Neutrophils in the interface between innate and adaptive immunity in response to *Haemophilus influenzae* type a

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Abstract

Haemophilus influenzae type a (Hia) is a bacterial human pathogen that has emerged as a significant cause of invasive disease in Canadian Indigenous populations. Protection against encapsulated bacteria such as Hia is mediated by the antigen-specific antibody response, in cooperation with innate immune mechanisms. Anti-capsular antibodies and complement opsonize bacteria for phagocytosis. Neutrophils are abundant innate immune cells primarily involved in bacterial clearance via opsonophagocytosis. Recent studies revealed they can modulate the adaptive immune response, but this function is poorly understood. Also, much is still unknown about the innate and adaptive immune responses to Hia and its polysaccharide capsule. As such, this study aimed to determine if Hia can activate the innate and adaptive immune responses through neutrophils and if these responses differ between encapsulated versus unencapsulated, or invasive versus non-invasive strains.

To answer this, HL-60-derived-neutrophils (dHL-60) cells, differentiated with 1.25% dimethyl sulfoxide over 9 days, were used to develop an opsonophagocytosis assay (OPA) and an *in vitro* model of infection. The dependency of the immune response on strain-specific features was examined by using clinical Hia isolates of a dominant sequence type (ST)-23, including Hia 11-139 (encapsulated, invasive), 14-61 (encapsulated, non-invasive), 13-0074 (unencapsulated, invasive), as well as a representative ST-4 isolate (Hia 13-240, encapsulated, invasive), and a nontypeable strain (NTHi 375, unencapsulated, non-invasive).

The killing of Hia strains in the OPA was assessed by counting colony-forming units that grew on supplemented brain heart infusion agar plates overnight following exposure to dHL-60 cells, pooled human serum, and baby rabbit complement. Analysis identified strain-specific differences in the killing of Hia and NTHi that was differentially mediated by innate and

adaptive immune components. Specifically, unencapsulated and non-invasive Hia strains were more susceptible to non-specific killing by the innate immune system while protective antibodies were vital in defense against Hia 11-139. Despite the use of multiple resistance mechanisms, NTHi 375 continuously had the highest killing among all isolates, ultimately proving the protective capabilities of the capsule.

For the *in vitro* infection model, dHL-60 cells were stimulated with Hia 11-139 or 14-61 for 72h, with gentamicin added at the 1-hour mark. Flow cytometry analysis of costimulatory molecule ICAM-1 and Fc receptors CD89, CD64, and CD16 revealed that stimulation with Hia significantly increased the surface expression of ICAM-1, CD89, and CD64 but decreased CD16 expression, identifying potential mechanisms of neutrophil-mediated defense against Hia. No difference in surface marker expression after stimulation with Hia 11-139 vs. 14-61 was detected, suggesting that neutrophil responses to encapsulated Hia can modulate both innate and adaptive immune responses to the pathogen. To summarize, this study provided evidence that neutrophils can bridge the two immune responses in host defense against Hia infection and identified strain specificity in the immune response to Hia.

Lay Summary

The Biology Department's mission statement 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 adds to our understanding of biology by elucidating the immune response to the important human bacterium *Haemophilus influenzae* type a (Hia). *Haemophilus influenzae* (Hi) can cause local infection or severe invasive disease such as meningitis or pneumonia. Hia is an encapsulated strain of Hi that is a significant cause of invasive disease in North American Indigenous populations, especially in young children and the immunocompromised. Neutrophils are instrumental instigators of opsonophagocytosis and have recently been identified to shape adaptive immune responses. This study suggests that neutrophil's may be able to activate the innate and adaptive immune responses after stimulation with Hia through opsonophagocytosis and the upregulation of various surface molecules. Insights into the role of neutrophils in innate and adaptive immune responses will aid in understanding neutrophil's expanding function in immunity and the pathogenesis of Hia.

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List of Abbreviations

Ab	Antibodies
ADCC	Antibody-dependent cell-mediated cytotoxicity
ANOVA	Analysis of variance
APRIL	A proliferation-inducing ligand
BAFF	B-cell activating factor
BHI	Brain heart infusion
BSA	Bovine serum albumin
CARD	Amino-terminal caspase recruitment domain
CD	Cluster of differentiation
CFU	Colony forming unit
dHL-60	HL-60-derived-neutrophils
DMSO	Dimethyl sulfoxide
Fc	crystallized fragment
FcR	Fc receptor
FITC	Fluorescein isothiocyanate
HA	Hemagglutinating
Hap	*Haemophilus* adherence and penetration protein
HI	Heat-inactivated
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 fibril
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB kinase
IRF3	Interferon regulatory factor 3
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MBL	Mannose-binding lectin
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NAD	Nicotine adenine dinucleotide
NET	Neutrophil extracellular trap

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NLRs	NOD-like receptors
NOD	Nucleotide oligomerization domain
NTHi	Nontypeable *Haemophilus influenzae*
OPA	Opsonophagocytosis assay
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PD	Protein D
PE	Phycoerythrin
PRR	Pattern recognition receptor
RC	Rabbit complement
ROS	Reactive oxygen species
ST	Sequence type
TAK	Transforming growth factor-beta-activated kinase 1
TRIF	TIR domain-containing adapter inducting IFN-β

1 - Literature Review

1.1 - Characteristics of *Haemophilus influenzae* and its virulence factors

1.1.1 Clinical significance and epidemiology

Haemophilus influenzae (Hi) is a human Gram-negative, coccobacillary bacterial pathogen that can cause local or invasive infection (Bennett et al. 2020). It is classified into two groups, unencapsulated and encapsulated, based on the presence of a polysaccharide capsule. Capsular antigens divide encapsulated strains into six serotypes (a-f), while unencapsulated strains are termed 'nontypeable' (NTHi) due to their lack of capsular antigens. Hi typically colonizes the upper respiratory tract and can cause meningitis, pneumonia, septic arthritis, osteomyelitis, bacteremia, and epiglottitis. Systemic infections by encapsulated strains usually occur in vulnerable populations such as young children, immunocompromised individuals, or the elderly (Ulanova and Tsang 2009, Bennett et al. 2020).

Before a vaccine was developed, serotype b (Hib) originally caused the majority of invasive disease and was the leading cause of bacterial meningitis worldwide, particularly in children under 5 years (Kelly et al. 2004, Agrawal and Murphy 2011). A polysaccharide conjugate vaccine was developed in the late 1980s that offered protection to children as young as two months old and decreased the incidence of invasive infection and respiratory carriage of Hib by 99% (Adams et al. 1993). However, the vaccine did not offer cross-protection to other serotypes, allowing for non-type b serotypes to occupy the ecological niche previously filled by Hib through capsule replacement mechanisms (Jin et al. 2007, Ulanova and Tsang 2009, Agrawal and Murphy 2011, Tsang et al. 2021). As a result, the epidemiology of invasive *H.*

influenzae disease has significantly changed. Moreover, infection rates of Hi serotypes vary by geographic region.

Now, NTHi causes the majority of disease by *H. influenzae.* Clinical presentations include otitis media, sinusitis, and community-acquired pneumonia (Agrawal and Murphy 2011). In the North American Arctic, western/northwestern Canada, Australia, and the southwest USA serotype a (Hia) has emerged as a significant cause of invasive disease, especially in Indigenous populations (Millar et al. 2005, Tsang et al. 2014, Ulanova and Tsang 2014). In fact, the incidence of disease by Hia has reached levels approaching the pre-vaccine Hib incidence rate (Tsang et al. 2014). Clinical presentations of Hia are analogous to those of Hib, except for epiglottitis. Meningitis and pneumonia are the most common clinical presentations, with invasive disease mostly affecting children from 6 months - 2 years of age at a 16% fatality rate in some regions (Jin et al. 2007, Ulanova and Tsang 2014).

1.1.2 Virulence factors

Hi possesses a multitude of virulence factors that aid in its pathogenicity by contributing to invasion and/or evasion of the host immune response. These include the lipooligosaccharide, bacterial adhesins, IgA protease, outer membrane proteins, and the polysaccharide capsule. Among these, the polysaccharide capsule is associated with the most virulent strains of Hi and is one of the most important virulence factors to consider.

1.1.2.1 Polysaccharide Capsule

The polysaccharide capsule is a critical determinant of virulence and functions in immune evasion, allowing for invasion of the bloodstream and the eventual spread of infection. The

capsule's influence is highlighted in animal studies of genetically defined unencapsulated mutants of *H. influenzae* which have reduced virulence (Moxon et al. 1984, Kroll and Moxon 1988). In Gram-negative bacteria such as Hi, the capsule is attached to the covalently linked lipids of the outer membrane, and it forms the outermost layer of most encapsulated strains (Cress et al. 2014). Bacteria with a polysaccharide capsule are susceptible to phagocytosis only when coated by antibodies and/or complement proteins that engage Fc and complement receptors to trigger bacterial uptake. However, the capsule has a hydrophilic, negatively charged structure capable of physically limiting access to cell surface antigens and protecting the bacteria from desiccation (Moxon and Kroll 1990, Cress et al. 2014). These structural characteristics create an antiphagocytic effect by reducing the surface tension between the phagocytic cell and bacterium, creating a mutual repulsion between the bacteria and phagocytic cell (Moxon and Kroll 1990).

Hi virulence is likely related to the varying capsular structures of the six serotypes, as depicted by differences in serum and complement resistance between serotypes (Jin et al. 2007, Nix et al. 2018). Hib, the most virulent strain, has a capsule composed of polyribose ribitol phosphate linked by phosphodiester bonds. Following Hib, serotype a is the most virulent, followed by types c and f (Zwahlen et al. 1989, Jin et al. 2007). The serotype a capsule is similar in structure to Hib and consists of a polymer of glucose and ribitol with phosphodiester linkages. It likely aids in host defense evasion in a similar mechanism to Hib, but the immune response to Hia infection is still unclear (Moxon and Kroll 1990, Ulanova 2014).

A major contributor to the capsules virulence is its ability to inhibit the complement system by acting as a mechanical barrier or binding complement components. The complement system is a vital component of the host immune system that contributes to the immune response to bacteria such as Hi (Hallstrom et al. 2010).

Another aspect of the capsules virulence is driven by the fact that it is not a protein, and thus a T-cell-independent antigen that cannot be presented on the major histocompatibility complex (MHC) T-cell receptor (Murphy and Weaver 2017). This means the adaptive immune response to encapsulated bacteria heavily relies on B-cells. Without the help of T-cells, weak antibody responses are induced, predominantly by the natural antibody IgM. Polysaccharides can engage surface B-cell receptors and induce antigen-specific B-cell responses from mature B-cells (Murphy and Weaver 2017). A subpopulation of spleen marginal zone B-cells is responsible for this response, but this response does not develop until children are 2-5 years of age, making them more susceptible to invasive infection by encapsulated bacteria such as Hi (Ulanova and Tsang 2014).

1.1.2.2 Other virulence factors

Other virulence factors include lipooligosaccharide, IgA protease, outer membrane proteins, and bacterial adhesins. Of these, the lipooligosaccharide (LOS) is instrumental in the virulence of Hi.

LOS is a truncated endotoxin expressed on the surface of all Hi strains (Schweda et al. 2007). LOS is similar in function and structure to LPS, but it lacks a repeating O-antigen side chain. LOS consists of an inner core region covalently attached to lipid A, which is the endotoxic component (Mansson et al. 2001, Morey et al. 2013). Lipid A is embedded in the bacterial outer membrane. It is recognized by pathogen recognition receptors of the innate immune system (e.g., TLR4) and induces a pro-inflammatory response (Li et al. 2008). Specifically, the LOS of Hi has been shown to induce an *in vitro* pro-inflammatory response in human monocytic cells through the upregulation of costimulatory (CD86), antigen-presenting molecules (HLA-ABC or HLA-

DR), and inflammatory cytokine production (Choi et al. 2014). It has been suggested that the role

of LOS in virulence may be more pronounced in NTHi strains due to the lack of capsule.

However, LOS is also known to contribute to the virulence of encapsulated Hi strains, suggesting

the role of LOS extends to encapsulated strains (Zwahlen et al. 1989). As such, LOS is an

important virulence factor to consider.

LOS acts as a virulence factor by enhancing host mimicry mechanisms, resistance to

complement/neutrophil-mediated killing, and adherence to and invasion of host cells. There is

extensive inter- and intra-strain heterogeneity in LOS glycoforms due to phase variation creating

multiple compositionally and structurally different glycoforms in the oligosaccharide region

(Swords et al. 2003). This variability allows for efficient adaptation to environmental stressors

and immune evasion by Hi as a result. In addition, these same glycoforms can mimic host

carbohydrate moieties to evade innate immune responses. Host mimicry (also known as

molecular mimicry) can also occur through the incorporation of N-acetylneuraminic acid and

sialic acid into the LOS (de Jong et al. 2022). Sialylated LOS confers resistance to complement-

mediated killing by preventing IgM binding and classical complement pathway activation as a

result (Swords et al. 2003, Oerlemans et al. 2019). In addition, LOS can utilize host receptors to

aid in bacterial invasion (Swords et al. 2003). For example, phosphorylcholine (ChoP) in the

oligosaccharide portion of the LOS can bind to the platelet-activating factor receptor on human

airway epithelial cells to gain entry (Swords et al. 2000).

IgA protease and bacterial adhesins are necessary virulence factors for immune evasion,

bacterial adherence, and invasion. The contribution to virulence by IgA protease is made clear by

the fact that IgA proteases are produced only by pathogenic bacteria (Mulks et al. 1978). IgA

protease is an enzyme from the autotransporter family of proteins and is produced by almost all

Hi strains. It cleaves human immunoglobulin A1 (IgA1) and secretory IgA at the hinge region, effectively inhibiting the function of IgA (Murphy et al. 2015). IgA is an antibody present on mucous membranes and functions in inhibiting bacterial adherence through agglutination and neutralizing microbial enzymes and toxins (Spahich and St. Geme 2011). As such, the protease functions in invasion, trafficking, and persistence of Hi through the inactivation of IgA (Murphy et al. 2015). The IgA1 proteases of individual strains vary considerably due to horizontal gene transfer to avoid immune detection. Two types currently exist and are defined based on the bond cleaved in the IgA1 hinge region. Serotypes a, b, d, and f express type 1, and serotypes c and e express type 2. NTHi strains express both or either type (Spahich and St. Geme 2011). Despite variable expression between strains, studies show that IgA protease expression upregulates during early colonization and persistence of Hi in the nasopharynx, highlighting its importance in virulence (Spahich and St. Geme 2011, Murphy et al. 2015).

Bacterial adhesins such as Hap (*Haemophilus* adherence and penetration protein), Hia (*H. influenzae* adhesin)/Hsf (*Haemophilus* surface fibril) are autotransporter proteins with a similar structure to IgA protease. They have been suggested to cooperate to enhance bacterial adhesion (Spahich and St. Geme 2011). Hap shares considerable homology with IgA1 protease and is expressed by serotypes a, b, f, e, and NTHi. Hap functions in epithelial cell adhesion, facilitates adherence to glycoproteins, and mediates bacterial aggregation and microcolony formation (Fink et al. 2003, Rodriguez et al. 2003). Hia shares 72% identity and 81% similarity with Hsf, and both proteins display the same binding pattern to different epithelial cell lines (Rodriguez et al. 2003). Hia and Hsf are found in non-typeable and encapsulated strains, respectively, and contribute to the adherence to epithelial cells to a greater degree than Hap. This is because the passenger domains of the C-terminus of Hia and Hsf remain cell-associated, which may enhance

adherence to host cells (Spahich and St. Geme 2011). In addition, Hia has two binding domains that interact with the same host receptor with differing affinities due to differences in the aspartic acid and glutamine side chains. The second binding domain functions in translocating the host cell closer to the bacteria, and the first higher affinity binding domain displaces the second through a ratcheting effect. Similar to Hia, Hsf has two binding pockets that recognize the same receptor whose identity is still unknown. The distal domain is replaced by the higher affinity proximal domain after host cell translocation through competitive displacement mechanisms (Cotter et al. 2005, Spahich and St. Geme 2011). Hsf is a much larger protein than Hia and protrudes past the capsule, which may explain why Hsf is found in encapsulated strains, but Hia is not (Spahich and St. Geme 2011). It's been proposed that the considerable structural similarities between the binding pockets of Hia and Hsf may mean they bind to the same host cell receptor in a proposed conserved mechanism. As a result, encapsulated strains containing Hsf may out-compete unencapsulated strains containing Hia for adhesion to respiratory surfaces (Cotter et al. 2005). Furthermore, the Hsf of encapsulated strains has additional functions that contribute to virulence, including the recruitment of vitronectin to the outer membrane of Hi. This functions in inhibiting the formation of the membrane attack complex of the complement system, which ultimately increases the survival of Hi (Langereis and de Jonge 2020).

Other bacterial adhesins include high molecular weight (HMW) proteins HMW1/HMW2 and hemagglutinating (HA) pili. HMW1/HMW2 are highly immunogenic proteins characterized by their high molecular weight and are expressed in 75% of NTHi strains and some Hia, Hie, and Hif strains. However, encapsulated strains that express these proteins usually have lost the ability to express their capsules through mutations (Rodriguez et al. 2003). HMW1 and HMW2 are important for binding to human epithelial cells through proteoglycans such as sialic acid. NTHi

strains lacking these proteins have a significantly decreased attachment, highlighting their importance in virulence (Noel et al. 1994). HA pili are expressed by a subset of NTHi strains and some encapsulated strains. HA pili promote bacterial adherence and colonization to eukaryotic cells through an interaction with sialic-containing lactosylceramide structures (Rodriguez et al. 2003). To avoid immune recognition, pili expression varies between strains due to point mutations, phase variations, horizontal gene transfer, and recombination. These mechanisms of evasion combined with the fact that Hi strains lacking HA pili were reported to have lower binding to mucin mean it's a significant virulence factor (Rodriguez et al. 2003).

Outer membrane proteins are the final group of virulence factors that aid in cell attachment and immune evasion. Proteins D, E, and F are highly conserved proteins that mediate cell attachment. Protein D (PD) is a surface-exposed lipoprotein found in almost all strains of encapsulated and unencapsulated Hi strains (Ruan et al. 1990, Forsgren and Riesbeck 2008, Duell et al. 2016). PD is thought to enhance the virulence of Hi through multiple functions. PD binds to the hinge region of IgD, which is thought to inhibit antibody secretions (Ruan et al. 1990). The glycerophosphodiesterase activity of PD allows for the hydrolysis of glycerophosphorylcholine into glycerol-3-phosphate and choline. In turn, choline is incorporated into the LOS, which increases adherence of Hi to human monocytes (Forsgren and Riesbeck 2008). PD also causes damage to the cilia of host cells, which promotes adherence and eventual internalization of Hi into human monocytes (Forsgren and Riesbeck 2008). In addition, NTHi mutants that lack PD expression notably had a lower ability to cause infection, further supporting these proposed functions (Janson et al. 1994). Protein E acts in concert with Hap to promote adhesion and entry of Hi into epithelial cells by binding the human extracellular matrix proteins laminin and vitronectin. It's also known to bind and store host hemin to support the nutrition and

survival of Hi during infection (Jubair et al. 2014, Duell et al. 2016). Similar to protein E, protein F binds to the globular domain of the alpha chain of laminin to promote host cell attachment. The binding of vitronectin by both proteins (E and F) also interferes with complement system activation by inhibiting C5b-C9 deposition and membrane attack complex formation (Duell et al. 2016).

Outer membrane proteins P2, P4, P5, and P6 are well-established virulence factors of NTHi. These proteins have multiple redundant features for repeat binding of host proteins to enhance the survival of Hi. P2 and P5 are porins that bind mucin, which allows for anchoring to the host mucosal epithelium (Duell et al. 2016). P2 is the most abundant outer membrane protein on NTHi and has high antigenic heterogeneity, leading to evasion of the antibody immune response. P5 can additionally bind human factor H, which regulates the alternative pathway through C3b. This function is identical to that of the recently discovered protein H, which is a virulence factor found on serotypes b, f, and NTHi (Fleury et al. 2014). P4 binds laminin and vitronectin in a similar manner to proteins E and F, leading to additional resistance to serum and complement as a result. P6 has an immunomodulatory effect and camouflages itself by utilizing multiple orientations on the outer membrane that allow for evasion of the antibody-mediated response (Duell et al. 2016).

1.1.3 Conclusions

Hi is a significant bacterial human pathogen capable of causing severe invasive disease. Due to serotype replacement mechanisms, Hia has emerged as the dominant strain in North American Indigenous populations, causing a significant disease burden in these communities, especially in young children. This is possible due to the multitude of virulence factors that aid in

host cell attachment, invasion of host cells, and evasion of the host immune system. Their antigenic heterogeneity and functional redundancy make Hi an efficient pathogen capable of causing disease. Therefore, it is important to characterize Hi's interaction with the host immune system to effectively understand its pathogenesis and develop potential treatments and therapies.

1.2 - Recognition of *Haemophilus influenzae* by the innate immune system

1.2.1 Basic characteristics of the innate immune system

The innate immune system is an immediate, non-specific, inborn response to pathogens. It is the body's first line of defense and is made up of anatomical barriers, chemical barriers, the complement system, and innate immune cells (Murphy and Weaver 2017). Anatomical barriers include the mucous membrane and skin, which prevent pathogens from initially entering the body. Chemical barriers include antimicrobial peptides and enzymes that break down bacteria, which are present in substances such as tears and saliva. If these initial barriers are breached, the complement system is activated, which is a group of plasma proteins present in blood and other body fluids (Murphy and Weaver 2017). An example of a source of complement is serum, the fluid phase of clotted blood. The complement system acts in conjunction with antibodies to trigger a cascade of events upon exposure to a pathogen, generating three main outcomes. These include inflammation, opsonization, and activation of a membrane attack complex. Complement-mediated killing is a major defense against Hi. As such, Hi has evolved a multitude of evasion mechanisms to the complement system (Hallstrom and Riesback 2010). Due to this, innate immune cells are usually needed to aid in defense against bacterial infection. Innate immune cells include macrophages, granulocytes, mast cells, natural killer cells, and dendritic cells.

Cellular defenses are initiated when sensor cells detect inflammatory inducers known as pathogen-associated molecular patterns (PAMPs) using pattern recognition receptors (PRRs) (Murphy and Weaver 2017). Innate immune cells respond with effector activity and/or inflammation following receptor activation. When an immune response is initiated, neutrophils are the innate immune cells that are the first to arrive at the site of infection.

1.2.2 Neutrophils

Neutrophils are the most abundant immune cell in the human body, accounting for fifty to seventy percent of all circulating leukocytes. Fifty-five to sixty percent of the bone marrow is dedicated to producing neutrophils from hematopoietic stem cells to eventually create their large circulating and marginal cell pools (Gorgens et al. 2013, Murphy and Weaver 2017). They mature over 2 weeks, undergoing six divisions, and maturing in stages from promyelocytes, myelocytes, metamyelocytes, band cells, to mature neutrophils (Rosales 2018). As a neutrophil matures, its nucleus changes from round to a banded, lobulated morphology. The proportion of bands increases as a sign of infection (Vietinghoff and Ley 2008). Neutrophils also develop tertiary granules and secretory vesicles that store substances needed for their antimicrobial functions. In an uninfected host, the neutrophil population is maintained at a constant number through apoptosis. When an inflammatory stimulus is received, the number of circulating neutrophils dramatically increases. Neutrophils rapidly migrate to the infection site, and cytokines such as granulocyte-colony stimulating factor (G-CSF) briefly extend their lifespan (Rosales 2018). Activated neutrophils use a variety of receptors to sense extracellular ligands and quickly trigger effector functions important for the innate immune response. There are receptors for the detection of proinflammatory mediators, cytokines, chemokines, opsonins, complement

proteins, and PAMPs. The three main effector functions of neutrophils are phagocytosis, degranulation, and neutrophil extracellular trap (NET) formation (Mayadas et al. 2014, Murphy and Weaver 2017, Rosales 2018).

Neutrophils primarily function as innate phagocytes. Due to their considerable number, their role in ingesting and killing bacteria is essential to the overall host immune response (Murphy and Weaver 2017). This is best exemplified in cases where deficiencies in neutrophils or their products make that patient more prone to recurrent and severe bacterial infections (Lin et al. 2008, Nguyen et al. 2017, Lehman and Segal 2020). This response is initiated when PRRs (such as Toll-like receptors) and/or opsonic receptors recognize a microbial surface and/or surface-bound opsonin, respectively. Phagocytosis is most efficient when it is facilitated by opsonization, in which case it can be termed opsonophagocytosis (Kobayashi et al. 2018). Phagocytosis occurs after actin polymerization allows for the advancement of the neutrophil plasma membrane, engulfing the microbe into a phagocytic vacuole where secretory granules, antimicrobial enzymes, and reactive oxygen species work to kill the microbe (Kessel et al. 2014).

