MOLECULAR DETERMINANTS OF IMMUNOGENICITY OF THE NONTYPEABLE *HAEMOPHILUS INFLUENZAE* HMW1 AND HMW2 ADHESINS

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A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2021

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ACKNOWLEDGMENTS

I wish to express my gratitude to everyone who has helped me throughout this long and challenging endeavor. I would like to thank my mentor, Dr. Joseph W. St. Geme III, who gave me the opportunity to explore new ideas and taught me to constantly challenge my own thinking. Joe's mentoring has helped me develop into an inquisitive and more thoughtful scientist. I'd also like to acknowledge past and current members of the St. Geme lab, who have been an incredible network of friendship and collaboration. First, Dr. Eric Porsch, who has been around for all of the highs and lows of my graduate career. Eric has been a phenomenal role model and mentor, and he has put up with more than he has ever needed to. Dr. Susan Grass, though far away, has also been an incredible source of knowledge and support, and her insight has been invaluable. Dr. Vanessa Muñoz, Alexandra Sacharock, Nina Montoya, Dan Morreale, and Kevin Hernandez have been the greatest possible group of fellow students to work with, and they are all remarkably talented scientists. None of this work would have been accomplished without their thoughtfulness and support. Many collaborators also helped make this work possible, including Dr. Hao Shen, Dr. Elizabeth Littauer, Ying Yang, Dr. Lynn Spruce, and Dr. Steve Seeholzer.

ABSTRACT

MOLECULAR DETERMINANTS OF IMMUNOGENICITY OF THE NONTYPEABLE *HAEMOPHILUS INFLUENZAE* HMW1 AND HMW2 ADHESINS

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Joseph W. St. Geme, III

Nontypeable *Haemophilus influenzae* (NTHi) is associated with significant morbidity in both children and adults, causing localized respiratory tract disease such as acute otitis media, sinusitis, and pneumonia and accounting for occasional serious invasive disease. The pathogenesis of NTHi disease begins with colonization of the upper respiratory tract, followed by contiguous spread to sites of infection. Existing strategies to prevent NTHi disease are limited to adenoidectomy and antibiotic prophylaxis; unfortunately, these approaches are relevant only for recurrent otitis media and often associated with short-term and long-term complications. Consequently, there is interest in developing a vaccine to prevent nasopharyngeal carriage, a prerequisite for disease. Among potential vaccine antigens is the High Molecular Weight (HMW) family of outer membrane adhesins, which includes HMW1 and HMW2. In this work, we evaluated the ability of HMW1 and HMW2 to stimulate an immune response and protection against NTHi nasopharyngeal colonization. We demonstrated that intranasal immunization with HMW1 and HMW2 is highly immunostimulatory and protects mice against colonization by heterologous strains of NTHi. Elements of this protective immunity include both a strain-specific antibody response and broad-acting Th17 response. To study the strain specificity of the antibody response in greater depth, we compared the immune response to HMW1 and HMW2 from the same strain and from

heterologous strains. Despite the high conservation of the HMW1 and HMW2 adhesins, these proteins show significant immunological differences and consistently produce strain-specific antibody responses. We found that these differences may be caused by protein glycosylation. Given the role of protein glycosylation in shaping the immunogenicity of the HMW1 and HMW2 adhesins, we investigated the mechanisms that influence HMW1 glycosylation by the HMW1C glycosyltransferase. We found that while there is no clear signal shaping their interaction, the stoichiometry between HMW1 and HMW1C is critical to shaping glycosylation specificity. Collectively, this body of work highlights the potential for HMW1 and HMW2 as vaccine antigens and reveals important insights into how these adhesins can stimulate protective immunity.

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1. INTRODUCTION

1.1 Nontypable *Haemophilus influenzae*

Haemophilus influenzae was first isolated from the sputum of influenza patients in 1892. Richard Pfeiffer, a German bacteriologist who worked to identify the organism on chocolate agar, touted it as the causative agent of influenza(Richard Pfeiffer, 1892). Serial isolation of *H. influenzae* from infected patients propagated this notion, until 1933, when the etiological agent of the flu was found to be viral. The bacteria was eventually placed in the genus *Haemophilus*, recognizing its affinity for blood and maintained the species name *influenzae* in reference to its history.

H. influenzae is a gram-negative bacterium and a member of the *Pasteurellaceae* family. The bacteria are nonmotile and non−spore-forming and typically appear as small coccobacilli, roughly 1×0.3 μm in size. *H. influenzae* is able to grow under both anaerobic and aerobic conditions; aerobic growth requires two supplements, known as factor X, derived from hemin or hemoglobin, and factor V, nicotinamide adenine dinucleotide (NAD).

Isolates of *H. influenzae* are typically classified based on the presence of a polysaccharide capsule. Encapsulated strains have one of six structurally and antigenically distinct capsular types that are designated serotypes a-f. In addition, strains can be nonencapsulated; these strains are defined on the basis of their failure to react with typing antisera against capsular serotypes a-f and are therefore referred to as *nontypeable H. influenzae* (NTHi)(Pittman, 1931).

Compared to encapsulated strains of *H. influenzae*, which are clonal, NTHi exhibits much greater genetic diversity and heterogeneity, much of which is due to higher rates of genetic recombination(Pinto et al., 2019). Traditional grouping techniques such as multilocus sequence typing (MLST) and ribotyping do not correlate with clinical or geographic information for most NTHi isolates, and clonality is difficult to clearly establish(LaCross et al., 2013). However, discrete subpopulations have been defined for nontypeable strains based on the presence of genes encoding select conserved surface structures, including lipooligosaccharide (LOS), the High Molecular Weight proteins (HMW1/HMW2), the *Haemophilus influenzae* adhesin (Hia), and the *Haemophilus* adhesion and penetration protein (Hap)(De Chiara et al., 2014).

NTHi stably colonizes the upper respiratory tract of about 80% of adults and children and is an important cause of morbidity. In children, NTHi is the most common cause of acute otitis media and otitis media with effusion, accounting for 40-50% of all cases(Block, 1997). NTHi also frequently causes purulent conjunctivitis, sinusitis, and community-acquired pneumonia(Timothy F. Murphy et al., 2009). In adults with underlying lung disease, including chronic obstructive pulmonary disease (COPD), chronic bronchitis, and cystic fibrosis (CF), NTHi often colonizes the lower respiratory tract and has been associated with acute inflammatory exacerbations(Bandi et al., 2001; Sethi et al., 2002). Most studies indicate that *H. influenzae* is responsible for 13%-50% of COPD exacerbations(Sethi, 2004). NTHi is increasingly being recognized as an important cause of serious invasive disease, including septicemia, meningitis, and endocarditis, especially in neonates, pregnant women, and immunocompromised individuals(Bender et al., 2010; O'Neill et al., 2003). The economic burden of NTHi

disease is estimated to be over $1 billion in the United States and several billion worldwide(Milne & Vander Hoorn, 2010; Talbird et al., 2010).

1.2. Pathogenesis of Nontypeable *Haemophilus influenzae* Disease

The pathogenesis of NTHi disease begins with adherence to the nasopharyngeal epithelium and colonization of the upper respiratory tract (Figure 1.1). Colonization is typically stable, and NTHi is maintained as a commensal in the upper respiratory tract for extended periods. However, NTHi is also an opportunistic pathogen. Under precipitating circumstances, colonization is followed by contiguous spread within the respiratory tract to sites of localized disease, including the middle ear, sinuses, conjunctiva, or lungs. NTHi first colonizes the nasopharynx in early infancy, at a rate of about 20% within the first year of life. The colonization rate gradually increases with age, and nearly half of all children are colonized before the age of 2 years(Howard et al., 1988; Spinola et al., 1986). Children can be asymptomatically colonized with a particular strain for weeks to months, going through cycles of strain acquisition and loss, and then will remain colonized into adulthood. The diversity of colonizing strains also decrease with age, as children will typically harbor multiple strains concurrently, whereas adults will typically carry one(Howard et al., 1988; Kuklinska & Kilian, 1984; Spinola et al., 1986; Trottier et al., 1989).

NTHi is typically acquired through exposure to airborne droplets or by direct contact with respiratory secretions. In order to successfully colonize the upper respiratory tract, NTHi must evade or disarm different components of upper respiratory immunity. Inhaled NTHi must first overcome innate mechanical host defenses, including

the mucociliary escalator, coughing, and sneezing. The mucociliary escalator prevents infection through rapid turnover of the mucous layer, clearing bound bacteria. Mucous turnover is driven by beating of the airway epithelial cell cilia moving, moving mucous and associated bacteria out of the airways(Finney et al., 2014). During initial interactions with the airway epithelium, NTHi can evade and impair mucociliary clearance. Certain NTHi outer membrane proteins, including P2 and P5, have demonstrated binding to sialic acid-containing oligosaccharides of human respiratory mucins(Davies et al., 1995; Miyamoto & Bakaletz, 1996; Reddy et al., 1996). This allows NTHi to overcome anionic characteristics of mucins that typically repel bacterial cells. Additionally, NTHi secretes ciliotoxic factors, including the lipid A portion of the lipooligosaccharide (LOS)(A. P. Johnson & Inzana, 1986) and the surface-exposed glycerophosphodiester phosphodiesterase protein D. When applied to different tracheal tissues *in vitro*, NTHi induces ciliostasis, gradual loss of cilia, and eventual sloughing of epithelial cells(Denny, 1974; A. P. Johnson & Inzana, 1986). The presence of higher molecular weight LOS during initial colonization has also been associated with enhanced adherence to epithelial cells as well as low grade inflammation at mucosal surfaces(J N Weiser, 1993). Consistent with these observed mechanisms, evidence suggests that NTHi preferentially binds to and invades non-ciliated and damaged epithelial cells in the airways(Ketterer et al., 1999; Petris et al., 2018).

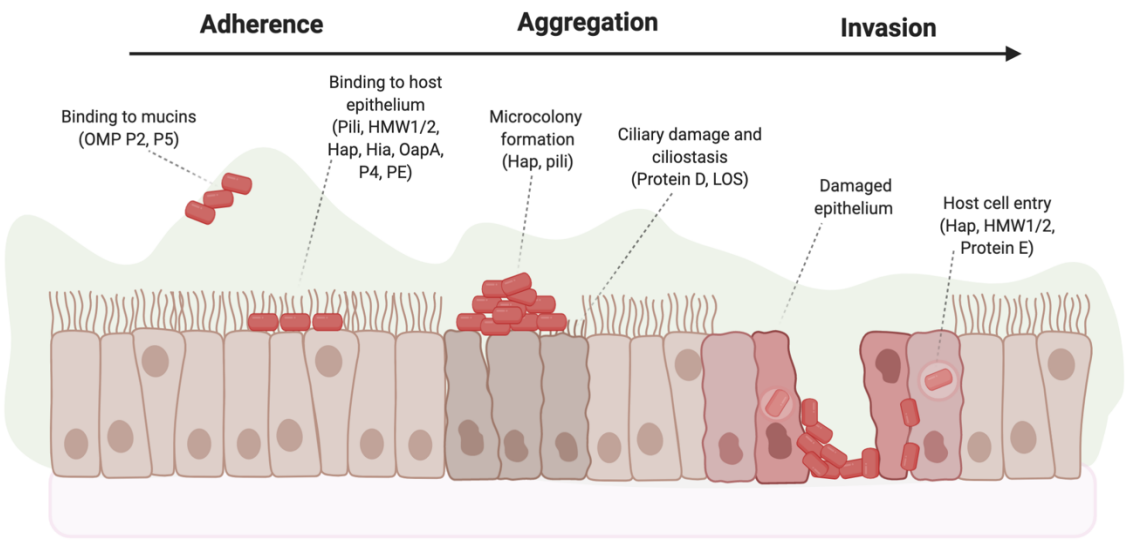


Figure 1. 1. The sequence of events in the pathogenesis of NTHi disease.
Pathogenesis begins with bacterial adherence to the upper respiratory tract mucosa. At each step in pathogenesis, various NTHi adhesins and outer membrane factors promote adherence and survival. Following stable adherence, NTHi forms aggregates which develop into microcolonies. Secretion of toxic factors and the resulting inflammation results in epithelial damage, promoting host cell invasion.

1.2.1 Adherence

To achieve tight adhesive interactions with the host respiratory epithelium, NTHi employs several key surface adhesins that mediate attachment to extracellular matrix (ECM) components and specific host cell plasma membrane receptors.

Pili

Some isolates of *H. influenzae*, including NTHi strains, express adhesive pili. Type IV pili (Tfp) are fibers of about 5-7nm in diameter, up to 4μm in length, and comprised of a single protein subunit assembled into a helical structure(Wall & Kaiser, 1999). Expression of Tfp in NTHi depends on the presence of the *pilABCD* locus as well

as the *comABCDEF* locus. PilA, the primary subunit of the NTHi Tfp, has been shown to mediate adherence to polarized respiratory tract epithelial cells and normal normal human bronchial epithelial cells *in vitro*(Jurcisek et al., 2007; Novotny & Bakaletz, 2016). PilA is thought to adhere specifically to cells that express the intercellular adhesion molecule I (ICAM1) surface receptor(Novotny & Bakaletz, 2016). Tfp are only detected *in vitro* under certain nutrient-restricted growth conditions, suggesting that their expression is regulated by environmental cues(Bakaletz et al., 2005; Jurcisek et al., 2007).Based on animal studies in chinchillas, PilA is expressed *in vivo,* and expression is required for stable respiratory tract colonization and biofilm formation in the chinchilla middle ear(Jurcisek et al., 2007).

So-called haemagglutinating pili represent a second type of pilus found in NTHi. These pili, which are characterized by their ability to mediate agglutination of human erythrocytes. Haemagglutinating pili are approximately 450nm in length and expressed peritrichously(Stull et al., 1984). These pili are encoded by the *hif* group of genes, which share homology with the chaperone-usher pilus assembly machinery genes of other gram-negative bacteria(van Ham et al., 1994). Within the five-gene locus (*hifA-E)*, HifA (*hifA*) comprises the major pilin subunit- repeating HifA subunits form the pilus shaft, and HifE (*hifE*), a tip-associated accessory protein, is thought to be the primary adhesive component(McCrea et al., 1997). Though primarily associated with encapsulated strains of *H. influenzae,* studies of NTHi clinical isolates suggest that approximately 15% carry an intact *hif* locus and express haemagglutinating pili(Geluk et al., 1998; Joseph W. St. Geme et al., 1996). During infection, *H. influenzae* isolates from the nasopharynx express these pili more often, whereas strains recovered from the bloodstream usually do not, suggesting that piliation is beneficial during the initial stages of colonization and

infection and may be advantageous to the organism in the bloodstream(Virkola et al., 2000).

Haemagglutinating pili promote specific adherence to human oropharyngeal epithelial cells and enhance adherence to human nasal and adenoidal tissue in organ culture(Farley et al., 1990; Loeb et al., 1988). Studies on human oropharyngeal epithelial cells *in vitro* have revealed that this adherence occurs through interactions with sialyl-lactosylceramide derivatives found on epithelial cells and erythrocytes(van Alphen et al., 1991). Haemagglutinating pili also facilitate interactions with human erythrocytes through the Anton antigen (AnWj). Beyond interactions with mucins and host cell plasma membranes, pili have been described to facilitate bacterial interactions with certain ECM components(Kubiet et al., 2000). Studies using murine heparin-binding growth-associated molecule have shown that piliation is required for binding with heparin-binding ECM proteins(Virkola et al., 2000). Piliated *H. influenzae* have been found to interact with glycosylated and collagenous ECM components, including fibronectin, laminin, and both type I and III collagens(Virkola et al., 1996).

Non-pilus Adhesins

NTHi is able to achieve substantial adherence independently of pili due to a variety of non-pilus surface adhesins. Given the relatively low occurrence of haemagglutinating pili among isolates, nonpilus adhesins are found in nearly all NTHi isolates, allowing many diverse strains to achieve adherence. Among major adhesins identified on the NTHi surface, several key structures belong to the autotransporter family of proteins (Figure 1.2). Classical autotransporters are synthesized as preproteins

with at least three functional domains, including an N-terminal signal sequence, an internal passenger domain, and a C-terminal outer membrane translocator domain (β-domain). The signal sequence directs export of the polypeptide across the inner membrane and is then cleaved. Subsequently, the β-domain is inserted into the outer membrane and folds into a β-barrel structure with a hydrophilic pore, allowing for extrusion of the passenger domain across the membrane to the surface, where it is anchored and can act as an adhesin. Trimeric autotransporters, the key autotransporter adhesins on the NTHi surface, include Hap, Hia/Hsf, and the HMW1/HMW2 adhesins.

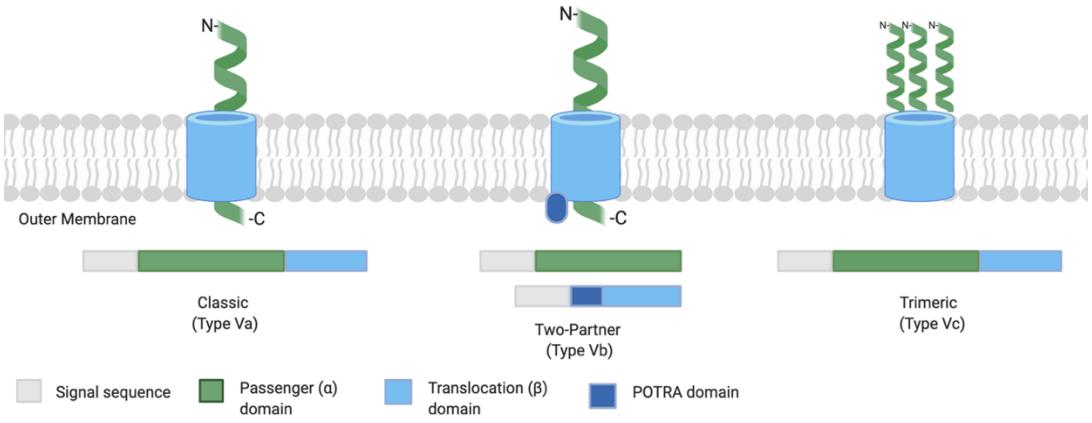


Figure 1. 2. Secretion of autotransporter adhesins.
Autotransporters are exported by a type V secretion system and include classical (Va), two-partner (Vb), and trimeric (Vc) autotransporters. Autotransporters are characterized by a signal sequence for secretion into the periplasm, an internal passenger domain which contains the adhesin, and a translocation domain which mediates outer membrane export. In type Vb systems, the translocation domain is a second protein.

Hap, *Haemophilus* adherence and penetration protein, is found ubiquitously among both nontypeable and encapsulated isolates of H. influenzae(Rodriguez et al., 2003). Hap shares homology with serine-type immunoglobulin A1 (IgA1) proteases expressed by Haemophilus and Neisseria species. Hap was first identified by its ability to facilitate in vitro adherence and invasion in assays with cultured human epithelial

cells(Joseph W. St. Geme et al., 1994). Hap also supports adhesive interactions by binding to ECM components, including fibronectin, laminin, and collagen IV, even in the absence of pili(Fink et al., 2002) .

Hap is synthesized as a 155-kDa preprotein with three primary domains: The N-terminal signal peptide, a 45-kDa outer membrane translocator domain (Hap_{β}), and a 110-kDa passenger domain (Hap_S). Hap_S contains the adhesive domains the protein. The C-terminal 511 residues of Hap_S contain the ECM binding domain, and the C-terminal 311 residues mediate interactions with host epithelial cells and contain the self-associating autotransporter (SAAT) domain, which allows Hap-Hap interactions, contributing to the formation of microcolonies and gradual biofilm development(Fink et al., 2002; Hendrixson & St. Geme, 1998).

Notably, Hap_S also contains an N-terminal serine protease domain. Under certain conditions, Hap_S is secreted and subsequently undergoes an autoproteolytic cleavage event, releasing soluble Hap_S from Hap_{β} into the environment(Meng et al., 2011). Hap_S-mediated adherence and NTHi aggregation are thus modulated by the presence of surface-anchored Hap_S. Hap autoproteolysis is inhibited by secretory leukocyte protease inhibitor (SLPI), a protein found in varying amounts in the upper and lower respiratory tract. Consequently, in the nasopharynx, Hap_S remains anchored to the bacterial surface and mediates increased levels of adherence, contributing to bacterial colonization.

Hia, the *Haemophilus influenzae adhesin*, is another high molecular weight adhesin, with a molecular mass of approximately 115-kDa, and is a trimeric autotransporter protein. First identified on NTHi strain 11, the *hia* gene is found in approximately 20% of NTHi clinical isolates(Stephen J. Barenkamp & St. Geme, 1996b;

Joseph W. St Geme et al., 1998). Encapsulated strains of *H. influenzae* ubiquitously express a primary adhesin homologous to *hia* knows as *Hsf* (*Haemophilus surface fibrils*), which is associated with the expression of short adhesive surface fibrils. *Hia* and *Hsf* share 81% similarity and 72% similarity based on amino acid sequences(Joseph W. St. Geme et al., 1996; Joseph W. St Geme et al., 1998). Hia has been shown to mediate high-affinity adhesive interactions with various human target cell types, including primary ciliated bronchial epithelia, type II pneumocytes, and Chang conjunctival cells(Baddal et al., 2015; Stephen J. Barenkamp & St. Geme, 1996b; J. W. St. Geme & Cutter, 2000). In the chinchilla model of OM, selection for increased Hia expression occurs during nasopharyngeal colonization, suggesting this adhesive activity contributes to stable colonization(Atack et al., 2015). Further, Hia expressing NTHi strains have been associated with cases of infant meningitis, suggesting a role for the adhesin in virulence and disease(Cardines et al., 2007).

Like other trimeric autotransporters, Hia is synthesized as a 115-kDa preprotein that consists of an N-terminal signal sequence, an internal passenger domain and a C-terminal β-domain. Unlike Hap, following export to the bacterial cell surface through the β-domain, the Hia passenger domain is uncleaved and remains surface anchored(J. W. St. Geme & Cutter, 2000). The Hia passenger domain is characterized by a repetitive domain architecture consisting of five distinct domain types(Meng et al., 2008). The adhesive activity of Hia is mediated through two binding domains, identified as HiaBD1 (residues 585-705 in strain 11) and HiaBD2 (residues 61-166 in strain 11). Both binding domains recognize the same host cell receptor, although with differing affinities. The identity of the receptor remains unknown(Yeo et al., 2004).

The ~80% of NTHi isolates that do not carry the *hia* gene instead express another set of adhesins known as the High Molecular Weight (HMW) adhesins. This adhesin family contains two members, HMW1 and HMW2. The HMW1 and HMW2 proteins are highly homologous, sharing 71% identity and 80% similarity overall. In their mature forms, HMW1 has a molecular mass of 125-kDa and HMW2 has a molecular mass of 120-kDa. Both adhesins mediate high levels of adherence to human epithelial cells, though they have different host cell specificities(S J Barenkamp & Leininger, 1992). Each adhesin recognizes a different host glycan. HMW1 interacts with a glycoprotein receptor containing N-linked oligosaccharide chains with sialic acid in an α-2,3 configuration(J. W. St. Geme, 1994). HMW2 binds to 2-6 linked N-acetylneuraminic acid(Atack et al., 2018). These complementary adhesin activities allow HMW-expressing NTHi to bind a variety of host cell types, proving an important advantage for NTHi during different stages of colonization and dissemination. *In vitro,* HMW1 and HMW2 have demonstrated adherence to a variety of human cell lines, including pharyngeal epithelial cells, Chang cells, HaCaT keratinocytes, and macrophages(Buscher et al., 2004; Joseph W. St. Geme et al., 1993; Vuong et al., 2013). Importantly, recent *in vivo* studies have revealed that the HMW1 and HMW2 directly facilitate upper respiratory tract colonization of rhesus macaques. In these studies, NTHi strains expressing *hmw1* and *hmw2* were able to efficiently outcompete an *hmw1hmw2* knockout strain. When introduced into the nares of macaques, the wild type *hmw*-expressing strain was recovered in greater density in both the nasopharynx and the oropharynx, and exhibited more stable colonization at both sites (Rempe et al., 2016).

