T.C.
BAHCESEHIR UNIVERSITY
GRADUATE SCHOOL
THE DEPARTMENT OF BIOENGINEERING

CHARACTERIZATION OF A NEW IMMUNE ESCAPE MECHANISM USED BY *SALMONELLA* TYPHIMURIUM

MASTER'S THESIS

NOURELIMEN HEDFI

ISTANBUL 2024

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ABSTRACT

CHARACTERIZATION OF A NEW IMMUNE ESCAPE MECHANISM USED BY *SALMONELLA* TYPHIMURIUM

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Master's Program in Bioengineering

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Gasdermin family members are important proteins of the innate immune system involved in a particular form of cell death called pyroptosis. While the precise structure and function of different Gasdermin proteins and the enzymes modulating them are being elucidated, the direct role of some Gasdermin proteins in host/pathogen interaction remain unknown. The non-canonical Caspase-4 inflammasome is an important pathway involved in the detection of the lipopolysaccharide of Gram-negative bacteria and the overactivation of this pathway leads to uncontrolled inflammation and sepsis. Bacteria, in turn, develop several immune escape mechanisms to inhibit their recognition and their killing by the components of the innate immune system. In this thesis, we aimed to investigate a novel immune evasion strategy employed by *Salmonella* Typhimurium (*S*Tm). Our study focused on Gasdermin B (GSDMB), a less characterized member of the Gasdermin family, and its modulation during *S*Tm infection. Specifically, we aimed to determine whether GSDMB undergoes ubiquitination in response to *S*Tm infection and to identify the bacterial ubiquitin ligase responsible for this post-translational modification. By using human cell lines, we demonstrated for the first time that GSDMB is ubiquitinated during *S*Tm infection. Bioinformatic analyses suggested that a type III ubiquitin ligase from *S*Tm could interact with Gasdermin B and trigger its ubiquitination. These findings provide insights into the role of GSDMB in bacterial infections and propose potential therapeutic targets for managing inflammation and sepsis caused by *Salmonella enterica*.

Key Words: Pyroptosis, Gasdermin, *Salmonella* Typhimurium, Ubiquitination

ÖZET

SALMONELLA TYPHIMURIUM TARAFINDAN KULLANILAN YENİ BİR İMMÜN KAÇIŞ MEKANİZMASININ BELİRLENMESİ

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Gasdermin ailesi üyeleri, doğal bağışıklık sistemine ait ve piroptoz adı verilen yeni bir hücre ölümü formunda rol oynayan önemli proteinlerdir. Farklı Gasdermin proteinleri ve bunları modüle eden enzimlerin yapısı ve işlevi açıklığa kavuşturulurken, bazı Gasderminlerin konak/patojen etkileşimindeki doğrudan rolü henüz bilinmemektedir. Kanonik olmayan Kaspaz-4 inflamazomu, Gram-negatif bakterilerin lipopolisakkaritinin tespitinde rol oynayan önemli bir yolaktır ve bu yolağın aşırı çalışması, kontrolsüz inflamasyona ve sepsise yol açar. Buna karşın, bakteriler de doğuştan gelen bağışıklık sisteminin bileşenleri tarafından tanınmalarını ve öldürülmelerini engellemek için çeşitli bağışıklık kaçış mekanizmaları geliştirir. Bu tezde *Salmonella* Typhimurium'un (*S*Tm) kullandığı yeni bir immün kaçış stratejisini araştırmayı amaçladık. Çalışmamız, Gasdermin ailesinin daha az karakterize edilmiş bir üyesi olan Gasdermin B'ye (GSDMB) ve bunun *S*Tm enfeksiyonu sırasındaki modülasyonuna odaklandı. Spesifik olarak, GSDMB'nin *S*Tm enfeksiyonuna yanıt olarak übikitinlenip übikitinlenmediğini belirlemeyi ve bu modifikasyondan sorumlu bakteriyel ubikitin ligazını tanımlamayı amaçladık. İnsan hücre hatları kullanarak, ilk kez GSDMB'nin *S*Tm enfeksiyonu sırasında übikitinlendiğini gösterdik. Biyoenformatik analizler, *S*Tm'nin bir tip III ubikuitin ligazının Gasdermin B ile etkileşime girebileceğini bulduk. Bu bulgular, GSDMB'nin bakteriyel enfeksiyonlardaki rolünü ortaya koymakta ve *Salmonella enteric*a'nın neden olduğu

inflamasyonu ve sepsisi önlemek için potansiyel terapötik hedefler önerilmesine olanak sağlmaktadır.

Anahtar Kelimeler: Piroptoz, Gasdermin, *Salmonella Typhimurium,* Übikitin

To my wonderful parents, Faten Weldi and Soufian Hedfi, for your endless love and support.

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF SYMBOLS/ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
CFU	Colony-forming unit
DAMPs	Damage-associated molecular patterns
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
GBP	Guanylate-Binding Proteins
GSDM	Gasdermin
GSDMA	Gasdermin A
GSDMA3	Gasdermin A3
GSDMB	Gasdermin B
GSDMC	Gasdermin C
GSDME	Gasdermin E
GSDM-CT	Gasdermin C-Terminal Domain
GSDM-NT	Gasdermin N-Terminal Domain
GZMA	Granzyme A
HEK293FT	Human Embryonic Kidney Cell Line
IFN	Interferon
IL-1β	Interleukin 1β
Ipah7.8	Invasion Plasmid Antigen H

Irgm2	Immunity-Related GTPase Family M Protein 2
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeat
MOI	Multiplicity of Infection
N-GSDMD:	N-Terminal Gasdermin D
NEL	Novel E3 Ligase
NK	Natural Killer
NLRP3	NLR Family Pyrin Domain-Containing 3
PBS	Phosphate Buffer Saline
PKN1	Protein Kinase N1
PJVK	Pejvakin
SlrP	Leucine-Rich Repeat Protein of *Salmonella* Typhimurium
SPI-1	*Salmonella* Pathogenicity Island 1
SPI-2	*Salmonella* Pathogenicity Island 2
SspH1	Salmonella Secreted Protein H1
SspH2	*Salmonella* Secreted Protein H2
SspH3	*Salmonella* Secreted Protein H3
STm	*Salmonella* Typhimurium
T3SS	Type III Secretion System
UB	Ubiquitin
WHO	World Health Organization

Chapter 1

Introduction

An estimated 300.000 deaths per year are believed to be caused by *Salmonella enterica* infections which represent a serious public health hazard primarily affecting third-world nations (Buckle et al., 2012). Hundreds of *Salmonella enterica* strains were identified based on the makeup of their surface antigen composition so far (Brenner et., al 2000). The most common way that leads to *Salmonella enterica* Typhimurium (*S*Tm)'s entry into the host organism is via oral consumption of contaminated food and water resources (Ohl & Miller, 2001).

In the case of an infection, such as *Salmonella enterica* infections, inflammation is frequently viewed as the primary host reaction used to drive out an invading pathogen (Galan, 2021). In fact, innate immune receptors have evolved to recognize bacterial-associated molecular patterns that are frequently exhibited by bacterial pathogens and trigger an inflammatory response (Creagh & O'Neill, 2006; Medzhitov, 2001). Nonetheless, this inflammatory response is, in certain cases, used by *Salmonella enterica* to promote their colonization of the intestinal tract and escape the innate immune response (Winter et al., 2010).

Salmonella enterica Typhimurium is believed to replicate mostly in the large intestine after entering the body through the oral tract and from there, it moves down the digestive tract. Once inside the large intestine, *Salmonella enterica* Typhimurim locates itself near the intestinal epithelium (Stecher et al., 2004) by using its flagella systems. The *Salmonella* pathogenicity island 1 (SPI-1) encoded type III protein secretion system (T3SS) is activated upon contact with the intestinal epithelium (Galán et al., 2014). This leads to the transmission of many bacterial effector proteins that can alter different host cells (Galán, 2021). The primary result of this first encounter is the induction of host-cell responses, which in turn cause bacteria to internalize and the infected cell to undergo transcriptional reprogramming that induces inflammation (Bruno et al., 2009). When *Salmonella* then enters a membrane-bound compartment, it uses effectors that are mostly expressed by a second T3SS contained within its pathogenicity island 2 (SPI-2), which is triggered by the intracellular environment (Jennings et al., 2017) to modify vesicle trafficking. *Salmonella* uses several immune

escape mechanisms to ensure its survival in the host organism. One of these mechanisms is through the manipulation of vesicle trafficking, which shapes an intracellular niche that becomes favorable for the bacteria's survival and reproduction (Winter et al., 2010).

Antibiotic resistance is the biggest challenge when it comes to treating severe *Salmonella* infections, which usually needs extensive medical supervision. Historically, the first-line of antibiotics used for *S.* Typhimurium infections were trimethoprim–sulfamethoxazole, ampicillin, and chloramphenicol (Crump et al., 2015). The Centre for Disease Control and Prevention (CDC) organization stated that one of the major causes of increased antibiotic resistance in many pathogenic bacteria, including *S.* Typhimurium, is the misuse of antibiotic, which according to the same source, was highly observed in developing countries. The spread of antibiotic-resistant *Salmonella* serovars has been effective in global distribution, as noted by various studies (Butaye et al., 2006; Michael et al., 2006; Alcaine et al., 2007; Mather et al., 2013).

If the infection is not treated on time, excessive bacterial growth as a result of the immune system evasion strategies employed by *Salmonella* Typhimurium, leads to a type of dysregulated and intensive host immune response to infection that causes septic shock which consists of a potentially fatal organ malfunction and sepsis (Singer et al., 2016). According to epidemiological studies, the mortality rate from sepsis could reach 20.6% (Zhou et al., 2017; Cheng et al., 2017). If patients have septic shock, the incidence could increase to 40%–50% (Lakshmikanth et al., 2016). Thus, it is crucial to identify new therapeutic targets for sepsis because there are currently only few approved therapies available. Early in sepsis, the host triggers an immunological defense response that causes immune cells to undergo a specific programmed death called pyroptosis (Zheng et al., 2021).

While both pyroptosis and apoptosis are forms of programmed cell death, their biological effects, processes, and cell morphologies differ. Apoptosis ends by non-inflammatory cell demise and is mediated by Caspases associated to apoptosis, such as Caspase-3/8/9 (Zheng et al., 2021). Apoptotic cells exhibit nuclear condensation,

DNA cleavage, and apoptotic bodies during the process. On the other hand, pyroptosis is mediated by inflammatory Caspases (such as Caspase-1/4/5 in human and -11 in mice), which result in nuclear condensation, DNA cleavage, and cell membrane rupture. The integrity of the cell membrane is compromised through the activation specific targets forming pores on the cell membrane and results in inflammatory necrosis (Jorgensen & Miao, 2015). When pyroptosis spirals out of control, neighboring cells and tissues experience inflammatory responses that intensify the inflammation. This ultimately triggers a systemic inflammatory response that can result in organ failure or septic shock (He et al., 2015).

Zychlinsky et al. originally described the first characteristics on the phenomena of pyroptosis in 1992. They found that macrophages infected with *Shigella flexneri* died due to Caspase-1 dependency instead of the more common Caspase-3 dependent cell death (Zychlinsky et al., 1992). A similar process was identified by Cooksen and Brennen in 2001 in *Salmonella* Typhimurium-infected macrophages, and they named this type of programmed cell death "pyroptosis" (Cookson & Brennan, 2001). In 2008, Fink et al. discovered that intracellular material was released during pyroptosis due to DNA fragmentation and cell membrane disruption, which resulted in a severe inflammatory response, as these materials act as damage associated molecular patterns that act on neighboring cells (Fink et al., 2008).

The mechanism by which Caspases initiate pyroptosis is still not fully understood. According to numerous research, the primary inflammatory Caspase substrate is Gasdermin D (GSDMD). The cleavage of GSDMD and the subsequent release of its N-terminus (1–275 aa) create pores in the plasma membrane and the activation of inflammatory Caspases are the signature final events of pyroptosis (Broz, 2015). The inflammatory Caspases, Caspase-1/4/5 in humans and Caspase-1/11 in mice, are activated in response to different endogenous and microbial stimuli. This leads to the cleavage of GSDMD, which in turn causes rupture of the cell membrane in neutrophils and macrophages (Schroder & Tschopp, 2010).

This process causes a large-scale release of cytokines and damage-associated molecular patterns (DAMPs), which in turn sets off a potent inflammatory response

that can be localized or systemic. Depending on the various stimuli and inflammatory Caspases, pyroptosis can be classified as either canonical or non-canonical. Conventional pyroptosis is triggered by a variety of DAMPs from the cell membrane that activate Caspase-1 through conventional inflammasomes like the NLRP3-ASC complex. Caspase-1 then releases the GSDMD N-terminus, which starts the pyroptotic process (Chen et al., 2019).

On the other hand, different inflammatory Caspases (human Caspase-4/5 and mouse Caspase-11) are activated by cytoplasmic bacteria through the recognition and binding of the lipopolysaccharide (LPS) on their wall and trigger the non-canonical inflammasome pathway. This pathway results in GSDMD cleavage via the non-canonical Caspase-4/5/11 activation that results in pyroptosis. Then, a later NLRP3 inflammasome mediated Caspase-1 cleavage of pro-GSDMD, pro-IL-1beta and pro-IL-18 (Man & Kanneganti, 2019; Aglietti & Dueber, 2017). In an LPS-induced septic shock mouse model, GSDMD deficiency was shown to protect the mice and enhance their survival rate (Kayagaki et al., 2015). As a result, pyroptosis triggered by the GSDMD N-terminus may be a significant factor in the development and mortality caused by inflammatory diseases. GSDMD is a member of the family Gasdermin. The conserved N- and C-terminal domains of this family's members have around 45% sequence homology overall (Tamura et al., 2007).

Interferons (IFNs), particularly IFN-γ, have been shown to play a pivotal role in the non-canonical inflammasome pathway (Aachoui et al., 2015). IFN-γ is necessary for priming Caspase-11, facilitating its ability to recognize cytosolic LPS and enhance the immune defense against intracellular bacteria through the transcriptional modulation of several critical non-canonical inflammasome components such as GBPs. This cytokine aids in the upregulation of immune responses, ensuring that the host can effectively manage and eventually clear infections.

Although its activation is important for host survival, this immune mechanism must be tightly regulated to prevent excessive inflammation, which can lead to detrimental conditions like sepsis. In this context, the proteins Irgm2 and Gate-16 in mice have been identified as modulators that fine-tune the immune response (Eren et

al., 2020). These proteins collaboratively suppress excessive activation of the non-canonical inflammasome by inhibiting the localization of Caspase-11 to intracellular bacteria, thus reducing pyroptosis and the release of cytokines that could otherwise lead to sepsis (Eren et al., 2020).

