Study of the Activation of the Inflammasome Protein Complex by *Haemophilus influenzae* Type a

A thesis presented to

The Faculty of Graduate Studies

of

Lakehead University

by

SIMRUN CHAHAL

In partial fulfillment of requirements
for the degree of
Master of Science in Biology

June 18, 2021

Abstract

Haemophilus influenzae (Hi) is a gram-negative bacterium capable of causing severe invasive infection such as meningitis and is classified by the presence or absence of polysaccharide capsule. The polysaccharide capsule is the most important virulence factor for encapsulated strains. Hi serotype a (Hia) is an encapsulated form of Hi that is recognized as an important emerging pathogen with incidence of invasive disease similar to Hi serotype b (Hib) prior to the introduction of the Hib vaccination. Despite this, not much is known about how Hia interacts with the innate immune system and how it induces an inflammatory response. Non-typeable Hi (NTHi) (the unencapsulated form of Hi) has recently been shown to activate the innate immune system through a multiprotein complex known as inflammasome. As such, this study aimed to investigate if Hia activates this innate immune complex and how this differs in encapsulated versus unencapsulated strains and invasive versus non-invasive strains.

THP-1 macrophages, pretreated in the presence or absence of caspase-1 inhibitor ac-YVAD-cmk, were stimulated with either the invasive encapsulated Hia strain 08-191, the mutant invasive unencapsulated Hia strain 13-0074, or the non-invasive NTHi strain 375 for one hour. Bacteria were then inactivated with gentamicin prior to incubation for an additional 17 hours. Supernatant was then collected and frozen while macrophages were harvested. Flow cytometry of harvested macrophages was used to measure THP-1 cell viability and ICAM-1 surface expression. THP-1 supernatant was thawed when three technical replicates were obtained and concentrations of both IL-1β and TNFα were measured via ELISA. It was found that both Hia strains and the NTHi strain induced a caspase-1 dependent increase in IL-1β secretion suggesting activation of inflammasome.

However, there were no differences among encapsulated and unencapsulated strains nor among invasive and non-invasive strains in any parameter measured in experiments using THP-1 cells pretreated with ac-YVAD-cmk or vehicle. This lack of differences may be due to the strains upregulating inflammasome-related gene products at similar levels as a result of homogeneity of Hi components between strains and the large number of redundancies within the innate immune system.

The role of viable Hi in activation of inflammasome was also analyzed in a similar method. Bacterial strains were heat inactivated at 65ºC for 30 minutes prior to stimulation of THP-1 macrophages. Cells stimulated with heat inactivated bacteria resulted in significantly decreased secretion of both IL-1β and TNFα in proportion to cells stimulated with viable bacteria. Stimulation with heat inactivated encapsulated Hia resulted in a proportionally greater release of both cytokines compared to both unencapsulated strains. As such, heat inactivation affects overall immunostimulatory activity of bacteria which is slightly mitigated by a polysaccharide capsule. This may be due to the capsule providing some form of thermostability; however, further study is required to fully understand the interaction.

Though this study provided evidence for inflammasome activation in Hia infection, it is only the first step. Future studies should look at roles of specific inflammasomes (and their components) in Hia infection, how other innate immune cells such as neutrophils or dendritic cells react to Hia, as well as investigation of these mechanisms in an *in vivo* model. Hia is an important pathogen and continued study is needed to better our understanding of how the innate immune response is involved in the pathogenesis of diseases caused by it.

Lay Summary

The mission statement of Lakehead University Department of Biology is: "Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms". This project aims to add to our understanding of biology through the study of the bacterium *Haemophilus influenzae* serotype a (Hia). This is a bacterium that poses a significant problem to those in Northern Canada, specifically the Indigenous population. Diseases associated with it include meningitis, pneumonia, and septic arthritis. Not much is understood of how specifically Hia interacts with the innate immune system, our frontline defense against pathogens. This study suggests that Hia activates the innate immune system through a multiprotein complex known as inflammasome and that viable *H. influenzae* may activate that protein complex and non-viable bacteria may activate it to a reduced effect.

Table of Contents

List of Figures

Abbreviations

ac-YVAD-cmk	N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone
AIM2	Absent in melanoma 2
ANOVA	Analysis of variance
ASC	Apoptosis-associated speck-like protein containing a CARD
BHI	Brain heart infusion
Ca^{2+}	Calcium ion
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
CLR	C-type lectin receptors
DAMP	Damage associated molecular pattern
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
Hi	*Haemophilus influenzae*
Hia	*Haemophilus influenzae* serotype a
Hib	*Haemophilus influenzae* serotype b
Hic	*Haemophilus influenzae* serotype c
Hid	*Haemophilus influenzae* serotype d
Hie	*Haemophilus influenzae* serotype e
Hif	*Haemophilus influenzae* serotype f
HMW	High molecular weight
Hsf	*Haemophilus surface* fibrils
ICAM-1	Intercellular adhesion molecule 1
IgA	Immunoglobulin A
IL-18	Interleukin-18
IL-1R	Interleukin-1 receptor
IL-1β	Interleukin-1 beta
IRAK4	IL-1 receptor associated kinase 4
IRF-3	Interferon regulatory factor 3

IκB	Inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells
IκK	IκB kinase
K+	Potassium ion
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response 88
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain
NTHi	Non-typeable *Haemophilus influenzae*
OD	Optical density
PAF	Platelet activating factor
PAMP	Pattern associated molecular pattern
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
RLR	Retinoic acid inducible gene-I-like receptors
ROS	Reactive oxygen species
TLR	Toll-like receptor
TNFR	TNFα receptor

TNFα	Tumor necrosis factor alpha
TRADD	TNFR type 1-associated death domain protein

Acknowledgements

Firstly, I would like to give my sincerest thanks to my supervisor Dr. Marina Ulanova for her vast knowledge, patience, kindness and most importantly, for allowing me the opportunity to work on such an interesting project with an amazing group of lab members. Working with her has allowed me to learn so much not only about immunology, but also about all aspects of life. I truly appreciate the opportunity that she has provided me.

Secondly, I would like to thank my committee members Drs. Neelam Khaper and Kam Leung. They have given me an immense amount of support and I appreciate all of their insightful comments, questions, and feedback throughout this process. I would also like to thank my external examiner, Dr. Aseem Kumar, for dedicating his time to my thesis and providing meaningful feedback.

Thirdly, I would like to thank all current and previous laboratory members at the Northern Ontario School of Medicine (NOSM) that I have had the opportunity to work with over the last three years for their help and friendship. I would like to especially thank Dr. Gabrielle Gaultier and Brenda Huska for their mentorship throughout my time at NOSM. I also gratefully acknowledge the Lakehead University Queen Elizabeth II Graduate Scholarship in Science and Technology, and the Doris K. Adams Memorial Award of Merit for their support as well as the the Faculty of Graduate Studies, and the Department of Biology for funding. Funding was also provided by the NSERC Discovery Grant.

Finally, I would also like to thank all of my friends and family for all of the love and support they have given me on this journey. I truly appreciate each and every one of you.

1 - Literature Review

1.1 - *Haemophilus influenzae* is an Important Human Pathogen

1.1.1 - *Haemophilus influenzae*: Disease and Epidemiology

Haemophilus influenzae (Hi) is a gram-negative coccobacillus in the *Pasteurellaceae* family (Kuhnert and Christensen 2008). The bacteria are characterized by the presence or absence of a polysaccharide capsule. Those species that possess the polysaccharide capsule are referred to as encapsulated and exist as one of six serotypes, a through f (i.e., Hia, Hib, Hic, Hid, Hie, and Hif), while those that lack the capsule are referred to as non-typeable (NTHi) (Moxon and Kroll 1990). Of these strains, Hib was the dominant strain in terms of invasive Hi disease and was one of the leading causes of childhood meningitis worldwide until a vaccine was introduced in the 1980s (Ulanova and Tsang 2014). After the introduction of the vaccine, the rate of Hib invasive disease decreased up to 99%, but disease caused by non-b serotypes increased both nationally and internationally (Ulanova and Tsang 2009). In the post-Hib-vaccine era, infection rates between Hi types are not consistent worldwide. In European countries, NTHi followed by Hif are the dominant cause of infections, however, in North America, NTHi followed by Hia are the most prevalent infections (Whittaker et al. 2017; Ulanova and Tsang 2014). Even among different populations in these continents there are differences in reported incidence of types of Hi infections. This is especially true for Indigenous populations in North America. Specifically, Hia has already been recognized as a pathogen capable of replacing Hib as a major cause of invasive disease in Indigenous populations (Tsang and Ulanova 2017).

Infections by Hi can manifest as either invasive diseases (such as meningitis or septicemia) or non-invasive disease (such as otitis media) (Ulanova and Tsang 2014; Rotondo et al. 2013). Invasive Hia disease presentation is quite similar to Hib in that it includes meningitis, pneumonia, septic arthritis, osteomyelitis, bacteremia, and epiglottitis (Tsang and Ulanova 2017). As a common bacterium within the respiratory tract, NTHi is commonly associated with exacerbations of both chronic obstructive pulmonary disease and cystic fibrosis (Clementi and Murphy 2011). Nonetheless, NTHi is also one of the leading causes of otitis media and has been associated with sinusitis and community-acquired pneumonia (Erwin and Smith 2007, Clementi and Murphy 2011).

1.1.2 - Major Virulence Factors

The pathogenicity of the Hi bacteria is mediated by the presence of specific virulence factors. The plethora of virulence factors of Hi can aid in adherence to host epithelia, invasion, survival, as well as evasion of the host immune system. Of these, some of the most important virulence factors of Hi are the presence of polysaccharide capsule and the lipooligosaccharide (LOS).

1.1.2.1 - Polysaccharide Capsule

As mentioned above, the polysaccharide capsule is a feature that defines encapsulated strains of Hi. This feature is also important in the evasion of the immune system by protecting the bacteria from physically interacting with the host immune system. The structure of the capsule allows for it to be both hydrophilic and negatively charged. These characteristics make it difficult for phagocytes to interact with

encapsulated Hi due to disruptions in surface tension as well as mutual repulsion due to similar charges of the cell membrane and capsule. Due to the difficult interaction between phagocytes and the capsule, phagocytosis of encapsulated bacteria is very inefficient (Moxon and Kroll 1990).

Along with phagocytes, the complement system is a crucial component of the innate immune system. Capsular polysaccharides of some bacterial species such as *Streptococcus pneumoniae* have been shown to provide a mechanical barrier to complement protein interactions. Specifically, C3b bound to the bacterial cell wall underneath the bacterial capsule is unable to be recognized by phagocytes. This would interfere with C5-C9 membrane attack complex formation, and block C3b-mediated lymphokine production (Moxon and Kroll 1990). Encapsulated forms of Hi have also been shown to bind Factor H, a complement regulatory protein. The binding of this regulatory protein inhibits activation of the alternative pathway of the complement system and promotes serum resistance (Hallström et al. 2008)

Adaptive immune responses to the capsule are mediated by T cell-independent pathways as the capsule is not a protein and thus, not able to be displayed on major histocompatibility complex (MHC) (receptors responsible for communication with T-cells). Therefore, the B-cell and innate immune responses are essential in battling encapsulated Hi infections. However, due to the capsule's structure of repeating sugars, only high avidity antibodies are capable of binding to bacterial capsule (Weintraub 2003). The spleen is the main contributor to the anti-capsular polysaccharide humoral immune response, specifically, a subpopulation of marginal zone B cells. However, this subpopulation does not mature in children until 2-5 years of age, thus making those

without the mature spleen subpopulations to be more susceptible to invasive disease caused by encapsulated forms of Hi (Ulanova and Tsang 2014). As most antibodies are unable to bind as effectively, the antibody-mediated activation of the complement is stunted (Moxon and Kroll 1990, Weintraub 2003). These mechanisms for complement evasion are necessary for survival of the bacteria in blood and invasive infection. Though the capsule is necessary for survival in the blood, different capsule types confer varying levels of protection to the bacteria. The Hib and Hia capsules are both made of ribitol components. This may allow for greater evasion of the immune system (Moxon and Kroll 1990).

To test the virulence associated with specific capsule types, experiments were conducted with mutant Hi strains that only differed in their expression of different polysaccharide capsules. In mice, Hib strains were shown to cause a greater degree of bacteremia and more cases of meningitis than other strains. Hia followed Hib as one of the more virulent strains through its ability to cause bacteremia and meningitis (Zwahlen et al 1989). Hib strains are believed to have the most virulent capsule due to the duplication of the *cap b* gene found in a majority (98%) of strains which confers additional virulence (Moxon 1992). Hia and Hib capsules are the most similar as they both have a five-carbon ribitol as their first functional group. This similarity may play a role in their increased virulence (Moxon and Kroll 1990).

1.1.2.2 - Lipooligosaccharide

LOS is a truncated form of lipopolysaccharide (LPS) that exists in other bacteria species (lacking repeating O-antigen and instead having a polyhexose chain) and is a very

important virulence determinant of bacterial pathogenesis in both unencapsulated and encapsulated strains of Hi. LOS displays a vast array of inter- and intra-strain heterogeneity depending on phase variation (on-off switching of genes to lose or gain specific structural elements), which functional group and moieties are attached to which heptose residue, and how many transferase enzymes act on other sugar residues (Schweda et al. 2007). Hi LOS has been shown to be a potent immunostimulatory molecule through its ability to illicit various innate immune responses such as the release of proinflammatory cytokines, and the upregulation of antigen-presenting molecules and proinflammatory molecules (Choi et al. 2014). As such, the LOS is a potent immunostimulatory molecule.

The LOS of Hi is essential in many steps of infection from adherence, to cell entry, to immune evasion. LOS can aid in adhesion interaction with the platelet activating factor (PAF). Phosphorylcholine of the LOS is able to interact with the PAF and mediate invasion into host cells via cytoskeletal rearrangement (formation of lamellipodia and filopodia that enfold adhered bacteria) (Swords et al. 2001). Activation of PAF can lead to a multifactorial host cell signal cascade. It is through this cascade that a G protein complex is activated which initiates a signal cascade to activate phosphatidylinositol-3-kinase (PI3K) (Swords et al. 2001). PI3K is involved in macropinocytosis which Hi can use to invade cells. Specifically, PI3K results in production of phosphatidylinositol (3,4,5)-trisphosphate patches in the cell membrane which are required to create the macropinosomes (the vesicle which will engulf large amounts of extracellular material) (Hoeller et al. 2013). Once within the cell by either of the aforementioned methods, Hi is protected from host defense mechanisms to a greater degree. It is also through the

binding of the PAF that NTHi is able to traffic past the blood-brain barrier and thus cause inflammation in the brain (Swords et al. 2001). LOS has also been shown to inhibit the movement of cilia in the respiratory tract and damage respiratory epithelium (Moxon and Wilson 1991). This damage may facilitate invasion (Moxon 1992).

LOS is one of the factors that provides mechanisms for host immune evasion. Specifically, LOS can be modified to mimic the host's cells through a variety of methods. One of these is the sialyation of the LOS. By incorporation of N-acetylneuraminic acid from the host, the LOS is able to present a host-like epitope and interfere with activation of innate and adaptive immune responses. Another mechanism for immune evasion is a shift to LOS containing digalactoside via phase-variation. Digalactoside does not appear to be expressed during carriage but after adherence. This digalactoside appears to play a similar role to LOS sialyation in that it allows the bacteria to mimic the host (Schweda et al. 2007).

1.1.2.3 - Other Virulence Factors

Though the polysaccharide capsule and LOS are some of the most important virulence factors, Hi has many more virulence factors that aid in adherence, invasion, and survival. One of these factors is the presence of high molecular weight (HMW) proteins HMW1 and HMW2. HMW1 and HMW2 are a pair of near identical proteins (80% identical) characterized by their high molecular weight (125 kDa and 120 kDa respectively) (St. Geme et al 1993). These proteins have been shown to interact with a variety of proteoglycans such as sialic acid. Though HMW1 and HMW2 are very similar in nature, they may have different affinities for different cells lines. This may indicate

that these proteins may be active in different steps of adhesion and colonization (St. Geme et al. 2002). NTHi strains lacking the HMW1 and HMW2 genes have shown significantly decreased ability to adhere to cells in the nasopharyngeal and respiratory tract. This combined with the fact that the HMW proteins are expressed in many isolates of NTHi indicate that they are important in adhesion of the bacteria to the host (St. Geme et al. 1993).

Hi adhesin plays a critical role in the adhesion of both non-typeable and encapsulated forms of Hi to the host (St. Geme 2002). Hi adhesin is in the autotransporter family of proteins. Unlike other members of this family, Hi adhesin undergoes little processing in its translocation and thus stays associated with the bacterial cell. This may play a role in increasing its adherence to epithelial cells (St. Geme and Cutter 2000). Adhesive activity of Hi adhesin is mediated by two homologous pockets located at the outer membrane translocator domain and at the N-terminus. These binding pockets appear to bind the same host cell receptors but at differing affinities. Though the primary pocket accounts for ~ 65-80% of the binding activity, the combination of both pockets appears to be essential for producing a stable interaction to avoid clearance methods of the host such as the mucociliary escalator and coughing (Laarmann et al. 2002).

Another virulence factor associated with adherence is *Haemophilus* surface fibrils (Hsf). These share significant similarity with the Hi adhesin autotransporter. As such, Hsf also has two binding domains of differing affinity. The different binding domains may work by binding to two receptors to increase avidity of the interaction of the Hi to the epithelial cells or the lower affinity domain may first bind to the epithelial cell and be replaced by the higher affinity domain. This rebinding of Hi to the epithelial cell by the

Hsf could act to move the Hi bacterium closer in a ratchet motion. Due to the homology between the binding domains of Hsf and Hi adhesin, it is predicted that they bind to the same receptors (most likely integrin) and thus encapsulated strains will compete with non-typeable strains in adherence to the host (Cotter et al 2005). Though Hsf plays a role in adherence of encapsulated strains to host epithelial cells, it may also play a role in host immune system evasion by binding components of the lytic pathway of the complement system and inhibiting them (Singh et al. 2015).

For Hi to successfully survive within a host, it needs to evade the host's immune responses against it. Many outer membrane proteins of Hi are associated with evasion. P2 is a porin that is heterogenous at both a DNA and protein level. This heterogenicity allows for evasion of antibodies and thus stunting of antibody-mediated complement activation (Hallström and Riesback 2010). The presence of another porin protein P6 leads to conformational changes in Hi outer membrane structure making it more resistant to membrane attack complex cytolytic activity (Murphy et al. 2006). Likewise, P5 may limit binding of immunoglobulin M and bind Factor H, thus playing a role in evasion of classical and alternative pathways of complement system (Rosadini et al. 2014).

Another virulence factor associated with host immune evasion is the immunoglobulin A (IgA) antibody which is a key component in the defense against pathogens in mucosal surfaces (Foxwell et al. 1998). As the respiratory tract contains these antibodies, Hi has developed a way to counter them through the use of IgA1 proteases. As the name suggests, these proteins cleave the IgA1 into fragments. Specifically, IgA1 proteases cleave the proline-rich hinge region of human IgA1. This separates the Fab and Fc regions of the antibody. The Fab region is responsible for

recognition of epitopes and will remain bound to the Hi. This has the ability to provide a "mask" for the Hi against the host. The Fc region interacts with various immune cells and, due to its separation from the Fab region, will have its effects muted (Spahich and St. Geme 2011). Not only do the proteases play a role in evasion of the host immune system, but they may also play a role in adhesion. The IgA1 proteases share a significant degree of homology to the Hap adhesion factor of Hi and may play a similar role to it (Foxwell et al. 1998). Two types of these IgA1 proteases exist based on the residue cleaved in the hinge region of IgA1 and are produced in different serotypes of Hi. Specifically, Hia, Hib, Hid, and Hif express Type 1 IgA1 proteases, Hic, and Hie express Type 2 IgA1 proteases, and NTHi strains are capable of expressing both types (Spahich and St. Geme 2011).

There is also an abundance of protein-related virulence factors that assist in the invasion of the host. Many of these proteins also play a role in adhesion such as HMW1 and 2, and the adhesin Hap which binds to fibronectin, collagen IV, and laminin and causes proteolytic damage to the extracellular matrix to facilitate invasion (Duell et al. 2016, Kenjale et al. 2009). Protein E is similar to Hap in that it causes damage to proteins in the extracellular matrix (e.g., vitronectin) to cause cytoskeletal rearrangement and facilitate entry into cells (Ikeda et al. 2015). Lastly, similar to LOS, Protein D, a glycerophosphodiester phosphodiesterase, is able to cause damage to cilia of host cells impairing clearance and facilitating entry into cells. Through its enzymatic activity, Protein D is also able to hydrolyze glycerophosphorylcholine from eukaryotic membrane-associated phospholipids in glycerol-3-phosphate and choline. This choline can be used in the production of LOS to aid in binding to PAF (Forsgren and Riesbeck 2008).

1.1.3 - Conclusions

As discussed in the above sections, Hi is an important human pathogen, with Hia presenting itself as an emerging pathogen in Indigenous populations. Once inside a host, it is able to use a plethora of different virulence factors to improve its survivability, adhesion, invasion into host cells, and evasion of the host immune system. Two of the major virulence factors are that of the polysaccharide capsule and the LOS which, when combined, play a major role in each of the aforementioned virulence functions. Understanding how Hi infections manifest is important for studying potential treatments and preventative measures. However, understanding how the host acts in response to the presence of Hi is also an important factor. As such, the following sections will discuss how the host responds to the Hi with the first line of defense, the innate immune system.

1.2 - Innate Immune System and Recognition of *Haemophilus influenzae*

1.2.1 - The Innate Immune System

The innate immune system is the body's first line of defense against invading organisms. It is equipped with a wide array of non-specific mechanisms to mount a quick response to pathogens and mitigate damage to the host. There are three major levels to the innate immune system: physical/chemical barriers, the complement system, and activation of innate immune cells. The physical and chemical barriers provide a method to avoid and resist infection by commensal organisms and pathogens. Examples of these include the presence of mucus to provide a physical barrier preventing interaction of pathogens with cells or the acidic environment of the stomach killing some organisms (Murphy and Weaver 5). The complement system is a tightly regulated network of

proteins present in the blood that, upon activation, causes a cascade resulting in the opsonization and lysis of pathogens. However, as mentioned above, Hi has a wide array of virulence factors (such as the presence of capsule or outer membrane proteins) that aid in the evasion of complement (Hollström and Riesbeck 2010). As such, one of the most important innate defenses against Hi infection is the cellular response.

The cellular arm of the innate immune system has many different types of cells with unique purposes. One of the mechanisms shared by many of these cells is the usage of pattern recognition receptors (PRRs) as a way to recognize different pathogen-associated molecular patterns (PAMPs) and produce the machinery needed to either deal with the pathogen or initiate a more specific adaptive immune response. The major types of these PRRs are the retinoic acid inducible gene-I-like receptors (RLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and the Toll-like receptors (TLRs). RLRs and CLRs are important facets of the innate response, but as they recognize intracellular viral RNA and lectins which are not found in Hi species, their response is not important for a host defense against Hi (Tako et al. 2011, Geijtenbeek and Gringhuis 2009). Nonetheless, TLRs and NLRs are incredibly important in the innate response to Hi.

TLRs are capable of recognizing extracellular and intracellular pathogens. This is due to the wide variety of TLRs that exist within cells. This variety not only allows for recognition of PAMPs in different cellular compartments, but also recognition of an array of different PAMPs. In mammals, there are 10 different TLRs, however not much is known about the ligands for TLR10 (Takeda et al. 2003, Murphy and Weaver 91). TLR5 is an extracellular TLR recognizing bacterial flagella (Takeda et al. 2003, Murphy and

Weaver 90). Though Hi is generally considered a non-flagellated bacterium, some forms of NTHi have recently been shown to express flagella (Carabarin-Lima et al 2017). As such, TLR5 may play a role in the host response in the limited amount of flagellated Hi strains but no studies have been conducted on Hi's activation of TLR5. TLR3, -7, and -8 are intracellular TLRs that recognize single and double stranded RNA (Takeda et al. 2003, Murphy and Weaver 91). Though these TLRs play a role in the host response to viruses, they may also play a limited role in Hi infections. In fact, TLR7 has been shown to be upregulated in response to Hi both *in vitro* and *in vivo* and Hi has been shown to activate TLR3, though the exact component of Hi responsible for TLR3 activation is not yet known (Sakai et al. 2007, Teng et al. 2010). Similar to TLR3, -7, and -8, TLR9 exists within the intracellular compartment, however, TLR9 recognizes unmethylated CpG dinucleotides commonly found in bacteria (Takeda et al. 2003, Murphy and Weaver 91). Interestingly, studies have shown that TLR9 is not involved in the response to Hi infection but TLR9 does recognize purified Hi DNA, therefore, Hi DNA may not be available in the cytosolic compartment in an appreciable amount to activate TLR9 during Hi infection (Wieland et al. 2010, Mogensen et al. 2006).TLR1, -2, and -6 are extracellular receptors that form dimers among themselves and allow for recognition of many PAMPs such as peptidoglycan (a component of bacteria walls) and various lipopeptides (Takeda et al. 2003, Murphy and Weaver 90). Specifically, it has been found that porins of Hi (e.g., P2 and P6) are able to activate TLR2 (Galdiero et al. 2004, Mogenson et al. 2006). Finally, TLR4 is capable of recognizing LPS and the truncated LOS found in Hi (Mogenson et al. 2006). In fact, TLR2 and TLR4 have both shown to be

crucial in the mucosal clearance of encapsulated Hi infections and important in the mucosal clearance of unencapsulated Hi (Zola et al. 2008).

Upon activation, these TLR activate very similar pathways (Takeda et al. 2003, Murphy and Weaver 92). Specifically, through the MyD88-dependent pathway, the dissociation of IκB and NFκB occurs allowing translocation of NFκB to the nucleus where it has various actions such as the production of inflammatory cytokines. Furthermore, through the MyD88-independent pathway, IRF-3 is translocated to the nucleus and various interferon-related genes are transcribed (aiding in viral immune responses) (Takeda et al. 2003, Murphy and Weaver 92-96). The activation of TLRs is incredibly important in induction of various proinflammatory genes to fight infection. However, another important result of activation of TLRs is the production of a specialized subfamily of NLR. This is further discussed in section 1.2.2.1.

NLRs are another PRR that are important in the recognition and activation of innate immunity to Hi infections. Two of the most well characterized NLRs are NOD1 and NOD2. Both NOD1 and NOD2 are comprised of a caspase activation and recruitment domain (CARD) (NOD2 possesses 2 CARDs), a NOD, and multiple leucine rich repeats (LRRs). Both of these receptors recognize different fragments of peptidoglycan. NOD1 recognizes γ-D-glutamyl-meso-diaminopimelic acid (present in many gram-negative and some gram-positive bacteria), whereas NOD2 recognizes muramyl dipeptide (present in both in gram-negative and gram-positive bacteria). As these are intracellular receptors, the peptidoglycan must enter the cells to be recognized by the NLRs. However, how the peptidoglycan enters the cell is not well understood. Upon activation, both NOD1 and NOD2 will oligomerize through CARD-CARD

interactions. Through oligomerization the receptors are able to activate the NFκB and MAPK pathways resulting in the production of proinflammatory cytokines and antimicrobial peptides (Caruso et al. 2014, Murphy and Weaver 96-98). Both NOD1 and NOD2 have been shown to play a role in the immune response to Hi (Lee et al. 2019). In fact, similar to TLR2 and TLR4, NOD1 was shown to be crucial in the clearance of encapsulated Hi strains and important in the clearance of unencapsulated strains (Zola et al. 2008). Nevertheless, another form of NLR complex has been shown to play a role in NTHi infection as well. These are the inflammasomes, multiprotein complexes responsible for the maturation of proinflammatory cytokines and induction of specialized cell death. These are discussed further in section 1.2.2.

1.2.1.1 - The Role of Macrophages in Innate Immunity

Macrophages are one of the chief immune cells in the response to pathogens and have many functions in both innate and adaptive immunity. Macrophages originate in the bone marrow as progenitor cells which are differentiated into monocytes. Upon migrating to tissues, monocytes are capable of differentiating further into macrophages or dendritic cells (Murphy and Weaver 4). It is upon differentiation that macrophages become much more adept at tackling pathogens. Macrophages have a wide array of PRRs on their surface and within their cellular compartments. Macrophages use PRRs to recognize various PAMPs allowing for quick non-specific responses (e.g., release of cytokines like tumor necrosis factor alpha (TNFα) and interleukin 1-beta (IL-1β)). Through recognition of these PAMPs, macrophages are also able to take in foreign material and destroy/neutralize pathogens through the process of phagocytosis. This can be done

through recognition via scavenger, mannose, complement, and Fc receptors (Xia and Triffitt 2006). Upon ingestion of the material, the specialized lysosomal compartments allow for effective protease and bactericidal activity. As one of their main functions is phagocytosis, they are dubbed professional phagocytes (Varol et al. 2015). Once the pathogen is internalized, macrophages can play another important role in immunity through antigen presentation. After the pathogen has been broken down in vesicles within the macrophage, small peptides from the pathogen can be displayed on MHCII receptors. MHCII are a site of communication with T cells and serve as an important bridge between the innate and adaptive immune systems (Murphy and Weaver 224). Through their ability to recognize, internalize, and then display Hi epitopes, macrophages are a key component of the immune system response to Hi infection.

1.2.2 - The Inflammasome

The inflammasomes are multiprotein complexes involved in the activation of proinflammatory caspases which can mature cytokines such as IL-1β and interleukin-18 (IL-18) (Rathinam et al. 2012). After recognition of the ligand, oligomerization and recruitment of the apoptosis-associated speck-like protein containing CARD (ASC) occurs. Through pyrin domain and CARD domain interactions, these molecules can cleave procaspase-1 into the mature caspase 1 (also known as interleukin-1 converting enzyme) (Broz and Dixit 2016). Multiple inflammasomes have been identified. Specifically, inflammasomes that may be responsible in the innate immune response to Hi are the NLR family pyrin domain containing protein 3 (NLRP3), absent in melanoma

2 (AIM2), and the non-canonical caspase 4/5/11 inflammasomes (Rotta Detto Loria et al. 2013, Yang et al. 2019, He et al. 2016).