HMW1 and HMW2 are encoded by homologous loci, *hmw1ABC* and *hmw2ABC.* These loci are found at unlinked locations in the NTHi chromosome. However, strains

that express HMW1 and HMW2 always carry both loci and do not carry the *hia* gene. Each *hmw* locus encodes an identical two-partner secretion system (Figure 1.3). In a two-partner secretion system, the first gene (*tpsA*) encodes the passenger domain. The second gene (*tpsB*) encodes an outer membrane pore through which the TpsA protein transits. The TpsA protein is secreted into the periplasm in a Sec-dependent manner, and the N- terminal signal sequence is cleaved. The resulting new N- terminus contains the TPS domain that is recognized by a periplasmic polypeptide-associated-translocator (POTRA) domain of the TpsB pore. Once on the surface the TpsA protein can remain anchored to the TpsB protein or be released into the extracellular space(Guérin et al., 2017a). *Hmw1A/hmw2A* encode the TpsA protein, which is the primary adhesin, HMW1 or HMW2. The second gene, *hmw1B/hmwB* encodes the HMW1B/HMW2B outer membrane pore(H. Li et al., 2007; Joseph W. St. Geme & Yeo, 2009). HMW1 and HMW2 remain anchored to HMW1B and HMW2B due to an intramolecular disulfide bond located in the C-terminal 20 residues(Buscher et al., 2006). However, following export through the outer membrane pore, the N-terminal region of the adhesin containing the TPS domain, known as the pro-piece, is cleaved from the mature adhesin and released into the extracellular environment(Yeo et al., 2007a). The role of this cleavage event remains unclear.

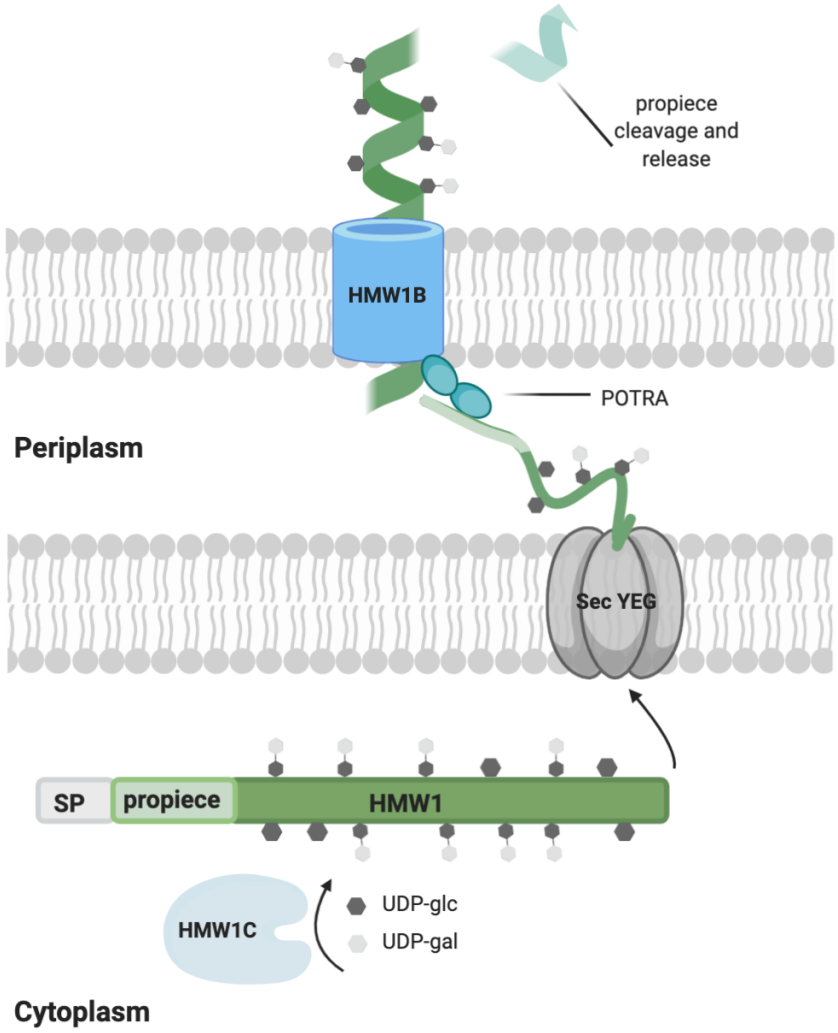


Figure 1. 3. Secretion of the hmw1abc two-partner system.
Nascent HMW1 (*hmw1a*) is first generated as a preproprotein, containing a signal peptide (SP) and propiece. HMW1 is modified by the HMW1C cytoplasmic N-glycosyltransferase. After export through the Sec YEG complex, the signal peptide is cleaved, and the propiece interacts with the POTRA domains of the HMW1B (*hmw1b*) translocon. HMW1 is exported through HMW1B and remains surface anchored, while the propiece is cleaved and released into the environment.

The final gene located in each locus, *hmw1C/hmw2C*, encodes a cytoplasmic N-linking glycosyltransferase, HMW1C and HMW2C. HMW1C and HMW2C modify nascently translated HMW1 and HMW2 in the NTHi cytoplasm. Modification occurs by sequentially adding UDP-glucose or UDP-galactose to multiple sites in a sequential

fashion so that each site is modified with either a mono- or di-hexose(Grass et al., 2003a). Glycans are added onto asparagine residues at the consensus site NX(S/T), where “X” indicates any amino acid except proline(Gross et al., 2008). The glycosylation of HMW1 and HMW2 is thought to play an important role in adhesin export, folding, stability, and function. Elimination of HMW1C results in degradation of HMW1, indicating that glycosylation is required for full adhesin stability. Moreover, unmodified HMW1 does not remain anchored to HMW1B and falls off the cell surface, suggesting that glycosylation is important for surface anchoring(Grass et al., 2003a; McCann & St. Geme, 2014b). As a result, expression of HMW1 without HMW1C eliminates HMW1-mediated adherence.

Other adhesins

In addition to the autotransporter proteins, NTHi expresses a variety of other protein surface adhesins that interact with a variety of host structures. These include the outer membrane proteins (OMPs) P5, P4, Protein E, and Opacity-associated protein A (OapA). OMP P5 is a fimbrial structure shown to be expressed by 100% of clinical NTHi isolates recovered from children with chronic otitis media. P5 interacts with human receptors CEACAM1 and ICAM-1 on epithelial cells(Hill et al., 2001; Novotny & Bakaletz, 2016). P5 has been shown to mediate adherence to bronchiolar epithelia and pneumocytes and facilitates nasopharyngeal colonization in chinchillas(Bookwalter et al., 2008; Euba et al., 2015). OMP P4 is a lipoprotein that interacts with ECM components to promote adherence, including laminin, fibronectin, and vitronectin(Su et al., 2016). Protein E is another membrane-bound lipoprotein which has been found in nearly all

NTHi strains. Protein E indirectly facilitates bacterial adherence to epithelial cells by first binding laminin and vitronectin(Hallströ et al., n.d.). OapA has been shown to drive adherence to Chang cells *in vitro* and contribute to colonization of infant rats *in vivo*(Prasadarao et al., 1999; Jeffrey N. Weiser et al., 1995).

1.2.2 Mechanisms of Persistence

Once NTHi establishes adhesive interactions with ECM and host respiratory epithelia, the organism employs several mechanisms to evade or disarm host immunity. Consequently, NTHi is able to exist as a commensal organism or alter the host environment to disseminate and cause disease.

Biofilm

As adherent NTHi aggregate on epithelial surfaces, biofilms begin to form. Biofilms are dense microbial communities existing in a matrix of extracellular polymeric substances such as extracellular DNA (eDNA), proteins, and polysaccharides. The extracellular polysaccharide matrix of NTHi biofilms is made up of LOS and related glycans containing phosphorylcholine, N-acetylneuraminic acid, and sialic acid moieties(Greiner et al., 2004; Hong et al., 2007). Biofilms *in vivo* have also been observed to contain double stranded eDNA and various outer membrane proteins, including pili, HMW1 and HMW2, Hap, P5, and P6(Jurcisek & Bakaletz, 2007; Webster et al., 2006). NTHi biofilm formation *in vivo* was first appreciated on the tympanostomy tubes of children with otitis media(Post, 2001). NTHi is now known to form biofilms on

middle ear mucosa and on airway epithelia of patients with COPD(Post, 2001; Starner et al., 2006). Biofilms containing NTHi have been detected in the adenoids of children with chronic otitis media(Hall-Stoodley et al., 2006; Hoa et al., 2010). Similarly, some evidence suggests that NTHi may form biofilms on lower airway tissue and form pulmonary biofilms in COPD(Pang et al., 2008). Taken together, these findings suggest that biofilm formation may contribute to prolonged NTHi infections and recurrent disease.

In a biofilm, bacteria are more effectively protected from various mechanisms of host recognition and clearance, contributing to NTHi persistence. The dense structure of the NTHi biofilm has been shown to prevent phagocytosis by immune cells and restrict antibody binding by creating steric hindrance and masking surface antigens(Langereis & Hermans, 2013). NTHi within a biofilm are also more resistant to clearance by neutrophil extracellular traps (NETs), such that NET material and dead neutrophils are incorporated into the biofilm to increase its mass(Hong et al., 2009; Juneau et al., 2011). Biofilm components such as eDNA further protect NTHi against neutrophils by binding and inhibiting the activity of cationic antimicrobial peptides(Jones et al., 2013). Further, the polysaccharide matrix dilutes concentrations of effector proteins to reduce their activity, including antibiotics used to treat NTHi infection(Slinger et al., 2006).

Intracellular invasion

While NTHi is classically considered an extracellular pathogen, it is also known to act as an opportunistic intracellular pathogen(J. W. St.Geme & Falkow, 1990). In addition, evidence suggests that NTHi can penetrate between host epithelial cells and

persist in intercellular spaces between airway cells, or undergo transcytosis to the subepithelial space(Van Schilfgaarde et al., 1999). Host cell invasion is thought to be an important mechanism for NTHi persistence and disease progression, as it allows NTHi to evade host immune factors and antibiotic interventions. Intracellular NTHi are thought to act as a reservoir of surviving bacteria, contributing to chronic infections. Intracellular NTHi have been detected in pulmonary tissue of patients with recurrent flares of COPD, in bronchial biopsies in patients with chronic bronchitis, and in adenoidal tissues of children with recurrent OM (Bandi et al., 2001; Ketterer et al., 1999). NTHi has been shown to invade and survive within a variety of cell types, including various epithelial cell types and macrophages(Craig et al., 2002).

Successful entry and survival within host cells involves multiple bacterial and host cell factors. Several NTHi surface adhesive proteins facilitate both adherence and internalization, including Hap, HMW1 and HMW2, and Protein E(Ahrén et al., 2001; Kenjale et al., 2009; Mell et al., 2016; Swords, Buscher, Ver Steeg Li, et al., 2000). Glycan surface structures also contribute to invasion- LOS interacts with the host platelet-activating factor receptor (PAFR) to facilitate entry, via phosphorylcholine moieties (ChoP) on LOS(Swords, Buscher, Ver Steeg Li, et al., 2000). The interaction of NTHi surface structures with host cells triggers changes in host cell signaling, initiating cytoskeletal rearrangements that result in NTHi internalization through PAFR-driven endocytosis or macropinocytosis[103,104]. Intracellular NTHi is thought to traffic through and between host cells to gain access to subepithelial compartments. Moreover, NTHi has been detected to survive within host lysosomes, suggesting the presence of mechanisms to withstand and neutralize hostile compartments(Clementi & Murphy, 2011).

Altered host cell signaling

During NTHi entry into the upper respiratory tract, the host is tasked with detecting the pathogen and mounting a rapid inflammatory response to minimize the chance for bacteria to colonize. Inflammatory responses result in increased antimicrobial activity that creates an unfavorable environment for bacterial survival. Typically, the binding of NTHi to host cell ligands including CEACAMs, Toll-like receptor (TLR) 2, TLR4, and ECM components will initiate proinflammatory signaling cascades(Mikami et al., 2005; Punturieri et al., 2006). Consequences include enhanced production of antimicrobial compounds, recruitment of phagocytic cells, and exfoliation of epithelial cells in order to rapidly reduce infectious burden and prevent stable colonization(Mulvey et al., 2000).

Some interactions of NTHi surface components with host cell ligands significantly alter canonical host cell signaling, dampening inflammatory responses and consequently promoting NTHi persistence. The binding of OMP P5 on the NTHi surface to host CEACAM1 promotes epithelial adhesion to ECM, combatting host cell shedding(Muenzner et al., 2005). The interaction between OMP P5 and host CEACAM triggers host cell expression of CD105, a growth factor receptor which that modulates integrin affinity to promote adherence(Muenzner et al., 2005). Similarly, NTHi binding to host cells activates the epidermal growth factor receptor (EGFR), which consequently downregulates activity of the p38 inflammatory kinase (Mikami et al., 2005). During NTHi internalization, the binding of ChoP on LOS with the host PAFR will further negatively

regulate TLR activation, reducing inflammation to promote intracellular survival(Erwin & Smith, 2007; Fukao & Koyasu, 2003).

Phase variation

Many NTHi virulence factors exhibit phase variation, allowing bacteria to combat pressure by the host immune system. Phase variation generates greater population diversity, creating more phenotypically distinct bacteria and promoting survival in different host environments(Moxon et al., 2006). Phase variation is often mediated by a number of simple sequence repeats (SSR) in promoters. SSRs lead to slipped-strand mispairing during DNA replication, resulting in altered numbers of SSRs, correlating with different amounts of gene expression(Poole et al., 2013; Power et al., 2009). The HMW1 and HMW2 adhesins exhibit phase variation due to 7-bp repeats in the *hmw1A* and *hmw2A* promoter regions; with increasing numbers of repeats, adhesin expression is reduced(Giufrè et al., 2008). Similarly, Hia expression is controlled by a variable polythymidine tract in the *hia* promoter(Atack et al., 2015). Pili generated by the *hif* locus vary due to TA repeats found within the *hifA* and *hifB* promoters(van Ham et al., 1993). Variation of LOS results from turning on and turning off different biosynthetic genes that contribute to the full molecule, generating LOS of different lengths and forms(Fox et al., 2014).

Phase variation aids NTHi in maintenance as a commensal in the nasopharynx or in the transition to disease. Clones expressing higher amounts of Hia are favored during nasopharyngeal colonization; however, in the presence of anti-Hia antibody, there is selection for organisms with reduced Hia expression(Atack et al., 2015). Similarly, on-

expression of the LOS modifying gene *licA* is associated with nasopharyngeal colonization rather than an invasive state(Poole et al., 2013). Certain LOS modifications, including terminal sialylation or addition of the oligosaccharide HepIII- β1,2-Glc, allow NTHi to evade opsonization by serum IgM and consequent neutrophil-mediated killing. Clinical isolates expressing this alternate form of LOS are therefore more often found to cause invasive disease, presumably due to enhanced survival in the bloodstream(Fox et al., 2014; Jackson et al., 2019).

Antigenic variation

Due to the high genetic heterogeneity among NTHi isolates, significant antigenic variation has also been observed for surface components. Antigenic differences in surface proteins further diversifies NTHi and allows for persistence despite significant host immune pressure. Antigen heterogeneity has been observed in many NTHi outer membrane proteins that interface with the host immune system, including OMPs P1, P2 and P5(Birgitta Duim et al., 1993; Hiltke et al., 2002, 2003; Munson et al., 1989). Amino acid changes in these proteins are often the result of point mutations, insertions and deletions, and high DNA recombination rates(S J Barenkamp et al., 1982). The most antigenic drift and resulting diversity occurs in immunodominant epitopes of proteins. Consequently, the host will generate highly strain-specific antibody responses, and these antibodies cannot mediate protection against a variant strain. This allows NTHi to evade complement-mediated killing and opsonization(Sethi et al., 2004; Troelstra et al., 1994). Antigenic drift can create reservoirs of diverse strains, allowing recurrent and prolonged infection to occur despite an efficient host immune response. Strains with

different OMP variants have been found simultaneously in the lungs of patients with cystic fibrosis and COPD, and exacerbations occur despite the development of anti-NTHi IgG and IgA(Staples et al., 2016). Several concurrent OMP P5 variants have been identified in patients with chronic bronchitis[120,121]. Similarly, in children with recurrent otitis media, different episodes are attributed to strains which have minor amino acid changes in OMPs and LOS structures(Timothy F Murphy et al., 1987).

IgA protease

NTHi expresses IgA proteases, a family of highly specific endopeptidases. IgA proteases classically function by cleaving human IgA_1, the most prevalent antibody expressed at human mucosal surfaces. Cleavage occurs at the antibody hinge region, separating the antigen-binding Fab domain from the Fc region. This cleavage promotes NTHi persistence by inhibiting antibody-driven antibacterial functions, including agglutination, opsonization, and inhibition of bacterial adherence to epithelia(Kilian et al., 1979; KILIAN et al., 1996).

Across strains, NTHi expresses four variants of IgA protease, encoded by an *igaA* or *igaB* locus. The resulting variants are A1, A2, B1, and B2(Timothy F Murphy et al., 2015). Nearly all strains have the *igaA* gene, and approximately 40% of strains also have *igaB*. These IgA proteases have been implicated in different pathogenesis functions, beyond IgA cleavage. IgaA1 and IgA2 are thought to facilitate adherence, invasion, and persistence; IgaB1 and IgaB2 do not influence adherence, but drive NTHi internalization into host cells. *IgaB* shares homology with genes encoding the type 2 IgA1 protease in *Neisseria*, which cleaves lysosome-associated membrane protein 1

(LAMP1) in host cells, disrupting endolysosome acidification and promoting intracellular survival in respiratory epithelial cells(Clementi et al., 2014; Lin et al., 1997; Timothy F Murphy et al., 2017).

Each of the four IgA protease variants is selectively expressed during NTHi infection(Timothy F Murphy et al., 2015). Phase variation within the *igaA* and *igaB* genes influences this selection, promoting survival of strains expressing different amounts through stages of NTHi colonization and disease(Gallo et al., 2018). Overall, expression of IgA protease is favored during stable nasopharyngeal colonization in humans, indicating that positive selection occurs to enable persistence(Poole et al., 2013). Strains expressing IgaB1 and IgaB2 are more often found among disease isolates, including those associated with otitis media and COPD, suggesting that selection for *igaB* may occur during disease progression rather than commensal behavior(Fernaays et al., 2006; Gallo et al., 2018).

1.2.3 Bacterial Competition

The upper respiratory tract houses a diverse range of microbes. Bacteria colonizing the upper respiratory tract face stress from the host environment as well as co-colonizing members of the microflora. Other commensals of the nasopharynx include other *Haemophilus* species, as well as *Streptococcus, Staphylococcus, Moraxella,* and *Neisseria* species(Biesbroek et al., 2014; Robinson, 2004). Competitive interactions with other commensal species and opportunistic pathogens can restrict the ability of NTHi to establish stable colonization. *Haemophilus haemolyticus*, a nonpathogenic commensal, restricts the ability of NTHi to adhere to respiratory epithelial cells *in vitro*(J. L. Pickering

et al., 2016). *H. haemolyticus* further restricts NTHi by producing haemophilin, a heme-sequestering compound that allows *H. haemolyticus* to limit NTHi heme acquisition. NTHi growth is then limited in the presence of *H. haemolyticus*(Atto et al., 2020). NTHi faces similar competition from resident *S. pneumoniae*, which may compete with NTHi for use of the PAFR to anchor to host epithelial cells(Cundell et al., 1995). *Streptococcus* also directly interferes with NTHi adherence by using neuraminidase to desialyate the NTHi LOS, limiting interactions with host epithelium(Shakhnovich et al., 2002). *S. pneumoniae* also creates a restrictive environment for NTHi through production of bactericidal concentrations of hydrogen peroxide(Pericone et al., 2000).

Conversely, some polymicrobial interactions enhance NTHi survival within the respiratory tract. *Moraxella catarrhalis*, for example, has been observed to form polymicrobial biofilms with NTHi. This biofilm can shield NTHi from complement-mediated killing and protects NTHi from peroxide production by *S. pneumoniae*(Bair & Campagnari, 2020; Tan et al., 2007).

1.2.4 Progression to Disease

Following the establishment of a stable colonizing population, NTHi is maintained in the upper respiratory tract. However, colonization is as a delicate balance between commensal and infectious states. Changes to the host immune environment, including immunodeficiencies in the host, allow NTHi to act as an opportunistic pathogen and disseminate to sites of localized disease. From the nasopharynx, NTHi can ascend up the Eustachian tube to infect the middle ear, move through the airways to infect the sinuses or lungs, or transverse epithelia to cause invasive disease. Generally, localized

respiratory tract disease, including otitis media and pneumonia, is preceded by increases in NTHi density in the nasopharynx(Chochua et al., 2016; Smith-Vaughan et al., 2006). Different conditions promote NTHi outgrowth, increasing the likelihood of disease progression.

Viral infection

In many instances, disseminated NTHi disease is preceded by a viral infection. Acute otitis media is frequently associated with upper respiratory viral infection, including infection caused by respiratory syncytial virus (RSV), influenza, adenovirus, or parainfluenza(Ruuskanen et al., 1989). Viral-induced predisposition to increased NTHi density and more severe otitis media has been demonstrated in animal models as well. In chinchillas, preceding adenoviral infection results in greater middle ear inflammation after NTHi inoculation(Suzuki & Bakaletz, 1994).

Upper respiratory viral infection promotes NTHi pathogenicity by altering the inflammatory state of the host and compromising host protective functions. Influenza-A viral infection has been shown to directly damage ciliated cells of the host respiratory epithelium, impairing mucociliary clearance in the Eustachian tube and middle ear, suggesting that infection may support NTHi binding and promote subsequent bacterial ascension from the nasopharynx(Carson et al., 1985; Park et al., 1993; Pittet et al., 2010). In chinchillas, RSV infection impairs β-defensin production in the upper respiratory tract, resulting in impaired NTHi clearance after inoculation(Bakaletz, 2010; McGillivary et al., 2009). Similarly, inflammation due to viral infection results in reduced neutrophil recruitment and oxidative burst activity and reduced macrophage activity,

leading to greater bacterial density in the airways and greater susceptibility to infection(McNamee & Harmsen, 2006; Sun & Metzger, 2008). Inflammation in the upper respiratory tract also results in upregulation of host surface proteins, which serve as receptors for NTHi adhesive interactions. During RSV infection, epithelial cells show increased surface expression of CEACAM1, ICAM, and PAFR(Avadhanula et al., 2006; Jiang et al., 1999). More recent studies suggest that infecting viruses may also directly interact with NTHi and contribute to pathogenesis. In *in vitro* studies, NTHi interacts with influenza viral particles, and these viral-bacterial particles exhibit greater adhesion to epithelial cells than bacteria alone(Rowe et al., 2019).

Host inflammation

The host immune response is important for modulating NTHi in the upper respiratory tract. However, the inflammatory responses generated in response to NTHi can also be deleterious to the host and contribute to the development of associated disease. In animal models of COPD, NTHi-induced activation of interferon production in macrophages results in cytokine production that diminishes bacterial clearance and increases lower lung inflammation(Yang et al., 2019). In infants, colonization with NTHi contributes to early immune system reprogramming and the development of asthma(Essilfie et al., 2011; McCann et al., 2016). Recent studies in neonatal mice have shown that nasopharyngeal colonization with NTHi during early life resulted in more severe airways disease, including increased infiltration of immune effector cells neutrophils, eosinophils, and inflammatory cytokines into the airways, coupled with

decreased expression of T-regulatory cells. The result in juvenile mice was more severe neutrophilic asthma, even after NTHi clearance(McCann et al., 2016).

1.3. Prevention or Nontypeable Haemophilus influenzae disease

Treatment of NTHi disease, including otitis media, pneumonia, sinusitis, and invasive disease, is primarily limited to antibiotic therapy. Antibiotic courses are often successful at eliminating disease; however, morbidity due to NTHi remains significant. Recurrent infections are common among children, and following repeated episode of acute otitis media, accumulated fluid can remain in the middle ear for months, causing hearing impairment that can result in deficiencies in language acquisition and speech development(Haapala et al., 2014, 2016; Kaspar et al., 2018). Repeated courses of antibiotics to treat NTHi infections may also select for antibiotic-resistant organisms. Along these lines, the prevalence of resistance to ampicillin has increased over time(Yanagihara et al., 2019). In young children, recurrent otitis media with effusion can also be treated by adenoidectomy and insertion of a tympanostomy tube, removing the nasopharyngeal reservoir for NTHi(Mattila et al., 2003). While the surgical procedure is effective, it places children at risk for significant complications.