It's crucial to note that while mouse models provide valuable insights into the functioning of the immune system, not all findings are directly translatable to humans. For example, the human homolog of Irgm2 does not play a role in sepsis, indicating species-specific differences in the regulation of immune responses (Eren et al., 2020). This discrepancy underscores the **importance of identifying and studying human-specific modulators of sepsis**.

After the discovery of Gasdermin D and its role in pyroptosis, the other members of this family started to be investigated in the context of infection. Gasdermin family of human genes consists of *GSDMA, GSDMB, GSDMC, GSDMD, GSDME* and *PJVK* genes. **Even though, other subfamily genes are retained in the mouse genome, the human *GSDMB* gene is absent** (Kovacs & Miao, 2017). Compared to other members of the Gasdermin family, which exhibit highly tissue-specific expression in the gastrointestinal tract, immune cells, and skin epithelium, GSDMB exhibits a significantly broader expression pattern (Saeki et al., 2009). According to genome-wide association studies (GWAS), there is a significant correlation between GSDMB and an increased risk of developing inflammatory diseases such type I diabetes, asthma, and Crohn's disease (Saleh et al., 2011, Zhao et al., 2015, Chao et al., 2017). Therefore, it would seem that inflammatory reactions that are out of control could be caused by disruption of GSDMB function (Rana et al., 2022).

Indeed, regardless of its pore-forming activity (which is still being explored), a recent study indicates that GSDMB aids in the repair of wounds in epithelial cells (Rana et al., 2022). Research has shown that cellular Caspases do not cleave and activate GSDMB in contrast to the majority of canonical GSDM family members. Instead, Granzyme A (GZMA), a serine protease, which comes from cytotoxic T lymphocytes and natural killer (NK) cells, activates GSDMB through site-specific cleavage of the inter-domain linker, freeing its N-terminus domain and causing

malfunctioning cells to undergo pyroptotic cell death (Zhou et al., 2020). The GSDMB C-terminus domain has a helical-bundle structure (Chao et al., 2017), just like in GSDMD (Liu et al., 2018), However *in vitro*, only the full-length GSDMB (not any other Gasdermin) was able to interact with bacterial wall specific lipids, pointing to a different activation mechanism (Chao et al., 2017).

These findings, along with the lack of research on GSDMB function, led us to the investigate and aim to identify the modulators of GSDMB that could be the human modulator of sepsis. Understanding the role of Gasdermin B could lead to the **development of novel therapeutics for inflammatory and cell death-related diseases mainly in the context of septic shock caused by *Salmonella enterica*.**

While conducting our research, we have come across an article by Hansen et al published in 2021, which has investigated and proved the ubiquitination of GSDMB that is caused by *Shigella flexneri* (Hansen et al., 2021). The group discovered that the activation of GSDMB by GZMA limits the growth of bacteria (Hansen et al., 2021). Remarkably, it was demonstrated within the same article that GSDMB directly lyses Gram-negative bacteria.

Shigella flexneri is a Gram-negative bacterium that causes shigellosis, it has been proved to deliver the type III secretion system (T3SS) effector protein Ipah7.8 (Hansen et al., 2021), which suppresses the GSDMB innate immunological defense mechanism. Ipah7.8 is a member of a family of bacterial ubiquitin E3 ligases that have a conserved novel E3 ligase (NEL) catalytic domain at the C-terminus, after a variable leucine-rich repeat (LRR) domain at the N-terminus (Rohde et al., 2007). The group has discovered that post-infection, Ipah7.8 targets and ubiquitinates GSDMB, which inhibits the bacterial lysis caused by the non-canonical pathway by degrading GSDMB (Hansen et al., 2021), proposing a new immune evasion system in bacterial infections.

This matter led us to identify homologous candidates to *Shigella flexneri*'s Ipah7.8 in *Salmonella enterica Typhimurium* as both species are Gram-negative bacteria that affect the gastrointestinal tract exhibiting a high genetic and functional similarities.

The overarching goal of this research is to **identify and characterize modulators that influence the Gasdermin B protein**, particularly during the cellular response to *Salmonella* infections. We propose the hypothesis that specific ubiquitin-ligases represent novel modulators that can regulate the activity of Gasdermin B. The identification and understanding of these interactions are critical, as they may provide new insights into the innate immune response and pave the way for innovative therapeutic strategies to cure sepsis.

Chapter 2

Literature Review

2.1 Structural and Functional Insights on the Gasdermin Family

Gasdermins are a family of pore-forming effector proteins that have been recently discovered and associated with pyroptosis which is a lytic pro-inflammatory form of cell death, this family of proteins consists of six paralogous members; GSDMA, GSDMB, GSDMC, GSDMD, GSDME and PJVK present in the genetic make-up of several organisms (Figure 1).

A flexible linker connects the cytotoxic N-terminal domain and the C-terminal repressor domain of Gasdermins. The cytotoxic domain can insert itself into cellular membranes and produce oligomeric pores through proteolytic cleavage between these two domains, which results in membrane rupture and causes cell death.

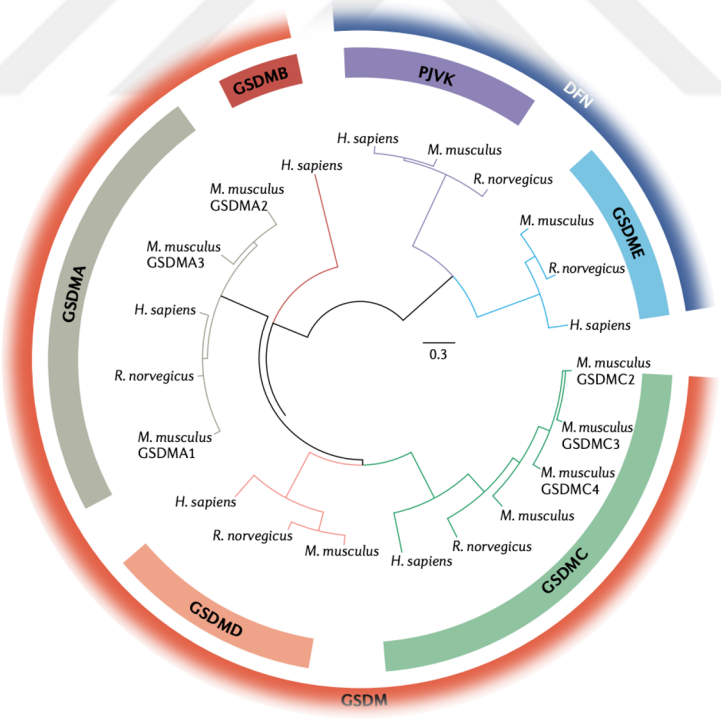

Figure 1. The Gasdermin family. A phylogenetic tree illustrating the divergence of the Gasdermin proteins in humans, mice, and rodents is shown. The six genes that

together encode the human Gasdermin protein (GSDM) family have a sequence similarity ranging from 23.9 to 49.4%. Picture taken from Broz et al., 2020.

2.1.1 Gasdermin Activation

Pyroptosis in toxin-stimulated or pathogen-infected macrophages was once thought to be a Caspase-1-dependent death in the early 2000s. Subsequent research revealed that not only Caspase-1 but also other pro-inflammatory Caspases, a class of proteases active within the inflammasome complex, primarily function through pyroptosis (Broz & Dixit, 2016). The canonical and non-canonical inflammatory pathways are two different but connected pathways that detect danger molecular signals elicited by pathogens or hosts, respectively, and trigger the activation of Caspase-1 and Caspase-4 in humans or Caspase-1 and Caspase-11 in mice.

The non-canonical inflammasome pathway, distinct from the canonical pathway that typically involves Caspase-1, primarily targets Caspase-11 in mice and its human equivalent, Caspase-4 (Kayagaki et al., 2011). This pathway is activated in response to intracellular LPS from invading Gram-negative bacteria. The activation of Caspase-11 is crucial for the effective production of IL-1β in macrophages infected with pathogens like *Escherichia coli*, *Citrobacter rodentium*, or *Vibrio cholerae*. Interestingly, in specific gene-targeted mice models, it was demonstrated that Caspase-11 is essential for Caspase-1 activation and the subsequent secretion of IL-1β, revealing a vital interaction between these Caspases in the inflammatory response (Kayagaki et al., 2011). All in all, Caspase-11's involvement in the non-canonical inflammasome highlights its unique role in recognizing cytosolic bacterial components, thereby linking pathogen detection to the inflammatory response.

The central linker region of GSDMD is cleaved by pro-inflammatory Caspases, Caspase-1 and Caspase-4/11, generating two key fragments: a 31-kDa N-terminal GSDMD-NT fragment possessing intrinsic pore-forming activity, and a 22-kDa C-terminal GSDMD-CT fragment (Shi et al., 2015). The latter binds to GSDMD-NT and acts as a repressor, inhibiting its activity. Overexpression of GSDMD-CT can prevent cell death, whereas expression of GSDMD-NT alone induces pyroptosis (Kayagaki et al., 2011; Shi et al., 2015). This cleavage process, which specifically targets the linker

between the amino-terminal and carboxy-terminal domains of GSDMD, is crucial as the removal of the inhibitory carboxy-terminal domain enables the Gasdermin-N domain to form membrane pores leading to cell rupture and death (Kayagaki et al., 2011). This critical mechanism was identified through genome-wide CRISPR-Cas9 nuclease screens in mouse bone marrow macrophages, which demonstrated that cells deficient in GSDMD could resist pyroptosis induced by cytosolic lipopolysaccharide (LPS) and other inflammasome activators (Kayagaki et al., 2011).

These findings have led to the development of a model where the intramolecular interaction between the domains of GSDMD becomes susceptible to disruption. This disruption occurs when GSDMD-NT binds to membrane phospholipids. This binding event releases the cytotoxic GSDMD-NT from its intramolecular autoinhibition, which is normally imposed by GSDMD-CT. It has been shown that once free, the GSDMD-NT can then induce pyroptosis by forming pores in the cell membrane (Broz, 2015).

This two-domain structure is shared by all members of the Gasdermin family—with the exception of Pejvakin (PJVK)— and it consists of an N-terminal cytotoxic domain and a C-terminal inhibitory domain connected by a linker. Additionally, ectopic expression of the N-terminal domain of GSDMA, GSDMB, GSDMC, or GSDME induces necrosis with morphology similar to GSDMD-induced pyroptosis (Ding et al., 2016).

With the exception of a splice variation of GSDMB (Panganiban et al., 2018), the Caspase-1 cleavage site seen in GSDMD is absent from all other Gasdermins, however alternative protease cleavage sites may be present. It has been reported that GSDME is cleaved at its linker region following Caspase-3 activation by apoptotic stimuli, as evidenced by the fact that both human and mouse GSDME exhibit a Caspase-3 cleavage motif in this region (Rogers et al., 2017).

This result led to the hypothesis that GSDME induces pyroptosis and membrane integrity loss after cells have already entered apoptosis (Rogers et al., 2017). This theory has now been refuted by subsequent research, which demonstrates that GSDME

can directly induce pyroptosis even in cell types with naturally high GSDME levels. Additionally, GSDME-deficient macrophages have the ability to undergo secondary necrosis of an unidentified program after entering apoptosis (Lee et al., 2018, Chen et al., 2019).

It is now known that there are several mechanisms besides the Caspase family that can activate Gasdermin. Neutrophil elastase, a serine protease critical to neutrophil maturation and anti-microbial activity, has been shown in two investigations to be able to cleave GSDMD in active neutrophils (Kambara et al., 2018). Despite the fact that elastase cleaves GSDMD at multiple locations upstream of the motif that inflammatory Caspases target, the cleavage nevertheless results in a functional pore-forming fragment (Kambara et al., 2018). This discovery led to an ongoing discussion on GSDMD's precise role in neutrophils. It has been discovered that GSDMs-deficient mice exhibit greater resistance to *Escherichia coli* infection and the scientists attributed this to the prolonged neutrophil lifespan (Kambara et al., 2018). On the other hand, it has been suggested that GSDMD is necessary for NETosis (Chen et al., 2018). Therefore, depending on the kind of infection and whether neutrophil death or survival is necessary for pathogen restriction, GSDMD may have both negative and positive effects on neutrophils life.

Recent insights challenge this conventional understanding and suggest a more nuanced mechanism underlying Gasdermin activation. Research indicates that the activation of Gasdermins may not strictly require the removal of the C-terminal Gasdermin (GSDM-CT) domain. Indeed, the presence or absence of this domain does not necessarily impede the ability of the protein to form pores in the cell membrane. Instead, specific mutations that disrupt the connection of the autoinhibitory linker domain have been shown to activate Gasdermin proteins. This suggests alternative pathways or triggers that can lead to Gasdermin activation, highlighting a complex regulation mechanism that goes beyond simple proteolysis (Ding et al., 2016).

The GSDMA3 crystal structure published recently (Ding et al., 2016) indicates that the α1 helix and β1–β2 hairpin loop of GSDMA3-NT, which enter deeply into a groove with a hydrophobic core within GSDMA3-CT, are important components of the

interdomain interaction. The C-terminal domains of GSDMA, GSDMA3, GSDMC, GSDMD, and GSDME all cause hydrophobic core alterations that result in pyroptosis (Ding et al., 2016). Additionally, a number of mutations that induce alopecia in GSDMA3 localize in the C-terminal domain and the interdomain interaction interface (Ding et al., 2016).

Therefore, it is plausible that physiological signaling pathways, such as phosphorylation or other post-translational alterations, might similarly relieve autoinhibition and induce Gasdermin activation independently of its cleavage.

2.1.2 Gasdermin Pore Formation

Gasdermin pore formation is the cause of plasma membrane rupture seen in pyroptotic cell death together with downstream NINJ1 activation (Aglietti & Dueber, 2017; Kayagaki et al., 2021). The Gasdermin N-terminus interacts directly with membrane lipids, as shown by in vitro binding assays (Ding et al., 2016). It has been demonstrated that GSDMD-NT preferentially binds to acidic phospholipids such cardiolipin and phosphoinositides, but it can also sporadically attach to phosphatidic acid and phosphatidylserine (Ding et al., 2016). Similar lipid-binding characteristics are shown by the N-terminal domains of other Gasdermins, including human GSDME, GSDMA, and murine GSDMA3. This suggests that the Gasdermin family as a whole has a similar membrane-targeting strategy.