Neutrophils have primary, secondary, and tertiary granules, and secretory vesicles (Lacy 2006). Degranulation of these granules occurs only after receptors, such as the G protein-coupled receptor, signal their movement to the cell membrane. From here, vesicle tethering and docking occur, creating a contact surface between the target membrane and the outer membrane of the granule. Priming then occurs and creates a fusion pore structure between the granule and target membrane. The contents of the granule are released when this fusion pore expands (Lacy 2006). Primary granules contain elastase, myeloperoxidase, cathepsins, bactericidal/permeability-increasing protein, and defensins which are important in anti-bacterial defense (Stock et al. 2018). Secondary and tertiary granules contain lactoferrin and matrix metalloproteinase, while

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secretory vesicles contain human serum albumin (Stock et al. 2018). In addition to degranulation, neutrophils intracellularly release a variety of antimicrobial proteins, enzymes, and reactive oxygen species (ROS) that aid in the killing of ingested bacteria (Lacy 2006). Reactive oxygen species are produced when receptor activation causes membrane-bound NADPH-dependent oxidase to generate superoxide. From this, secondary products such as hypochlorous acid and peroxynitrite are created (Nguyen et al. 2017). These products are cytotoxic and damage proteins, membrane lipids, and nucleic acids to destroy microbes (Kobayashi et al. 2018).

Neutrophil extracellular traps (NETs) are produced when the bacteria are too large to be phagocytosed, and instead, web-like structures consisting of DNA fibers, proteins, and granule contents are released (Rosales 2018). Death by NETs is called NETosis and additionally requires ROS production. NETs have been observed to be produced in response to NTHi, however, NTHi was resistant to NETosis due to LOS oligosaccharide moieties, so it is unknown if they have a bactericidal effect on Hi strains (Juneau et al. 2011).

Neutrophils were originally considered to be short-lived cells with limited functions in the acute inflammatory response as described above. Recent research has revealed they may have a lifespan of up to 5 days once activated, broadening their potential to contribute to the immune response (Pillay et al. 2010). In addition, it's been discovered they have a wide array of functions previously undescribed (Amulic et al. 2012, Kolaczkowska and Kubes 2013, Mayadas et al. 2015, Rosales 2018). Specifically, they have the potential to either directly or indirectly modulate the adaptive immune system, making them highly versatile immune cells (Mantovani et al. 2011, Scapini and Cassatella 2014, Li et al. 2019). This crosstalk is mediated by cell-derived soluble factors and/or contact-dependent mechanisms which allow neutrophils to activate B-cells and initiate their proliferation. For example, human neutrophils have been identified to

produce cytokines such as B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) that promote B-cell proliferation and differentiation (Scapini et al. 2008). In addition, the vast repertoire of cytokines produced by neutrophils has the potential to modulate adaptive immune responses and lymphocytes in ways that are not fully understood (Mantovani et al. 2011, Tamassia et al. 2018, Cassatella et al. 2019, Costa et al. 2019). Their ability to modulate B-cell survival, maturation, and differentiation is important in defense against Hi, as the polysaccharide capsule is a T-cell independent antigen. Neutrophils have also been shown to migrate to lymph nodes during infection, where they can modulate the cellular and humoral immune responses (Abadie et al. 2005, Chtanova et al. 2008, Rosales 2020). Here, neutrophils function as antigen-presenting cells by carrying bacterial antigens to dendritic cells and macrophages or by expressing major histocompatibility complex II molecules and costimulatory molecules (Blomgran and Ernst 2011, Kolaczkowska and Kubes 2013, Hampton et al. 2015).

1.2.3 Pattern recognition receptors

Pattern recognition receptors (PRRs) are germ-line encoded receptors that recognize conserved microbial molecular patterns known as pathogen-associated molecular patterns (PAMPs) expressed on invading pathogens (Murphy and Weaver 2017, Abbas et al. 2018). The microbial patterns recognized are often essential for microbe survival, such as the mannose-rich oligosaccharide, peptidoglycans, and LPS. PRR activation causes immune cells to respond with effector activity in target tissues and initiate the inflammatory response through cytokine and chemokine production (Amarante-Mendes et al. 2018). PRRs are expressed by most cells, including neutrophils, and are classified based on cellular location as either free receptors in serum, membrane-bound phagocytic receptors (C-type lectin receptors (CLRs)), membrane-

bound signaling receptors (Toll-like receptors (TLRs)), cytoplasmic signaling receptors (NOD-like receptors (NLRs)), or retinoic acid-inducible gene-I-like receptors (RLRs)) (Murphy and Weaver 2017). CLRs and RLRs are present on neutrophils, but they are primarily involved in antifungal and viral defense, respectively, meaning they do not have an influential role in the immune response against Hi. However, the activity of the serum receptors, TLRs, and NLRs are all applicable to the immune response to Hi.

Free receptors in serum include ficolins, pentraxins, collectins, and complement receptors. Ficolins recognize bacterial carbohydrate molecules such as acetylated polysaccharides, sialic acid, lipopolysaccharides, and bacterial peptidoglycans. They are sub-divided into L-ficolin, M-ficolin, and H-ficolin (Zhang and Ali 2008, Bidula et al. 2019). They function as opsonins that activate the lectin-complement pathway and enhance phagocytosis by cells such as neutrophils. Pentraxins are serum proteins with pentameric structure and include C-reactive protein and serum amyloid P (Du Clos 2013). Known ligands include the C-polysaccharide of *S. pneumoniae* and the phosphocholine of NTHi LOS (Langereis et al. 2019). After recognition of these ligands, the pentraxins interact with the complement system and with Fc gamma and alpha receptors, favoring opsonization via the classical pathway of complement and enhancing phagocytosis, signaling, and cytokine production by neutrophils (Du Clos 2013, Langereis et al. 2019). Collectins, such as mannose-binding lectin (MBL) and surfactant proteins (A, B, D), are C-type lectins containing collagen that bind to glycoconjugates of the microbial cell wall or envelope. For example, surfactant protein D binds to LPS, which results in increased bacterial agglutination and phagocytosis of several strains of Gram-negative bacteria by human neutrophils *in vitro* through the lectin pathway activation and subsequent opsonization (Kuan et al. 1992, Lim et al. 1994, Crouch 1999).

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TLRs are membrane (surface or intracellular) associated receptors with a considerable role in the innate immune response due to their variety and number. TLRs 1-10 are known to exist in mammals and all except TLR3 are expressed by human neutrophils (Hayashi et al. 2003). TLRs recognize a broad number of ligands that are specific to the type of TLR. In general, they recognize common microbial molecules including bacterial LPS and peptidoglycans. Their activation can initiate several signaling cascades to induce inflammation and activation of phagocytes (Murphy and Weaver 2017, Abbas et al. 2018). All TLRs except TLR3 signal using the MyD88 pathway (Abbas et al. 2018). TLR engagement and activation of the MyD88 pathway results in the phosphorylation of the NF-κB inhibitor protein (IκB). This allows for the translocation of NF-κB into the nucleus, where it mediates numerous functions including the transcription of proinflammatory cytokines. TLR3 signals using the TIR domain-containing adapter inducting IFN-β (TRIF)-dependent pathway, which causes the transcription factor IRF3 to enter the nucleus and induce type I interferon gene expression (Kawasaki and Kawai 2014). Interestingly, TLR4 is capable of activating both pathways, making it a critical TLR in the inflammatory response.

TLRs -3, -7, -8, and -9 are intracellular receptors designed to recognize microbial nucleic acids, internalized bacteria, or viruses. TLR -3, -7, and -8 bind to endosomal double or single-stranded RNA derived from bacteria and viruses, and TLR9 binds to unmethylated CpG motifs found in bacterial DNA (Abbas et al. 2018). TLR7 and -8 traditionally recognize viruses, but studies have shown that TLR7 also has the potential to recognize phagosomal bacteria and is upregulated *in vitro* and *in vivo* with the aid of TLR2 after exposure to NTHi (Sakai et al. 2007, Mancuso et al. 2009). This mechanism was suggested to be important in mixed viral/bacterial infections in which bacterial infection enhances the host antiviral response and increases the

production of type I interferons (Sakai et al. 2007). Although TLR9 may recognize *H. influenzae* after its internalization, studies found it has no role in defense against NTHi and Hib, potentially due to the low stimulatory potency of Hi bacterial DNA (Mogensen et al. 2006, Wieland et al. 2010).

TLRs -1, -2, -4, -5, -6, and -10 are cell surface receptors that mainly recognize microbial cell wall components (Kawasaki and Kawai 2014, Abbas et al. 2018). TLRs -1, -2, and -6 recognize structures such as triacyl lipopeptides, diacyl lipopeptides, zymosan, peptidoglycans, lipoteichoic acids, LPS, and mannan. Of these, TLR2 has been identified to induce cytokine production after recognizing Hib porins and NTHi lipoprotein P6 (Galdiero et al. 2004, Lugade et al. 2011). TLR2 expression and function are closely related to TLR4, as depicted in mouse models with acute Hi infection (Lorenz et al. 2005). TLR4 is one of the best characterized TLRs to date and is extremely important in the immune response to Gram-negative bacteria such as Hi (Wang et al. 2002, El-Zayat et al. 2019). TLR4 recognizes Gram-negative bacteria components such as lipid A of LPS. An *in vivo* mouse model revealed TLR4 is capable of directly recognizing NTHi LOS and was essential for neutrophil influx, cytokine production, and bacterial clearance (Wang et al. 2002, Lorenz et al. 2005). In another mouse model, TLR2 and TLR4 were both essential in the clearance of encapsulated Hi strains (Zola et al. 2008). TLR5 recognizes bacterial flagellum which is absent in Hi strains. Accordingly, studies with encapsulated Hib failed to activate TLR5 on human epithelial cells *in vitro* (Zhang et al. 2005). TLR10 is the most recently discovered TLR and has no known ligand (Fore et al. 2020). It has been identified to play a significant role in the inflammatory response to the Gram-positive bacterium, *Listeria monocytogenes*, through the help of TLR2, suggesting it may be able to detect other intracellular pathogens (Regan et al. 2013).

NLRs are cytosolic receptors that detect microbial products in the cytoplasm and are expressed by monocytes, macrophages, neutrophils, and lymphocytes. NLRs can initiate the same inflammatory responses as TLRs via NF-κB and a variety of other downstream intracellular signaling pathways (Murphy and Weaver 2017). NLRs are composed of a central NOD domain critical for activation, a leucine-rich repeat (LRR) domain responsible for recognizing PAMPs, and an amino-terminal caspase recruitment domain (CARD) that induces signaling. NOD1 and NOD2 are the best characterized NLRs that recognize fragments of the bacterial cell wall peptidoglycans (Franchi et al. 2009, Murphy and Weaver 2017). NOD1 recognizes gamma-glutamyl diaminopimelic acid, which is a breakdown product of Gram-negative bacteria peptidoglycans and some Gram-positive peptidoglycans. NOD2 recognizes muramyl dipeptide (MDP), a component of most bacterial peptidoglycans (Franchi et al. 2009). Following the binding of a ligand to NOD1 or -2, a CARD-containing serine-threonine kinase is recruited (RIP2), which in turn activates TAK1 and IKK, leading to NF-κB and MAPK activation and the expression of pro-inflammatory cytokines, chemokines, and antibacterial peptides (Murphy and Weaver 2017). Both NOD1 and NOD2 are critical in defense against Hi. Previous studies found that effective neutrophil influx and clearance of encapsulated Hib strains required TLR4, TLR2, and NOD1 expression, but the deficiency of one receptor did not affect the clearance of capsule-deficient Hi (Zola et al. 2008). This emphasizes the different signaling requirements to clear the infection by encapsulated vs unencapsulated bacteria. NOD2 has a role in defense against NTHi as it is upregulated with LOS stimulation *in vitro* and involved in the inflammatory reaction produced after the cytoplasmic release of internalized NTHi (Choi et al. 2014, Woo et al. 2014).

1.2.4 The complement system

1.2.4.1 Definition and sources of complement in the body

    The complement system is an integral component of the innate immune system and is crucial in the immune response to bacteria. It is a complex protein network present in blood and other bodily fluids that is activated upon exposure to bacteria cells and results in opsonization, direct bacterial killing by the membrane attack complex (MAC), and inflammatory responses. Complement was originally classified as a part of serum, the fluid phase of clotted blood (Murphy and Weaver 2017). However, complement proteins can also exist as membrane-associated proteins and are widely distributed in the body. Complement proteins in serum are mainly produced by liver hepatocytes but can also be produced locally by cell types such as epithelial cells, macrophages, and monocytes in tissues (Lubbers et al. 2017). In addition, inflammation can cause complement to increase at the infection site. This occurs when respiratory tract inflammation increases the permeability of the mucosa, allowing for plasma to enter the airway lumen (Hallstrom and Riesbeck 2010). The complement system is especially important in defense against Gram-negative bacteria, as their thin peptidoglycan layer and outer lipid membrane allow for quick insertion of the MAC into the cell membrane (Heesterbeek et al. 2018). The importance of complement is further elucidated by patients with genetic deficiencies in complement that are more prone to recurrent infection by Gram-negative bacteria (Heesterbeek et al. 2018).

1.2.4.2 Three pathways of activation

    The complement system can be activated by three pathways, known as the lectin pathway, the classical pathway, and the alternative pathway (Hallstrom and Riesback 2010,

Murphy and Weaver 2017). During the initial detection of Hi by the complement system, the

classical and alternative pathways likely dominate (Winkelstein et al. 1992, Brouwer et al. 2008,

Hallstrom and Riesbeck et al. 2010). The lectin pathway is activated by the binding of mannan-

binding lectin (MBL) or ficolins to surface mannose-containing carbohydrates on pathogens. An

in vitro study found that although MBL was capable of binding to Gram-negative bacteria such

as *E. coli*, opsonophagocytosis by neutrophils did not significantly increase as a result, meaning

this pathway may not be necessary in the response to Gram-negative bacteria (Brouwer et al.

2008). The classical pathway is activated when complement component C1 (in complex with

recognition protein C1q and proteases C1r and C1s) either recognizes a microbial surface

directly or binds to the Fc region of antigen:antibody complexes. The latter provides a link

between innate and adaptive immunity and is particularly useful when natural antibodies

recognize common microbial membrane constituents (such as phosphocholine) or antigen-

specific antibodies produced during the adaptive immune response recognize the bacterial

surface (Ochsenbein and Zinkernagel 2000). Natural antibodies are polyreactive antibodies

present in the absence of infection found in serum. IgM is the antibody class most efficient at

binding C1q and activating the classical pathway, however, this can also be achieved by IgG

(Nesargikar et al. 2012). The alternative pathway is initiated by spontaneous hydrolysis and

activation of complement component C3, which generates the alternative pathway fluid-phase

C3 convertase (C3(H_2O)Bb). The alternative pathway can also be activated via the lectin and

classical pathways when C3b generated by either of these pathways binds factor B, leading to the

creation of the second C3 convertase (C3bBb). The two C3 convertases are short-lived but are

stabilized by plasma proteins such as properdin (Murphy and Weaver 2017). Complement

activation can also occur by many non-traditional mechanisms. For example, properdin has been

identified to directly bind C3b *in vitro* through interaction with LOS on bacteria, leading to the activation of the alternative pathway (Markiewski et al. 2008). Neutrophil-derived properdin was also shown to bind to apoptotic T-cells, which led to activation of the alternative pathway and eventual C3b deposition (Kemper et al. 2008).

All pathways converge to generate enzymatic activity by C3 convertase, which is a multi-subunit protein with protease activity, and follow the same terminal pathway. C3 convertase binds to the pathogen surface, where it cleaves C3, creating C3b and C3a. From here, C3b is covalently bound to the pathogen surface while C3a is released. Following this, C3b binds to the C3 convertase, generating the C5 convertase and creating C5a and C5b. These molecules all work together to generate the three main effector activities including phagocytosis, inflammation, and formation of the MAC (Murphy and Weaver 2017). C3b is deposited in large quantities on the microbial surface and acts as an opsonin to enable phagocytosis by cells with complement receptors. Deficiencies in C3 and a component of its convertase (C4b) have been associated with invasive Hib disease, highlighting its importance in opsonization (Winkelstein and Moxon 1992). C5b binds with C6-C9 to form the MAC. The MAC is a pore in the membrane which permits fluid influx by osmosis. This leads to a disruption of cellular homeostasis, the membrane proton gradient, and allows enzymes to penetrate the cell, eventually causing cell lysis (Kondos et al. 2010). C3a, C4a, and C5a are anaphylatoxins that are highly related proinflammatory molecules that act as chemoattractants and generate inflammation. Inflammation results in increased vascular permeability, smooth muscle contraction, adhesion molecule activation, and immune cell recruitment (such as macrophages or neutrophils) (Klos et al. 2010). In addition, they cause the release of inflammatory molecules such as histamine and tumor necrosis factor-α from mast cells. This response by anaphylatoxins is necessary to recruit

other aspects of the immune response due to the evolution of multiple bacterial evasion strategies to complement-dependent killing (Haas and Strijp 2007).

The complement system is tightly regulated by membrane-bound and soluble regulators. This ensures that complement activity, along with its amplification potential, is confined to the pathogen surface and does not cause any damage to the host tissues (Dunkelberger and Song 2010, Murphy and Weaver 2017). Host cells have intrinsic membrane-bound regulators such as the decay-accelerating factor and CR1 to inhibit activity by the C3/C5 convertases or MAC. Factor H (FH), FH-like-protein-1, and vitronectin are examples of soluble regulators that either inhibit the production of C3-convertases or MAC formation (Dunkelberger and Song 2010, Hallstrom and Riesbeck 2010). In addition, inactivation of the anaphylatoxins is important in controlling complement and is achieved by serum carboxypeptidases that cleave the N-terminal arginine of the anaphylatoxins (Dunkelberger and Song 2010).

The importance of the complement system is further emphasized by the fact that Gram-negative bacteria have evolved several mechanisms to resist the actions of complement. Hi resists complement using physical barriers and soluble complement regulators (Hallstrom and Riesbeck 2010). The polysaccharide capsule contributes to complement resistance by creating a physical barrier that results in greater serum resistance and reduced C3 deposition, opsonization, MAC formation, and phagocytosis. In particular, the capsule affects the opsonic potential of a key complement component, C3b. This is done by inhibiting C3 activation, adherence to the bacterial surface, and accessibility to phagocytic cells (Hallstrom and Riesbeck 2010). Specifically, *E. coli* Ki and group B *Streptococcus* Type III polysaccharide capsules contain N-acetylneuraminic acid, which favours the inactivation of C3b, a major complement protein responsible for initiating many effector functions of complement (Maruvada et al. 2008, Hyams

et al. 2010). This occurs because N-acetylneuraminic acid contains a binding site for factor H, a regulatory protein of the complement system. The binding of factor H to N-acetylneuraminic acid interrupts the binding of C3b to the bacterial surface. Capsular polysaccharides of *S. pneumoniae* have also been shown to hide C3b on the cell wall beneath the capsule, limiting its recognition by phagocytes (Winkelstein 1981). Although the capsule is an effective evasion mechanism, complement can still lead to bacterial killing when the capsule is coated with antibodies and C3b.

Another evasion strategy used by encapsulated Hi strains and NTHi is to bind Factor H and FHL-1, two major regulators of the alternative pathways (Fleury et al. 2014). Previous studies found unencapsulated and encapsulated Hi strains can directly bind to factor H *in vitro* (Hallstrom et al. 2008). Factor H binds to the surface of Hi and maintains its complement regulatory activity. As a result, C3b is inactivated, and the complement amplification loop is broken down, leading to decreased opsonophagocytosis of the bacteria. In addition, the LOS of NTHi is highly variable between strains and is often sialylated. Sialylated LOS has an increased affinity for factor H. Encapsulated strains of Hi have been shown to use a surface lipoprotein (protein H) to bind factor H *in vitro* (Fleury et al. 2014). Interestingly, the amount of bound factor H varied between encapsulated strains and strains with low binding were more sensitive to killing by serum (Hallstrom and Riesbeck 2010). Lastly, the virulence factors Hsf and protein E of Hi have been shown to bind vitronectin. This leads to the inactivation of the terminal pathway of complement and limits MAC formation (Hallstrom and Riesbeck 2010).

1.3 - ***Haemophilus influenzae*** **and the adaptive immune response**

1.3.1 Basic characteristics of the adaptive immune response

The adaptive immune response is a pathogen-specific, slower response, with the ability to generate immunological memory to prevent reinfection. The actions of the adaptive immune system are largely mediated by the two major types of lymphocytes, known as B lymphocytes (B-cells) and T lymphocytes (T-cells). Both cell types recognize antigens through their highly variable antigen receptors but have different overall roles in the immune response. B-cells mature in the bone marrow and express B-cell receptors (also known as membrane immunoglobulin) and are responsible for producing antibodies, also known as the humoral immune response (Murphy and Weaver 2017). B-cells can differentiate into plasma cells, the effector cells that release antigen-specific antibodies, or memory cells that participate in immunological memory. T-cells mature in the thymus and express T-cell receptors. T-cell receptors recognize specific antigens presented to them by the major histocompatibility complex (MHC) molecules and initiate the cellular immune response. T-cells are classified as either cytotoxic T-cells that kill infected cells, helper T-cells that provide signals to other cells, or regulatory T-cells that suppress the activity of other lymphocytes (Murphy and Weaver 2017). Although T-cells are extremely valuable to the host immune response, the T-cell response is more important in defense against intracellular bacteria. Also, the polysaccharide capsule is a T-cell independent antigen, meaning that it can not be recognized by T-cell receptors (Nagata and Koide 2010). Due to this, the primary focus will be on the humoral immune response.

Immediately following B-cell receptor binding, B-cells will proliferate and differentiate into plasma cells that secrete antibodies with the same specificity as the original antigen. The hallmark of the adaptive immune response is the clonal expansion of lymphocytes to create mature lymphocytes that express receptors for a unique antigen (LeBien and Tedder 2008). This occurs by somatic gene rearrangements of incomplete receptor gene segments, resulting in

combinatorial diversity and junction diversity that generates thousands of different receptor chains. Although this process generates a strong, specific response to the pathogen, it is limited by its 4–6-day maturation period, meaning the response is not fully effective until a week after infection (Murphy and Weaver 2017).

Antibodies are of vital importance as they are secreted in copious quantities by the B-cell effector cells (plasma cells). They have a wide array of functions, including acting as a bridge between innate and adaptive immune responses. Natural antibodies are non-specific antibodies present without prior antigen exposure. However, antigen-specific antibodies are also generated by plasma cells of adaptive immunity after exposure to an antigen, creating a much stronger effect as discussed above. The antibody is composed of constant and variable regions and is made up of two light and heavy chains (Janeway et al. 2001). The variable region (also known as the Fab region) includes the ends of the light and heavy chains and determines the antigen specificity. The constant region (also known as the Fc region) determines antibody class, which can be divided into five major classes, IgM, IgG, IgA, IgD, and IgE. The major classes all have unique immune functions and distributions within the body. IgM is a low-affinity membrane-bound or soluble antibody present in the blood and lymph. It is extremely important during the beginning stages of the adaptive immune response, including efficiently activating the complement system (Liu et al. 2019, Keyt et al. 2020). IgG is the dominant antibody in plasma, blood, and other bodily fluids and mainly functions as an opsonin to activate opsonophagocytosis. IgG is also present on B-cells and its function is poorly understood, but it is thought to regulate B-cell development and homeostasis (Gutzeit et al. 2018). IgA is commonly found in secretions and on mucosal surfaces. It is important in passive immune protection at birth through breast milk and can stimulate effector functions such as phagocytosis (Janeway et al.

2001). Despite this, it has been reported to be a poor complement activator because it does not activate the classical pathway (Woof and Kerr 2005). IgE is distributed throughout the blood and extracellular fluid in low levels and functions in stimulating allergic reactions via mast cells (Janeway et al. 2001).

IgM, IgG, and IgA are primarily involved in host antibacterial defense. IgM and IgG are the predominant antibodies in serum that participate in the opsonization of bacteria (Gonzalez-Quintela et al. 2008). IgA is also present in serum in large amounts, but its functional roles remain incompletely understood (Leong et al. 2014). However, IgA has been observed to opsonize *Staphylococcus aureu*s and induce phagocytosis by polymorphonuclear leukocytes *in vitro* (Gorter et al. 1987). An increase in IgA, IgM, and IgG serum levels was also observed after nasopharyngeal colonization with NTHi using an experimental human model, suggesting they are important in the opsonization of NTHi (Winokur et al. 2013).

Fc receptors (FcRs) are surface molecules that interact with the crystallized fragment (Fc) of pathogen-bound immunoglobulin molecules and are necessary to activate the desired effector functions by the cells that express them. FcRs are present on cells such as monocytes, macrophages, and neutrophils. Their wide distribution on innate and adaptive immune cells and various functions mean FcRs function as a link between the two main arms of immunity (Paul 2013, Mkaddem et al. 2019).