Various types of inflammasomes have been identified from the NLR family. These include the NLRP1 and NLRP3 (Broz and Dixit 2016). The most extensively studied inflammasome in the NLR family is the NLRP3 inflammasome. This inflammasome has been shown to be activated by several gram-negative bacteria including *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, and *Haemophilus ducreyi* (Hua et al. 2015, Duncan et al. 2009, Deng et al. 2016, Li et al. 2013). NLRP3 inflammasome has also been shown to be activated by NTHi, most likely through activation of PRRs via its multiple virulence factors and then through damage to the cell causing lysosomal leakage, potassium ion efflux, and oxidative stress (activating both inflammasome signals as discussed later) (Rotta Detto Loria et al. 2013). The NLRP3 inflammasome responds to a variety of stimuli, however unlike most inflammasomes, to become activated, production of the inflammasome components must first be induced with an additional signal. This is the basis of the two-hit system for NLRP3 inflammasome activation (Sutterwala et al. 2014). This two-hit system is discussed in detail in the following two sections.

Another type of inflammasome is the AIM2 inflammasome. This inflammasome uses AIM2-like receptors (a PRR) to recognize intracellular double stranded DNA (e.g., bacterial DNA) and construct the inflammasome complex to mature caspase-1. However, similar to the NLRP3 inflammasome, AIM2 inflammasome needs a proinflammatory signal to promote the expression of the components required in the inflammasome complex. The AIM2 inflammasome is induced by type I interferons (Lugrin and

Martinon 2017). It has recently been shown that Hi DNA induces these interferons and thus, the AIM2 inflammasome might be involved in the innate immune response to Hi (Yang et al. 2019).

Inflammasomes can be defined as either canonical or non-canonical. Canonical inflammasomes are inflammasomes that interact with procaspase-1 such as those in the NLR family (e.g., NLRP1, NLRP3, NLRC4) and the AIM2 inflammasome. Unlike canonical inflammasomes, the non-canonical inflammasome does not interact with procaspase-1. An example of this is the caspase-4/5/11 inflammasome (caspases 4 and 5 exist in humans and caspase 11 exists in mice). The caspases involved in this inflammasome are able to directly recognize LPS shed from gram-negative bacteria outer membrane vesicles (Yi 2018). Once detected, the caspases will oligomerize and open potassium ion (K^+) channels (a stimulus for NLRP3 activation) and cleave gasdermin D (inducing pyroptosis) (He et al. 2016). IL-1β production was shown to be dependent on this inflammasome via TRIF signaling in gram-negative bacteria such as Hi and thus may play an important role in inflammasome activation by the strains used in this experiment (Rathinam et al. 2012).

1.2.2.1 - Signal 1: Priming of the NLRP3 Inflammasome

Priming is necessary in the induction of an NLRP3 inflammasome response as basal levels of NLRP3 complex proteins are not sufficient to form the inflammasome (Sutterwala et al. 2014). This priming is mediated by the transcription factor NFκB, which is involved in the upregulation of many proinflammatory genes such as cytokines, chemokines, and adhesion molecules (Lawrence 2009). NFκB is found in the cytosol

attached to IκBα which inhibits the translocation to the nucleus. To promote translocation, IκBα must be removed through phosphorylation by IκB kinase (IKK). IKK is produced as the result of a variety of signal cascades. Specifically, IKK can be produced through the signaling cascades created when PRRs (such as TLRs and NLRs) and cytokine receptors (such as the TNF receptor (TNFR) and IL-1 receptors (IL-1R)) are activated. Once the appropriate ligand binds to the PRR or cytokine receptor, one of the MyD88-dependent, IL-1 receptor associated kinase 4 (IRAK4), tumor necrosis factor receptor type 1-associated death domain protein (TRADD) or RIP2 pathways will result in the production of IKK (Figure 1) (He et al 2016, Sutterwala et al 2014). As the amount of NFκB increases from priming, more NLRP3 inflammasome components as well as more procaspase-1, pro-IL-1β, and pro-IL-18 are produced causing a greater response to signal 2 of the NLRP3 inflammasome.

Although transcriptional priming is necessary for a more robust activation of NLRP3 inflammasome, posttranslational changes to NLRP3 can result in a quicker activation time albeit with a lower concentration of activated NLRP3. Juliana et al. demonstrated that NLRP3 activation could be completed in the absence of *de novo* protein synthesis through the use of posttranslational modification the LRR region by deubiquitination (Juliana et al. 2012). These modifications are done by activation of the TRIF and IRAK1 pathways by PRR and cytokine receptor activation. Through these, the deubiquitinase BRCC3 can play its role in posttranslationally modifying the NLRP3 so that the LRR region can mediate oligomerization. It is theorized that this priming step is an essential regulatory point for inflammasome activation. If NLRP3 were constitutively expressed at higher concentrations, excess inflammatory activation from signal 2 could

harm the host. As such, signal 1 of the NLRP3 serves an important regulatory function (Sutterwala et al 2014).

As mentioned previously, Hi is able to activate a plethora of PRRs. Specifically, it has been shown to activate TLR2, -3, and -4 as well as NOD1 and NOD2 (Galdiero et al. 2004, Teng et al. 2010, Zola et al. 2008, Lee et al. 2019). Hi has also been shown to induce secretion of cytokines such as TNFα and IL-1β (Rotta Detto Loria et al. 2013). These PRRs and cytokine receptors are capable of activating NfκB nuclear translocation as mentioned above. As such, the Hi strains to be used in the experimental work of this study should provide ample stimulation of signal 1 of the inflammasome.

1.2.2.2 - Signal 2: Activation of the NLRP3 Inflammasome

After the priming signals have been achieved, the NLRP3 inflammasome is able to become activated. Unlike other PRRs, NLRP3 is activated not by PAMPs, but by damage-associated molecular patterns (DAMPs). The specific cellular signal and its origin are fairly controversial in the literature and many studies have been conducted to provide evidence for the signals and origins of activation of the NLRP3 pathway. Studies have shown that K^+ efflux, calcium ion (Ca^{2+}) flux, mitochondrial dysfunction/ reactive oxygen species (ROS) production, and/or lysosomal damage might be the DAMPs that are capable of activating the NLRP3 inflammasome (either on their own or through an integration of the signals) (Figure 2) (He et al. 2016).

A well-established NLRP3 activation mechanism is the efflux of K^+ out of the cell. Studies have shown that increasing extracellular K^+ (and thus preventing K^+ movement out of the cell) has blocked the activation of NLRP3 (Muñoz-Planillo et al.

2013, Pétrilli et al. 2007). This was also seen in studies investigating NTHi and the NLRP3 inflammasome where incubation with potassium chloride (a K+ efflux inhibitor) limited IL-1β secretion (Rotta Detto Loria et al. 2013). K^+ efflux may be caused by the activation of the non-canonical caspase-4/5 inflammasome by intracellular LPS leading to opening of the pannexin-1 ATP channel. ATP released from the cell is able to activate P2X7R K^+ channels causing the efflux (He et al 2016). ATP may also be released upon cell death (Ayna et al. 2012). Through the use of K^+ ionophores, decreasing intracellular K^+ has also been shown to activate NLRP3 alone (Pétrilli et al. 2007). K^+ efflux is a common outcome of various NLRP3 activators such as bacterial toxins and particulate matter, and as such, is thought to be a common pathway in NLRP3 activation (He et al. 2016).

K^+ efflux appears to be one of the main driving forces of NLRP3 activation, however, some recent studies have revealed that Ca^{2+} may also play a role in activation of the NLRP3 inflammasome. Elevated levels of Ca^{2+} influx as well as efflux of Ca^{2+} into the cytosol from within organelles such as the endoplasmic reticulum have been shown to activate NLRP3 (Lee et al. 2012). The exact mechanism by which Ca^{2+} plays a role in NLRP3 activation is fairly controversial. Some studies have provided evidence that the NLRP3 activation was seen due to increases in extracellular Ca^{2+} causing an increase in particulate matter (Muñoz-Planillo et al. 2013). As the extracellular Ca^{2+} crystalizes, it will be taken into phagocytes by phagocytosis. This may lead to lysosomal destabilization. This could mean that Ca^{2+} cannot activate NLRP3 alone but plays a role in activation through lysosomal damage by particulate matter (which may cause K^+ efflux) (Muñoz-Planillo et al. 2013). Elevated Ca^{2+} could not activate inflammasome

when extracellular K+ was increased indicating that K^+ was either more important in the activation of NLRP3 or that Ca^{2+} is not a sufficient stimulus to activate NLRP3 alone (Muñoz-Planillo et al. 2013). Other studies reveal the mechanism by which Ca^{2+} may play a role in NLRP3 activation is by reducing cAMP, an inhibitor of NLRP3. Specifically, a calcium sensing receptor is activated thereby promoting inflammasome assembly and decreasing intracellular cAMP levels (Lee et al. 2012). Another mechanism could be through Ca^{2+} flux causing mitochondrial dysfunction and the production of ROS (Muñoz-Planillo et al. 2013). The exact nature of Ca^{2+} flux in terms of NLRP3 activation requires further study.

Mitochondrial dysfunction and ROS may also play a role in activation of NLRP3. Through the use of ROS, mitochondria, and oxidase inhibitors, it was shown that ROS produced by NADPH oxidases may be responsible for NLRP3 activation; though, when tested in cells lacking the NADPH oxidases, NLRP3 was still shown to have been activated (Dostert et al. 2008, van Bruggen et al. 2010). Studies indicate that cell death and mitochondrial dysfunction result in the release of mitochondrial DNA which binds NLRP3 and may cause its activation (i.e., not mediated by ROS production) (Shimada et al. 2012). Other studies have shown that after mitochondrial damage, intracellular nicotinamide adenine dinucleotide (NAD) levels decrease causing a signaling cascade resulting in the movement of ASC adapter molecules to NLRP3 within the cytoplasm. This colocalization may be necessary for NLRP3 activation (Misawa et al. 2013). Clearly, the exact mechanism of mitochondrial dysfunction and ROS production mediated NLRP3 activation is not yet known, however, evidence suggests they may play a role in activation, either through DAMP signals or through colocalization of the NLRP3

protein complex. In fact, inhibition of ROS production with N-acetyl-L-cysteine lead to drastic inhibition of IL-1β secretion in NTHi treated cells signifying a role of ROS in NLRP3 activation (Rotta Detto Loria et al. 2013).

A final model for NLRP3 activation is through lysosomal damage from crystals and particulates causing leakage of lysosomal products. Specifically, NLRP3 inflammasome activation by lysosomal damage seems to be mediated by the release of cathepsins, lysosomal cysteine proteases (Chu et al. 2009). Indeed, inhibition of lysosomal leakage with glibenclamide lead to decreases in IL-1β secretion when cells were incubated with NTHi (Rotta Detto Loria et al. 2013). However, studies have also shown that NLRP3 activation still occurs when cathepsin-deficient macrophages are stimulated with NLRP3 activators (Dostert et al. 2009). In fact, lysosomal damage and cathepsin release may be an additional redundancy to K^+ efflux alongside non-canonical inflammasome and cell death-mediated ATP release, though the exact mechanism behind how cathepsin causes the efflux is unknown (Muñoz-Planillo et al. 2013). However, other recent studies have shown that cathepsin B is required for NLRP3 activation as cathepsin B deficient mice were unable to activate inflammasome (Chevriaux et al. 2020). Regardless, lysosomal damage serves as a justifiable model for NLRP3 activation.

NLRP3 activation is mediated by multiple different DAMPs. The exact mechanism behind how these DAMPs cause the activation is not clear. K^+ efflux appears to be the most well-established model for NLRP3 activation while the role of other DAMPs is not fully understood. Nonetheless K^+ efflux, Ca^{2+} flux, mitochondrial dysfunction/ ROS production, and lysosomal damage appear to be interconnected to a large degree. The exact mechanism might involve the signals converging with

redundancies built in (Jo et al. 2016). More research must be conducted to define this process.

As indicated, there may be several signals involved in the activation of signal 2 of the NLRP3 inflammasome. Many gram-negative bacteria have been shown to activate this inflammasome including *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Haemophilus ducreyi*, and NTHi (Hua et al. 2015, Duncan et al. 2009, Deng et al. 2016, Li et al. 2013, Rotta Detto Loria et al. 2013). These studies show that capsular polysaccharide and LPS are important virulence factors for gram-negative bacteria in the activation of inflammasome. These bacteria have been shown to elicit K^+ efflux and increase cathepsin B levels. As such, one of these DAMPs may be methods by which Hia will activate signal 2 of the NLRP3 inflammasome.

1.2.2.3 - Result of Inflammasome Activation: Role of Interleukins and Pyroptosis

After inflammasomes are activated, several outcomes are achieved both within the cell and extracellularly. Within the cell, caspase-1 works to both mature IL-1β and IL-18 and to initiate cell death. Extracellularly, the different ILs will have effects on other cells in the vicinity of activation as well as systemic effects on the host. Caspase-1, upon being activated through interactions with NLRP3, acts on both pro-IL-1β and pro-IL-18. However, without a mechanism to secrete these proteins, the effects of IL-1β and IL-18 are stunted. One of the methods by which these cytokines are secreted is through a specialized form of programmed cell death. As this is initiated by the inflammasomes thus differing from apoptosis, this specialized form of cell death is referred to pyroptosis. During pyroptosis, pores are formed in the cell membrane allowing for fluid intake and

cell rupture limiting intracellular replication of pathogens. Caspase-1 is responsible for the production of these pores. Specifically, caspase-1 is able to cleave the N-terminal of gasdermin D, allowing for pore-initiation (Tsuchiya et al. 2019). As described previously, caspases-4, -5, and -11 are also able to cleave the N terminal of gasdermin D (He et al. 2016). Unlike these other caspases, caspase-1 may also play a role in apoptosis. Tsuchiya et al. found that, in the absence of gasdermin D, caspase-1 is capable of interacting with the Bid-caspase-9-caspase-3 axis which may be relevant in areas of the body where a severe immune response may be detrimental such as cortical areas in the brain (Tsuchiya et al. 2019).

Once mature, IL-1β and IL-18 have various effects after being secreted from the cell. One such effect is that the IL-1β will activate the translocation of NFκB in other cells through its interaction with the IL-1R. By doing this, it is able to activate signal 1 of NLRP3 activation and prime the cell for the production of more IL-1β and IL-18 (He et al. 2016). Along with these, IL-1β induces the transcription of a multitude of other proinflammatory genes such as cytokines (e.g., IL-6 and IL-8), COX2, chemokines, and antimicrobial peptides, as such, it is important in inflammation (von Moltke et al. 2013). Alongside the production of proinflammatory molecules, IL-1β stimulates the production of acute phase proteins in the liver (activating complement opsonization), promote Th17 and Th2 differentiation, induce fever through interactions with the hypothalamus in the brain, and mobilize neutrophils and thus increase the number of phagocytic cells (von Moltke et al. 2013, Murphy and Weaver 120). IL-18 also has important immune functions. Through interactions with its receptor, IL-18 will induce the production of IFN-γ, an important antiviral molecule. It can also induce differentiation of CD4+ T cells

into Th1 cells as well as Th2 and Th17 (von Moltke et al. 2013). Obviously, the products of inflammasome activation have important proinflammatory actions throughout the body and are crucial components of the innate immune system.

1.3 - Conclusion

This section has reviewed how Hi uses a variety of virulence factors to help it survive in the host through adhering to the host, invading host cells, and evading the host immune system. These virulence factors include the polysaccharide capsule of encapsulated strains of Hi, LOS, IgA1 proteases, and various adhesins. It has also been described how the host's innate immune system can act to quickly identify the threat and initiate a general inflammatory response. Specifically, this section focused on how Hi was recognized by various PRRs and how, once activated, PRRs will initiate signal cascades resulting in the release of cytokines. One of the methods a host can use to fight off pathogens by utilizing PRRs is through the use of inflammasome. Inflammasomes are an important protein complex and are responsible for the maturation of several cytokines. Activation of the inflammasome is a critical aspect of the innate immune system and inflammation leading to systemic effects such as inducing fever, acute phase protein production, and stimulating an initiation of the adaptive immune response while also producing important local effects such as pyroptosis for infection management (Murphy and Weaver 120).

The polysaccharide capsule is one of the most important virulent factors. As such, it may play a role in activation of the innate immune system and the inflammatory response. As encapsulated and unencapsulated forms of Hi share many of the same

virulence factors (either identical or homologous), there is reason to believe that encapsulated forms would activate some of the same immune defenses as unencapsulated forms. As such, this study will aim to investigate the role of inflammasome in encapsulated Hi infection.

2 – Rationale, Research Questions, Hypothesis, and Objectives

2.1 – Rationale and Research Questions

Hia is an important emerging pathogen, especially in Indigenous populations, with incidence of invasive disease as high as Hib prior to the introduction of the vaccination in some areas (Tsang and Ulanova 2017, Cerqueira et al. 2019). Hia infections manifest as inflammation (e.g., meningitis or sinusitis). The inflammasome is an important innate immune mechanism that mediates inflammation through the maturation of cytokines such as IL-1β and through pyroptosis. The inflammasome has been shown to be activated by several other gram-negative bacteria such as *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, and *Pseudomonas aeruginosa* (Hua et al. 2015, Duncan et al. 2009, Deng et al. 2016). Recent studies have also shown activation of the NLRP3 inflammasome by NTHi and other species of *Haemophilus* (Rotta Detto Loria et al. 2013, Li et al. 2013). These have also shown that viable bacteria are required to activate the inflammasome. However, there is no published literature regarding the innate immune response (and thus inflammation) to Hia. The polysaccharide capsule is one of the most important virulence factors for encapsulated strains of Hi and thus likely plays a role in the activation of the innate immune system and the inflammatory response to encapsulated Hi strains. The lungs are constantly exposed to inhaled pathogens and large amounts of inflammation could damage the host. As such, discrimination between viable and non-viable bacteria may be an important regulatory step in the inflammatory response as to not mount a large immune response to a pathogen with limited pathogenicity (and prevent harm to the host). As such, this study aims to answer the following questions:

1) Is an encapsulated invasive strain of Hia able to elicit an inflammasome response?
2) If so, how does the activation of this response differ when compared to both an unencapsulated invasive Hia strain and an unencapsulated non-invasive NTHi strain?
3) Does the viability of the encapsulated Hia affect its ability to activate the inflammasome response?
4) If so, does this differ when compared to both an unencapsulated invasive Hia strain and an unencapsulated non-invasive NTHi strain?

2.2 – Research Hypotheses

Encapsulated Hia strains will cause an increased innate immune response in THP-1 macrophages and activate caspase-1 mediated inflammasome to a greater degree than unencapsulated Hi strains.

Viable Hi will be required to initiate an inflammasome response in THP-1 macrophages.

2.3 – Objectives

1) Develop an experiment system to optimize the conditions of the *in vitro* infection model of THP-1 macrophages with various strains of Hi.
2) Determine the appropriate pretreatment conditions for THP-1 macrophages with selective caspase-1 inhibitor.
3) Examine and compare the effects of an encapsulated invasive Hia strain, an unencapsulated invasive Hia strain, and a unencapsulated NTHi strain on select proinflammatory markers of the innate immune system to elucidate the effect of these strains on the inflammasome complex.

4) Examine the effect of heat inactivation of these stains on the same innate immune markers to elucidate the effect of bacterial viability on the inflammasome complex.

3 - Materials and Methods

3.1 - Cell Culture Conditions

Human THP-1 monocytic leukemia cell line (ATCC, Manassas, VA, USA) was stored in liquid nitrogen until thawed for culturing and then used from passage numbers 4 to 20. Cells were maintained at 37ºC in 5% CO_2 and in RPMI-1640 medium (Sigma-Aldrich, Oakville, ON)) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (R&D Systems, Inc., Minneapolis, MN, USA) and 1% antibiotic-antimycotic (Gibco, Eugene, OR, USA). Cells were seeded in T-25 flasks (Corning Incorporated, Corning, NY, USA) and passaged every four-five days when cell concentration reached approximately $1x10^6$ cells/mL. Cell number and viability were determined using Trypan Blue exclusion assays with a hemocytometer. Cells with viability lower than 90% were discarded and not used for experiments. Cells were plated in six-well plates at a concentration of $2.5x10^5$ cells/mL. Macrophage differentiation was induced with the addition of 20 ng/mL of phorbol myristate acetate (PMA) (Sigma-Aldrich, Oakville, ON) in the medium and incubation for 24 hours at 37ºC in 5% CO_2. PMA was stored in stock solutions of 10 µg/mL in dimethyl sulfoxide (DMSO) at -20ºC. After the 24 hour incubation, supernatant was removed and discarded. Cells were then washed with 1 mL RPMI-1640 medium which was then removed and discarded. 2 mL of fresh RPMI-1640 medium supplemented with 10% FBS was added to each well of the six-well plate. The plates were incubated at 37ºC in 5% CO_2. Macrophage differentiation was confirmed using flow cytometry with Alexa Fluor 488-conjugated anti-CD11b antibody on the FL1 channel as described in section 3.7.1.

3.2 - Pretreatment with Caspase-1 Inhibitor

After 24 hours of incubation with fresh medium, supernatant was removed and replaced with 2 mL RPMI media supplemented with 10% FBS. THP-1 macrophages were treated with 20 µL of 10 mM of the specific caspase-1 inhibitor, ac-YVAD-cmk (N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone) (Sigma-Aldrich, Oakville, ON), for a final concentration of 100 µM. The 10 mM ac-YVAD-cmk stock was were stored in DMSO at -20ºC. This was added directly to the medium in the six-well plate. Plates were gently swirled to facilitate mixing without disturbing the cells. Cells were incubated for 24 hours at 37ºC in 5% CO_2. Cells treated in the absence of inhibitor were exposed to only 20 µL of vehicle i.e., DMSO (for a final percentage of 1% DMSO in RPMI-1640 medium supplemented with 10% FBS).

3.3 - *Haemophilus influenzae* Strains and *in vitro* Infection Model

Hia strains 08-191 and 13-0074, along with NTHi strain 375 were used (these were kindly provided by Dr. Raymond Tsang (National Microbiology Laboratory, Canada)). Hia 08-191 is an invasive strain isolated from the blood of an adult First Nations man from Northwestern Ontario (Kelly et al. 2011). Hia 13-0074 is another invasive strain that is a capsular deficient mutant strain of Hia confirmed by genotypic analysis belonging to the same clonal type as Hia 08-191 (sequency type (ST)-23) (Dr. Tsang: personal communication). NTHi 375 is a non-invasive strain and was isolated from the middle ear of a pediatric patient with otitis media (Mell et al. 2014). Stock suspensions were stored in 1.5 mL aliquots containing bacteria, 750 µL brain heart infusion (BHI) (Teknova, Hollister, CA, USA) broth, and 750 µL 50% glycerol at -80 ºC. Using sterile

inoculation loops, small amounts of frozen stock bacteria were streaked onto fresh BHI plates (prepared with 1.5% agar, 10 μg/mL of hemin chloride, and 5 μg/mL NAD). These were left to grow for 16 hours at 37ºC in 5% CO_2. Hemin chloride and NAD stock solutions were stored at 10 mg/mL and 5 mg/mL at 21ºC and -20ºC, respectively. Isolated colonies were taken from the culture plates and suspended in 3 mL of BHI broth with 10 μg/mL of hemin chloride, and 5 μg/mL NAD. The optical density (OD) of these suspensions were determined using spectrophotometry. Bacterial suspensions were then diluted to 0.1 OD at a wavelength of 600 nm (OD600). 500 μL of 0.1 OD600 bacterial suspension was added to 10 mL of fresh BHI broth with 10 μg/mL of hemin chloride, and 5 μg/mL NAD and were incubated until mid-log phase (approximately five hours) in a shaking incubator at 37ºC at 150-200 rpm. To achieve a multiplicity of infection (MOI) of 10, $5x10^6$ bacteria were washed twice with PBS and then resuspended in 200 uL of PBS before being added to corresponding wells containing $5x10^5$ THP-1 macrophages. Cells were incubated at 37ºC in 5% CO_2. After one hour, bacteria were killed by adding 220 μL of 1mg/mL gentamicin (prepared in sterile water) to achieve a final concentration of 100 μg/mL. Cells were incubated with gentamicin-killed bacteria for a further 17 hours. For experiments that required non-viable bacteria, bacteria were heat-inactivated for 30 minutes at 65ºC in a block heater prior to addition into the macrophages in the six-well plate. Bacterial killing by heat inactivation was confirmed as described in section 3.7.2. Macrophages require two signals for activation of inflammasome (Netea et al. 2009). As such a proper positive control would need to activate both signals. For positive control, cells were incubated with 100 ng/mL *Escherichia coli* (*E. coli*) LPS (Invitrogen, Carlsbad, CA, USA) for 17.5 hours (to activate signal 1 through TLRs) prior to addition

of 5 mM ATP (Sigma-Aldrich, Oakville, ON) prepared in sterile distilled H_2O for 30 minutes (to activate signal 2 through DAMPs) (Schneider et al. 2013, Alhazmi 2018). After addition of ATP, 4 µL of 3 M NaOH was used to balance the pH. For negative control, 200 µL PBS was added to macrophages.

3.4 - Flow Cytometry Analysis of Cell Death and Surface Expression of ICAM-1

Following the 18-hour stimulation with bacteria or controls, cells were harvested from the six-well plate after placing on ice for three minutes and then washed with 1 mL PBS. For measurement of intercellular adhesion molecule (ICAM)-1 surface expression, cells were suspended in 100 µL PBS supplemented with 1% bovine serum albumin (w/v) (PBS/BSA) and with 1 µg/mL phycoerythrin-conjugated antibody against ICAM-1 (Mouse anti-human CD54) (BD Biosciences, Mississauga, ON) and incubated for one hour at 4ºC. Cells were washed twice with 1 mL of PBS and analyzed using flow cytometry on the FACSCalibur with Cell Quest Pro software (BD Biosciences, Mississauga, ON) on the FL-2 channel. Geometric means of fluorescent intensity (MFI) were acquired. To measure the amount of cell death, cells not stained with anti-ICAM-1 antibody were incubated in 100 µl of PBS/BSA with 1 µg/mL propidium iodide (PI) at 4ºC for one minute. Cells were analyzed using flow cytometry on the FACSCalibur with Cell Quest Pro software on the FL-2 channel. Percent of cell death was calculated by comparing number of cells with more permeable membranes (i.e., dead cells that allow for PI to intercalate with their DNA) to those that did not have permeable membranes (i.e., live cells) (**Figure 3**).

3.5 - ELISA Assays

Following the 18-hour stimulation with bacteria or controls, cell supernatant was collected and stored at -80ºC until analysis. Concentrations of both IL-1β and TNFα in cell supernatants were determined using Human IL-1β Uncoated and Human TNFα Uncoated ELISA kits (Invitrogen, Carlsbad, CA, USA). The ELISA kits were used according to the manufacturer's specifications. Supernatant samples were diluted 40x (those treated with Hi or LPS/ATP) or 2x (negative controls) in the 1X ELISA/ELISPOT diluent provided in the ELISA kit. Supernatant samples were incubated in antibody-coated wells overnight (18 hours) for maximum sensitivity. Samples were run in duplicate on the ELISA plates and means of these duplicates were used for analysis.

3.6 - Statistics

Statistical differences was determined using single-factor analysis of variance (ANOVA) or two-factor ANOVA using R software (Vienna, Austria). To determine homogeneity in variance Bartlett's test was conducted. The Anderson-Darling test was used to determine normality of residuals. Should any dataset not pass either Bartlett's or the Anderson-Darling tests ($p<0.05$) (i.e., non-normal residuals or heterogeneity in variance), the data was transformed using a square root function to better meet the assumptions of ANOVA. To determine specific differences in means, a post-hoc Tukey's honestly significant difference test was conducted. P values of less than 0.05 were considered statistically different.

3.7 - Optimization

3.7.1 - Monocyte Differentiation

To obtain the most optimal/consistent results, a series of optimization experiments were run. Compared to monocytes, macrophages produce a greater immune response to specific stimuli. They express more surface molecules and are more phagocytic (Starr et al. 2018). Based on literature and the work of others in our group, PMA is one of the most effective reagents at producing THP-1 monocyte-derived macrophages closely resembling peripheral blood monocyte-derived macrophages (Chanput et al. 2014, Khan et al. 2017). PMA activates protein kinase C and is associated with rewiring of the MAPK signaling and production of specific kinases resulting in macrophage differentiation (Schwende et al. 1996, Richter et al. 2016). As such, THP-1 cells were differentiated from monocytic cells to macrophages with the use of PMA for these experiments. To confirm that the concentration of PMA and the incubation time with the chemical was sufficient to induce differentiation, flow cytometry was used. Specifically, the amount of CD11b (a known macrophage differentiation marker associated with phagocytosis and cellular adhesion) was used to determine if macrophage differentiation was induced as per Starr et al. 2018 (Solovjov et al. 2005).

To test differentiation, THP-1 cells were incubated with either 20 ng/mL PMA or 0.2% DMSO (vehicle) for 24 hours 37ºC in 5% CO_2. After 24 hours, cells were washed consistent with the above protocol and incubated with fresh media for 48 hours at 37ºC in 5% CO_2. Supernatant of adherent cells was removed and replaced with 1 mL of PBS. For non-adherent cells, cell solution was removed and centrifuged at 500 x g for five minutes. This supernatant was then removed without disturbing the cell pellets and the pellets

were resuspended in 1 mL PBS. The resuspended cell solutions were put in their respective wells on the six-well plate. Plates were then put on ice for three minutes. Cells were scraped off and collected. Once collected, cells were washed twice with 1 mL of PBS. Each sample was split in half. One half was incubated with 1 μg/mL Alexa Fluor 488-conjugated antibody against CD11b (Human Anti-Mouse) (Cedarlane Laboratories, Burlington, ON) for one hour at 4ºC. The other half was incubated without any antibody (but an equal amount of PBS added) for one hour at 4ºC. Cells were washed twice with PBS and analyzed using flow cytometry on the FACSCalibur with Cell Quest Pro software. It was concluded that incubation with 20 ng/mL PMA for 24 hours was sufficient to induce differentiation of THP-1 monocytes to macrophages.

3.7.2 – Bacterial Culture, Inactivation, and MOI

3.7.2. Bacterial Culture

Various aspects of the bacterial infection needed to be optimized for these experiments. Specifically, the concentration of bacteria in suspension; what concentration of gentamicin would inactivate bacteria; what temperature and at what incubation length would heat inactivate the bacteria; and which MOI would be appropriate in eliciting an inflammatory response needed to be determined. To determine the amount of bacterial suspension needed to achieve an MOI of 10, Hi strains were grown for 16 hours on BHI-agar plates and diluted to 0.1 OD600 as above. The 0.1 OD600 suspension was then serially diluted by a factor of one million. Nine 10 μL drops of the diluted bacterial suspension were then placed equally spaced on a BHI-agar plate. These were then incubated for 16 hours at 37ºC. After 16 hours, the number of colonies grown at the sites

of each 10 μL drop were counted. Colony numbers were averaged and multiplied by $1x10^6$ to determine the concentration of bacteria in a 0.1 OD600 suspension in colony forming units/mL. Using this concentration, the amount of bacterial suspension that needed to be added to the macrophages at any MOI could be determined. This information was determined from the work by previous members in the Ulanova group (thank you to Kayla Colledanchise and Lynnea Lobert).