Only the cytoplasmic part of the plasma membrane contains phosphoinositides. Accordingly, the N-terminal Gasdermin (GSDM-NT) domain can only induce pyroptosis within cells and adding an active Gasdermin extracellularly does not result in membrane lysis (Ding et al., 2016). Cardiolipins are found in the inner membrane of the mitochondria in both bacteria and eukaryotes. They have a negatively charged head similar to that of phosphoinositides. Severe toxicity is shown by GSDM-NT domain expression in *Escherichia coli*, and *Bacillus megaterium* protoplasts, both can be lysed by recombinant GSDM-NT protein (Ding et al., 2016). It has been demonstrated that recombinant GSDMD-NT inhibits bacterial growth and that GSDMA3-NT and GSDMD-NT alone or free from intramolecular inhibition can

disrupt mitochondria (Lin et al., 2015). However, it is still unclear how the Gasdermin pore-forming domain can reach the inner membrane location in both scenarios. Other membrane lipids may potentially affect the activity of the GSDM-NT. For example, sphingomyelin can significantly increase GSDMD-NT binding to liposomes, while cholesterol inclusion in the lipid membrane significantly decreases GSDMD-NT association (Mulvihill et al., 2018).

Interestingly, the full-length GSDMB and cleaved GSDMB-NT have comparable lipid-binding capabilities to the membrane, indicating that the GSDMB-CT domain does not obstruct GSDMB's ability to bind lipids (Chao et al., 2017). This is different than for the majority of other Gasdermins, where lipid binding can only be observed with their free GSDM-NT domains (Chao et al., 2017). Apart from its binding to phosphoinositide, GSDMB also exhibits specific binding to sulfatide (Chao et al., 2017). Yet, the physiological significance of this binding is still to be established.

It is apparent that the majority of Gasdermins, if not all of them, use a similar process to create pores in the membrane given the substantial sequence similarity among all GSDM-NT domains. A complete understanding of Gasdermin pore generation can be obtained using the high-resolution crystal structure of full-length GSDMA3 (Ding et al., 2016) and the recently discovered cryo-electron microscopy structure of a GSDMA3-NT pore retrieved from the reconstituted liposomes (Ruan et al., 2018). The GSDMA3-NT domain assumes an extended twisted β-sheet structure in the GSDMA3 crystal structure, surrounded by several helices (Ruan et al., 2018). This unique globular fold is not present in other pore-forming proteins that are currently known to exist.

From one end of the β-sheet, a lengthy loop extends to the helical GSDM-CT domain. Helix α1 and a small β-hairpin on the concave side of the β-sheet structure within the GSDM-NT domain interact closely with the GSDM-CT domain (Ding et al., 2016). A short helix supported by two flexible loops emerges from the globular fold at the other end of the β-sheet to engage with a different region of the GSDM-CT domain (Ding et al., 2016). The full-length GSDMA3 is locked into an autoinhibited state by the two interdomain contacts. The GSDM-NT domain is free to generate membrane pores

when the autoinhibition is disrupted, releasing the GSDM-CT domain from the concave surface. The structure of GSDMA3-NT within the pore exhibits significant conformational alterations as compared to the autoinhibited state, primarily in two structural elements (Ruan et al., 2018). Together with its surrounding loops, the short helix that makes contact with the GSDM-CT domain in the autoinhibited structure refolds into two β-strands, creating a β-hairpin. Another β-strand and its loops refold into β-strands and produce an additional β-hairpin in the neighbouring region (Ruan et al., 2018). In the core β-sheet structure, each of the four newly produced β-strands merges with an already-existing β-strand. The Gasdermin N-terminal domain's core globular fold is separated from the lengthy, four-stranded amphiphilic β-sheet formed by the two anti-parallel β-hairpins. The GSDM-NT domain forms a ring-shaped pore as a result of these conformational changes, which also create three oligomerization interfaces. The amphiphilic β-sheets bundle together to form a membrane-inserting β-barrel (Ruan et al., 2018). Mutations in the β-barrel or at the oligomerization interfaces, like in GSDMD E15K and L192D (Ding et al., 2016), significantly compromise the GSDM-NT domain's ability to form pores.

About 26–28 GSDM-NT protomers with a significant 27-fold symmetry are present in each of the GSDMA3 pores (Ruan et al., 2018). The GSDMA3 pore has inner and outer diameters that are roughly 18 and 28 nm, respectively (Ding et al., 2016). With an inner diameter ranging from 10 to 20 nm (Ding et al., 2016; Ruan et al., 2018). GSDMD pores on the other hand, are more heterogeneous, indicating that the stoichiometry of pores generated by various GSDM-NT domains varies depending on the situation.

Through cryo-electron microscopy, a potential lipid-binding site has been identified in the structure of the GSDMA3 pore (Ruan et al., 2018). The head group of cardiolipin, which is employed to reconstitute the GSDMA3 pore, can be used to represent the additional electron densities found in a deep and positively charged pocket between helix α1 and the membrane-inserting β-sheet. The GSDM-CT domain in the autoinhibited GSDMA3 structure completely masks the surface of this pocket (Ding et al., 2016). Since interdomain cleavage (in the case of GSDMD and GSDME) (Ding et al., 2016, Wang et al., 2017) does not activate the autoinhibitory interaction, it is

still unclear how the charged phospholipid heads can enter the fully buried pocket and cause further conformational changes.

When the canonical inflammasome is activated, mature IL-1β passes through the GSDMD pore (Evavold et al., 2018). The pore-forming conformational changes are initiated when the GSDM-NT domain inserts itself into the membrane phospholipids. This ability of GSDM-NT to integrate into cellular membranes and create pores is critical not just for the release of IL-1β but also signifies the unique action of Gasdermins, particularly Gasdermin D (GSDMD). Unlike other proteins that might contribute to cytokine secretion, GSDMD uniquely forms a pore large enough to allow the secretion of pro-inflammatory cytokines such as IL-1β (Evavold et al., 2018).

The formation of these pores by GSDMD is not only a central event in pyroptosis but also a critical factor in various inflammatory diseases. Conditions such as atherosclerosis, Alzheimer's disease, and autoinflammatory syndromes are often exacerbated by overproduction of IL-1β (Allali-Boumara et al., 2023). Consequently, targeting the activity of GSDMD and its ability to form pores could provide a novel therapeutic approach to manage IL-1β-mediated diseases.

This has spurred interest in developing inhibitors that can selectively modulate the activity of GSDMD to reduce the adverse effects of inflammation while retaining beneficial immune responses. In this sense, recent research published by Kayagaki et al., 2023 further elucidates the inhibition of downstream NINJ1 activation which was shown to be a mediator of pyroptotic membrane rupture (Kayagaki et al., 2021) limits the tissue injury caused by lytic cell death (Figure 2).

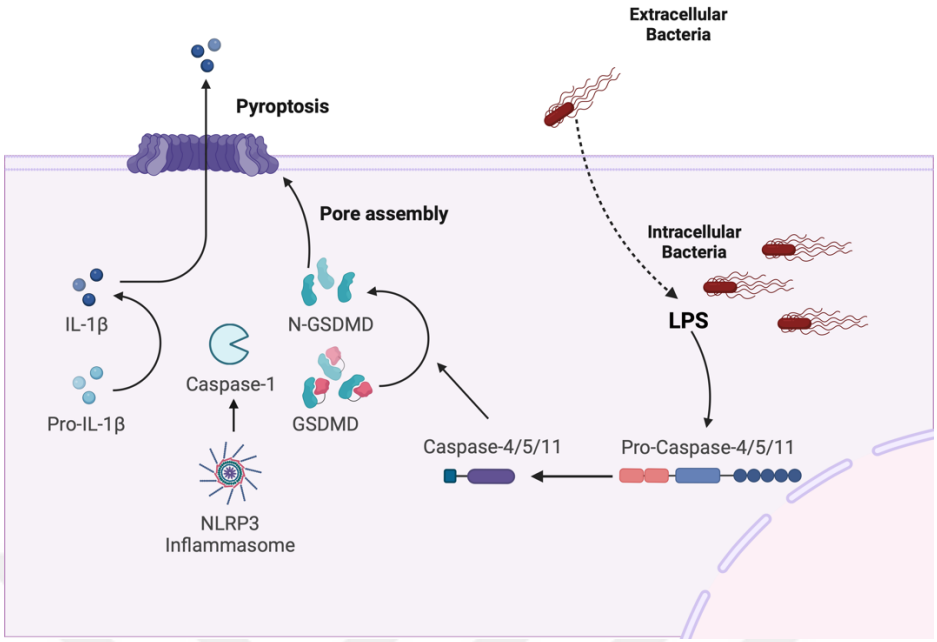

Figure 2. The non-canonical inflammasome. Intracellular lipopolysaccharide (LPS) binding to (Caspases-4, -5, and -11) and the initiation of pyroptosis is shown. The N-terminal p30 domain of Gasdermin D (GSDMD) is released from the autoinhibitory GSDMD-C domain by the inflammatory Caspases upon activation. Following its binding to lipids in the plasma membrane, activated N-GSDMD creates sizable oligomeric holes that allow the escape of cellular contents and cause cell death. Abbreviations: NLRP3, NLR family pyrin domain-containing 3; IL-1β, interleukin 1β. Created with BioRender.com.

2.2 Gasdermin B

Since the discovery of Gasdermins, GSDMD has been the most and best investigated Gasdermin to date. Most of the information available on Gasdermins is based on our knowledge on GSDMD. The intriguing findings from studies on the composition and operations of GSDMD brought additional focus to the other members of the GSDM family including GSDMB and their possible roles in various cellular processes.

2.2.1 Gasdermin B Structure and Function

Due to its ability to operate in a variety of clinical conditions, including enterobacteria infections (Hansen et al., 2021), asthma (Das et al., 2016), inflammatory bowel diseases (Chen et al., 2019), and cancer (Ivanov et al., 2022), GSDMB has been lately proved to play complex biological roles similar to GSDMD.

GSDMB is expressed in a variety of organs, primarily the respiratory system, lymphoid tissues, and the gastrointestinal tract (Saeki et al., 2009). It is also expressed in a variety of tumor types, such as the breast, stomach, and bladder (Sarrió et al., 2021). Depending on the biological setting, GSDMB either promotes pro-tumor or anti-tumor actions (Sarrió et al., 2021). In breast and gastric carcinomas, GSDMB is often co-expressed with the HER2/Erb2 oncogene (Hergueta-Redondo et al., 2016, Gámez-Chiachio et al., 2022). Overexpression of GSDMB facilitates carcinogenesis (Sarrio et al., 2022), invasion, metastasis, and resistance to treatment (Hergueta-Redondo et al., 2016). Interestingly, if GSDMB's pore-forming pyroptotic function is triggered in cancer cells, it may also have an antitumor effect.

GSDMB is located at chromosome 17q21 and consists of 411 amino acids (Das et al., 2017). The conformation of GSDMB is different from other Gasdermin family members. A recent study has shown that GSDMB has six isoforms ranging from 18 to 50 kDa and encoding for six different proteins in different cell types (Oltra et al., 2023). Five of them, have different lengths and linker sequences between their NT and CT domains, whilst isoform 5 is composed solely of a CT domain (Oltra et al., 2023).

The linker sequence connecting the C- and N-terminal domains of GSDMB is less stable than that of the other GSDMs (Chao et al., 2017). The function of GSDMs depends on the cleavage of their N- and C-terminal domains because, as previously indicated, the GSDM-CT domain of GSDMD, GSDMA, and GSDMA3 suppresses the GSDM-NT domain binding to phospholipids. Conversely, GSDMB's entire length and its NT domain can both bind to phospholipids. Accordingly, GSDMB's CT domain does not inhibit its NT domain's capacity to bind lipids (Kim et al., 2017). The binding of GSDMB to phosphatidylinositol phosphates and sulfatides rather than phosphoserine and cardiolipin is another characteristic that sets it apart from other Gasdermins.

Research has demonstrated that cellular Caspases do not necessarily cleave and activate GSDMB. Rather, Granzyme A (GZMA), a serine protease derived from natural killer (NK) cells and cytotoxic T lymphocytes, cleaves the inter-domain linker at certain sites to activate GSDMB by releasing its N-terminus domain and inducing pyroptotic cell death in defective cells (Zhou et al., 2020).

With the aid of perforin, Granzyme A penetrates the target cells and activates full-length GSDMB, causing the release of GSDMB-NT fragments that oligomerize forming pores on the lipid bilayer membrane. While the apoptotic executioner Caspases cleaves GSDMB at the after 88DNVD91 within its NT domain, Granzyme A cleaves it at the Lys244 of its linker region (Zhou et al., 2020; Chao et al., 2017).

2.2.2 Gasdermin B and Associated Diseases

GSDMB displays a substantially larger expression pattern in different tissues in comparison to other members of the Gasdermin family that show high tissue-specific expression in the gastrointestinal tract, immune cells, and skin epithelium (Saeki et al., 2009).

The development and progression of inflammatory illnesses, including type I diabetes, asthma, and Crohn's disease, is significantly correlated with GSDMB expression according to genome-wide association studies (GWAS) (Saleh et al., 2011, Zhao et al., 2015, Chao et al., 2017). Thus, it would appear that disruption of GSDMB function may be the source of uncontrollably high inflammatory reactions (Rana et al., 2020).

It has been observed that in 60% of instances of human epidermal growth factor 2-positive breast cancer, GSDMB promotes the invasion, development, and metastasis of cancer cells. Additionally, it has been shown that delivering an antibody targeting GSDMB intracellularly can decrease the metastasis, migration, and therapy resistance of human epidermal growth factor 2-positive breast cancer. The pro-tumor effects of GSDMB, such as migration, metastasis, and resistance to therapy, have been reduced

in HER2 positive breast cancer cells by intracellular delivery of an anti-GSDMB antibody (Molina-Crespo et al., 2019).
Given that GSDMB was shown to promote cancer cell metastasis, these findings point out the possibility of using it as a screening marker for breast cancer. Additionally, GSDMB targeting can be used as a treatment for human epidermal growth factor 2-positive breast cancer (Molina-Crespo et al., 2019).

In another study by Komiyama et al., GSDMB's expression was investigated by comparing stomach tissue samples from people with gastric cancer and healthy donors. They found that the majority of healthy stomach tissues zero to very low levels of GSDMB expression on the other hand, GSDMB was overexpressed in gastric malignancies compared to healthy tissues, suggesting that GSDMB may be important in controlling the proliferation of cancer cells (Komiyama et al., 2010). Consequently, it has also been found that the tissues of colon and hepatocellular carcinoma also exhibit overexpression of GSDMB when compared to healthy colon and kidney tissue (Saeki et al., 2009). Similar pattern of overexpression was reported in cervical squamous cell carcinoma tissues (Lutkowska et al., 2017).