Upon infection, antibodies will detect pathogens and aggregate on bacterial surfaces, which causes cross-linking of FcRs. The crosslinking leads to dual tyrosine phosphorylation of the ITAM (immunoreceptor tyrosine-based activation motif) or ITIM (immunoreceptor tyrosine-based inhibition motif) sequence depending on activating or inhibiting signaling. Following this, Syk kinase is recruited and activated (Futosi et al. 2013). Next, PI3K is activated, and adaptor

molecules are recruited, which results in a sustained increase in cytosolic calcium by inositol triphosphate and diacylglycerol. Subsequent cellular responses are determined by cell type (Abbas et al. 2018, Breedveld et al. 2019). Of note, FcRs can recognize bacteria coated with non-specific or specific antibodies and activate crucial host defenses, including opsonophagocytosis and antibody-dependent cellular cytotoxicity (ADCC). Opsonophagocytosis is initiated when a phagocyte recognizes an antibody bound to a pathogen via FcRs which triggers the uptake and destruction of the bacteria. ADCC is traditionally known as an anti-tumor cell killing mechanism that causes cell lysis through the release of toxic substances (such as perforin and granzymes). Natural killer cells have been extensively studied as mediators of ADCC responses, but ADCC has occasionally been found to occur in neutrophils in response to virus-infected cells and bacteria such as *Chlamydia trachomatis* (Naglak et al. 2017, Worley et al. 2018).

1.3.2 Neutrophil Fc receptors

Neutrophils are known to express FcRs for IgG (Fcγ) and IgA (Fcα) (Futosi et al. 2013). The FcRs expressed for IgG include FcγRI (CD64), FcγRIIA (CD32a), FcγRIIB (CD32b), FcγRIIC (CD32c), FcγRIIIA (CD16a) and FcγRIIIB (CD16b). All are activating receptors, except FcγRIIB and FcγRIIIB, which are inhibitory (Wang and Jonsson 2019). FcγRI is a high-affinity receptor that is upregulated during infection and is suggested to enhance neutrophils' ability to phagocytose IgG-opsonized bacteria, produce ROS, and trigger ADCC. However, much is still unknown about FcγRI and its functions. FcγRII(A, C) are low-affinity receptors that participate in phagocytosis, degranulation, ROS production, and NET formation. FcγRIIB has variable expression on neutrophils and inhibits various cell functions including degranulation and phagocytosis. FcγRIII(A-B) are low-affinity FcRs exclusively expressed on neutrophils and

their functional relevancy is not fully understood. Neutrophils express low levels of FcγRIIIA (CD16A), which is thought to mediate neutrophil activation and phagocytosis (Golay et al. 2019). In contrast, resting and activated neutrophils express high levels of FcγRIIIB (CD16B). FcγRIIIB has no signaling capacity on its own and its functional role on neutrophils remains unknown (Wang and Jonsson 2019). Human neutrophils are also known to express FcαRI that recognizes monomeric serum IgA (Monteiro et al. 1990). Receptor ligation leads to inflammatory processes and neutrophil ADCC, however, its function is still poorly understood (Borrok et al. 2015, Brandsma et al. 2019, Wang and Jonsson 2019).

1.4 - Conclusion

The innate immune system is the first line of defense against bacterial infections and works quickly to eliminate infections via mechanical/chemical barriers, the complement system, and cellular defenses. The complement system is one of the most important defense mechanisms against Gram-negative bacteria such as Hi, as exhibited by the many evasion mechanisms Hi and other bacteria have against this system. It can kill Hi through a combination of opsonophagocytosis, MAC formation, and the production of inflammation. Neutrophils are the most abundant innate immune cells with a wide array of functions. They use surface receptors such as PRRs and FcRs to stimulate the release of molecules such as cytokines. Recent evidence has shown that neutrophils activate and modulate aspects of adaptive immunity. The adaptive immune response is necessary to create a specific, strong response to the invading pathogen using B-cells and T-cells. However, the adaptive immune response requires signals from the innate immune system and takes much longer to develop, which is why the innate and adaptive immune responses must be properly integrated. FcRs on neutrophils link innate and adaptive

immune responses by recognizing Ig-opsonized bacteria and initiating neutrophil effector functions. Neutrophils' ability to act as this bridge between innate and adaptive immune responses makes them significant players in the overall immune response to Hi, but their function in adaptive immunity is still poorly understood. Therefore, this study will aim to investigate the role of neutrophils in activating the innate and adaptive immune responses to Hia.

2 - Rationale, Research Questions, Hypotheses, Objectives

2.1 Rationale and Research Questions

Despite the use of a vaccine for Hib, infection by *H. influenzae* remains an important concern, particularly in North American Indigenous populations where disease burden by Hia is reaching levels approaching the pre-vaccine Hib incidence rate (Tsang et al. 2020). Neutrophils are essential to innate immune functions such as opsonophagocytosis and their potency and efficacy make them indispensable factors to consider in the immune response to Hi. Their essential role in bacterial clearance is illustrated in cases of severe neutropenia, in which bacterial infections reoccur and increase in severity and clinical morbidity (Bodey et al. 1966, Malech and Nauseef 1997, Vento and Cainelli 2003). Accumulating evidence has suggested neutrophils can shape adaptive immune responses despite being an innate immune cell, meaning they are important players in the overall pathogenesis of numerous bacterial infections (Leliefeld et al. 2015, Mantovani et al. 2011, Li et al. 2019, Rosales 2020). However, the innate and adaptive immune responses to Hia and neutrophil's role in shaping these responses are still poorly understood. The polysaccharide capsule is a critical virulence factor of Hia and is necessary to consider in the immune response to Hia. As such, it is important to consider strain-specific features, including capsule presence or absence, that may be associated with enhanced virulence and greater disease burden by Hia to better understand its pathogenesis. From this, the following questions were posed:

1) Can *H. influenzae* type a activate the innate and adaptive immune responses via neutrophils?

2) Do the innate and adaptive immune responses differ after stimulation with invasive vs. non-invasive and encapsulated vs. unencapsulated *H. influenzae* strains?

2.2 Research Hypotheses

It is hypothesized that invasive, encapsulated strains of Hia will have the greatest resistance to killing by opsonophagocytosis *in vitro* and will stimulate the lowest activation of the innate and adaptive immune response by neutrophils due to the suppressive nature of the polysaccharide capsule

2.3 Research Objectives

1. Develop and optimize an opsonophagocytosis assay (OPA) with HL-60-derived neutrophils to measure and observe differences in the opsonophagocytosis of various Hia clinical isolates and NTHi

2. Develop and optimize an *in vitro* model of infection to measure differences in surface expression of immunologically important molecules by HL-60-derived neutrophils when stimulated with various Hia isolates and NTHi

3. Use these experimental systems to measure and compare the activation of the innate and adaptive immune responses by encapsulated vs. unencapsulated, or invasive vs. non-invasive Hi strains

3 - Materials and Methods

3.1 HL-60 cell culture conditions and differentiation

The human HL-60 myeloblastic leukemia cell line was stored in liquid nitrogen until thawed for culturing. Undifferentiated cells were maintained at a cell density between 1×10^5 and 1×10^6 viable cells/mL in RPMI 1640 medium (Sigma-Aldrich, Oakville, ON) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (R&D systems, Inc., Minneapolis, MN, USA), and 1% antibiotic-antimycotic (Life Technologies Corporation, NY, USA). Cells were incubated at 37ºC in 5% CO_2 in T-25 flasks (Corning Incorporated, Corning, NY, USA). Undifferentiated cells were passaged every 3-4 days and were maintained up to passage twenty. Before beginning the differentiation process, cultures were grown for two passages to establish their growth and grown to a density of 5-8 $\times$ 10^5 cells/mL. Cell count and viability were determined with a hemocytometer using a 1:1 dilution factor with 0.4% Trypan blue solution (GE Healthcare Bio-Sciences, Pasching, Austria). Cells with viability lower than 90% were not used in experiments. To induce differentiation, cells were diluted to a density of 1×10^5 cells/mL in RPMI 1640 medium supplemented with 20% heat-inactivated FBS, 1% antibiotic-antimycotic, and 1.25% dimethyl sulfoxide (DMSO) (Fisher BioReagents, PA, USA). Medium was replaced every two days and cell concentration was kept below 1×10^6 cells/mL. Previous work in our lab established that the cells were fully differentiated after 9 days of induction, which was confirmed as described in Section 3.2.

3.2 Confirmation of differentiation

Cell differentiation was confirmed microscopically and using flow cytometry. When the cells were fully differentiated, morphological alterations were confirmed visually. Morphological assessment was exclusively used after the differentiation methods had been well established by flow cytometry.

Approximately 0.5×10^6 of undifferentiated and differentiated cells were harvested by centrifugation for 5 min at 500 xg for flow cytometry analysis. Differentiated and undifferentiated cells were washed with ice-cold 1X phosphate buffered saline (PBS) (Fisher BioReagents, PA, USA) supplemented with 10% FBS. Cells were immunostained with Alexa Fluor 488 fluorochrome-conjugated primary antibodies against CD11b at a concentration of 0.1 µg/mL. All samples were incubated in the dark for 1h at 4ºC. Cells were then washed three times with ice cold 1XPBS with 10% FBS by centrifugation (400 xg for 5 min) and resuspended in 500 µL of ice-cold PBS with 10% FBS for flow cytometry analysis. Cells were kept in the dark on ice for one hour before analysis. Flow cytometry analysis was on the SONY SA3800 spectral cell analyzer with SA3800 Software (Sony Corporation, CA, USA), acquiring 10,000 total events.

3.3 *Haemophilus influenzae* strains and bacterial culture conditions

Hia strains 11-139, 13-240, 14-61, 13-0074, and NTHi strain 375 were used (Table 1). Of these strains, Hia 11-139, 13-240, and 14-61 are encapsulated while Hia 13-0074 and NTHi 375 are not. Hia 11-139 is an encapsulated, invasive, sequence type (ST)-23 strain from Canada that has been previously described (Tsang et al. 2013, Nix et al. 2018). Hia 13-240 is an encapsulated, invasive, ST-4 strain with enhanced virulence due to a IS*1016-bexA* partial

deletion. Hia 14-61 is an encapsulated, non-invasive ST-23 strain, isolated from the ear of a 6-month-old patient in Ontario (Tsang et al. 2013). Hia 13-0074 is an invasive mutant isolate that has lost expression of its capsule, with the same sequence type as Hia 11-139 (R.S.W. Tsang: personal communication). NTHi 375 is a nontypeable, non-invasive strain isolated from the middle ear of a pediatric patient with otitis media (Mell et al. 2014).

Bacteria were taken from 1.5 mL frozen stock suspensions made in 750 µL brain heart infusion (BHI) (Teknova, Hollister, CA, USA) broth, and 750 µL 50% glycerol (stored at -80ºC). Bacteria were grown for 16 hours on BHI plates (1.5% agar supplemented with 1 µg/mL nicotine adenine dinucleotide (NAD, stored at -20ºC) and 10 µg/mL hemin (stored at 21ºC) at 37ºC and 5% CO_2. Isolated colonies were transferred to 3 mL of growth factor supplemented BHI broth and the optical density (OD) at 600 nm was determined using a spectrophotometer. The bacterial suspension was adjusted to 0.1 OD and 500 µL of the 0.1 OD bacterial suspension was added to 10 mL of fresh growth factor supplemented BHI broth. The bacteria were then grown to log phase (5-6 hours) in a 37ºC incubator with shaking at 125 rpm. Bacteria was harvested in log-phase and adjusted to 0.1 OD at 600nm. Bacteria were diluted to the desired concentration using concentrations at an optimal density of 0.1 at 600 nm calculated from previously established growth curves (see appendix 1).

3.4 Opsonophagocytosis assay (OPA)

The methods used for the OPA were based upon those described by Winter and Barenkamp (Winter and Barenkamp 2003). The OPA was initially optimized for use with Hia as described in Section 5.2.

dHL-60 cells were harvested by centrifugation (150 xg for 8 min at room temperature) and resuspended in growth medium. Cell number and viability were assessed, and the experiment proceeded if cell viability was above 90%. The desired number of cells was centrifuged under the same conditions. The supernatant was removed, and cells were resuspended in Hanks' buffer without Ca^{2+} and Mg^{2+} at 5 mL per 50 mL of centrifuged cell culture. Resuspended cells were kept at 37ºC in a 5% CO_2 atmosphere until immediately before use in the functional assay.

To prepare for the OPA, bacteria in mid-log phase at 0.1 OD were used to create a working solution of 2.5×10^5 colony forming units (CFU)/mL in opsonophagocytosis buffer which consisted of Veronal-buffered saline (Lonza, Walkersville, USA) with 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, Oakville, ON) and 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Bacteria were washed twice with opsonophagocytosis buffer and maintained at room temperature for less than 1 hour before use in the OPA as described below.

For the functional assay, pooled human serum was used as a serum source to limit variability. It was created by combining the serum samples of 25 healthy adults from the area surrounding Thunder Bay, Ontario, Canada that have been described previously (Nix et al. 2015, Gaultier et al. 2022). The serum was stored at -80ºC and thawed to room temperature before use. The serum was heat-inactivated (HI) by submerging the vial in a 56ºC water bath for 30 minutes. HI serum was serially diluted (2-fold) in 50 µL of opsonophagocytosis buffer in a dilution plate until a dilution of 1:16 was reached. After serum was diluted, 10µL of each dilution was added to the microtiter plate. Then, 20 µL of bacterial suspension (approx. 5×10^3 CFU) prepared as described previously was added to each well with diluted serum. The plate was then incubated at 37ºC in 5% CO_2 for 15 min. During the 15 min incubation period, dHL-60 cells were centrifuged

again (150 xg for 8 min at room temperature), the supernatant was discarded, and the pellet was gently resuspended in 2 mL of opsonophagocytosis buffer for use in the assay. Following the incubation period, 15 µL of 3-week-old baby rabbit serum (Pel-Freeze, Rogers, Arkansas) as a complement source was added to each well. Immediately following this, 60 µL of dHL-60 cells (approx. 5×10^4 cells) were added to each well. The plate was incubated again at 37ºC for 90 min with horizontal shaking (220 rpm) to promote the phagocytic process. Directly after, 10 µL aliquots from each well were plated onto supplemented BHI agar plates with multiple replicates. Plates were incubated for 16h at 37ºC in 5% CO_2 and bacterial CFU were counted the following day. Each additional control included is shown in appendix 2, in which the desired component was replaced with the nutrient equivalent non-immunogenic component (heat-inactivated rabbit complement replaced serum or complement and buffer replaced dHL-60 cells).

3.5 *In vitro* model of infection

The methods used for the *in vitro* model of infection were developed based on previous studies and experience in our lab, as well as various *in vitro* studies involving HL-60 cells (Muller et al. 2008, Boros-Majewska et al. 2015). The *in vitro* model was initially optimized for use with Hia as described in Section 5.5.

dHL-60 cells were harvested by centrifugation (500xg for 5 min at room temperature) and resuspended in RPMI with 20% FBS. Cell number and viability were assessed, and the experiment proceeded if viability was above 90%. Using the 0.1 OD bacterial suspensions prepared as described previously, the desired number of bacteria was washed twice with PBS and resuspended in 200 µL of PBS. This new suspension was then added to wells containing 5×10^5 dHL-60 cells and incubated for 1 hour at 37ºC, 5% CO_2. After 1 hour, bacteria were killed

by adding 220 µL of 1 mg/mL gentamicin prepared in sterile water (final concentration 100 µg/mL). The plate was then incubated for an additional 71h. For the positive control, cells were incubated with 100 ng/mL and 1 µg/mL *Escherichia coli* (*E. coli*) LPS (Invitrogen, Carlsbad, CA, USA). The negative control was incubated with 200 µL of PBS. After 72h, bacterial killing was confirmed by plating 10 µL aliquots from each well onto supplemented BHI agar plates and observing growth overnight.

3.6 Flow cytometry analysis of the surface expression of molecules on dHL-60 cells

Following 72h stimulation, the six-well plate was placed on ice for 3 minutes and the cells were harvested by centrifugation and washed with ice-cold 1XPBS. Cell pellets were then resuspended in 1XPBS supplemented with 10% FBS and stained with 1 µg/mL phycoerythrin (PE)-conjugated mouse-antihuman ICAM-1 (BD Biosciences, Mississauga, ON) or CD64 (Invitrogen, CA, USA) antibody and/or 1 µg/mL fluorescein isothiocyanate (FITC)-conjugated mouse-antihuman CD16 (Invitrogen, CA, USA) or CD89 (Invitrogen, CA, USA) antibody for one hour at 4ºC in the dark. Immediately after, the cells were washed an additional three times with 1XPBS and analyzed using flow cytometry on the SONY SA3800 spectral cell analyzer with SA3800 Software (Sony Corporation, CA, USA). The desired population was gated based on light scattering properties and 10,000 gated events were collected. Cell death was measured by adding 1µg/mL propidium iodide (PI) to each sample immediately before analysis. Cells were gated on the PI-negative population to obtain % PI-negative cells. Mean fluorescence intensity was then recorded as a representative of the entire population and PI-negative cells. A PE- or FITC- conjugated isotype control (Invitrogen, CA, USA) was used to exclude non-specific antibody binding from analysis.

3.7 Statistics

Statistical differences were determined using SPSS software (IBM, Armonk, NY). Data is a representation of at least 3 independent experiments (n=3) and the statistical test used is specified in the figure legend. For the OPA, percent killing was determined by taking the difference between the CFU count in the treatment well and the negative control CFU count (only bacteria) as a percentage of the entire negative control CFU count (see appendix 3). Linear regression analysis was used to examine the dependency of percent killing on serum dilution. For differences between strains, a one-way ANOVA with a Tukey post-hoc test was done. For the *in vitro* infection model, a one-way ANOVA with Tukey post-hoc test was used to find significant differences between treatments. An independent samples t-test was used to find any specific differences between strains.

4 - Results

4.1 Incubation in growth medium supplemented with 1.25% DMSO over 9 days is sufficient to induce differentiation of HL-60 cells to neutrophils

Before the two assays could be developed to answer the research questions, the differentiation of HL-60 cells needed to be well established. This was critical to provide reproducible yields of neutrophils suitable for experimental use. DMSO has been reported to induce sufficient granulocytic differentiation of HL-60 cells (Jacob et al. 2002, Fleck et al. 2005). To induce differentiation, the growth medium of HL-60 cells was supplemented with 1.25% DMSO over a 9-day period consistent with the protocol described in Section 3.1. HL-60 cells were assessed for differentiation in two ways, morphologically and by utilizing flow cytometry. Morphological assessment was done microscopically. Cells were observed to shift from a large, round shape to small and irregular, which was consistent with literature (Figure 1) (Fleck et al. 2005).

HL-60 cell differentiation was also confirmed with flow cytometry analysis of a widely used marker of differentiation, CD11b (Romero-Steiner et al. 1997, Fleck et al. 2003). Differentiated and undifferentiated cells were used to examine differences in the expression of this marker. Unstained differentiated cells were used as a negative control to assess the autofluorescence of the cells. There was a significant increase in CD11b expression in differentiated cells compared to undifferentiated cells when looking at mean fluorescence intensity (MFI) ($p < 0.001$). In addition, histogram analysis showed a distinct shift in cell population when comparing differentiated and undifferentiated cells (Figure 2). From this, it can be concluded that the HL-60 cells were sufficiently differentiated.

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4.2 Optimization of the opsonophagocytosis assay

The OPA was used to analyze the susceptibility to killing by opsonophagocytosis and complement-mediated mechanisms by different Hia strains and compare it to NTHi. Before this could be done, the opsonophagocytosis assay was adapted from Winter and Barenkamp (2003). Specifically, the effector-to-target cell ratio and complement source were optimized. Winter and Barenkamp (2003) used an effector-to-target cell ratio of 400:1 to ensure maximum phagocytosis. This study used a ratio of 10:1 and no differences were seen when the ratio was increased to 100:1 or 400:1 (data not shown).

In addition, baby rabbit complement was used as the complement source because it supports complement-dependent bactericidal activity with human antibodies. Multiple concentrations of rabbit complement were tested (12.5% to 14.3%) to determine the optimal concentration in this system, as rabbit complement concentrations in OPAs vary between studies (Romero-Steiner et al. 2006, Winter and Barenkamp 2003). The optimal concentration would produce a noticeable effect on the killing of Hia without completely inhibiting growth. It was found that 14.3% was sufficient in this assay (data not shown).

4.3 Serum antibodies are essential to the killing of Hia 11-139 but not Hia 14-61

Bacterial CFU were counted and used to calculate the percent killing of each Hia strain (Table 1). The negative control consisted of bacteria, buffer, and heat-inactivated rabbit complement (the nutrient equivalent non-immunogenic component). Percent killing was calculated using the negative control CFU count as a reference, in which the difference in CFU count between the control and treatment was taken as a percentage of the whole negative control (see appendix 3). Killing was expected to be less efficient as immune components were removed or diluted.

Hia 11-139 and 14-61 had similar sensitivities to killing in the complete OPA (killing ranged from 14.76% to 38.66% and 19.63% to 31.52% for Hia 11-139 and 14-61, respectively). The killing of Hia 11-139 and 14-61 was also strongly correlated to serum dilution ($r = 0.675$ and $r = 0.773$ for Hia 11-139 and 14-61, respectively) (Figure 3). That is, as serum (the source of antibodies) was diluted, the percent killing decreased.

Hia 11-139 had the lowest percent killing when complement was removed from the system (19.35%, 11.27%, 4.36% at serum dilutions 1:2, 1:4, 1:8, respectively). When dHL-60 cells were removed, killing ranged from 22.32% to 19.38% to -3.12% at serum dilutions 1:2,1:4, and 1:8, respectively. In both cases, the killing of Hia 11-139 remained strongly correlated to serum dilution ($r = 0.692$) and no killing was observed at the highest serum dilution. Removing serum from the system also resulted in no killing of Hia 11-139 (-0.981%) (Figure 4). These results suggest that the killing of Hia 11-139 is heavily mediated by opsonization by antibodies, subsequently followed by activation of the classical complement pathway.

Like Hia 11-139, killing of Hia 14-61 was the lowest when complement was removed (ranged from 10.83% to 20.01%). Removing dHL-60 cells resulted in a minor decrease in percent killing from the complete OPA (ranged from 15.04% to 27.88%). However, in direct contrast to Hia 11-139, the percent killing of Hia 14-61 was weakly correlated to serum dilution when either complement ($r = 0.391$) or dHL-60 cells ($r = 0.379$) were removed from the system. In addition, when serum was removed completely, the killing of Hia 14-61 remained high (19.71%) (Figure 4). These results suggest that the killing of Hia 14-61 is most efficient when antibodies opsonize bacteria for phagocytosis. However, efficient killing can still occur in the absence of antibodies, likely by the alternative complement pathway and opsonophagocytosis via neutrophil complement receptors.

This shows a clear distinction between the killing mechanisms of invasive vs. non-invasive Hia strains, in which invasive strains depend on antibodies for efficient killing while non-invasive strains do not.

4.4 Differences in sensitivity to killing between ST-23 and ST-4 isolates

Hia 13-240 is an encapsulated, invasive isolate representative of ST-4. Hia of ST-4 do not circulate in the area. Hia 13-240 contains a IS*1016-bexA* partial deletion associated with severe disease and a higher case-fatality rate (Kapogiannis et al. 2005, Tsang et al. 2013). Hia 13-240 had the lowest percent killing compared to other Hia strains when serum, complement, and dHL-60 cells were present (ranged from 17.59% to 25.16%) (Figure 3, Figure 5). However, percent killing of Hia 13-240 slightly increased as complement (ranged from 19.62% to 28.25%) or dHL-60 (ranged from 30.47% to 33.62%) were removed. In fact, its killing was significantly higher than the other invasive, encapsulated strain (Hia 11-139) when neutrophils were removed (Figure 5). This suggests Hia 13-240 utilizes an unknown virulence factor that increases its survival in the presence of neutrophils.

Percent killing of Hia 13-240 consistently showed a weak positive correlation between percent killing and serum dilution (r = 0.106, 0.326, 0.137) for all treatments (Figure 3). Also, when serum was removed, killing remained high (25.98%), suggesting serum did not contribute to killing (Figure 4).

4.5 Unencapsulated isolates have similar sensitivity to killing

The killing of Hia 13-0074 followed the expected trend, in which killing was less efficient with the removal of immune components (Figure 3). In the complete OPA, percent

killing was comparable to Hia 11-139 and 14-61 (ranged from 24.11% to 31.1%). Removing complement or dHL-60 cells caused a similar decrease in killing. Nevertheless, killing remained high (ranged from 17.53% to 29.55% and 16.8% to 26% when complement or dHL-60 cells were removed, respectively).

Distinctly, the killing of Hia 13-0074 showed no correlation to serum dilution ($r = 0.16$ in the complete OPA), suggesting killing is not dependent on serum antibodies in the presence of complement and dHL-60 cells, unlike Hia 11-139 and Hia 14-61 (Figure 3). Also, when serum was removed from the system, killing remained high (28.37%), meaning that complement and dHL-60 cells can mediate efficient killing without antibodies (Figure 4). However, when complement or dHL-60 cells were removed, the correlation of percent killing to serum dilution increased ($r=0.295$ and 0.303, respectively). These results suggest the killing of Hia 13-0074 primarily depends on complement activity and opsonophagocytosis by dHL-60 cells. Like Hia 14-61, efficient killing of Hia 13-0074 can occur in the absence of antibodies but additional help from antibodies may be required with the lack of complement or dHL-60 cells.