3.7.2.2 Bacterial Inactivation

Gentamicin is a commonly used aminoglycoside originally isolated from *Micromonospora*. Its bacterial killing action is mediated by inhibition of protein synthesis by irreversibly binding to the bacterial 30S ribosome (Gonzalez and Spencer 1998). As such, gentamicin was used to kill the bacteria in this experiment. Prior to initiating experiments involving bacterial stimulation, the concentration that would be sufficient enough to kill the bacteria was determined. This protocol was based on various methods from the literature as well as experience from previous members of the Ulanova group (Li et al. 2013, Ahrén et al. 2001, Buyck et al. 2013, Alhazmi 2018). To confirm this, Hi strains were grown for 16 hours on BHI-agar plates and diluted to 0.1 OD600 as above. 2 mL of growth factor supplemented BHI broth was placed in each well of a six well plate. $5x10^8$ Hia 08-191, Hia 13-0074, or NTHi 375 were added to corresponding wells. After one hour of incubation at 37ºC in 5% CO_2, 220 μL of 1 mg/mL gentamicin was added to one well corresponding to each strain and 220 μL sterile dH_2O was added to the other wells. The plates were then incubated for 16 hours at 37ºC in 5% CO_2. The contents of each well were collected and centrifuged at 1000 x g. BHI broth was removed and 500

μL of fresh BHI broth was used to suspend the bacteria. These bacterial suspensions were plated using the drop-plate technique described above. After 16 hours incubation 37ºC in 5% CO_2, plates were inspected for bacterial growth at the sites of the 10 μL drops. This procedure was repeated with 2 mL RPMI-1640 medium supplemented with 10% FBS in each well in place of BHI broth. It was found that incubation with 100 μg/mL gentamicin for one hour was sufficient to kill Hi.

Heat inactivation protocols vary in the literature using different incubation lengths and incubation temperatures (Li et al. 2013, Kirkham et al. 2013, Juneau et al. 2011, Hartwig et al. 2016). Heat inactivation was confirmed in a similar method to gentamicin killing. Hi strains were grown on BHI-agar plates for 16 hours. Colonies were harvested and diluted in growth factor supplemented with BHI broth to 0.1 OD600 as above and allowed to reach mid-log phase. Bacterial suspensions were once again diluted to 0.1 OD600. Bacterial suspensions were then incubated at 65ºC or 37ºC in a block heater for 30 minutes. These bacterial suspensions were plated using the drop-plate technique described above. After 16 hours incubation at 37ºC in 5% CO_2, plates were inspected for bacterial growth at the sites of the 10 μL drops. No growth for bacteria incubated at 65ºC was found indicating that an incubation of 65ºC for 30 minutes was sufficient to heat inactivate bacteria. This process was repeated with bacterial suspensions incubated at 60ºC in a block heater for one hour. There were no differences in growth results between the bacteria heat inactivated at 65ºC for 30 minutes for 60ºC for one hour (data not shown). Due to lack of differences between heat inactivation methods, incubation at 65ºC for 30 minutes was chosen for sake of time.

3.7.2.3 Bacterial MOI

The MOI of bacteria that would elicit an inflammatory response while maintaining cell death to a minimum also needed to be determined. This was done by testing an array of MOIs. Methods regarding THP-1 cell maintenance and bacterial infection and killing were consistent with the above protocol, however, MOIs of 1, 10, 100, and 1000 were used. 100 ng/ml *E. coli* LPS with ATP and PBS were used as positive and negative controls respectively. Cell death was measured through PI incubation and flow cytometry analysis as noted above. ICAM-1 surface expression as well as IL-1β and TNFα concentrations in supernatant were measured. Data were analyzed using two factor ANOVA as described above.

3.7.3 – Treatment with Caspase-1 Inhibitor

Another aspect of the experiment that required optimization was the concentration and incubation time of ac-YVAD-cmk and method of measuring ac-YVAD-cmk inhibitory action. Ac-YVAD-cmk is a selective and irreversible caspase-1 inhibitor though it has weak inhibitory activity against caspase-4 and -5. It is preferred over other caspase inhibitors due to higher specificity against caspase-1 (e.g., z-YVAD-fmk is sometimes used but is a pan-caspase inhibitor with activity against caspase-1, -3, -7, and -8) (Lipinska et al. 2014). Similar to the above optimization, multiple concentrations of ac-YVAD-cmk were used, specifically, concentrations of 0, 40, and 100 μM. Ac-YVAD-cmk was added either 5h or 24h prior to incubation with 100 ng/mL *E. coli* LPS for 18 hours and 5 mM ATP for the final 30 minutes. These concentrations and incubations times were based on information from the manufacturer, literature search, and work from

previous members of the Ulanova group (Lipinska et al. 2014, Chen et al. 2013, Anantharajah et al. 2015, Alhazmi 2018).

After the addition of ac-YVAD-cmk to the cell media, stimulation of inflammasome was needed to test ac-YVAD-cmk inhibitory action. To measure inhibitory action of ac-YVAD-cmk, the concentration IL-1β, one of the downstream products of caspase-1 was measured via ELISA. TNFα secretion, ICAM-1 surface expression, and cell viability were also measured to ensure that differences in IL-1β secretion were a direct effect of caspase-1 inhibition. Data were analyzed using one factor ANOVA. Tests of assumptions and post-hoc tests were conducted as above.

The above optimization results revealed partial inhibition of IL-1β by the vehicle (DMSO). As such a dose response of DMSO on THP-1 macrophage was conducted with 24h pretreatment with 0%, 0.4%, or 1% DMSO prior to 17.5 hour incubation with 100 ng/mL *E. coli* LPS and 30 minute incubation with 5 mM ATP. ICAM-1 levels were measured via flow cytometry and IL-1β and TNFα levels were measured via ELISA. Data were analyzed using one factor ANOVA. Tests of assumptions and post-hoc tests were conducted as above.

4 – Results

4.1 – Incubation with 20 ng/mL PMA for 24 hours is Sufficient to Induce Differentiation of THP-1 Monocytes to Macrophages

Through its interactions with protein kinase C and MAPK, PMA is one of the most effective monocyte-to-macrophage differentiation reagents (Chanput et al. 2014, Khan et al 2017). To confirm differentiation of THP-1 monocytes to macrophages, THP-1 monocytes were incubated with either 0.2% DMSO or with 20 ng/mL PMA and surface levels of CD11b were measured as described in section 3.7.1. THP-1 cells treated with PMA showed much greater surface expression of CD11b whereas THP-1 cells not treated with PMA showed much lower CD11b surface expression (**Figure 4**). From these results it was concluded that incubation with 20 ng/mL of PMA for 24 hours was sufficient to induce differentiation of THP-1 monocytic cells to macrophage-like cells.

4.2 - 100 µg/mL Gentamicin or 30 Minutes at 65ºC is Sufficient to inactivate *Haemophilus influenzae in vitro*

Previous studies involving other species of *Haemophilus* and NTHi and inflammasome found that viable bacteria are needed to stimulate inflammasome activation though prolonged exposure can lead to issues with cell viability (Loria et al. 2013, Li et al. 2013). Thus, the viability of Hi after chemical and heat inactivation needed to be confirmed. Bacteria incubated with 100 µg/mL gentamicin for 17 hours showed no growth when incubated in both BHI broth supplemented with growth factors (**Figure 5a**) and RPMI 1640 media supplemented with 10% FBS (**Figure 5b**). Likewise, bacteria showed no growth on BHI-agar plates after the bacteria had been heat inactivated at 65ºC for 30 minutes (**Figure 6**).

4.3 – *H. influenzae* Multiplicity of Infection of 10 is Appropriate for Stimulation of Inflammatory Markers Without Affecting Cell Viability in THP-1 Macrophages

Before any experiments could be done to answer the research question, an *in vitro* infection model involving Hi and THP-1 cells needed to be optimized. To optimize the conditions for Hi stimulation, THP-1 macrophages were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at an MOI of 1, 10, 100, or 1000 for one hour prior to inactivation with gentamicin and further incubation for 17 hours. For positive control, cells were stimulated with 100 ng/mL *E. coli* LPS (and 5 mM ATP for 30 minutes). For negative control, cells were incubated with PBS. Cells were stained with anti-ICAM-1 antibody or PI and, using flow cytometry, ICAM-1 levels (**Figure 7**) and cell viability (**Figure 8**) were measured. Cell supernatants were collected and ELISAs for IL-1β (**Figure 9**) and TNFα (**Figure 10**) were conducted.

Strains of Hi did not significantly differ from one another in their effect on cell viability, or IL-1β release (**Figure 8**, **Figure 9**). However, Hia 08-191 induced significantly higher ICAM-1 levels than both Hia 13-0074 ($p<0.001$) and NTHi 375 ($p<0.01$) (**Figure 7**). NTHi 375 induced a significantly higher TNFα secretion when compared to both Hia 08-191 ($p<0.05$) and Hia 13-0074 ($p<0.05$) (**Figure 10**). All MOIs showed similar trends of increased ICAM-1 levels, IL-1β secretion, and TNFα as MOI increased, however, at MOI 1000, there was a decrease in all of these parameters (**Figure 7**, **Figure 9**, **Figure 10**). Inversely, as MOI increased, cell viability decreased significantly with up to 17% mean decrease in cell viability when comparing MOI 1 to 1000 (**Figure 8**). Though MOI 1 and 10 were not significantly different in any parameter

measured, MOI 10 did appear to induce an increase in ICAM-1 levels, IL-1β secretion, and TNFα secretion (**Figure 7**, **Figure 9**, **Figure 10**). These findings suggested that an MOI 10 would provide a sufficient stimulus of the inflammatory response for THP-1 macrophages while maintaining relatively high cell viability in future experiments.

4.4 - 24 Hour Pretreatment with 100 μM ac-YVAD-cmk is Sufficient to Inhibit Caspase-1 Dependent IL-1β Secretion in THP-1 Macrophages

Ac-YVAD-cmk has been shown to inhibit caspase-1 (and thus products of inflammasome activation) in THP-1 cells (Xing et al. 2016). To verify the inhibitory action of ac-YVAD-cmk on inflammasome in our model and optimize pretreatment conditions, THP-1 macrophages were incubated for five or 24 hours with either 40 μM ac-YVAD-cmk, 100 μM ac-YVAD-cmk, 1% DMSO (0 μM ac-YVAD-cmk), or no reagent. Cells were then stimulated with 100 ng/mL *E. coli* LPS for 18 hours (with incubation with 5 mM ATP for 30 minutes). For negative control, cells were incubated with PBS. ICAM-1 levels (**Figure 11**), cell viability (**Figure 12**), IL-1β secretion (**Figure 13**) and TNFα secretion (**Figure 14**) were measured as stated previously.

Inhibitory action by ac-YVAD-cmk was evident as both 24 hour and five hour pretreatments with ac-YVAD-cmk significantly decreased IL-1β release when compared to vehicle control (DMSO) ($p<0.001$ for all comparisons) and positive control (no pretreatment) ($p<0.001$ for all comparisons) (**Figure 13**). Ac-YVAD-cmk was not seen to have an effect on either ICAM-1 MFI, cell viability, or TNFα release (**Figure 11**, **Figure 12**, **Figure 14)**. Longer incubations with the caspase inhibitor resulted in significantly reduced IL-1β secretion compared to shorter incubations ($p<0.05$ between 100 μM

pretreatments and $p<0.01$ between 40 μM pretreatments) whereas different concentrations did not significantly differ from one another (**Figure 13**).

Our findings showed that macrophages pretreated with only DMSO induced significantly lower IL-1β levels than samples with no pretreatment ($p<0.001$) (**Figure 13**). To further investigate the inhibitory effect of DMSO, THP-1 macrophages were incubated with 0%, 0.4%, and 1.0% DMSO (corresponding with no pretreatment, 40 μM and 100 μM ac-YVAD-cmk in the previous experiment respectively) for 24 hours. Cells were then stimulated with LPS/ATP as above. ICAM-1 levels (**Figure 15**), IL-1β secretion (**Figure 16**) and TNFα secretion (**Figure 17**) were measured as stated previously. Release of IL-1β was significantly reduced in samples pretreated with DMSO compared to those that were not ($p<0.001$ for both comparisons) (**Figure 16**). Differences in ICAM-1-levels and TNFα release between pretreatments were not significant (**Figure 15**, **Figure 17**). Using the data from both of these experiments, it was concluded that ac-YVAD-cmk inhibited IL-1β in a caspase-1 dependent manner and a 24h pretreatment with 100 μM ac-YVAD-cmk would be sufficient to inhibit caspase-1 in future experiments.

4.5 - Encapsulated and Unencapsulated *Haemophilus influenzae* Type a Strains Initiate IL-1β Secretion in a Caspase-1 Dependent Manner in THP-1 Macrophages

The previous results had shown that both unencapsulated and encapsulated Hia strains as well as NTHi were able to elicit a significantly increased amount of IL-1β secretion when compared to the negative control (cell treated with PBS). The next steps to address the research questions were to see if this increase in IL-1β secretion was caspase-1

dependent. Using the now optimized experimental model this could be tested. THP-1 macrophages were pretreated with either 1% DMSO or 100 μM ac-YVAD-cmk for 24 hours. Macrophages were then stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at an MOI of 10; with 100 ng/mL LPS (with 5 mM ATP as above); or incubated with PBS for one hour. 100 μg/mL gentamicin was then added to macrophages and cells were incubated for 17 hours. Cells were stained with anti-ICAM-1 antibody or PI and, using flow cytometry, ICAM-1 levels (**Figure 18**) and cell viability (**Figure 19**) were measured. Cell supernatants were collected and ELISAs for IL-1β (**Figure 20**) and TNFα (**Figure 21**) were conducted.

All Hi strains induced significantly higher levels of ICAM-1 and secretion of both IL-1β and TNFα than the negative control (PBS) (**Figure 18**, **Figure 20**, **Figure 21**). IL-1β secretion was significantly decreased in samples pretreated with caspase-1 inhibitor compared to those pretreated with vehicle (DMSO) ($p<0.001$) (**Figure 20**). Significant differences between ac-YVAD-cmk and vehicle pretreated macrophages were not present in the other inflammatory markers measured or cell viability (**Figure 18**, **Figure 19**, **Figure 21**). No significant differences were found between any of the Hi strains used in terms of ICAM-1 levels, cell viability, IL-1β secretion or TNFα secretion (**Figure 18**, **Figure 19**, **Figure 20**, **Figure 21)**. These findings indicate that caspase-1 is important in IL-1β secretion by macrophages stimulated with Hia.

4.6 – Heat-Inactivated *H. influenzae* Induces Lower Cytokine Release Compared to Non-Heat-Inactivated *H. influenzae* in THP-1 Macrophages

After conducting the above experiments showing the caspase-1 dependent nature of IL-1β secretion by THP-1 macrophages stimulated with both encapsulated and unencapsulated Hia strains, the next research questions could be addressed. Specifically, this aimed to investigate the role of bacterial viability in IL-1β secretion of THP-1 macrophages. To test this, Hia 08-191, Hia 13-0074, and NTHi 375 were incubated at either 37ºC or 65ºC for 30 minutes prior to addition to THP-1 macrophages at an MOI of 10 for one hour. 100 μg/mL gentamicin was then added before further incubation for 17 hours. Stimulation of macrophages with either 100 ng/mL *E. coli* LPS (with 5 mM ATP for 30 minutes) or incubation with PBS served as positive and negative controls respectively. Cells were harvested and stained with anti-ICAM-1 antibody or PI and, using flow cytometry, ICAM-1 levels (**Figure 22**) and cell viability (**Figure 23**) were measured. Cell supernatants were collected and ELISAs for IL-1β (**Figure 24**) and TNFα (**Figure 25**) were conducted.

Macrophages stimulated with any Hi strain showed significantly increased ICAM-1 expression ($p<0.001$) and secretion of IL-1β ($p<0.001$) and TNFα ($p<0.001$) when compared to the negative control (PBS) (**Figure 22**, **Figure 24**, **Figure 25**). Analysis of raw data did not show statistical differences of bacterial heat inactivation on ICAM-1 levels, cell viability, IL-1β secretion, or TNFα secretion (**Figure 22**, **Figure 23**, Figure **24**, **Figure 25**). All three experiments showed similar trends in cytokine release, however, due to the large degree of variability between individual trials, statistically significant effects may have been masked. Therefore, cytokine release was normalized as

percent of release by macrophages stimulated with viable bacteria. Statistical analysis as described in section 3.6 was conducted on the normalized data for both IL-1β secretion (**Figure 26**) and TNFα secretion (**Figure 27**). Significant differences were apparent in the normalized data. Specifically, heat-inactivated bacteria had significantly decreased IL-1β ($p<0.001$) and TNFα ($p<0.001$) secretion compared to viable bacteria (**Figure 26**, **Figure 27**). Also, both heat inactivated Hia 13-0074 ($p<0.01$) and NTHi ($p<0.001$) showed significantly decreased secretion of both cytokines measured compared to heat inactivated Hia 08-191 (**Figure 26**, **Figure 27**). These findings show that heat-inactivation of Hi leads to decreased cytokine release in THP-1 macrophages when compared to those stimulated with viable bacteria.

5 – Discussion

Though invasive disease caused by Hib has decreased since the introduction of the vaccine, a decrease in Hib carriage may have allowed non-Hib strains to occupy a previously unavailable ecological niche and cause an increase in non-Hib infection and thus non-Hib invasive disease such as meningitis, pneumonia, and epiglottitis (Ulanova and Tsang 2009, Cerqueira et al. 2009, Tsang and Ulanova 2017). NTHi and Hia are the two most prevalent causes of invasive Hi disease in Canada (Ulanova and Tsang 2014). Moreover, in some Indigenous population areas, incidence of Hia invasive infection is similar to incidence of Hib invasive infection in the pre-Hib vaccine era (Tsang and Ulanova 2017). However, there is no published literature regarding the innate immune response to Hia. Recently, NTHi and other species of *Haemophilus* have been shown to activate the inflammasome, a multiprotein complex involved in the innate immune response to DAMPs associated with infections by pathogens (Rotta Detto Loria et al. 2013, Li et al. 2013). The polysaccharide capsule is one of the most important virulence factors of Hia contributing to the development of the aforementioned invasive disease and likely plays a role in the activation of the innate immune system and inflammatory response to encapsulated Hia strains. This led to the research questions outlined in section 2.1.

5.1 – Experimental Model Justification

To answer the research questions, an experimental model needed to be constructed to test our hypotheses. The infection model used was modelled after the work of Rotta Detto Loria et al. (2013), Li et al. (2013), as well as work with inflammasome

activation done by previous members of the Ulanova group (Alhazmi 2018). This model used three different strains of Hi. Specifically, it used Hia 08-191, Hia 13-0074, and NTHi 375. Hia 08-191 is an encapsulated, invasive strain isolated from an Indigenous man from Northwestern Ontario (Kelly et al. 2011). Like most isolates in Canada, this is an ST-23 strain (Kelly et al. 2011, Ulanova and Tsang 2014). Similar to Hia 08-191, Hia 13-0074 is an invasive ST-23 strain, however, unlike Hia 08-191, this is a capsular deficient mutant strain (confirmed with genotypic analysis) (Dr. Tsang: personal communication). Finally, NTHi is an unencapsulated non-invasive strain (Mell et al. 2014). The use of these strains will allow us to see differences in the capacity to activate the inflammasome complex/inflammatory response between unencapsulated and encapsulated strains as well invasive and non-invasive strains.

These strains were used to stimulate THP-1 macrophages. These are an immortalized monocytic cell line that closely resemble peripheral blood monocytes (as well as monocyte-derived macrophages when differentiated) but with more consistency between trials among other advantages allowing for a suitable and reliable model for studying monocyte and macrophage responses (Chanput et al. 2014). In fact, this cell line has been used in multiple studies of inflammasome activation (Deng et al. 2016, Duncan et al. 2009, Li et al. 2013, Netea et al. 2009, Hua et al. 2015). THP-1 cells were differentiated to macrophages as macrophages express more surface molecules and are more phagocytic (Starr et al. 2018). Of the many surface molecules expressed at higher levels, it has been shown that macrophages express higher levels of CD14 (a co-receptor for TLR4) as well as have a higher rate of translocation of NFκB to the nucleus; two important factors for signal 1 of NLRP3 inflammasome activation (Forrester et al. 2018,

Takashiba et al. 1999). The differentiation of THP-1 monocytes to macrophages was confirmed by the presence of CD11b in flow cytometry analysis as discussed in section 3.7.1 (**Figure 4**).

The final aspects of the model were the use of suitable controls and a caspase-1 inhibitor. Two controls were used in these experiments: a positive and negative control. The positive control was incubation with 100 ng/mL *E. coli* LPS and incubation with 5 mM ATP for the final 30 minutes. LPS has been shown to activate TLR4 and initiate signal 1 of NLRP3 inflammasome activation and is one of the crucial TLRs in the mucosal clearance of encapsulated Hi through recognition of LOS (Mogenson et al. 2006, Zola et al. 2008). It is also important in activation of the non-canonical inflammasome through TRIF signaling. ATP causes robust stimulation of the second signal of inflammasome activation within 30 minutes to an hour, however, longer incubations can lead to cell death (Schneider et al. 2013). *E. coli* LPS with ATP has been confirmed to activate inflammasome in the literature as well as within our group (Netea et al. 2009, Schneider et al. 2013, Alhazmi 2018). This positive control would allow us to see if there were discrepancies in any specific trial in any of the experiments and confirm stimulation of cells. The negative control used was PBS, the vehicle for both the positive control as well as the Hi strains. The negative control allowed us to see if there were any effects of vehicle on the stimulation of cells.

The levels of three proinflammatory markers were measured in this model. ICAM-1 is critical in leukocyte recruitment as well as a co-activation signal for T-cell activation. Though it is expressed at low levels in macrophages, in the presence of proinflammatory cytokines (such as TNFα or IFN-γ) or viral and/or bacterial infection,

NFκB is translocated to the nucleus and increases expression of ICAM-1 (Roebuck and Finnegan 1999). ICAM-1 has been shown to be upregulated when cells were exposed to LOS of Hi as well as whole Hi (Choi et al. 2014, Humlicek et al. 2004, Avadhanula et al. 2006). As such, ICAM-1 expression could be used as a measure of inflammatory activity induced by Hi infection of THP-1 macrophages.

Along with surface expression of ICAM-1, the proinflammatory cytokines, TNFα and IL-1β, were also used as a measure of the inflammatory activity caused by Hi stimulation. TNFα has a plethora of proinflammatory effects including local effects such as increasing vascular permeability or apoptosis to systemic effects such as initiating a fever response (Murphy and Weaver 111). TNFα exerts these effects through activation of secondary proteins such as the transcription factors NFκB and AP-1, mitochondrial proteins, and protein kinases (Idriss and Naismith 2000). Similarly, IL-1β is able to activate NFκB through interaction with the IL-1R and through this will increase proinflammatory gene products such as other cytokines, chemokines, and antimicrobial peptides. Systemically, IL-1β will result in increase in acute phase proteins, induce fever, and promote differentiation and mobilization of various leukocytes (von Moltke et al. 2013). Both of these cytokines have been shown to be upregulated by virulence factors of Hi and whole Hi infection (Choi et al. 2014, Diab et al. 1997, Rotta Detto Loria et al. 2013). Thus, these could also be used as measures of inflammatory activity induced by Hi infection of THP-1 macrophages.

The use of these specific proinflammatory markers allows for insight into the possible mechanism of initiation of the inflammatory response by the innate immune system to Hi. Specifically, it allowed us to see if the inflammasome was activated by Hi.

IL-1β is the caspase-1 dependent product of inflammasome activation as described in section 1.2.2. As such, measurement of IL-1β levels could help determine if the inflammasome is activated upon stimulation of THP-1 cells with Hi. However, to confirm this, caspase-1 inhibition had to be used. Reduction of IL-1β secretion in caspase-1 inhibited THP-1 cells would show that IL-1β is dependent on caspase-1 and thus the activation of inflammasome. ICAM-1 and TNFα levels were measured as these are both caspase-1 independent proinflammatory markers that are controlled by NFκB and would serve as controls. Specifically, if these markers were not decreased in inhibited cells such as IL-1β, it would further provide evidence of inflammasome-dependent IL-1β secretion. Similar models have been used to model inflammasome activation in the literature (Rotta Detto Loria et al. 2013, Li et al. 2013, Hua et al. 2015, Deng et al. 2016, Alhazmi 2018).

5.2 – Discussion of Model Optimization

To optimize the experimental model many experiments needed to be performed. Firstly, appropriate concentrations of gentamicin and heating conditions for heat inactivation needed to be found. Gentamicin concentration and protocol is fairly standard in the literature as well as within our group and was confirmed prior to use in experiments (**Figure 5**) (Li et al. 2013, Ahrén et al. 2001, Buyck et al. 2013, Alhazmi 2018). However, heat inactivation of bacteria is not as standardized. Multiple different temperatures and incubation times have been found to heat inactivate Hi (Rotta Detto Loria et al. 2013, Kirkham et al. 2013, Juneau et al. 2011, Hartwig et al. 2016). As such two heat inactivation methods (65 ºC for 30 minutes and 60 ºC for one hour) were compared. No differences were found between the two heat inactivation methods with

any of the bacterial strains used (i.e., both conditions resulted in no bacterial growth after plating). It was found that incubation with 100 μg/mL gentamicin for one hour and incubation of bacterial suspensions in a 65 ºC for 30 minutes were sufficient to kill bacteria and halt growth.

Secondly, an appropriate MOI needed to be determined. As discussed in section 4.1, increasing MOI increased proinflammatory markers until an MOI of 1000 where there were decreases in all three markers. As MOI increased, cell viability decreased. This increase in cell death associated with THP-1 macrophages stimulated with bacteria at MOI 1000 may have caused the lower levels of proinflammatory markers. This may be due to a survival mechanism by Hi. It has been shown that Hi may cause cell necrosis when phagocytosed by leukocytes to increase persistence in infection (Naylor et al. 2007). As such, an overwhelming amount of Hi may have caused necrosis to a greater degree with a MOI 1000. Interestingly, Hia 08-191 induced a higher level of ICAM-1 than other strains and NTHi induced a greater secretion of TNFα than other strains in this optimization experiment. This may have been due to natural variability in the experiment as similar results were not seen in other experiments with the strains. Further evidence for this is due to the fact that both ICAM-1 and TNFα are regulated by NFκB and differences between strains were not consistent among these parameters measured. Using this information and the results from the experiment, a MOI of 10 was established to be appropriate going forward due to its ability to increase inflammatory markers while limiting cell death.

Finally, confirmation of the inhibition of caspase-1 needed be conducted. Initially, caspase-1 concentration was measured using a Human Caspase-1/ICE Quantakine®

ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). However, it was found that caspase-1 was increased in cell supernatants treated with ac-YVAD-cmk (data not shown). Upon investigation into the mechanism of ac-YVAD-cmk, it was found that ac-YVAD-cmk inhibits caspase-1 activity by binding to the active site and thus measurement of downstream products needed to be conducted to confirm inhibitory action (Sigma-Aldrich: personal communication). All concentrations and incubations had a significant inhibitory effect on IL-1β production (**Figure 13**). Both longer incubation times and greater concentrations resulted in increased inhibitory effect (though only longer incubation times had significant effects). These results coincide with inhibitory action of ac-YVAD-cmk as seen in the literature and within our group (Lipinska et al. 2014, Chen et al. 2013, Anantharajah et al. 2015, Alhazmi 2018). The vehicle used for ac-YVAD-cmk dilution, DMSO, was also found to have an inhibitory effect on IL-1β secretion (**Figure 13**). DMSO has been shown to be able to inhibit the NLRP3 inflammasome. It has been theorized that DMSO may act as an ROS scavenger, preventing activation of inflammasome, and thus lower levels of IL-1β (Ahn et al. 2014). Interestingly, DMSO has been shown to inhibit NFκB and the ICAM-1 gene at *in vivo* indicating that DMSO may also inhibit inflammasome production at the genetic level, however, inhibition of ICAM-1 levels were not seen in this study (**Figure 11**, **Figure 15**) (Chang et al. 1999). Regardless, inhibitory action by ac-YVAD-cmk was much greater than that of DMSO and thus was concluded to be an appropriate inhibitor to test for inflammasome activity.

5.3 – Inflammasome Activation by Hia

The inflammasome is a multiprotein complex responsible for maturation of IL-1β and IL-18 and for initiating pyroptosis. It can be activated by a plethora of DAMPs (He et al. 2016, Sutterwala et al. 2014). Studies have found that several gram-negative bacteria are capable of activating the inflammasome. These include *Pseudomonas aeruginosa, Neisseria gonorrhoeae*, *Klebsiella pneumoniae*, and *Haemophilus ducreyi* (Deng et al. 2016, Duncan et al. 2009, Hua et al. 2015, Li et al. 2013). Recently, NTHi has also been shown to activate the NLRP3 inflammasome (Rotta Detto Loria et al. 2013). Therefore, the inflammasome may be involved in the inflammatory response to Hia infection. Our findings show that stimulation with both encapsulated and unencapsulated Hia strains increased IL-1β secretion. Moreover, this increase was attenuated by the caspase-1 inhibitor ac-YVAD-cmk without changes in either ICAM-1 levels or TNFα secretion. Interestingly, there were no differences among the encapsulated and unencapsulated Hia strains nor were there differences among either Hia strain and the unencapsulated, non-invasive NTHi 375. The innate immune system is capable of recognizing a wide variety of virulence factors and components of Hi via PRRs. These include recognition of peptidoglycan, lipoproteins, and porins by TLR2, LOS by TLR4, purified Hi DNA within the cytosol by TLR9 (though this may not be important in an inflammatory response to Hi due to low availability of cytosolic Hi DNA during infections), cytosolic peptidoglycan fragments by NOD1 and 2, and recognition of yet unknown components by TLR3 (Galdiero et al. 2004, Wieland et al. 2010, Mogenson et al. 2006, Teng et al. 2010, Zola et al. 2008, Lee et al. 2019). These all are capable of increasing nuclear translocation of NFκB and thus initiate signal 1 of the NLRP3 inflammasome. Lack of

differences among strains in activation of inflammasome may be due to similar levels of upregulation of inflammasome-related genes due to homogeneity of components among Hi strains. This differs from other encapsulated gram-negative bacteria like *Klebsiella pneumoniae* where lack of capsular polysaccharide in mutant strains lead to a significant decrease in IL-1β secretion in monocytes when compared to wild type strains (Hua et al. 2015).