In addition to its overexpression in cancer, numerous genome-wide association studies (GWAS) demonstrated the strong correlation between the number of single nucleotide polymorphisms (SNPs) regulating GSDMB expression and inflammatory and autoimmune pathologies, including asthma. However, it is surprising to note that the risk alleles for autoimmune diseases and asthma are consistently opposite, indicating different immunopathogenic mechanisms for these conditions.

Asthma severity, exacerbations, and susceptibility are all correlated with the majority of SNP alleles that upregulate GSDMB mRNA in leukocyte cells and, to a lesser extent, in bronchial cells (Li et al., 2021). Furthermore, transcription of pyroptotic variants of GSDMB (isoforms-3–4) is permitted by the rs11078928 asthma-risk allele (T) (Panganiban et al., 2018), indicating that an excess of pyroptosis in asthma is harmful. On the other hand, GSDMB may have non-pyroptotic functions in this illness. When GSDMB was nuclearly localized, it caused the overexpression of TGF-β1 and 5-lipoxygenase genes, which in turn led to airway remodelling, rather than increasing

cell death, as demonstrated by the asthmatic phenotype in knock-in mice that universally express isoform-3 (pyroptosis-proficient variation) (Das et al., 2016).
The SNP risk alleles for IBD typically downregulate total GSDMB mRNA in gut/immune cells, in contrast to asthma (Söderman et al., 2015). However, exon 6-pyroptotic GSDMB variants are not transcribed by the rs11078928 risk allele (C), which favors noncytotoxic isoforms (Morrison et al., 2013). These findings imply that IBD could be brought on by ineffective pyroptosis. In contrast, IBD-inflamed tissues were found to have higher levels of GSDMB protein (cleaved and uncleaved) in comparison to non-affected tissues and healthy individual controls (Rana et al., 2022).

Additionally, two prevalent IBD-risk SNPs (rs2305479 and rs2305480) cause missense changes within GSDMB-CT, which may dramatically modify other GSDMB functions and/or NT-CT autoinhibition (Chao et al., 2017). In particular, IBD-associated GSDMB mutations block the processes by which wildtype GSDMB causes efficient wound healing (increased proliferation, migration, and reduced adhesion). Additionally, it was demonstrated that the immunosuppressant methotrexate increases the expression of GSDMB and the location of uncleaved GSDMB on the gut epithelium's cell membrane (Rana et al., 2022).

According to further studies, GZMA cleavage efficacy is impacted by matching mutations in the pyroptotic-proficient isoform-3 (Gong et al., 2021), which may control cell death activity in IBD. In an unexpected turn of events, Chen et al. suggest that GSDMB increases non-canonical GSDMD-pyroptosis by activating Caspase-4, rather than causing pyroptosis in IBD on its own. GSDMB enhances Caspase-4 activity, which is necessary for the cleavage of GSDMD in non-canonical pyroptosis, by directly binding to the CARD domain of Caspase-4. (Chen et al., 2019)

In conclusion, it is now evident that GSDMB contributes significantly to various disorders in addition to malignancies. However, **further research is required to identify the precise pathways via which GSDMB impedes these illnesses and any potential connections to cellular death.**

2.2.3 Immune Escape Through Gasdermin B Modulation

Shigella flexneri, a highly virulent bacterium causing bacillary dysentery, employs multiple sophisticated mechanisms to evade the host immune response, particularly through modulation of Gasdermin B (GSDMB) and Gasdermin D (GSDMD) pathways. *Shigella flexneri* secretes IpaH7.8, a bacterial ubiquitin ligase, which specifically targets human GSDMD for proteasomal degradation, thereby preventing pyroptosis and facilitating bacterial survival within host cells (Luchetti et al., 2021). This inhibition of GSDMD-dependent pyroptosis highlights a key virulence strategy by which *Shigella* circumvents the innate immune response.

In addition to GSDMD, *Shigella* also targets GSDMB to evade immune defences. The degradation of GSDMB by the *Shigella* effector IpaH9.8 disrupts the function of cytotoxic lymphocytes and natural killer (NK) cells, which rely on GSDMB to exert their bactericidal functions (Hansen et al., 2021). This dual targeting of Gasdermins by *Shigella* effectors underscores the bacterium's ability to undermine multiple layers of the host immune defence, ensuring its survival and proliferation.

Moreover, *Shigella*'s evasion tactics extend to the modulation of inflammasome pathways. The type III secretion system (T3SS) effector OspC3 modifies Caspase-4 and Caspase-11 through ADP-riboxanation, thereby blocking their ability to cleave GSDMD and initiate pyroptosis (Li et al., 2021).

These strategies illustrate a sophisticated evolutionary arms race between host defense mechanisms and bacterial evasion tactics. The ability of *Shigella* to inhibit key components of the pyroptosis pathway through targeted degradation and modification of Gasdermins represents a critical aspect of its pathogenicity and highlights potential targets for therapeutic intervention (Wandel et al., 2017). **Despite the physiological importance of these escape mechanisms, whether a similar strategy is employed by *Salmonella* Typhimurium to evade host immunity through the modulation of Gasdermin B remains unknow.**

2.3 *Salmonella* Typhimurium

The genus *Salmonella* was first identified in 1885 while American veterinary pathologist Daniel Elmer Salmon and Theobald Smith were looking for the reason for common hog cholera. Smith suggested that sick pigs contained a novel kind of bacteria, previously known as *S. cholerae-suis*, as the causal agent. However, Salmon took credit for the finding even though Smith was the true discoverer, and the organism was later named in his honor. Subsequent investigations, however, showed that this bacterium is not the agent they were looking for (which was ultimately proved to be a virus), as it rarely causes gastrointestinal symptoms in pigs (Schultz, 2010).

Salmonella is a genus of bacteria that belong to the Enterobacteriaceae family. It is closely linked to the genus *Escherichia* and is a Gram-negative bacterium. *Salmonella* also has a rod-shaped morphology. These bacteria have diameters ranging from around 0.7 to 1.5 μm and lengths ranging from 2 to 5 μm. Being facultative anaerobes, their motility is primarily peritrichous. This genus describes main intracellular pathogens that cause a variety of clinical symptoms when an infection develops among humans (Murray et al., 1999, Coburn et al., 2007).

The World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France, defines and updates the classification of this genus. *Salmonella enterica* and *Salmonella bongori* are the two species that make up the genus. *S. enterica* can be further subdivided into six subspecies: *S. enterica subsp. houtenae, S. enterica subsp. salamae, S. enterica subsp. arizona, S. enterica subsp. diarizonae, and S. enterica subsp. indica* (Grimont & Weill, 2007). The CDC, reports that there are about 40,000 cases of salmonellosis (*Salmonella* infections) in the United States alone each year. However, the true number is thought to be at least thirty times higher because many milder cases are not diagnosed or reported. Acute salmonellosis is a disease that primarily affects children, elderly, and immunocompromised patients. An estimated 300,000 deaths per year are attributed to *Salmonella enterica* infections (Buckle et al., 2012).

Over the past few years, an extensive amount of research has been done on the pathogenesis cause by *S.* Typhimurium. The five *Salmonella* pathogenicity islands (SPIs) have been identified to date, which are thought to significantly influence host-cell interactions (Galán et al., 2014). The SPI-1 encodes a number of effector proteins, the majority of which cause actin cytoskeletal rearrangements and subsequent bacterial internalization to initiate the invasion of epithelial cells through the use of a type III secretion system (T3SS), known as T3SS-1 and also encoded within SPI-1, these effectors are translocated into the host cell (Lostroh & Lee, 2001). SPI-2 is formed by two parts: the ttrRSBCA operon, which is involved in tetrathionate reduction, and seven open reading frames (ORFs) with an unknown function. According to preliminary findings, these genes are not substantially involved in systemic infections in mice (Hensel et al., 1999). On the other hand, recent data has linked the expression of these genes to a growth advantage over the microbiota (Winter et al., 2010). The ability of *Salmonella* to survive and multiply inside host cells (epithelial cells and macrophages) within the SCV was first shown to be critically dependent on this island (Ochman et al., 1996).

Since the other three SPIs have not been examined as thoroughly as SPI-1 and SPI-2, there is less knowledge about their functions to date. It has been demonstrated that *Salmonella* may manipulate vesicle trafficking to evade innate immune responses, creating an intracellular niche that is favorable to the bacteria's survival and reproduction (Winter et al., 2010). On the other hand, it is important to stress the point that the accumulation of *Salmonella* Typhimurium growth and immune system evasion in the gastrointestinal tract may result in sepsis, a dysregulated host response to infection that when advanced results in a septic shock which consists of a potentially lethal organ malfunction (Singer et al., 2016). Epidemiological studies have shown that sepsis death rates can reach 20.6% (Zhou et al., 2017, Cheng et al., 2017); in patients who experience septic shock, these rates can rise to 40%–50% (Lakshmikanth et al., 2016).

Salmonella Typhimurium (*S*Tm) interacts with the innate immune system through distinct inflammasome pathways depending on its growth phase and the host's

genetic makeup. In wild-type (WT) mice, *S*Tm in the exponential phase expresses flagellin, which activates the NLRC4 inflammasome. This leads to the production of pro-inflammatory cytokines, enhancing the immune response to clear the infection (Kayagaki et al., 2011; Aachoui et al., 2015; Casson et al., 2015; Eren et al., 2020).

As *S*Tm transitions to the late exponential phase, it activates the non-canonical inflammasome pathway mediated by Caspase-11. This pathway is critical for detecting cytosolic lipopolysaccharide (LPS) from Gram-negative bacteria, leading to a robust inflammatory response that helps control the infection (Kayagaki et al., 2011; Aachoui et al., 2015; Casson et al., 2015; Eren et al., 2020).

Guanylate-binding proteins (GBPs) play a crucial role in the immune response to *S*Tm by facilitating the detection and destruction of intracellular pathogens. These GTPases are induced by interferons and are essential for the activation of the non-canonical Caspase-11 inflammasome during infections with vacuolar Gram-negative bacteria. GBPs promote the lysis of pathogen-containing vacuoles, releasing the bacteria into the cytosol, where their LPS can be detected by the inflammasome (Meunier et al., 2014; Eren et al., 2020; Santos et al., 2020). This GBP-mediated vacuole lysis is a critical step in ensuring efficient recognition and response to intracellular pathogens.

These mechanisms highlight the complex interplay between bacterial pathogens and the host immune system in controlling infections.

In conclusion, while the IFN pathway and proteins like Irgm2 and Gate-16 are vital for controlling the immune response in mice, the differences in immune system regulation between mice and humans necessitate focused research on human-specific modulators. Discovering and characterizing these factors, such as Gasdermin B, could pave the way for innovative treatments that prevent sepsis while preserving the body's ability to fight infections. Such advancements are crucial for enhancing the efficacy of medical interventions and improving outcomes in sepsis management.

2.4 Hypothesis

Although we are exposed to a variety of pathogens continuously, the presence of sophisticated cytosolic pathogen associated recognition pattern receptors and the activation of innate immune defenses through inflammation and pyroptosis ensure our survival. This protective immune response becomes deleterious for the host when it is overactivated and can lead to sepsis.

To prevent and stop sepsis, it is crucial to understand the molecular mechanisms of pathogen detection and elimination. Our research group aims to **identify new regulators of the non-canonical Caspase-4/11 inflammasome and to understand how pathogens evade this pathway by using *Salmonella* Typhimurium as a model.**

In the light of recent findings, the importance of all Gasdermin family members, especially Gasdermin B, continues to grow. While new therapeutic approaches based on targeting Gasdermin B in HER-2 positive cancer cells is still ongoing, recent findings suggest that Gasdermin B have also a very important role in the detection of bacteria (Molina- Crespo et al., 2019). Gasdermin B was demonstrated (1) to be human specific; (2) to interact with the non-canonical inflammasome receptor Caspase-4; and (3) to bind to bacterial membranes directly and kill them. However, **the exact mechanism through which Gasdermin B triggers bacterial death is still unknown**. All these results suggest that **Gasdermin B may be the master regulator of the non-canonical inflammasome pathway in human and have a crucial role in bacteria elimination**.

Given growing evidence linking GSDMB to pyroptosis and its known involvement in a variety of diseases, including asthma and IBD, we set out to investigate and identify GSDMB modulators in the hopes of proposing new possible treatments for conditions related to inflammation and cell death, particularly in the context of septic shock brought on by *Salmonella enterica*.

Similar to many interactions, the host/pathogen interaction is very dynamic. Bacteria develop several immune escape mechanisms to overcome host's immune system and survive. A recent example of immune system modulation by bacteria was shown by the lately published study published by Hansen et. al. GSDMB has the ability to directly lyses Gram negative *Shigella flexneri* as an innate immune response to infection (Hansen et al., 2021). In turn, *Shigella*'s type III secretion system (T3SS) effector protein Ipah7.8 (Hansen et al., 2021) ubiquitinates GSDMB, suppressing the innate immune defense mechanism (Hansen et al., 2021). Since both *Shigella flexneri* and *Salmonella* Typhimurium are Gram-negative bacteria that exhibit considerable genetic and phenotypic similarities, we sought to determine **whether *Salmonella* Typhimurium could use a similar mechanism to modulate Gasdermin B**.

In this thesis, we hypothesize that ***Salmonella* Typhimurium could escape innate immune defenses by triggering the degradation of GSDMB through ubiquitination**. By using a combination of bioinformatic and wet lab techniques, we (1) investigate whether Gasdermin B is ubiquitinated during *Salmonella* infection and (2) propose/identify a potential bacterial ubiquitin ligase that could modify GSDMB post-translationally during infection.

The finding of this thesis, will allow a better understanding of innate escape mechanisms used by *Salmonella* Typhimurium, clarify the role of Gasdermin B in the non-canonical inflammasome activation and open the way to new possible treatments for conditions related to inflammation and cell death, particularly in the context of septic shock triggered by *Salmonella enterica*.

Chapter 3

Materials and Methods

3.1 Materials

Below is given detailed description of different materials used in this study.

3.1.1 Cell Culture

Human embryonic kidney cell line (HEK293FT) was used in this research, the cell line was kindly provided by Prof. Dr. Nesrin Özören, AKIL Lab, Boğaziçi Univesity, Turkey.