NTHi 375 consistently had the highest percent killing among all the Hi strains tested. In the complete OPA, killing ranged from 33.47% to 33.69% (Figure 3). Like Hia 13-0074, killing remained high despite the removal of immune components. This may be because both strains are unencapsulated, so they are not able to evade the innate immune response as well as those with a capsule. However, unlike Hia 13-0074, killing did not decrease with the removal of complement or dHL-60 cells. When complement was removed, killing of NTHi ranged from 33.82% to 37.98% and was significantly higher than the killing of Hia 11-139 ($p<0.01$) (Figure 5). Percent killing of NTHi 375 was the highest (ranged from 34.04% to 39.82%) and significantly higher than the killing of Hia 11-139 ($p<0.001$) when dHL-60 cells were removed (Figure 5). This may

be attributed to NTHi's capacity to survive intracellularly or ability to create biofilms from neutrophil NETs *in vitro* (Hong et al. 2009, Langereis et al. 2013).

Additionally, percent killing of NTHi 375 consistently showed no correlation or a weak correlation to serum dilution (r=0.015, r=0.166, r=0.274) (Figure 3). However, when serum was completely removed, killing of NTHi decreased to 25.07% (Figure 4). This suggests NTHi 375 exhibits resistance to serum antibodies due to extensive heterogeneity in its cell wall components. However, antibodies are still necessary in its destruction and mediate efficient killing via the classical complement pathway. These results indicate a clear difference in the individual killing mechanisms of Hia vs. NTHi, in which the killing of Hia strains (11-139, 14-61, and 13-0074) require all components (serum, complement, and dHL-60 cells) for the most efficient killing while NTHi do not.

4.6 Optimization of flow cytometry analysis for the *in vitro* model of infection

To measure the expression of key surface receptors involved in innate and adaptive immune responses using flow cytometry, an *in vitro* assay was required. Before surface receptor expression could be quantified, extensive optimization was required on the SONY spectral cell analyzer. As discussed above, HL-60 cells are known to significantly increase CD11b surface expression after differentiation with DMSO, making CD11b a common marker of differentiation (Ustyantseva et al. 2019). Additionally, CD11b expression has been reported to increase with neutrophil activation (Martin et al. 2022). For this reason, CD11b expression on dHL-60 cells with and without stimulation was used as a marker to identify the correct population (differentiated, activated cells) on the side (SSC) vs forward (FSC) scatter plot. The side scatter measurement was expected to change with activation because neutrophil granularity increases

with activation (Ustyantseva et al. 2019). As such, multiple gates needed to be created to create

one on the SSC vs. FSC plot that fully encompassed neutrophils and their activated state.

Hia 11-139 was used to measure differences in CD11b expression with and without

stimulation. 0.5×10^6 neutrophils were stimulated at a multiplicity of infection (MOI) of 0.001,

10, and 100 for 72h, with 100 µg/mL gentamicin added at the 1h mark. LPS 100 ng/mL was used

as a positive control to confirm stimulation and PBS was used as a negative control. An

unstained sample was used to exclude auto-fluorescent cells from gating using SONY's

automatic autofluorescence finder tool. After 72h, the harvested cells were immunostained with

0.1 µg/mL Alexa Fluor 488-conjugated CD11b and 1 µg/mL PE-conjugated ICAM-1. Each

sample was incubated in the dark at 4ºC for 1h and flow cytometry analysis was done directly

after. ICAM-1 was used during optimization because it is a common co-stimulatory and

inflammatory molecule with high expression on neutrophils (Woodfin et al. 2016, Bui et al.

2020).

First, an SSC vs CD11b density plot was used to identify events with high CD11b

fluorescence (Gate U - purple) and observe any differences between the negative control (PBS)

and treatments (MOI 0.001 to 100) (Figure 6A). The events in gate U were visualized on the

SSC versus FSC plot to confirm the cell population. Using gate U as a guide, the appropriate cell

population was gated on to create gate A (red), which was defined as the population of interest

(Figure 6B). Data collected from MOI 100 was used to initially create gate A because it was

expected to have the highest number of activated neutrophils. The state of neutrophil activation

would change the side scatter properties such as granularity. As such, it was important to use

MOI 100 rather than the unstimulated control to ensure that the gate was not an underestimation

of all relevant events (Figure 7). Once gate A was defined, 10,000 gated events were collected and further analyzed.

Additional gates were created to examine cell viability. Cell viability was determined by incubating samples with 1 µg/mL propidium iodide (PI) immediately before analysis. The 10,000 events gated in gate A were then used to define PI-negative and PI-positive gates (Figure 8). The PI-negative gate was first created using a PI vs. FSC density plot showing all events in gate A, using the unstained control to discriminate background autofluorescence from fluorescence excited by PI (Figure 8A).

Following this, a PI-positive gate was created using a PI vs. ICAM-1 dot plot that encompassed all PI fluorescence above the PI-negative gate and excluded the events identified by the unstained and isotype controls (Figure 8B). The purpose of the PI-positive gate was to confirm the accuracy of the PI-negative gate and visualize ICAM-1 positive events that were also PI-positive. When examining markers other than ICAM-1, an additional plot of PI vs. that marker would be created to create a PI-positive gate and ensure the PI-negative gate was accurate. In addition, the mean fluorescence intensity of PI was confirmed to be low in the PI-negative and high in the PI-positive gate. Both gates were then visualized on a histogram showing PI fluorescence events to additionally confirm that the PI-negative gate was on the lower end of the histogram peak while the PI-positive gate was on the higher end, indicating high PI fluorescence (Figure 8C). In addition, both gates were backgated onto the SSC vs FSC plot to compare the light-scattering properties of events in each gate (Figure 8D).

Mean fluorescence intensity of events for CD11b-positive and ICAM-1-positive cells in gates A and PI-negative cells were compared and minimal differences were seen between the

two cell populations (Figure 9). This suggested most cells expressing ICAM-1 and CD11b were alive (PI-negative).

4.7 Stimulation with Hia 11-139 and 14-61 upregulates the expression of ICAM-1, CD89, and CD64 and downregulates CD16 expression on dHL-60 cells

After optimization of the flow cytometer parameters, the *in vitro* model of infection with dHL-60 and Hia was used to measure neutrophil surface receptor expression. Two strains of Hia were used, Hia 11-139 (encapsulated, invasive) and Hia 14-61 (encapsulated, non-invasive). For the positive control, cells were stimulated with *E. coli* lipopolysaccharide (LPS). *E. coli* LPS has been reported to produce a pro-inflammatory response in primary granulocytes and HL-60 cells (Poplutz et al. 2014, Snall et al. 2016). However, reports on the strength of LPS as a stimulus of HL-60 cells have been varied (Fleck et al. 2005). As such, it was necessary to determine which concentration would produce a noticeable effect on neutrophil surface marker expression. Due to this, 100 ng/mL or 1 µg/mL LPS was used to confirm stimulation and elucidate possible dose-responses. For the negative control, cells were incubated with PBS.

To optimize conditions, Hia 11-139 was used to stimulate $5×10^5$ dHL-60 cells at an MOI of 0.001, 0.01, 0.1, 1, 10, 100, and 200 for 72h to observe possible dose-dependent responses in each marker of interest. Following the determination of the optimal conditions, Hia 14-61 was used to stimulate at an MOI of 1 and 100, with LPS 100 ng/mL used as the positive control. After 72h stimulation with Hia, harvested cells were immunostained with fluorochrome-labelled antibodies for ICAM-1, CD89, CD64, and CD16. Directly after, flow cytometry analysis was done to quantify their surface expression on dHL-60 cells.

Mean fluorescence intensity was recorded as a representative of the entire population (Gate A, red) (Figure 7). Cell viability was examined using the PI-negative gate. The population of PI-negative cells significantly decreased with stimulation with Hia and LPS compared to the unstimulated control ($p<0.05$) (Figure 10). Percent PI-negative cells also decreased with increasing MOI, but this difference was not significant. There was no significant difference between Hia strains, meaning they caused the same amount of cell death.

Hia 11-139 and 14-61 induced a dose-dependent increase in ICAM-1 expression compared to the unstimulated control ($p<0.001$). For Hia 11-139, this increase was significant between MOI 0.001 and MOI 0.1 ($p<0.05$) and between *E. coli* LPS 100ng/mL and 1µg/mL ($p=0.002$) (Figure 11). Stimulation with MOI 100 and 200 resulted in a dramatic increase in ICAM-1 expression that was significantly different from all treatments ($p<0.001$). The same trend was seen after stimulation with Hia 14-61, in which ICAM-1 expression significantly increased between MOI 1 and 100 ($p<0.001$) (Figure 12). These results suggest Hia and LPS elicit a proinflammatory response in neutrophils in a dose-dependent manner via immune cell recruitment and subsequent inflammatory cytokine production.

For Hia 11-139, MOI 1 and 10 significantly increased CD89 expression compared to the unstimulated control ($p<0.01$) (Figure 13). Hia 14-61 at MOI 1 additionally increased CD89 expression compared to the unstimulated control, although this increase was not significant (Figure 14). However, significance may have been masked by inter-trial variability. In both cases, CD89 expression decreased at MOI 100, which could be associated with lower cell viability with higher stimulation (64.95% compared to 57.97% PI-negative cells for MOI 1 and 100, respectively) (Figure 8). Stimulation with LPS at 100 ng/mL and 1 µg/mL also increased CD89 expression compared to the unstimulated control, but this did not reach significance.

Stimulation with Hia 11-139 resulted in a dose-dependent decrease in CD16 expression (Figure 15). Unstimulated cells had significantly higher CD16 expression than any stimulated cells ($p<0.05$) other than MOI 0.001. MOI 0.001 was likely an insufficient degree of stimulation to produce a significant difference in CD16 expression. Stimulation with MOI 100 and 200 resulted in a significant decrease in CD16 expression compared to all treatments except MOI 10 ($p<0.01$). In fact, the expression of CD16 was negligible after stimulation with MOI 100 (MFI = 27.87) and 200 (MFI = -8.53). Increasing the LPS dose from 100 ng/mL to 1 µg/mL decreased CD16 expression, although variability may have masked any statistical significance. Hia 14-61 followed the same trend, in which MOI 100 significantly decreased CD16 expression compared to all treatments ($p<0.001$) (Figure 16). This indicates that CD16 expression decreases with neutrophil activation.

Stimulation of dHL-60 cells with Hia 11-139 and 14-61 resulted in a significant increase in CD64 expression at MOI 100 compared to all other treatments except MOI 0.01 ($p<0.01$ and $p<0.05$ for Hia 11-139 and 14-61, respectively) (Figure 17-Figure 18). Stimulation with LPS (100 ng/mL and 1µg/mL) and any bacterial dose lower than MOI 100 did not cause significant variation in CD64 expression compared to the unstimulated control.

4.8 Hia 11-139 and Hia 14-61 do not produce significantly different effects on ICAM-1, CD89, CD16, and CD64 expression

Two encapsulated strains that differed in their ability to cause invasive disease were used to evaluate strain-specific differences in neutrophil marker expression. There were no significant differences found in ICAM-1, CD89, CD16, and CD64 levels after stimulation with Hia 11-139 (invasive) vs. Hia 14-61 (non-invasive) (Figure 19). However, Hia 14-61 consistently induced

slightly higher expression of CD89, although this did not reach significance and could be

attributed to high variability between experiments. These findings suggest invasive isolates and

non-invasive Hia isolates have the same capacity to alter neutrophil surface receptor expression

5 - Discussion

Infection by *H. influenzae* remains an important concern despite the use of a vaccine for serotype b (Hib) for pediatric immunization. Through serotype replacement mechanisms, serotype a (Hia) and nontypeable (NTHi) strains have emerged as significant causes of invasive disease in Canada. Hia is the second most virulent strain (after Hib) and likely utilizes its capsule to evade immune responses in a similar mechanism to Hib, however much is still unknown about the immune response to Hia. Recurrent infection with encapsulated bacteria usually results from defects in opsonization and antibody production (Vinuesa et al. 2001). Correspondingly, the effective elimination of encapsulated bacteria such as Hi relies on non-specific innate immune mechanisms and specific anti-capsular antibodies.

The innate immune system is an immediate, non-antigen-specific response to a pathogen that primarily involves physical/chemical barriers, the complement system, and innate immune cells for defense. The adaptive immune response is an antigen-specific response that involves cell-mediated and humoral defenses. Proper integration of the two immune responses is essential for the effective clearance of bacteria (Iwasaki and Medzhitov 2015). Neutrophils are innate immune cells that exhibit functional plasticity that allow them to participate in innate and adaptive immune responses, but their function in adaptive immunity and defense against Hi is still poorly understood (Leliefeld et al. 2015, Rosales 2018). It's necessary to elucidate the mechanisms of protection against Hia in order to develop effective vaccines and/or therapies for disease. In this study, an OPA and *in vitro* model of infection were developed to elucidate the immune responses to Hia, with an emphasis on neutrophils' role in activating and connecting the innate and adaptive immune responses.

5.1 Optimization of the differentiation conditions of HL-60 cells

Optimization of the *in vitro* assays required a source of functional phagocytes. Neutrophils can not be cryopreserved and are impossible to expand *in vitro*. As such, neutrophils must be sourced from one of two sources: human normal peripheral blood neutrophils or cell lines. Sourcing the neutrophils from the peripheral blood of normal donors introduces donor-dependent variation and requires implausible volumes of blood from a single donor (Blanter et al. 2021). As a result, HL-60 cells have been increasingly used in bioassays to replace neutrophils for convenience and assay reproducibility purposes (Collins et al. 1977, Fleck et al. 2005).

The HL-60 cell line is a continuous human myeloid cell line isolated from the peripheral blood of a patient with acute myeloblastic leukemia in 1977 (Birnie 1988). They can differentiate into three cell lineages (monocytic, eosinophilic, granulocytic) based on different environmental and chemical conditions that regulate the cell cycle. Optimal differentiation is affected by many factors, including the concentration of the inducer, duration of exposure to the inducer, and cell passage number (Fleck et al. 2005). Nonuniformity in phagocytic preparation between laboratories means differentiation conditions must be well established before their use as neutrophils in bioassays to limit variation between experiments. One commonly used reagent, DMSO, can differentiate the cells into neutrophils after 5 to 10 days of exposure (Collins et al. 1978, Bunce et al. 1983). The exact mechanism by which DMSO induces differentiation is unknown, but it is suspected to involve perturbation of protein kinase C activity, an increase in membrane fluidity, the elevation of calcium uptake, and an alteration in the expression of the c-myc and p53 genes (Tsiftsoglou et al. 2003).

It was found that supplementing the growth medium with 1.25% DMSO for a total of 9 days was sufficient to induce differentiation to neutrophils. This was confirmed when the cells displayed morphological alterations consistent with literature describing neutrophil differentiation of HL-60 cells. Specifically, the cells shifted from a large, ovoid shape to a small and irregular one (Fleck et al. 2005).

Another commonly used method of determining differentiation is to measure the expression of differentiation marker CD11b using flow cytometry. CD11b is a receptor for iC3b, which is an opsonin that can be deposited on encapsulated bacteria such as Hi. As such, measuring CD11b expression can indicate differentiation to neutrophils and determine if the dHL-60 cells can effectively be used to recognize opsonins (Fleck et al. 2005). Undifferentiated cells displayed low levels of CD11b which is consistent with literature that describes low-level spontaneous differentiation in HL-60 cells (Fleck et al. 2003). After differentiation, CD11b expression significantly increased, which confirmed the HL-60 cells were sufficiently differentiated and confirmed the original morphological assessment.

5.2 Opsonophagocytosis assay optimization and justification

The objective was to optimize an OPA with dHL-60 cells to measure the susceptibility of Hia to complement-mediated killing and opsonophagocytosis.

Five strains of Hi were analyzed, including Hia 11-139, Hia 14-61, Hia 13-240, Hia 13-0074, and NTHi 375. Of these, Hia 11-139, Hia 14-61, and Hia 13-0074 belong to sequence type (ST)-23, which is the dominant circulating ST in Canada (Tsang et al. 2013). Hia 13-240 belongs to ST-4, which is a virulent strain associated with severe disease. It is important to investigate the immune response to both STs to reveal protective mechanisms of defense that extend to strains

that do not circulate in the area. The strains also vary in encapsulation and ability to cause invasive disease. Hia 11-139 and Hia 13-240 are encapsulated, invasive strains while Hia 14-61 is an encapsulated, non-invasive strain. Hia 13-0074 is an unencapsulated invasive mutant strain that has lost its ability to express the polysaccharide capsule on its surface. NTHi 375 is a non-invasive representative nontypeable strain that has recently had its entire genome sequenced (Mell et al. 2014). The polysaccharide capsule is a critical virulence factor of Hia and evaluation of its participation in the immune response is necessary to gather information on the entire immune response.

Previously, OPAs have been difficult to standardize due to high variability between experiments. High variability can be attributed to several disadvantages, including the requirement for a high effector-to-target cell ratio and individual optimization of the appropriate rabbit complement concentrations and controls. It was first necessary to optimize each of these components to limit variability and ensure optimal results.

Previous studies reported a less distinct phagocytic effect at low effector-to-target cell ratios (1:1). In addition, maximum phagocytosis was observed at a ratio of 400:1 (Romero-Steiner et al. 2006, Yaseen et al. 2017). A ratio of 10:1 was observed to be optimal for this study, as it limited the use of large volumes of dHL-60 cells and did not differ in phagocytosis efficiency.

Variability was limited by using pooled human serum. The pooled human serum was created from multiple serum samples collected from 25 healthy adults in the area surrounding Thunder Bay, Ontario, Canada. The serum was heat-inactivated to inactivate its complement proteins and served as the source of antibodies that contributed to opsonizing activity in the assay. Secondly, baby rabbit complement served as the complement source to limit variability

and ensure consistence of the complement source. Rabbit complement supports complement-dependent bacteriolysis, has no intrinsic antibodies, and has naturally occurring bactericidal activity for Hi (Erwin et al. 2000).

To effectively evaluate the effect of each immunogenic component on Hia, multiple controls were included (Figure 3). The negative control (only bacteria) represented uninhibited bacteria growth and was used in the calculation of percent killing. Additional controls included no complement, no dHL-60 cells, and no serum controls. It was found to be most effective to replace the missing component (either complement, dHL-60 cells, serum, or all) with the nutrient-equivalent immunogenic component (in this case, HI rabbit complement) to avoid stunting bacteria growth, as buffer may not provide a suitable environment for bacteria growth.

5.3 Discussion of opsonophagocytosis assay components

The OPA combines dHL-60 phagocytes, pooled human serum (antibodies), and rabbit complement to measure the killing of bacteria in a complement-dependent manner. The complement system represents an efficient mechanism of clearing bacteria that can be non-specific or antibody specific, making it a critical bridge between innate and adaptive immune responses. As such, complement-mediated killing is important in protection against encapsulated bacteria that require opsonization before phagocytosis can occur.

In the complete OPA system, antibodies, complement, and phagocytes work together to destroy bacteria. When antibodies are present in conjunction with complement and neutrophils, specific antibodies opsonize the pathogen. Neutrophil Fc receptors and/or complement receptors detect antibody:antigen complexes on the pathogen surface, which initiates opsonophagocytosis or the classical complement pathway. The alternative pathway can be activated independently of antibodies in response to bacteria through spontaneous hydrolysis and activation of complement

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component C3. The classical pathway also has the potential to indirectly activate the alternative pathway. Both pathways result in either opsonization, MAC formation, and/or the release of inflammatory molecules (Janeway et al. 2001). The activities of complement are then increased by multiple amplification loops. The alternative pathway amplifies the classical pathway by using an alternative C3 convertase to opsonize bacteria with C3b (Janeway et al. 2001). In addition, the antimicrobial peptides and proteins released by neutrophils enhance complement-dependent lysis of pathogens (Miajlovic et al. 2014). Neutrophils also have the ability to be activated independently of the complement system through PRRs (such as TLR4) that recognize common microbial molecular patterns. PRR activation and the resulting signaling cascade results in phagocytosis, degranulation, and cytokine release. The exact killing mechanisms of Hi in the system are shown in Figure 20.

Antibodies present in serum include antibodies in the natural antibody repertoire, such as anti-capsular antibodies and antibodies with specificities for other Hi surface antigens (Makela 1991). In serum, the dominant antibody isotypes are IgM, IgG, and IgA (Gonzalez-Quintela et al. 2008). These antibodies can be strain-specific or bind to common antigens that exist on multiple Hi strains. Anti-capsular antibodies in serum play a major role in host defense, while antibodies for other Hi exposed surface antigens (such as outer membrane proteins) vary in their effectiveness. In order to be recognized by neutrophil FcRs, antibodies must be bound to the surface of Hi, which is why anti-capsular antibodies are essential in defense against encapsulated strains of Hi (Makela 1991, Miajlovic et al. 2014). Serum resistance is a common mechanism of virulence many bacteria use to persist in the host and cause bacteraemia. For Hi, the polysaccharide capsule, LOS, or other virulence factors confer resistance to serum but Hi retains some sensitivity to killing by serum (Torres et al. 2021).

Removing any of the components should result in less efficient killing of the bacteria. When the serum is removed, killing can occur via complement and neutrophil activation (Figure 20D). Complement will coat the bacteria and neutrophils can recognize complement or common bacterial molecular structures with complement receptors or pattern recognition receptors, respectively. Classical and alternative complement pathways can also be activated through direct recognition of a bacterial surface or spontaneous activation (Janeway et al. 2001). When the complement source is removed, only antibodies and neutrophils remain, and neutrophils provide the only source of killing (Figure 20B). Killing occurs when neutrophil FcRs or PRRs recognize the antibody:antigen complexes or common microbial molecular patterns. When the phagocyte source is removed, only antibodies and complement remain (Figure 20C). Killing occurs by antibody-dependent, direct, or spontaneous activation of the complement system. This will occur via the classical and alternative pathways. Without phagocytes, bacterial lysis via the membrane attack complex formation is the only source of bacterial killing.

5.4 Differences in sensitivity to killing

Hia 11-139 (invasive) and Hia 14-61 (non-invasive) are two encapsulated strains that circulate in the area (ST-23) that vary in their ability to cause invasive disease. Invasive infection is defined as the isolation of Hia from a normally sterile site of the body, such as the bloodstream, and is often associated with severe disease. Hia isolates may differ in their ability to cause disease due to variations in their capsular material. For example, several invasive Hib isolates have been shown to contain five or more copies of the cap locus, causing them to be heavily capsulated (Cerquetti et al. 2005, Chandran et al. 2013).

Although Hia 11-139 and 14-61 had similar sensitivity to killing when serum, complement, and dHL-60 cells were present, removal of the individual components revealed

differences in sensitivity to immune components. Efficient killing of Hia 11-139 heavily relied on opsonization by serum antibodies and complement, suggesting invasive strains require antibody-mediated complement activity for killing. Neutrophil-mediated opsonophagocytosis is also important in killing Hia 11-139, although to a lesser degree. This agrees with multiple studies that evaluated the immune response to invasive Hi strains, in which opsonization by antibodies (anti-capsular and non-capsular) was important in killing invasive Hi and NTHi strains (Shenep 1983, Rubin 1988, Nix et al. 2018, Dudukina et al. 2020). The importance of the classical pathway was confirmed in a study that found IgG and IgM antibodies against LOS significantly contributed to the serum bactericidal activity and killing of encapsulated Hi via the classical complement pathway (Choi et al. 2015).

Contradictory to Hia 11-139, deficiencies in serum resulted in alternative mechanisms of destruction of Hia 14-61. For Hia 14-61, killing was likely mediated by the alternative complement pathway, as diluting serum did not affect killing when only complement and serum were present. This suggested that efficient killing of Hia 14-61 could occur through MAC-dependent lysis of bacteria via the alternative complement pathway. In agreement with this observation, previous studies revealed Hia 11-139 was more sensitive to the complement-dependent bactericidal activity of serum antibodies compared to Hia 14-61 (Nix et al. 2018). These results provide a clear distinction between the killing mechanisms of invasive vs. invasive Hia strains of the same sequence type, in which antibody-mediated killing is more important for the destruction of invasive (Hia 11-139) than non-invasive (14-61) strains.

Interestingly, the pooled serum did not exhibit bactericidal activity to a representative of ST-4 (Hia 13-240), which does not circulate in the region and contains an IS*1016-bexA* partial deletion associated with enhanced virulence. This could mean the pooled human serum from the

region only contains antibodies specific to strains circulating in the area (ST-23). However, this disagrees with a study by Nix et al. 2018, in which Hia 11-139 and Hia 13-240 showed similar sensitivity to the serum bactericidal effect, which was attributed to the presence of serum anti-capsular antibody. Instead, its lack of sensitivity to serum may be attributed to additional virulence factors that enhance its survival. Hia 11-139 and Hia 13-240 had similar sensitivity to killing in an OPA when serum, complement, and neutrophils were present. However, the killing of Hia 13-240 was significantly higher than Hia 11-139 with the removal of dHL-60 cells. Hia 13-240 may have additional virulence factors that increase its survival in the presence of neutrophils. For example, many Gram-negative bacteria are known to survive in neutrophils after phagocytosis, such as *Bordetella pertussis, Chlamydia species, Legionella pneumophila* (Urban et al. 2006).

Hia 13-0074 is an invasive, unencapsulated strain that has lost its ability to express its capsule due to a mutation (R.S.W. Tsang, personal communication). Reasons for this mutation are unclear, although a study involving mutated *Neisseria meningitidis* isolates with a loss or reduction in capsule polysaccharide production suggested this may confer survival advantages such as colonization efficacy (Ispasanie et al. 2018).