Though activation of signal 1 is important in NLRP3 inflammasome activation, DAMPs must be recognized by the cell in order for signal 2 activation and thus activation of the inflammasome. As there were no significant differences in IL-1β secretion among strains in this study, the Hi capsule may not play a role in initiating DAMPs. NTHi has been shown to activate the NLRP3 inflammasome through K^+ efflux, lysosomal damage, and ROS production (Rotta Detto Loria et al. 2013). However, there is another DAMP associated with NLRP3 activation, specifically Ca^{2+} flux within the cell. Intracellular calcium is reduced in patients infected with Hi (due to the calcium requirement for recognition, internalization, and killing of pathogens). However, when calcium was added to monocyte-derived macrophages of COPD patients stimulated with NTHi, it led to increases in secretion of various cytokines including IL-1β (Provost et al. 2015). This could be due to increase calcium flux causing NLRP3 activation and maturation of IL-1β via caspase-1 though no studies have been conducted testing the importance of calcium in inflammasome activation to Hi specifically. As such, more research is needed in this regard. NLRP3 inflammasome activation is the consequence of multiple different factors due to its two-hit nature. As such, differences in in IL-1β secretion due to the capsule might be masked due to the large number of redundancies within both signal 1 and 2. As

such, further studies on the effect of Hi capsule and other components of Hi on individual components of both signal 1 and 2 of NLRP3 inflammasome (both PRRs and DAMPs) may be required to unmask differences in activation.

Though NLRP3 inflammasome has been implicated in NTHi infection, other inflammasomes might also play a role (Rotta Detto Loria et al. 2013). AIM2 inflammasome, like NLRP3, is a two-hit protein complex. The first signal is dependent on type I interferon production and the second signal is dependent on DNA within the cytosolic compartment (Lugrin and Martinon 2017). NTHi has recently been shown to both elicit these interferons and upregulate AIM2 proteins (Yang et al. 2019, Chen et al. 2018). As such, AIM2 might also play a role in Hia infection. Another inflammasome that has been implicated in NTHi infection is the non-canonical caspase-4/5/11 inflammasome. After recognition of LPS/LOS by TLR4 or RNA by TLR3, the TRIF pathway will be activated leading to caspase oligomerization, K^+ efflux, and gasdermin D cleavage inducing pyroptosis and NLRP3 activation (Rathinam et al. 2012, Yi 2018, He et al. 2016). Rathinam et al. (2012) found that the TRIF pathway and caspase 11 were crucial in a mouse model of NTHi infection. It is theorized that LOS from outer membrane vesicles of Hi endocytosed by phagocytes and induction of type I interferon play a major role in the activation of this non-canonical inflammasome (Vanaja et al. 2016, Rathinam et al. 2012). As such, the non-canonical inflammasome may play a role in Hia infection through TLR3 and TLR4 activation. There is a wide array of inflammasomes. Lack of differences between the strains in this study may be due to differential activation of various inflammasomes. As such, studies involving

measurement of various inflammasome proteins are needed to fully elucidate potential differences in these strains on activation of different inflammasome complexes.

Using this knowledge this is a proposed model for inflammasome activation by Hi (**Figure 28**). Upon infection of host by Hi, various PRRs will recognize extracellular Hi. Specifically, LOS will be recognized by TLR4, peptidoglycan, lipoproteins, and porins will be recognized by TLR2. Upon internalization, peptidoglycan fragments will be recognized by NOD1 and 2 and a currently unknown component of Hi will activate TLR3. Activation of TLR3 and TLR4 will activate the TRIF pathway leading to non-canonical inflammasome formation. This activation will result in K^+ efflux and non-caspase-1 mediated IL-1β secretion and pyroptosis. Upon activation of the other PRRs, various signal pathways will converge on IκBα phosphorylation by IKK allowing NFκB nuclear translocation. NFκB within the nucleus will transcribe a plethora of proteins including those required for the NLRP3 and AIM2 inflammasomes. Internalized Hi will lead to DAMPs such as K^+ efflux, ROS production, and lysosomal damage. These signals will activate the NLRP3 inflammasome. Hi DNA in the cytosolic compartment will activate the AIM2 inflammasome. Activation of these inflammasomes will result in cleavage of procaspase-1 to caspase-1 which will cleave gasdermin D, pro-IL-18, and pro-IL-1β. This will result in pyroptosis as well as both systemic and local effects of the matured cytokines.

5.4 – Heat Inactivation of Hi and the Inflammasome

Along with evidence for inflammasome activation by Hia, we also found that, when normalized to the IL-1β secretion of macrophages stimulated with viable bacteria,

heat inactivated bacteria induced significantly reduced IL-1β secretion in THP-1 macrophages (averaging a 45% decrease among strains). This effect was mirrored in TNFα secretion; however, ICAM-1 and cell viability were not affected by heat inactivation of Hi. This indicates that heat inactivation of bacteria may have affected immunostimulatory activity of the bacteria in general and not only affected its ability to activate the inflammasome. This is interesting as it contradicts most studies showing that NTHi and *Haemophilus ducreyi* are unable to activate the inflammasome in any appreciable capacity when heat inactivated (Rotta Detto Loria et al. 2013, Li et al. 2013). However, TNFα secretion in these studies was also reduced to a minor degree similar to this study. Though Li et al. used a similar heat inactivation method (60ºC for one hour prior to stimulation), Rotta Detto Loria et al. used a more aggressive heat inactivation method involving multiple rounds of freeze-thaw between temperatures of -80ºC and 60ºC. Bacterial RNA has been identified as a labile PAMP that is capable of signaling viability of the pathogen to the innate immune system (known as viability-associated or vita-PAMPs) (Sander et al. 2011). When this RNA reaches the cytosolic compartment, it can activate the NLRP3 inflammasome (Sander et al. 2011). As such, Hi RNA may have still been active to some degree in heat inactivated treatments and thus produced the IL-1β release seen in our results. A more aggressive inactivation protocol (as seen in Rotta Detto Loria et al. 2013) may inactivate Hi RNA to a greater degree resulting in lower IL-1β secretion. TNFα secretion is controlled by NFκB nuclear translocation (i.e., activation of PRRs and cytokine receptors) (Parameswaran and Patial 2010, Liu et al. 2017). There were similar differences in TNFα and IL-1β, therefore, these changes may have been due to differences in PRR and cytokine receptor activity. Specifically, heat inactivation may

have affected more than bacterial RNA and destroyed other bacterial components resulting in reduced recognition of Hi by macrophages.

Reductions in IL-1β and TNFα secretion were not consistent among strains in macrophages stimulated with heat inactivated bacteria. Specifically, heat inactivated Hia 08-191 (the encapsulated strain) conserved a significantly higher proportion of cytokine release compared to both unencapsulated strains (Hia 13-0074 and NTHi 375). As such, the capsule may have provided some thermo-protective effect on components of Hi. However, ICAM-1 is also mediated by NFκB and there were no differences in ICAM-1 levels in macrophages stimulated with heat inactivated or viable bacteria in this study (Roebuck and Finnegan 1999). Clearly, more work needs to be done to elucidate the exact effect of heat inactivation on activation of the inflammasome and the inflammatory response by Hia.

5.5 – Limitations and Future Studies

One of the largest limitations in this study is the use of the *in vitro* model. The innate immune system utilizes many different cell types to recognize and mount responses to pathogens. Moreover, the interaction between some of these cells may be crucial in the response to bacterial pathogens such as Hi. As such, the expansion of the ideas presented here through the use of other cell types such as dendritic cell lines and peripheral blood monocytes in *in vitro* as well as *in vivo* models may be important in understanding the true effect of encapsulated Hi on the innate immune system. Another limitation to this study was that due to time constraints, more replicates could not be obtained. Variability (especially in the heat-inactivated experiments) and the use of

ANOVA with low statistical power was a direct result of this. To further legitimize these results, more replicates are needed. With additional time, a more aggressive bacterial inactivation could also be used to ensure minimal bacterial RNA.

Though this study did elucidate the ability for encapsulated strains to activate the inflammasome complex, it did not state definitively the role of Hi capsular polysaccharide in this pathway. The use of purified polysaccharide capsule (free of any LOS or other virulence factor contamination) could shed more light on their role in inflammasome activation especially if compared to purified LOS. Furthermore, while this study provides evidence for activation of inflammasome by Hia, it did not reveal the specific inflammasomes involved in the recognition of encapsulated and unencapsulated invasive strains of Hi. Further analysis with measurement of different proteins associated with NLRP3, AIM2, and the non-canonical caspase 4/5/11 inflammasomes via western blotting would need to be performed to confirm involvement of these inflammasomes.

6 – Conclusions

To summarize, the aim of this study was to fill the gaps in our knowledge on Hia induced inflammatory response mediated by the innate immune system. Although there have been multiple studies regarding Hia on a clinical and epidemiological level (e.g., Ulanova and Tsang 2009, Nix et al. 2015, Nix et al. 2018, Cerqueira et al. 2019, Gaultier et al. 2019, and Kubinec et al. 2020), there were no studies found relating to the pathogenesis of Hia through the innate immune system. This is an important area of study due to severe invasive Hia disease presenting as inflammation such as meningitis and pneumonia (Ulanova and Tsang 2014). Though NTHi has been shown to activate the inflammasome, not much is known about the role of the polysaccharide capsule in innate immunity, the most important virulence factors of encapsulated strains (Rotta Detto Loria et al. 2013, Moxon and Kroll 1990). This study revealed that both encapsulated and unencapsulated invasive Hia strains were capable of inducing production of mature IL-1β in a capsase-1 dependent manner indicative of inflammasome activation. Interestingly, there were no significant differences between these two Hia strains indicating that the polysaccharide capsule may not play a significant role in inflammasome activation. Furthermore, though there were differences in mature IL-1β production in macrophages stimulated with viable or heat-inactivated Hi when the data was normalized, these differences may have been due to reduction in overall immunostimulatory activity of heat inactivated Hi (as seen by mirrored differences in TNFα secretion). Interestingly, encapsulated Hia showed proportionally more IL-1β and TNFα secretion than the other two strains. This may be due to the presence of capsule providing some form of thermostability. The results of this study are an important first step in understanding how

Hia interacts with the innate immune system and causes an inflammatory response and paves the way for more in-depths studies looking at the inflammatory response to Hia.

Figures

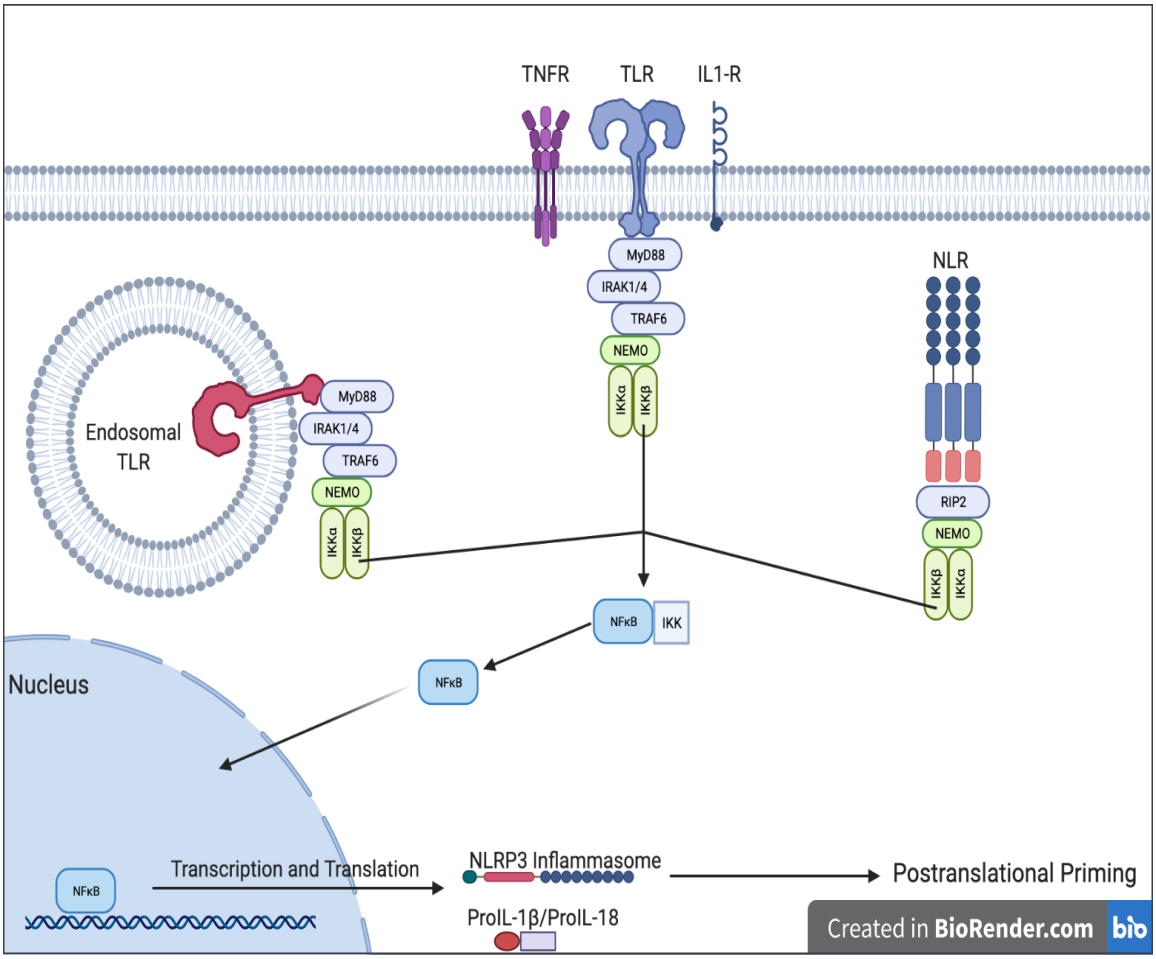


Figure 1: Signal 1 of the NLRP3 Inflammasome

Various signals can result in stimulation of the production of NLRP3 inflammasome components (signal 1 of NLRP3 inflammasome activation). These signals culminate in the production of IKK, which dephosphorylates IKβ, an inhibitor of NFκB. NFκB can then translocate to the nucleus where, as a transcription factor, it can transcribe the genes for the components of the NLRP3 inflammasome, such as procaspase-1, and the inactive cytokines pro-IL-1β and pro-IL-18. Receptors capable of initiating the signal cascade for NFκB production include TLRs, NLRs, TNFRs, and IL-1Rs. Upon recognition of its various ligands, TLRs activate the MyD88-dependent resulting in IKK production.

NLRs, upon recognition of peptidoglycan fragments, will activate the RIP2 pathway and produce IKK. TNFR recognizes TNFα and activates the TRADD pathway to produce IKK. Finally, upon recognition of IL-1β by IL-1R, the IRAK4 pathway will produce IKK. Basal NLRP3 inflammasome component levels are too low prior to activation of signal 1. Without the increased production of these components, the inflammasome response to DAMPs would be severely dampened (He et al 2016, Sutterwala et al 2014).

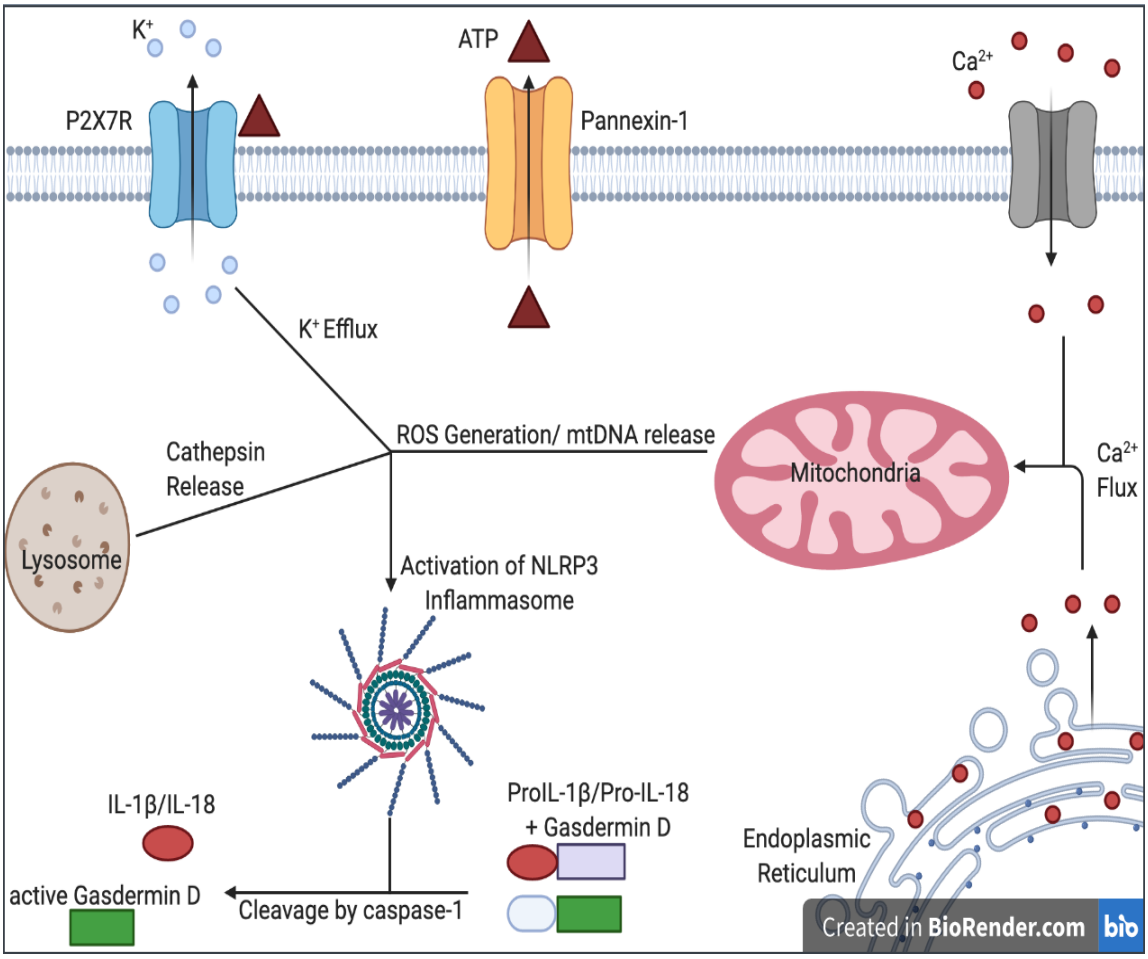


Figure 2: Signal 2 of the NLRP3 Inflammasome

The exact mechanism of NLRP3 signal 2 activation appears to be mediated by various DAMPs. Most literature indicates that efflux of K^+ from the cytosol drives activation. Cell death and LPS-mediated pannexin-1 channel opening can lead to ATP release from the cell (Ayna et al. 2012, He et al. 2016). The ATP is able to open the P2X7R K^+ channel resulting in the efflux of the cation. This efflux is detected by the NLRP3 inflammasome resulting in activation (He et al. 2016). Another cation that may play a role in NLRP3 activation is Ca^{2+}. Ca^{2+} flux into the cytosol from both outside of the cell and from within the endoplasmic reticulum have been associated with NLRP3 activation. Specifically, calcium sensing receptors may sense elevated Ca^{2+} and decrease cAMP

levels (an inhibitor of NLRP3) while also promoting assembly of the inflammasome complex (Lee et al. 2012). Ca^{2+} may also promote mitochondrial dysfunction and ROS production (Muñoz-Planillo et al. 2013). The exact mechanism behind the role of the mitochondria in NLRP3 activation is unclear. Release of mitochondrial DNA or ROS may cause cascades resulting in ASC molecules moving closer to the NLRP3 (Shimada et al. 2012, Misawa et al. 2013). The final DAMP that may play a role in NLRP3 activation is lysosomal damage resulting in cathepsin release though this may be through activating K^+ efflux (He et al. 2016, Chu et al. 2009). These various DAMPs may play a role in NLRP3 activation leading to the maturation of cytokines IL-18 and IL-1β and activation of gasdermin D.

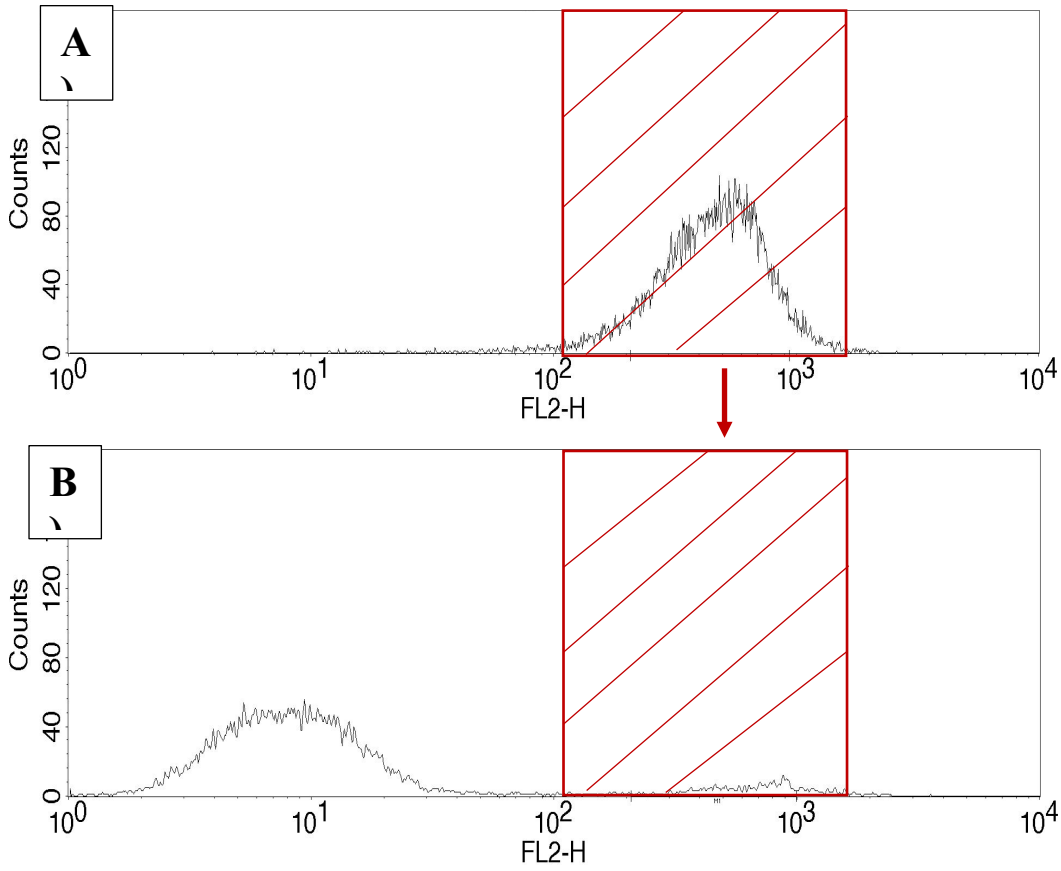


Figure 3: Propidium Iodide Can be Used to Measure Cell Viability Using Flow Cytometry

Live cells exclude PI from entering their membranes. However, in dead cells, PI is able to penetrate cellular membranes where it can intercalate with the DNA. Once bound to the DNA, the PI can be excited by specific wavelengths of light and fluoresces (Coder 2001). This fluorescence was detected to calculate cell viability. To establish fluorescence level of dead cells, THP-1 macrophages were incubated at 56 ºC for 30 minutes prior to incubation with 1 µg/mL PI for one minute and flow cytometry of 10 000 gated events (FL-2 channel) (**A**) (Incubation at 65 ºC as used in bacterial killing resulted in complete cell lysis and accurate readings were unobtainable on the flow cytometer). The results were expressed as mean fluorescence intensity. Cell death was confirmed using Trypan

Blue exclusion assays with a hemocytometer. The histogram produced with the dead cells established a fluorescent range where cells permeated by PI fluoresced (hatched area). Using the fluorescent range established in (**A**), the number of PI permeated (i.e., dead) cells could be established for subsequent macrophages used in experiments. An example of this being used is shown in (**B**) where calculation of cell viability of THP-1 macrophages incubated with 200 μL PBS for 18 hours is shown. The number of PI permeated cells could be divided by the number of live cells to determine the percent of dead cells.

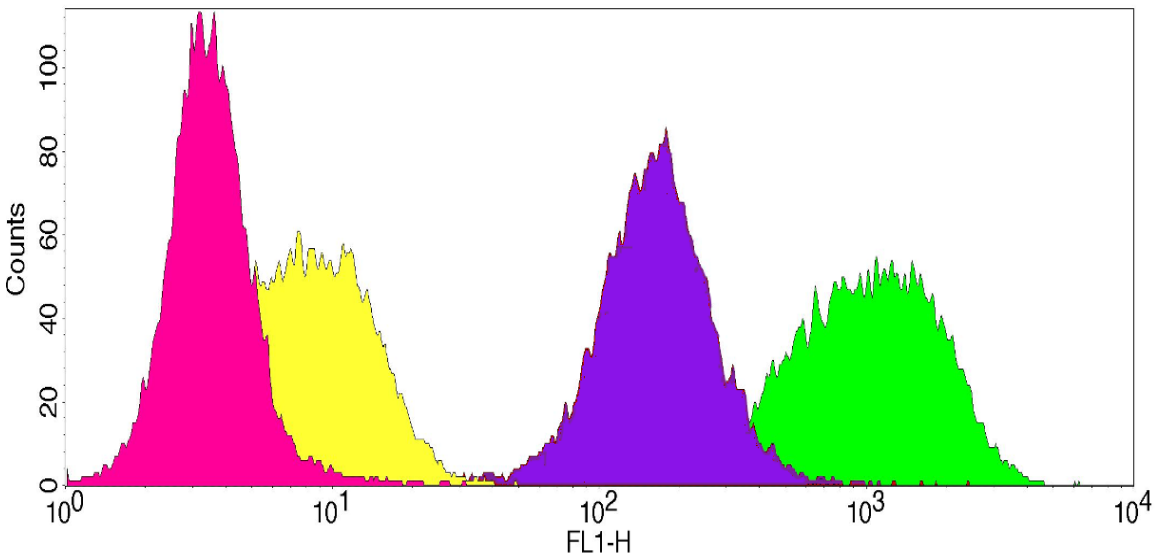


Figure 4: Incubation with 20 ng/mL PMA for 24 Hours is Sufficient to Differentiate THP-1 Monocytes to Macrophages

THP-1 monocytes were incubated with either 20 ng/mL PMA or 0.2% DMSO for 24 hours in RPMI 1640 medium as described in section 3.7.1. After 48 hours, cells were harvested and stained with 1 µg/mL human Alexa Fluor 488-conjugated anti-mouse CD11b antibody. Unstained cells were incubated PBS. CD11b levels were assessed via flow cytometry using 10, 000 gated events (FL-1 channel). Histograms depicting mean fluorescence intensity of one experiment were overlaid on one another. Pink represents unstained monocytes incubated with 0.2% DMSO, yellow represents unstained monocytes incubated with 20 ng/mL PMA, purple represents monocytes incubated with 0.2% DMSO stained with CD11b antibody, and green represents monocytes incubated with 20 ng/mL PMA for 24 hours and stained withcCD11b antibody. This was process was repeated two additional times with similar results.

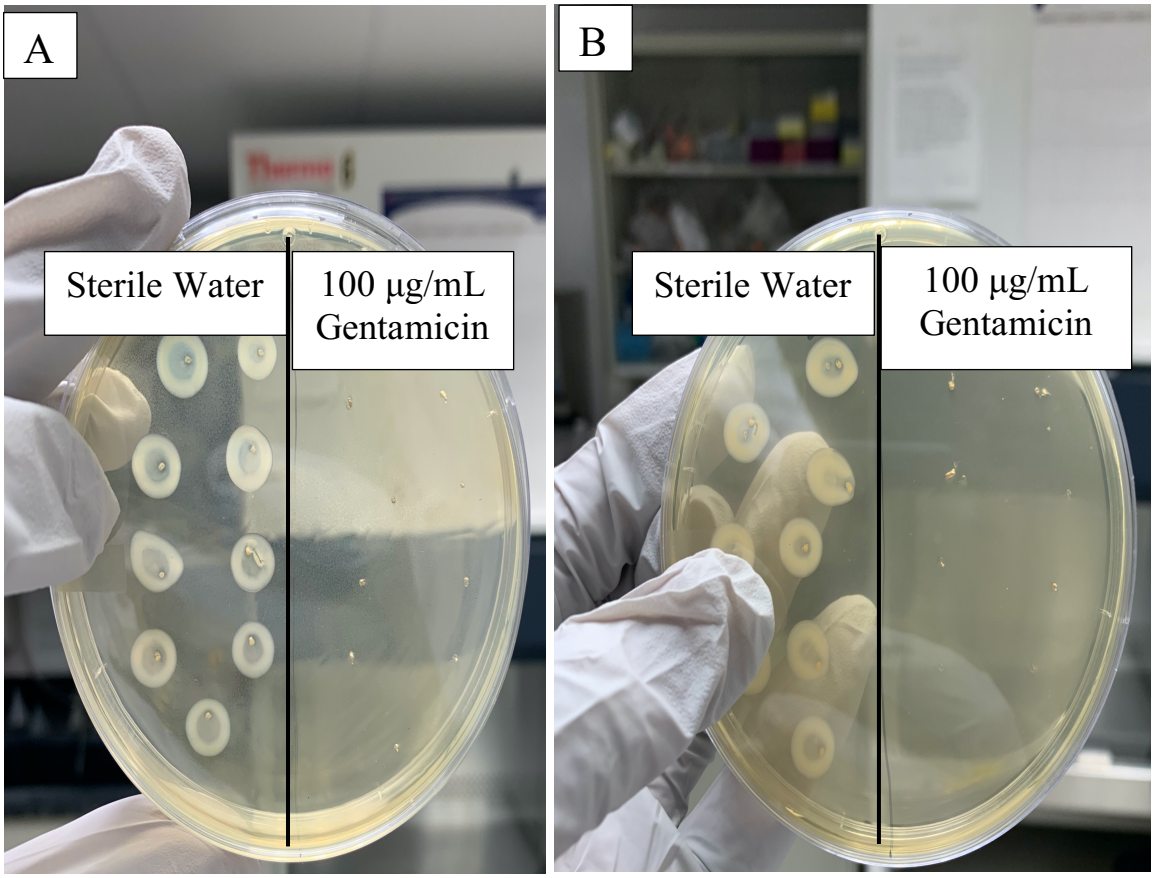


Figure 5: The Inactivating Effect of Gentamicin on Hi

Approximately $5x10^8$ Hia 08-191 bacteria were added to 2 mL of either BHI broth supplemented with 10 µg/mL hemin chloride and 5 µg/mL NAD or RPMI 1640 media supplemented with 10% FBS upon reaching mid-log phase. After one hour of incubation at 37ºC in 5% CO_2, 100 µg/mL gentamicin or vehicle (sterile water) was added. Bacteria was further incubated 16 hours. Contents of wells were collected, centrifuged, and resuspended in fresh BHI broth supplemented with 10 µg/mL hemin chloride and 5 µg/mL NAD and drop-plated on BHI-agar plates supplemented with 10 µg/mL hemin chloride and 5 µg/mL NAD. These were incubated at 37ºC in 5% CO_2 for 16 hours and growth was assessed by presence or absence of bacterial colonies. There were no signs of

growth in bacteria incubated with gentamicin in either supplemented BHI broth (**A**) or supplemented RPMI media (**B**). This process was repeated with Hia 13-0074 and NTHi 375. No growth was seen for either bacterial strain.