Table 1

Solutions and Buffers Used in Cell Culture

Solution	**Main Source**
Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine, phenol red,and high glucose	PAN BIOtech
Phosphate buffer saline (PBS) 10X	PAN BIOtech
Trypsin-EDTA (0.25%)	PAN BIOtech
Fetal Bovine Serum (FBS) 10%	GIBCO, UK
Non-essential amino acids (NEAA)	GIBCO, UK
Penicillin/Streptomycin	PAN BIOtech
Dimethyl sulfoxide (DMSO) 10%	SIGMA-ALDRICH

3.1.2 Calcium Phosphate Transfection

Table 2

Solutions and Buffers Used for Transfection

2M $CaCl_2$	2.22 g of $CaCl_2$ in 10 ml ddH_2O (filtered)
2X HBS Buffer	2.5 ml of 50 mM HEPES pH 7.5 0.04 g of 10 mM KCl 0.11 g of 12 mM D-Glucose 0.82 g of 280 mM NaCl 1.5 mM Na_2HPO4 Completed till 50 mL of ddH_2O
Chloroquine	Sigma C6628-25G
MG132	Thermo Scientific J63250
IFN-γ	PeproTech 300-02

3.1.3 Bacterial Culture

Table 3

Solutions Used for Bacterial Cultures

LB Broth	20 g of LB Lenox (SIGMA-ALDRICH) in 1L of ddH_2O
LB Miller Broth	10 g of LB Lennox (SIGMA-ALDRICH) 5 g NaCl in 1L of ddH_2O
LB Miller Agar	10 g of LB Lennox (SIGMA-ALDRICH) 4 g NaCl 15 g of Agar in 1L of ddH_2O
Ampicillin	SIGMA-ALDRICH, 100 mg/ml stock solution
Streptomycin	BioFroxx, 100mg/ml stock solution

Salmonella enterica Typhimurium SL1344 was used in this study (Assoc. Prof. Nefise Akçelik, Ankara University, Turkey). *E. coli* DH5alpha strain was used for transformation of plasmids.

3.1.4 Infections

We used Gentamicin solution 10mg/mL from SIGMA-ALDRICH G1272 for the infection procedure.

3.1.5 Immunoprecipitation Assay

Buffers and solutions for immuno-precipitation assay were provided with the Sigma-Aldrich FLAG Immunoprecipitation Kit (catalog number: 1001540500 FLAGPT1-1KT).

3.1.6 Colony Forming Unit Assay (CFU)

Table 4

Buffers Used for the CFU

0.1% Triton-X solution	5 µl Triton-X into 5ml PBS
10 mM H_2O_2	39.1 µl H_2O_2 into 5ml PBS

3.1.7 Western Blotting

Table 5

Solutions Used for Western Blotting

30% acrylamide	BioFroxx
Tris-base	SIGMA-ALDRICH
SDS	Wisent Inc.
APS	SIGMA-ALDRICH

TEMED	BioMatik
Glycine	BioFroxx
Methanol	ISOLAB
Tween 20	BioFroxx
NaCl	Biochemistry
BSA	BioFroxx
Protein Ladder	Thermo Scientific
RIPA	Thermo Scientific
Protease Inhibitor Coktail (PIC)	Thermo Scientific
ECL Kit	Advansta Inc.

Table 6

Buffers Used for Western Blotting

12% Resolving gel	3,2 ml ddH_2O 2,5 ml Tris 1.5M Ph 6.8 4 ml 30% Acrylamide 100 µl 10% SDS 100 µl 10% APS 10 µl TEMED
4% Stacking gel	3 ml ddH_2O 1.25 ml Tris 0.5M pH 6.8 0,67 ml 30% Acrylamide 50 µl 10% SDS 50 µl 10% APS 5 µl TEMED
Running buffer	100 ml 10X SDS Buffer (EcoTech) 900 ml of ddH_2O
Transfer buffer	5.82 g Tris-base 2.93 g Glycine 200 ml Methanol

	ddH_2O till 1 L
10X TBS	24 g Tris base 88 g NaCl
1X TBST	100 ml TBS 10X 900 mL ddH_2O 1 ml Tween-20
5% BSA	2.5 g Bovine Serum Albumin 50 ml 1X TBST
Stripping buffer	20 ml 10% SDS 12.5 ml Tris base pH 6.8 800 µl beta-mercaptoethanol 67.7 ml ddH_2O

3.1.8 Plasmids

Table 7

Plasmids Used in this Study and their Sources

pcDNA3-hGB-Flag	This study (Genescript)
pcDNA3-SlrP-Flag (pIZ1725)	Kindly provided by Prof. Francisco Ramos-Morales, Sevilla University, Spain
pcDNA3-hUB-HA	Kindly provided by Prof. Dr. Nesrin Özören, Boğaziçi University, Turkey (initially Prof. Dr. Gabriel Nunez, Michigan University, USA)
pcDNA3-HA	Kindly provided by Prof. Dr. Nesrin Özören, Boğaziçi University, Turkey (initially Prof. Dr. Gabriel Nunez, Michigan University, USA)
pcDNA3-Flag	Kindly provided by Prof. Dr. Nesrin Özören, Boğaziçi University, Turkey (initially Prof. Dr. Gabriel Nunez, Michigan University, USA)

3.1.9 Equipment and Consumables

Table 8

Equipment Used in the Laboratory and their Sources

Shakers	Grant, UK
Water bath	Nüve, turkey
Centrifuges	Eppendorf, Germany
Freezers	Eppendorf, Germany
Incubator	Nüve, Turkey
Autoclave	Nüve, Turkey

Microplate reader	Thermo Scientific
Microscopes	Olympus, Japan
BioRad chemiDoc MP	BioRad, USA
BioRad Trans-blot Turbo	BioRad, USA
Laminar flow hood	Nüve, Turkey
BioRad qPCR Thermocycler	BioRad, USA

Table 9

Consumables Used in the Laboratory and their Sources

Pipette tips	ISOLAB Laborgeräte GmbH, Germany
Micropipettes	Eppendorf, Germany
Serological Pipettes	ISOLAB Laborgeräte GmbH, Germany
Eppendorf tubes	ISOLAB Laborgeräte GmbH, Germany
Conical Sterile Polypropylene Falcon tubes	NEST, Turkey
Cell Culture well plated	NEST, Turkey
10 cm Petri dishes	Thermo Scientific
0.22µ Filters	ISOLAB Laborgeräte GmbH, Germany

Cell counting chamber	ISOLAB Laborgeräte GmbH, Germany
Immun-blot 0.2µm PVDF-membranes	BioRad, USA
Western Blot filter papers	BioRad, USA

3.2 Methods

3.2.1 Cell Culture

Human embryonic kidney cells were used in this research (HEK293FT) they are adherent cell lines and were cultured in DMEM with 1% NEAA to help the proliferation, 1 mM Penicillin/Streptomycin to avoid contaminations, and 10% FBS and incubated at 37°C with 5% CO_2 (called full DMEM).

When the cells were about 80% confluent, the supernatant was discarded, and 2 ml of trypsin-EDTA solution was added to detach the cells from the surface. After 3 minutes of incubation at 37°C, 2 ml of full DMEM was added to deactivate the enzymatic activity of trypsin. The cells were then collected in a 15 ml falcon tube and centrifuged at 2000 rpm for 2 minutes. The supernatant was then discarded, and the cell pellet was resuspended in full DMEM and transferred to a new flask.

3.2.2 Cell Freezing

Cell stocks were frozen using FBS supplemented with 10% DMSO. We first put them in -20°C for 20 minutes before transferring them to -80°C.

3.2.3 Calcium Phosphate (CaPO4) Transfection

600.000 cells in 6 well plates or 5.000.000 cells in 10 cm petri dishes were plated in full medium and incubated O/N, at 37°C with 5% CO_2. After the incubation time was over, the cells were checked for their confluency, 70% confluency was optimum for HEK293FT cells according to our optimization trials.

The next day, after the old media are aspirated, 1 ml (in 6 well plates) / 5 ml (10 cm petri dishes) of $DMEM^{-/-}$ (DMEM only, without any supplements) and 25 µM chloroquine were added onto the cells. $CaPO_4$/plasmid mixtures were prepared by adding the indicated plasmid concentration in water to a final volume of 220,5 µl. 30.5 µl of ice-cold 2M $CaCl_2$ was added to the transfection mixture drop by drop, and the mixture was then incubated at room temperature for 5 minutes. The incubation was followed by the addition on 250 µl ice-cold 2X HBS buffer drop by drop, then the final mixture is incubated for 20 minutes before being added to the cells.

The cells are then incubated with the transfection mixture for 5 hours at 37°C with 5% CO_2. 5 hours post-transfection, the cells are washed 2 to 3 times with pre-warmed PBS to remove the $CaPO_4$ aggregates before adding full DMEM.

In the case we were to infect the cells post transfection, we would prime the cells with interferon gamma (IFN-γ) at 10 ng/ml right after changing the media to full DMEM in order to activate the non-canonical pathway, which is our interest in this study. For indicated experiments, the degradation of our proteins of interest was prevented by the addition of the proteasome inhibitor MG132 at 1 µM right after changing the media to full DMEM.

3.2.4 Immunoprecipitation Assay

24 hours post transfection, the cells were washed with PBS, then harvested in a mixture of IP Lysis buffer containing protease inhibitor cocktail (PIC) to prevent the degradation of our proteins of interest. The cell lysates were incubated for at least 16 hours at -80°C to ensure the cellular membrane lysis. Cell lysate was cleared by centrifugation.

Sigma-Aldrich FLAG Immunoprecipitation Kit protocol was used to immunoprecipitated the Flag-tagged protein. The total cell lysate was harvested and incubated overnight at 4°C in beads containing Flag-tag antibodies with agitation. The next morning, the mixture was centrifuged, and the antigen-antibody beads complex was washed multiple times. 2X Laemmli buffer from the kit was added onto the complex and the mixture was then desaturated through boiling at 95°C before being used in downstream applications (Western Blotting in our case).

3.2.5 Plasmid Purification

Two different kits were used for the purification of our plasmids:

Table 10

Plasmid Purification Kits Used

NucleoSpin Plasmid Mini Prep	MACHEREY-NAGEL, GERMANY
NucleoBond Xtra Midi kit for transfection-grade plasmid DNA	MACHEREY-NAGEL, GERMANY

Transformed bacteria containing the plasmids were stored in 50% glycerol stocks at -80°C. One night before the purification, an LB/Ampicillin bacterial culture (30 ml for Miniprep/ 200ml for Midiprep) was started and incubated at 37°C O/N in a shaker. The bacterial cells were harvested post-incubation through centrifugation, then, the kit protocol is followed to obtain the purified plasmids.

For Miniprep, the pellet was resuspended in 250 µl of A1 buffer before adding 500 µl of buffer A2 and incubating 5 minutes at room temperature. 600 µl of buffer A3 was added with mixing until complete neutralization followed by a 5-minute centrifugation at ≥15000 rpm. Another 1 min centrifugation is made after transferring the supernatant to the column. The samples were then washed with 600 µl of buffer A4 before a 1-

minute centrifugation to dry the silica membrane. DNA was then eluted in 30 µl nuclease-free water, and the concentration was assessed using a spectrophotometer before storage at -20°C.

For Midiprep, the pellet was resuspended in 8 ml of Resuspension Buffer before adding 8ml of Lysis buffer and incubating 5 minutes at room temperature. Meanwhile, the filters that were placed onto the columns are equilibrated with 12 ml of the Equilibration buffer. After the incubation time was over, the reaction mixture was neutralized with 8 ml of Neutralization buffer before loading it onto the filter. The filter was equilibrated once again with 5 ml of Equilibration Buffer, then the filter was removed, and the column was washed with 8 ml of Wash Buffer. Consequently, the plasmids were eluted with the Elution Buffer that is pre-heated at 50°C, followed by the addition of 3.5 ml Isopropanol, vortexing and centrifugation at maximum speed at 4°C. The supernatant was then discarded and a total of 2 ml of 70% Ethanol was added, followed by a shorter centrifugation at maximum speed. The supernatant was then discarded, the pellet is dried at room temperature, then resuspend in nuclease free water.

As a final step for both Miniprep and Midiprep, the concentration and purity of the plasmids were checked using a plate reader and then stored at -20°C.

3.2.6 Infection

*S*Tm SL1344 bacterial cultures were opened one night before the infection in a streptomycin containing LB Miller solution. Bacteria were kept growing for 16 hours at 37°C overnight and the next day, 1:50 dilutions were prepared and incubated at 37°C until OD reaches 1,2-1,5 to make sure that the bacteria are in the late stationary phase in order to ensure the triggering of Caspase-4 activation. The required amount of bacteria to have the indicated multiples of infection (MOI) was then centrifuged, washed with pre-warmed PBS before being gently resuspended in pre-warmed $DMEM^{-/-}$.

The media of the cells to be infected was changed into DMEM$^{-/-}$ and bacteria were added into the cell plate walls. The cell plates are then centrifuged to ensure synchronization and incubated at 37°C with 5% CO_2. 30 minutes post-infection, 100 ng/µl Gentamycin was added to the infected cells and 1-hour post-infection, the whole cellular media was discarded, the infected cells were washed with pre-warmed PBS until we ensure the removal of all extracellular bacteria. Then the cells were incubated again in full DMEM with 10 µg/ml of Gentamycin (Meunier et al. 2014; Eren et al., 2020; Santos et al., 2020). The cells are harvested 4 hours post-infection to be used in downstream processes.

3.2.7 Colony Forming Assay (CFU)

After performing the infection procedure as previously described, cells infected with intracellular bacteria were harvested in ice-cold PBS, centrifuged and 400 µl of 0.1% Triton-X solution was added to the cellular pellet to lyse the cells and release intracellular bacteria without lysing them. The pellet was dissociated gently before being incubated at RT for 5 minutes.

The cells were then centrifuged, and the pellets were dissociated in LB Miller with or without the addition of H_2O_2. The microtubes containing the bacteria were incubated for 45 minutes at 37°C, centrifuged and different dilutions were made and seeded in LB Miller Agar plates, or used for kinetic studies.

3.2.8 Western Blotting

The total cell lysates were heated at 95°C for 15 minutes to denature the proteins. Subsequently, the samples were loaded to a 12% acrylamide gel and run at 100V for about 2 hours to separate the proteins. Protein transfer onto the membrane was carried out using either the Turbo Mini Transfer System or the Wet Transfer method, utilizing methanol activated 0,2 µm PVDF membranes for both.

After transferring, the membrane was blocked with 5% BSA blocking buffer for 30 minutes at RT, then treated O/N at 4°C with the primary antibody (anti-FLAG: M2, Sigma aldrich #F1804, 1/5000; anti-Caspase-4: Novus #NB12010454, 1/200; anti-HA: Invitrogen #26183, 1/1000; anti-Ubiquitin: Cell Signaling Technologies #3936, 1/1000; anti-GAPDH: GenTex #GTX100118, 1/10000), with agitation. The following day, the membrane was washed with TBST and incubated with the secondary antibody at RT for one hour (goat anti-mouse-HRP: Southern Biotech #1034-05, 1/10000; goat anti-rat-HRP: GE Healthcare #NA935V, 1/3000; goat anti-rabbit-HRP: Southern Biotech #4030-05, 1/10000), followed by TBST washing to remove unbound secondary antibodies. Finally, the membrane is visualized using ECL solutions and imaged with a BIO-RAD gel imaging system.