As expected, Hia 13-0074 experienced greater killing than the encapsulated isolates tested (Hia 11-139 and 14-61). In addition, removing complement or dHL-60 cells resulted in a minor decrease in percent killing that remained higher than Hia 11-139 and Hia 14-61. This was likely because Hia without a capsule is more susceptible to killing than encapsulated strains. The capsule is a major virulence factor that acts as a mechanical barrier that mediates resistance to surface binding of antibodies and complement (Taylor and Roberts 2005). These results agree with a study on a mutant *Klebsiella pneumoniae* strain that had increased sensitivity to neutrophil

antimicrobial peptides and decreased survival in an *in vitro* antimicrobial peptide sensitivity assay (Campos et al. 2004). Like Hia 14-61, Hia 13-0074 was sensitive to serum, but its presence was not necessary for efficient killing. Although Hia 13-0074 is unencapsulated like NTHi 375, in contrast to NTHi 375, Hia 13-0074 has not had the opportunity to evolve resistance mechanisms to the host immune system through natural selection, making it more susceptible to killing by each immune component. Despite this, killing of Hia 13-0074 remained lower than NTHi 375, suggesting Hia 13-0074 retained some Hia virulence factors that contributed to its survival.

A representative of a nontypeable strain, NTHi 375, consistently had the highest percent killing out of all the isolates tested in the OPA. This could be explained by NTHi's exposed outer membrane rendering it more susceptible to killing by aspects of the host immune system, as the capsule ultimately confers greater resistance to the host immune system. Unlike the other unencapsulated strain (Hia 13-0074), the killing of NTHi did not differ with serum dilutions, suggesting it is relatively resistant to the actions of serum. These observations agree with a study by Choi et al. 2015, in which the same isolate showed resistance to serum. Similarly, percent killing of NTHi 375 did not decrease when complement was removed from the system. This can be explained by its lack of capsule, as NTHi strains are constantly under selective pressure to develop new mechanisms of resistance to serum and complement (Duell et al. 2016). Resistance is mediated by NTHi's ability to exhibit extensive heterogeneity in LOS glycoforms, form biofilms, and undergo phase variation of its outer membrane proteins (Langereis and Weiser 2014, Duell et al. 2016). In addition, increased serum resistance by NTHi has been correlated to the increased binding of complement-inhibitory proteins and disease severity as a result (Hallstrom et al. 2010). NTHi has the ability to resist the alternative complement pathway

through outer membrane proteins binding to factor H (such as P5) (Rosadini et al. 2014). Thus, NTHi's multitude of virulence factors contribute to serum and complement resistance.

In spite of its resistance to serum and complement, antibodies were important in the killing of NTHi, as percent killing decreased by 10% when serum was removed. This suggested that protective antibody in serum was able to mediate complement and neutrophil activation. Protective antibodies to NTHi may be present in pooled human serum due to previous natural infection by NTHi (Shurin et al. 1980). Previous studies revealed that NTHi outer membrane proteins and LOS are the principal targets of protective antibodies and cause significant antibody-mediated bactericidal activity of NTHi through opsonophagocytosis and complement activation (Barenkamp et al. 1986, Akkoyunlu et al. 1996, Choi et al. 2015, Thomas et al. 2018). Also, it was shown that classical complement pathway inhibition resulted in 100% bacterial survival of NTHi, confirming that antibody-mediated complement activation is necessary for the destruction of NTHi (Choi et al. 2015). As a result, antibodies specific to NTHi surface molecules are vital in its destruction.

Like Hia 13-240, the highest percent killing of NTHi 375 occurred with the removal of neutrophils. NTHi has been shown to live intracellularly in macrophages and use them to survive *in vitro* (Craig et al. 2001). In addition, NTHi strains have been known to use nucleic acid derived from neutrophil extracellular traps to form biofilms and persist *in vivo* and *in vitro* (Hong et al. 2009, Juneau et al. 2011). As a result, NETs do not mediate clearance of NTHi and instead enhance their survival. It is unknown if Hia 13-240 and NTHi 375 use similar mechanisms to mediate survival and future work is needed to elucidate possible mechanisms of survival of Hia in the presence of neutrophils.

In summary, the OPA revealed strain-specific sensitivities to killing. The killing of Hia 11-139 was heavily dependent on serum antibodies, while Hia 14-61, 13-240, and 13-0074 were not. There was high percent killing of unencapsulated isolates Hia 13-0074 and NTHi 375. However, the killing of Hia 13-0074 was attributed to complement and neutrophil activity and decreased with their removal. The percent killing of NTHi was consistently high and was shown to be primarily mediated by antibody-dependent complement activation. Interestingly, survival mechanisms of NTHi 375 and Hia 13-240 were revealed in the presence of neutrophils, which could be attributed to biofilm formation via NETs or intracellular survival. This data provides a clear distinction in the susceptibility of multiple Hia isolates and NTHi to killing by opsonophagocytosis. In addition, it elucidated multiple distinct killing mechanisms of each isolate despite similarities in capsulation, sequence type, and ability to cause invasive disease.

5.5 Discussion of *in vitro* model of infection optimization and justification

To achieve the second research objective outlined in section 2.3, an *in vitro* model of infection was optimized. Optimization of the appropriate controls and multiplicity of infection (MOI) had to be completed before the model could be used.

Positive and negative controls were included in each experiment to properly assess quantitative differences in surface markers. The negative control was used to determine if stimulation significantly changed marker expression on dHL-60 cells and consisted of PBS only. As a positive control, *E. coli* LPS, a strong agonist of TLR4 signaling, was used at a concentration of 100 ng/mL and 1 μg/mL (Maeshima and Fernandez 2013, Bertani and Ruiz 2019). LPS is an outer membrane component of Gram-negative bacteria whose structure consists of lipid A, the core oligosaccharide, and the O-antigen. Lipid A is a highly immunogenic

endotoxin and is a cognate ligand for Toll-like receptor 4 (TLR4) (Li et al. 2008). Its recognition by TLR4 on neutrophils activates the transcription factor NF-κB through MyD88-dependent and TRIF-dependent pathways, causing a signaling cascade that activates aspects of the innate and adaptive immune systems through cytokine release and cell surface molecule upregulation (Maeshima and Fernandez 2013). Hia contains LOS, which is an O-deacylated, truncated form of LPS. Similarly to LPS, LOS is a potent immunostimulatory molecule that is recognized by TLR4 via lipid A, which subsequently activates NF-κB. LOS has been shown to induce a similar pro-inflammatory response as LPS through the upregulation of co-stimulatory molecules, antigen-presenting molecules, and inflammatory cytokines, although to a lesser degree than *E. coli* LPS (Choi et al. 2014). However, similarities in mechanisms of action between LOS and LPS make LPS a reliable positive control in this experiment.

The final component that required optimization was the MOI of bacteria. Two strains were used in the *in vitro* assay, including Hia 11-139 and Hia 14-61. Initial optimization was done using Hia 11-139. The entire dose-response was gathered for each marker of interest (ICAM-1, CD89, CD64, CD16) by stimulating dHL-60 cells at an MOI of 0.001, 0.01, 0.1, 1, 10, 100, and 200 with Hia 11-139. There is no published literature on the expression of these markers on dHL-60 cells after stimulation with bacteria. Accordingly, it was necessary to gather data for each MOI to give a more accurate representation of their expression. ICAM-1, CD89, and CD16 all showed significant differences from the negative control after stimulation with MOI 1. MOI 100 produced a response significantly different from MOI 1 in ICAM-1, CD16, and CD64 expression. Based on these results, it was concluded that Hia 14-61 could be analyzed at MOI 1 and 100 to represent the lower and higher end of activation, respectively, and examine differences in receptor expression between strains.

5.6 Flow cytometry analysis of surface molecules on dHL-60 cells after Hia stimulation

Neutrophils express a wide variety of surface receptors linked to innate and adaptive immune responses that initiate multiple intracellular signaling processes which directly and indirectly eliminate pathogens. For example, FcR and TLR ligation leads to a downstream signaling cascade via ITAM phosphorylation and the NF-κB pathway, respectively. Each signaling cascade has multiple outcomes, including cytokine production, cell proliferation, cell activation, and the enhancement of cell survival (Futosi et al. 2013). When neutrophils are activated by an inflammatory stimulus, their phenotype alters, and multiple cell surface markers change their expression. Flow cytometry analysis was used to evaluate ICAM-1, CD89, CD64, and CD16 expression levels before and after stimulation, which are all cell surface molecules involved in innate and adaptive immune responses. Evaluating their expression levels will help to elucidate the mechanisms of activation of the immune responses by neutrophils when stimulated with Hia.

Two strains were used, including Hia 11-139 and Hia 14-61. Hia 11-139 is an encapsulated, invasive strain while Hia 14-61 is an encapsulated, non-invasive strain (Nix et al. 2018, Tsang et al. 2013). Both strains belong to sequence type (ST)-23, which is the dominant sequence type in Canada. Evaluating the immune response to Hia isolates that have the same molecular-genetic characteristics assessed by multi-locus sequence typing but differ in their ability to cause invasive disease is a key step in defining the immune response to Hia.

Propidium iodide staining was used to evaluate cell viability after stimulation with Hia 11-139, 14-61, and LPS. As expected, cell viability was significantly different than the negative control and decreased with increasing stimulation for both Hia and LPS. No difference was seen

in cell viability after stimulation with Hia 11-139 vs. Hia 14-61, meaning invasive Hia does not affect neutrophil cell death differently than non-invasive Hia.

Host cell death during bacterial infections occurs through multiple mechanisms, including phagocytosis-induced cell death and pre-mature bacterial-induced apoptosis. Phagocytosis-induced cell death occurs after neutrophils kill ingested bacteria, which allows for the removal of neutrophils and the resolution of infection (Koybayashi et al. 2018). Therefore, increased cell death could indicate the increased phagocytosis of bacteria. However, *E. coli* and Hi are known to induce pre-mature neutrophil apoptosis after phagocytosis by increasing the production of reactive oxygen species. Oxygen-dependent apoptosis is induced when NAD and ATP are depleted or the protein c-*jun* is activated via the NF-κB pathway (Watson et al. 1996, Naylor et al. 2007, Geering and Simon 2011, Kobayashi et al. 2018). LPS has also been shown to induce apoptosis of bone marrow-derived macrophages *in vitro* through autocrine production of TNF-α and NO (Xaus et al. 2000). Thus, decreased cell viability could also be attributed to Hi and LPS causing the pre-mature death of neutrophils.

ICAM-1 is a cell surface adhesion receptor and co-stimulatory molecule responsible for regulating a variety of immune responses, including inflammation, leukocyte recruitment, and T-cell stimulation (Bui et al. 2020). ICAM-1 is expressed at a low level on various immune cell types but is significantly upregulated after bacterial stimulation through the translocation of the NF-κB protein to the nucleus (Lisby et al. 1989, Roeback and Finnegan 1999). As such, its upregulation could be used as a measurement of the innate and adaptive immune responses induced by Hi infection. Studies on its presence on neutrophils usually focused on its role in neutrophil adhesion and aggregation (Sans et al. 2001, Wang et al. 1997). Reports on the mechanisms behind ICAM-1 expression and its anti-bacterial function on neutrophils are scant.

In this study, stimulation of dHL-60 cells with Hia and LPS caused a dose-dependent increase in the expression of ICAM-1 compared to the unstimulated control, suggesting Hia and LPS elicit a dose-dependent proinflammatory response in neutrophils. These results agree with a previous study that showed an increase in ICAM-1 expression on dHL-60 cells after stimulation with the intracellular bacteria, *Anaplasma phagocytophilum*, where ICAM-1 was suggested to function in leukocyte-leukocyte interactions through the ligation of CD62L and β_2 integrins (Choi et al. 2003). Prior research also documented an upregulation in ICAM-1 expression on macrophages in a TLR4-dependent PI3K signaling manner after stimulation with LPS, which was correlated to enhanced ROS production and phagocytosis (Zhong et al. 2021). Altogether, heightened ICAM-1 expression on neutrophils might indicate an important role of this receptor in the immune response to Hia through the enhancement of neutrophil phagocytosis, ROS production, and leukocyte interactions. The mechanism in which it regulates neutrophil phagocytosis and ROS production remains to be understood.

The remaining surface markers evaluated were FcRs. FcRs are surface molecules capable of interacting with the crystallized fragment (Fc) of antigen-bound immunoglobulin molecules. They are activated by antibodies bound to pathogens and modulate several immune responses (Paul 2013). FcRs act as a link between the two main arms of immunity by binding immune complexes formed by natural or antigen-specific antibodies and initiating multiple effector functions by innate immune cells. Natural antibodies are germline-encoded non-specific antibodies present in circulation prior to antigen exposure. Antigen-specific antibodies are generated by plasma cells of adaptive immunity after exposure to an antigen. Neutrophils express multiple FcRs, including receptors for IgG (Fcγ) and IgA (Fcα) (Wang and Jonsson 2019, Bakema and Egmond 2011). Neutrophil FcRs can facilitate phagocytosis of opsonized bacteria

but also mediate other inflammatory processes through mechanisms that are still poorly understood (Futosi et al. 2013).

CD89 is the FcR for IgA (FcαR) and is known to be constitutively expressed on neutrophils (van Gool and van Egmond 2020). Activation of CD89 results in phagocytosis, degranulation, superoxide production, extracellular trap formation, the release of cytokines, and antigen presentation by neutrophils. Of note, CD89 on the surface of blood phagocytes has been reported to directly bind to and facilitate the killing of *S. pneumoniae* and *E. coli* independently of IgA and C-reactive protein ligands (de Tymowski et al. 2019). This means CD89 can be involved in innate and adaptive immune responses through early, direct recognition of bacteria, or late-phase recognition of antibody-bound bacteria and non-opsonized bacteria combined. This wide range of functions makes CD89 capable of modulating innate and adaptive immune responses.

Previous studies have identified CD89 expression on dHL-60 cells without stimulation and an increase in its expression on blood neutrophils after bacterial stimulation (Otten et al. 2005, de Tymowski et al. 2019). However, CD89 expression on dHL-60 cells after bacterial stimulation has never been reported. Initial stimulation of dHL-60 cells with Hia 11-139 showed a significant increase in CD89 expression at MOI 1 and 10 compared to the unstimulated control. Hia 14-61 also induced an increase in CD89 expression at MOI 1 that did not reach significance, but significance could have been masked by the variability between experiments. Stimulation with LPS increased CD89 expression, although this was not significantly different than the unstimulated control. These results agreed with studies in which CD89 surface expression increased on isolated human monocytes with increasing doses of LPS (0.5ng/mL – 500 ng/mL) (Shen et al. 1994). Increased CD89 expression on neutrophils could lead to enhanced

phagocytosis, as enhanced phagocytosis of *E. coli, Streptococcus pneumoniae,* and

Staphylococcus aureus has been observed by neutrophils after activation of CD89 (van Gool and

van Egmond 2020). Based on this, increased CD89 expression with Hia stimulation could be

correlated to neutrophil activation and result in enhanced phagocytosis towards Hia.

At an MOI higher than MOI 10, CD89 expression decreased, which could be due to

increased neutrophil apoptosis. An *in vitro* study found that LPS stimulation of peripheral blood

neutrophils caused CD89 expression to initially increase but led to significant neutrophil death in

a concentration-dependent manner (Wehrli et al. 2014). This means depending on the

inflammatory environment, CD89 can regulate neutrophil survival. Neutrophil apoptosis after

CD89 engagement was shown to be the result of NETosis induced by the phagocytosis of IgA-

opsonized bacteria (Aleyd et al. 2014). NETosis is defined as neutrophil cell death after the

generation and release of neutrophil extracellular traps (NETs) that trap and kill bacteria. Based

on this, a decrease in CD89 expression with higher stimulation could indicate increased cell

death of neutrophils expressing CD89.

CD64 is the high-affinity FcR I for IgG (FcγRI) and is normally expressed at a low level

on resting human neutrophils. Its expression is known to significantly increase with increasing

bacterial infection in response to microbial cell wall components (LPS) and inflammatory

cytokines such as G-CSF, and interferon-γ (Qureshi et al. 2001, Sack 2017). CD64 expression

varies by infection type, in which Gram-negative bacteria induce higher expression of CD64 than

Gram-positive bacteria (Sack 2017). Additionally, an increase in the expression of CD64 by

neutrophils is associated with complications from sepsis and bacterial infection (Allen et al.

2002, Davis 2005, Wang et al. 2015). The functional relevancy of CD64 on neutrophils is still

poorly understood but it is thought that CD64 engagement and the following signaling cascade

causes the increased bactericidal activity of neutrophils through degranulation, activation of the oxidative burst, immune complex clearance, cytokine release, bacterial phagocytosis, and antigen presentation (Sack 2017).

Although the engagement of CD64 leads to similar effector functions as CD89, the efficiency of the activated functions may differ and depend on neutrophil maturation. Otten et al. (2005) reported that neutrophil-mediated tumor cell killing depended on both CD89 and CD64 expression on immature neutrophils, but mature neutrophils expressed only CD89. CD89 was thought to activate more efficient signaling pathways as it induced higher levels of MAPK phosphorylation and intracellular free calcium levels. These receptors may initiate different killing mechanisms by neutrophils that remain to be defined, as CD89 can be expressed without the FcR γ-chain and may interact with additional proteins (Otten et al. 2005, Brandsma et al. 2019). Accordingly, it was important to investigate the expression of each FcR after infection with Hia.

In this study, dHL-60 cells were observed to express low levels of CD64 without stimulation as well as after stimulation with Hia 11-139 and Hia 14-61 at low MOIs. However, CD64 expression was significantly heightened after stimulation with Hia 11-139 and 14-61 at MOI 100. Despite this, stimulation with LPS 100 ng/mL and 1 μg/mL did not significantly upregulate CD64 expression compared to the unstimulated control. The constant low level of CD64 expression with low stimulation can be attributed to the differentiation stage of dHL-60 cells. Previous studies with HL-60 cells revealed undifferentiated cells express low levels of CD64 on their surface (Verbrugge et al. 2006). HL-60 differentiation with DMSO generates mature neutrophils, but the process leaves a small portion of HL-60 cells undifferentiated (Gupta et al. 2015). Evaluation of CD11b expression on dHL-60 cells confirms this, as a small

population of cells expressing low levels of CD11b was seen despite differentiation (Figure 2). In addition, previous studies of neutrophil granulocytes revealed resting neutrophils have a negligible level of CD64 on their surface that can be detected by flow cytometry (Davis 2005). Therefore, it can be deduced that with low stimulation, not all neutrophils were activated. CD64 was expressed in low amounts after stimulation with Hia at any MOI lower than 100 due to its presence on undifferentiated HL-60 cells and resting neutrophils.

Significant upregulation of CD64 suggests stimulation with MOI 100 and higher induces an increased inflammatory response in neutrophils that is frequently observed in patients with sepsis or systemic inflammatory response syndrome. This agrees with a study that used flow cytometric analysis of polymorphonuclear cells from systemic inflammatory response syndrome patients (Qureshi et al. 2001). It revealed a supranormal increase in CD64 expression that was accompanied by abnormal and dysregulated neutrophil activity that is characteristic of sepsis. Increased CD64 expression was associated with increased cross-linking of CD64 by antigen-antibody complexes, leading to enhanced phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), ROS production, and cytokine release (Qureshi et al. 2001). Based on CD64's proposed functional roles, it can be said that CD64 enhances neutrophil effector functions such as phagocytosis and ROS production and could reflect the magnitude of the immune response to Hi. However, due to CD64's association with sepsis in clinical studies, its increase potentially reflects the dysregulation of neutrophil effector functions, but this hypothesis warrants to be determined by further research.

CD16 is the low-affinity FcR III for IgG (FcγRIII) and is expressed in two forms - CD16A and CD16B. CD16A is a trans-membranous form expressed on macrophages and natural killer cells with low or no expression on resting and activated neutrophils. CD16B exists as a

monomeric GPI-anchored form exclusively expressed on neutrophils. CD16B is highly

expressed on neutrophils and is subject to shedding with neutrophil activation (Wang and

Jonsson 2019). Therefore, it can be deduced that CD16B is primarily observed on dHL-60 cells.

CD16B's role in neutrophils is still poorly understood, but it is thought to activate secretory

processes in neutrophils (Fossati et al. 2001).

Despite both being receptors for IgG, CD64 and CD16 have varied functional roles and

expression on neutrophils. Treatment of neutrophils purified from patient peripheral blood

showed no increase in CD16 expression after treatment with the inflammatory cytokine, IFN-γ,

despite an increase in CD64 expression (Goulding 1992). In addition, cells expressing higher

levels of CD16B than CD64 differed in effector function efficiencies such as phagocytosis of

opsonized bacteria and antigen-presenting ability. Therefore, it is worth investigating which

receptor is primarily involved in neutrophil responses to Hia.

CD16 has been reported to be expressed in unstimulated dHL-60 cells, but its expression

on stimulated dHL-60 cells has never been reported (Takeda et al. 2003). In this study, the

expression of CD16 was observed to significantly decrease on dHL-60 neutrophils after

stimulation with Hia 11-139 and 14-61 compared to unstimulated cells. Also, CD16 expression

decreased with increasing bacterial and LPS stimulation. A significant decrease in CD16

expression was seen after stimulation with Hia 11-139 and 14-61 at MOI 100. These results

agree with a study in which stimulation with LPS was observed to cause loss of CD16 expression

on human monocytes (Pangiagua et al. 2015).

In humans, CD16B is co-expressed with another Fcγ receptor, CD32A, and CD16B acts

as a decoy receptor to limit binding to CD32A in resting neutrophils. Neutrophil functions are

dependent on ITAM-dependent strong signaling by CD32A. Therefore, signaling by CD32A is

regulated in resting neutrophils to avoid tissue injury. CD16B has been reported to extend out further from the cell surface membrane on resting neutrophils, making it more likely to capture circulating immune complexes than CD32A, rendering CD32A inactive when neutrophils are inactive (Selvaraj et al. 2004). Activated neutrophils have been observed to shed CD16 from their surface to allow for increased avidity and higher engagement of CD32A (Wang and Jonsson 2019). Shedding was proposed as the primary mechanism of the decrease of surface CD16, as internalization of CD16 has never been observed and an *in vitro* study with natural killer cells revealed minimal internalization of CD16 (Romee et al. 2013). In this case, loss of expression of CD16 with increasing MOI and LPS dose could indicate increased neutrophil activation and CD16 shedding as a result. Notably, CD32A often induces stronger signals and results in augmented phagocytosis and ADCC of IgG-coated target cells by neutrophils (Treffers et al. 2019, Wang and Jonsson 2019). ADCC is initiated by FcR receptor cross-linking and downstream signal propagation that results in the release of granzymes, Fas signaling initiation, phagocytosis, and ROS production (Zahavi et al. 2018). From this, these results could also indicate CD32A has the potential to enhance neutrophil phagocytosis and ADCC via IgG:antigen complexes and mediate defense against Hia after CD16B is shed.

To conclude, all markers of interest were expressed on dHL-60 cells and significantly affected by stimulation with Hia. ICAM-1 increased in a dose-dependent manner, which implied it is functionally involved in the inflammatory immune response to Hia. CD89 expression increased after stimulation with MOI 1 and 10 before decreasing again, which may be due to increased neutrophil NETosis due to CD89 engagement. CD64 was expressed at low levels until a large stimulus was applied (MOI 100 and greater), suggesting CD64 expression can be used as a measure of the magnitude of the immune response and potentially reflects neutrophil

dysregulation. CD16 expression decreased with stimulation, suggesting that this protein was shed as neutrophils were activated. No differences were seen in marker expression after stimulation with Hia 11-139 vs. Hia 14-61.

5.7 Limitations and future studies

One limitation of this study was the use of dHL-60 cells as the source of neutrophils. Although dHL-60 cells exhibit neutrophil-like qualities and are the most frequently used cell line for neutrophil research, they are not a perfect neutrophil model. For example, dHL-60 cells do not express all neutrophil phagocytosis receptors (such as CD32) and lack some neutrophil granules (Blanter et al. 2021). Due to this, the results must be confirmed using primary cells or an *in vivo* model to gain a holistic view of neutrophil responses. *In vivo* models provide an advantage over *in vitro* models because they capture the complexity of the entire immune system, including cell interactions and biochemical processes that occur during the immune response to bacteria.

One limitation of the OPA was the high variability between trials. This could be associated with manual colony counting, which is labor-intensive and contributes to human error. As a result, the development of the assay for a large number of serum samples is implausible. For proper standardization, a serum titer with >50% killing should be established. Assay standardization also reduces the volume of serum used, which is important for the evaluation of pediatric Hia infections, in which there is limited access to serum. The assay with an established serum titer could then be used to evaluate the efficiency of specific antibodies in mediating the opsonophagocytic activity of Hia and NTHi to aid in developing a vaccine for those affected by Hia disease.

Future work should aim to elucidate the exact mechanisms of neutrophil killing of Hia. Although this study did provide evidence of opsonophagocytosis, it is unknown if neutrophils use other mechanisms, such as NETosis, to contribute to the killing of Hia. This could be quantified *in vitro* using fluorescence microscopy. In addition, neutrophil ADCC-mediated killing of bacteria is still poorly defined in literature and mechanisms of action should be further characterized using flow cytometry cellular cytotoxicity assays.