Currently, vaccination is only available against type b encapsulated *H. influenzae* (Hib), which used to be a common cause of Hib invasive disease, such as meningitis and sepsis. Hib vaccines are conjugate vaccines, using purified capsule polysaccharide conjugated to an immunogenic carrier protein. Consequently, these vaccines afford no protection against nontypeable strains. The only NTHi protein used in a licensed vaccine application to date is protein D, which is used as an immunogenic carrier protein in the 10-valent pneumococcal conjugate vaccine Synflorix (GlaxoSmithKline), which targets

the *S. pneumoniae* polysaccharide capsule. Since the introduction of Synflorix in 2008, studies have reported modest reductions in NTHi acute otitis media. However, there has been no significant impact on nasopharyngeal carriage of NTHi(Clarke et al., 2017; Amanda Jane Leach et al., 2015; Van Den Bergh et al., 2012). Presently, there are no treatment or vaccine strategies directed at NTHi, including vaccines that prevent nasopharyngeal colonization. This represents a significant need and an active area of research in NTHi disease.

1.3.1 NTHi vaccine development

Vaccine approaches

Efforts to develop a vaccine against NTHi have thus far primarily focused on preventing otitis media and COPD. The high genetic diversity among NTHi isolates and prevalence of antigen heterogeneity pose significant challenges to identifying effective vaccine antigens. Ideal vaccine antigens are immunogenic in humans during NTHi infection and capable of stimulating a robust immune response during immunization. Historically, the presence of NTHi-specific antibodies has been considered a correlate of protective immunity. Moreover, candidate antigens are typically surface exposed and identified as important for bacterial infection, as antibodies targeting these antigens are more likely to mediate antibacterial functions at mucosal surfaces, including opsonization, restriction of bacterial adherence, and agglutination(Cerutti et al., n.d.; Roche et al., 2015).

Many children develop convalescent antibody directed at NTHi outer membrane proteins following acute otitis media, and these antibodies can often mediate bactericidal activity(Kaur et al., 2011; Khan et al., 2012). Moreover, colonization with NTHi is considered an immunizing event, as children develop natural antibody against NTHi components even without acute otitis media. Naturally-acquired antibodies during colonization and infection, especially against conserved targets, can contribute to a reduced colonization density(Zola et al., 2009). However, despite the presence of these antibodies, children are not completely protected and still experience infection with new strains. The antibodies that typically arise during epitopes of acute otitis media are typically highly strain specific even though they are reactive against conserved OMPs(Bernstein et al., 1992; Shurin et al., 1980). As a result, the developed antibody response is typically protective against recurrent infection by the same strain but does not grant broad immunity.

Increasingly, cell-mediated immunity is being identified as a driver of protection in the airways. Emerging evidence implicates that Th17 cells and the resulting production of IL-17 mediate immunity to extracellular bacteria at exposed mucosal surfaces including the airways, skin, and gut. Th17 cells, first characterized in 2005, are a subset of $CD4^+$ effector T-cells that develop as a lineage distinct from Th1 and Th2 lymphocytes(Harrington et al., 2005). Production of IL-17 at mucosal surfaces stimulates production of antimicrobial peptides, chemotactic factors, and granulopoetic factors. This results in increased recruitment of macrophages and neutrophils and enhanced cytotoxicity and phagocytosis(Hoshino et al., 2000; Kao et al., 2004; Lu et al., 2008). IL-17 production has been implicated in host defense against various mucosal pathogens including *Streptococcus pneumoniae* and *Bordetella pertussis*(Archer et al., 2016;

Higgins et al., 2006). Th17 cells are thought to be important in driving natural immunity as well. People with mutations impacting Th17 development and IL-17 function experience greater susceptibility to colonization and infection by mucosal pathogens(J. Chen et al., 2010; Milner et al., 2008). Similarly, Th17 memory developed after mucosal exposure to *S. pneumoniae* mediates protection against re-colonization in mice, as neutralization of CD4 or IL-17A abrogates protection(Malley et al., 2005). Generation of Th17 memory and production of a robust IL-17 may therefore be important for protection against nasopharyngeal colonization by NTHi.

Immunization with Th17 antigens is increasingly of interest in vaccine design. Unlike antibodies, Th17 immune memory can contributes to protective immunity against highly variable antigens. Variable antigens often lead to variant-specific antibody responses due to changes in conformational epitopes. However, the presence of common major histocompatibility complex class (MHC-II) epitopes can result in conserved Th17 responses, driving protective immunity against heterologous strains(Kuipers et al., 2017b). Generation of Th17 immunity therefore allows for protective immunity independent of antibody-driven specificity and has been shown to be effective against heterologous strains of *Streptococcus* and *Klebsiella*(K. Chen et al., 2011; Kuipers et al., 2017a). Recent work has also shown that intrapulmonary immunization with Th17 antigens identified in NTHi can stimulate immunity against direct lower airways infection by heterologous strains of NTHi, independent of antibody reactivity, raising the potential that stimulation of cellular immunity may be an important consideration for developing a vaccine(W. Li et al., 2018).

Candidate vaccines

Several NTHi surface antigens have been explored as vaccine antigens and evaluated for their impact on nasal colonization. Because protein D has shown a potential impact on otitis media in the pneumococcal conjugate vaccine, it has been highly investigated as an NTHi-specific vaccine antigen. Although protein D is not directly involved in adherence, it is surface exposed, and its phosphodiesterase activity supports adherence and invasion(Swords, Buscher, Ver Steeg Ii, et al., 2000). Moreover, the ciliotoxicity of protein D makes it an important virulence factor in the progression of otitis media. Protein D is highly conserved and found in both encapsulated and nontypable strains of *H. influenzae*(Birgitta Duim et al., 1997). The immunogenicity of protein D has also been well-studied. During experimental NTHi otitis media in rats, protein D was identified as an antibody target of systemic IgG and IgA antibodies(Akkoyunlu & Forsgren, 1996). Immunization with recombinant protein D, by either systemic or mucosal routes, has also been shown to stimulate antibody responses in rats, mice, and chinchillas(Akkoyunlu & Forsgren, 1996; Behrouzi et al., 2016; LO et al., 1999). Further, the resulting antibody response has demonstrated anti-NTHi activity in opsonophagocytosis assays *in vitro*, and passive transfer of anti-protein D antibody to chinchillas enhances protection against otitis media *in vivo*(LO et al., 1999; Motlagh et al., 2016). Importantly, the effects of protein D abrogation *in vivo* have only been observed in the middle ear, with no effects on adherence and fitness in the nasopharynx in chinchilla models(R. W. Johnson et al., 2011). Similar observations have recently been made in mouse models of NTHi acute otitis media, in which immunization with protein D reduces bacterial loads in middle ear washes and ear bullae, but not in the nares(Michel et al., 2018). This discrepancy in protection suggests that a protein D-targeted immune response may only be valuable in the context of the middle ear, rather than mitigating the preceding nasopharyngeal colonization.

The OMP P5 fimbrial structure is another highly conserved outer membrane component and is found in nearly 100% of NTHi otitis media isolates(Novotny & Bakaletz, 2013). P5 exhibits high variability in surface-exposed regions, with variant P5-carrying strains appearing during chronic bronchitis and across anatomical sites(B Duim et al., 1997). Serum antibodies against P5 have been identified in children recovering from NTHi acute otitis media(Novotny et al., 2002). However, the development of an antibody response in children is highly variable, and this may be due to the P5 variances across infecting strains(Bronson et al., 1997). Antibodies directed against immunodominant epitopes of P5 are poorly protective against experimental otitis media, though protection has been observed for antibody against subdominant epitopes(Novotny & Bakaletz, 2003). In chinchillas, immunization with a synthetic P5-derived peptide, LB1, which contains these protective subdominant epitopes, results in antibody capable of promoting nasopharyngeal clearance of the homologous strain(Kennedy et al., 2000). Protection against heterologous strains conferred by LB1 immunization has thus far only been observed after conjugation to a T-cell promoting carrier(Kyd et al., 2003).

P6 is another significant peptidoglycan-associated lipoprotein that is thought to be an important NTHi antigen and a promising candidate. P6 is found in nearly all strains of *H. influenzae*, with highly conserved epitopes across isolates(Green et al., 1990; T. F. Murphy et al., 1986). Following nasal colonization with NTHi or acute otitis media, children develop serum IgG that is directed at P6 and capable of enhancing bactericidal activity against NTHi, suggesting that P6 is highly immunogenic during natural infection(Pichichero et al., 2010). Further, children who develop lower levels of anti-P6 antibody are more susceptible to otitis media, suggesting an important role for a P6

immune response(Bernstein et al., 1997). In mouse studies, immunization with P6 stimulates IgA and IgG, and improves nasopharyngeal clearance 3 days after NTHi infection with the homologous strain(Hotomi et al., 2002). This protective immunity is thought to be due to a combination of antibody and Th17 cells induced by immunization, though it is unclear whether mice are protected against heterologous strains(Noda et al., 2011).

Protein E (PE), another ubiquitous NTHi adhesin, has been considered due to its high conservation and vital interactions with host ECM. Intranasal immunization with PE, as a fusion protein with the type IV pilus component PilA, stimulates an antibody response that is capable of dispersing NTHi biofilms and interfering with PE-vitronectin interactions(Novotny et al., 2015; Ysebaert et al., 2019). While PE is immunogenic, the PilA subunit is poorly immunogenic and unable to mediate protective immunity on its own. The PE-PilA fusion vaccine is able to reduce bacterial burden in the murine nasopharynx by a heterologous strain 1 or 2 days after inoculation(Ysebaert et al., 2019).

Hap has been of great interest as a vaccine antigen because its expressed by all NTHi isolates and is an adhesin involved in key steps of nasopharyngeal colonization, including interactions with epithelial cells and microcolony formation. Hap is well-conserved among isolates, as sequences from differing strains are about 80% similar to each other. Purified Hap_S, the adhesive component of Hap, is highly immunogenic in mice, and the resulting antibodies are capable for interfering with NTHi Hap-mediated adherence to epithelial cells. Moreover, mice immunized with Hap_S are protected against nasopharyngeal colonization by a heterologous NTHi strain 3 days after inoculation(Cutter et al., 2002). Though immunized mice in these studies developed

robust antibody, greater antibody titers did not correlate with increased levels of protection, suggesting that the mechanism of this protection is yet to be defined.

Though Hia is only expressed by 25% of NTHi isolates, it is a critical adhesin for the subset of strains that do not express HMW1 and HMW2 and therefore under consideration as a vaccine candidate. During nasopharyngeal colonization, humans develop Hia antibodies, and these antibodies mediate bactericidal activity against Hia-expressing NTHi strains(Nix et al., 2015). In chinchillas, immunization with recombinant Hia results in serum containing Hia-specific IgG that enhances opsonophagocytosis of the homologous strain and heterologous strains; however, the opsonophagocytic titer for the homologous strain was significantly higher than the titer for heterologous strains, suggesting some degree of strain-specificity(Winter & Barenkamp, 2009, 2014). No studies to date have immunized animals solely with Hia, presumably due to the low percentage of isolates expressing the adhesin. However, engineered outer membrane-vesicles expressing Hia in combination with other outer-membrane adhesins stimulate antibody in chinchillas capable of broadly enhancing opsonophagocytosis and afford protection against experimental otitis media(Winter & Barenkamp, 2017).

Like Hia, HMW1 and HMW2 are of interest due to their importance in facilitating adherence. Moreover, because there is direct evidence that HMW1 and HMW2 facilitate nasopharyngeal colonization in rhesus macaques, they are very likely to drive colonization in humans and are therefore very promising as vaccine targets(Rempe et al., 2016). HMW1 and HMW2 are highly immunogenic during colonization and infection. In children recovering from acute otitis media caused by NTHi, HMW1 and HMW2 are immunodominant targets of convalescent sera(Stephen J. Barenkamp & Bodor, 1990). Similarly, rhesus macaques colonized with HMW-expressing strains develop antibody

responses against HMW1 and HMW2, and antibodies are present in adults, suggesting that nasal colonization is an immunizing event(Rempe et al., 2016). The development of HMW-specific antibodies in humans also coincides with increased serum bactericidal activity(Stephen J. Barenkamp & Bodor, 1990). However, enhancement of opsonophagocytosis appears to be primarily against the homologous infecting strain, rather than heterologous strains(Winter & Barenkamp, 2016). Immunization with a combination of purified HMW1 and HMW2 is protective in the chinchilla model of acute otitis media(S J Barenkamp, 1996).

1.4. Thesis Overview

Chapter 2 further explores the capacity of the HMW1 and HMW2 adhesins to serve as vaccine antigens, with a focus on preventing nasopharyngeal colonization. This work explores the significance of intranasal immunization versus subcutaneous immunization, and then characterizes the systemic antibody and cell-mediated immune responses generated in mice after immunization. Chapter 3 investigates the determinants of immunogenicity of HMW1 and HMW2 and evaluates important immunological and functional differences between the two adhesins. Chapter 4 broadens the current mechanistic understanding of HMW1C-driven glycosylation of HMW1. Chapter 5 summarizes the implications of this work and explores future directions.

2. IMMUNIZATION WITH THE HMW1 AND HMW2 ADHESINS PROTECTS AGAINST COLONIZATION BY HETEROLOGOUS STRAINS OF NONTYPEABLE HAEMOPHILUS INFLUENZAE

2.1. Introduction

Nontypeable (nonencapsulated) *Haemophilus influenzae* (NTHi) is a common cause of acute otitis media (AOM), otitis media with effusion, sinusitis, and exacerbations of underlying lung disease in children (Timothy F. Murphy et al., 2009). NTHi is also frequently associated with community-acquired pneumonia and exacerbations of chronic obstructive pulmonary disease (COPD) in adults (Sethi et al., 2002).

The pathogenesis of NTHi disease begins with colonization of the nasopharynx. Colonization occurs early in life and is common throughout childhood and into adulthood (Trottier et al., 1989). NTHi spreads contiguously within the respiratory tract to cause localized disease, typically in the setting of a viral respiratory infection or allergic disease (Amanda J. Leach et al., 1994; Rao et al., 1999; Smith-Vaughan et al., 2006). Recent evidence indicates that colonization in early infancy may predispose to development of neutrophilic asthma and allergic airway disease (McCann et al., 2016). Given the burden of NTHi disease and the association between early life NTHi colonization and asthma, there is interest in developing a vaccine against NTHi.

Currently, vaccines against *H. influenzae* are limited to polysaccharide conjugate vaccines targeting the capsule of type b strains (Hib). While these vaccines have been largely successful in eliminating Hib invasive disease, there has been no effect on strains of NTHi, which lack a capsule. Efforts to identify highly conserved antigens in NTHi and develop a vaccine against NTHi have been significantly more difficult. While

Hib and other encapsulated strains of *H. influenzae* are clonal, NTHi strains exhibit much greater genetic diversity and heterogeneity (Pinto et al., 2019). Across NTHi strains, common surface antigens exhibit high degrees of antigenic variation, resulting in variable and strain-specific antibody responses. In studies of mice, antibodies acquired during NTHi infection, including IgG in nasal washes directed against outer membrane proteins, are associated with reduced nasopharyngeal colonization density following subsequent challenge by NTHi (Zola et al., 2009). This suggests a protective role for antibodies at the mucosal surface. However, evidence in children indicates that despite the presence of antibody against an infecting strain, children remain susceptible to subsequent infections by new strains, suggesting that the antibody response may only protect against infection by the same strain.

In combination with antibody responses, Th17 cells are being increasingly recognized as key mediators of protection against airway infection by respiratory pathogens. Production of IL-17A mediates many Th17 effector functions at mucosal surfaces, including production of antimicrobial peptides, proinflammatory cytokines, chemotactic factors, and granulopoietic factors. These effector functions result in increased recruitment of macrophages and neutrophils and enhanced cytotoxicity and phagocytosis (Hoshino et al., 2000; Kao et al., 2004; Lu et al., 2008). There is also evidence that Th17 cells drive humoral immunity, including generation of antibody in mucosal secretions (Cao et al., 2012; Jaffar et al., 2009). IL-17 production is known to be essential for defense against various mucosal pathogens, including *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae,* and *Bordetella pertussis* (Archer et al., 2016; Higgins et al., 2006; Wu et al., 2007). In studies of *S. aureus*, clearance of bacteria colonizing the nasopharynx is driven by Th17 responses, including IL-17-mediated neutrophil influx and antimicrobial peptide production (Archer et

al., 2013, 2016). Importantly, protection studies in *S. aureus* suggest that IL-17 driven protection is largely serotype independent. Thus far, it is unclear whether IL-17 production directly contributes to host defense against nasopharyngeal colonization by NTHi.

NTHi colonization of the nasopharynx begins with bacterial adherence to respiratory epithelial cells, which is dependent on NTHi adhesive proteins. The HMW1 and HMW2 high molecular weight proteins are surface-exposed glycoproteins and are the predominant adhesins in approximately 75-80% of NTHi strains (S J Barenkamp & Leininger, 1992; Buscher et al., 2004; Grass et al., 2003b; Joseph W. St. Geme et al., 1993; Joseph W. St Geme et al., 1998). HMW1 and HMW2 also facilitate upper respiratory tract colonization in rhesus macaques, as highlighted in studies comparing a wild type strain and an isogenic mutant lacking both of these proteins (Rempe et al., 2016). HMW1 and HMW2 are highly homologous to each other, sharing approximately 70% identity and 80% similarity. HMW1 and HMW2 are also highly homologous among diverse NTHi strains (Davis et al., 2014; Shahini Shams Abadi et al., 2016).

In children recovering from acute otitis media, HMW1 and HMW2 are the major targets of the serum antibody response to infection. Similarly, rhesus macaques colonized with HMW1/HMW2-expressing strains develop serum antibody responses against HMW1 and HMW2, and serum antibodies against HMW1 and HMW2 are present in adults, suggesting that these adhesins are highly immunogenic. The development of HMW1/HMW2-specific antibodies in humans also coincides with increased serum bactericidal activity (Stephen J. Barenkamp & Bodor, 1990). However, antibody-mediated killing appears to be directed primarily against the homologous infecting strain, rather than broadly-acting against heterologous strains (Winter & Barenkamp, 2016). The conservation of HMW1 and HMW2 among diverse strains, the

immunogenicity of HMW1 and HMW2, the surface localization of HMW1 and HMW2, and the role of HMW1 and HMW2 in adherence to respiratory epithelial cells and in colonization make these adhesins promising antigens for a vaccine.

In this study, we evaluated whether immunization with HMW1 and HMW2 protects against nasopharyngeal colonization. Using a mouse model of immunization and nasopharyngeal challenge, we found that immunization with the HMW1 and HMW2 proteins results in protection against colonization by homologous and heterologous strains, despite a highly strain-specific antibody response. Protection against nasopharyngeal colonization is mediated by both antibody and T-cell responses.

2.2 Materials and Methods

2.2.1. Bacterial Strains and Culture

The bacterial strains used in the present study are described in Table 2.1. NTHi strain 12 is the prototype strain from which the *hmw1* and *hmw2* loci were first cloned and from which the HMW1 and HMW2 proteins were first characterized. NTHi strains were grown on chocolate agar (BD Biosciences, San Jose, CA, USA) or brain heart infusion (BHI) agar supplemented with 0.1% (vol/vol) lysed horse blood as a source of hemin and 3.5 μg/ml NAD (BHIs), with 500 μg/ml streptomycin as appropriate. Agar plates were incubated overnight at 37°C with 5% CO_2. Spontaneous streptomycin-resistant derivatives of NTHi were generated by spreading a dense bacterial suspension onto agar plates containing 500 μg/ml streptomycin and then recovering survivors. For each set of isogenic strains, growth studies determined that the parent and derivative strain had similar growth rates. In preparation for intranasal inoculation, NTHi strains

were resuspended in BHIs broth to an optical density at 600 nm (OD_{600}) of 0.2. Cultures were grown shaking at 250 rpm to an OD_{600} of 0.8, pelleted, washed with phosphate-buffered saline (PBS) (Lonza Biologics, Basel, Switzerland), and resuspended in PBS.

Table 2. 1: Bacterial strains used in Chapter 2

Strain	Description	Source or reference
12	Nontypeable *Haemophilus influenzae*, expresses $HMW1_{12}$ and $HMW2_{12}$	Clinical isolate, middle ear fluid, acute otitis media
12*hmw1hmw2*	12 *hmw1::kan hmw2::kan*, expresses neither $HMW1_{12}$ nor $HMW2_{12}$	(Joseph W. St. Geme et al., 1993)
12*hmw1*	12 *hmw1::kan*, expresses only $HMW2_{12}$	(Joseph W. St. Geme et al., 1993)
12*hmw2*	12 *hmw2::kan,* expresses only $HMW1_{12}$	(Joseph W. St. Geme et al., 1993)
5	Nontypeable *Haemophilus influenzae*, expresses $HMW1_5$ and $HMW2_5$	Clinical isolate, middle ear fluid, acute otitis media
5*hmw1hmw2*	5 *hmw1::kan hmw2::kan*, expresses neither $HMW1_5$ nor $HMW2_5$	(Joseph W. St. Geme et al., 1993)
5*hmw1*	5 *hmw1::kan*, expresses only $HMW2_5$	(Joseph W. St. Geme et al., 1993)
5*hmw2*	5 *hmw2::kan,* expresses only $HMW1_5$	(Joseph W. St. Geme et al., 1993)
15	Nontypeable *Haemophilus influenzae*, expresses $HMW1_{15}$ and $HMW2_{15}$	Clinical isolate, middle ear fluid, acute otitis media
NT127	Nontypeable *Haemophilus influenzae*, expresses neither HMW1 or HMW2 and instead expresses the Hia adhesin	Clinical isolate, blood, meningitis (Provided by Hao Shen, University of Pennsylvania)

2.2.2. Cell lines

The cell lines used in this study were obtained from the American Tissue Culture Collection (ATCC). Cells were maintained at 37°C with 7.5% CO_2. Chang cells (human conjunctiva; ATCC CCL-20.2) were maintained in Eagle minimal essential media (MEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% nonessential amino acids (NEAA) and 10% fetal calf serum (FCS). HaCaT cells (derived from human keratinocytes)(Boukamp et al., 1988) were maintained in MEM + 0.1% NEAA + 10% FCS.

2.2.3. Purification of HMW1 and HMW2

HMW1 and HMW2 were purified from the NTHi surface as described previously (Rempe et al., 2016). NTHi mutants expressing HMW1 only or HMW2 only were inoculated into 10 liters of BHIs broth and grown 12-14 hours shaking at 37°C. The culture was then centrifuged for 20 min at 8000 rpm, the supernatant was discarded, and the pellet was frozen at −80°C overnight. To release HMW1 or HMW2 from the bacterial surface, the frozen pellet was thawed on ice and resuspended in 200 ml of extraction buffer (0.5 M NaCl, 10 mM EDTA, 10 mM Tris pH 7.5, 50 μM 1,10-phenanthroline) in the presence of protease inhibitors (Roche, Basel, Switzerland). The resuspended bacteria were incubated at 4°C for 1 hr and then centrifuged for 15 min at 8500 rpm. The supernatant containing the released HMW1 or HMW2 was saved, and the pellet was discarded. The supernatants were dialyzed overnight into 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), 75mM NaCl pH 6.0 and then stepwise dialyzed

in MES with 250mM NaCl, in MES with 125mM NaCl, and then in 80mM NaCl. Dialyzed protein was loaded onto a Resource S cation exchange chromatography column (GE Life Sciences, Marlboro, MA, USA). Bound protein was eluted with 20 mM MES, 1 M NaCl pH 6.0. Fractions containing HMW1 or HMW2 were combined, concentrated, and then loaded onto a HiLoad SuperDex 16/60 200pg size-exclusion column (GE Life Sciences) equilibrated with 20 mM MES, 150 mM NaCl pH 6.0, 5% glycerol. The fractions containing HMW1 or HMW2 were pooled, resolved on SDS-PAGE gels, and stained with Coomassie blue to ensure purity.

2.2.4. Antibody measurement by ELISA

Purified protein (2.5 μg/ml in 0.1 M carbonate buffer, pH 9.6) was coated onto 96-well plates by incubation at 4°C overnight. Plates were washed with PBS-0.1% Tween 20 and blocked with 2% nonfat milk in PBS. Serial dilutions of mouse sera were applied to wells in triplicate. The plates were incubated at 37^{o}C for 1 hr. Mouse IgG was detected using a 1:2000 dilution of rabbit anti-mouse IgG conjugated to horseradish peroxidase (Sigma). For detection, the wells were incubated with 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) ELISA substrate (Rockland, Pottstown, PA, USA), and absorbance was recorded at 655 nm.