3.2.9 Bioinformatic Analysis

NCBI platform was used for sequence homology studies. To simulate the GSDMB-SlrP protein interaction, PDB database was used to obtain the three-dimensional structures of SlrP- Theodoxin (PDB 4PUF; Zouhir et al., 2014) and GSDMB- IpaH7.8 (PDB 8GTJ; Zhong et al., 2023). Using PyMol software, water molecules and structures other than amino acids were cleared and Gasdermin B and SlrP structures were obtained as monomers. Using the Haddock software, Gasdermin B and SlrP interaction was simulated (Honorato et al., 2021; van Zundert et al., 2016). Results obtained from this stimulation were analyzed by using PyMol, Chimera X and interaction residues were determined by using PDBsum.

3.2.10 Total RNA Isolation

Total RNA from Gasdermin B-FLAG transfected and FLAG transfected control HEK293FT cells were isolated using the Thermo Scientific GeneJET RNA Purification kit. After thawing the samples stored at -80°C in lysis buffer, they were centrifuged at ≥15000 rpm for 10 minutes at 4°C, 100 µl of Chloroform was added to the supernatant before incubating at room temperature for 2 minutes. A second

centrifugation of 15 minutes was followed by adding 500 µl of 70% Ethanol to 200 µl of the supernatant obtained this time before mixing. In the GeneJET RNA Purification Column inserted in a collection tube, 700 µl of the mix was transferred before centrifugating for 1 min at ≥8000 rpm speed at 4°C. The flow-through was discarded at this step and the samples were washed three times with 700 µl of wash buffer 1, 600µl of wash buffer 2, and 250 µl of wash buffer 3 separated by a 1-minute centrifugation at ≥15000 rpm after each wash buffer. The samples were then dried by an empty centrifugation for 1 minute at ≥15000 rpm. The elution of the samples was performed by adding 50 µl of nuclease free water, incubate for 1 minute at room temperature before centrifuging for 2 minutes at ≥15000 rpm to get the purified RNA samples. The RNA samples were then used for quantitative polymerase chain reaction.

3.2.11 Quantitative Polymerase Chain Reaction

Total RNA from Gasdermin B transfected, HEK293FT was utilized to perform qPCR using iTaq™ Universal SYBR® Green One-Step Kit and specific forward and reverse primers described below:

Name of the primer	**Sequence (5’ to 3’)**
CP11- GB qPCRForw	ATGTAGACTCAACGGGAGAGTT-
CP12- GB qPCRRev	GTAGCCAGATACTGCTGGGATA
CP13- GAPDH qPCRForw	ACAACTTTGGTATCGTGGAAGG
CP14- GAPDH qPCRRev	GCCATCACGCCACAGTTTC

3.2.12 Sequencing

Plasmids were sent to Macrogen Europe for sequencing. CMV-F (5’-CGCAAATGGGCGGTAGGCGTG-3’) and BGH-R (5’-TAGAAGGCACAGTCGAGG-3’) primers synthesized by Macrogen Europe were used. Sequencing results were analysed with Snapgene software.

3.2.13 Bacteria Gram Staining

SIGMA ALDRICH Millipore 77730-gram staining kit was used. Fresh SL1344 were fixed on a microscope slide, incubated with respectively Crystal Violet, Iodine Solution, decolored and then incubated with Safranin before being visualized under the microscope.

3.2.14 Bacterial Kinetic Analysis

SL1344 were incubated for 16 hours at 37°C with agitation in a streptomycin rich LB Miller Agar broth, then, the liquid culture was diluted (1:50). A microplate reader was used to measure the absorbance of the bacterial culture at 600 nm for 24 hours with a rate of 1 read per minute while incubated at 37°C with agitation.

3.2.15 Data Analysis

Graphics were generated and statistical analyses were performed using Prism GraphPad v10 software. A 2-tailed Student's t-test was performed.

Chapter 4

Results

4.1 *S*Tm Characterization

To identify bacterial innate immune system escape mechanism and to elucidate Gasdermin B's involvement in the detection and elimination of cytosolic bacteria within the non-canonical inflammasome, we decided to use *Salmonella enterica* Typhimurium SL1344 known to activate the non-canonical inflammasome in stationary phase from previous studies of our group and others and which is an important treat of human health. We obtained this strain and in order to assess its purity and characterize its growth, Gram staining and kinetic analysis of bacterial growth were performed (Figure 3).

Microscope analysis of Gram staining of a fresh bacterial *Salmonella enterica* Typhimurium SL1344 culture showed that all cells were pink, and rod-shaped proving that we had a pure *Salmonella* culture in our hands (Figure 3A). When kinetic assay was performed on SL1344 to elucidate and time the different growth stages of our strain, we found that the stationary phase of SL1344 starts when the optical density (OD) is 0,6 post 1:50 dilution (Figure 3B).

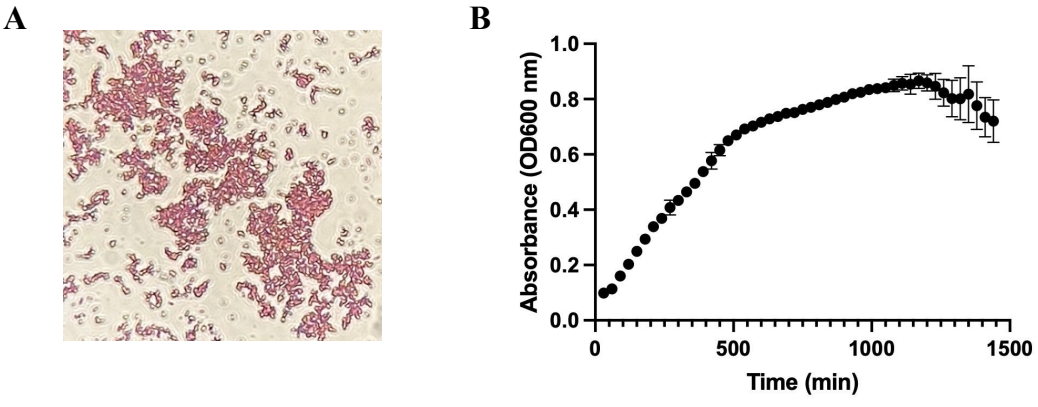

Figure 3. STm Characterization. ***A.*** *Representative picture of Salmonella Typhimurium SL1344 after Gram staining. Picture was taken with an inverted microscope with the 100X objective.* ***B.*** *Kinetic analysis of bacterial growth. OD at*

600 nm was measured every 30 minutes during 24 hours in three biological triplicates from a starting culture of 1/50 dilution of the overnight culture.

4.2 Expression of Gasdermin B

Since the endogenous Gasdermin B levels are very low and there is not any good quality antibody that recognizes Gasdermin B, pcDNA3-Gasdermin B-FLAG was obtained, and Gasdermin B-FLAG was ectopically expressed in human cells. To ensure that the pcDNA3-Gasdermin B-FLAG plasmid indeed contained wildtype human Gasdermin B and a FLAG tag, we sent the plasmid DNA to sequencing. The sequencing results showed the presence of full-length Gasdermin B, free of mutation and with a C-terminal FLAG tag (Figure 4A, Appendices A).

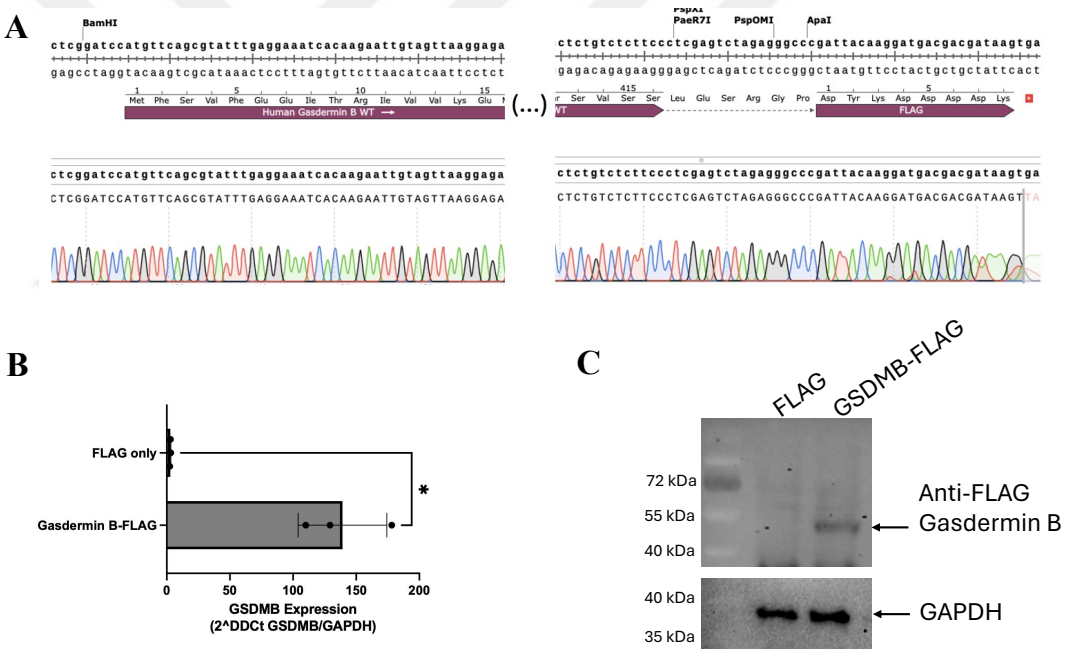

Figure 4. Expression of Gasdermin B. ***A.*** *Sequence of Gasdermin B-FLAG. The N-terminus and C-terminus of the cDNA are shown.* ***B.*** *qPCR analysis of Gasdermin B expression in HEK293FT cells transfected with 1 µg of pcDNA3-FLAG or pcDNA3-GSDMB-FLAG plasmids.* ***C.*** *Western blot analysis of Gasdermin B-FLAG protein by using anti-FLAG. GAPDH was used as a housekeeping gene.*

In order to validate the expression of the recombinant GSDMB protein, we have transfected HEK293FT cells with FLAG-tagged GSDMB plasmid and measured

GSDMB level both at the RNA and protein levels (Figure 4B, 4C). qPCR analysis revealed a statistically significant increase (approximately 140-fold) in GSDMB expression normalized to GAPDH in GSDMB-FLAG transfected cells compared to control cells transfected with only FLAG with a p-value of 0.02 (Figure 4B).
Similarly, Western blot analysis with anti-FLAG antibody showed the presence of recombinant GSDMB-FLAG protein at ~52 kDa in pcDNA3-Gasdermin B-FLAG transfected cells whereas it was absent in pcDNA3-FLAG transfected cells (Figure 4C, Appendices B).

Altogether, these results suggest that Gasdermin B was successfully expressed in HEK293FT cell lines with our transfection protocol.

4.2 Gasdermin B Directly Interacts with Caspase-4 in *S*Tm Infection

Since our aim is to identify the modulation of GSDMB during *S*Tm infection, we hypothesized that Gasdermin B modulator protein might interact with it directly or indirectly. We established a model of infection in HEK293FT cell lines by transfecting Gasdermin B and infecting transfected cells with late logarithmic-phase *S*Tm and immunoprecipitated Gasdermin B and its interaction partners (Figure 5A).

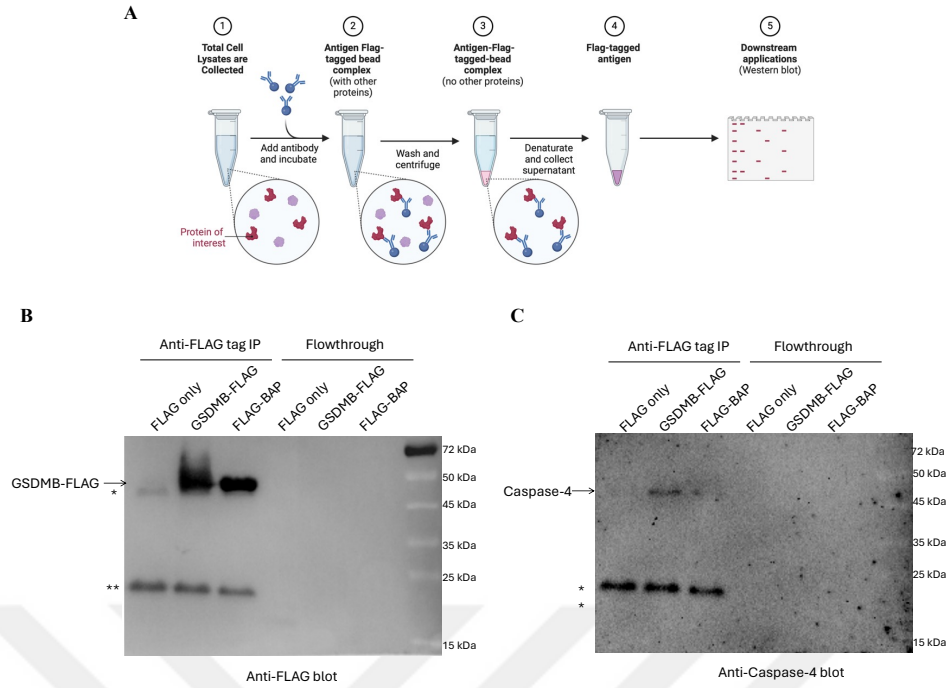

Figure 5. Immunoprecipitation of Gasdermin B. ***A****. Immunoprecipiattion protocol. HEK293FT cells were transfected with FLAG or GSDMB-FLAG and immunoprecipitated with anti-FLAG antibody.* ***B.*** *Anti-FLAG blot to visualize Gasdermin B-FLAG in the immunoprecipitation fraction (anti-FLAG tag IP) and fraction that did not bind to anti-FLAG (flowthrough).* ***C****. Anti-Caspase-4 blot to determine whether Gasdermin B interacts with Caspase-4. FLAG-BAP was used an internal control for immunoprecipitation. *heavy chains of the anti-FLAG antibody. **light chains of the anti-FLAG antibody.*

In a previous publication, Gasdermin B was found to interact with the LPS sensor Caspase-4 (Chen et al., 2019). To validate our model, we verified whether Gasdermin B interacts with Caspase-4 in our model. We could detect GSDMB at ~52 kDa demonstrating that Gasdermin B was successfully immunoprecipitated in GSDMB expressing cells (Figure 5B). Immunoprecipitation of GSDMB transfected HEK293FT cells with an MOI of 25 of *S*Tm SL1344 strain post IFN-γ priming resulted in a Caspase-4 band at ~50 kDa (Figure 5C). Taken together, these results validated our transfection, infection and immunoprecipitation protocols and confirmed that GSDMB interacts with Caspase-4 during *S*Tm infection.