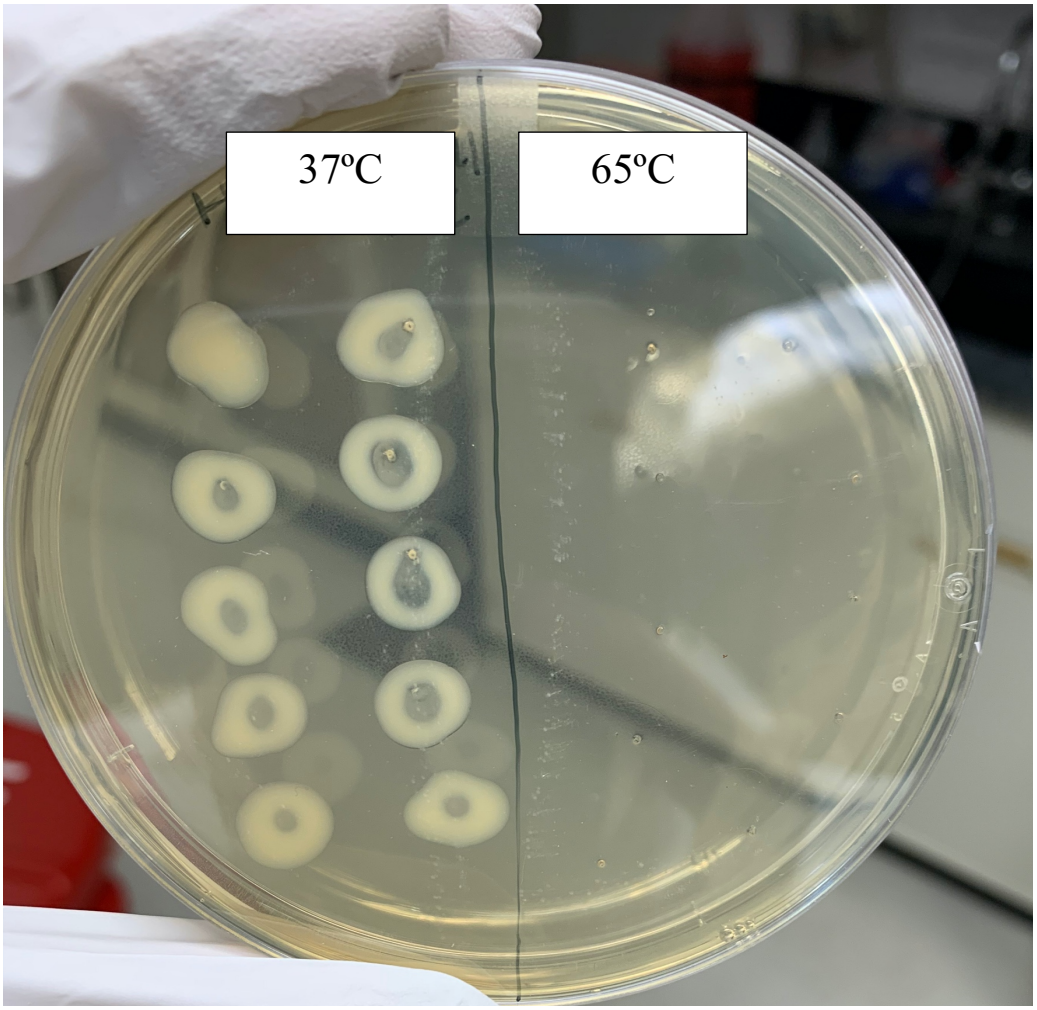


Figure 6: The Inactivating Effect of Heat on Hi

Hia 08-191 was grown to mid-log phase in BHI broth supplemented with 10 µg/mL hemin chloride and 5 µg/mL NAD and diluted to 0.1 OD600. This was then incubated at 65ºC or 37ºC for 30 minutes. Bacteria were drop plated onto a supplemented BHI-agar plate and incubated for 16 hours and growth was assessed by presence or absence of bacterial colonies. No growth was seen in heat inactivated bacteria. This process was repeated with Hia 13-0074 and NTHi 375. There were no signs of growth found for either

bacterial strain when heat inactivated. This process was also repeated with incubation at 60ºC for one hour with similar results.

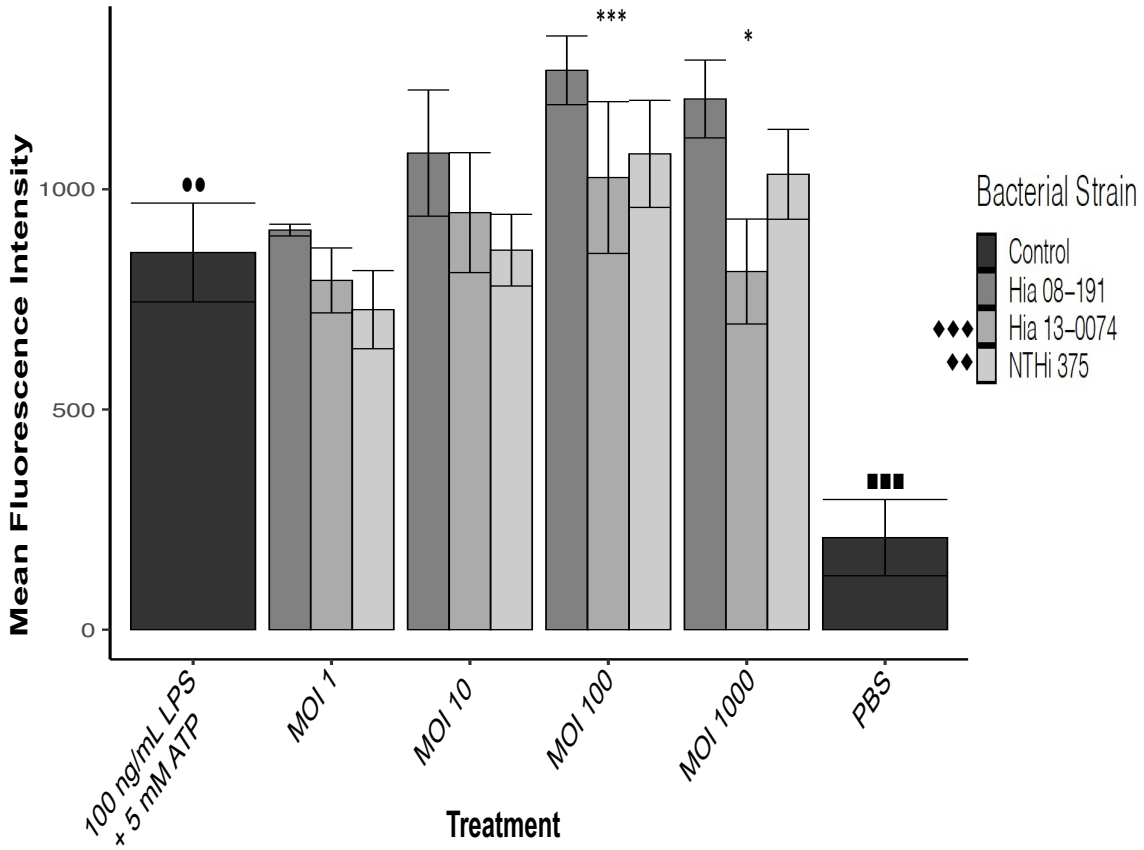


Figure 7: The Effect of Multiplicity of Infection (MOI) on ICAM-1 Levels of THP-1 Macrophages

THP-1 macrophages were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOIs of 1, 10, 100, or 1000; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.7.2.2. ICAM-1 levels were assessed in harvested cells incubated with 1 μg/mL mouse-antihuman phycoerythrin-conjugated ICAM-1 antibody for one hour via flow cytometry using 10,000 gated events (FL-2 channel). The results were expressed as mean fluorescence intensity. Bars represent mean ± SD of three independent experiments. ■■■ $p<0.001$, difference compared to samples stimulated with bacteria or LPS/ATP. * $p<0.05$, ***

p<0.001, difference compared to MOI 1. ●●● p<0.001, difference compared to MOI 100.

◆◆ p<0.01, ◆◆◆ p<0.001, difference compared to Hia 08-191 at all MOIs.

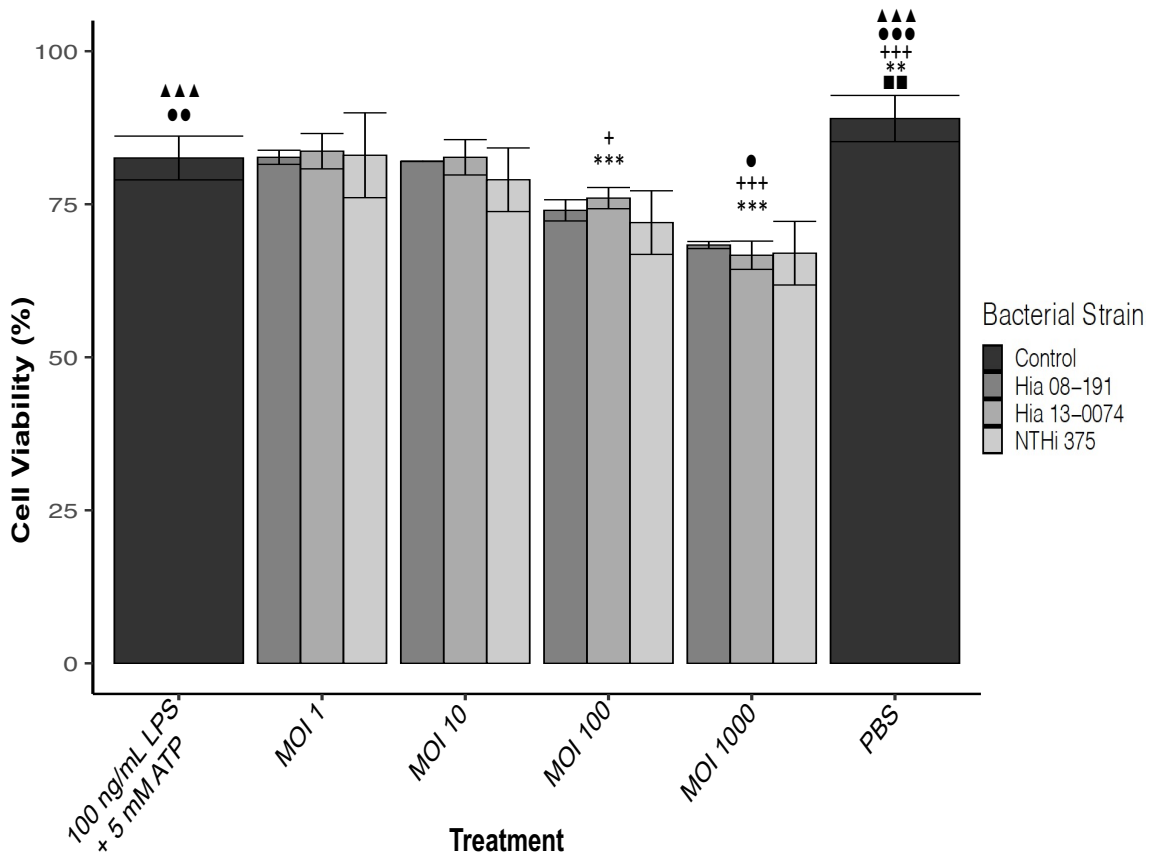


Figure 8: The Effect of Multiplicity of Infection (MOI) on Viability of THP-1 Macrophages

THP-1 macrophages were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOIs of 1, 10, 100, or 1000; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.7.2.2. Cell viability was assessed in harvested cells incubated with 1 µg/mL PI for one minute via flow cytometry using 10,000 gated events (FL-2 channel). Bars represent mean ± SD of three independent experiments. ■■■ $p<0.001$, difference compared to samples stimulated with LPS/ATP. ** $p<0.01$, *** $p<0.001$, difference compared to MOI 1. + $p<0.05$, +++ $p<0.001$, difference compared to MOI 10. ● $p<0.05$, ●●● $p<0.001$, difference compared

to MOI 100. ▲▲▲ $p<0.001$, difference compared to MOI 1000. There were no significant differences among strains on cell viability.

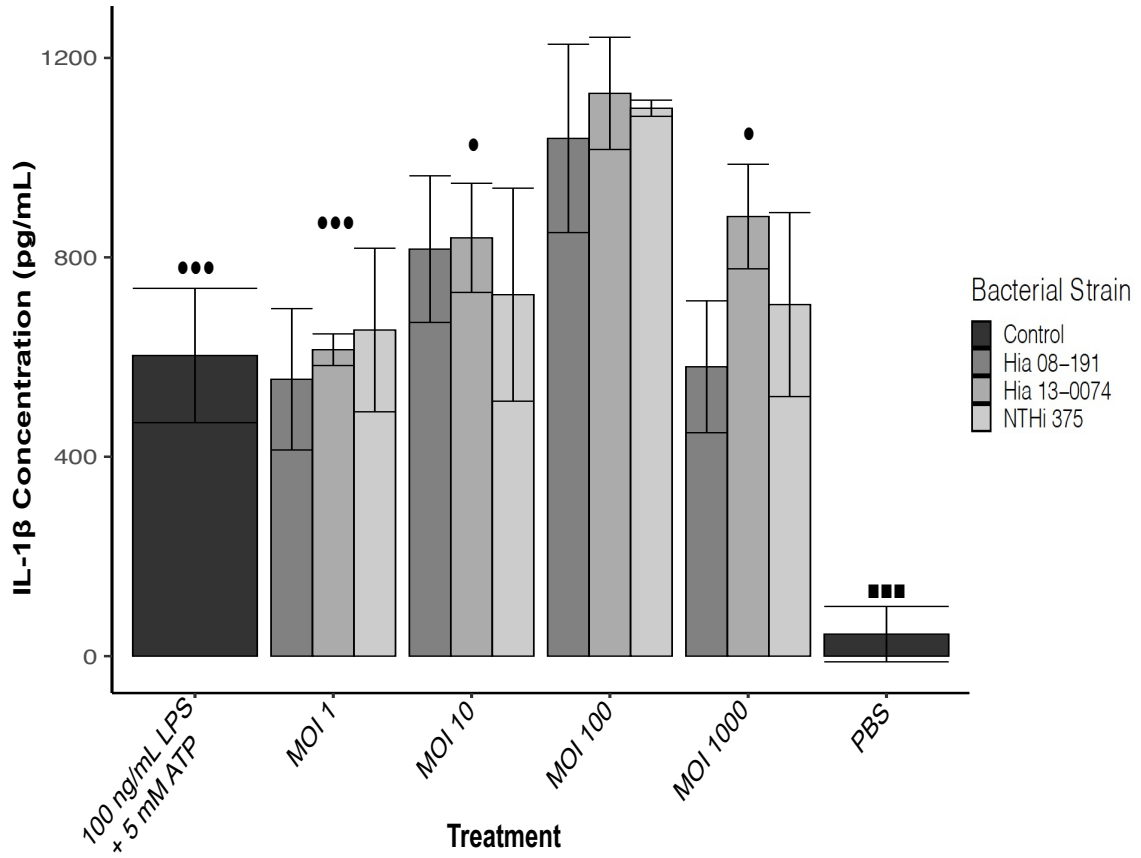


Figure 9: The Effect of Multiplicity of Infection (MOI) on IL-1β Secretion by THP-1 Macrophages

THP-1 macrophages were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOIs of 1, 10, 100, or 1000; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.7.2.2. IL-1β concentration in cell supernatants was measured with ELISA. Bars represent mean ± SD of three independent experiments. ■■■ $p<0.001$, difference compared to samples stimulated with bacteria or LPS/ATP. ● $p<0.05$, ●●● $p<0.001$, difference compared to MOI 100. There were no significant differences among strains on IL-1β secretion.

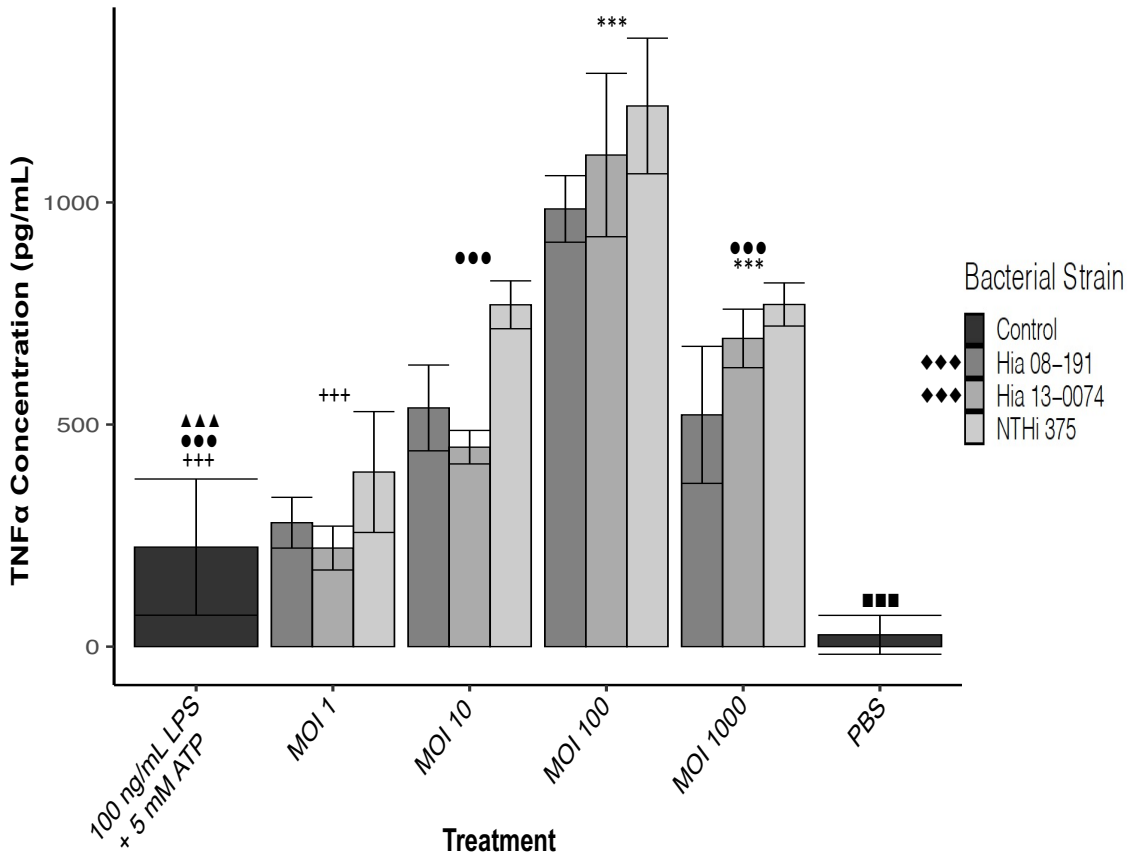
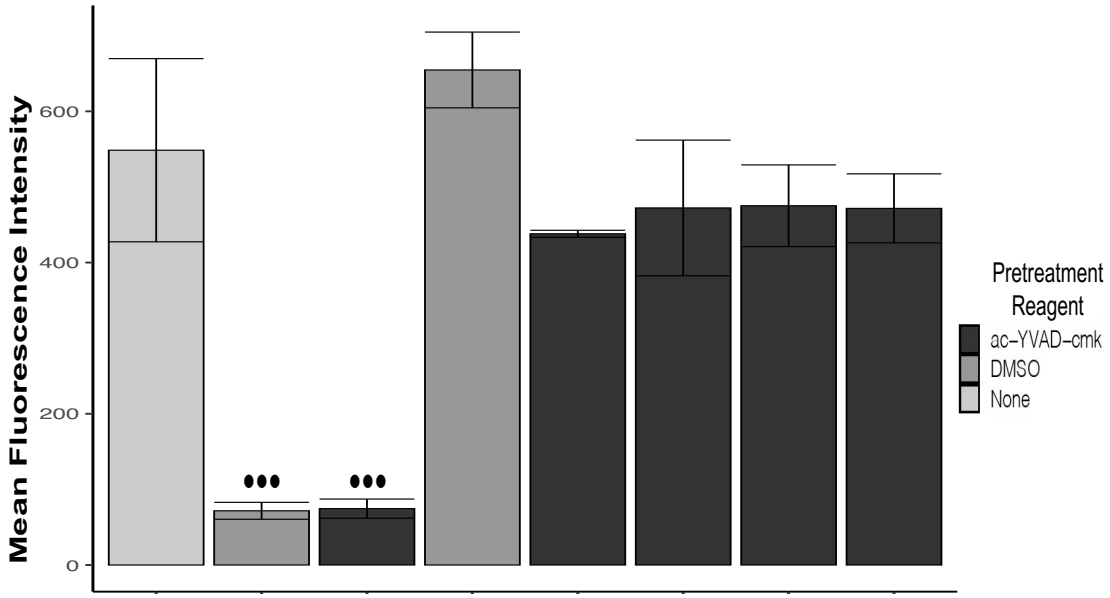


Figure 10: The Effect of Multiplicity of Infection (MOI) on TNFα Secretion by THP-1 Macrophages

THP-1 macrophages were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOIs of 1, 10, 100, or 1000; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.7.2.2. TNFα concentration in cell supernatants was measured with ELISA. Bars represent mean ± SD of three independent experiments. ■■■ $p<0.001$, difference compared to samples stimulated with bacteria or LPS/ATP. *** $p<0.001$, difference compared to MOI 1. +++ $p<0.001$, difference compared to MOI 10. ●●● $p<0.001$, difference compared to MOI 100. ▲▲▲ $p<0.001$, difference compared to MOI 1000. ◆◆◆ $p<0.001$, difference compared to NTHi 375 at all MOIs.

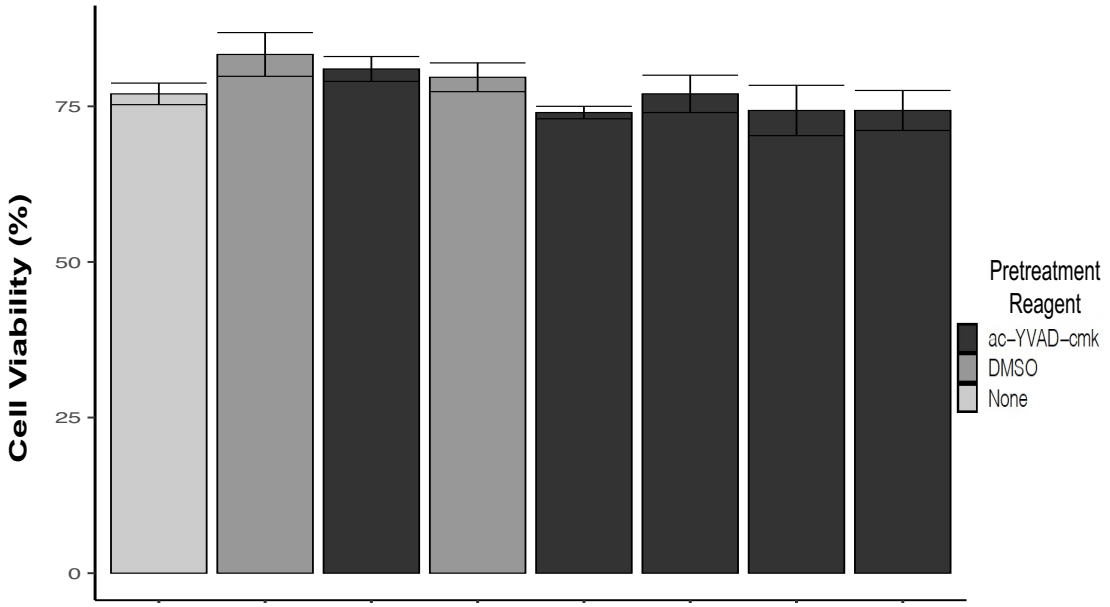


Pre-treatment Time	-	24h	24h	24h	24h	24h	5h	5h
ac-YVAD-cmk (μM)	0	0	100	0	40	100	40	100
DMSO (%)	0	1	1	1	0.4	1	0.4	1
LPS/ATP	+	-	-	+	+	+	+	+
PBS	-	+	+	-	-	-	-	-

Figure 11: The Effect of ac-YVAD-cmk Concentration on ICAM-1 Levels of THP-1 Macrophages

THP-1 macrophages were pretreated with either 0% DMSO, 1% DMSO, 40 μM ac-YVAD-cmk, or 100 μM ac-YVAD-cmk for either five hours or 24 hours and stimulated with 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes) or incubated with PBS for 18 hours as described in section 3.7.3. ICAM-1 levels were assessed in harvested cells incubated with 1 μg/mL mouse-antihuman phycoerythrin-conjugated ICAM-1 antibody for one hour via flow cytometry using 10,000 gated events (FL-2 channel). The results were expressed as mean fluorescence intensity. Bars represent mean ± SD of three

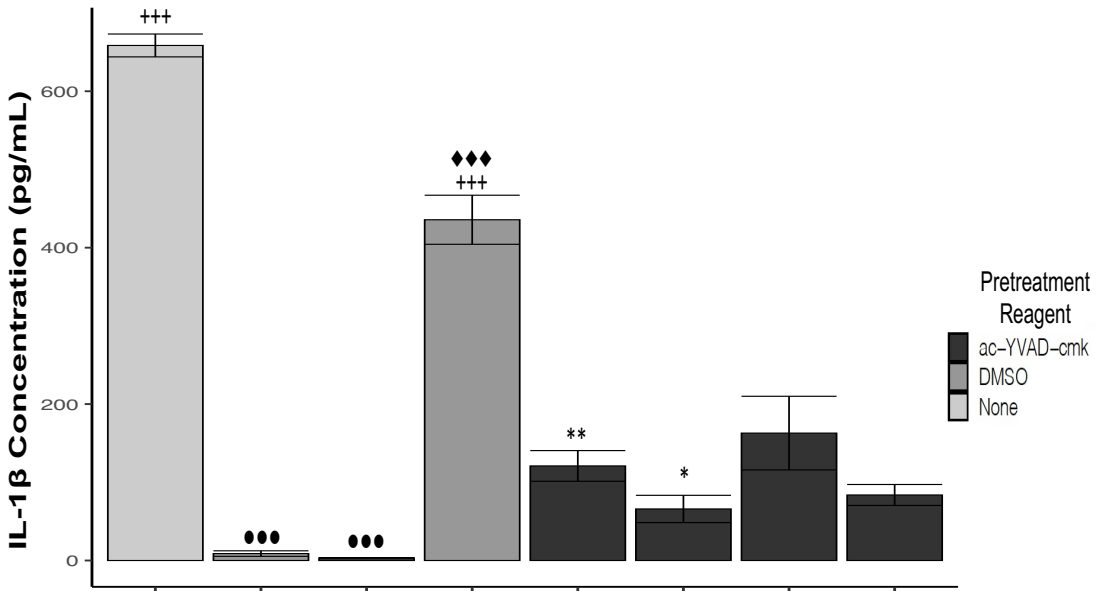
independent experiments. ●●● $p<0.001$, difference compared to samples stimulated with LPS/ATP.



Pre-treatment Time	-	24h	24h	24h	24h	24h	5h	5h
ac-YVAD-cmk (μM)	0	0	100	0	40	100	40	100
DMSO (%)	0	1	1	1	0.4	1	0.4	1
LPS/ATP	+	-	-	+	+	+	+	+
PBS	-	+	+	-	-	-	-	-

Figure 12: The Effect of ac-YVAD-cmk Concentration on Viability of THP-1 Macrophages

THP-1 macrophages were pretreated with either 0% DMSO, 1% DMSO, 40 μM ac-YVAD-cmk, or 100 μM ac-YVAD-cmk for either five hours or 24 hours and stimulated with 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes) or incubated with PBS for 18 hours as described in section 3.7.3. Cell viability was assessed in harvested cells incubated with 1 μg/mL PI for one minute via flow cytometry using 10,000 gated events (FL-2 channel). Bars represent mean ± SD of three independent experiments.

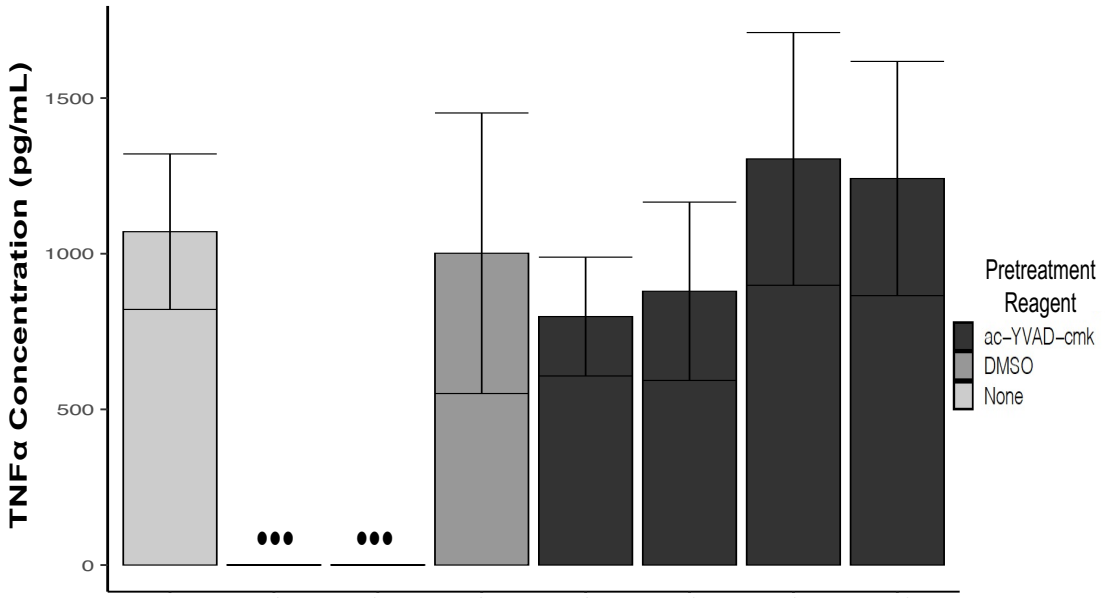


Pre-treatment Time	-	24h	24h	24h	24h	24h	5h	5h
ac-YVAD-cmk (μM)	0	0	100	0	40	100	40	100
DMSO (%)	0	1	1	1	0.4	1	0.4	1
LPS/ATP	+	-	-	+	+	+	+	+
PBS	-	+	+	-	-	-	-	-

Figure 13: The Effect of ac-YVAD-cmk Concentration on IL-1β Secretion by THP-1 Macrophages

THP-1 macrophages were pretreated with either 0% DMSO, 1% DMSO, 40 μM ac-YVAD-cmk, or 100 μM ac-YVAD-cmk for either five hours or 24 hours and stimulated with 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes) or incubated with PBS for 18 hours as described in section 3.7.3. IL-1β concentration in cell supernatants was measured with ELISA. Bars represent mean ± SD of three independent experiments. * $p<0.05$, ** $p<0.01$, difference compared to one hour pretreatment at the same concentration. ●●● $p<0.001$, difference compared to samples stimulated with LPS/ATP. ++ $p<0.01$, +++ $p<0.001$, difference compared to samples pretreated with ac-YVAD-cmk

and stimulated with LPS/ATP. ◆◆◆ $p<0.001$, difference compared to samples not pretreated with any reagent.