2.2.5. Antibody measurement by flow

NTHi strains were inoculated into 3mL of BHIs broth at OD_{600} = 0.200 and grown shaking at 37°C until OD_{600} = 0.800. Bacteria were then spun down and resuspended in 1X PBS to OD_{600} = 0.600. Bacteria were subsequently fixed in 1X TBS+1%

formaldehyde for 1 hr, then incubated with the appropriate immune serum. Following incubation, bacteria were washed in 1X PBS and incubated with fluorescent anti-mouse IgG (Rockland) for 1 hr, washed. Bacterial suspensions were brought up to 1mL total volume and stained with propidium iodide (PI) to identify bacteria. Fluorescence was analyzed using a BD Accuri C6 flow cytometer and BD Accuri C6 plus software (BD Biosciences). 50,000 PI+ events were recoded per sample.

2.2.6. Bacterial Adherence

Quantitative adherence assays were performed as described previously (Rempe et al., 2016). Approximately $1.8x10^5$ cells were seeded into 24-well tissue culture plates and incubated overnight. Epithelial monolayers were inoculated with ~$2x10^7$ CFU of NTHi in the presence or absence of immune serum, and the plates were centrifuged at 165 x *g* for 5 min to facilitate contact between the bacteria and the epithelial cells. After incubation for 30 min at 37°C in a 5% CO_2 atmosphere, monolayers were rinsed with PBS to remove non-adherent bacteria. Trypsin-EDTA (0.25% trypsin, 0.5% EDTA) (Sigma) was added to the wells to release epithelial cells and adherent bacteria. Dilutions of adherent organisms were plated on BHIs agar and incubated overnight to determine the number of adherent bacteria per monolayer. To image epithelial cell monolayers, cells were seeded onto glass coverslips. Following inoculation and incubation with bacteria, nonadherent bacteria were washed away with PBS, and monolayers were stained with Giemsa.

2.2.7. Animal Immunizations

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Children's Hospital of Philadelphia. Groups of 6-8 week-old female BALB/c mice were immunized intranasally or subcutaneously with purified HMW1 and HMW2. For subcutaneous immunizations, mice were given 10 µg of purified protein diluted in PBS, conjugated to a suspension of Imject alum as an adjuvant (Thermo Scientific, Waltham, MA, USA). Subcutaneous immunizations were given in a 100 µL volume (Cutter et al., 2002). Intranasal immunizations were performed as described by Cutter *et al,* with purified protein diluted in PBS to a concentration of 15 µg/40 µL with 0.1 µg *E. coli* labile toxin mutant LT(R192G/L211A) (dmLT) as a mucosal adjuvant (S. Leach et al., 2012). Purified dmLT was received as a gift from Dr. John Clements at Tulane University (Clements & Norton, 2018). Prior to intranasal immunization, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (75 mg/kg + 7.5 mg/kg body weight). Intranasal immunizations were delivered by pipetting 20 µL directly into each nostril. Following immunization, mice were placed in a supine position for 3-5 mins. Mice were immunized on weeks 0, 1, 3, and 5. Two weeks following the final immunization, blood was collected by terminal cardiac puncture, and serum was isolated from whole blood.

2.2.8. Intranasal Challenge

Two weeks following the final immunization, mice were challenged intranasally with ~$1x10^8$ CFU of streptomycin-resistant NTHi in 10 µL. Mice were anesthetized as

described for intranasal immunization, and 5 µL of the bacterial suspension was delivered into each nostril. Following inoculation, mice were placed in a supine position for 3-5 min. Three days following challenge, animals were sacrificed. Nasal washes were collected by inserting a catheter into the trachea and flushing PBS through the nose. Nasopharyngeal tissue was harvested, weighed, homogenized, diluted, and plated. Recovered NTHi was plated onto BHIs-agar containing 500 µg/mL streptomycin and incubated at 37°C for 48 hours to determine colony counts.

2.2.9. Serum Transfer

For the passive transfer of serum, naïve mice were injected intravenously with 200 µL of pooled serum from immunized donor animals. Mice were challenged intranasally with NTHi 24 hr following the transfer. 3 days following challenge, mice were sacrificed to determine nasopharyngeal colonization density.

2.2.10. Antibody-mediated *in vivo* depletion of IL-17A

Mice were immunized as described. Two weeks following immunization, mice were given monoclonal anti-IL-17A antibody (clone 1F17) or the IgG isotype control antibody (clone MOPC-21) (BioXCell, Lebanon, NH, USA). Antibody was administered in a 1mL intraperitoneal injection at a concentration of 0.500 mg/mL in sterile PBS. Mice were given antibody 1 day prior to intranasal challenge with NTHi (day -1). At 3 dpi, mice were sacrificed to determine colonization density, as described.

2.2.11. Agglutination

Bacteria were resuspended from chocolate agar plates in 3mL BHIs to OD_{600}= 0.200, grown to OD_{600}= 0.800, then resuspended in 1X PBS. A 100 µL volume of the bacterial suspension was incubated with pooled, heat-inactivated serum for 1hr at room temperature. Bacteria were then fixed by adding formaldehyde to a final concentration of 1% and incubating at room temperature for 1hr. Following fixation, the samples were stained with crystal violet for imaging or diluted in PBS for analysis by flow cytometry.

2.2.12. Measurement of agglutination by flow cytometry

Bacteria were fixed and stained with propidium iodide to gate on bacterial cells. Bacterial populations were then evaluated in the forward scatter (FSC-A) and side scatter (SSC-A) dot plot to measure aggregate size, and the FSC-A count plot to determine aggregate quantity (Habets et al., 2017). Samples were analyzed in duplicate, and 50,000 PI+ events were measured for each sample. Data were analyzed using FlowJo ver. 10 (BD).

2.2.13. Imaging

Bacterial aggregates and epithelial monolayers were visualized by light microscopy. Images were acquired at a magnification of 400X using a Leica DM2500 microscope.

2.2.14. Statistical analysis

Data were analyzed in GraphPad Prism (version 8.0) software (GraphPad Software, Inc, La Jolla, CA). Statistical significance was determined using two-way Analysis of Variance (ANOVA), the Tukey-Kramer nonparametric test, and Student's t tests when appropriate. For nasopharyngeal colonization studies, the Kruskal–Wallis test was used to evaluate variance among all groups. If significant variance was found between groups, the Mann–Whitney test was used to determine significant differences between individual groups.

2.3. Results

2.3.1. Immunization with HMW1 and HMW2 stimulates serum antibody

To evaluate the antibody response following immunization with HMW1 and HMW2, we immunized groups of mice by either the intranasal or subcutaneous route. Mice were immunized with HMW1 and HMW2 purified from NTHi strain 12 ($HMW1_{12}$ and $HMW2_{12}$) or NTHi strain 5 ($HMW1_5$ and $HMW2_5$). As shown in Figure 2.1, animals immunized by either the subcutaneous or intranasal route developed a robust serum IgG response based on assessment of pooled serum. Measurement of total IgG binding to whole bacteria by flow cytometry (Figure 2.1A) revealed that immune serum was specific for NTHi expressing the HMW1 and HMW2 proteins. Antibodies generated by immunization with $HMW1_{12}$+$HMW2_{12}$ were specific for NTHi strain 12 and showed no significant binding to heterologous HMW1/HMW2-expressing strains (strain 5 and strain 15). Similarly, antibodies generated by immunization with $HMW1_5$+$HMW2_5$ were specific for strain 5. This specificity was present with both subcutaneous and intranasal

immunization. There was no significant antibody reactivity with strains that do not possess the HMW1 and HMW2 proteins, including *hmw1hmw2* mutants of strain 12 and strain 5 and strain NT127, which lacks the *hmw1* and *hmw2* loci. In ELISAs using purified HMW1 and HMW2, sera also exhibited reactivity with the HMW1 and HMW2 adhesins, including variable cross reactivity with HMW1 and HMW2 from heterologous strains (Figure 2.1B). There was no significant reactivity in serum from animals immunized with only adjuvant (alum or dmLT). These studies demonstrate that serum antibodies against HMW1 and HMW2 exhibited specificity for the parent strain from which HMW1 and HMW2 were purified in assays with whole bacteria and variable cross-reactivity with purified proteins from heterologous strains.

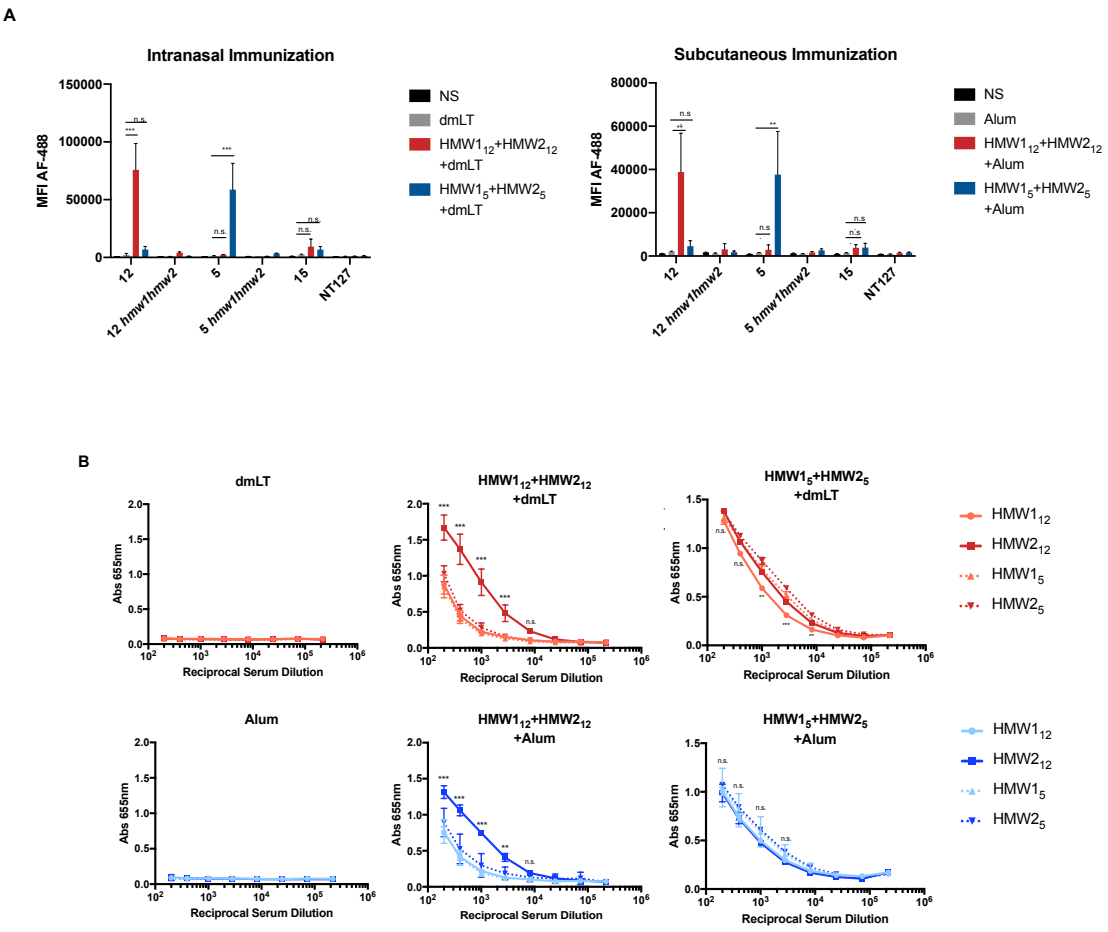


Figure 2. 1. Immunization with HMW1 and HMW2 stimulates systemic IgG.
Immunization with HMW1+HMW2 and dmLT (intranasal) or HMW1+HMW2 and alum (subcutaneous) results in systemic IgG responses. (**A**) IgG binding to whole NTHi was evaluated by flow cytometry. IgG binding is expressed as mean antibody fluorescence intensity (MFI) based on 50,000 events per sample. (**B**) Binding to purified HMW1 and HMW2 by ELISA. Serum pooled from 5 immunized animals per group. Control groups immunized with dmLT in PBS (intranasal) or alum in PBS (subcutaneous). Data are expressed as mean ± SD from three independent experiments. Statistical significance was determined using two-way ANOVA with Tukey's correction for multiple comparisons. n.s. indicates not significant. * $p < 0.05$, ** $p < 0.01$, *** $p< 0.001$.

2.3.2. Immunization with HMW1 and HMW2 stimulates protection against nasopharyngeal colonization by homologous and heterologous strains

Following immunization with HMW1$_{12}$+HMW2$_{12}$, HMW1$_5$+HMW2$_5$, or adjuvant alone by either the subcutaneous or intranasal route, animals were challenged

intranasally with either the parent strain, the heterologous HMW1/HMW2-expressing strain, the 12*hmw1hmw2* mutant, or strain NT127. Three days following the challenge, the density of colonization in animals immunized with $HMW1_{12}$+$HMW2_{12}$ or $HMW1_5$+$HMW2_5$ was markedly reduced, based on CFUs recovered from homogenized nasal tissue (Figure 2.2).

Compared to animals immunized with dmLT in PBS, the density of colonization of mice immunized intranasally with $HMW1_{12}$+$HMW2_{12}$ was reduced by approximately 2 logs. The density of colonization of animals immunized subcutaneously with $HMW1_{12}$+$HMW2_{12}$ was reduced by 0.5-1 log compared to alum in PBS, though this change was not statistically significant (Figure 2.2A). Animals immunized with $HMW1_{12}$+$HMW2_{12}$ were also protected against nasopharyngeal colonization with NTHi strain 5, with larger reductions in colonization density detected in the intranasally-immunized group. Immunization with $HMW1_5$+$HMW2_5$ was also protective against both strain 5 and strain 12 (Figure 2.2B). Intranasal immunization consistently resulted in greater reductions in colonization density compared to subcutaneous immunization, suggesting a role for local immune factors in the nasopharynx. Colonization density was not reduced in any animals challenged with strain 12*hmw1hmw2* or strain NT127. These results suggest that mice were protected against nasopharyngeal colonization by heterologous strains, despite an antibody response that appears to be strain specific.

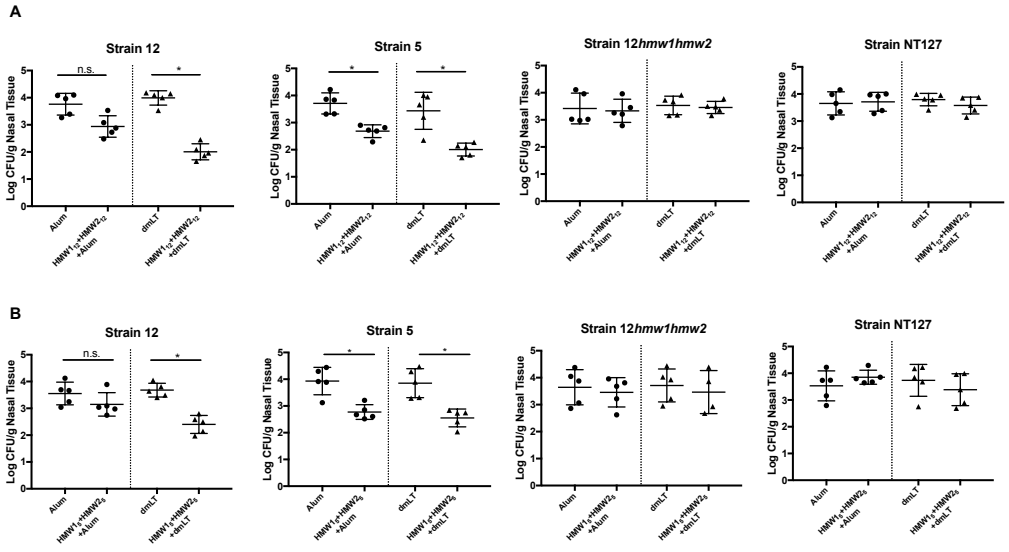


Figure 2. 2. Immunization with HMW1+HMW2 reduces nasopharyngeal colonization density. Mice were immunized with purified $HMW1_{12}$+$HMW2_{12}$ (**A**) or $HMW1_5$+$HMW2_5$ (**B**) and subsequently challenged with NTHi strain 12, strain 5, strain 12*hmw1hmw2* or strain NT127. Inoculums were approximately 10^8 CFU. Density of colonization represented as log(recovered CFU/g nasal tissue). Data represent means ± SD of 5 animals per group. Significance determined using the Kruskall-Wallis test. If a significant variance was identified, the Mann-Whitney test was used to compare individual groups to adjuvant controls. n.s., Not Significant. * $p < 0.05$.

2.3.3. HMW1 and HMW2 antibodies promote NTHi agglutination

To study the specificity of antibody-driven antibacterial behaviors, we first measured agglutinating activity of HMW1 and HMW2 antibodies, employing a flow-cytometry based assay developed by Habets *et al* (Habets et al., 2017). NTHi strains were exposed to immune serum or adjuvant-only serum. Following incubation, bacteria were fixed, stained, and evaluated using flow cytometry. Agglutination was detected based on increases in the forward scatter signal (FSC-A), which indicate increases in particle size. In the presence of $HMW1_{12}$+$HMW2_{12}$ immune serum, the mean FSC-A of strain 12 increased when compared to adjuvant-only serum (Figure 2.3A-B). The formation of aggregates was confirmed by staining bacteria with crystal violet and

imaging with bright field microscopy (Figure 2.3C). Serum generated against $HMW1_{12}$+$HMW2_{12}$ was associated with agglutination of NTHi strain 12 but not strain 5 or strain 15, again suggesting strain specificity of the antibody response. Similarly, immune serum generated against $HMW1_5$+$HMW2_5$ only agglutinated strain 5. Antibodies against strain 12 or strain 5 HMW1 and HMW2 proteins failed to agglutinate strain 12*hmw1hmw2*, strain 5*hmw1hmw2*, or strain NT127, indicating that agglutinating activity was due to HMW1 and HMW2-specific antibodies. Detectable agglutination was proportional to the concentration of immune serum, suggesting that the agglutination reaction is serum-dependent (Figure 2.3B). These results indicate that immunization with HMW1 and HMW2 results in serum antibodies that mediate strain-specific bacterial agglutination.

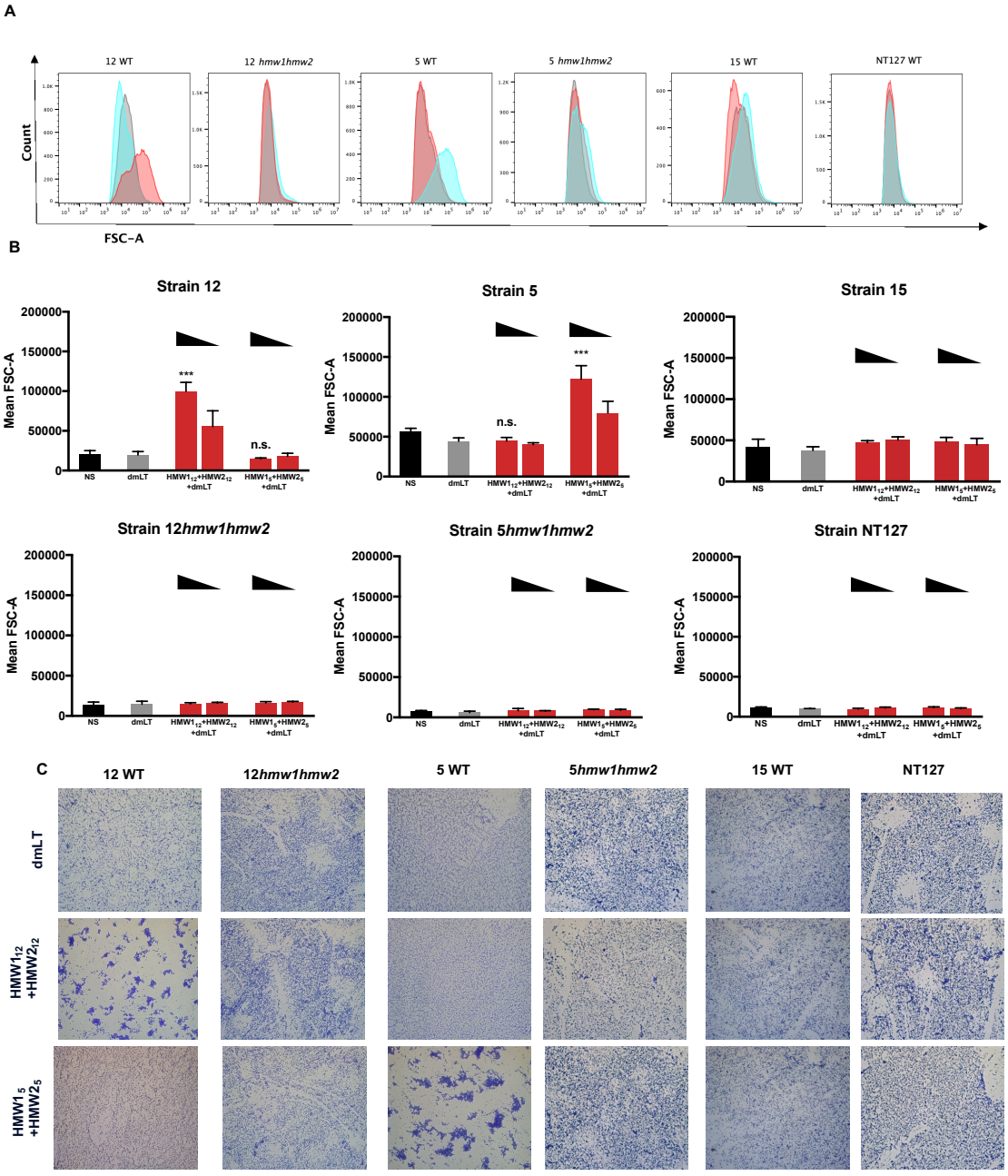


Figure 2. 3. Agglutination of NTHi by serum from HMW1+HMW2 immunized animals.
NTHi were incubated with pooled serum from groups of 5 mice immunized with HMW1+HMW2. (**A**) Agglutination was measured by flow cytometry and is represented as histograms of the FSC-A signal. NTHi were incubated with 2% serum from animals immunized with dmLT alone (gray), $HMW1_{12}$+$HMW2_2$+dmLT (red), or $HMW1_5$+$HMW2_5$+dmLT (blue). Mean FSC-A is quantified in (**B**) and is based on 50,000 events per sample. Error bars represent means ± SD from three independent experiments. Statistical significance determined using two-way ANOVA with Tukey's correction for multiple comparisons. n.s., Not Significant. * $p < 0.05$, ** $p < 0.01$, *** $p< 0.001$. (**C**) Representative images of agglutination imaged by light microscopy following crystal violet staining. Images shown at 400x total magnification. NS, No Serum.

2.3.4. HMW1 and HMW2 antibodies restrict HMW2-mediated adherence

To further address the functional activity of the antibody response to the HMW1 and HMW2 proteins, we assayed the ability of immune sera to inhibit HMW1 and HMW2-mediated adherence to epithelial cells. NTHi strains were inoculated onto monolayers of HaCaT cells in the presence or absence of pooled immune serum. In the presence of 0.2% serum derived against $HMW1_{12}$+$HMW2_{12}$, adherence of strain 12 was reduced by approximately 20%; when serum was increased to 2%, adherence was further reduced, demonstrating a dose-dependent effect (Figure 2.4A, solid bars). Similarly, strain 5 adherence was reduced in the presence of serum generated against $HMW1_5$+ $HMW2_5$ (Figure 2.4B, solid bars) and was proportional to the amount of serum present. Strain 12 and strain 5 bacterial adherence was not reduced in the presence of serum generated against proteins from the heterologous strain or in the presence of adjuvant-only serum. Immune serum derived against proteins from either parent strain had no impact on strain 12*hmw1hmw2* or strain 5*hmw1hmw2*, which are unable to adhere to HaCaT cells. As shown in Figure 2.4C, direct visualization of adherence by light microscopy after Giemsa staining was consistent with the results of quantitative adherence assays. Antibodies against adhesins can reduce bacterial adherence, and consequently colonization, due to either agglutination of bacteria or direct interference with the receptor-ligand interaction or both (Mitsi et al., 2017; Roche et al., 2015). To evaluate whether agglutination was the sole mechanism of reduced adherence, we treated total serum with papain, digesting intact IgG in serum to produce non-agglutinating $F(ab)_2$ fragments. Papain-digested serum retained the ability to inhibit adherence (Figure 2.4A-B, striped bars). Together, these results suggest that HMW1

and HMW2 antibodies are capable of promoting antibacterial function in a strain-specific manner, through both direct agglutination and blocking of adhesin-receptor interactions.