4.3 Gasdermin B is Ubiquitinated during *S*Tm Infection

To directly address our general hypothesis; whether or not GSDMB is ubiquitinated during *S*Tm infection, we have co-transfected HEK93FT cells with GSDMB-FLAG and HA-Ubiquitin. 24 hours post-transfection, the cells were infected with a MOI of 25 of SL1344 *S*Tm and GSDMB was immunoprecipitated four hours after infection.

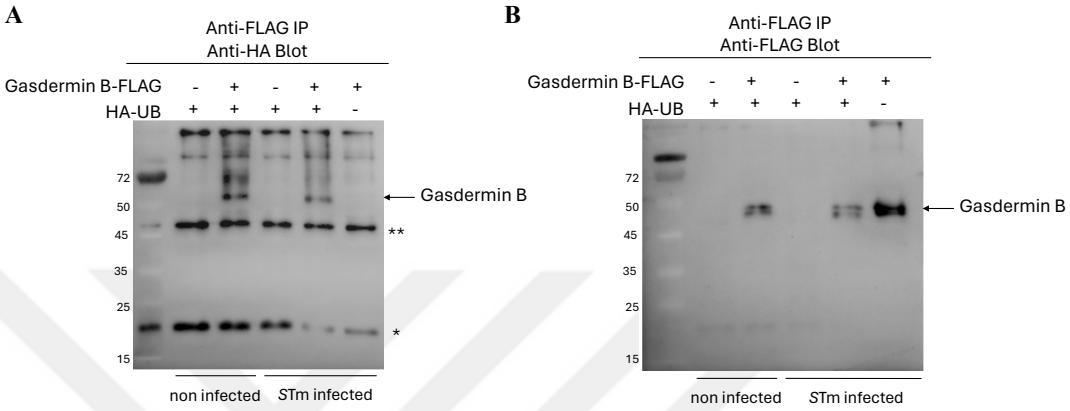

Figure 6. Ubiquitination of Gasdermin B during STm infection. HEK293FT cells transfected with HA-Ubiquitin, FLAG-Gasdermin B were infected with STm and immunoprecipitated with anti-FLAG antibody. ***A.*** *Revelation with anti-HA antibody.* ***B.*** *Revelation with anti-FLAG antibody.*

We could detect that GSDMB is ubiquitinated (at ~63 kDa) in *S*Tm infected cells and in non-infected cells after incubating the membrane at anti-HA (Figure 6A). Given that ubiquitin has a molecular weight of 8.5 kDa and ubiquitinated proteins are indeed expected to have a larger molecular weight compared to the non-modified form, it is safe to conclude that the increase in the molecular weight of GSDMB when ubiquitinated is normal. To ensure that the ubiquitinated protein is indeed the FLAG-tagged GSDMB, we have revealed with an anti-FLAG antibody (Figure 6B).

4.4 Bioinformatic Analysis of *S*Tm Ubiquitin Ligases

Since Gasdermin B was ubiquitinated, we aimed to determine the bacterial ubiquitin responsible for this post-translational modification. Literature research revealed that *S*Tm contains five different ubiquitin ligases: SlrP (Bernal-Bayard &Ramos- Morales

2007), SspH1 (Rohde et al., 2009), SspH2 (Quezada et al., 2009), SspH3 (Herod et al., 2022) and SopA (Zhang et al., 2006, Diao et al., 2008) ubiquitin ligases. Each ubiquitin ligase is secreted by different secretion system and have different target host proteins (Table 11). To this day, none of these ubiquitin ligases were shown to modulate any Gasdermin proteins including Gasdermin B.

Table 11

Salmonella Typhimurium's Ubiquitin Ligases

Candidate Ubiquitin Ligase	**Structural Protein Family**	**Secretor T3SS**	**Target host protein**	**References**
SlrP	Novel T3 Ligase	SPI-1 and SPI-2	Thioredoxin	(Bernal-Bayard &Ramos- Morales 2007)
SspH1	Novel T3 Ligase	SPI-2	PKN1	(Rohde et al., 2009)
SspH2	Novel T3 Ligase	SPI-2	Nod1, STG	(Quezada et al., 2009)
SspH3	Novel T3 Ligase	Unknown	Unknown	(Herod et al., 2022)
SopA	HECT	SPI-1	Unknown	(Zhang et al., 2006, Diao et al., 2008)

In order to identify which of this/these ubiquitin ligase(s) could ubiquitinate Gasdermin B during infection, we analyzed the structure of these enzymes. Since Gasdermin B was previously demonstrated to be ubiquitinated by Shigella flexneri's IpaH7.8 enzyme (Hansen et al., 2021), we hypothesized that *S*Tm's ubiquitin ligase that targets Gasdermin B might have a structural homology with Shigella's IpaH7.8. thus, we conducted a bioinformatic analysis and compared the protein sequence of *S*Tm ubiquitin ligases with the structure of IpaH7.8 (Figure 7). Among the other ubiquitin ligases, SlrP had the highest structural homology with IpaH7.8 (40,24%,

Figure 7). Based on these results, we proposed that SlrP might ubiquitinate Gasdermin B during *Salmonella* infection to escape immune clearance.

A

Candidate Ubiquitin Ligase	NCBI Accession code	Percent Identity to IpaH7.8	E-value
SlrP	WP_212634585.1	40.24%	2e-109
SspH1	WP_000481981	39.32%	5e-128
SspH2	WP_023228915.1	43.76%	3e-127
SspH3	WP_250292904.1	41.36%	1e-125
SopA	NP_461011.1	N/A	N/A

B

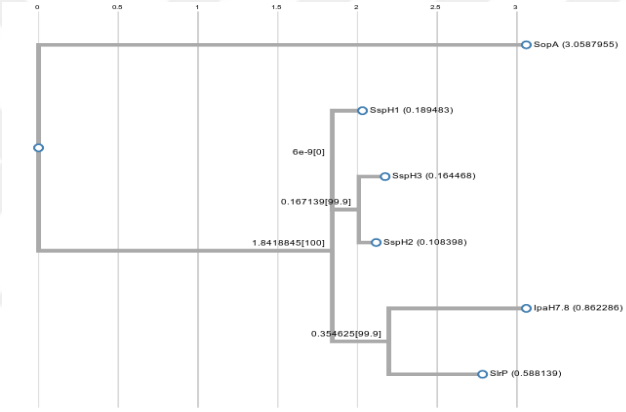

Figure 7. Identification of Salmonella ubiquitin ligases. ***A.*** *Results of protein sequence identity analysis of different ubiquitin ligases.* ***B.*** *Phylogenetic tree of STm ubiquitin ligases and Shigella flexneri's IpaH7.8 ubiquitin ligase.*

4.5 Bioinformatic Analysis of Gasdermin B and SlrP Interaction

Since SlrP was the most promising candidate for Gasdermin B modulation, we aimed to detemine whether these two proteins could interact with each other bioinformatically. The three-dimensional structures of Gasdermin B-IpaH7.8 LRR and SlrP-Thioredoxin were downloaded from the PDB database. Using the PyMol

program, water molecules and non-amino acid structures were removed from these structures, and the Gasdermin B and SlrP structures were obtained as monomers. The interaction between Gasdermin B and SlrP was simulated using the Haddock program. The obtained data were analysed using PyMol and Chimera X programs, and interaction regions were identified with the PDBsum program (Figure 8).

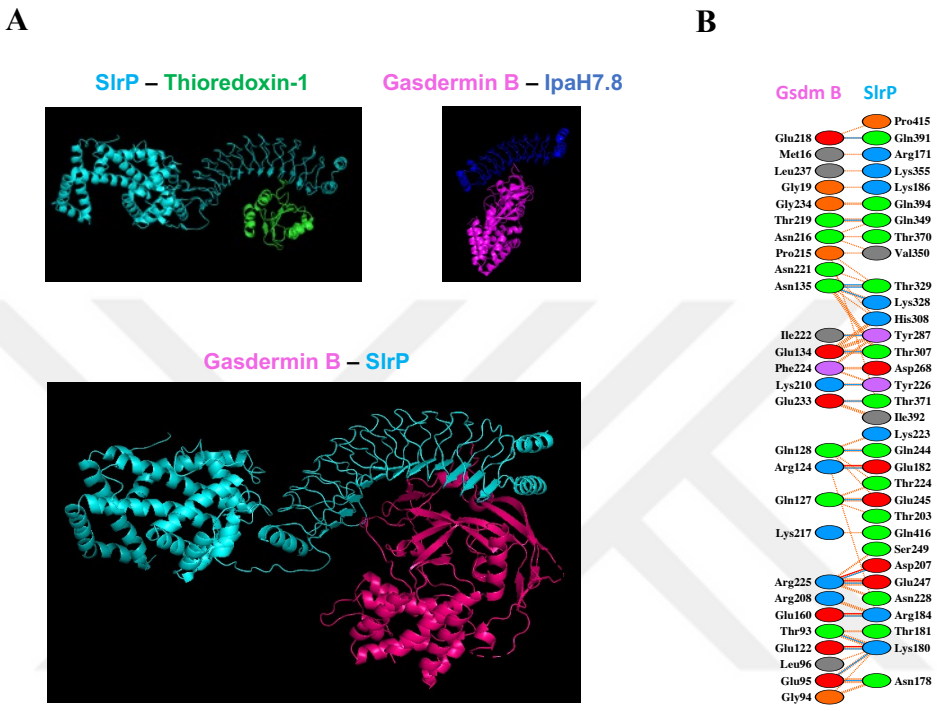

Figure 8. Bioinformatic analysis of Gasdermin B and SlrP interaction. ***A.*** *Representation of Gasdermin B and SlrP docking. Docking of Gasdermin B and SlrP resulted in 39 models of interaction. One of these models is represented.* ***B.*** *Amino acids of Gasdermin B and SlrP interacting with each other.*

The molecular docking of SlrP and Gasdermin B suggests that SlrP and Gasdermin B could interact with each other in 39 different ways (Figure 8). In all these models, Gasdermin B interacted with SlrP's LRR domain (Figure 8A). Gasdermin B and SlrP interacted with each other through 27 amino acids in Gasdermin B and 33 amino acids

of SlrP (Figure 8B). These bioinformatic analysis suggest that Gasdermin B and SlrP might interact with each other.

To validate this interaction in wet laboratory, we aimed to immunoprecipitated Gasdermin B and SlrP. However, since they are not antibodies against both proteins and they are both were FLAG-tagged, we needed to clone on one of the proteins into a vector containing another tag. However, due to time limitation and because our first attempt was not successful, we could not obtain that construct and validate our results by co-immunoprecipitation.

4.6 SlrP Sequencing and Cellular Expression

Since SlrP was the strongest candidate to modulate Gasdermin B, we obtained the plasmid coding SlrP and verified its sequence (Figure 9).

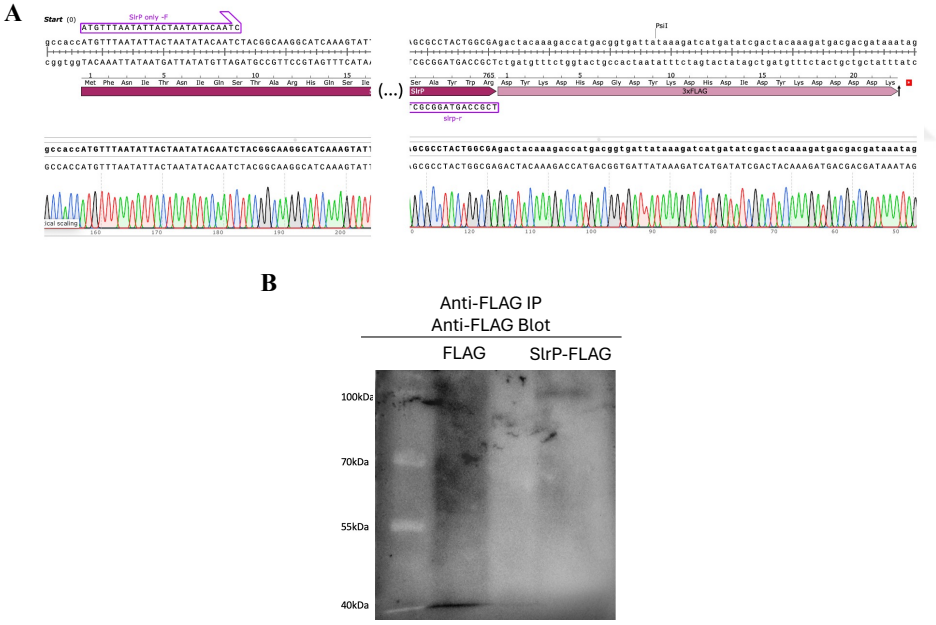

Figure 9. SlrP expression. ***A.*** *SlrP plasmid was sequenced and revealed a WT SlrP with a C-terminal 3X FLAG tag.* ***B.*** *SlrP was slightly expressed after anti-FLAG immunoprecipitation.*

The sequencing results confirmed that the plasmid contained SL1344's wildtype SlrP cDNA with a C-terminal 3X Flag-tag (Figure 9A). To confirm SlrP protein expression,

cells were transfected and analysed with Western blot. However, SlrP could not be detected (not shown). To concentrate the proteins, cell lysates were immunoprecipitated with anti-FLAG and revealed with FLAG antibody. Although of poor quality, a band of ~100kDa was visualized in SlrP transfected cells, which was in accordance with the predicted size of SlrP (Figure 9B).

4.7 GSDMB is Ubiquitinated of in SlrP Overexpressing Cells

To investigate whether SlrP is the bacterial ubiquitin ligase responsible for GSDMB ubiquitination post-*S*Tm infection, we have co-transfected HEK293FT with SlrP-Flag and GSDMB-Flag and primed the cells with IFN- γ. Cells were infected 24 hours post-transfection with an MOI of 25 of *S*Tm and the proteosome inhibitor MG132 was added to prevent the possible degradation of the ubiquitinated GSDMB (Figure 10).