Both assays used in this study should be applied to other Hia isolates that do not circulate in the area, vary in capsulation, and vary in their ability to cause invasive disease to develop knowledge on Hia's evasion of the immune system and pathogenesis. This study should also be applied to other strains of NTHi. NTHi strains exhibit high genetic variability, so these findings can not be generalized to all NTHi strains. In addition, IgM has been identified as a key serum antibody responsible for bactericidal activity against encapsulated Hia and NTHi (Choi et al. 2015). An FcR for IgM on neutrophils has never been reported. As such, it would be interesting to investigate the presence of an FcR for IgM on neutrophils to determine if IgM-dependent opsonophagocytosis is a plausible mechanism for the destruction of Hia.

6 - Conclusions

In conclusion, this research has led to the successful development of an OPA and *in vitro* model of infection using dHL-60 cells and Hia. The aim of this study was to elucidate mechanisms of activation of the innate and adaptive immune responses to Hia and to determine if these responses differed between encapsulated versus unencapsulated, or invasive versus non-invasive strains. It was demonstrated that neutrophils can modulate innate and adaptive immune responses to Hia. The OPA revealed strain-specific sensitivies to killing. Specifically, encapsulated strains (Hia 11-139, 14-61, 13-240) had lower percent killing compared to the unencapsulated strains (Hia 13-0074 and NTHi 375). The killing of Hia 11-139 was heavily dependent on antibodies, while the efficient killing of Hia 14-61, 13-240, and 13-0074 were mediated by complement and/or neutrophil activation. Hia 13-240 exhibited resistance to killing by serum and neutrophils compared to the other Hia strains, which may be attributed to an additional unknown virulence factor. Notably, NTHi 375 exhibited multiple resistance mechanisms to antibodies and complement. Regardless, NTHi 375 had the highest killing compared to the other Hi isolates, which was likely mediated by the initiation of the classical complement pathway by antibodies directed towards the cell wall components of NTHi. The *in vitro* assay revealed significant upregulation in ICAM-1, CD89, and CD64, and downregulation in CD16 expression after stimulation with Hia. Although these differences were not strain-specific, this study revealed potential mechanisms behind the activation of neutrophil effector functions against Hia. The results of this study are crucial in understanding the innate and adaptive immune responses to Hia. Furthermore, this study is an important step in defining neutrophils' functional capabilities in bacterial infection by Hia.