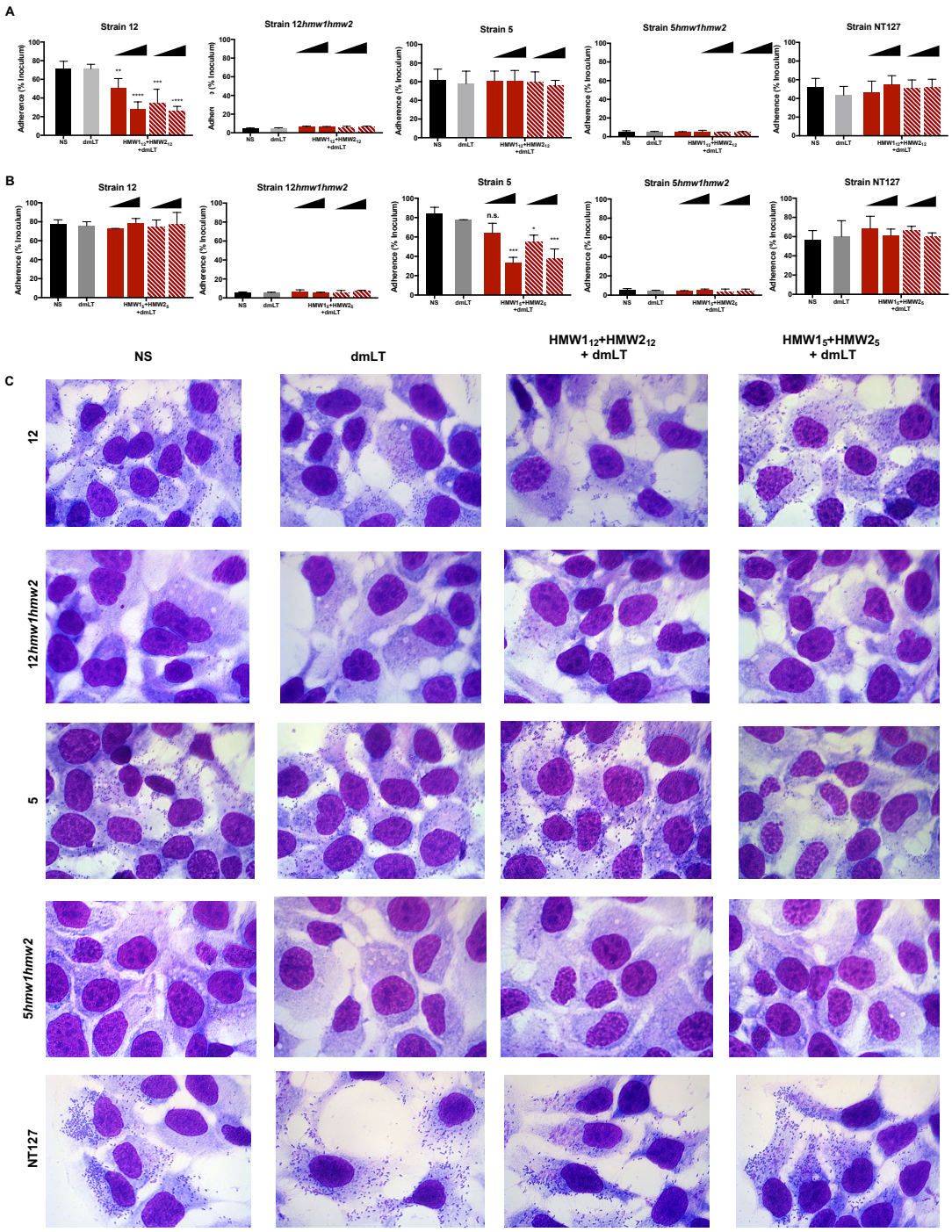


Figure 2. 4. HMW1 and HMW2 antibodies reduce bacterial adherence.
Bacteria were inoculated onto HaCaT cells in the presence or absence of immune serum derived against $HMW1_{12}$+$HMW2_{12}$ (**A**) or $HMW1_5$+$HMW2_5$ (**B**). Solid bars represent whole pooled serum. Striped bars represent pooled serum digested with papain. Data expressed as means ± SD of three independent experiments. NS, No Serum. Statistical significance determined using two-way ANOVA with Tukey's correction for multiple comparisons. Significance is determined relative to the dmLT serum condition. n.s., Not Significant. * $p < 0.05$, ** $p < 0.01$, *** $p< 0.001$.(**C**) Representative images of bacterial adherence to HaCaT monolayers. Images shown at 400X total magnification.

2.3.5. Passive transfer of antibodies mediates protection against colonization by the homologous but not heterologous NTHi strain

To evaluate the contribution of antibodies in the absence of other immune factors, we conducted passive-transfer studies. Donor animals were immunized intranasally with purified $HMW1_{12}$+$HMW2_{12}$, purified $HMW1_5$+$HMW2_5$, or dmLT. Immune serum was administered to naïve animals, and recipient animals were then challenged with NTHi and assessed for colonization (Figure 2.5). The density of colonization with strain 12 in animals receiving $HMW1_{12}$+$HMW2_{12}$-derived serum was reduced approximately 1.5-logs compared to animals that received serum derived against dmLT alone. There was no significant reduction in density of colonization with strain 12 in animals that received $HMW1_5$+$HMW2_5$-derived serum. Similarly, the density of colonization with strain 5 in animals that received serum generated against $HMW1_5$+$HMW2_5$ was reduced ~1.2-logs compared to control animals, but the density of colonization with strain 12 in these animals was unaffected. There were slight reductions (<0.5-log) in colonization density in animals that received serum derived against the heterologous strain, though these decreases were not statistically significant. Transfer of sera against purified $HMW1_{12}$+$HMW2_{12}$ or purified $HMW1_5$+$HMW2_5$ had no effect on colonization by an *hmw1hmw2* mutant. Thus, passive receipt of antibodies granted protection against the homologous strain but was not sufficient to mediate protection against a heterologous strain, despite expression of HMW1 and HMW2.

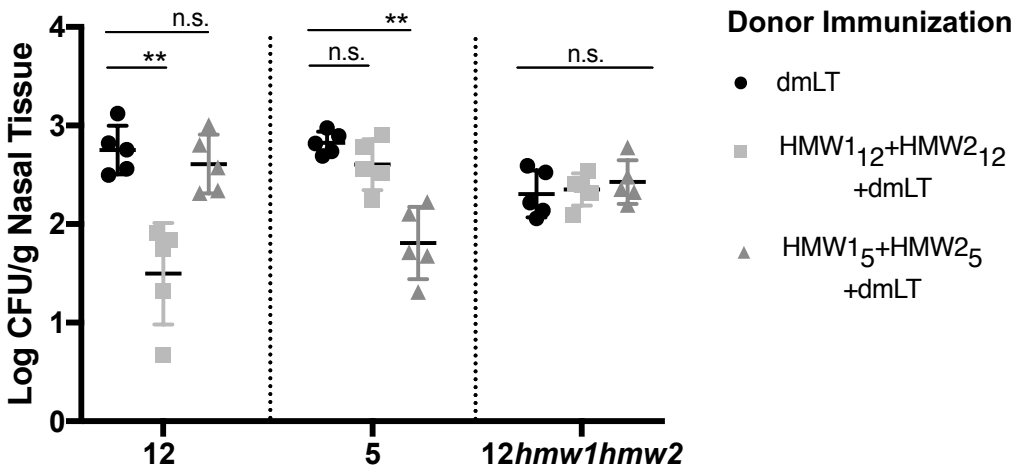


Figure 2. 5. Passive transfer of antibodies mediates homologous protection.
Mice were passively immunized with immune serum from mice immunized with dmLT (circles), HMW1$_{12}$+HMW2$_{12}$ (squares) or HMW1$_5$+HMW2$_5$ (triangles) and subsequently challenged with NTHi strain 12, strain 5, strain 12*hmw1hmw2*. 3 days following challenge, nasopharyngeal colonization densities were determined. Data represent means ± SD of N=5 animals per group. Significance determined using the Kruskall-Wallis test. If a significant variance was identified, the Mann-Whitney test was used to compare individual groups to adjuvant controls. n.s., Not Significant. * $p < 0.05$, ** $p < 0.01$.

2.3.6. IL-17A is required for protection against heterologous strains

To determine whether an IL-17 response facilitates protection against nasopharyngeal colonization, we performed an antibody-mediated *in vivo* depletion of IL-17A. Mice were immunized intranasally with HMW1$_{12}$+HMW2$_{12}$, HMW1$_5$+HMW2$_5$, or dmLT alone. One day prior to intranasal inoculation (-1 dpi), mice were given anti-IL-17A antibody (BioXCell; clone 1F17) or an IgG isotype control antibody (BioXCell; clone MOPC-21). Mice were then challenged with the parent strain or the heterologous HMW-expressing strain, and colonization density was determined 3 days later (Figure 2.6).

Among groups given the IgG isotype control antibody, mice immunized with purified protein were colonized at lower densities than mice immunized with dmLT alone, as expected. Administration of anti-IL-17A antibody had no significant impact on nasopharyngeal colonization by the homologous strain in animals immunized with either

$HMW1_{12}$+$HMW2_{12}$ or $HMW1_5$+$HMW2_5$. However, anti-IL-17A treatment reduced the degree of protection observed against heterologous strains. Compared to control animals immunized with dmLT, animals immunized with $HMW1_5$+$HMW2_5$ were colonized by strain 12 at approximately 1.4-logs lower density when given the IgG isotype control. When given anti-IL-17A, animals immunized with $HMW1_5$+$HMW2_5$ were colonized by strain 12 at 0.05-logs lower density, a difference that was not statistically significant compared to the dmLT-immunized control. Likewise, animals that were given MOPC-21 and then immunized with $HMW1_{12}$+$HMW2_{12}$ were colonized by strain 5 at 1.3-logs lower density, but animals that were given ani-IL-17A and then immunized with $HMW1_{12}$+$HMW2_{12}$ had no significant reduction in strain 5 colonization density. These results suggest that IL-17A is critical for protection against heterologous strains and that IL-17A may facilitate protection independent of antibody strain specificity.

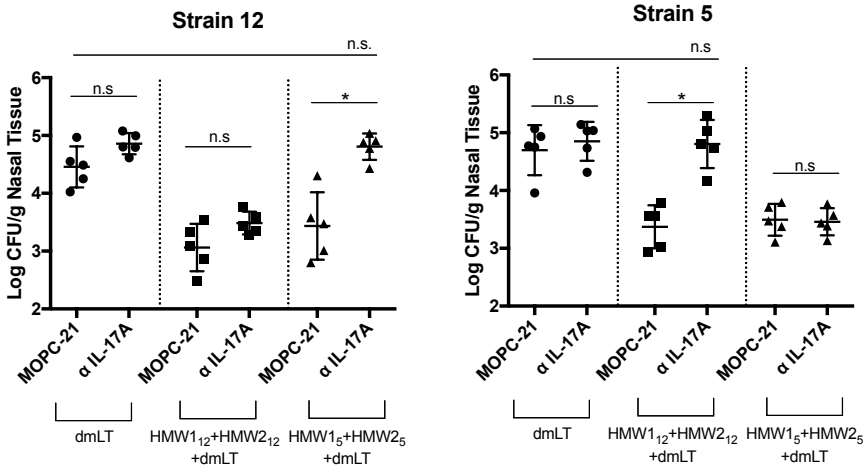


Figure 2. 6. IL-17A mediates heterologous protection.
Mice were immunized with dmLT, $HMW1_{12}$+$HMW2_{12}$, or $HMW1_5$+$HMW2_5$. 1 day prior to challenge, groups were administered anti-IL-17A antibody or IgG isotype control (MOPC-21). Mice were then challenged intranasally with NTHi strain 12, strain 5, strain 12*hmw1hmw2*. 3 days following challenge, nasopharyngeal colonization densities were determined. Data represent means ± SD of 5 animals per group. Significance determined using the Kruskall-Wallis test. If a significant variance was identified, the Mann-Whitney test was used to compare individual groups to adjuvant controls. n.s., Not Significant. * $p < 0.05$.

2.4. Discussion

In this study, we establish that immunization with the HMW1 and HMW2 proteins stimulates protective immunity against nasopharyngeal colonization by NTHi. Importantly, the induced immunity protects against the parent strain as well as heterologous HMW1/HMW2-expressing strains, suggesting broad protection. Following immunization with purified HMW1+HMW2, serum antibody responses were highly strain specific, and passive delivery of antibody alone was insufficient for significant heterologous protection. Consistent with this observation, we found that heterologous protection against nasopharyngeal colonization is IL-17A dependent. Immunization with HMW1 and HMW2 likely stimulates broadly reactive cellular immunity in addition to serum antibody. In the absence of IL-17A production, as shown by antibody knockdown of IL-17A, animals immunized with HMW1+HMW2 were still protected against the homologous strain. Similarly, passive-transfer experiments revealed that antibodies facilitate much greater protection against the homologous strain than the heterologous strain. During nasopharyngeal challenge, the presence of antibodies may directly reduce the ability of some strains to adhere to host epithelium, and also enhance opsonophagocytosis by host cells through direct binding and agglutination. Complete protection against nasopharyngeal colonization is likely to involve contributions of both antibody and cell-mediated arms of the immune response.

Emerging evidence has begun to implicate IL-17 in protection against NTHi. Li W *et al* recently showed that Th17 cells drive antibody-dependent protection against pulmonary infection by NTHi, and that Th17 cells are capable of mediating protection against heterologous strains despite serotype-specific antibody responses (W. Li et al., 2018). Similarly, intranasal immunization with NTHi outer membrane vesicles (OMVs) has been shown to stimulate Th17 memory and subsequent protection against

heterologous strains (Noda et al., 2011; Roier et al., 2012). While several immunogenic outer membrane proteins have been identified from OMVs, including the HMW1 and HMW2 adhesins, it has not been clear exactly which proteins directly contribute to IL-17 production and are capable of stimulating protective immunity in the nasopharynx.

In our studies, animals immunized with HMW1+HMW2 by the subcutaneous or intranasal route developed comparable amounts of NTHi-specific serum antibody. However, based on recovered CFU, our data suggest that intranasally immunized mice were protected to a greater degree, with greater reductions in colonization relative to adjuvant controls. This result suggests an advantage to intranasal immunization and a role for local immune factors in mediating heterologous protection in the upper airways. This finding is consistent with other studies evaluating mucosal immunization, which have observed enhanced protection compared to immunization at distal sites (O'Hara et al., 2020; Roier et al., 2012). The delivery of antigens to intranasal lymphoid tissue likely stimulates local Th17 development, facilitating cell-mediated protection in combination with antibody-mediated systemic immunity. In our studies, animals immunized subcutaneously with HMW1 and HMW2 were still protected against full levels of colonization by either the parent strain or the heterologous strain, suggesting development of Th17 responses in the nasopharynx without local antigen delivery. Recent evidence suggests that parenteral immunization with bacterial antigens can stimulate the development of nasal Th17 memory cell populations, though the mechanism through which this occurs is unclear (O'Hara et al., 2020). These findings suggest that the route of antigen delivery is an important consideration in NTHi vaccine design, and that intranasal immunization may be more effective.

Despite the high degrees of homology of the HMW1 and HMW2 proteins across strains, the antibody responses following immunization were remarkably strain specific.

Based on amino acid sequences, $HMW1_{12}$ and $HMW1_5$ share 73.08% identity, and $HMW2_{12}$ and $HMW2_5$ share 69.66% identity (table 2). In our studies, while antibodies bind to whole bacteria in a strain-specific pattern, binding assays to purified proteins revealed that cross-reactive antibodies are present in immune sera, suggesting that strain specificity stems from factors beyond amino acid sequence, an ongoing area of investigation.

Table 2. 2. Identity of the HMW1 and HMW2 adhesins.

$HMW1_{12}$	100.0	**69.76**	**73.08**	**67.24**
$HMW2_{12}$	69.76	100.0	69.66	74.59
$HMW1_5$	73.08	69.66	100.0	79.18
$HMW2_5$	67.24	74.59	79.18	100.0

Percent identity matrix – generated using Clustal2.1

Our data also suggest that the HMW1 and HMW2 proteins may have important immunological differences within a strain. Though animals were immunized with a 1:1 mixture of $HMW1_{12}$+$HMW2_{12}$, the antibody response appeared to heavily favor $HMW2_{12}$. While $HMW1_{12}$ and $HMW2_{12}$ share ~70% identity, they contain two regions of significant divergence, including an $HMW1_{12}$-specific region of 62 amino acids that is absent from $HMW2_{12}$ and a ~360 amino acid region that harbors binding activity and accounts for the differences in HMW1 and HMW2 binding specificity. These regions could explain the observed differential immunogenicity between $HMW1_{12}$ and $HMW2_{12}$. Interestingly, immunization with $HMW1_5$ and $HMW2_5$ generated an antibody response that did not heavily favor either protein, suggesting that the differences are not solely due to the HMW1 and HMW2 binding domains.

Collectively, these data implicate the HMW1 and HMW2 adhesins as promising

antigens for a vaccine against nasopharyngeal colonization by NTHi. Given the high prevalence of HMW1/HMW2-expressing NTHi clinical isolates and the protective immunity afforded by these proteins, they may be valuable for inclusion in a broadly protective vaccine.

Acknowledgments

This work was supported by the National Institute of Allergy and Infectious Diseases (NIAID) under the training grant in Microbial Pathogenesis and Genomics (1-T32-AI-141393-01).

We thank Dr. John Clements for providing the purified dmLT used in these studies and Dr. Elizabeth Q. Littauer, Dr. Paul J. Planet, and Ying Yang for assistance with the animal work.

This work has been submitted for publication as referenced below:
Kadry N.A., Porsch E.A., Shen H., and St. Geme III, J.W. 2020. “Immunization with HMW1 and HMW2 adhesins protects against colonization by heterologous strains of nontypeable *Haemophilus influenzae*.” Proceedings of the National Academy of Sciences.

3. DETERMINANTS OF IMMUNOGENICITY OF THE HMW1 AND HMW2 ADHESINS

3.1. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is a major source of morbidity in children and adults, causing localized respiratory tract disease such as acute otitis media, sinusitis, and pneumonia and also accounting for occasional serious invasive disease. The pathogenesis of NTHi disease begins with colonization of the upper respiratory tract. Most individuals become colonized during infancy and have intermittent asymptomatic colonization throughout childhood and adulthood (McCann et al., 2016). Under certain circumstances, NTHi will spread contiguously within the respiratory tract to produce localized disease or enter the bloodstream to cause invasive disease.

Current strategies to prevent NTHi disease are limited. Examples include adenoidectomy and antibiotic prophylaxis to prevent recurrent otitis media, approaches that are often inadequate and that may be associated with short-term and long-term complications. Consequently, there is interest in developing a vaccine, ideally preventing colonization as the essential first step in the pathogenic process. Among potential vaccine antigens are the HMW1 and HMW2 high molecular weight adhesins. These proteins are present in approximately 75% of NTHi clinical isolates and are the primary targets of serum antibody in children recovering from NTHi disease. Recent work has established that rhesus macaques colonized with NTHi develop HMW1- and HMW2-specific antibodies, suggesting that colonization is an immunizing event (Rempe et al., 2016). The appearance of HMW1 and HMW2-specific IgG in human serum has been correlated with the development of complement-dependent serum bactericidal activity against NTHi (Stephen J. Barenkamp & Bodor, 1990; Winter & Barenkamp, 2006).

Moreover, in children with acute otitis media, higher concentrations of detectable NTHi-specific antibodies in middle ear fluids at the time of diagnosis, including antibodies directed against HMW1 and HMW2, have been associated with faster clearance (Faden et al., 1989; Sloyer et al., 1976).

In recent work, we have established that intranasal immunization with a mixture of the HMW1 and HMW2 adhesins can stimulate protective immunity against nasopharyngeal colonization by heterologous strains of NTHi, through stimulation of both antibody and T-cell driven responses. Importantly, antibody responses appear to mediate strain specific immunity and drive protection against the parent strain, and the Th17 response is required for protection against heterologous strains (Chapter 2). In order to further develop the HMW1 and HMW2 proteins as vaccine antigens, it is important to clarify the determinants of their immunogenicity.

HMW1 and HMW2 are highly homologous glycoproteins, sharing approximately 71% identity and 80% similarity to each other. These proteins are highly conserved among diverse NTHi strains, with HMW1 homologs typically at least 70% similar to each other and HMW2 homologs typically at least 70% similar to each other (Rodriguez et al., 2003; Shahini Shams Abadi et al., 2016). HMW1 and HMW2 are encoded by distinct genetic loci that are located in physically separate regions on the chromosome and contain 2 accessory genes that encode the HMW1B/2B and HMW1C/2C accessory proteins. The HMW1B and HMW2B proteins form an outer membrane pore and facilitate translocation of the HMW1 and HMW2 across the outer membrane. HMW1C and HMW2C are cytoplasmic glycosyltransferases that mediate the N-linked glycosylation of HMW1 and HMW2. The HMW1 and HMW2 adhesins are glycosylated at asparagine residues (N) at the sequence N-X-S-T (X≠Proline) with either single or di-hexose

residues (Joseph W. St Geme & Grass, 1998; Szymanski et al., 1999). In the absence of HMW1C/HMW2C-mediated glycosylation, HMW1 and HMW2 are more susceptible to degradation during the secretion process and are freely released from the cell surface (Grass et al., 2003b). Proper modification by HMW1C/HMW2C is also important for proper folding and adhesive activity of mature HMW1 and HMW2 (unpublished data).

In this study, we sought to compare the immune response developed to HMW1 versus HMW2 in a mouse model of intranasal immunization. Though HMW1 and HMW2 share significant homology, we found that they stimulate antibody responses with differing target specificities. We present evidence that HMW1C-driven glycosylation of HMW1 may shape the immunogenicity of HMW1, driving immunological differences between HMW1 and HMW2 across strains and altering recognition of NTHi by host antibodies.

3.2. Materials and Methods

3.2.1. Bacterial Strains and Culture

The bacterial strains used in the present study are described in Table 3.1. NTHi strains were grown on chocolate agar (BD Biosciences, San Jose, CA, USA) or brain heart infusion (BHI) agar supplemented with 0.1% (vol/vol) lysed horse blood as a source of hemin and 3.5 μg/ml NAD (BHIs), with 500 μg/ml streptomycin as appropriate. Agar plates were incubated overnight at 37°C with 5% CO_2. Spontaneous streptomycin-resistant derivatives of NTHi were generated by spreading a dense bacterial suspension onto agar plates containing 500 μg/ml streptomycin and then recovering survivors. For each set of isogenic strains, growth studies determined that the parent and derivative strain had similar growth rates. In preparation for intranasal inoculation, NTHi strains

were resuspended in BHIs broth to an optical density at 600 nm (OD_{600}) of 0.2. Cultures were grown shaking at 250 rpm to an OD_{600} of 0.8, pelleted, washed with phosphate-buffered saline (PBS) (Lonza Biologics, Basel, Switzerland), and resuspended in PBS.

E. coli strain BL21 was used to express $HMW1_{12}$ modified by alternative glycosyltransferases. *E. coli* was grown on Luria-Bertani Broth (LB) plates or in LB broth supplemented with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol as appropriate. Plates were incubated at 37°C with 5% CO_2, and liquid cultures were grown at 37°C with agitation at 250 rpm.

3.2.2. Cell lines

The cell lines used in this study were obtained from the American Tissue Culture Collection (ATCC). Cells were maintained at 37°C with 7.5% CO_2. Chang cells (human conjunctiva; ATCC CCL-20.2)
were maintained in Eagle minimal essential media (MEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% nonessential amino acids (NEAA) and 10% fetal calf serum (FCS).