Pre-treatment Time	-	24h	24h	24h	24h	24h	5h	5h
ac-YVAD-cmk (μM)	0	0	100	0	40	100	40	100
DMSO (%)	0	1	1	1	0.4	1	0.4	1
LPS/ATP	+	-	-	+	+	+	+	+
PBS	-	+	+	-	-	-	-	-

Figure 14: The Effect of ac-YVAD-cmk Concentration on TNFα Secretion by THP-1 Macrophages

THP-1 macrophages were pretreated with either 0% DMSO, 1% DMSO, 40 μM ac-YVAD-cmk, or 100 μM ac-YVAD-cmk for either five hours or 24 hours and stimulated with 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes) or incubated with PBS for 18 hours as described in section 3.7.3. TNFα concentration in cell supernatants was measured with ELISA. Bars represent mean ± SD of three independent experiments. ●●● $p<0.001$, difference compared to samples stimulated with LPS/ATP.

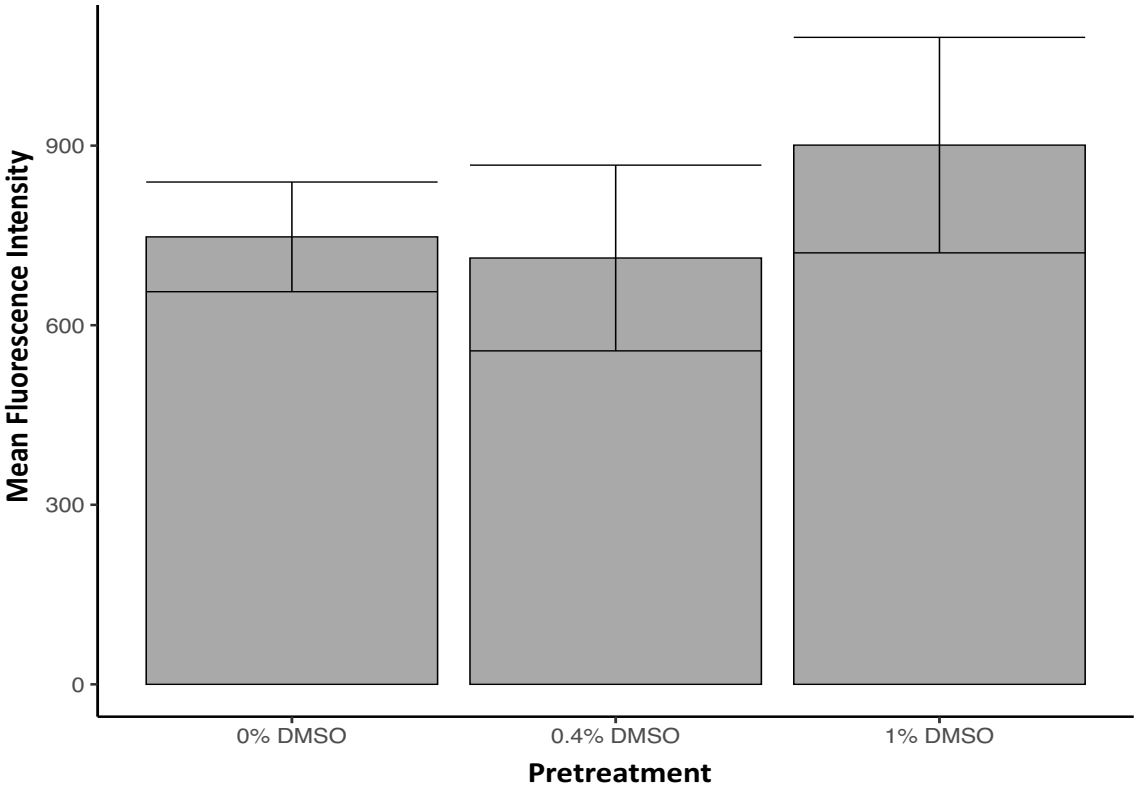


Figure 15: The Effect of DMSO Concentration on ICAM-1 Levels of THP-1 Macrophages

THP-1 macrophages were pretreated with either 0%, 0.4% or 1% DMSO for 24 hours and stimulated with 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes) for 18 hours as described in section 3.7.3. ICAM-1 levels were assessed in harvested cells incubated with 1 µg/mL mouse-antihuman phycoerythrin-conjugated ICAM-1 antibody for one hour via flow cytometry using 10,000 gated events (FL-2 channel). The results were expressed as mean fluorescence intensity. Bars represent mean ± SD of three independent experiments.

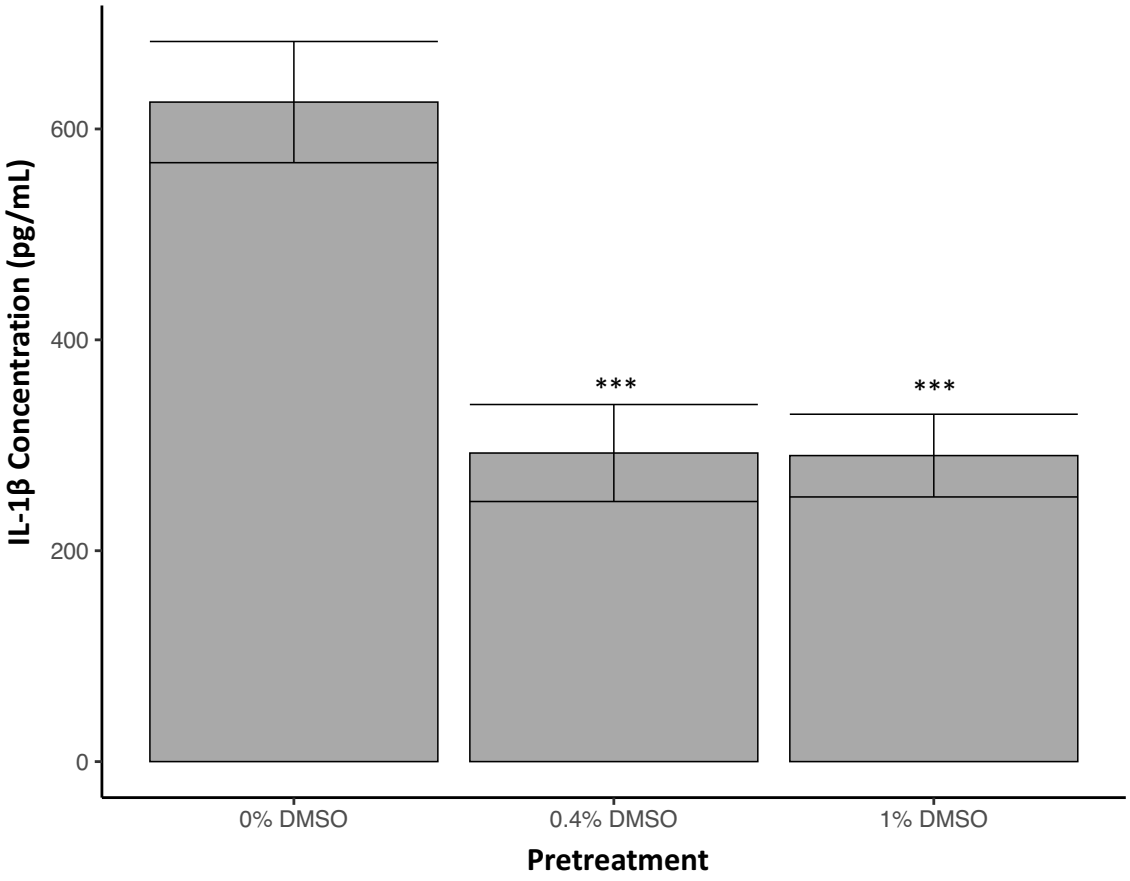


Figure 16: The Effect of DMSO Concentration on IL-1β Secretion by THP-1 Macrophages

THP-1 macrophages were pretreated with either 0%, 0.4% or 1% DMSO for 24 hours and stimulated with 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes) for 18 hours as described in section 3.7.3. IL-1β concentration in cell supernatants was measured with ELISA. Bars represent mean ± SD of three independent experiments. *** $p<0.001$ indicates significant decrease of IL-1β concentration compared to samples not pretreated with DMSO.

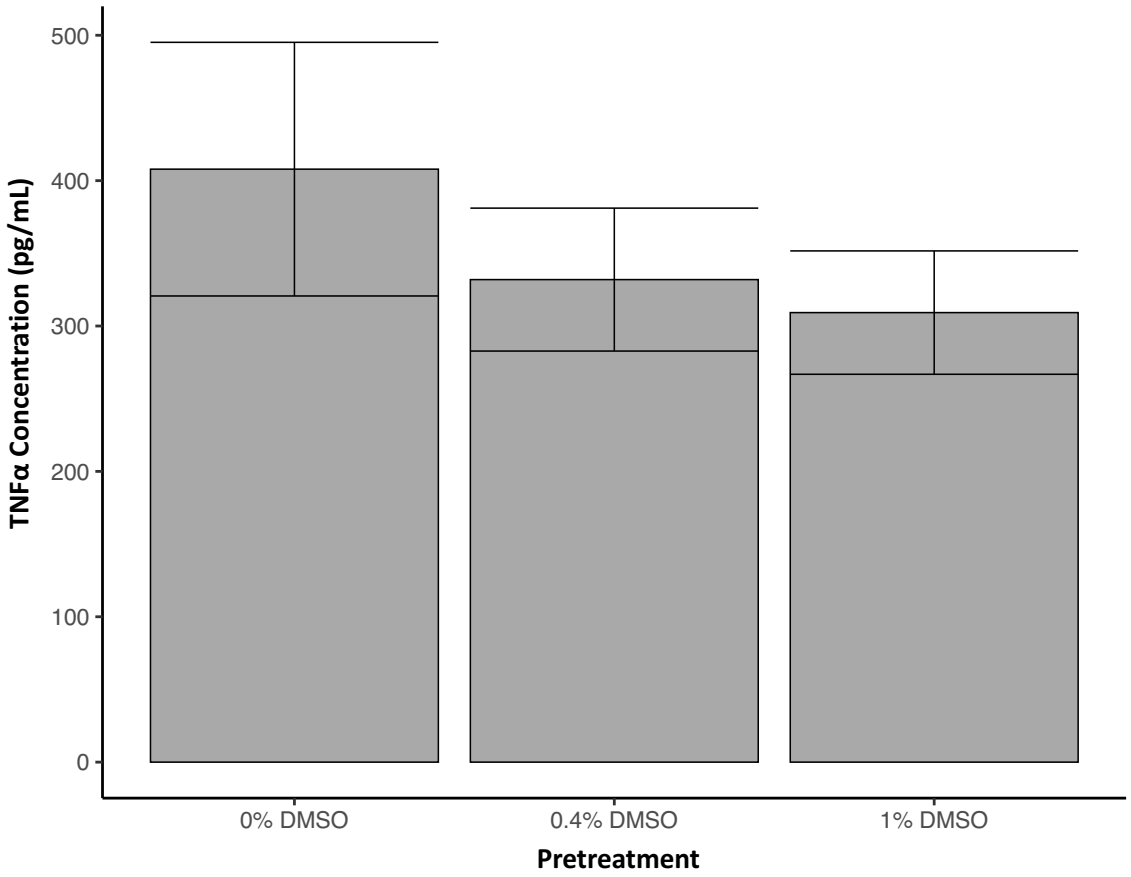


Figure 17: The Effect of DMSO Concentration on TNFα Secretion by THP-1 Macrophages

THP-1 macrophages were pretreated with either 0%, 0.4% or 1% DMSO for 24 hours and stimulated with 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes) for 18 hours as described in section 3.7.3. TNFα concentration in cell supernatants was measured with ELISA. Bars represent mean ± SD of three independent experiments.

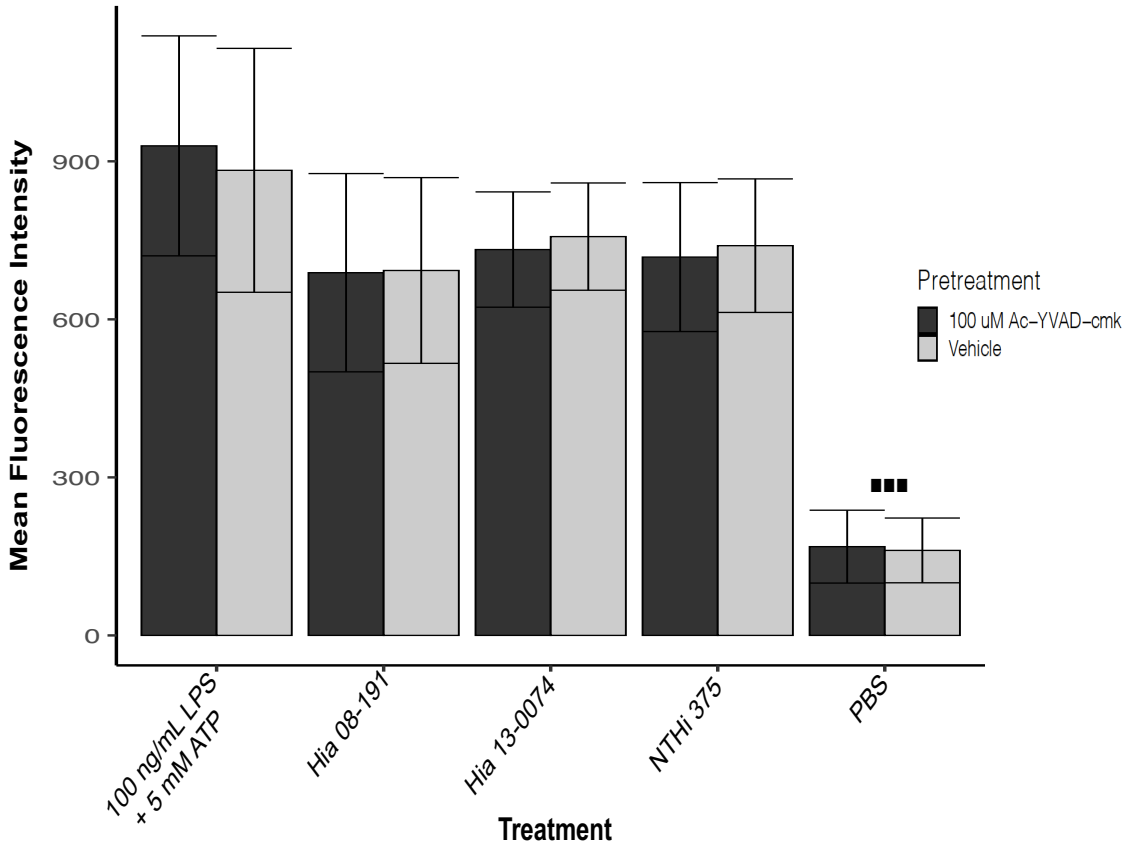


Figure 18: The Effect of Pretreatment of THP-1 Macrophages with Caspase-1 Inhibitor on ICAM-1 Levels in Response to *H. influenzae*

THP-1 macrophages were pretreated with either 100 μM ac-YVAD-cmk or vehicle (1% DMSO) for 24 hours. Cells were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOI of 10; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.3. ICAM-1 levels were assessed in harvested cells incubated with 1 μg/mL mouse-antihuman phycoerythrin-conjugated ICAM-1 antibody for one hour via flow cytometry using 10,000 gated events (FL-2 channel). The results were expressed as mean fluorescence intensity. Bars represent mean ± SD of three independent experiments. ■■■ $p<0.001$, difference compared to samples stimulated with bacteria or LPS/ATP**.** No significant

differences were found among strains. There were no significant differences in pretreatment condition on ICAM-1 levels.

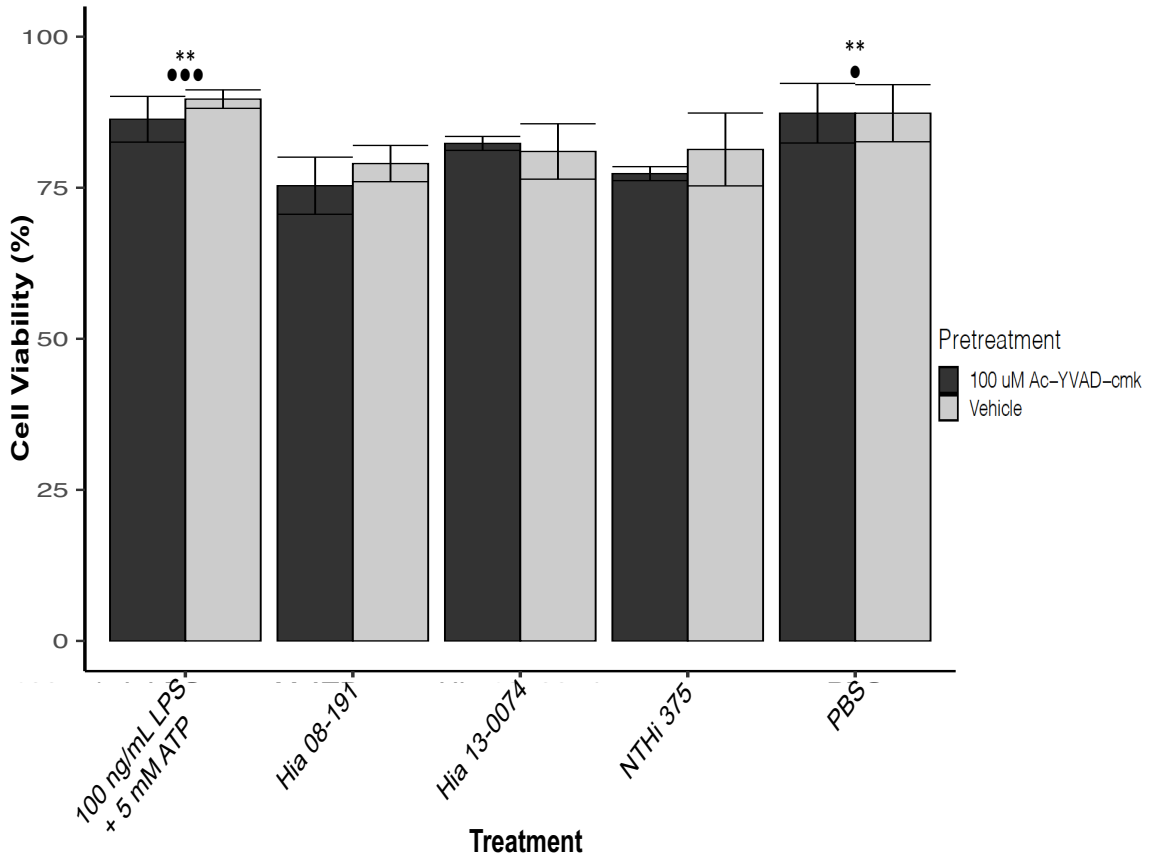


Figure 19: The Effect of Pretreatment of THP-1 Macrophages with Caspase-1 Inhibitor on Cell Viability in Response to *H. influenzae*

THP-1 macrophages were pretreated with either 100 μM ac-YVAD-cmk or vehicle (1% DMSO) for 24 hours. Cells were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOI of 10; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.3. Cell viability was assessed in harvested cells incubated with 1 μg/mL PI for one minute via flow cytometry using 10,000 gated events (FL-2 channel). Bars represent mean ± SD of three independent experiments. ● $p<0.05$, ●●● $p<0.001$, difference compared to samples stimulated with Hia 08-191. * $p<0.05$, ** $p<0.01$ difference compared to samples stimulated with NTHi 375. No significant differences were found among strains. There were no significant differences in pretreatment condition on cell viability.

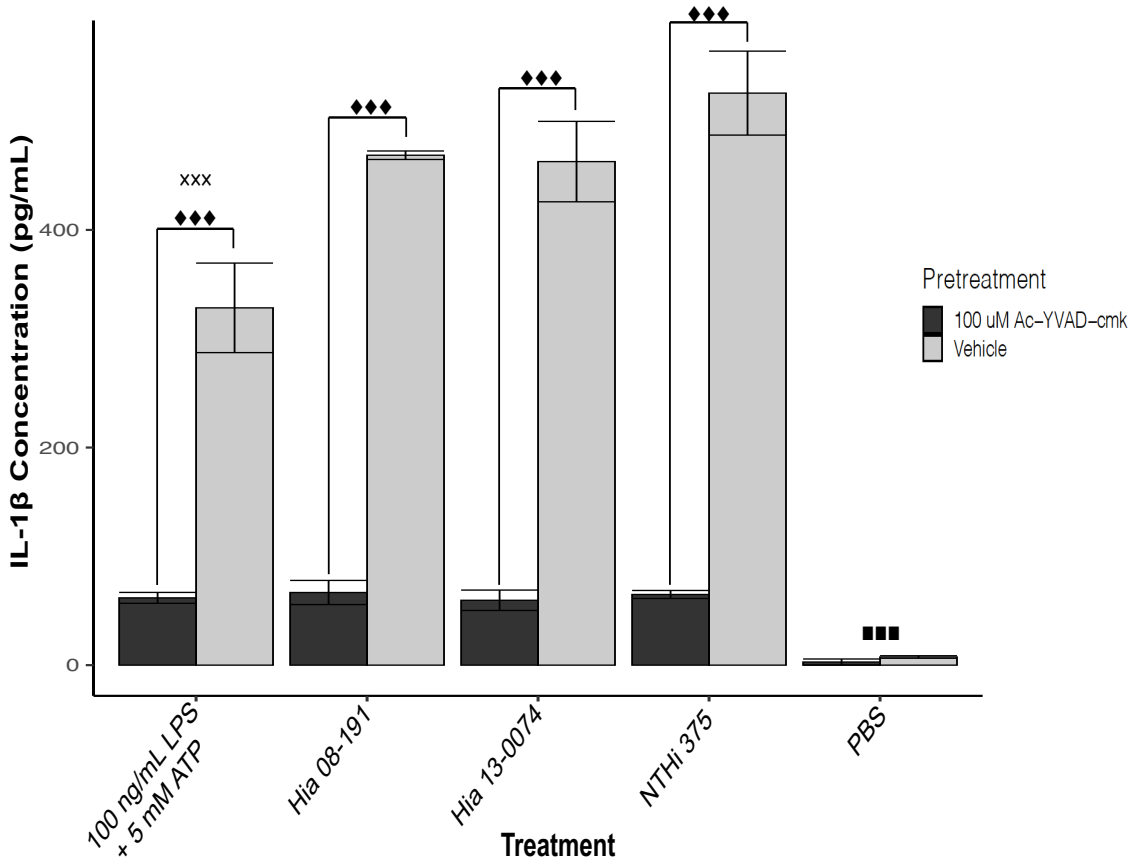


Figure 20: The Effect of Pretreatment of THP-1 Macrophages with Caspase-1 Inhibitor on IL-1β Secretion in Response to *H. influenzae*

THP-1 macrophages were pretreated with either 100 μM ac-YVAD-cmk or vehicle (1% DMSO) for 24 hours. Cells were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOI of 10; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.3. IL-1β concentration in cell supernatants was measured with ELISA. Bars represent mean ± SD of three independent experiments. ◆◆◆ $p<0.001$, differences among pretreatment conditions. ××× $p<0.001$, difference compared to samples stimulated with bacteria. ■■■

$p<0.001$, difference compared to samples stimulated with bacteria or LPS/ATP. No significant differences were found among strains.

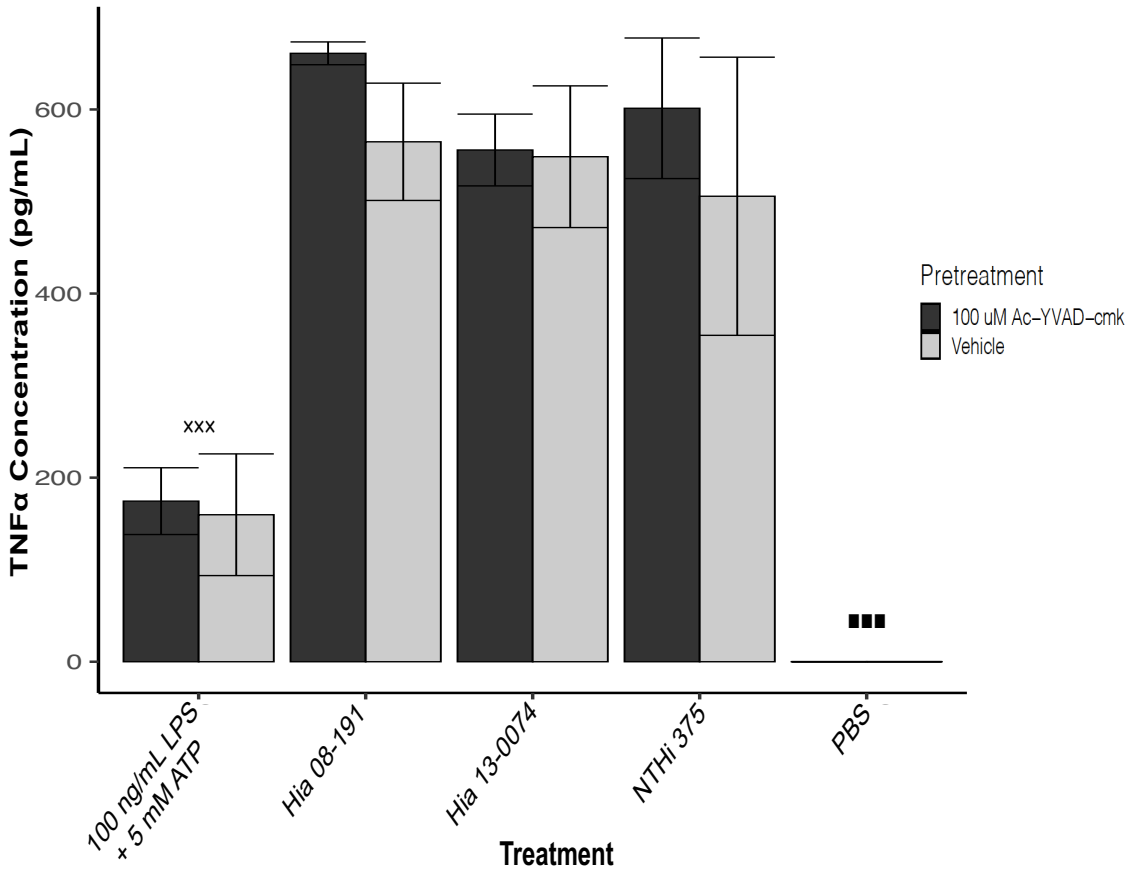


Figure 21: The Effect of Pretreatment of THP-1 Macrophages with Caspase-1 Inhibitor on TNFα Secretion in Response to *H. influenzae*

THP-1 macrophages were pretreated with either 100 μM ac-YVAD-cmk or vehicle (1% DMSO) for 24 hours. Cells were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOI of 10; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.3. TNFα concentration in cell supernatants was measured with ELISA. Bars represent mean ± SD of three independent experiments. ××× $p<0.001$, difference compared to samples stimulated with bacteria. ■■■ $p<0.001$, difference compared to samples stimulated with bacteria or LPS/ATP. No significant differences were found among strains.

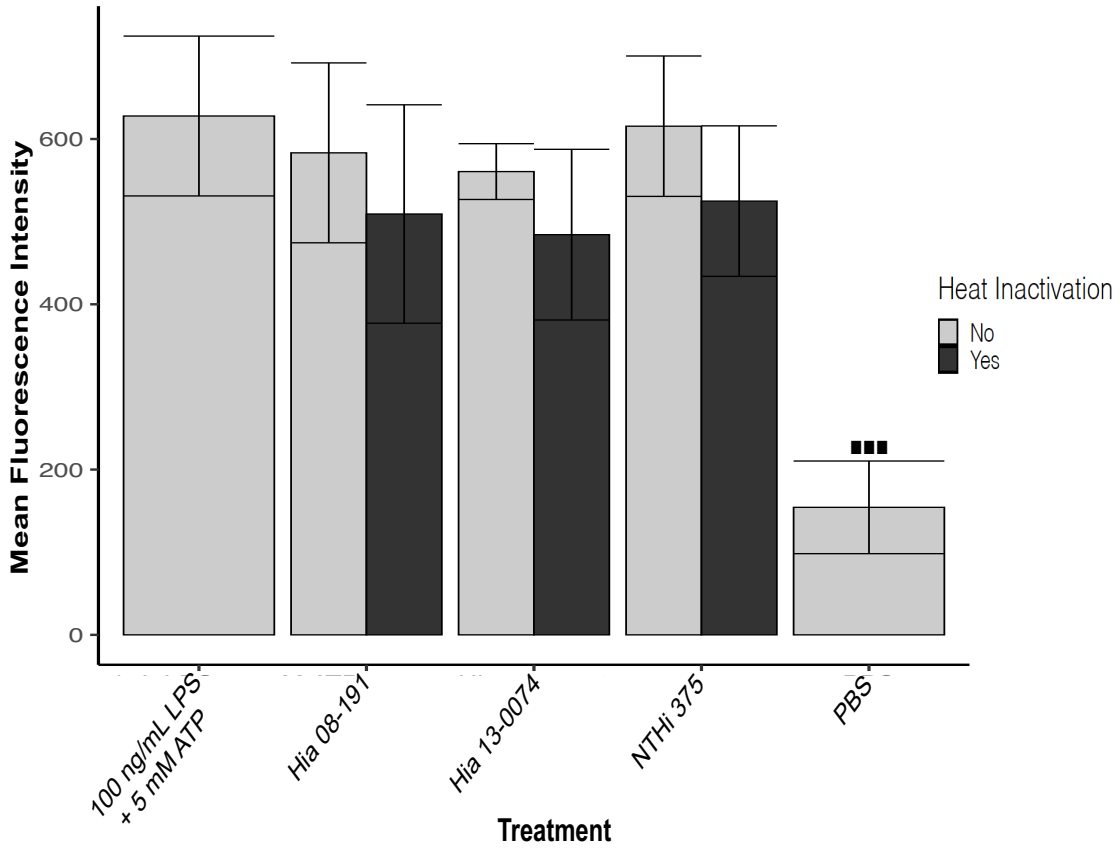


Figure 22: The Effect of Heat Inactivation of Hi on ICAM-1 Levels in THP-1 Macrophages

THP-1 macrophages were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOI of 10 incubated at 37 ºC or 65 ºC for 30 minutes; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.3. ICAM-1 levels were assessed in harvested cells incubated with 1 µg/mL mouse-antihuman phycoerythrin-conjugated ICAM-1 antibody for one hour with via flow cytometry using 10,000 gated events (FL-2 channel). The results were expressed as mean fluorescence intensity. Bars represent mean ± SD of three independent experiments. ■■■ $p<0.001$, difference compared to samples stimulated with bacteria or LPS/ATP. There

were no significant differences in ICAM-1 levels between strains. Heat inactivation of bacteria did not have a significant effect on ICAM-1 levels.

