practices.
4.5 Conclusion

In this study we investigated the role of inflammasome activation and pyroptosis in pathogenesis of systemic *Salmonella* infection. We found that during severe systemic infection pathogenesis is mediated by systemic inflammation as well as DIC. Both are mediated through inflammasome activation and pyroptosis. Systemic inflammation is mediated mainly through Naip, caspase 1 and GSDMD. DIC is mediated through Naip/NLRC4 inflammasome when there is presence of flagellin and SPI1 whereas it is mediated through NLRP3 inflammasome in the absence of flagellin and SPI1. Deficiency of caspase 1 which acts downstream of both NLRC4 and NLRP3 inflammasome protects the mice mortality during severe systemic *Salmonella* infection.
CHAPTER 5. FUTURE DIRECTIONS

Growing issue of multi-drug resistance and lack of new therapeutic strategies are major public health concerns in current era. Inability to develop new effective antimicrobials is another challenge especially for gram-negative bacteria because of their formidable double layered envelope. Although several studies have been performed to identify the rules or features of molecules which are essential to effective cross this double layered envelope, only little success have been achieved. To this regard, as mentioned in the chapter two we studied how fluoroquinolones class of antibiotics are distributed inside the *E. coli* after they enter through the membrane. We aimed to identify whether the distribution is homogenous or not and whether the measurement of whole cell accumulation is a good indicator of compound accumulation or not because only the amount present in the target region can actually perform its inhibitory activities. Similarly, in chapter three we studied novel approaches to breach the double layered cell envelope of gram-negative bacteria. We probed the “trojan-horse” technique to conjugate a nutrient molecule with a desired molecule for its effective delivery inside the cell. Moreover, in chapter four we studied the host pathogen interaction during the bacterial infection by using *Salmonella* Typhimurium as a gram-negative pathogen. We studied how the inflammasome activation and pyroptosis plays a role in pathogenesis of systemic *Salmonella* infection.

In chapter two we studied how fluoroquinolones are distributed inside the *E. coli* and found some interesting observations. We observed that periplasm of the bacteria holds a lot of intracellular fluoroquinolones and the concentration in the periplasm is even higher than the external concentration. This suggested the importance of appropriate measurement
in appropriate compartment of the cell rather than whole cell accumulation. We found out that the efficacy of the antibiotics correlates with the target compartment accumulation rather than whole cell accumulation. So, to study whether a molecule is good accumulator as a feature to be good antimicrobial, we need to study the target region accumulation instead of whole cell accumulation. We can extend this study by using more group of antibiotics that are effective in different kinds of bacteria to set some rules about the features that make good accumulators. We only used the fluoroquinolones class of antibiotics which have cytoplasmic target. We can also do a similar study to other similar class of antibiotics as well as the ones that have periplasmic target like beta-lactams. We can use new technologies like mass spectrometry even if the molecules are not fluorescent. Also, in this study we could not determine the compound associated with the membrane because of the longer time it takes to separate the membrane and assuming during that processing time the membrane associated compound can be in equilibrium with other compartments. We can use some of the newest technologies to find out the how much the membrane contributes to the accumulation, which will give even more precise distribution of compounds in the bacterial cell.

In the chapter 3, we studied on novel approaches for the effective delivery of small molecules across the double layered cell envelope in Gram-negative bacteria. We probed the “trojan-horse” technique and used biotin as a nutrient molecule to conjugate with a fluorescent molecule called Atto565 and studied whether the biotinylation helps in the increased penetration and accumulation of Atto565 in *E. coli*. We observed that
biotinylation helps in the increased penetration through outer membrane and cause high accumulation of the fluorescent molecule in periplasm. Interestingly, we found that the increased permeation through outer membrane is not through the biotin transporter but is through the outer membrane porin OmpC. We could not identify the exact mechanism of this facilitated entry through OmpC due to biotin conjugation. Next project could be to identify this mechanism which could help us understand more about the permeation of molecules through porins. Apart from this we could also probe different other nutrient molecules to potentially conjugate with small molecules for their effective delivery as a “trojan-horse” approach. Moreover, in chapter 3 we only explored the conjugation with fluorescent molecule to develop a proof of concept, we still need to explore more using some real antimicrobial molecules to see if we observe the increased penetration in that case. We used fluorescent molecule because of the ease of measurement using fluorescent spectroscopy, however, we can still use other molecules and use other techniques like mass spectrometry for the measurement.

In chapter 4, we studied about the role of inflammasome activation and pyroptosis in pathogenesis of systemic Salmonella infection. We found that during systemic infection, inflammasome activation and pyroptosis contributes to systemic inflammation and DIC. Both of these have severe consequences leading to lethality. We found that deficiency of inflammasome components can protect from severe systemic inflammation. Deficiency in Naip, Caspase 1 and GSDMD lead to significant reduction in plasma cytokine levels. However, how inflammasome independent cytokines are also released in plasma
downstream of Naip and caspase 1 needs to be further evaluated to understand the mechanism. Similarly, deficiency in caspase 1 and GSDMD also lead to protection against DIC. However, even after protection from severe inflammation and DIC, the lethality was only elongated but not protected completely. Further studies are needed to find out the cause of lethality other than cytokine storm and DIC. We can do the bacterial titer studies to find out the systemic dissemination of bacteria in various vital organs. Similarly, we can also do several tests for the biomarkers of different organ failure to find out what leads to the lethal prognosis.

Moreover, multiple virulence factors and multiple inflammasomes can be involved during bacterial infection. We identified the involvement of flagellin and SPI1 through Naip/NLRC4 inflammasome during early infection. However, during later phase of infection or when these virulence factors were not present, other virulence factors and inflammasome come into play. We found the role of NLRP3 and caspase 11 inflammasomes during long infection by *Salmonella* which do not have SPI1 and flagellin. However, further studies are still essential to determine the involvement of other kinds of inflammasomes. Moreover, we could not identify exactly what activates NLRP3 during longer infection although one of the possibilities could be caspase 11 mediated potassium efflux. Also, since the effect was more obvious when SPI1 was not present, some of the activation of NLRP3 could be through SPI2 mediated effectors. Further knockout strains and studies will be essential to identify the activator of NLRP3 during longer infection when SPI1 is not present. Similarly, apart from *Salmonella* we can study several others
flagellated Gram-negative pathogens if the role of inflammasome activation and pyroptosis during the systemic infection by them is similar or its species specific.
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**Periplasmic Targets for the Development of Effective Antimicrobials against Gram-Negative Bacteria**

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Gasdermin D (GSDMD) as a new target for the treatment of infection


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**Awards and Honors**

1. **Graduate student research achievement award:** Department of Chemistry, University of Kentucky, December 2021 for outstanding research progress among the department.

2. **Dean’s competitive fellowship:** Tuition and stipend fellowship for Fall semester 2021, College of Arts and Sciences, University of Kentucky

3. **Graduate/Postdoctoral Annual Meeting (Travel) Award:** American Society for Biochemistry and Molecular Biology (ASBMB) for annual meeting 2021

4. **Max Steckler fellowship:** Outstanding oral qualifier award during the PhD candidacy qualification exam, December 2019

5. **Fast start award 2019:** Department of Chemistry, University of Kentucky, normally presented to a graduate student in his or her first or second year in the Department of Chemistry, who has made outstanding initial overall progress towards his or her degree.