Table 3. 1. Bacterial strains used in Chapter 3

Strain	Relevant genotype or description	Source or reference
Nontypeable H. influenzae		
12	Nontypeable *H. influenzae* strain, produces $HMW1_{12}$ and $HMW2_{12}$	Clinical isolate, middle ear fluid, acute otitis media
12*hmw1hmw2*	12 *hmw1::kan hmw2::kan*, produces neither $HMW1_{12}$ nor $HMW2_{12}$	(Joseph W. St. Geme et al., 1993)
12*hmw1*	12 *hmw1::kan*, produces only $HMW2_{12}$	(Joseph W. St. Geme et al., 1993)
12*hmw2*	12 *hmw2::kan,* produces only $HMW1_{12}$	(Joseph W. St. Geme et al., 1993)
5	Nontypeable *H. influenzae* strain, produces $HMW1_5$ and $HMW2_5$	Clinical isolate, middle ear fluid, acute otitis media
5*hmw1hmw2*	5 *hmw1::kan hmw2::kan*, produces neither $HMW1_5$ nor $HMW2_5$	(Joseph W. St. Geme et al., 1993)
5*hmw1*	5 *hmw1::kan*, produces only $HMW2_5$	(Joseph W. St. Geme et al., 1993)
5*hmw2*	5 *hmw2::kan,* produces only $HMW1_5$	(Joseph W. St. Geme et al., 1993)
NT127	Nontypeable *H. influenzae* strain, produces neither HMW1 or HMW2 and instead produces the Hia adhesin	Clinical isolate, blood, meningitis (Provided by Hao Shen, University of Pennsylvania)
Rd KW20	Laboratory strain, formerly serotype d, lacking *hmw* genes and non-adherent *in vitro*	(Fleischmann et al., 1995)
Rd/$HMW1_{12}$	Derivative of strain Rd KW20 that contains the intact $hmw1_{12}$ locus and produces $HMW1_{12}$	(S J Barenkamp & Leininger, 1992)
Rd/$HMW1_{12}$*hmw1C*	Derivative of Rd/$HMW1_{12}$ that contains the $hmw1_{12}$ locus with a kanamycin cassette inserted at the *Xho*I site in the chromosomal copy of *hmw1C*	(Grass et al., 2003b)
Rd/$HMW1_{12}$*hmw1C* +pHMW1C	Derivative of Rd/$HMW1_{12}$*hmw1C recA* that contains pACYC184 *hmw1C*	This study
E. coli		
BL21 + pHMW1AB +pEtpC	pT7-7 $hmw1A_{12}$/$hmw1B_{12}$ pACYC184 *etpC*	Katherine Rempe
BL21 + pHMW1A +pHMW1C_{Kk}	pT7-7 $hmw1A_{12}$ pACYC184 $hmw1C_{Kk}$	(Rempe et al., 2015)

3.2.3. Purification of HMW1 and HMW2

HMW1 and HMW2 were purified from the surface of NTHi or *E. coli* as described previously (Rempe et al., 2016). NTHi mutants producing HMW1 only or HMW2 only were inoculated into 10 liters of BHIs broth and incubated for 12-14 hours shaking at 37°C. Alternatively, to purify protein from the surface of *E. coli* expressing HMW1, bacteria were inoculated into 10 liters of LB with antibiotics as appropriate and incubated for 12-14 hours shaking at 37°C. Following incubation, the cultures were centrifuged for 20 min at 8000 rpm, the supernatant was discarded, and the pellet was frozen at −80°C overnight. To release HMW1 or HMW2 from the bacterial surface, the frozen pellet was thawed on ice and resuspended in 200 ml of extraction buffer (0.5 M NaCl, 10 mM EDTA, 10 mM Tris pH 7.5, 50 μM 1,10-phenanthroline) in the presence of protease inhibitors (Roche, Basel, Switzerland). The resuspended bacteria were incubated at 4°C for 1 hr and then centrifuged for 15 min at 8500 rpm. The supernatant containing the released HMW1 or HMW2 was saved, and the pellet was discarded. The supernatants were dialyzed overnight into 20 mM 2-(N-morpholino) ethanesulfonic acid (MES), 75mM NaCl pH 6.0 and then stepwise dialyzed in MES with 250mM NaCl, in MES with 125mM NaCl, and then in 80mM NaCl. Dialyzed protein was loaded onto a Resource S cation exchange chromatography column (GE Life Sciences, Marlboro, MA, USA). Bound protein was eluted with 20 mM MES, 1 M NaCl pH 6.0. Fractions containing HMW1 or HMW2 were combined, concentrated, and then loaded onto a HiLoad SuperDex 16/60 200pg size-exclusion column (GE Life Sciences) equilibrated with 20 mM MES, 150 mM NaCl pH 6.0, 5% glycerol. The fractions containing HMW1 or HMW2 were pooled, resolved on SDS-PAGE gels, and stained with Coomassie blue to ensure purity.

To isolate HMW1 from the cytoplasm, bacteria were grown overnight, centrifuged, and frozen as described. Pellets were then thawed and resuspended in resuspension buffer (0.5M NaCl, 10mM EDTA, 10mM Tris pH 7.5, 50 μM 1, 10 phenanthroline) and were sonicated until the suspension reached maximum clarity. The sonicate was then centrifuged for 10 minutes at 18,598 x g. The supernatant containing the released HMW1 was saved, and the pellet was discarded. The supernatant was then dialyzed into 20mM MES, 10mM NaCl pH 6.0 and was loaded on a RESOURCE-S ionic exchange AKTA column. The bound protein was eluted with 20mM MES, 1M NaCl pH 6.0. HMW1-containing fractions were then concentrated and separated by size-exclusion chromatography.

3.2.4. Animal Immunizations

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Children's Hospital of Philadelphia. Groups of 6-8 week-old female BALB/c mice were immunized intranasally with purified HMW1 and HMW2. Intranasal immunizations were performed as described by Cutter *et al,* with purified protein diluted in PBS to a concentration of 15 μg/40 μL with 0.1 μg *E. coli* labile toxin mutant LT(R192G/L211A) (dmLT) as a mucosal adjuvant (S. Leach et al., 2012). Purified dmLT was received as a gift from Dr. John Clements at Tulane University (Clements & Norton, 2018). Prior to intranasal immunization, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (75 mg/kg + 7.5 mg/kg body weight). Immunizations were delivered by pipetting 20 μL directly into each nostril. Following immunization, mice were placed in a supine position for 3-5 mins. Mice

were immunized on weeks 0, 1, 3, and 5. Two weeks following the final immunization, mice were either challenged with NTHi or sacrificed by terminal cardiac puncture for serum collection.

3.2.5. Intranasal Challenge

Two weeks following immunization, mice were challenged intranasally with ~$1x10^8$ CFU of streptomycin-resistant NTHi in 10 µL. Mice were anesthetized as described for intranasal immunization, and 5 µL of the bacterial suspension was delivered into each nostril. Following inoculation, mice were placed in a supine position for 3-5 min. Three days following challenge, animals were sacrificed. Nasal washes were collected by inserting a catheter into the trachea and flushing PBS through the nose. Nasopharyngeal tissue was harvested, weighed, homogenized, diluted, and plated. Recovered NTHi was plated onto BHIs-agar containing 500 µg/mL streptomycin and incubated at 37°C for 48 hours to determine colony counts.

3.2.6. Antibody measurement by ELISA

Purified protein (2.5 µg/ml in 0.1 M carbonate buffer, pH 9.6) was coated onto 96-well plates by incubation at 4°C overnight. Plates were washed with PBS-0.1% Tween 20 and blocked with 2% nonfat milk in PBS. Serial dilutions of mouse sera were applied to wells in triplicate. The plates were incubated at 37°C for 1 hr. Mouse IgG was detected using a 1:2000 dilution of rabbit anti-mouse IgG conjugated to horseradish peroxidase (Sigma). For detection, the wells were incubated with 100 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) ELISA substrate (Rockland, Pottstown, PA, USA), and

absorbance was recorded at 655 nm.

3.2.7. Antibody measurement by flow

NTHi strains were inoculated into 3mL of BHIs broth at OD_{600} = 0.200 and grown shaking at 37°C until OD_{600} = 0.800. Bacteria were then spun down and resuspended in 1X PBS to OD_{600} = 0.600. Bacteria were subsequently fixed in 1X TBS+1% formaldehyde for 1 hr, then incubated with the appropriate immune serum. Following incubation, bacteria were washed in 1X PBS and incubated with fluorescent anti-mouse IgG (Rockland) for 1 hr, washed. Bacterial suspensions were brought up to 1mL total volume and stained with propidium iodide (PI) to identify bacteria. Fluorescence was analyzed using a BD Accuri C6 flow cytometer and BD Accuri C6 plus software (BD Biosciences). 50,000 PI+ events were recoded per sample.

3.2.8. Bacterial Adherence

Approximately $1.8x10^5$ cells were seeded onto glass coverslips placed in 24-well tissue culture plates and incubated overnight. Epithelial monolayers were inoculated with ~$2x10^7$ CFU of NTHi in the presence or absence of immune serum, and the plates were centrifuged at 165 x *g* for 5 min to facilitate contact between the bacteria and the epithelial cells. After incubation for 30 min at 37°C in a 5% CO_2 atmosphere, monolayers were rinsed with PBS to remove non-adherent bacteria. Monolayers were then stained with Giemsa for imaging.

3.2.9. Agglutination

Bacteria were resuspended from chocolate agar plates in 3mL BHIs to OD_{600}= 0.200, grown to OD_{600}= 0.800, then resuspended in 1X PBS. A 100 µL volume of the bacterial suspension was incubated with pooled, heat-inactivated serum for 1hr at room temperature. Bacteria were then fixed by adding formaldehyde to a final concentration of 1% and incubating at room temperature for 1hr. Following fixation, the samples were laid onto slides for imaging or diluted in PBS for analysis by flow cytometry.

3.2.10. Measurement of agglutination by flow cytometry

Bacteria were fixed and stained with propidium iodide to gate on bacterial cells. Bacterial populations were then evaluated in the forward scatter (FSC-A) and side scatter (SSC-A) dot plot to measure aggregate size, and the FSC-A count plot to determine aggregate quantity (Habets et al., 2017). Samples were analyzed in duplicate, and 50,000 PI+ events were measured for each sample. Data were analyzed using FlowJo ver. 10 (BD).

3.2.11. Imaging

Bacterial aggregates and epithelial monolayers were visualized by light microscopy. Images were acquired at a magnification of 400X using a Leica DM2500 microscope.

3.2.12. Statistical analysis

Data were analyzed in GraphPad Prism (version 8.0) software (GraphPad Software, Inc, La Jolla, CA). Statistical significance was determined using two-way Analysis of Variance (ANOVA), the Tukey-Kramer nonparametric test, and Student's t tests when appropriate. For nasopharyngeal colonization studies, the Kruskal–Wallis test was used to evaluate variance among all groups. If significant variance was found between groups, the Mann–Whitney test was used to determine significant differences between individual groups.

3.3. Results

3.3.1. HMW1 and HMW2 stimulate antibody responses with differing specificities

To evaluate the antibody responses generated against HMW1 and HMW2, we immunized groups of mice with HMW1 or HMW2 purified from NTHi strain 12 ($HMW1_{12}$, $HMW2_{12}$, $HMW1_{12}$+$HMW2_{12}$) or NTHi strain 5 ($HMW1_5$, $HMW2_5$, $HMW1_5$+$HMW2_5$). As shown in Figure 3.1, mice in all immunization groups developed a serum IgG response based on assessment of pooled serum. Measurement of total IgG binding to whole bacteria by flow cytometry (Figure 3.1A) revealed that serum antibodies were specific for NTHi strains expressing the HMW1 and HMW2 proteins. Immunization with proteins from NTHi strain 12, including $HMW1_{12}$, $HMW2_{12,}$ or a mixture of $HMW1_{12}$+$HMW2_{12,}$ resulted in an antibody response that preferentially bound to the parent strain. No significant binding was detected to isogenic mutant strains that do not produce the HMW1 and HMW2 adhesins (12*hmw1hmw2* and 5*hmw1hmw2*) or to a heterologous strain that does not carry the *hmw1* and *hmw2* loci (NT127). Despite the significant

amino acid homology among the HMW1/HMW2 adhesins across strains, no significant binding was detected to a heterologous HMW1/HMW2-expressing strain (strain 5 or strain 15). Similarly, antibodies generated against $HMW1_5$, $HMW2_5$, or $HMW1_5$+$HMW2_5$ were highly specific for strain 5, with no significant binding to strain 12.

To evaluate adhesin specificity independent of differences in adhesin amount, we measured IgG binding to purified protein. Evaluating IgG binding to surface-purified proteins (Figure 3.1B) revealed greater differences in immune response to the HMW1 and HMW2 adhesins. Consistent with the analysis of antibody binding to whole bacteria, serum from animals immunized with $HMW1_{12}$ alone preferentially bound to $HMW1_{12}$ and $HMW2_{12}$, with significantly reduced binding to $HMW1_5$ or $HMW2_5$, suggestive of specificity for the parent strain. Serum from animals immunized with $HMW2_{12}$ bound to the HMW1 and HMW2 proteins from strain 12 and strain 5 with comparable affinity. Serum generated against the mixture of $HMW1_{12}$+$HMW2_{12}$ resulted in antibody that exhibited preferential binding to $HMW2_{12}$, with significantly reduced levels of binding to $HMW1_{12}$, $HMW1_5$, and $HMW2_5$. Serum generated against $HMW2_5$ or a mixture of $HMW1_5$+$HMW2_5$ showed comparable binding to all variants, and serum against $HMW1_5$ showed reduced affinity for $HMW2_{12}$.

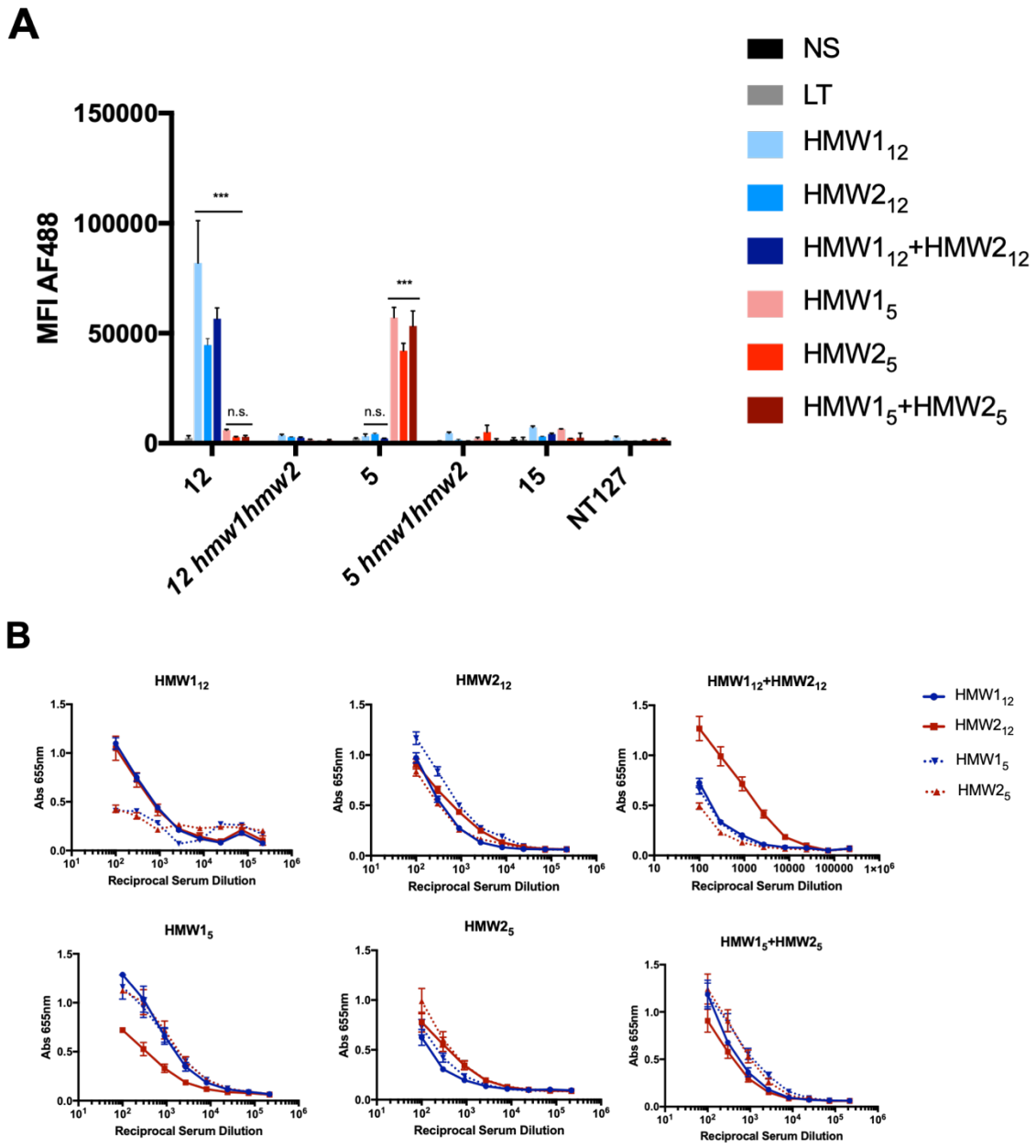


Figure 3. 1. Immunization with HMW1 or HMW2 from heterologous NTHi strains results in strain specific IgG.

Mice were intranasally immunized with mixtures of the HMW1 and/or HMW2 proteins. **(A)** Serum IgG binding to whole NTHi was evaluated by flow cytometry. IgG binding is expressed as mean antibody fluorescence (MFI) based on 50,000 events per sample. Statistical significance determined using two-way ANOVA with Tukey's correction for multiple comparisons. Significance determined relative to dmLT serum condition. n.s., Not Significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(B)** Binding of serum IgG to purified HMW1 and HMW2 by ELISA. Serum pooled from 5 immunized animals per group. Data represent mean ± SD from three independent experiments. NS, no serum. LT, dmLT in PBS.

Despite the remarkably high homology across the HMW1 and HMW2 adhesins, both HMW1 and HMW2 appear to stimulate antibody responses with different specificities. HMW1 and HMW2 proteins from different strains can also stimulate very different antibody responses. Together, these data reveal significant immunostimulatory differences among the HMW1 and HMW2 adhesins, apparently independent of amino acid sequence homology. Further, while immunization stimulates antibodies that are cross reactive in assays with purified HMW1 and HMW2, the binding of these antibodies to whole bacteria is strain specific.

3.3.2. HMW1 and HMW2 stimulate protective immunity against nasopharyngeal colonization

To evaluate whether differences in adhesin antibody responses influence protective immunity, mice were immunized with proteins from strain 12 (Figure 3.2A) or strain 5 (Figure 3.2B) and subsequently challenged with the parent strain or the heterologous HMW1/HMW2-expressing strain. Compared to adjuvant-only controls, mice immunized with mixtures of $HMW1_{12}$+$HMW2_{12}$ or $HMW1_5$+$HMW2_5$ were protected against nasopharyngeal colonization by both the parent strain and the heterologous strain, consistent with previous data (Chapter 2). Similarly, animals immunized individually with $HMW2_{12}$ or $HMW1_5$ were protected against colonization by both strain 12 and strain 5, with ~1.5-log reductions in colonization density. Animals immunized with $HMW1_{12}$ were protected against strain 12 but were not significantly protected against strain 5. Immunization with $HMW2_5$ alone resulted in reduced colonization density of both strains relative to controls, although these differences were not statistically

significant. These results suggest that variants of HMW1 and HMW2 may have altered capacity to stimulate protective immunity.

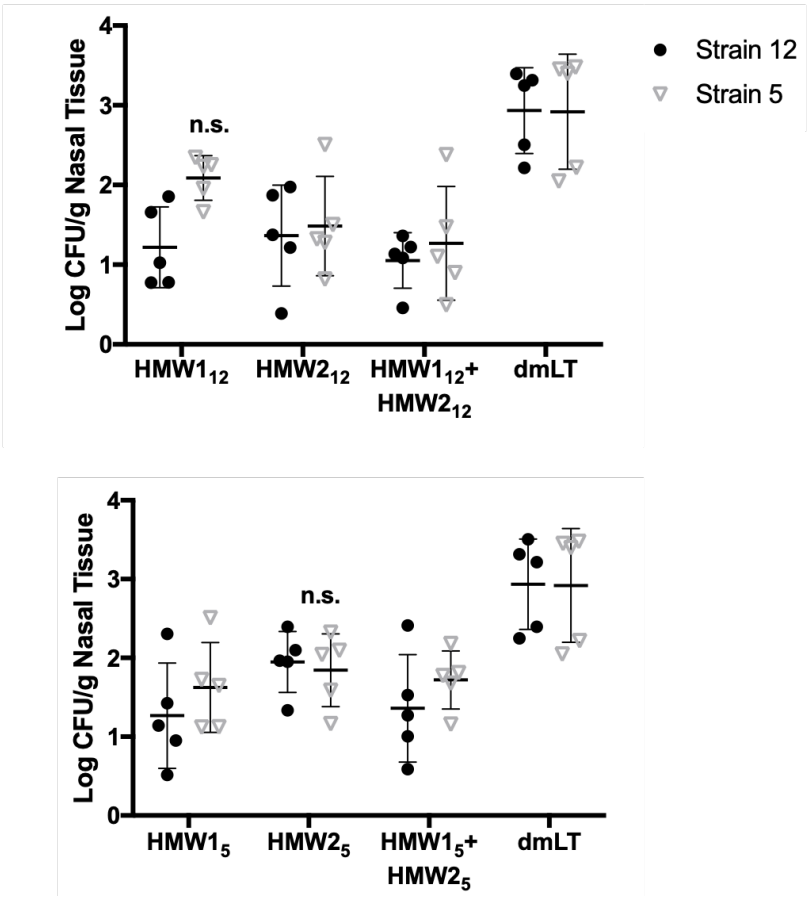


Figure 3. 2. Immunization with HMW1 or HMW2 reduces nasopharyngeal colonization density. Mice were immunized with combinations of purified $HMW1_{12}$ and $HMW2_{12}$ (**A**) or $HMW1_5$ and $HMW2_5$ (**B**) and subsequently challenged with NTHi strain 12 or strain 5. Inoculums were approximately 10^8 CFU. Density of colonization represented as log(recovered CFU/g nasal tissue). Data represent means ± SD of 5 animals per group. Significance determined using the Kruskall-Wallis test. If a significant variance was identified, the Mann-Whitney test was used to compare individual groups to adjuvant (dmLT) controls. n.s., Not Significant.

3.3.3. The glycosylation of HMW1 influences binding by serum antibody

We next sought to determine whether differences in the pattern of adhesin glycosylation affect antibody binding specificity. To generate differentially glycosylated variants of $HMW1_{12}$, we produced HMW1 in the presence of homologs of the HMW1C

glycosyltransferase, including the *E. coli* EtpC protein and the *Kingella kingae* $HMW1C_{KK}$ protein, which can mediate glycosylation HMW1, though resulting in a different pattern of glycosylation (Rempe et al., 2015). Previous work has established that these HMW1 variants, designated $HMW1_{KK1C}$ and $HMW1_{EtpC}$, are glycosylated at fewer sites relative to HMW1 modified by a chromosomal copy of HMW1C ($HMW1_{WT}$). Because we were unable to produce EtpC in strain Rd/$HMW1_{12}$, we produced HMW1AB alongside EtpC in *E. coli*, resulting in $HMW1_{EtpC}$ on the bacterial surface. $HMW1C_{KK}$ is not detectable on the bacterial surface; therefore, this variant was purified from the cytoplasm of *E. coli*. To generate a hyper-glycosylated variant of $HMW1_{12}$, we created a derivative of RdHMW1 in which *hmw1C* is expressed on a high copy plasmid (pHMW1C). The increased production of the HMW1C glycosyltransferase relative to the HMW1 substrate results in hyper-modification of HMW1 relative to $HMW1_{WT}$ (Elango & Schulz, 2020). This hyper-modified variant is designated $HMW1_{HMW1C}$. These variant forms of $HMW1_{12}$ are summarized in Table 2.

Table 3. 2. Purified $HMW1_{12}$ glycosylation variants

Variant	*Source of isolation*	*Glycosyltransferase*	*Relative modification*
$HMW1_{WT}$	Rd/$HMW1_{12}$ surface	Chromosomal HMW1C	Native
$HMW1_{KK1C}$	*E. coli* cytoplasm	$HMW1C_{KK}$	Reduced
$HMW1_{EtpC}$	*E. coli* surface	EtpC	Reduced
$HMW1_{HMW1C}$	Rd/$HMW1_{12}$ surface	pHMW1C	Increased

ELISAs were performed using serum generated against the individual HMW1 and HMW2 variants as well as HMW1+HMW2 mixtures. With all of the sera, binding was

consistently highest to $HMW1_{WT}$ (Figure 3.3, black bars). Antibody binding was significantly reduced to $HMW1_{KK1C}$ and $HMW1_{EtpC}$. Binding to $HMW1_{HMW1C}$ was also reduced, although there was variability depending on the source of the serum. Binding to $HMW1_{HMW1C}$ was lowest with serum from animals immunized with $HMW1_{12}$ and highest with serum from animals immunized with $HMW2_{12}$ or the strain 5 proteins. Differences in binding across serum groups again suggests that immunostimulatory differences exist among the HMW1 and HMW2 adhesins, resulting in antibody responses that recognize vastly different epitopes.