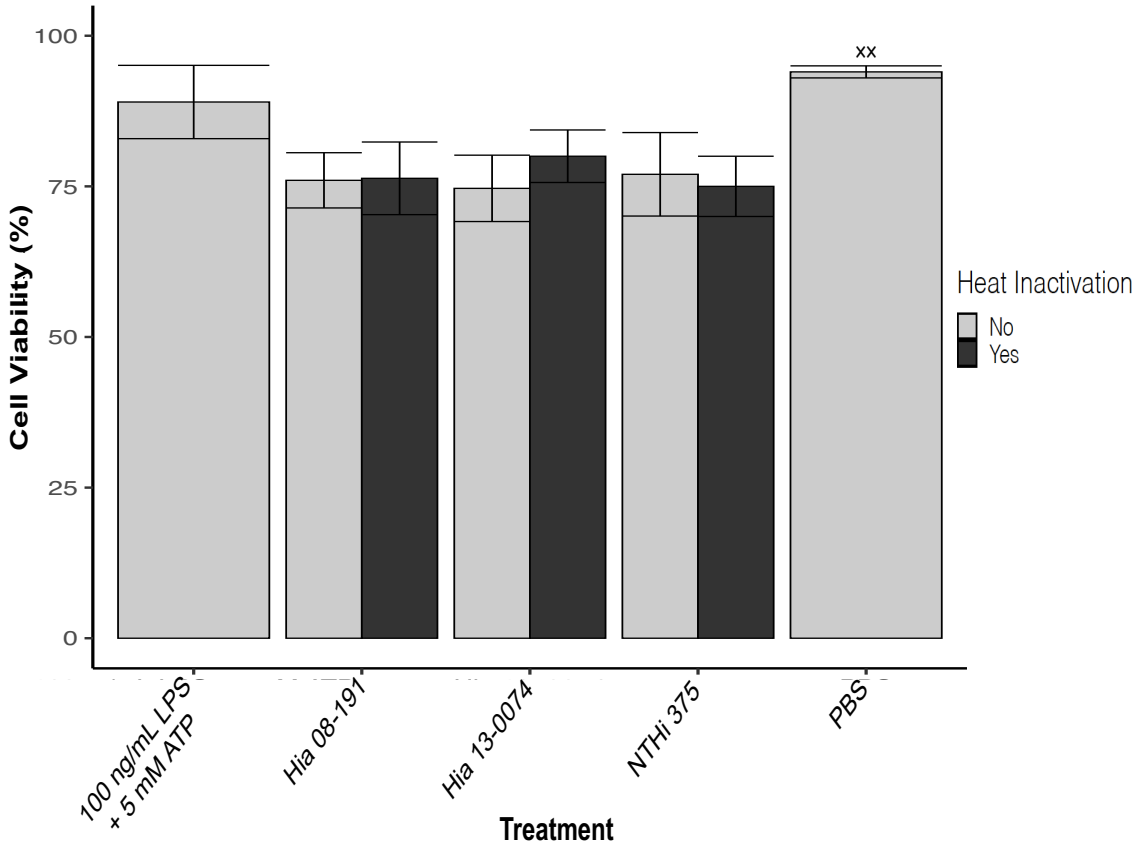


Figure 23: The Effect of Heat Inactivation of Hi on THP-1 Macrophage Viability

THP-1 macrophages were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOI of 10 incubated at 37 ºC or 65 ºC for 30 minutes; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.3. Cell viability was assessed in harvested cells incubated with 1 μg/mL PI for one minute via flow cytometry using 10,000 gated events (FL-2 channel). Bars represent mean ± SD of three independent experiments. ×× $p<0.01$, difference compared to samples stimulated with bacteria. There were no significant differences among strains. Heat inactivation of bacteria did not have a significant effect on cell viability.

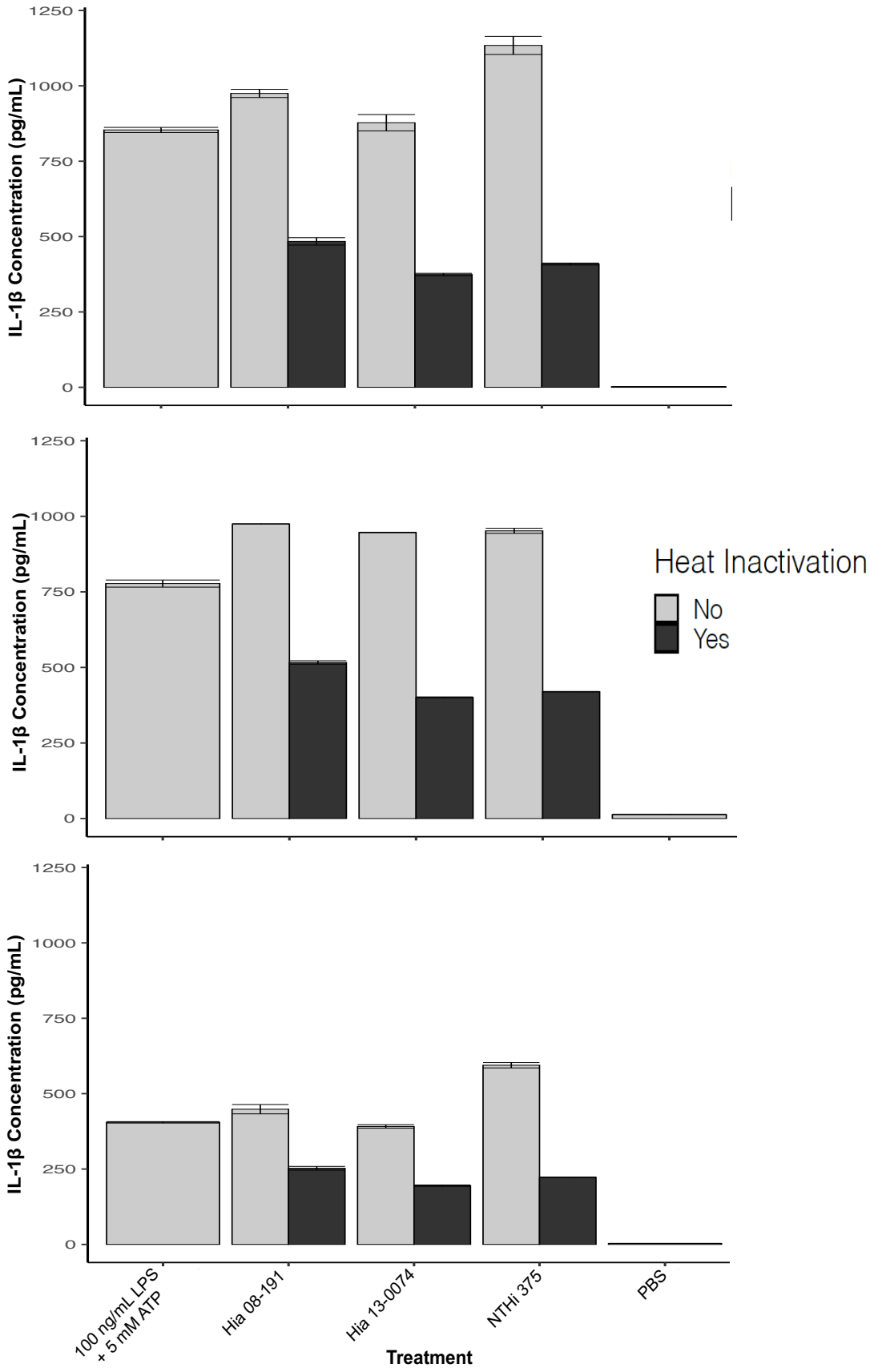

IL-1β Concentration (pg/mL)
1250
1000
750
500
250
0
Heat Inactivation
No
Yes
100 ng/mL LPS + 5 mM ATP
Hia 08-191
Hia 13-0074
NTHi 375
PBS
Treatment

Figure 24: The Effect of Heat Inactivation of Hi on IL-1β Secretion by THP-1 Macrophages: Data of Three Individual Experiments

THP-1 macrophages were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOI of 10 incubated at 37 ºC or 65 ºC for 30 minutes; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.3. IL-1β concentration in cell supernatants was measured with ELISA. Bars represent mean ± SD of ELISA plate duplicates of one independent experiment.

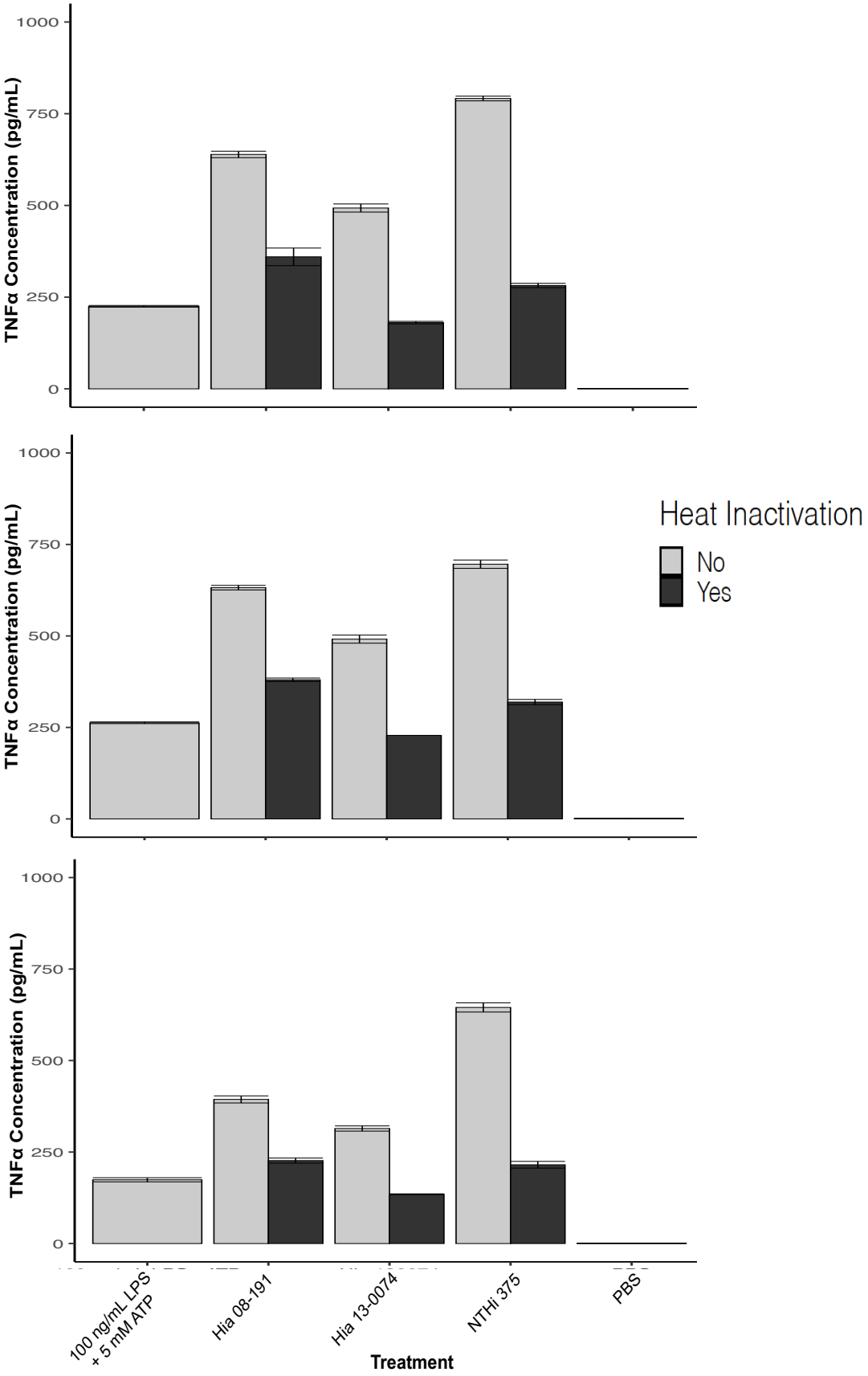

TNFα Concentration (pg/mL)
1000
750
500
250
0
Heat Inactivation
No
Yes
100 ng/mL LPS + 5 mM ATP
Hia 08-191
Hia 13-0074
NTHi 375
PBS
Treatment

Figure 25: The Effect of Heat Inactivation of Hi on TNFα Secretion by THP-1 Macrophage: Data of Three Individual Experiments

THP-1 macrophages were stimulated with either Hia 08-191, Hia 13-0074, or NTHi 375 at MOI of 10 incubated at 37 ºC or 65 ºC for 30 minutes; 100 ng/mL *E. coli* LPS (with 5 mM ATP for the final 30 minutes); or incubated with PBS for 18 hours as described in section 3.3. IL-1β concentration in cell supernatants was measured with ELISA. Bars represent mean ± SD of ELISA plate duplicates of one independent experiment.

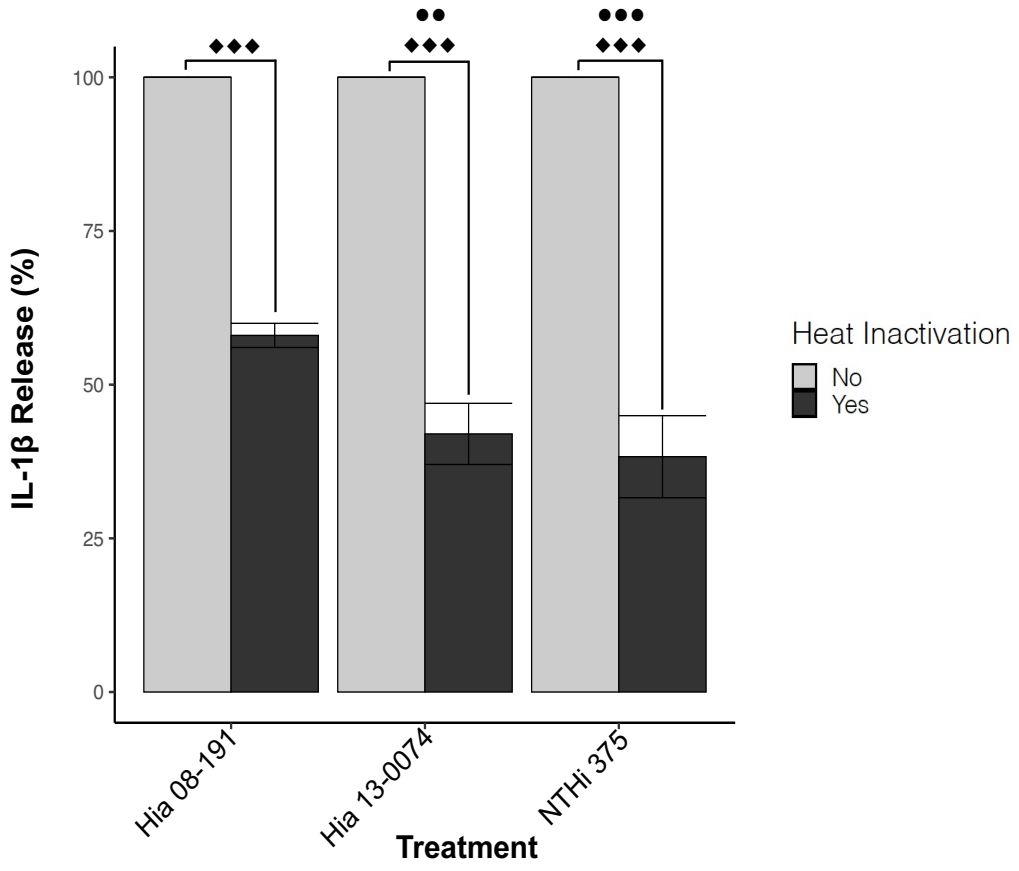


Figure 26: The Normalized Effect of Heat Inactivation of Hi on IL-1β Secretion in THP-1 Macrophages

H. influenzae strains Hia 08-191, Hia 13-0074, or NTHi 375 were incubated at 37 ºC or 65 ºC for 30 minutes prior being used to stimulate THP-1 macrophages at MOI of 10 as described in section 3.3. IL-1β concentration in cell supernatants was measured with ELISA. IL-1β concentration induced by viable bacteria is expressed as 100%. Bars represent mean ± SD of three independent experiments. ◆◆◆ $p<0.001$, differences among heat inactivated and non-heat inactivated bacterial samples. ●● $p<0.01$, ●●● $p<0.001$, difference compared to samples stimulated with Hia 08-191.

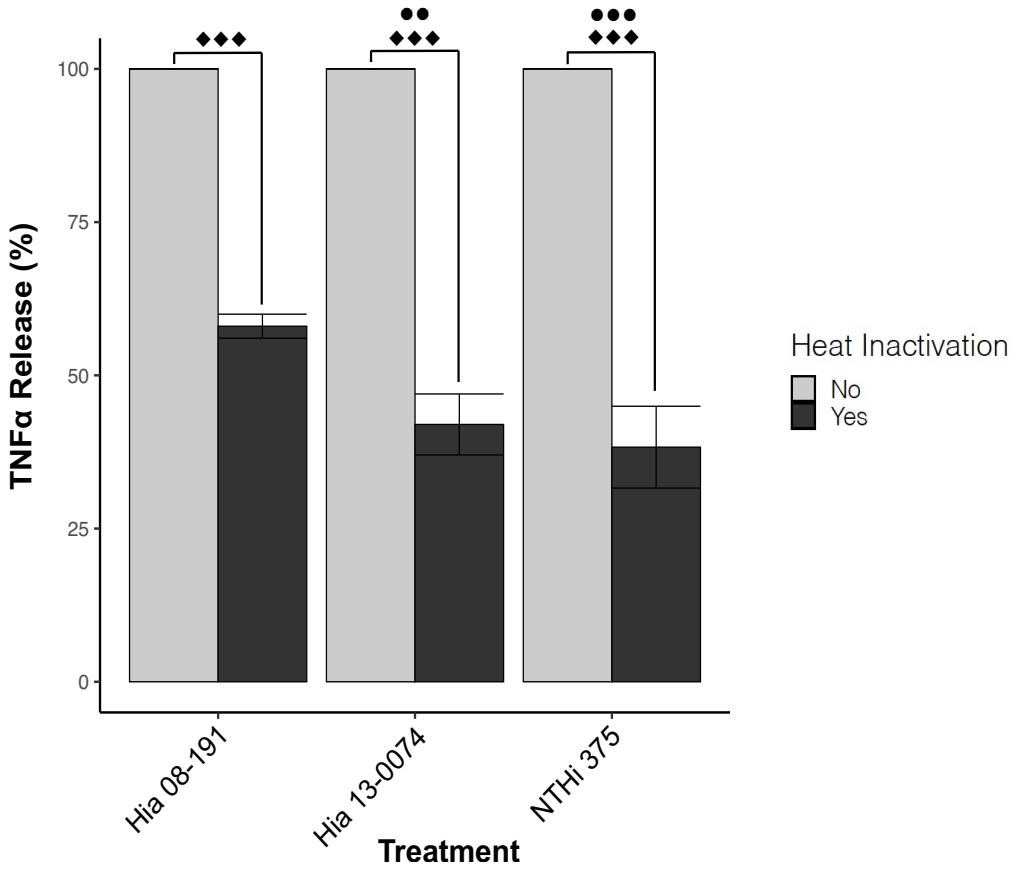


Figure 27: The Normalized Effect of Heat Inactivation of Hi on TNFα Secretion in THP-1 Macrophages

H. influenzae strains Hia 08-191, Hia 13-0074, or NTHi 375 were incubated at 37 ºC or 65 ºC for 30 minutes prior being used to stimulate THP-1 macrophages at MOI of 10 as described in section 3.3. TNFα concentration in cell supernatants was measured with ELISA. TNFα concentration induced by viable bacteria is expressed as 100%. Bars represent mean ± SD of three independent experiments. ◆◆◆ $p<0.001$, differences among heat inactivated and non-heat inactivated bacterial samples. ●● $p<0.01$, ●●● $p<0.001$, difference compared to samples stimulated with Hia 08-191.

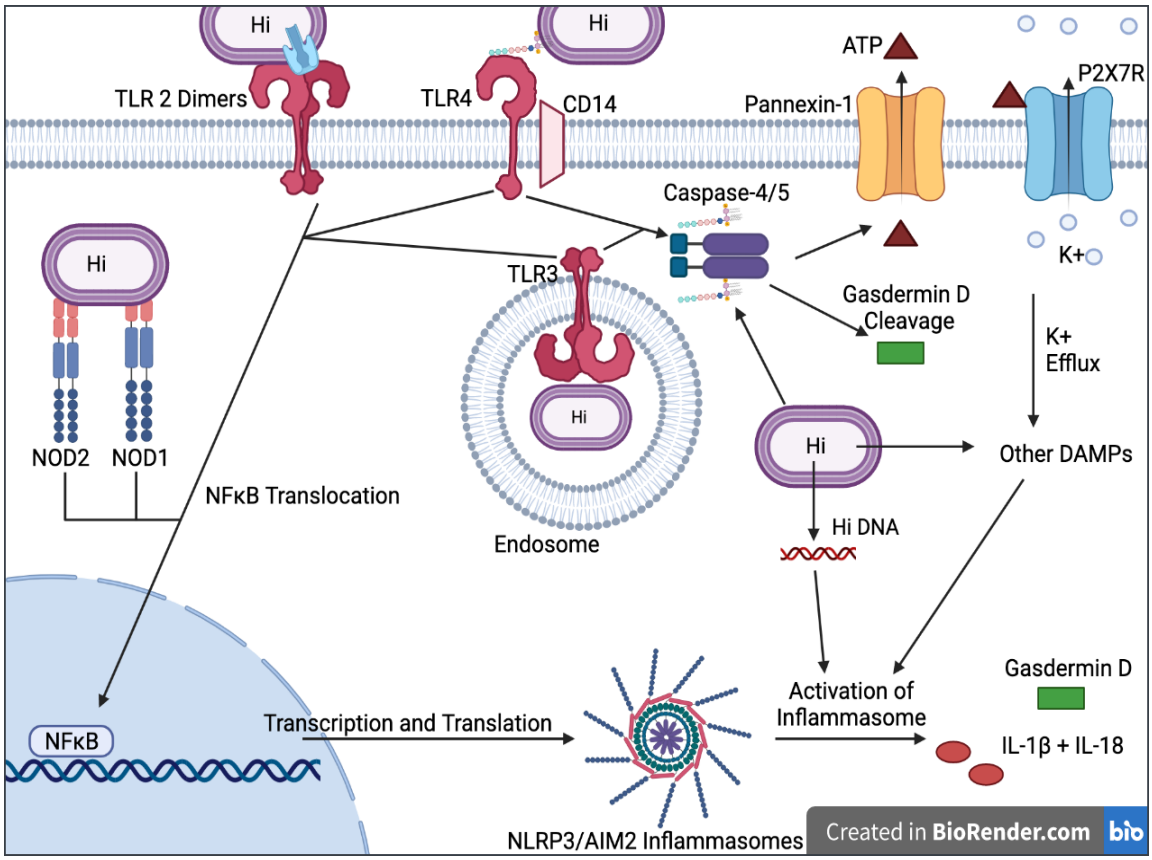


Figure 28: Proposed Model of Inflammasome Activation by Hi

The following is a proposed model of activation of inflammasome by the innate immune system in response to Hi infection. Various PRRs will recognize both extracellular and intracellular Hi. Specifically, extracellular Hi will be recognized by TLR4 and TLR2 dimers via its LOS as well as peptidoglycan, lipoproteins, and porins respectively. Intracellularly, Hi peptidoglycan fragments will be recognized by NOD1 and 2 and a currently unknown component of Hi will activate TLR3. Activation of TLR3 and TLR4 will also result in TRIF pathway activation and non-canonical inflammasome (caspase 4/5) formation. The non-canonical inflammasome will recognize LOS within the cytosol and open the pannexin-1 ATP channel resulting in K+ efflux. The non-canonical inflammasome will also cleave gasdermin D in a caspase-1 independent manner (leading to pyroptosis). Activation of all of the PRRs will result in IκBα phosphorylation by IKK

allowing NFκB nuclear translocation. The transcription factor NFκB will result in the production of inflammasome related gene products including NLRP3 inflammasome, IL-1β and IL-18, and gasdermin D. Along with the K^+ efflux as a result from non-canonical inflammasome activation, Hi will cause various other DAMPs (such as lysosomal damage and ROS production) activating NLRP3 inflammasome. Cytosolic Hi DNA will activate AIM2 inflammasome. Activation of these inflammasomes will result in cleavage of procaspase-1 to caspase-1 which will cleave gasdermin D, pro-IL-18, and pro-IL-1β. This will result in pyroptosis as well as both systemic and local effects of the matured cytokines.

References

Ahn, Huijeong, et al. “Dimethyl Sulfoxide Inhibits NLRP3 Inflammasome Activation.” *Immunobiology*, vol. 219, no. 4, 14 Nov. 2014, pp. 315–322., doi:10.1016/j.imbio.2013.11.003.

Ahrén, Irini Lazou, et al. “Protein D Expression Promotes the Adherence and Internalization of Non-Typeable Haemophilus Influenzae into Human Monocytic Cells.” *Microbial Pathogenesis*, vol. 31, no. 3, 2001, pp. 151–158., doi:10.1006/mpat.2001.0456.

Alhazmi, Alaa. “Exploration of Inflammasomes as Targets for Therapy of Pseudomonas Aeruginosa Infection.” 2018. Lakehead University, PhD Dissertation.

Alhazmi, Alaa, et al. “Syk Inhibitor R406 Downregulates Inflammation in an in Vitro Model of Pseudomonas Aeruginosa Infection.” *Canadian Journal of Physiology and Pharmacology*, vol. 96, no. 2, Feb. 2018, pp. 182–190., doi:10.1139/cjpp-2017-0307.

Anantharajah, Ahalieyah, et al. “Correlation between Cytotoxicity Induced ByPseudomonas Aeruginosaclinical Isolates from Acute Infections and IL-1β Secretion in a Model of Human THP-1 Monocytes.” *Pathogens and Disease*, vol. 73, no. 7, Oct. 2015, doi:10.1093/femspd/ftv049.

Avadhanula, Vasanthi, et al. “Nontypeable Haemophilus Influenzae Adheres to Intercellular Adhesion Molecule 1 (ICAM-1) on Respiratory Epithelial Cells and Upregulates ICAM-1 Expression.” *Infection and Immunity*, vol. 74, no. 2, Feb. 2006, pp. 830–838., doi:10.1128/iai.74.2.830-838.2006.

Ayna, Gizem, et al. “ATP Release from Dying Autophagic Cells and Their Phagocytosis Are Crucial for Inflammasome Activation in Macrophages.” *PLoS ONE*, vol. 7, no. 6, 29 June 2012, doi:10.1371/journal.pone.0040069.

Broz, Petr, and Vishva M. Dixit. “Inflammasomes: Mechanism of Assembly, Regulation and Signalling.” *Nature Reviews Immunology*, vol. 16, no. 7, July 2016, pp. 407–420., doi:10.1038/nri.2016.58.

Buyck, Julien M., et al. “Pharmacodynamic Evaluation of the Intracellular Activity of Antibiotics towards Pseudomonas Aeruginosa PAO1 in a Model of THP-1 Human Monocytes.” *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 5, May 2013, pp. 2310–2318., doi:10.1128/aac.02609-12.

Carabarin-Lima, Alejandro, et al. “Flagellar Expression in Clinical Isolates of Non-Typeable Haemophilus Influenzae.” *Journal of Medical Microbiology*, vol. 66, no. 5, 1 May 2017, pp. 592–600., doi:10.1099/jmm.0.000471.

Caruso, Roberta, et al. “NOD1 And NOD2: Signaling, Host Defense, and Inflammatory Disease.” *Immunity*, vol. 41, no. 6, 18 Dec. 2014, pp. 898–908., doi:10.1016/j.immuni.2014.12.010.

Cerqueira, Ashley, et al. “Continuing Surveillance of Invasive Haemophilus Influenzae Disease in Northwestern Ontario Emphasizes the Importance of Serotype a and Non-Typeable Strains as Causes of Serious Disease: a Canadian Immunization Research Network (CIRN) Study.” *Canadian Journal of Microbiology*, vol. 65, no. 11, Nov. 2019, pp. 805–813., doi:10.1139/cjm-2019-0210.

Chang, Cheow K., et al. “Inhibitory Effect of Dimethyl Sulfoxide on Nuclear Factor-KB Activation and Intercellular Adhesion Molecule 1 Gene Expression in Septic Rats.” *Journal of Surgical Research*, vol. 82, no. 2, Apr. 1999, pp. 294–299., doi:10.1006/jsre.1998.5527.

Chanput, Wasaporn, et al. “THP-1 Cell Line: An in Vitro Cell Model for Immune Modulation Approach.” *International Immunopharmacology*, vol. 23, no. 1, Nov. 2014, pp. 37–45., doi:10.1016/j.intimp.2014.08.002.

Chen, Alice C-H., et al. “Multiple Inflammasomes May Regulate the Interleukin-1-Driven Inflammation in Protracted Bacterial Bronchitis.” *ERJ Open Research*, vol. 4, no. 1, 23 Mar. 2018, pp. 00130–2017., doi:10.1183/23120541.00130-2017.