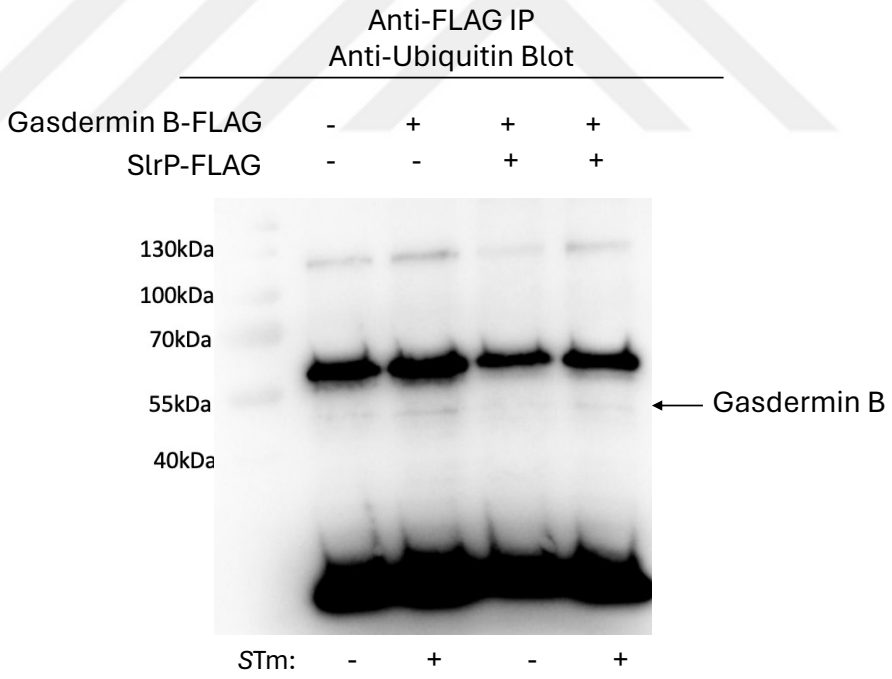

Figure 10. GSDMB is Ubiquitinated in SlrP Overexpressing Cells. HEK293FT cells were transfected with GSDMB and SlrP and treated with IFNg. Infection was performed when indicated with MOI 25 of STm and 1 μM MG132 was added for 4 hours to prevent proteasomal degradation.

A faint ubiquitinated Gasdermin B band was observed in all conditions (Figure 10). However, the ubiquitination levels were different between each condition. There was a noticeable increase in the ubiquitinated GSDMB levels in *S*Tm infected samples compared to the non-infected controls. These results suggest that SlrP might be the ubiquitin ligase that triggers Gasdemin B ubiquitination during *Salmonella* infection.

4.8 GSDMB Expression in SlrP Overexpressing Cells

To determine whether the overexpression of SlrP affects the cellular expression of GSDMB on a protein-level, we have co-transfected HEK93FT cells with a similar amount of GSDMB-FLAG plasmid and a gradually increasing amount of SlrP-FLAG plasmid, immunoprecipitated the Flag-tagged proteins (Figure 11).

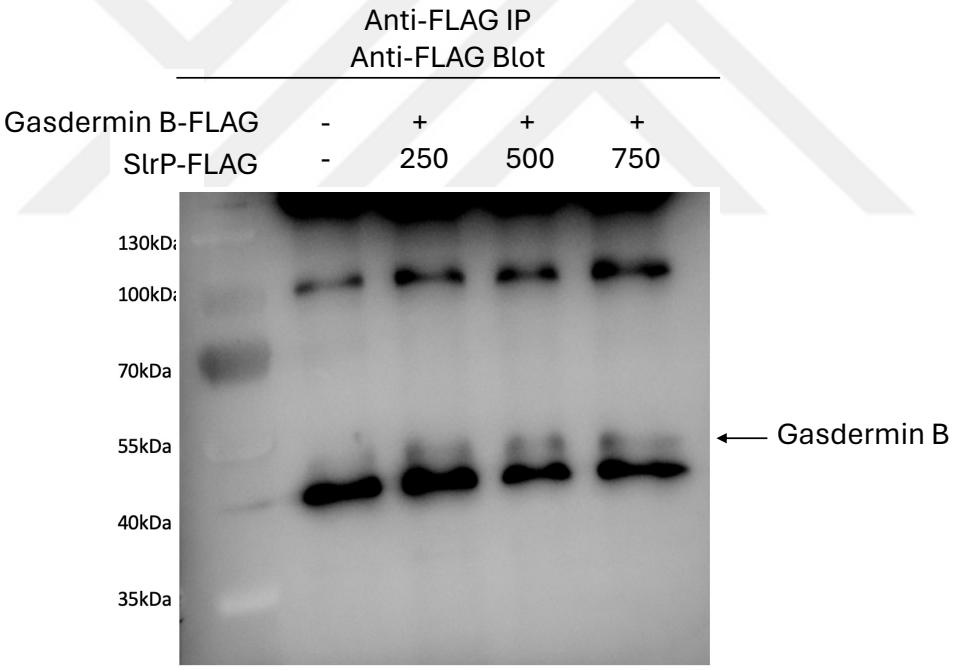

Figure 11. GSDMB expression in SlrP overexpressing cells. HEK293FT cells were co-transfected with 1µg Gasdermin B and increasing concentration of SlrP (250, 500 and 750 ng). Samples were immunoprecipitated and visualized with anti-FLAG antibody. The Gasdermin B band is shown.

Gasdermin B levels were the same for different concentrations of SlrP (Figure 11). Under these conditions, SlrP does not seem to affect Gasdermin B protein's expression. A control with Gasdermin B transfected cels without SlrP is needed to firmly affirm that SlrP does not have a role in Gasdermin B degradation.

4.9 Gasdermin B Killing Activity on Intracellular *S*Tm

Since Hansen et al., 2021, has proved the capability of GSDMB to bind and kill *Shigella flexneri* directly, we sought to investigate GSDMB to directly kill *S*Tm. To verify this hypothesis, we have transfected HEK293FT with either GSDMB-Flag or FLAG-only plasmids and 24 hours after transfection, we infected both conditions with *S*Tm with an MOI of 25, harvested the intracellular bacteria and seeded them in agar plates to quantify the number of viable *S*Tm recovered from these cells through CFU assay (Figure 12).

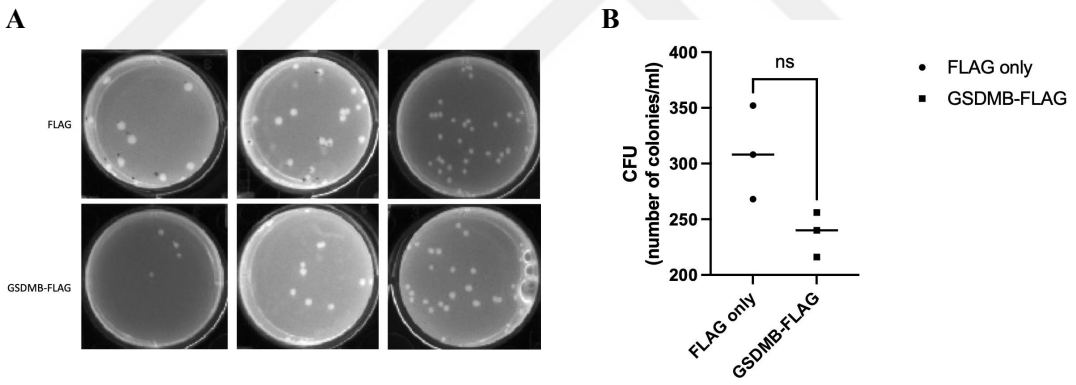

Figure 12. CFU assay of STm recovered from GSDMB or FLAG transfected cells. ***A.*** *Picture of the plates.* ***B.*** *Quantification of results.*

While approximatively 300 colonies/ml were recovered from FLAG transfected cells, only 250 colonies/ml were obtained from Gasdermin B transfected cells. Although the difference was not statistically different, the expression of Gasdermin B seemed to decrease the viability of intracellular *Salmonella* Typhimurium (Figure 12). According to these results, Gasdermin B might have a killing activity on *S*Tm. Further confirmation is required.

Chapter 5

Discussion and Conclusions

Our research aimed to elucidate the ubiquitination of Gasdermin B (GSDMB) during *Salmonella* Typhimurium (STm) infection and identify the specific bacterial ubiquitin ligase responsible for this post-translational modification. The ubiquitination of GSDMB was successfully demonstrated through co-transfection experiments using Flag-tagged GSDMB and HA-tagged ubiquitin (Figure 6). Immunoprecipitation assays confirmed the presence of ubiquitinated GSDMB (at ~63 kDa) in both infected and non-infected cells, aligning with the expected molecular weight increase due to ubiquitination. These results corroborate previous findings on the ubiquitination of other Gasdermin family members and reinforce the role of ubiquitination in modulating GSDMB activity during bacterial infection.

Through literature search and bioinformatic analyses, we identified SlrP, a type III ubiquitin ligase from *S*Tm, as a potential candidate responsible for GSDMB ubiquitination. SlrP exhibits significant homology with *Shigella flexneri*'s IpaH7.8, which has been shown to ubiquitinate GSDMB (Table 11, Figure 7). This suggests a conserved mechanism of immune evasion among different Gram-negative bacteria, highlighting the evolutionary pressure on these pathogens to develop sophisticated strategies to modulate host immune responses.

The similarity between the ubiquitination mechanisms in *S*Tm and *Shigella flexneri* underscores the importance of GSDMB as a target for bacterial immune evasion. The identification of SlrP as a functional analog to IpaH7.8 in *S*Tm supports the hypothesis that ubiquitination of GSDMB is a common strategy employed by different pathogens to suppress host immune defenses. Bioinformatical analysis of SlrP and Gasdermin B interaction revealed that Gasdermin B might interact with LRR domain of SlrP (Figure 8). However, because both constructs were FLAG tagged and our cloning was unsuccessful, we could not prove this interaction *in vitro* by co-immunoprecipitation.

Nonetheless, co-expression of GSDMB and SlrP showed ubiquitination of Gasdermin B (Figure 10). Although the bands were faint, ubiquitination of Gasdermin B was visualized in conditions where Gasdermin B was expressed alone, together with SlrP or with *S*Tm infection. However, ubiquitination levels were more elevated in the case of *S*Tm infection and SlrP overexpression suggesting that Gasdermin B could be ubiquitinated even in non-infected cells by endogenous ubiquitin ligases but this ubiquitination level increases during *S*Tm infection underlying the presence of bacterial ubiquitin ligases that modulate Gasdermin B. To clarify whether Gasdermin B is directly ubiquitinated by SlrP, co-incubation of SlrP with Gasdermin B is required in further investigations.

To determine whether the ubiquitination observed changes Gasdermin B stability, Gasdermin B was incubated with increasing doses of SlrP (Figure 11). In all conditions, same level of Gasdermin B protein was observed. However, since the SlrP expression is very low in our experimental setting (Figure 9) and in the absence of Gasdermin B protein without SlrP, we could not conclude whether SlrP triggers Gasdermin B degradation. SlrP expression could not be confirmed with a sharp band suggesting that either the antibody is not of good quality or there is another cellular mechanism limiting SlrP overexpression. Another technical limitation was the detection of Gasdermin B that had a size similar to the heavy chain of antibodies. Gasdermin B could not be detected without immunoprecipitation and after immunoprecipitation, the heavy chains of the antibodies appeared. We propose to use gradient gels to separate Gasdemin B from the heavy chain, refresh all antibodies that were used and choose a new elution method after immunoprecipitation to elute only the FLAG-proteins and their interactors without releasing the antibodies from the beads.

Ubiquitination may also alter GSDMB's function rather than its stability. In that case, the exact functional consequences of GSDMB ubiquitination remain to be fully elucidated, but it is plausible that this modification could either inhibit its pore-forming activity or alter its interaction with other cellular proteins, thereby modulating the host immune response.

It is worthy to note that our Gasdermin B plasmid encodes for the isoform 1, meaning that the outcome of this study is mainly specific the isoform 1 of Gasdermin B, whether other Gasdermin B isoforms are ubiquitinated upon *S*Tm infection needs to be further investigated. In addition to that, we have faced multiple challenges in optimizing the western blotting procedure, we have encountered multiple issues with the PDVF membrane and most of the antibodies that were in hand were old and thus not as effective as it should have been.

These results suggest that Gasdermin B is ubiquitinated during STm infection and SlrP might be the ubiquitin ligase responsible for this post-translational modification.

Finally, we investigated whether overexpression of Gasdermin B could kill bacteria directly or indirectly. When colony forming units were quantified from intracellular bacteria from FLAG only or Gasdermin B-FLAG expressing cells, we observed a tendency of decrease in living bacteria in cells overexpressing Gasdermin B (Figure 12). These results suggest that Gasdermin B may have a role in bacteria killing either directly by binding to the bacterial membranes and lysing them or indirectly through the activation of the non-canonical inflammasome components including GBP proteins. Since our experiments were performed in HEK293FT cells that do not express many inflammasome component, the first mechanism is plausible. The experimental system we used can also explain why the difference is not significance because Gasdermin B may need other components to be addressed to the bacterial membrane and/or to lyse them. Further experiments are needed to clarify the role of Gasdermin B is the non-canonical inflammasome, determine whether it kills intracellular bacteria and prove that it is modulated by bacterial enzymes.

Overall, this thesis advances our understanding of the molecular interactions between *S*Tm and the host immune system, specifically through the ubiquitination of GSDMB. Our key findings include: (1) demonstration of GSDMB ubiquitination in response to *S*Tm infection and (2) identification of SlrP as the *S*Tm ubiquitin ligase responsible for GSDMB ubiquitination.

This study is the first work in our knowledge to show possible GSDMB ubiquitination by *S*Tm's SlrP ubiquitin ligase. SlrP was previously shown to have a role in NLRP3 inhibition during anorexia (Rao et al., 2017; O'Neill, 2017). However, no evidence on SlrP-mediated regulation of GSDMB, nor the non-canonical Caspase-4 inflammasome was reported to date.

These findings provide a foundation for further research into the role of GSDMB in bacterial infections and its potential as a therapeutic target for managing inflammation and sepsis caused by *Salmonella enterica*. Future studies should focus on elucidating the precise functional consequences of GSDMB ubiquitination and exploring potential inhibitors that could prevent bacterial modulation of this critical immune protein. Detailed studies to explore how ubiquitination affects GSDMB's pore-forming activity and its interactions with other proteins are essential. Investigating potential inhibitors of SlrP-mediated ubiquitination could be a novel approach to boost host defenses against *S*Tm. Additionally, extending the research to other Gram-negative pathogens will help understand the broader implications of GSDMB ubiquitination in bacterial immune evasion strategies.

In conclusion, our study sheds light on a novel immune evasion strategy employed by *Salmonella* Typhimurium, providing significant insights into the role of GSDMB in bacterial infections and proposing new avenues for therapeutic interventions. By understanding these molecular interactions, we can develop innovative treatments to manage bacterial infections, inflammation, and sepsis more effectively.

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