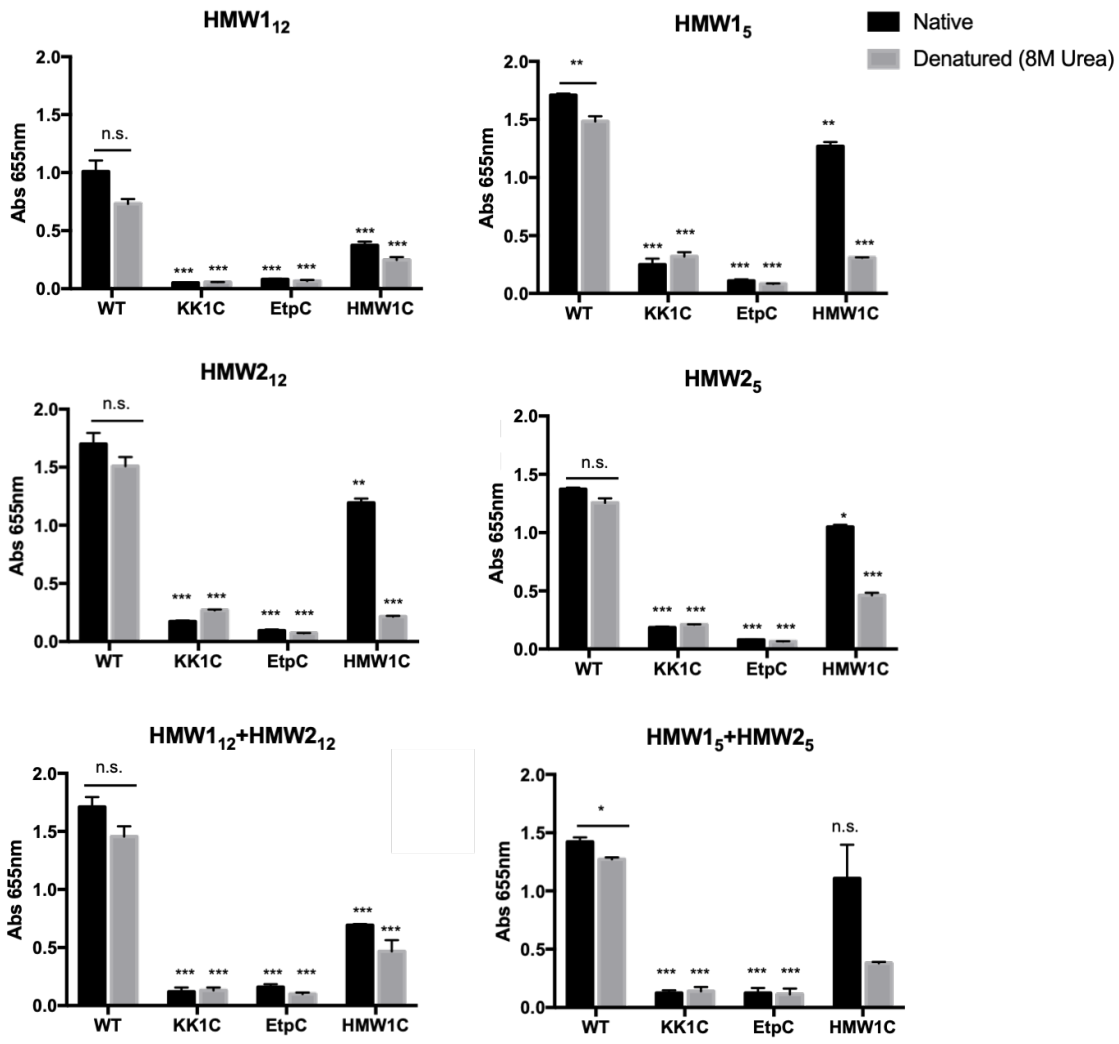


Figure 3. 3. Glycosylation of HMW1$_{12}$ impacts serum antibody binding.
Serum IgG binding to glycosylation variants of purified HMW1$_{12}$ was evaluated by ELISA. Variants are indicated by their modifying glycosyltransferase. Proteins were coated onto plates under native (black bars) or denaturing (gray bars) conditions. 8M urea was used to promote denaturing. Serum pooled from 5 immunized animals per group. Data represent mean ± SD from three independent experiments. Statistical significance shown relative to WT protein in each condition, unless indicated. n.s., not significant. * p<0.05, ** p<0.01, ***p<0.001.

Reduced antibody binding to purified glycosylation variants of HMW1$_{12}$ could be due to changes in existing glycopeptides or changes in global structure of the adhesin. When HMW1 is modified by EtpC, the protein shows a modified structure. Based on circular dichroism, HMW1$_{WT}$ adopts a structure that is 1.86% α-helix and 49.03% β-

sheet, while EtpC-modified HMW1 is 52.46% α-helix and 9.17% β-sheet (Rempe and St. Geme III, unpublished data). To evaluate antibody binding without an effect of structural changes, ELISAs were performed in a chaotropic condition, using 8M urea to denature coating proteins (Hnasko et al., 2011). In the denaturing condition (Figure 3.3, gray bars), IgG binding to $HMW1_{WT}$ was slightly reduced across all serum groups, though this reduction was not always statistically significant. The low levels of binding to $HMW1_{EtpC}$ and $HMW1_{KK1C}$ were not significantly changed. Binding to $HMW1_{HMW1C}$ was significantly reduced in serum derived against $HMW2_{12}$, $HMW1_{5}$, $HMW2_{5}$, and $HMW2_{5}$+$HMW2_{5}$. Importantly, binding to all glycosylation variants remained lower than binding to $HMW1_{WT}$ in the denaturing condition. These findings suggest that in the absence of structural epitopes, binding to $HMW1_{12}$ by serum antibody is reduced when the pattern of glycosylation is altered.

3.3.4. Differential modification of $HMW1_{12}$ on the NTHi surface prevents agglutination in the presence of antibody

In previous work, we found that antibodies generated against HMW1+HMW2 promote strain-specific agglutination of NTHi (Chapter 2). We therefore sought to establish whether changes in protein glycosylation could influence the strain-specificity of antibody binding and consequent agglutination of NTHi strains. Agglutination of NTHi derivates of Rd/$HMW1_{12}$ was evaluated in the presence of serum generated against $HMW1_{12}$ or $HMW1_{5}$ (Figure 3.4). Agglutination was measured by flow cytometry and determined using mean forward scatter measurements (FSC-A) to determine average bacterial particle size (Habets et al., 2017) (Figure 3.4A). The formation of aggregates was confirmed by light microscopy (Figure 3.4B). As expected, strain Rd/$HMW1_{12}$

agglutinated very efficiently in the presence of serum derived against the strain 12 proteins but not the strain 5 proteins. In the absence of the HMW1C glycosyltransferase (Δ*hmw1C*), $HMW1_{12}$ is rapidly degraded and released from the surface of NTHi; therefore, this strain does not agglutinate due to a lack of surface adhesin (Joseph W. St Geme & Grass, 1998). The strain expressing $HMW1_{HMW1C}$ (+pHMW1C) showed a significant defect in agglutination in the presence of antibodies generated against $HMW1_{12}$+$HMW2_{12}$. The presence of functional HMW1 on the surface was confirmed by imaging bacterial adherence to Chang epithelial cells (Figure 3.4C). These data suggest that changes in the pattern of HMW1 modification, either through a loss or an increase of modification, influence the ability of antibodies to bind HMW1 and subsequently promote bacterial agglutination.

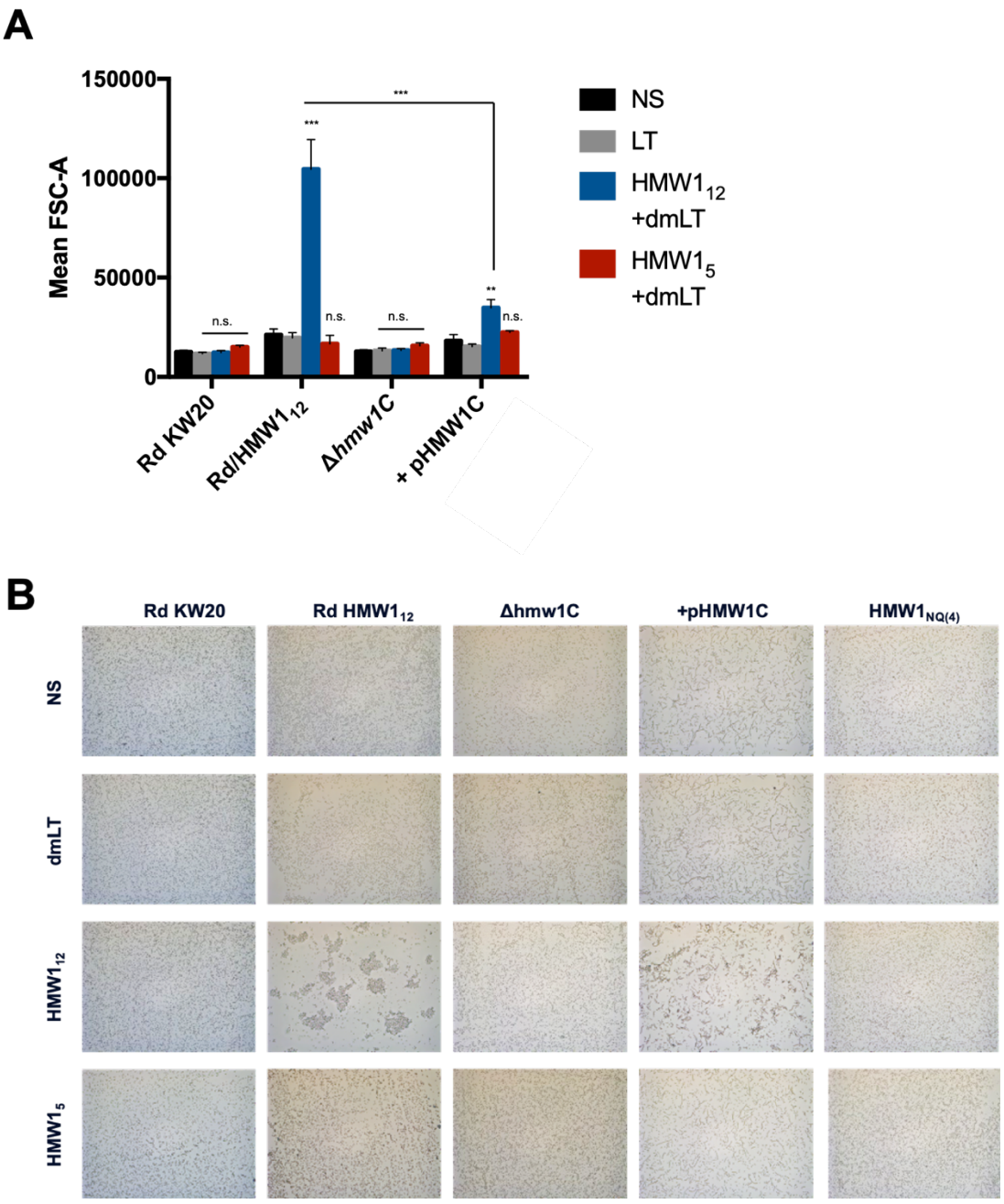
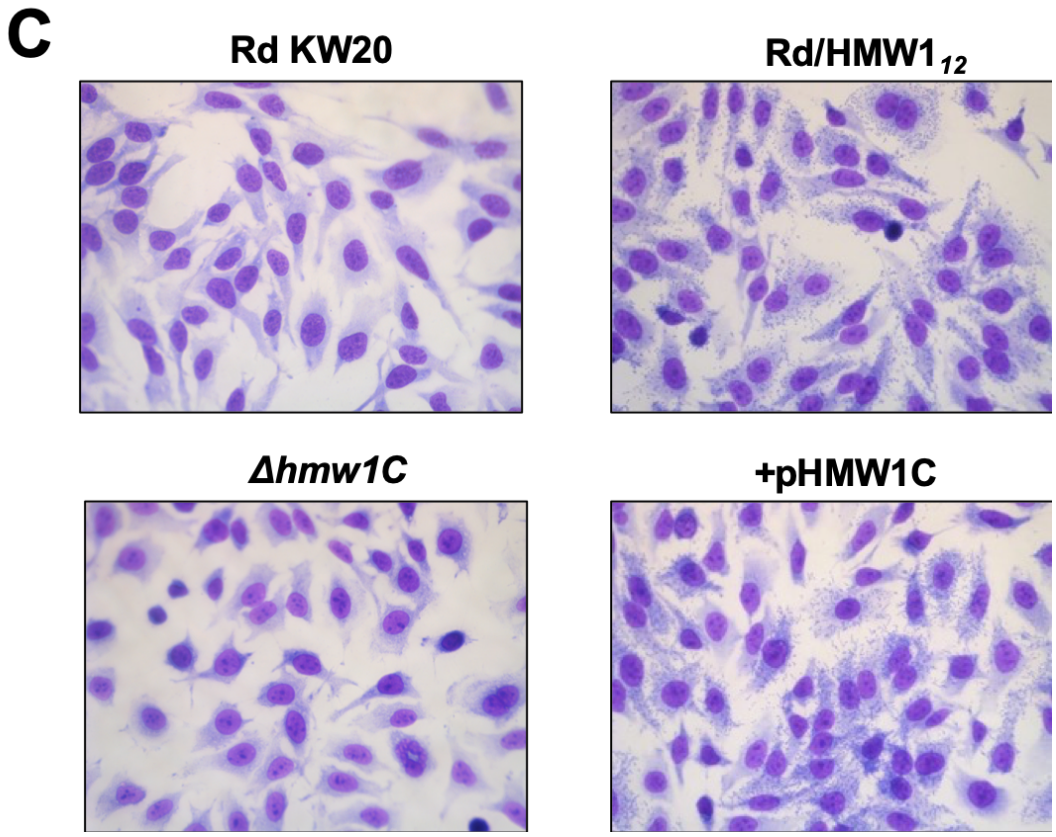


Figure 3. 4. Agglutination of NTHi by serum from immunized animals.
NTHi were incubated with pooled serum from groups of 5 mice immunized with HMW1+HMW2. (**A**) Agglutination was measured by flow cytometry and is represented by the mean FSC-A signal. NTHi were incubated with 2% serum from animals immunized with dmLT alone (gray), $HMW1_{12}$+dmLT (blue), or $HMW1_5$+dmLT (red). Data based on 50,000 events per sample. Error bars represent means ± SD from three independent experiments. Statistical significance determined using two-way ANOVA with Tukey's correction for multiple comparisons. Significance determined relative to dmLT serum condition unless indicated. n.s., Not Significant. * $p < 0.05$, ** $p < 0.01$, *** $p< 0.001$. (**B**) Representative images of agglutination imaged by light microscopy. (**C**) Bacterial adherence to epithelial cell monolayers was imaged following staining with Giemsa. All images shown at 400x total magnification. NS, No Serum.



3.4. Discussion

Bacterial glycoproteins are being recognized increasingly as important modulators of pathogen interactions with the host immune system. Glycan modifications of bacterial proteins often contribute to induction of immune responses. In many bacterial species, surface-exposed glycans are highly variable across strains, resulting in vast differences in intraspecies antigenicity. Consequently, glycosylation often confers serospecificity. In encapsulated bacterial species, including *E. coli* and *Streptococcus pnuemoniae*, the presence of distinct surface capsular polysaccharides results in hundreds of distinct serotypes. Similarly, glycosylation of flagellin in *Campylobacter* species is responsible for creating serotypes across strains and driving antibody reactivity (Alm et al., 1992; Szymanski et al., 1999). Differential glycosylation of HMW1

and HMW2 on the bacterial surface, both within and across strains, could similarly create broad serospecificity across NTHi strains.

An important consequence of serospecificity is evasion of host antibody responses. Antibodies generated in response to one strain are frequently ineffective against heterologous strains. Variation in glycosylation linkages or patterns can therefore allow different members of a species or population to evade recognition by host antibodies. In many cases, variation among bacterial glycans can also serve to create molecular mimicry of host glycans, promoting survival of commensal bacteria (Comstock & Kasper, 2006; Cusick et al., 2012; Poole et al., 2018). Following exacerbations of COPD driven by NTHi, adults tend to develop highly strain specific mucosal antibody responses, and these antibodies do not provide protection against exacerbations with newly acquired heterologous strains (Sethi et al., 2004). Similarly, the mucosal antibody response to NTHi nasopharyngeal colonization is thought to be highly strain specific, as antibodies generated against a colonizing strain do not mediate protection against colonization by a new strain (J. Pickering et al., 2014). In this work we demonstrate that NTHi expressing $HMW1_{12}$ agglutinate in the presence of $HMW1_{12}$ antibodies and that this response is strain specific. Expressing a hyperglycosylated variant of $HMW1_{12}$ allowed NTHi to evade agglutination by $HMW1_{12}$ antibodies, suggesting that changes to glycosylation prevent agglutination, despite no changes to the HMW1 amino acid sequence. Glycan variation, therefore, appears to serve as a mechanism of immune evasion for NTHi and likely contributes to the strain specificity of antibody-mediated agglutination.

Throughout this work, we demonstrate that differentially glycosylated $HMW1_{12}$ is antigenically distinct from $HMW1_{WT}$. Work studying HMW1 modification by HMW1C *ex vivo* has shown that systematic alterations in the relative amounts of HMW1 and

HMW1C quantitatively changes site-specific glycosylation of HMW1 (Elango & Schulz, 2020). Variable repeat elements identified in the *hmw1C* promoter may drive phase-variable expression of HMW1C, similar to the way that variation in the number of 7-base pair repeats in the promoter regions of *hmw1a* and *hmw2a* results in variation in levels of HMW1 and HMW2 (Giufrè et al., 2008). Taken together, these findings suggest that variation in the relative amounts of HMW1/HMW2 and glycosyltransferase could serve as a source of large variation of HMW1 and HMW2 modification across strains.

Changes in the glycosylation pattern of $HMW1_{12}$ appear to influence antibody binding even after chemical denaturation, suggesting that the source of altered recognition is independent of the structural changes related to glycosylation. This could indicate that immunogenic epitopes on HMW1 include the N-Glc fragments, alone or as parts of short glycopeptides. The immunogenicity of bacterial glycoproteins has been observed in other organisms. In *Mycobacterium tuberculosis*, O-linked mannosylation of the Apa complex is required for recalling immune responses in immunized guinea pigs; moreover, immune stimulation is dependent on both the presence of glycans and the extent of glycosylation, suggesting that the glycans play a critical role in shaping the immunogenicity of Apa (Horn et al., 1999; Romain et al., 1999). Changes to the extent of HMW1 glycosylation could therefore have an effect on recalling immune responses generated against $HMW1_{WT}$. Antibodies generated against different HMW1 or HMW2 proteins also exhibited differential binding to $HMW1_{WT}$ and the HMW1 glycosylation variants. This finding further suggests that glycosylation could influence both the antibody response generated and the ability of HMW1 to be recognized by antibody.

Notably, immunization with HMW1 alone or HMW2 alone was protective against nasopharyngeal colonization by heterologous strains of NTHi, despite the strain specificity of the antibody response. This result is consistent with observations described

in Chapter 2, where we demonstrated that heterologous protection is driven by a Th17 response rather than antibodies. It is possible that the glycosylation of HMW1 and HMW2 is important in shaping the specificity of the antibody response but does not influence the cell-mediated response. When antibodies against HMW1+HMW2 are the sole source of immunity against NTHi, mice are only protected against nasopharyngeal colonization by the homologous strain; similarly, depletion of IL-17A results in a loss of heterologous protection (Chapter 2). Significant differences in the mouse model of immunization and nasopharyngeal challenge may only be detected in a similar set of studies isolating antibody-driven immunity.

NTHi strains exhibit remarkable clonal diversity compared to encapsulated strains of *H. influenzae*. Because there is no polysaccharide capsule, much of the diversity stems from exposed surface antigens exhibiting high degrees of antigenic drift and recombination. Among NTHi surface antigens, most variation has been identified in the immunodominant epitopes of proteins, resulting in highly strain specific antibody (Sethi et al., 2004; Troelstra et al., 1994). Consequently, the vast antigenic variance of NTHi strains has been one of the greatest barriers to identifying candidate vaccine antigens. Our studies demonstrate a novel role for HMW1C-mediated glycosylation in further enhancing the antigenic diversity of NTHi strains and promoting evasion of antibodies. Given the promising potential of the HMW1 and HMW2 adhesins as vaccine candidates, the pattern of glycosylation may be an important factor to consider.

4. DETERMINANTS OF HMW1C GLYCOSYLTRANSFERASE SPECIFICITY

4.1. Introduction

The HMW1 and HMW2 high molecular weight proteins in NTHi are a family of adhesins that are secreted by a two-partner (TPS) secretion system. Each adhesin is produced by a homologous TPS locus, *hmw1abc* and *hmw2abc*. The *hmw1a/hmw2a* genes encode the TpsA proteins, the HMW1 and HMW2 adhesins, respectively. The *hmw1b/hmw2b* genes encode the TpsB proteins, the HMW1B and HMW2B outer membrane pore forming proteins (H. Li et al., 2007; Joseph W. St. Geme & Yeo, 2009). In addition to the primary TPS components, the *hmw1* and *hmw2* loci contain a third accessory gene, the *hmw1c* and *hmw2c* genes. These genes encode HMW1C and HMW2C, which are highly homologous glycosyltransferases that glycosylate HMW1 and HMW2, respectively (Grass et al., 2003a).

The HMW1C and HMW2C glycosyltransferases are cytoplasmic glycosyltransferases that mediate the asparagine (N)-linked glycosylation of nascently translated HMW1 and HMW2 in the cytoplasm. Modification occurs by sequentially adding UDP-glucose or UDP-galactose to multiple sites on nascent preproHMW1 in a sequential fashion, so that each glycosylation site is modified with either a mono- or di-hexose glycan rather than a complex polysaccharide (Grass et al., 2003a). Glycans are added onto asparagine residues at the conserved consensus site NXS/T, where “X” indicates any amino acid except proline (Gross et al., 2008). Based on mass spectrometry analyses of purified mature HMW1, there are at least 31 residues that are

modified with glucose (Glc) galactose (Gal), Glc-Glc, or Glc-Gal residues (Gross et al., 2008). The resulting modifications result in approximately 7-8kDa of the mature protein total mass.

Modification by HMW1C is required for normal HMW1 processing, secretion, and function. Elimination of HMW1C results in rapid breakdown of HMW1, indicating that glycosylation promotes stability and protects against premature degradation. Moreover, unmodified HMW1 that is not immediately degraded does not remain anchored to HMW1B and is rapidly released into the environment, suggesting that glycosylation is important for tethering (Grass et al., 2003a; Gross et al., 2008). As a result, production of HMW1 without HMW1C eliminates HMW1-mediated adherence.

Although HMW1C functions as an N-glycosyltransferase, homology analyses initially suggested that it was a member of the GT41 member of O-linked (O-GlcNAc) glycosyltransferases. However, GT41 O-GlcNAc transferases contain a characteristic tetratricopeptide repeat (TPR) fold at the N-terminus. HMW1C lacks the TPR fold and instead contains an N-terminal all α-helical domain (AAD) at the N terminus (Kawai et al., 2011). HMW1C is therefore considered the prototypic member of a novel family of cytoplasmic N-glycosyltransferases (McCann & St. Geme, 2014a).

HMW1C-mediated glycosylation differs significantly from other N-linked glycosylation systems identified in bacteria. Among the best-described bacterial N-linked glycosyltransferase systems is the *pgl* system, which was first identified in *Campylobacter jejuni* (Szymanski et al., 1999). In this system, UDP N-acetylglucosamine (GlcNAc) is converted into a branched oligosaccharide linked to a undecaprenyl phosphate (Und-P) lipid carrier. This process is mediated by a sequential

series of enzymes, including a C6 dehydratase (PglF), an aminotransferase (PglE), an acetyltransferase (PglD), followed by glycosyltransferases that extend the polysaccharide (PglC, PglA, PglJ, PglH, and PglI) (Glover et al., 2005, 2006; Schoenhofen et al., 2006; Wacker et al., 2002). The sugar chain is built in the cytoplasm and is then flipped into the periplasm. Once in the periplasm, the oligosaccharide chain is transferred *en bloc* to the NXS/T consensus sequence on over 60 different periplasmic and outer-membrane bound substrate proteins (Nothaft & Szymanski, 2013; Wacker et al., 2002). Thus, the *pgl* system serves as a general N-linked glycosylation pathway for many proteins. In contrast, HMW1C transfers UDP-hexoses and is the only enzyme involved, catalyzing both hexose-hexose and hexose-peptide bonds. Importantly, HMW1C-mediated glycosylation is not a general modification pathway. HMW1 is the only known target of HMW1C in NTHi, suggesting a remarkable degree of target specificity. Moreover, HMW1C appears to exhibit site-selection specificity, as only a subset of available NXST sequons are modified on HMW1.