Chen, Qixing, et al. “Alarmin HNP-1 Promotes Pyroptosis and IL-1β Release through Different Roles of NLRP3 Inflammasome via P2X7 in LPS-Primed Macrophages.” *Innate Immunity*, vol. 20, no. 3, 19 Mar. 2013, pp. 290–300., doi:10.1177/1753425913490575.

Chevriaux, Angélique, et al. “Cathepsin B Is Required for NLRP3 Inflammasome Activation in Macrophages, Through NLRP3 Interaction.” *Frontiers in Cell and Developmental Biology*, vol. 8, 31 Mar. 2020, doi:10.3389/fcell.2020.00167.

Choi, Joshua, et al. “Activation of Innate Immune Responses by Haemophilus Influenzae Lipooligosaccharide.” *Clinical and Vaccine Immunology*, vol. 21, no. 5, 26 Mar. 2014, pp. 769–776., doi:10.1128/cvi.00063-14.

Chu, Jessica, et al. “Cholesterol-Dependent Cytolysins Induce Rapid Release of Mature IL-1β from Murine Macrophages in a NLRP3 Inflammasome and Cathepsin B-Dependent Manner.” *Journal of Leukocyte Biology*, vol. 86, no. 5, 12 Aug. 2009, pp. 1227–1238., doi:10.1189/jlb.0309164.

Clementi, Cara F., and Timothy F. Murphy. “Non-Typeable Haemophilus Influenzae Invasion and Persistence in the Human Respiratory Tract.” *Frontiers in Cellular and Infection Microbiology*, vol. 1, 18 Nov. 2011, doi:10.3389/fcimb.2011.00001.

Coder, David M. “Assessment of Cell Viability.” *Current Protocols in Cytometry*, vol. 15, no. 1, 15 May 2001, doi:10.1002/0471142956.cy0902s15.

Cotter, Shane E., et al. “Architecture and Adhesive Activity of the Haemophilus Influenzae Hsf Adhesin.” *Journal of Bacteriology*, vol. 187, no. 13, July 2005, pp. 4656–4664., doi:10.1128/JB.187.13.4656-4664.2005

Deng, Qiuchan, et al. “Pseudomonas Aeruginosa Triggers Macrophage Autophagy To Escape Intracellular Killing by Activation of the NLRP3 Inflammasome.” *Infection and Immunity*, vol. 84, no. 1, Jan. 2016, pp. 56–66., doi:10.1128/iai.00945-15.

Diab, A., et al. “Haemophilus Influenzae and Streptococcus Pneumoniae Induce Different Intracerebral MRNA Cytokine Patterns during the Course of Experimental Bacterial Meningitis.” *Clinical & Experimental Immunology*, vol. 109, no. 2, 1997, pp. 233–241., doi:10.1046/j.1365-2249.1997.4441343.x.

Dostert, Catherine, et al. “Innate Immune Activation Through Nalp3 Inflammasome Sensing of Asbestos and Silica.” *Science*, vol. 320, no. 5876, 2 May 2008, pp. 674–677., doi:10.1126/science.1156995.

Dostert, Catherine, et al. “Malarial Hemozoin Is a Nalp3 Inflammasome Activating Danger Signal.” *PLoS ONE*, vol. 4, no. 8, 4 Aug. 2009, doi:10.1371/journal.pone.0006510.

Duell, Benjamin Luke, et al. “Host-Pathogen Interactions of Nontypeable Haemophilus Influenzae: from Commensal to Pathogen.” *FEBS Letters*, vol. 590, no. 21, 2016, pp. 3840–3853., doi:10.1002/1873-3468.12351

Duncan, Joseph A., et al. “Neisseria GonorrhoeaeActivates the Proteinase Cathepsin B to Mediate the Signaling Activities of the NLRP3 and ASC-Containing Inflammasome.” *The Journal of Immunology*, vol. 182, no. 10, 15 May 2009, pp. 6460–6469., doi:10.4049/jimmunol.0802696.

Erwin, Alice L., and Arnold L. Smith. “Nontypeable Haemophilus Influenzae: Understanding Virulence and Commensal Behavior.” *Trends in Microbiology*, vol. 15, no. 8, 2007, pp. 355–362., doi:10.1016/j.tim.2007.06.004.

Forrester, Megan A., et al. “Similarities and Differences in Surface Receptor Expression by THP-1 Monocytes and Differentiated Macrophages Polarized Using Seven Different Conditioning Regimens.” *Cellular Immunology*, vol. 332, Oct. 2018, pp. 58–76., doi:10.1016/j.cellimm.2018.07.008.

Forsgren, Arne, and Kristian Riesbeck. “Protein D of Haemophilus Influenzae: A Protective Nontypeable H. Influenzae Antigen and a Carrier for Pneumococcal Conjugate Vaccines.” *Clinical Infectious Diseases*, vol. 46, no. 5, 1 Mar. 2008, pp. 726–731., doi:10.1086/527396.

Foxwell, A. Ruth, et al. “Nontypeable Haemophilus Influenzae: Pathogenesis and Prevention.” *Microbiology and Molecular Biology Reviews*, vol. 62, no. 2, June 1998, pp. 294–308., doi:10.1128/mmbr.62.2.294-308.1998.

Galdiero, Marilena, et al. “Haemophilus Influenzae Porin Induces Toll-Like Receptor 2-Mediated Cytokine Production in Human Monocytes and Mouse Macrophages.” *Infection and Immunity*, vol. 72, no. 2, Feb. 2004, pp. 1204–1209., doi:10.1128/iai.72.2.1204-1209.2004.

Gaultier, Gabrielle Nicole, et al. “Natural Immunity against Haemophilus Influenzae Type a and B-Cell Subpopulations in Adult Patients with Severe Chronic Kidney Disease.” *Vaccine*, vol. 37, no. 28, 12 May 2019, pp. 3677–3684., doi:10.1016/j.vaccine.2019.05.036.

Geijtenbeek, Teunis B.H., and Sonia I. Gringhuis. “Signalling through C-Type Lectin Receptors: Shaping Immune Responses.” *Nature Reviews Immunology*, vol. 9, no. 7, 12 June 2009, pp. 465–479., doi:10.1038/nri2569.

Gonzalez, Luis S., and Jeanne P. Spencer. “Aminoglycosides: A Practical Review.” *American Family Physician*, vol. 58, no. 5, Nov. 1998, pp. 1811–1820.

Hallström, Teresia, et al. “Haemophilus Influenzae Interacts with the Human Complement Inhibitor Factor H.” *The Journal of Immunology*, vol. 181, no. 1, 1 July 2008, pp. 537–545., doi:https://doi.org/10.4049/jimmunol.181.1.537.

Hallström, Teresia, and Kristian Riesbeck. “Haemophilus Influenzae and the Complement System.” *Trends in Microbiology*, vol. 18, no. 6, 1 June 2010, pp. 258–265., doi:10.1016/j.tim.2010.03.007.

Hartwig, Stacey M., et al. “Non-Typeable Haemophilus Influenzae Protects Human Airway Epithelial Cells from a Subsequent Respiratory Syncytial Virus Challenge.” *Virology*, vol. 498, Nov. 2016, pp. 128–135., doi:10.1016/j.virol.2016.08.020.

He, Yuan, et al. “Mechanism and Regulation of NLRP3 Inflammasome Activation.” *Trends in Biochemical Sciences*, vol. 41, no. 12, 2016, pp. 1012–1021., doi:10.1016/j.tibs.2016.09.002.

Hoeller, O., et al. “Two Distinct Functions for PI3-Kinases in Macropinocytosis.” *Journal of Cell Science*, vol. 126, no. 18, 15 Sept. 2013, pp. 4296–4307., doi:10.1242/jcs.134015.

Hua, Kuo-Feng, et al. “Capsular Polysaccharide Is Involved in NLRP3 Inflammasome Activation by Klebsiella Pneumoniae Serotype K1.” *Infection and Immunity*, vol. 83, no. 9, 15 June 2015, pp. 3396–3409., doi:10.1128/iai.00125-15.

Humlicek, Alicia L., et al. “Modulation of Airway Inflammation and Bacterial Clearance by Epithelial Cell ICAM-1.” *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 287, no. 3, Sept. 2004, pp. L598–L607., doi:10.1152/ajplung.00073.2004.

Idriss, Haitham T., and James H. Naismith. “TNF? and the TNF Receptor Superfamily: Structure-Function Relationship(s).” *Microscopy Research and Technique*, vol. 50, no. 3, 1 Aug. 2000, pp. 184–195., doi:10.1002/1097-0029(20000801)50:3<184::aid-jemt2>3.0.co;2-h.

Ikeda, Masaki, et al. “Nontypeable Haemophilus Influenzae Exploits the Interaction between Protein-E and Vitronectin for the Adherence and Invasion to Bronchial Epithelial Cells.” *BMC Microbiology*, vol. 15, no. 1, 14 Nov. 2015, doi:10.1186/s12866-015-0600-8

Jo, Eun-Kyeong, et al. “Molecular Mechanisms Regulating NLRP3 Inflammasome Activation.” *Cellular & Molecular Immunology*, vol. 13, no. 2, Mar. 2016, pp. 148–159., doi:10.1038/cmi.2015.95.

Juliana, Christine, et al. “Non-Transcriptional Priming and Deubiquitination Regulate NLRP3 Inflammasome Activation.” *Journal of Biological Chemistry*, vol. 287, no. 43, 19 Oct. 2012, pp. 36617–36622., doi:10.1074/jbc.m112.407130.

Juneau, Richard A., et al. “Nontypeable Haemophilus Influenzae Initiates Formation of Neutrophil Extracellular Traps.” *Infection and Immunity*, vol. 79, no. 1, Jan. 2011, pp. 431–438., doi:10.1128/iai.00660-10.

Kelly, Len, et al. “Invasive Disease Caused by Haemophilus Influenzae Type a in Northern Ontario First Nations Communities.” *Journal of Medical Microbiology*, vol. 60, no. 3, 1 Mar. 2011, pp. 384–390., doi:10.1099/jmm.0.026914-0.

Kenjale, Roma, et al. “Structural Determinants of Autoproteolysis of the Haemophilus Influenzae Hap Autotransporter.” *Infection and Immunity*, vol. 77, no. 11, 6 Aug. 2009, pp. 4704–4713., doi:10.1128/iai.00598-09.

Khan, Farooq-Ahmad, et al. “Design, Synthesis and Immunological Evaluation of Novel Amphiphilic Desmuramyl Peptides.” *European Journal of Medicinal Chemistry*, vol. 141, Dec. 2017, pp. 26–36., doi:10.1016/j.ejmech.2017.09.070.

Kirkham, Lea-Ann S, et al. “A Practical Method for Preparation of Pneumococcal and Nontypeable Haemophilus Influenzae Inocula That Preserves Viability and Immunostimulatory Activity.” *BMC Research Notes*, vol. 6, no. 522, 9 Dec. 2013, doi:10.1186/1756-0500-6-522.

Kubinec, Chelsea J, et al. “Invasive Haemophilus Influenzae Disease in Northwestern Ontario First Nations Communities: Case Series.” *International Journal of Case Reports*, vol. 4, no. 140, 2020, p. 140., doi:10.28933/ijcr-2020-05-2805.

Kuhnert, Peter, and Henrik Christensen. *Pasteurellaceae: Biology, Genomics, and Molecular Aspects*. Caister Academic Press, 2008.

Laarmann, Sven, et al. “The Haemophilus Influenzae Hia Autotransporter Harbours Two Adhesive Pockets That Reside in the Passenger Domain and Recognize the Same Host Cell Receptor.” *Molecular Microbiology*, vol. 46, no. 3, 31 Oct. 2002, pp. 731–743., doi:10.1046/j.1365-2958.2002.03189.x

Lawrence, Toby. “The Nuclear Factor NFκB Pathway in Inflammation.” *Cold Spring Harbor Perspectives in Biology*, vol. 1, no. 6, Dec. 2009, doi:10.1101/cshperspect.a001651.

Lee, Geun-Shik, et al. “The Calcium-Sensing Receptor Regulates the NLRP3 Inflammasome through Ca2+ and CAMP.” *Nature*, vol. 492, no. 7427, 6 Dec. 2012, pp. 123–127., doi:10.1038/nature11588.

Lee, Jasmine, et al. “NOD1/NOD2-Mediated Recognition of Non-Typeable Haemophilus Influenzae Activates Innate Immunity during Otitis Media.” *Innate Immunity*, vol. 25, no. 8, 2019, pp. 503–512., doi:10.1177/1753425919872266.

Li, Wei, et al. “Haemophilus Ducreyi Infection Induces Activation of the NLRP3 Inflammasome in Nonpolarized but Not in Polarized Human Macrophages.” *Infection and Immunity*, vol. 81, no. 8, Aug. 2013, pp. 2997–3008., doi:10.1128/iai.00354-13.

Lipinska, Katarzyna, et al. “Applying Caspase-1 Inhibitors for Inflammasome Assays in Human Whole Blood.” *Journal of Immunological Methods*, vol. 411, Sept. 2014, pp. 66–69., doi:10.1016/j.jim.2014.05.018.

Liu, Ting, et al. “NF-KB Signaling in Inflammation.” *Signal Transduction and Targeted Therapy*, vol. 2, no. 1, 14 July 2017, doi:10.1038/sigtrans.2017.23.

Lugrin, Jérôme, and Fabio Martinon. “The AIM2 Inflammasome: Sensor of Pathogens and Cellular Perturbations.” *Immunological Reviews*, vol. 281, no. 1, 16 Dec. 2017, pp. 99–114., doi:10.1111/imr.12618.

Mell, Joshua Chang, et al. “Complete Genome Sequence of Haemophilus Influenzae Strain 375 from the Middle Ear of a Pediatric Patient with Otitis Media.” *Genome Announcements*, vol. 2, no. 6, 4 Dec. 2014, doi:10.1128/genomea.01245-14.

Misawa, Takuma, et al. “Microtubule-Driven Spatial Arrangement of Mitochondria Promotes Activation of the NLRP3 Inflammasome.” *Nature Immunology*, vol. 14, no. 5, May 2013, pp. 454–460., doi:10.1038/ni.2550.

Mogensen, T. H., et al. “Live Streptococcus Pneumoniae, Haemophilus Influenzae, and Neisseria Meningitidis Activate the Inflammatory Response through Toll-like Receptors 2, 4, and 9 in Species-Specific Patterns.” *Journal of Leukocyte Biology*, vol. 80, no. 2, 26 May 2006, pp. 267–277., doi:10.1189/jlb.1105626.

Moxon, E. Richard. “Molecular Basis of Invasive Haemophilus Influenzae Type b Disease.” *Journal of Infectious Diseases*, vol. 165, no. Supplement 1, 1992, pp. S77–81., doi:10.1093/infdis/165-supplement_1-s77.

Moxon E.R. and Kroll J.S. “The Role of Bacterial Polysaccharide Capsules as Virulence Factors.” *Bacterial Capsules*, by Jann K., Jann B., Springer-Verlag, 1990, pp. 65–85.

Moxon, E. Richard, and R. Wilson. “The Role of Haemophilus Influenzae in the Pathogenesis of Pneumonia.” *Reviews of Infectious Diseases*, vol. 13, Supplement 6, 1991, pp. S518–S527., doi:10.1093/clinids/13.supplement_6.s518.

Muñoz-Planillo, Raúl, et al. “K+ Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter.” *Immunity*, vol. 38, no. 6, 27 June 2013, pp. 1142–1153., doi:10.1016/j.immuni.2013.05.016.

Murphy, Kenneth, and Casey Weaver. *Janeway's Immunobiology*. 9th ed., Garland Science, Taylor & Francis Group, LLC, 2017.

Murphy, Timothy F., et al. “Construction of a Mutant and Characterization of the Role of the Vaccine Antigen P6 in Outer Membrane Integrity of Nontypeable Haemophilus Influenzae.” *Infection and Immunity*, vol. 74, no. 9, 2 June 2006, pp. 5169–5176., doi:10.1128/iai.00692-06.

Naylor, Emma J., et al. “Haemophilus InfluenzaeInduces Neutrophil Necrosis.” *American Journal of Respiratory Cell and Molecular Biology*, vol. 37, no. 2, 15 Jan. 2007, pp. 135–143., doi:10.1165/rcmb.2006-0375oc.

Netea, Mihai G., et al. “Differential Requirement for the Activation of the Inflammasome for Processing and Release of IL-1β in Monocytes and Macrophages.” *Blood*, vol. 113, no. 10, 5 Mar. 2009, pp. 2324–2335., doi:10.1182/blood-2008-03-146720.

Nix, Eli B., et al. “Naturally Acquired Antibodies against Haemophilus Influenzae Type a in Aboriginal Adults, Canada.” *Emerging Infectious Diseases*, vol. 21, no. 2, 15 Aug. 2015, pp. 273–279., doi:10.3201/eid2102.140722.

Nix, Eli B., et al. “Characterization of Natural Bactericidal Antibody against Haemophilus Influenzae Type a in Canadian First Nations: A Canadian Immunization Research Network (CIRN) Clinical Trials Network (CTN) Study.” *PLOS ONE*, vol. 13, no. 8, 15 Aug. 2018, doi:10.1371/journal.pone.0201282.

Ospina, Maria B., et al. “Incidence and Prevalence of Chronic Obstructive Pulmonary Disease among Aboriginal Peoples in Alberta, Canada.” *Plos One*, vol. 10, no. 4, 13 Apr. 2015, pp. 1–13., doi:10.1371/journal.pone.0123204.

Parameswaran, Narayanan, and Sonika Patial. “Tumor Necrosis Factor-α Signaling in Macrophages.” *Critical Reviews™ in Eukaryotic Gene Expression*, vol. 20, no. 2, 2010, pp. 87–103., doi:10.1615/critreveukargeneexpr.v20.i2.10.

Pétrilli, V, et al. “Activation of the NALP3 Inflammasome Is Triggered by Low Intracellular Potassium Concentration.” *Cell Death & Differentiation*, vol. 14, no. 9, Sept. 2007, pp. 1583–1589., doi:10.1038/sj.cdd.4402195.

Provost, Karin A., et al. “Calcium Restores the Macrophage Response to NontypeableHaemophilus Influenzaein Chronic Obstructive Pulmonary Disease.” *American Journal of Respiratory Cell and Molecular Biology*, vol. 52, no. 6, June 2015, pp. 728–737., doi:10.1165/rcmb.2014-0172oc.

Rathinam, Vijay A.K., et al. “TRIF Licenses Caspase-11-Dependent NLRP3 Inflammasome Activation by Gram-Negative Bacteria.” *Cell*, vol. 150, no. 3, 3 Aug. 2012, pp. 606–619., doi:10.1016/j.cell.2012.07.007.

Richter, Erik, et al. “Induction of Macrophage Function in Human THP-1 Cells Is Associated with Rewiring of MAPK Signaling and Activation of MAP3K7 (TAK1) Protein Kinase.” *Frontiers in Cell and Developmental Biology*, vol. 4, 30 Mar. 2016, doi:10.3389/fcell.2016.00021.

Roebuck, Kenneth A., and Alison Finnegan. “Regulation of Intercellular Adhesion Molecule-1 (CD54) Gene Expression.” *Journal of Leukocyte Biology*, vol. 66, no. 6, Dec. 1999, pp. 876–888., doi:10.1002/jlb.66.6.876.

Rosadini, Charles V., et al. “Outer Membrane Protein P5 Is Required for Resistance of Nontypeable Haemophilus Influenzae to Both the Classical and Alternative Complement Pathways.” *Infection and Immunity*, vol. 82, no. 2, Feb. 2014, pp. 640–649., doi:10.1128/iai.01224-13.

Rotondo, Jenny L., et al. “The Epidemiology of Invasive Disease Due to Haemophilus Influenzae Serotype a in the Canadian North from 2000 to 2010.” *International Journal of Circumpolar Health*, vol. 72, no. 1, 5 Aug. 2013, doi:10.3402/ijch.v72i0.21142.

Rotta Detto Loria, Johannes, et al. “Nontypeable Haemophilus Influenzae Infection Upregulates the NLRP3 Inflammasome and Leads to Caspase-1-Dependent Secretion of Interleukin-1β — A Possible Pathway of Exacerbations in COPD.” *PLoS ONE*, vol. 8, no. 6, 26 June 2013, doi:10.1371/journal.pone.0066818.

Sakai, Akihiro, et al. “The Bacterium, Nontypeable Haemophilus Influenzae, Enhances Host Antiviral Response by Inducing Toll-like Receptor 7 Expression.” *The FEBS Journal*, vol. 274, no. 14, 3 July 2007, pp. 3655–3668., doi:10.1111/j.1742-4658.2007.05899.x.

Sander, Leif E, et al. “Detection of Prokaryotic MRNA Signifies Microbial Viability and Promotes Immunity.” *Nature*, vol. 474, 22 May 2011, pp. 385–389., doi:10.1038/nature10072.

Schneider, Katharina S., et al. “Inflammasome Activation and Inhibition in Primary Murine Bone Marrow-Derived Cells, and Assays for IL-1α, IL-1β, and Caspase-1.” *Methods in Molecular Biology*, vol. 1040, 2013, pp. 117–135., doi:10.1007/978-1-62703-523-1_10.

Schweda, Elke K.H., et al. “Expression and Structural Diversity of the Lipopolysaccharide of Haemophilus Influenzae: Implication in Virulence.” *International Journal of Medical Microbiology*, vol. 297, no. 5, Sept. 2007, pp. 297–306., doi:10.1016/j.ijmm.2007.03.007.

Schwende, Heike, et al. “Differences in the State of Differentiation of THP-1 Cells Induced by Phorbol Ester and 1,25-Dihydroxyvitamin D3.” *Journal of Leukocyte Biology*, vol. 59, no. 4, 1 Apr. 1996, pp. 555–561., doi:10.1002/jlb.59.4.555.

Shimada, Kenichi, et al. “Oxidized Mitochondrial DNA Activates the NLRP3 Inflammasome during Apoptosis.” *Immunity*, vol. 36, no. 3, 23 Mar. 2012, pp. 401–414., doi:10.1016/j.immuni.2012.01.009.

Singh, Birendra, et al. “Haemophilus Influenzae Surface Fibril (Hsf) Is a Unique Twisted Hairpin-like Trimeric Autotransporter.” *International Journal of Medical Microbiology*, vol. 305, no. 1, 2015, pp. 27–37., doi:10.1016/j.ijmm.2014.10.004.

Solovjov, Dmitry A., et al. “Distinct Roles for the α and β Subunits in the Functions of Integrin αMβ2.” *Journal of Biological Chemistry*, vol. 280, no. 2, 14 Jan. 2005, pp. 1336–1345., doi:10.1074/jbc.m406968200.

Spahich, Nicole A., and Joseph W. St. Geme. “Structure and Function of the Haemophilus Influenzae Autotransporters.” *Frontiers in Cellular and Infection Microbiology*, vol. 1, 28 Sept. 2011, pp. 1–9., doi:10.3389/fcimb.2011.00005.

Starr, Tregei, et al. “The Phorbol 12-Myristate-13-Acetate Differentiation Protocol Is Critical to the Interaction of THP-1 Macrophages with Salmonella Typhimurium.”

Plos One, vol. 13, no. 3, 14 Mar. 2018, pp. 1–13., doi:10.1371/journal.pone.0193601.

St. Geme, Joseph W. "Molecular and Cellular Determinants of Non-Typeable Haemophilus Influenzae Adherence and Invasion." *Cellular Microbiology*, vol. 4, no. 4, 7 Jan. 2002, pp. 191–200., doi:10.1046/j.1462-5822.2002.00180.x.

St. Geme, Joseph W., et al. "High-Molecular-Weight Proteins of Nontypable Haemophilus Influenzae Mediate Attachment to Human Epithelial Cells." *Proceedings of the National Academy of Sciences*, vol. 90, no. 7, Apr. 1993, pp. 2875–2879., doi:10.1073/pnas.90.7.2875

St. Geme, Joseph W., and David Cutter. "The Haemophilus Influenzae Hia Adhesin Is an Autotransporter Protein That Remains Uncleaved at the C Terminus and Fully Cell Associated." *Journal of Bacteriology*, vol. 182, no. 21, Nov. 2000, pp. 6005–6013., doi:10.1128/jb.182.21.6005-6013.2000.

Sutterwala, Fayyaz S., et al. "Mechanism of NLRP3 Inflammasome Activation." *Annals of the New York Academy of Sciences*, vol. 1319, no. 1, 19 June 2014, pp. 82–95., doi:10.1111/nyas.12458.

Swords, W. Edward, et al. "Binding of the Non-Typeable Haemophilus Influenzae Lipooligosaccharide to the PAF Receptor Initiates Host Cell Signalling." *Cellular Microbiology*, vol. 3, no. 8, 10 Apr. 2001, pp. 525–536., doi:10.1046/j.1462-5822.2001.00132.x.

Takashiba, Shogo, et al. "Differentiation of Monocytes to Macrophages Primes Cells for Lipopolysaccharide Stimulation via Accumulation of Cytoplasmic Nuclear Factor KB." *Infection and Immunity*, vol. 67, no. 11, 1999, pp. 5573–5578., doi:10.1128/iai.67.11.5573-5578.1999.

Takeda, Kiyoshi, et al. "Toll-Like Receptors." *Annual Review of Immunology*, vol. 23, 9 Jan. 2003, pp. 335–376., doi:10.1146/annurev.immunol.21.120601.141126.

Tako, Hiroki, et al. "RIG-I-like Receptors: Cytoplasmic Sensors for Non-Self RNA." *Immunological Reviews*, vol. 243, 26 Aug. 2011, pp. 91–98., doi:10.1111/j.1600-065X.2011.01052.x.

Teng, Fang, et al. "Toll-like Receptor 3 Is Involved in Airway Epithelial Cell Response to Nontypeable Haemophilus Influenzae." *Cellular Immunology*, vol. 260, no. 2, 2010, pp. 98–104., doi:10.1016/j.cellimm.2009.09.008.

Tsang, Raymond S.w., and Marina Ulanova. "The Changing Epidemiology of Invasive Haemophilus Influenzae Disease: Emergence and Global Presence of Serotype a Strains That May Require a New Vaccine for Control." *Vaccine*, vol. 35, no. 33, 1 June 2017, pp. 4270–4275., doi:10.1016/j.vaccine.2017.06.001.

Tsuchiya, Kohsuke, et al. “Caspase-1 Initiates Apoptosis in the Absence of Gasdermin D.” *Nature Communications*, vol. 10, no. 1, 7 May 2019, doi:10.1038/s41467-019-09753-2.

Ulanova, Marina, and Raymond S.W. Tsang. “Invasive Haemophilus Influenzae Disease: Changing Epidemiology and Host–Parasite Interactions in the 21st Century.” *Infection, Genetics and Evolution*, vol. 9, no. 4, July 2009, pp. 594–605., doi:10.1016/j.meegid.2009.03.001.

Ulanova, Marina, and Raymond S W Tsang. “Haemophilus Influenzae Serotype a as a Cause of Serious Invasive Infections.” *The Lancet Infectious Diseases*, vol. 14, no. 1, Jan. 2014, pp. 70–82., doi:10.1016/s1473-3099(13)70170-1.

Vanaja, Sivapriya Kailasan, et al. “Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation.” *Cell*, vol. 165, no. 5, 19 May 2016, pp. 1106–1119., doi:10.1016/j.cell.2016.04.015.

van Bruggen, Robin Van, et al. “Human NLRP3 Inflammasome Activation Is Nox1-4 Independent.” *Blood*, vol. 115, no. 26, 1 July 2010, pp. 5398–5400., doi:10.1182/blood-2009-10-250803.

Varol, Chen, et al. “Macrophages: Development and Tissue Specialization.” *Annual Review of Immunology*, vol. 33, no. 1, 2015, pp. 643–675., doi:10.1146/annurev-immunol-032414-112220.

von Moltke, Jakob, et al. “Recognition of Bacteria by Inflammasomes.” *Annual Review of Immunology*, vol. 31, no. 1, Mar. 2013, pp. 73–106., doi:10.1146/annurev-immunol-032712-095944.

Weintraub, Andrej. “Immunology of Bacterial Polysaccharide Antigens.” *Carbohydrate Research*, vol. 338, no. 23, 13 July 2003, pp. 2539–2547., doi:10.1016/j.carres.2003.07.008.

Whittaker, Robert, et al. “Epidemiology of Invasive Haemophilus Influenzae Disease, Europe, 2007–2014.” *Emerging Infectious Diseases*, vol. 23, no. 3, Mar. 2017, pp. 396–404., doi:10.3201/eid2303.161552.

Wieland, Catharina W., et al. “Toll-like Receptor 9 Is Not Important for Host Defense against Haemophilus Influenzae.” *Immunobiology*, vol. 215, no. 11, Nov. 2010, pp. 910–914., doi:10.1016/j.imbio.2009.10.004.

Xia, Zhidao, and James T Triffitt. “A Review on Macrophage Responses to Biomaterials.” *Biomedical Materials*, vol. 1, no. 1, 2006, doi:10.1088/1748-6041/1/1/r01.

Xing, Y, et al. “TLR and NLRP3 Inflammasome-Dependent Innate Immune Responses to Tumor-Derived Autophagosomes (DRibbles).” *Cell Death & Disease*, vol. 7, no. 8, 2016, doi:10.1038/cddis.2016.206.

Yang, Shenghui, et al. “Type I Interferon Induced by DNA of Nontypeable Haemophilus Influenza Modulates Inflammatory Cytokine Profile to Promote Susceptibility to This Bacterium.” *International Immunopharmacology*, vol. 74, Sept. 2019, doi:10.1016/j.intimp.2019.105710.

Yi, Young-Su. “Regulatory Roles of the Caspase-11 Non-Canonical Inflammasome in Inflammatory Diseases.” *Immune Network*, vol. 18, no. 6, Dec. 2018, doi:10.4110/in.2018.18.e41.

Zola, Tracey A., et al. “Mucosal Clearance of Capsule-Expressing Bacteria Requires Both TLR and Nucleotide-Binding Oligomerization Domain 1 Signaling.” *The Journal of Immunology*, vol. 181, no. 11, 1 Dec. 2008, pp. 7909–7916., doi:10.4049/jimmunol.181.11.7909.

Zwahlen, André, et al. “The Molecular Basis of Pathogenicity in Haemophilus Influenzae: Comparative Virulence of Genetically-Related Capsular Transformants and Correlation with Changes at the Capsulation Locus Cap.” *Microbial Pathogenesis*, vol. 7, no. 3, 1989, pp. 225–235., doi:10.1016/0882-4010(89)90058-2.