7 – Figures
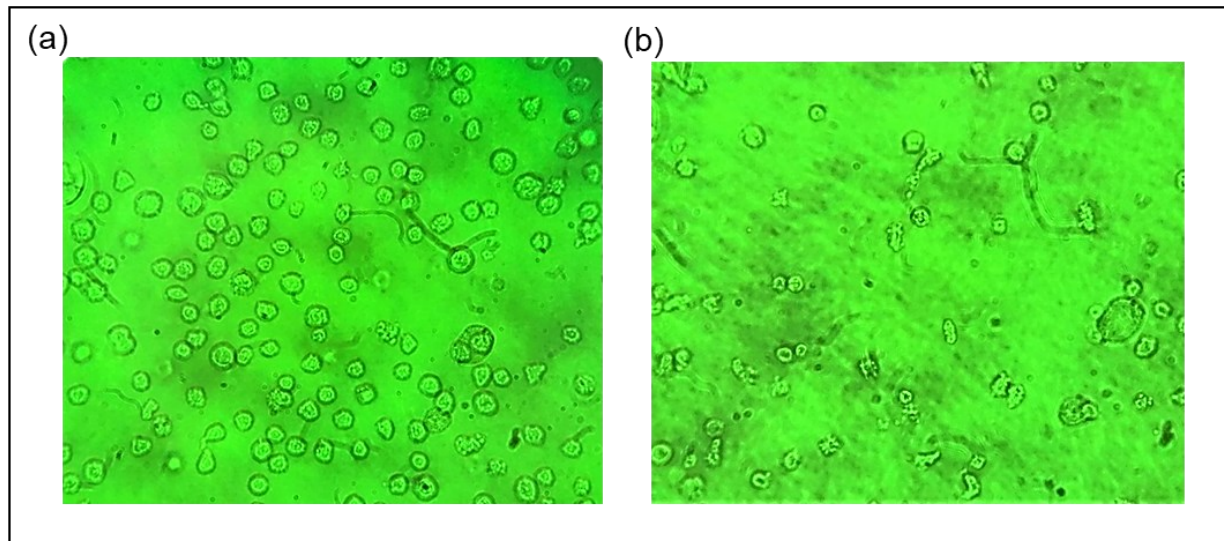
Figure 1. Microscopic view (40X) of HL-60 cells zero (a) and nine (b) days after incubation with 1.25% DMSO supplemented growth medium. Exponentially growing cells were diluted to 1×10^5 cells/mL in RPMI 1640 medium with 20% heat-inactivated FBS, 1% antibiotic-antimycotic, and 1.25% DMSO. Cells were incubated at 37ºC with 5% CO_2, medium was replaced every two days, and the cell concentration was maintained below 1×10^6 cells/mL. Cells were fully differentiated to neutrophils nine days after induction and morphology was assessed microscopically, in which cells were observed to shift from large and ovoid at day zero **(a)** to small and irregular at day nine **(b)**.
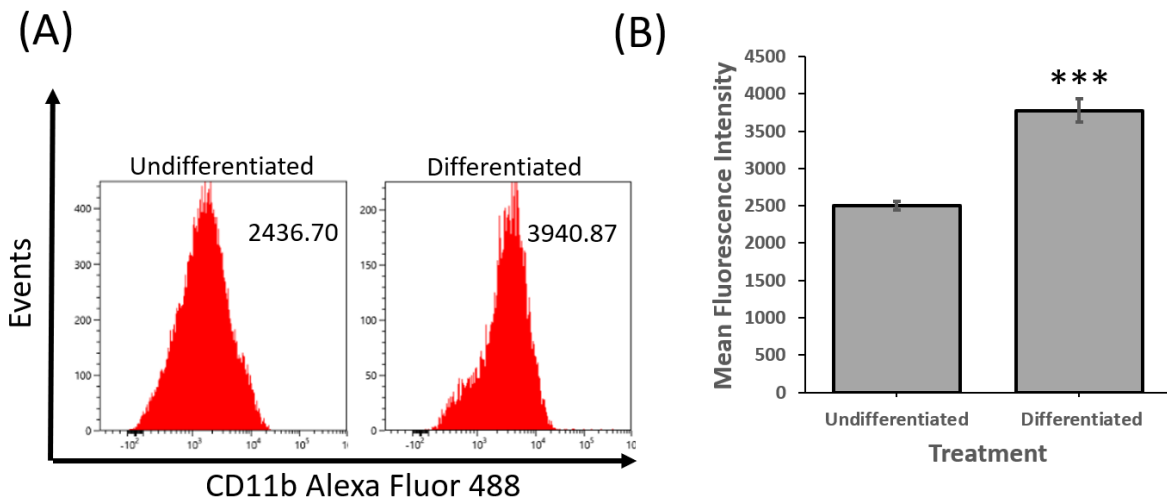
Figure 2. Flow cytometry analysis of the expression of CD11b in differentiated and undifferentiated HL-60 cells. HL-60 cells were induced to differentiate into neutrophils over a 9-day period using 1.25% DMSO in growth medium. Undifferentiated cells were grown without DMSO and split every 2-3 days. Cells (1×10^6) were stained with 0.1 μg/mL Alexa Fluor 488 fluorochrome-conjugated primary antibodies against CD11b. (A) Histograms of one representative experiment showing CD11b expression in undifferentiated and differentiated cells, with mean fluorescence intensity (MFI) shown. (B) Data are expressed as the MFI with bars representing the standard deviation (n=3) ($p<0.001$).
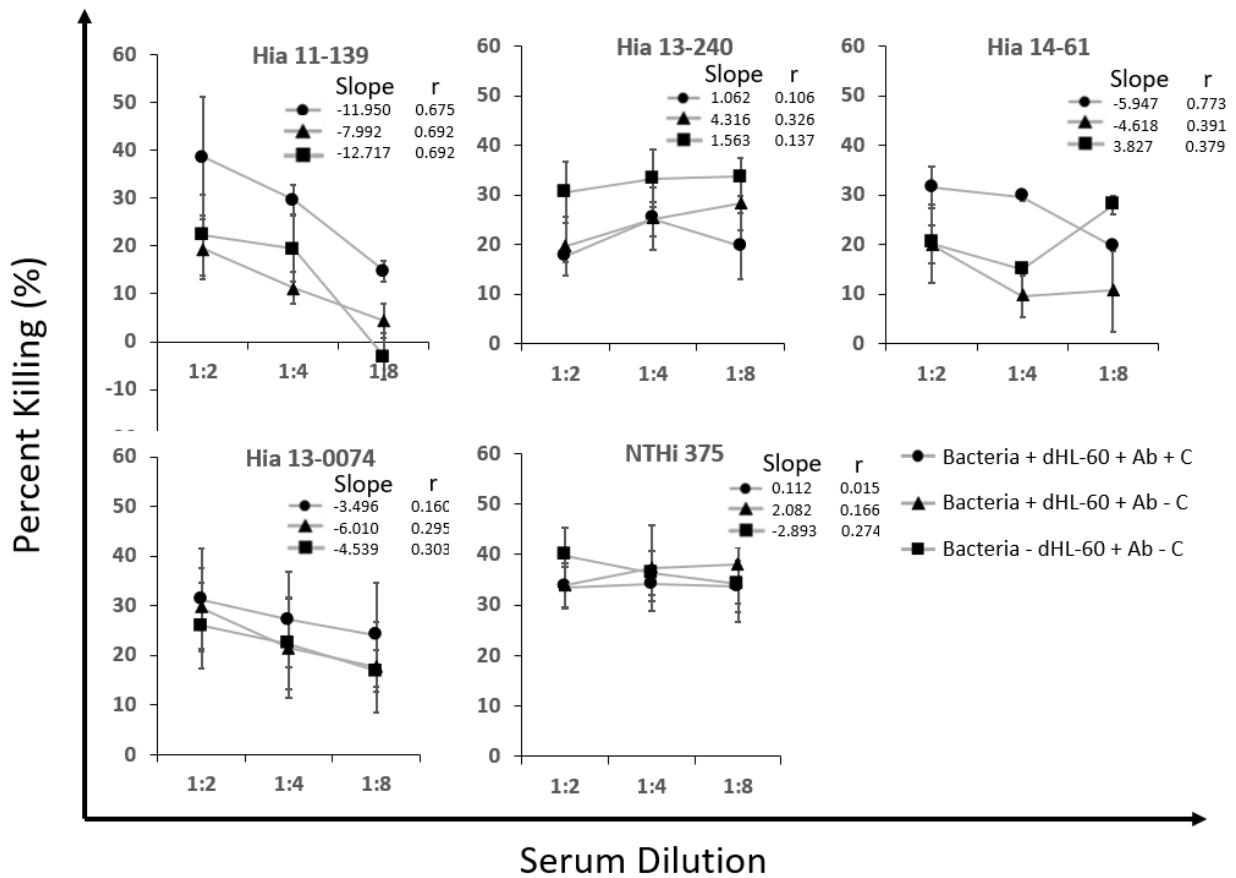
Figure 3. Percent killing of Hia strains after exposure to multiple immunogenic components in an OPA over multiple dilutions of pooled human serum. Bacteria were incubated with pooled serum, rabbit complement, and dHL-60 cells and 10 µL aliquots were plated and incubated overnight at 37°C in 5% CO_2. Percent killing is displayed as the mean of multiple independent trials (n=3), with bars representing the standard error of the mean (SEM). The legend indicates the reaction mixture components, in which Ab = antibody, and C = complement. Slope and Pearson r from linear regression analysis is shown.
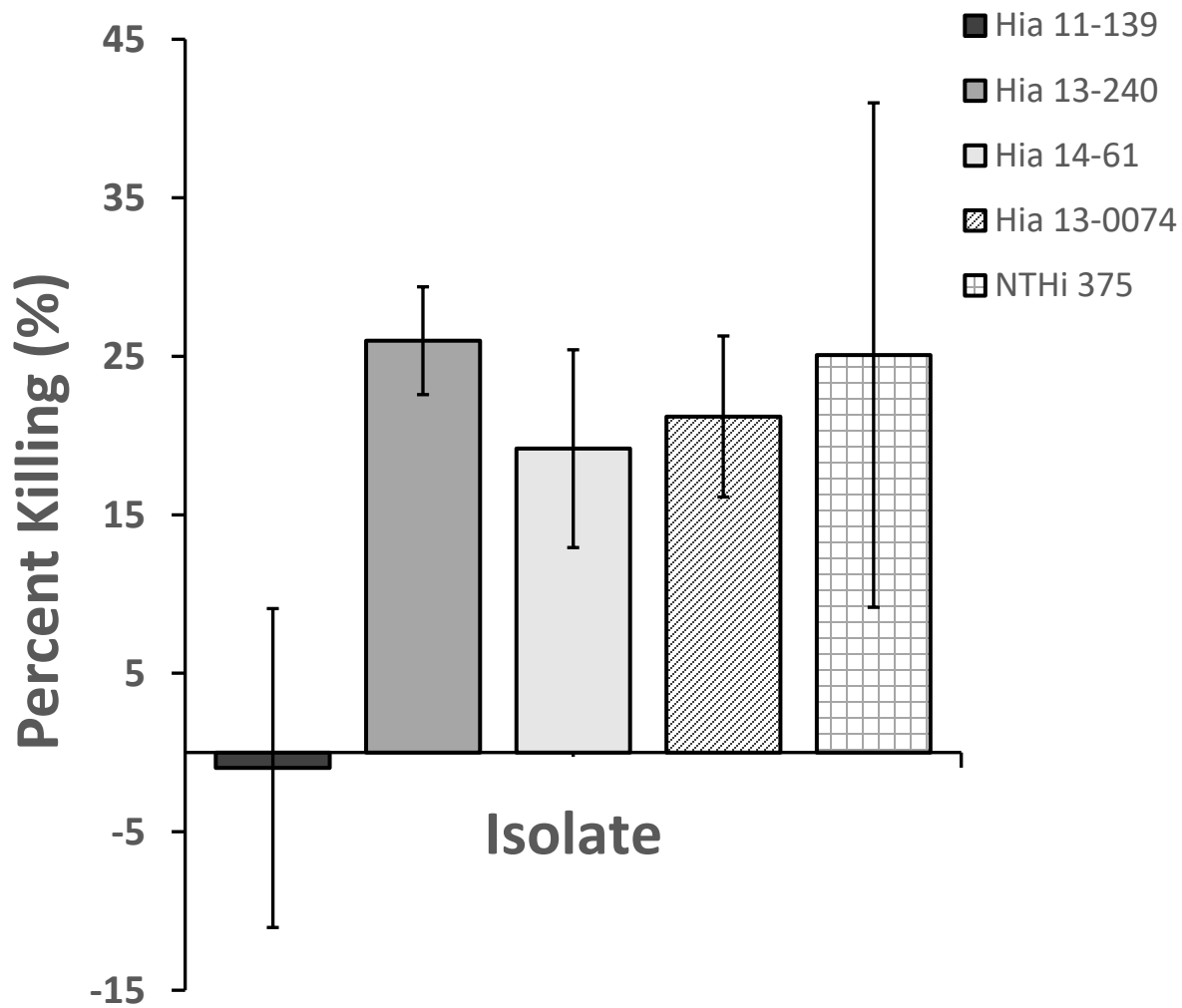
Figure 4. Percent killing of Hia strains after exposure to neutrophils and complement in an OPA. Bacteria were incubated with dHL-60 cells and rabbit complement for 90 minutes and 10 µL aliquots were plated and incubated overnight at 37ºC in 5% CO_2. Serum was replaced with heat-inactivated rabbit complement. Percent killing is displayed as the mean of multiple independent trials (n=3), with bars representing the standard error of the mean (SEM).
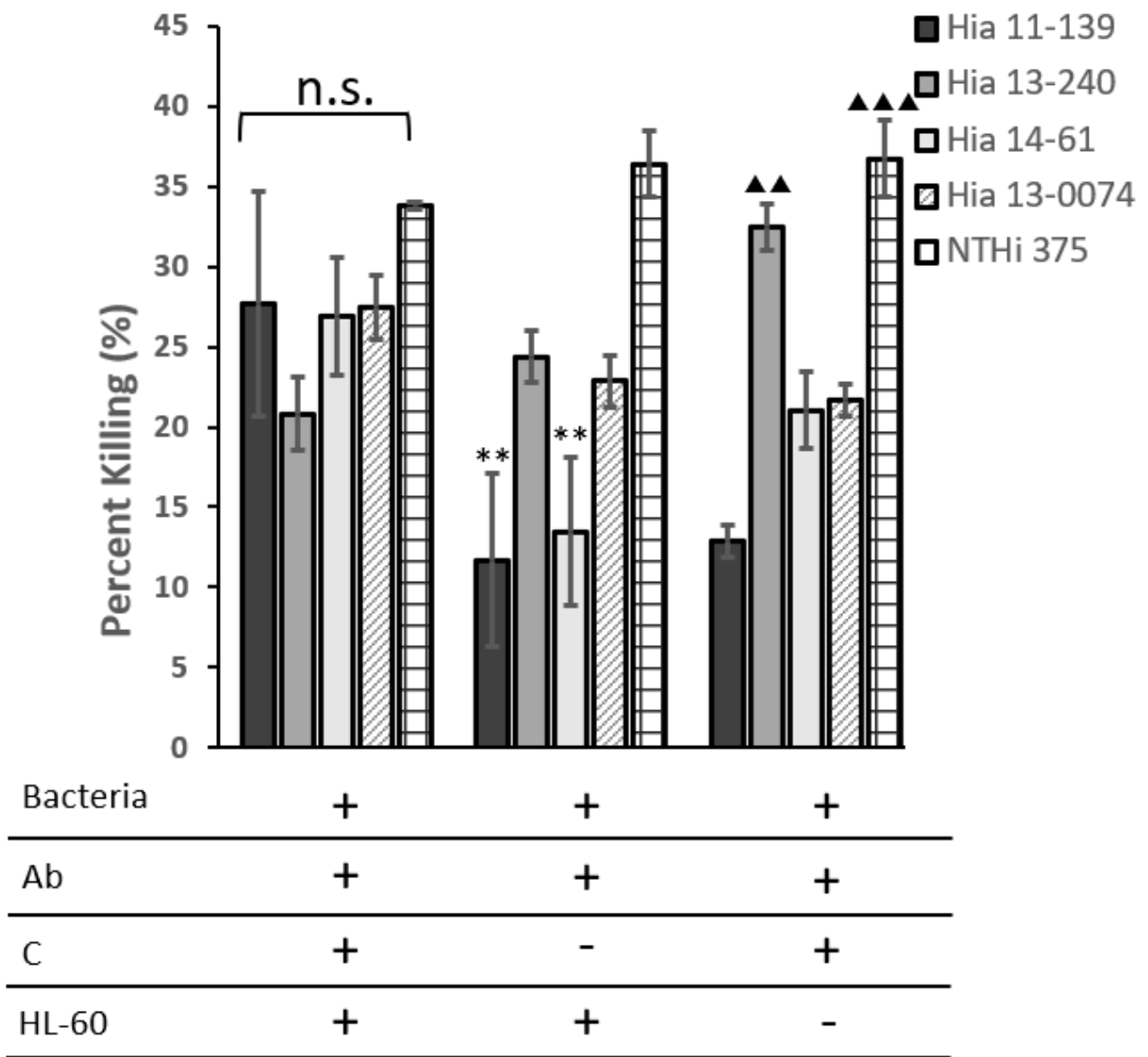
Figure 5. Percent killing of Hia strains after exposure to neutrophils, complement, and serum in an OPA. Bacteria were incubated with various system components at multiple serum dilutions in an OPA and 10 µL aliquots were plated and incubated overnight at 37ºC in 5% CO_2. Percent killing is displayed as the mean percent killing over 3 serum dilutions of 3 independent trials, with bars representing the standard error of the mean (SEM). The legend indicates the reaction mixture components, in which Ab = antibody, and C = complement.
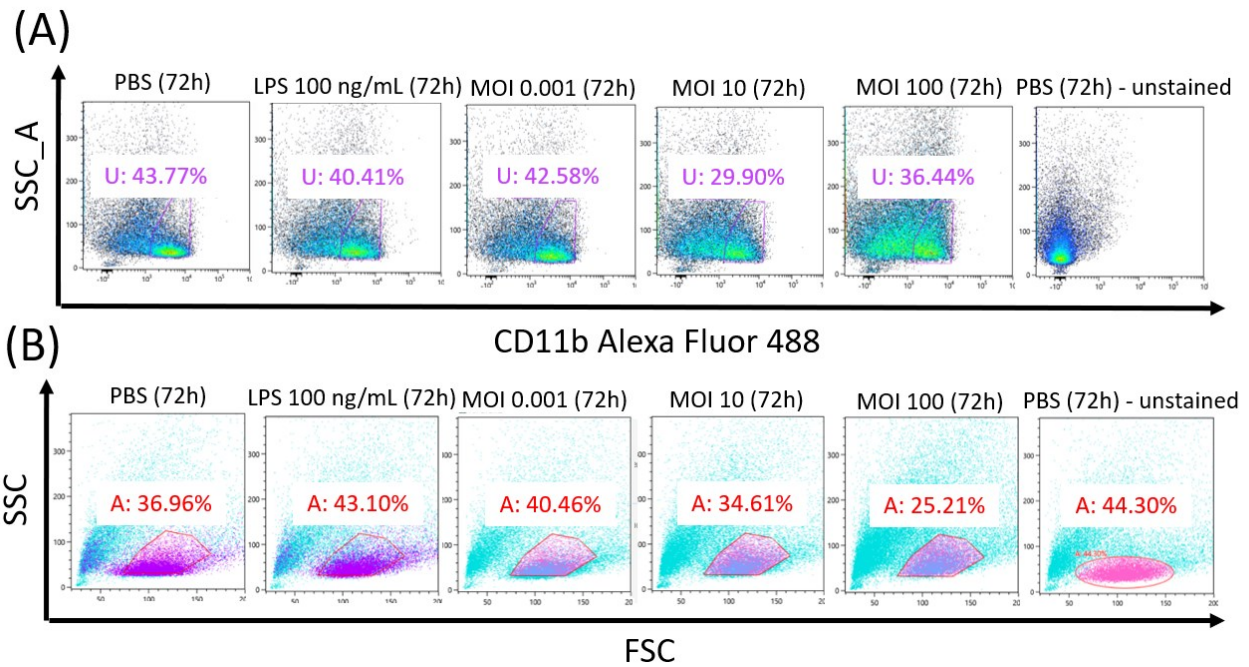
Figure 6. Flow cytometry analysis of CD11b expression on dHL-60 cells after stimulation. 0.5 x 10^6 cells were stimulated with Hia 11-139 at an MOI of 0.1, 1, 10, and 100, with 100 µg/mL gentamicin added at the 1-hour mark. PBS was added to the negative control and the positive control was stimulated with 100 ng/mL *E. coli* LPS. Harvested cells were stained with 0.1 µg/mL mouse-antihuman Alexa Fluor 488-conjugated CD11b antibody and 1 µg/mL PE-conjugated ICAM-1 for one hour. (A): Density dot plots showing all events, with a gate around high CD11b expression (Gate U - purple). (B): SSC vs FSC dots plots showing all events, with Gate U shown in purple and Gate A (red) used to gate the desired cell population.
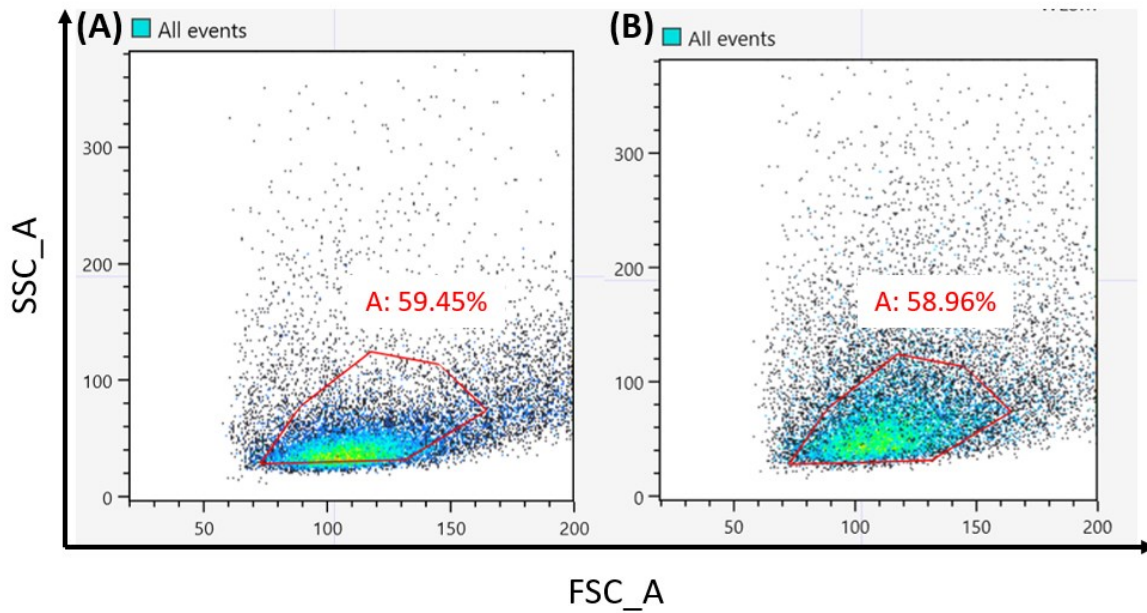
Figure 7. Flow cytometry light scattering (forward scatter, FSC, side scatter, SSC) of a representative experiment showing gating of **(A):** unstimulated (PBS control) and **(B):** stimulated (Hia 11-139 MOI 100) dHL-60 neutrophils. Flow cytometry analysis was done using 10,000 gated events on the SONY SA3800 Flow Cytometer and the desired cell population was gated, represented by A.
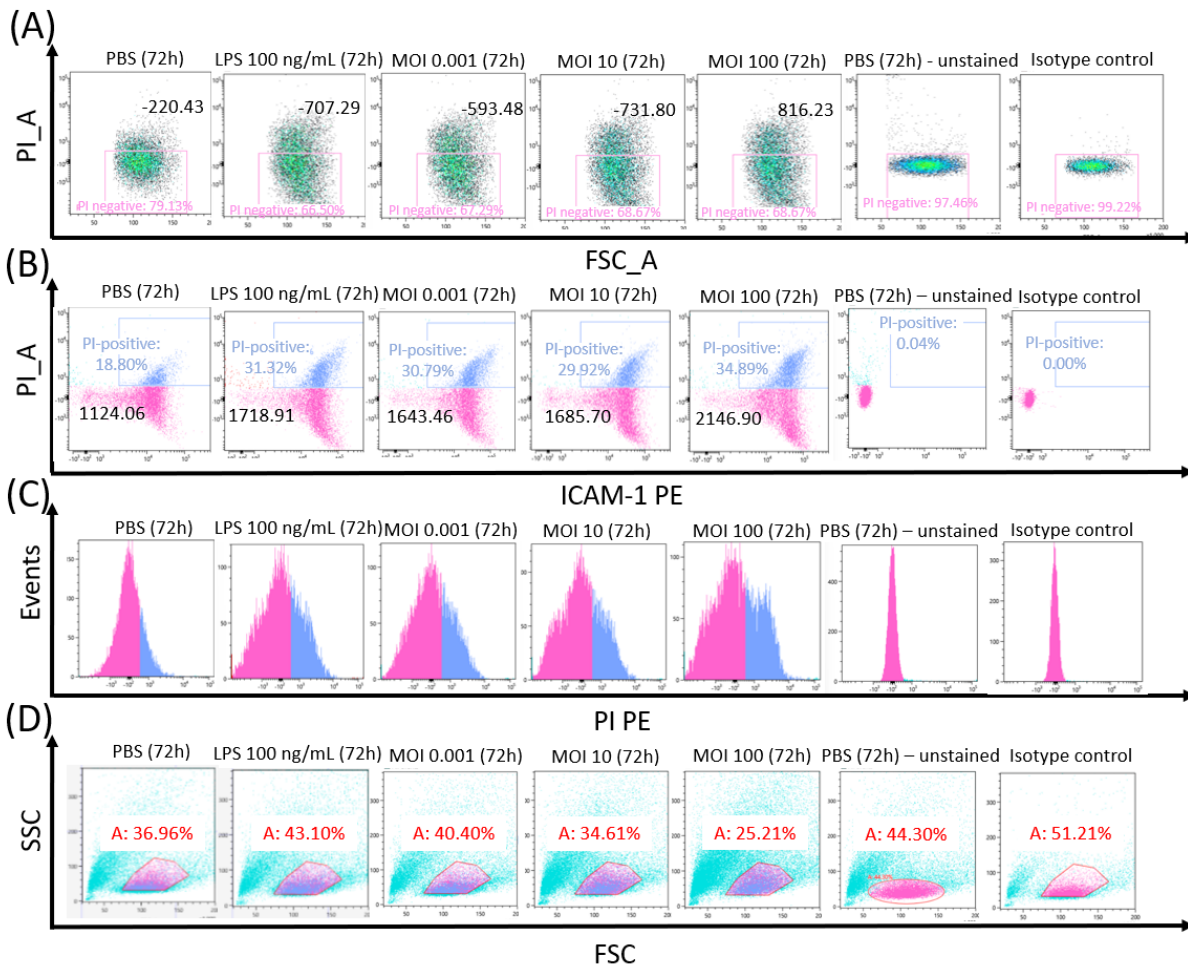
Figure 8. Flow cytometry analysis of dHL-60 cell viability after stimulation. 0.5 x 10^6 cells were stimulated with Hia 11-139 at an MOI of 0.1, 1, 10, and 400, with 100 µg/mL gentamicin added at the 1-hour mark. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL or 1 µg/mL. Harvested cells were stained with 1 µg/mL PE-conjugated ICAM-1 antibody and propidium iodide (PI) at 4ºC for one minute. (A): PI vs FSC dot plots showing events in gate A, with PI mean fluorescence intensity in the PI-negative gate (Pink) shown. (B): PI vs ICAM-1 PE dot plots showing events in gate A, with PI MFI in the PI-positive gate (Blue) shown. (C): Histograms of PI expression showing events in gate A, with PI-negative events shown in pink while PI-positive events are in blue.
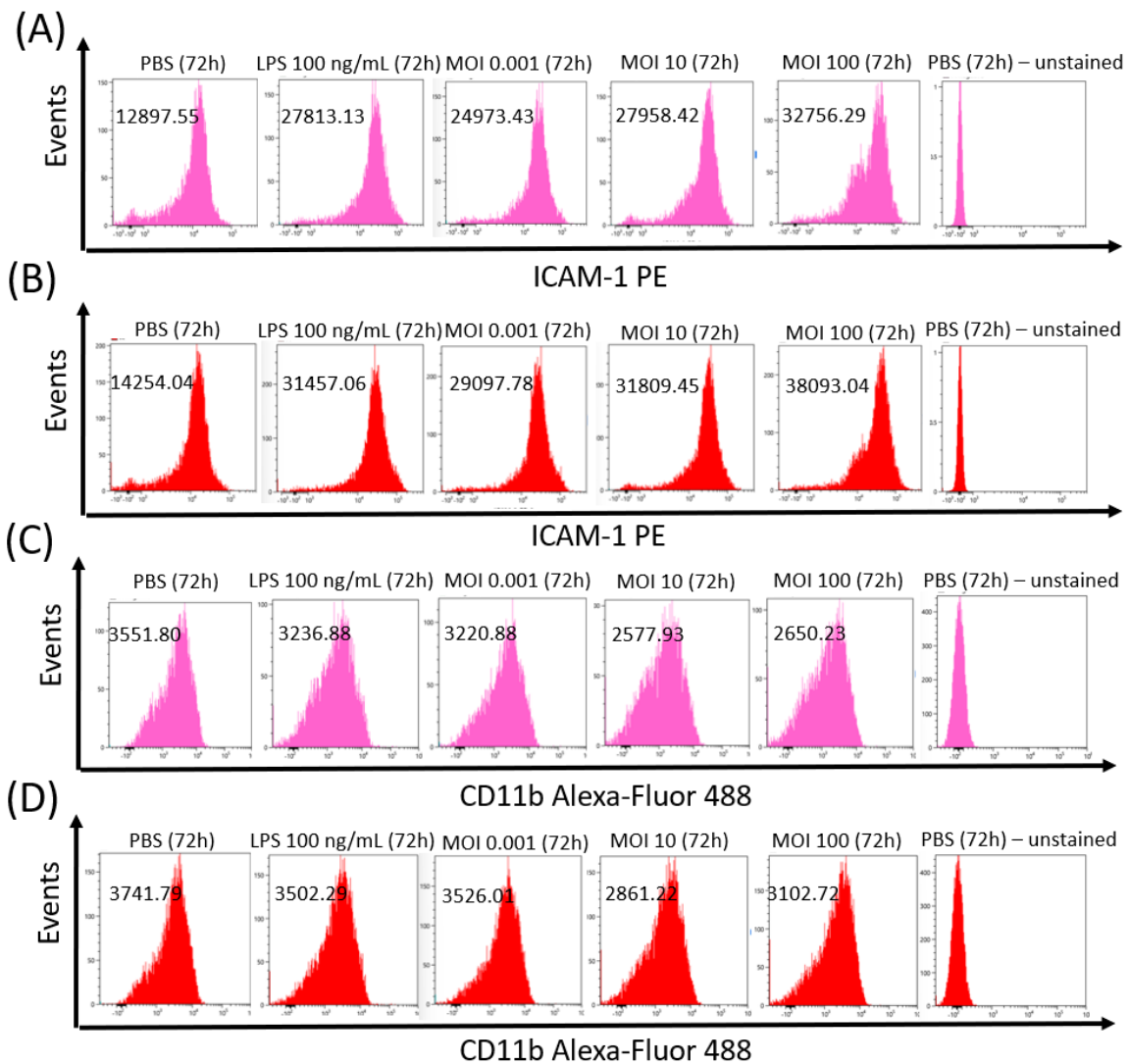
Figure 9. Flow cytometry analysis of ICAM-1 (A-B) and CD11b (C-D) expression on dHL-60 neutrophils after stimulation. 0.5×10^6 cells were stimulated with Hia 11-139 at an MOI of 0.1, 1, 10, and 400, with 100 µg/mL gentamicin added at the 1-hour mark. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL or 1 µg/mL. Harvested cells were stained with 0.1 µg/mL Alexa Fluor 488-conjugated CD11b antibody and PE-conjugated ICAM-1 antibody for one hour. (A): Histograms showing ICAM-1 events in the PI-negative gate (Pink), with MFI in the PI-negative gate shown. (B): Histograms showing ICAM-1 events in gate A (Red), with MFI in the A gate shown. (C): Histograms showing CD11b events in the PI-negative gate (Pink), with MFI in the PI-negative gate shown. (D): Histograms showing CD11b events in gate A (Red), with MFI in the A gate shown.
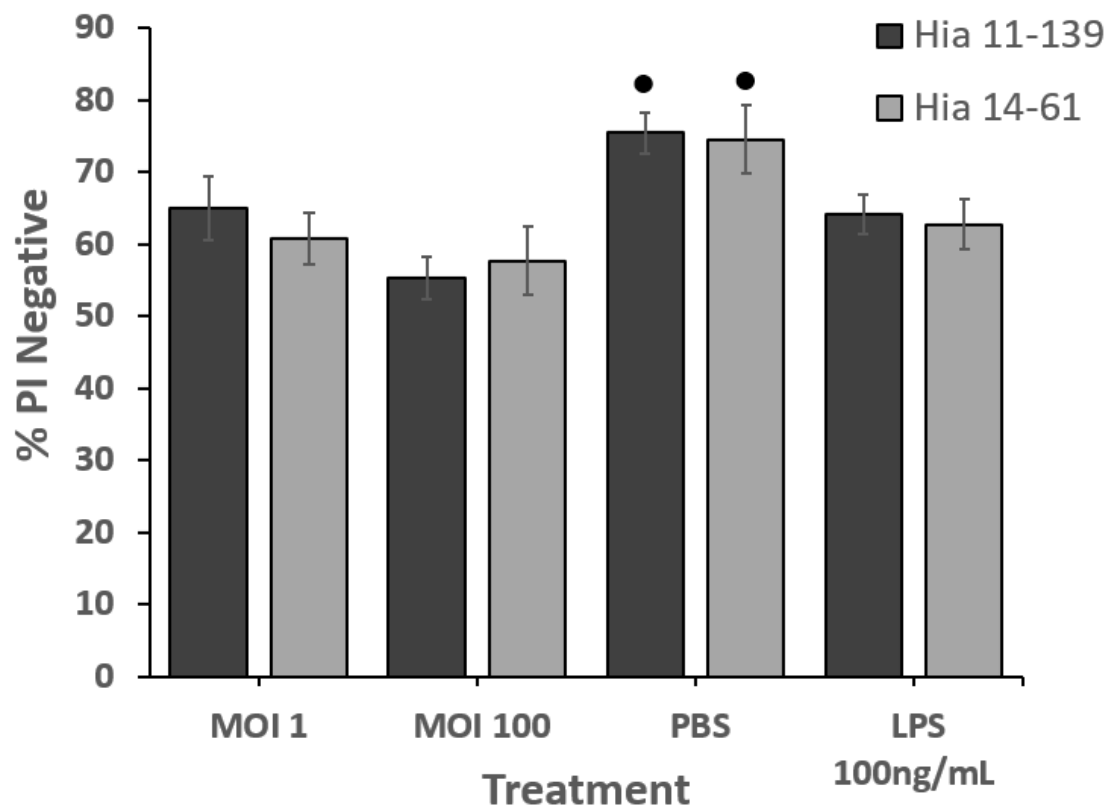
Figure 10. dHL-60 cell viability after 72h stimulation with Hia 11-139 and 14-61. PBS was used as a negative control and *E. coli* LPS 100 ng/mL was used as the positive control. Stained cells were incubated with 1 μg/mL propidium iodide (PI) at 4ºC for one minute. Cells were gated on the negative PI population to identify live cells as described previously. Results are displayed as the mean percentage of at least 3 independent experiments (n=3). •p<0.05 compared to all treatments in their respective category (one way ANOVA analysis with Tukey post-hoc test).
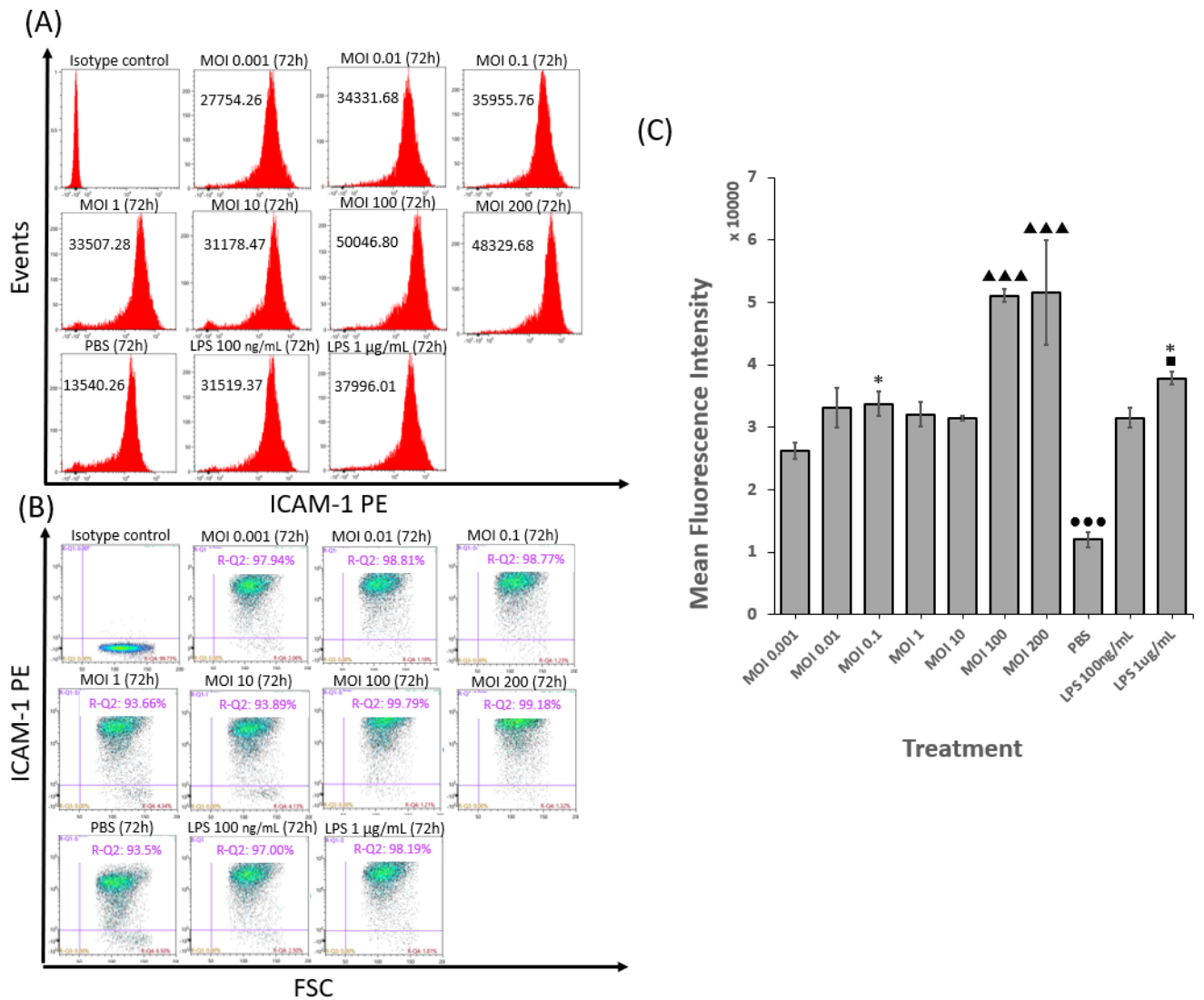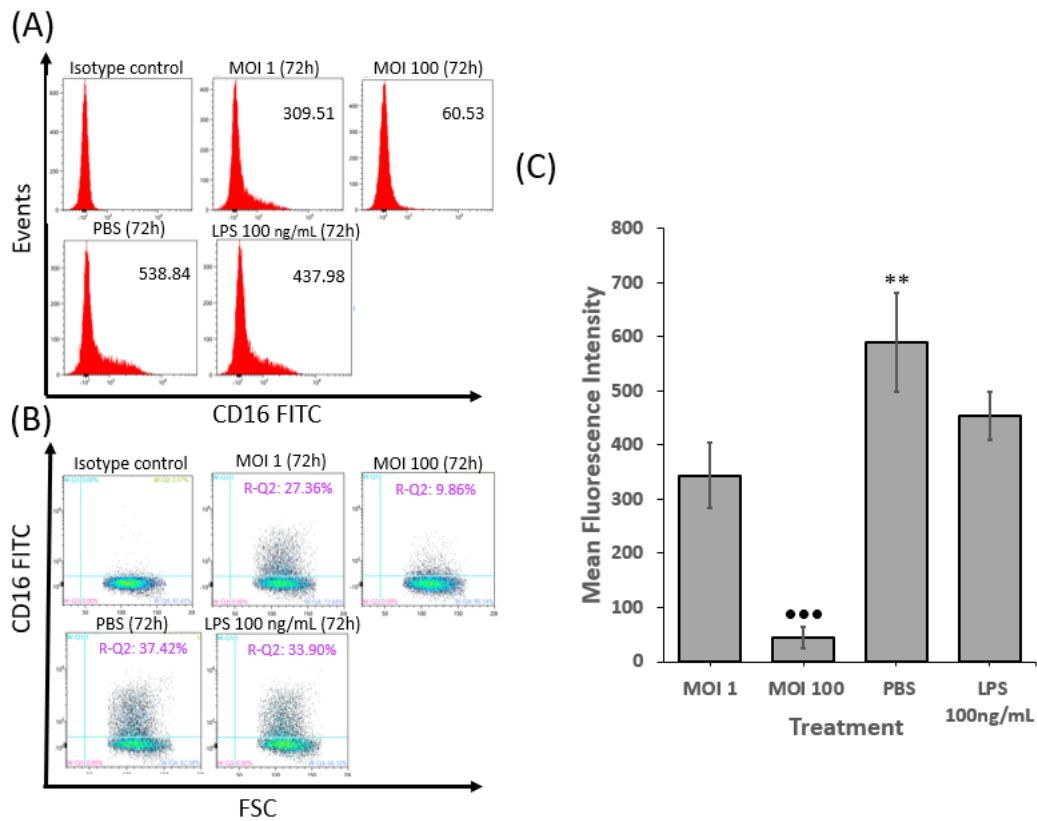
Figure 11. Flow cytometry analysis of ICAM-1 expression on dHL-60 cells stimulated with Hia 11-139. 0.5 x 10^6 cells were stimulated with bacteria at an MOI of 0.001, 0.01, 0.1, 1, 10, 100, and 200, with 100 µg/mL gentamicin added after 1-hour. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL or 1 µg/mL. The isotype control was used to discriminate non-specific binding from specific antibody binding to ICAM-1. Harvested cells were stained with 1 µg/mL mouse-antihuman PE-conjugated ICAM-1 antibody for one hour. (A): Representative histograms are shown using all gated events (gate A – red). (B): Representative dot plots are shown, with ICAM-1 positive cells represented by gate R-Q2 (Purple). (C): Mean fluorescence intensity (MFI) ± SD, n=3. ●●●p<0.001 compared to all treatments, ■p<0.05 compared to LPS 100 ng/mL, ▲▲▲ p<0.001 compared to all treatments except MOI 100, 200 *p<0.05 compared to MOI 0.001 (one-way ANOVA with a Tukey post-hoc test).
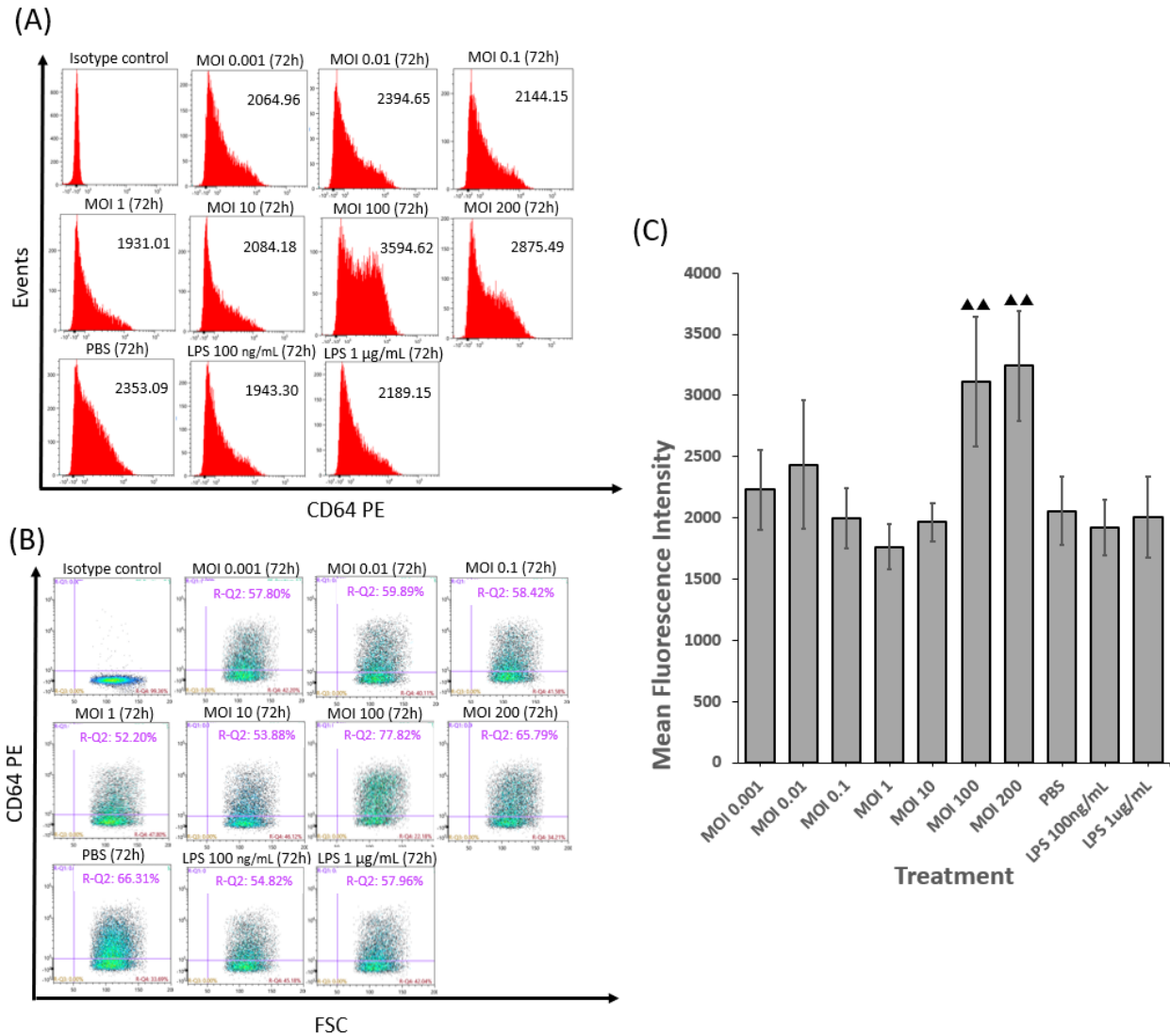
Figure 12. Flow cytometry analysis of ICAM-1 expression on dHL-60 cells stimulated with Hia 14-61. 0.5 x 10^6 cells were stimulated with bacteria at an MOI of 1 and 100, with 100 µg/mL gentamicin added after 1-hour. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL. The isotype control was used to discriminate non-specific binding from specific antibody binding to ICAM-1. Harvested cells were stained with 1 µg/mL mouse-antihuman PE-conjugated ICAM-1 antibody for one hour. **(A):** Representative histograms are shown using all gated events (gate A – red). **(B):** Representative dot plots are shown, with ICAM-1 positive cells represented by gate R-Q2 (Purple). **(C):** Mean fluorescence intensity (MFI) ± SD, n=3. ●●●$p<0.001$ compared to all treatments (one-way ANOVA with a Tukey post-hoc test).
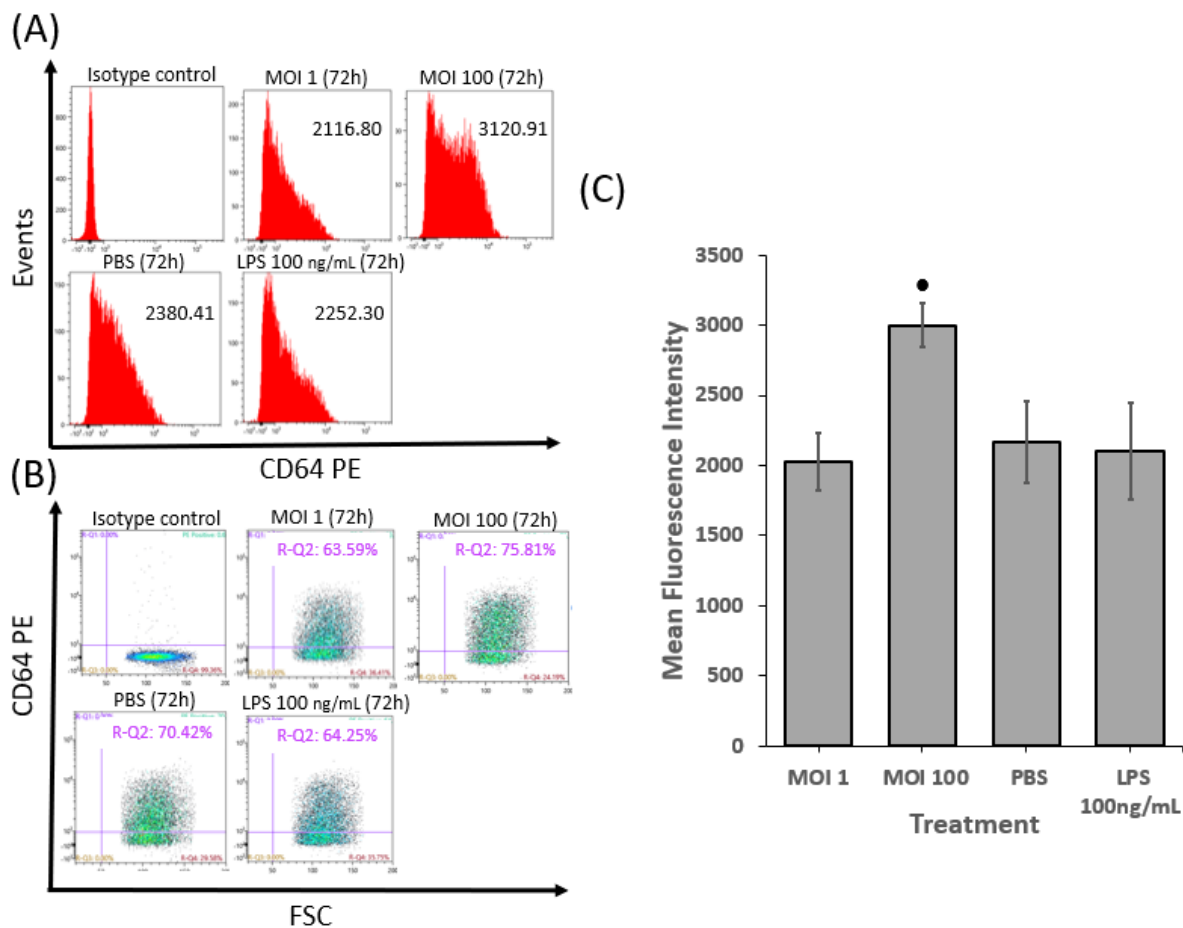
Figure 13. Flow cytometry analysis of CD89 expression on dHL-60 cells stimulated with Hia 11-139. 0.5 x 10^6 cells were stimulated with bacteria at an MOI of 0.001, 0.01, 0.1, 1, 10, 100, and 200, with 100 µg/mL gentamicin added after 1-hour. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL or 1 µg/mL. The isotype control was used to discriminate non-specific binding from specific antibody binding to CD89. Harvested cells were stained with 1 µg/mL mouse-antihuman FITC-conjugated CD89 antibody for one hour. **(A):** Representative histograms are shown using all gated events (gate A – red). **(B):** Representative dot plots are shown, with CD89 positive cells represented by gate R-Q2 (Purple). **(C):** Mean fluorescence intensity (MFI) ± SD, n=3. ✖✖ $p<0.01$ compared to PBS (one-way ANOVA with a Tukey post-hoc test).
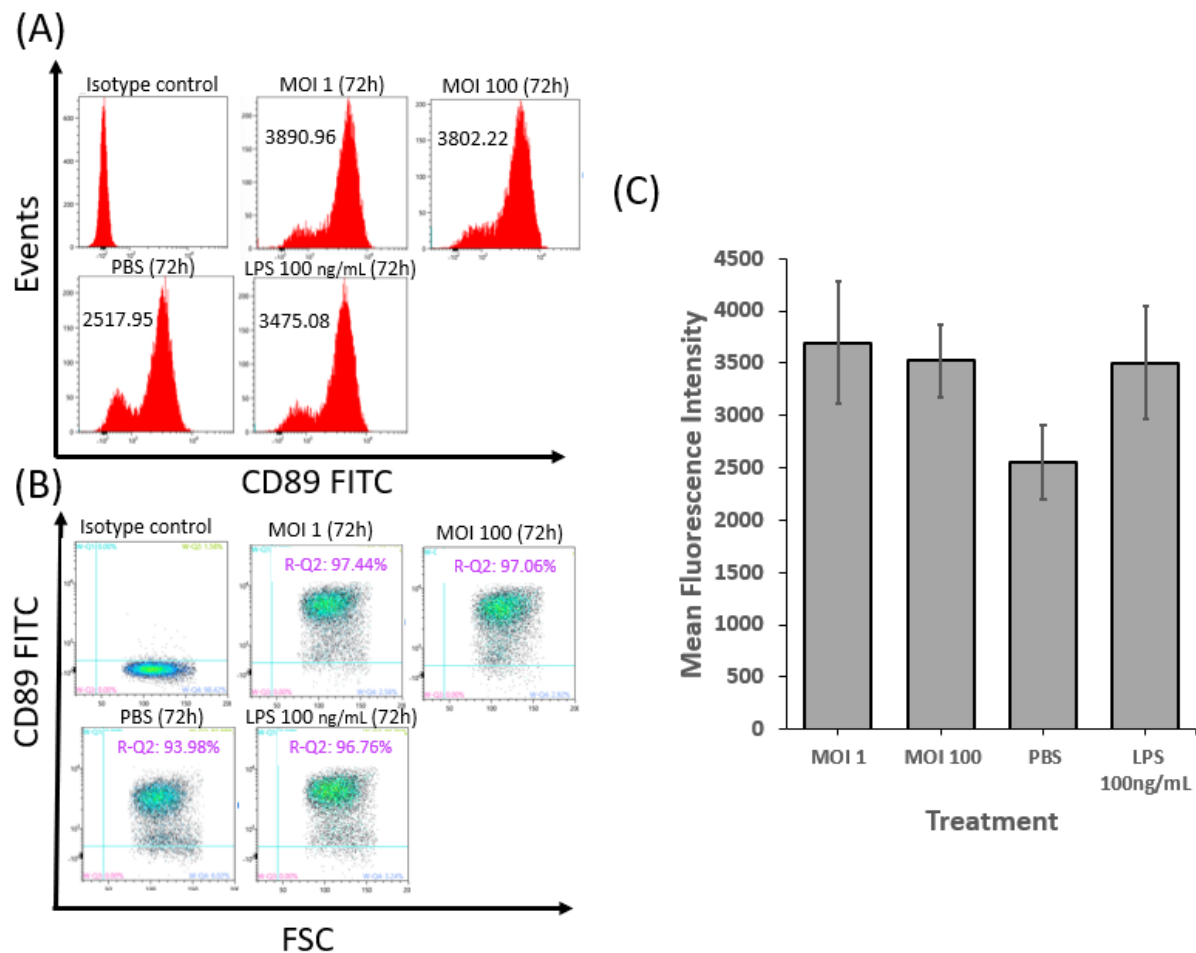
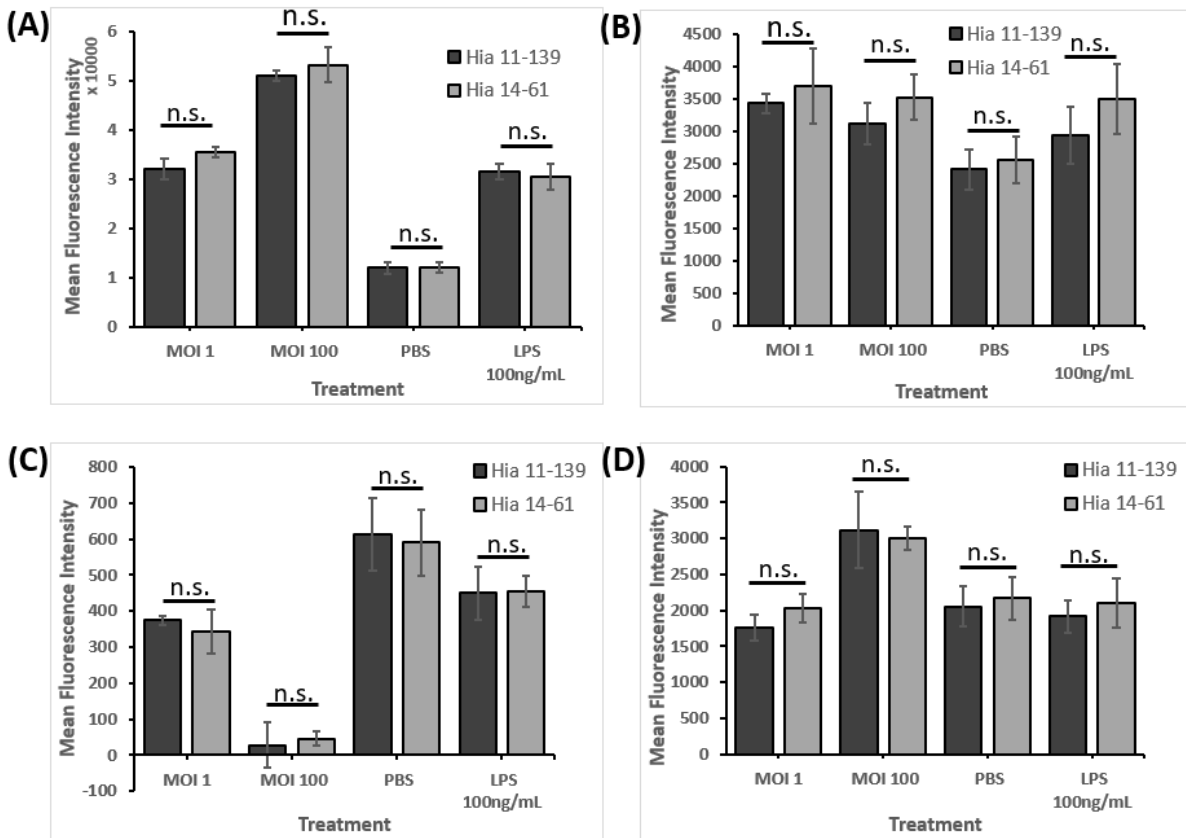Figure 14. Flow cytometry analysis of CD89 expression on dHL-60 cells stimulated with Hia 14-61. 0.5 x 10^6 cells were stimulated with bacteria at an MOI of 1 and 100, with 100 µg/mL gentamicin added after 1-hour. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL. The isotype control was used to discriminate non-specific binding from specific antibody binding to CD89. Harvested cells were stained with 1 µg/mL mouse-antihuman FITC-conjugated CD89 antibody for one hour. **(A):** Representative histograms are shown using all gated events (gate A – red). **(B):** Representative dot plots are shown, with CD89 positive cells represented by gate R-Q2 (Purple). **(C):** Mean fluorescence intensity (MFI) ± SD, n=3.

Figure 15. Flow cytometry analysis of CD16 expression on dHL-60 cells stimulated with Hia 11-139. 0.5×10^6 cells were stimulated with bacteria at an MOI of 0.001, 0.01, 0.1, 1, 10, 100, and 200, with 100 µg/mL gentamicin added after 1-hour. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL or 1 µg/mL. The isotype control was used to discriminate non-specific binding from specific antibody binding to CD16. Harvested cells were stained with 1 µg/mL mouse-antihuman FITC-conjugated CD16 antibody for one hour. **(A):** Representative histograms are shown using all gated events (gate A – red). **(B):** Representative dot plots are shown, with CD16 positive cells represented by gate R-Q2 (Purple). **(C):** Mean fluorescence intensity (MFI) ± SD, n=3. ●$p < 0.05$ compared to all treatments except MOI 0.001, ▲▲ $p < 0.01$ compared to all treatments except MOI 10, 100, 200 (one-way ANOVA with a Tukey post-hoc test).

Figure 16. Flow cytometry analysis of CD16 expression on dHL-60 cells stimulated with Hia 14-61. 0.5×10^6 cells were stimulated with bacteria at an MOI 1 and 100, with 100 µg/mL gentamicin added after 1-hour. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL. The isotype control was used to discriminate non-specific binding from specific antibody binding to CD16. Harvested cells were stained with 1 µg/mL mouse-antihuman FITC-conjugated CD16 antibody for one hour. **(A):** Representative histograms are shown using all gated events (gate A – red). **(B):** Representative dot plots are shown, with CD16 positive cells represented by gate R-Q2 (Purple). **(C):** Mean fluorescence intensity (MFI) ± SD, n=3. ●●●p<0.001 compared to all treatments. **p<0.01 compared to MOI 1,100 (one-way ANOVA with a Tukey post-hoc test).

Figure 17. Flow cytometry analysis of CD64 expression on dHL-60 cells stimulated with Hia 11-139. 0.5×10^6 cells were stimulated with bacteria at an MOI of 0.001, 0.01, 0.1, 1, 10, 100, and 200, with 100 µg/mL gentamicin added after 1-hour. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL or 1 µg/mL. The isotype control was used to discriminate non-specific binding from specific antibody binding to CD64. Harvested cells were stained with 1 µg/mL mouse-antihuman PE-conjugated CD64 antibody for one hour. **(A):** Representative histograms are shown using all gated events (gate A – red). **(B):** Representative dot plots are shown, with CD64 positive cells represented by gate R-Q2 (Purple). **(C):** Mean fluorescence intensity (MFI) ± SD, n=3. ▲▲ $p<0.01$ compared to all treatments except MOI 0.01 (one-way ANOVA with a Tukey post-hoc test).

Figure 18. Flow cytometry analysis of CD64 expression on dHL-60 cells stimulated with Hia 14-61. 0.5 x 10^6 cells were stimulated with bacteria at an MOI of 1 and 100, with 100 µg/mL gentamicin added after 1-hour. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL. The isotype control was used to discriminate non-specific binding from specific antibody binding to CD64. Harvested cells were stained with 1 µg/mL mouse-antihuman PE-conjugated CD64 antibody for one hour. **(A):** Representative histograms are shown using all gated events (gate A – red). **(B):** Representative dot plots are shown, with CD64 positive cells represented by gate R-Q2 (Purple). **(C):** Mean fluorescence intensity (MFI) ± SD, n=3. •p<0.05 compared to all treatments (one-way ANOVA with a Tukey post-hoc test).

Figure 19. Flow cytometry analysis of **(A)** ICAM-1, **(B)** CD89, **(C)** CD16, **(D)** CD64, on dHL-60 cells after stimulation with Hia 11-139 and 14-61 at an MOI of 1 and 100, with 100 μg/mL gentamicin added at the 1-hour mark. PBS was added to the negative control and the positive control was stimulated with *E. coli* LPS 100 ng/mL. Harvested cells were stained with 1 μg/mL mouse-antihuman PE-conjugated ICAM-1/CD64 or FITC-conjugated CD89/CD16 antibody for 1-hour. Mean fluorescence intensity (MFI) ± SD of all gated cells is shown. Data represents the average MFI of at least 3 independent experiments. *p<0.05 (independent samples t-test).
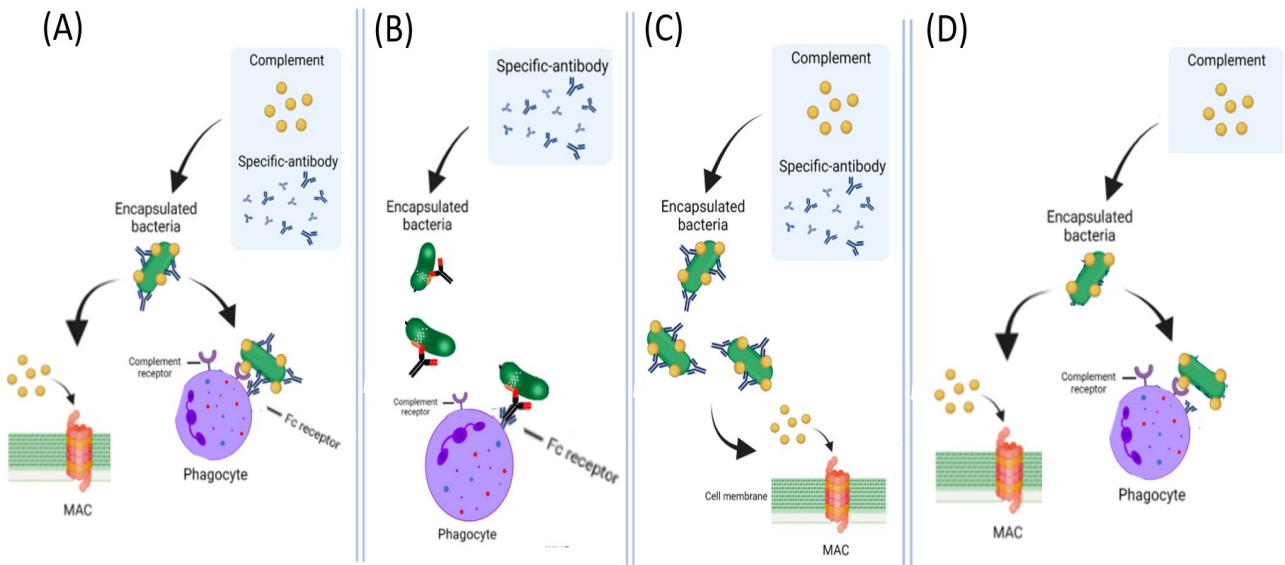
Figure 20. Mechanisms of bacterial killing in the OPA. (A): Killing by opsonophagocytosis occurs following the coating of the bacteria with complement and serum antibodies. Bacterial lysis additionally occurs when the membrane attack complex (MAC) is formed on the bacterial surface following complement activation. (B): When complement is removed, killing occurs by opsonization followed by phagocytosis. (C): When neutrophils are removed, killing occurs by antibody-dependent (classical pathway) and spontaneous activation (alternative pathway) of the complement system, which leads to bacteriolysis through membrane attack complex formation. (D): When serum is removed, the alternative complement pathway and neutrophils kill bacteria through antibody-independent pathways. The figure was adapted from Toh et al. (2021).

8 - Tables

Table 1. Hi clinical isolates examined and their characteristics. Hi was taken from frozen stock (stored at -80ºC) and grown on BHI plates supplemented with 1 µg/mL (NAD) and 10 mg/mL hemin at 37ºC and 5% CO_2 for 16 hours. Isolated Hia colonies were transferred to supplemented BHI broth and the sample was diluted to create an OD = 0.1 solution. The solution was used to obtain the desired concentration of bacteria.

Strain	Isolate	Sequence Type (ST)	Characteristics
Hia	11-139	ST-23	Invasive, encapsulated
Hia	13-0074	ST-23	Invasive, unencapsulated (mutated)
Hia	13-240	ST-4	Invasive, encapsulated
Hia	14-61	ST-23	Non-invasive, encapsulated
NTHi	375	N/A	Non-invasive, encapsulated

Table 2. Hia CFU counts on supplemented BHI agar plates for each isolate. Hia isolates were incubated with various system components at multiple dilutions of serum and 10 µL aliquots were plated and incubated overnight at 37ºC in 5% CO_2. CFU counts are displayed from multiple independent trials, with n=3. Reaction mixture components are indicated, with Ab representing antibody, C as complement, and HI C as heat-inactivated complement. Due to inter-trial variation in growth capacity, percent killing was calculated for each trial using the equation included in appendix 3 and the final percent killing value was averaged over the 3 trials.

Isolate	Serum Dilution	Hia + Ab + C + dHL-60	Hia + Ab + dHL-60	Hia + Ab + C	Hia + HL-60	Hia + HI C + buffer
Hia 11-139	None	-	-	-	53, 31, 32	37, 26, 55
	1:2	22, 11, 46	26, 20, 51	29, 16, 51	-	-
	1:4	26, 17, 42	31, 24, 51	31, 17, 50	-	-
	1:8	31, 23, 47	33, 26, 54	41, 24, 59	-	-
Hia 14-61	None	-	-	-	25, 25, 34	28, 30, 50
	1:2	17, 22, 36	24, 24, 36	25, 26, 32	-	-
	1:4	20, 21, 35	27, 25, 45	23, 26, 27	-	-
	1:8	23, 23, 41	30, 24, 40	21, 21, 36	-	-
Hia 13-240	None	-	-	-	47, 65, 54, 83	73, 83, 72, 105
	1:2	58, 71, 61, 85	66, 75, 51, 74	45, 64, 41, 87	-	-
	1:4	57, 54, 55, 85	67, 58, 48, 74	40, 58, 44, 86	-	-
	1:8	68, 52, 61, 86	62, 61, 44, 71	44, 52, 48, 81	-	-
Hia 13-0074	None	-	-	-	75, 74, 90, 122	95, 118, 86, 164
	1:2	83, 49, 70, 106	72, 62, 77, 103	140, 75, 78, 110	-	-
	1:4	84, 55, 73, 116	75, 70, 91, 113	77, 82, 119	-	-
	1:8	76, 55, 83, 131	77, 73, 91, 131	75, 130	-	-
NTHi 375	None				136, 211, 158	236, 237, 202
	1:2	140, 170, 138	130, 177, 140	99, 152, 158		
	1:4	138, 167, 138	131, 180, 111	111, 164, 158	-	-
	1:8	146, 167, 132	136, 171, 110	112, 190, 147	-	-

9 – References

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10 - Appendices

Appendix 1 - Bacterial counts for Hia strains

Table 3. Bacterial counts (CFU/mL) for each Hia strain. Hia was taken from frozen stock (stored at -80ºC) and grown on BHI plates supplemented with 1 µg/mL (NAD) and 10 mg/mL hemin at 37ºC and 5% CO_2 for 16 hours. Isolated Hia colonies were transferred to supplemented BHI broth and the sample was diluted to create an OD = 0.1 solution using the following CFU/mL counts.

Strain	CFU/mL ($\times 10^8$)
Hia 11-139	3.05
Hia 13-240	1.59
Hia 14-61	3.03
Hia 13-0074	4.84
NTHi 375	3.1

Appendix 2 - 96 well plate organization

1-2: serum, 3-4: HI serum, 5: No serum, 6: No HL-60
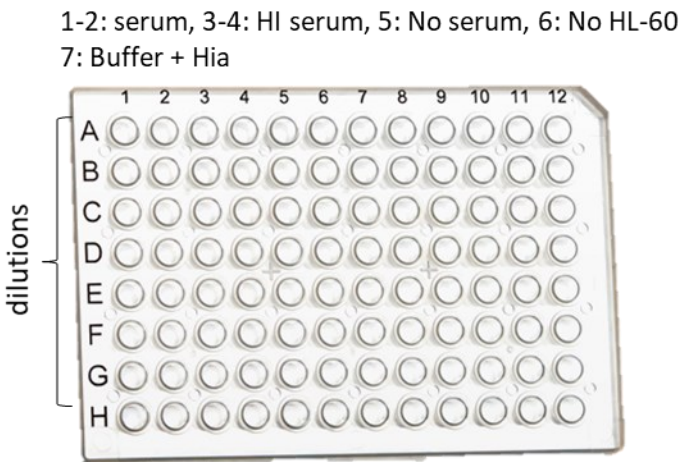7: Buffer + Hia

Figure 21. A sample of the 96-well plate used for the OPA. Position and composition of various controls are indicated and included in columns 1-7. Serum was serially diluted (2-fold) in rows A-E.

Appendix 3 - Calculating percent opsonophagocytosis

Formula for the determination of percent opsonophagocytosis:

$$\frac{(\text{CFU Hia} + \text{buffer control well}) - (\text{CFU Hia} + \text{Ab} + \text{C} + \text{HL} - 60 \text{ well})}{\text{CFU Hia} + \text{buffer control well}} \times 100\%$$