In vitro, HMW1C is capable of modifying a variety of peptides containing the NXST sequon, including both peptides derived from HMW1 and peptides from other proteins (Naegeli et al., 2014). Moreover, heterologous expression of HMW1C in *E. coli* results in glycosylation of other proteins (Gawthorne et al., 2014). Together, these observations suggest that while HMW1C is capable of off-target modification, the specificity for HMW1 is tightly controlled in NTHi. The determinants of this specificity have thus far been undefined.

There are several potential factors that could shape the specificity of HMW1C for HMW1 in the NTHi cytoplasm. The genomic proximity of *hmw1a* and *hmw1c* could result in spatial proximity of HMW1C to HMW1, resulting in preferred modification of HMW1. HMW1 may also contain a yet-unidentified sequence that promotes HMW1C-driven

modification. Finally, the stoichiometry between HMW1C and available HMW1 substrate may restrict the ability of HMW1C to modify other proteins in the NTHi cell. In this study, we evaluated these possibilities and their influence on HMW1C modification. While there appears to be no clear sequence that is required for recognition of HMW1 by HMW1C, we found that the relative amount of available HMW1C in the NTHi cytoplasm influences target specificity, revealing a mechanism for target selection.

4.2. Materials and Methods

4.2.1 Bacterial Strains and Culture.

The bacterial strains used in the present study are described in Table 4.1. NTHi strains were grown on chocolate agar (BD Biosciences, San Jose, CA, USA) or brain heart infusion (BHI) agar supplemented with 0.1% (vol/vol) lysed horse blood as a source of hemin and 3.5 μg/ml NAD, referred to as supplemented BHI (BHIs). 2μg/ml chloramphenicol was added as appropriate. Agar plates were incubated overnight at 37°C with 5% CO_2. Liquid cultures were grown at 37°C with agitation (250rpm).

Table 4. 1. Bacterial strains used in Chapter 4

Strain	Relevant genotype or description	Source or reference
Nontypeable H. influenzae		
12	Nontypeable *H. influenzae* strain, produces HMW1 and HMW2	Clinical isolate, middle ear fluid, acute otitis media
12Δ*hmw1C*Δ*hmw2C*	12 *hmw1c::kan hmw2c::cam*, produces neither HMW1C nor HMW2C	(Grass et al., 2003b)
12Δ*hmw1C*	12 *hmw1c::kan*, produces HMW2C	(Grass et al., 2003b)
12Δ*hmw2C*	12 *hmw2c::cam,* produces HMW1C	(Grass et al., 2003b)
Rd KW20 (Rd)	Laboratory strain, formerly serotype d, lacking *hmw* genes and non-adherent *in vitro*	(Fleischmann et al., 1995)
Rd/HMW1	Derivative of strain Rd KW20 that contains the intact *hmw1* locus and produces HMW1	(S J Barenkamp & Leininger, 1992)
Rd/HMW1$_{HapSS}$	Derivative of Rd/HMW1 that expresses HMW1 with the typical signal peptide of the Hap adhesin	This study
Rd/HMW1 *hmw1c::kan*	Derivative of Rd/HMW1 that contains the *hmw1* locus with a kanamycin cassette inserted at the *Xho*I site in the chromosomal copy of *hmw1C*	(Grass et al., 2003b)
Rd/HMW1 *hmw1C::kan* +pHMW1C	Derivative of Rd/HMW1Δ*hmw1c* Δ*recA* that contains pACYC184 *hmw1c*	This study

4.2.2. Strain construction.

Variants of Rd/HMW1 were generated by first constructing *hmw1* variants on plasmids in *E. coli*. Plasmids were isolated and linearized by restriction digest. Linearized DNA containing the variant *hmw1* were then transformed into Rd/HMW1 using the MII/MIV method of NTHi transformation (Steinhart & Herriott, 1968). To select for positive transformants, a kanamycin resistance casette (*kan*) was included in the construct in the SpeI restriction site, upstream of *hmw1a*. To introduce plasmids into Rd/HMW1, the recombinase mutation *recA* was introduced, followed by transformation of intact plasmid by MII/MIV (Stuy, 1989).

4.2.3. Cell lines.

The cell lines used in this study were obtained from the American Tissue Culture Collection (ATCC). Cells were maintained at 37°C with 7.5% CO_2. Chang cells (human conjunctiva; ATCC CCL-20.2) were maintained in Eagle minimal essential media (MEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% nonessential amino acids (NEAA) and 10% fetal calf serum (FCS).

4.2.4. Purification of HMW1.

HMW1 was purified from the NTHi surface as described previously (Rempe et al., 2016). NTHi mutants expressing HMW1 or HMW1 + pHMW1C were inoculated into 10 liters of BHIs broth and grown 12-14 hours shaking at 37°C with antibiotics as appropriate. The culture was then centrifuged for 20 min at 8000 rpm, the supernatant was discarded, and the pellet was frozen at −80°C overnight. To release HMW1 from the bacterial surface, the frozen pellet was thawed on ice and resuspended in 200 ml of

extraction buffer (0.5 M NaCl, 10 mM EDTA, 10 mM Tris pH 7.5, 50 μM 1,10-phenanthroline) in the presence of protease inhibitors (Roche, Basel, Switzerland). The resuspended bacteria were incubated at 4°C for 1 hr and then centrifuged for 15 min at 8500 rpm. The supernatant containing the released HMW1 was saved, and the pellet was discarded. The supernatants were dialyzed overnight into 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), 75mM NaCl pH 6.0 and then stepwise dialyzed in MES with 250mM NaCl, in MES with 125mM NaCl, and then in 80mM NaCl. Dialyzed protein was loaded onto a Resource S cation exchange chromatography column (GE Life Sciences, Marlboro, MA, USA). Bound protein was eluted with 20 mM MES, 1 M NaCl pH 6.0. Fractions containing HMW1 were combined, concentrated, and then loaded onto a HiLoad SuperDex 16/60 200pg size-exclusion column (GE Life Sciences) equilibrated with 20 mM MES, 150 mM NaCl pH 6.0, 5% glycerol. The fractions containing HMW1 were pooled, resolved on SDS-PAGE gels, and stained with Coomassie blue to ensure purity.

4.2.5. Western blotting.

Proteins were transferred from SDS-PAGE gels to nitrocellulose membranes and blocked using 5% nonfat milk in PBS for 30 minutes at room temperature. Membranes were then incubated overnight with guinea pig antiserum generated against mature HMW1 (GP104) or HMW1C (GP64) at a 1:2000 dilution. Following incubation, membranes were washed with TBS +0.1% Tween. Membranes were then incubated with an anti-guinea pig IgG horseradish peroxidase conjugate at a 1:5000 dilution for 30 minutes at room temperature, washed, and developed.

4.2.6. In-gel protein digestion.

Coomassie stained samples were excised from gels, cut into 1 mm cubes destained with 50% methanol/1.25% acetic acid, reduced with 5 mM Dithiothreitol (Thermo Fisher Scientific San Jose, CA), and alkylated with 20 mM iodoacetamide (Sigma) (Shevchenko et al., 1996).(Gel pieces were then washed with 20 mM ammonium bicarbonate (Sigma) and dehydrated with acetonitrile (Fisher). Trypsin (Promega) (5ng/mL in 20 mM ammonium bicarbonate) was added to the gel pieces, and proteolysis was allowed to proceed overnight at 37oC. Peptides were extracted with 0.3% triflouroacetic acid and then 50% acetonitrile. Extracts were combined, and the volume was reduced by vacuum centrifugation.

4.2.7. Mass spectrometry analysis.

Tryptic digests were analyzed by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) on a hybrid LTQ Orbitrap Elite mass spectrometer (Thermo) coupled with a nanoLC Ultra (Eksigent). Peptides were separated by reverse phase (RP)-HPLC on a nanocapillary column, 75 μm ID × 15 cm Reprosil-pur 3 um, 120A (Dr. Maisch, Germany) in a nanoflex chip system (Eksigent). Mobile phase A consisted of 1% methanol (Fisher)/0.1% formic acid (Thermo), and mobile phase B consisted of 1% methanol/0.1% formic acid/80% acetonitrile. Peptides were eluted into the mass spectrometer at 300 nL/min, with each RP-LC run comprising a 90 minute gradient from 10 to 25% B in 65 min, 25 to 40% B in 25 min. The mass spectrometer was set to repetitively scan m/z from 300 to 1800 (R = 240,000 for LTQ-Orbitrap Elite) followed by data-dependent MS/MS scans on the twenty most abundant ions, with a minimum signal of 1500, dynamic exclusion with a repeat count of 1, repeat duration of 30s, exclusion size of 500 and duration of 60s, isolation width of 2.0, normalized collision

energy of 33, and waveform injection and dynamic exclusion enabled. FTMS full scan maximum fill time was 500 ms, and ion trap MSn fill time was 50 ms; microscans were set at one. FT preview mode, charge state screening, and monoisotopic precursor selection were all enabled with rejection of unassigned and 1+ charge states.

4.2.8. Spectra identification.

The tandem mass spectra were extracted using ProteoWizard (v3.0.5047). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using SEQUEST (Thermo Fisher Scientific; version 1.0). SEQUEST was set up to search the UniProt *Haemophilus influenzae* proteome appended with common contaminants, assuming a full tryptic digestion with the possibility of two missed cleavages. SEQUEST was searched with a fragment ion mass tolerance of 1 Da and a parent ion tolerance of 15 ppm. S-carbamamidomethyl of cysteine was specified in SEQUEST as a fixed modification. Oxidation of methionine and hexose on asparagine were specified in SEQUEST as variable modifications. Results were visualized using Scaffold (Proteome Software, Inc). SIgnalP was used to identify all potential glycosylated sequons in mature HMW1 (Technical Universtiy of Denmark).

4.2.9. Bacterial Adherence.

Quantitative adherence assays were performed as described previously (Rempe et al., 2016). Approximately $1.8x10^5$ cells were seeded into 24-well tissue culture plates and incubated overnight. Epithelial monolayers were inoculated with ~$2x10^7$ CFU of NTHi in the presence or absence of immune serum, and the plates were centrifuged at

165 x *g* for 5 min to facilitate contact between the bacteria and the epithelial cells. After incubation for 30 min at 37°C in a 5% CO_2 atmosphere, monolayers were rinsed with PBS to remove non-adherent bacteria. Trypsin-EDTA (0.25% trypsin, 0.5% EDTA) (Sigma) was added to the wells to release epithelial cells and adherent bacteria. Dilutions of adherent organisms were plated on BHIs agar and incubated overnight to determine the number of adherent bacteria per monolayer. To image epithelial cell monolayers, cells were seeded onto glass coverslips. Percent adherence was calculated as the number of adherent CFU in relation to the total inoculum CFU per strain.

4.2.10. Statistical analysis.

Data were analyzed in GraphPad Prism (version 8.0) software (GraphPad Software, Inc, La Jolla, CA). Statistical significance was determined using two-way Analysis of Variance (ANOVA), the Tukey-Kramer nonparametric test, and Student's t tests when appropriate.

4.3. Results

4.3.1. The chromosomal proximity of *hmw1a* and *hmw1c* is not required for interactions between HMW1 and HMW1C.

To evaluate the role of genomic proximity between *hmw1a* and *hmw1c* in targeting, we took advantage of the fact that *hmw1abc* and *hmw2abc* are located in conserved, but unlinked locations in the NTHi chromosome (Buscher et al., 2004). We examined the ability of HMW2C to modify HMW1 by generating a derivative of NTHi strain 12 in which *hmw1c* is insertionally inactivated. Under these conditions, HMW1 can

only be modified by HMW2C, which is highly homologous and functionally equivalent to HMW1C (McCann & St. Geme, 2014a). We determined whether HMW1 was modified based on HMW1-dependent NTHi adherence to Chang cell monolayers. If HMW1 is not modified by HMW1C, the adhesin is rapidly degraded and released from the cell surface, resulting in nonadherent bacteria (Joseph W. St Geme & Grass, 1998). As shown in Figure 4.1, expression of HMW1C or HMW2C is sufficient to produce functional HMW1, suggesting that modification occurs. This result indicates that *hmw1c* does not need to be expressed in the same genetic locus as *hmw1a* in order to modify HMW1.

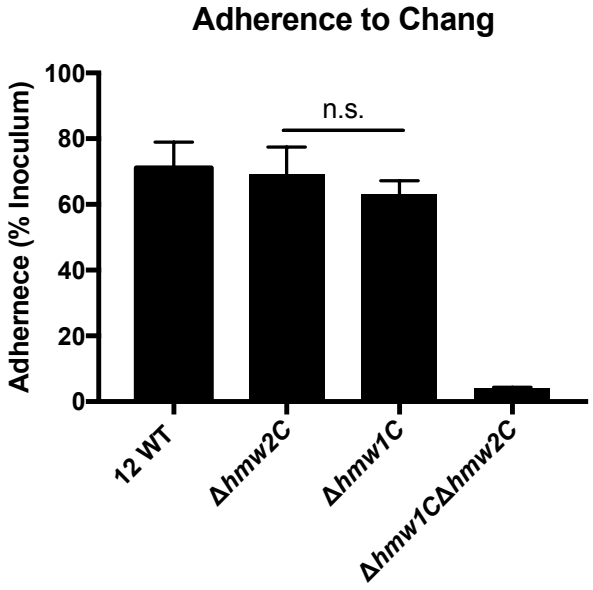


Figure 4. 1. Chromosomal proximity of hmw1a and hmw1c is not required for HMW1 modification. Bacteria were grown in liquid BHIs and inoculated onto monolayers of Chang epithelial cells. Adherent bacteria are represented as the recovered CFU percentage of the inoculum. Inoculums were approximately $2x10^7$ CFU/mL. Statistical significance determined from 3 independent experiments using two-way ANOVA. n.s., not significant.

4.3.2. Targets of HMW1C-like glycosyltransferases do not contain a clear shared motif.

To examine the possibility that HMW1 contains an unidentified sequence to promote recognition and modification by HMW1C, we compared amino acid sequences of known targets of HMW1C and related glycosyltransferases. Figure 4.2A lists identified N-glycosyltransferases homologous to HMW1C and their target proteins. Based on the amino acid sequences of the target proteins, there are no regions of significant homology across the four target proteins that could be indicative of a recognition sequence (Figure 4.2B). It is therefore unlikely that HMW1C and related glycosyltransferases use a shared sequence motif to achieve specificity for their target proteins.

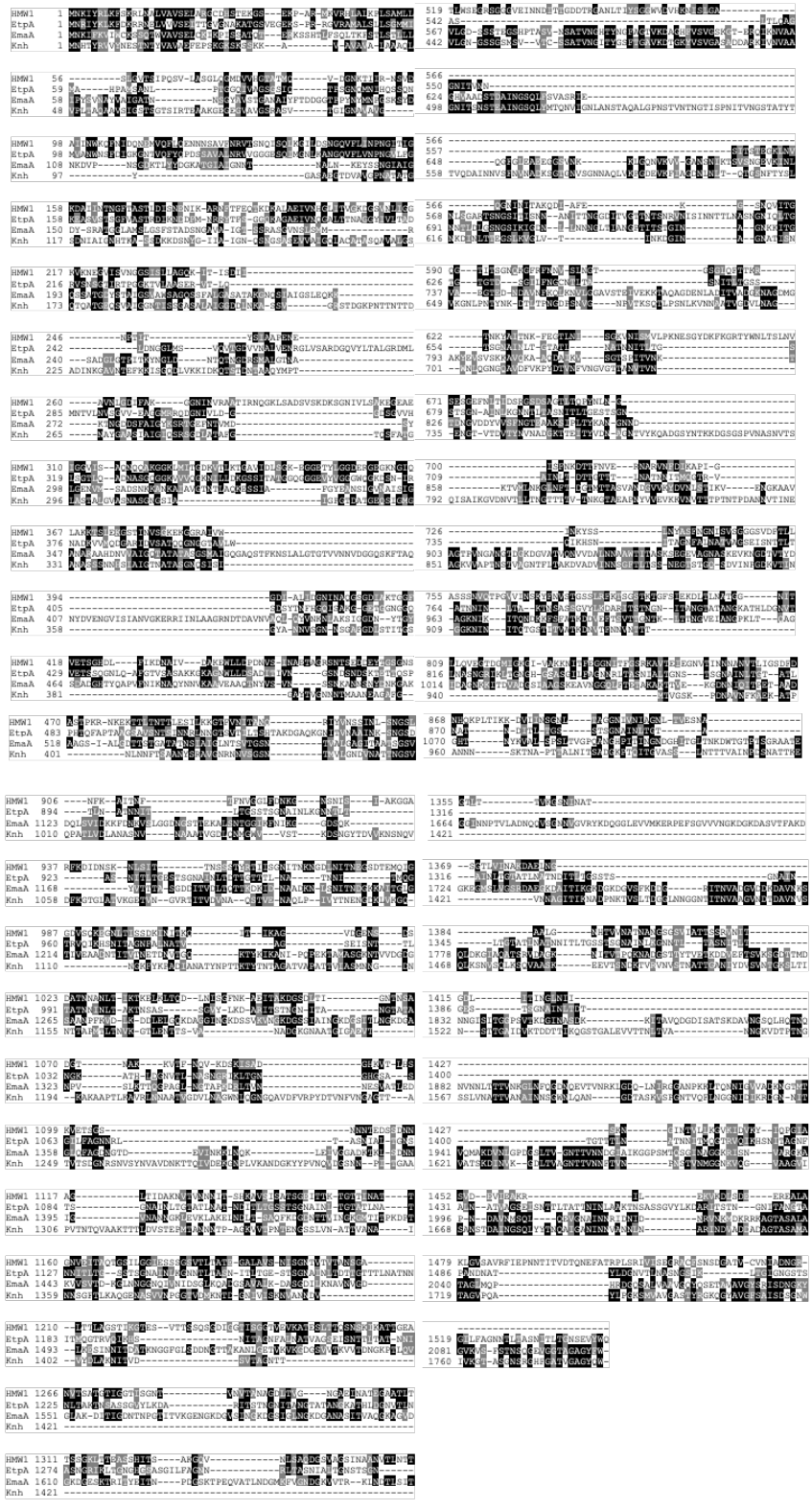
A

Organism	Glycosyltransferase	Target
Nontypeable *Haemophilus influenzae* (NTHi)	HMW1C	HMW1
Enterotoxigenic *Escherichia coli* (ETEC)	EtpC	EtpA
Aggregatibacter aphrophilus	$HMW1C_{Aa}$	EmaA
Kingella kingae	$HMW1C_{Kk}$	Knh

.

Figure 4. 2. HMW1 homologues do not contain a shared motif.
(A) Homologues of HMW1C in other bacterial species were identified using BLAST search (NCBI). Target proteins were identified and confirmed based on genetic context and through literature searches (B) Amino acid sequence alignment proteins modified by HMW1C-like proteins.

B



4.3.3. The HMW1 extended signal peptide is not required for HMW1C-mediated glycosylation.

Many TpsA proteins, including HMW1, are first translated with extended, atypical signal peptides to aid in secretion through the Sec-dependent pathway. The HMW1 signal peptide is 68 amino acids long, compared to the average length of approximately 25 amino acids seen across secreted proteins in gram-negative bacteria. Extended signal peptides, seen frequently in autotransporter proteins, have been shown to slow down export and delay signal peptide cleavage, presumably to prevent non-productive folding of the passenger domain in the periplasm (Guérin et al., 2017b; Szabady et al., 2005). We therefore sought to determine whether the extended signal peptide of HMW1 contributes to recognition and modification by HMW1C. To this end, we generated a variant of HMW1 in which the atypical signal peptide has been replaced with a shorter 25aa-signal peptide from the NTHi adhesin Hap. NTHi expressing this variant of HMW1, $HMW1_{HapSS}$, produced wild-type levels of mature HMW1 (Figure 4.3A), and were able to mediate full levels of adherence to Chang epithelial cells (Figure 4.3B).

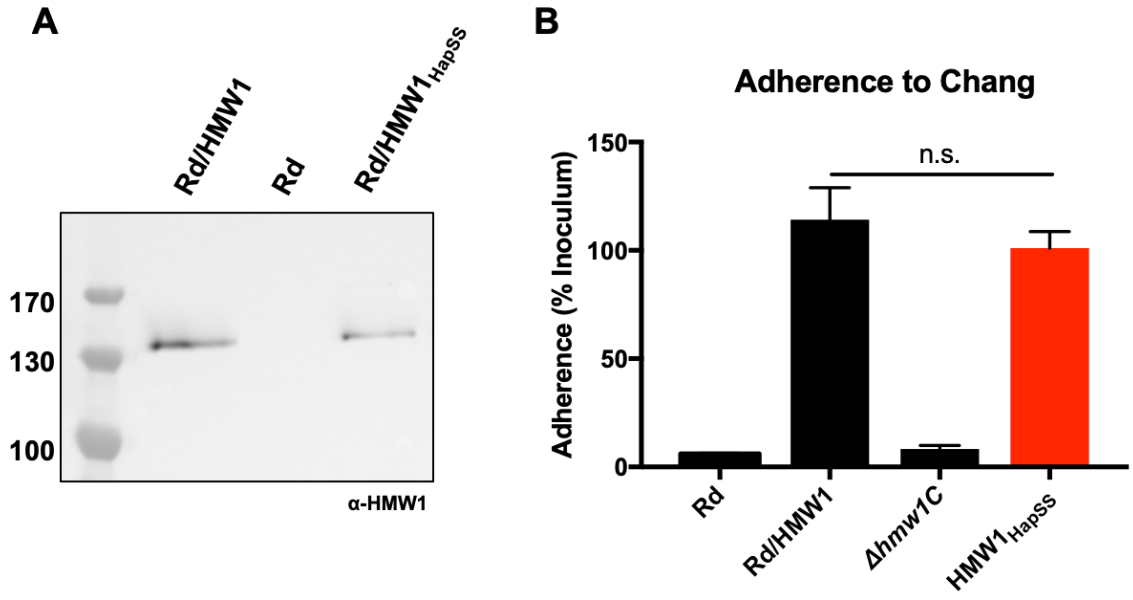


Figure 4. 3. The HMW1 signal peptide is not required for glycosylation.
The extended HMW1 signal peptide was replaced with the signal peptide from Hap (A). Western blot of whole cell sonicates is shown using guinea pig serum generated against mature HMW1. (B) Adherence of bacterial strains to Chang epithelial cell monolayers. Adherent bacteria represented as the recovered CFU percentage of the inoculum. Statistical significance determined using two-way ANOVA. n.s., not significant. Representative image shown.

4.3.4. The HMW1 propiece is not required for glycosylation by HMW1C.

When nascent HMW1 is produced in the cytoplasm, it is a preproprotein with two targeting regions: the signal peptide, corresponding to amino acids 1-68, and the propiece, corresponding to residues 69-441 (Yeo et al., 2007b). The signal peptide directs the adhesin into the periplasm, and the propiece contains the TpsA secretion domain and is responsible for facilitating interactions with the HMW1B outer membrane pore during secretion (Grass et al., 2015). The propiece could play an additional role in serving as a docking site for HMW1C in the cytoplasm. To investigate this possibility, we examined whether the propiece (PP) is required in the NTHi cytoplasm in order for HMW1C-mediated glycosylation to occur. Deletion of the propiece ($HMW1_{\Delta PP}$) resulted

in production of mature HMW1, with no difference in size compared to wild-type protein in whole cell sonicates, suggesting this variant is glycosylated comparably to wild type HMW1 (Figure 4.4). Moreover, inactivation of *hmw1C* in a strain producing $HMW1_{\Delta PP}$ caused a reduction in protein size, indicating a loss of glycosylation. This result suggests that in the presence of HMW1C, $HMW1_{\Delta PP}$ contains sufficient sequence for recognition by HMW1C and modification. Therefore, the propiece is not required for modification of HMW1 by HMW1C.

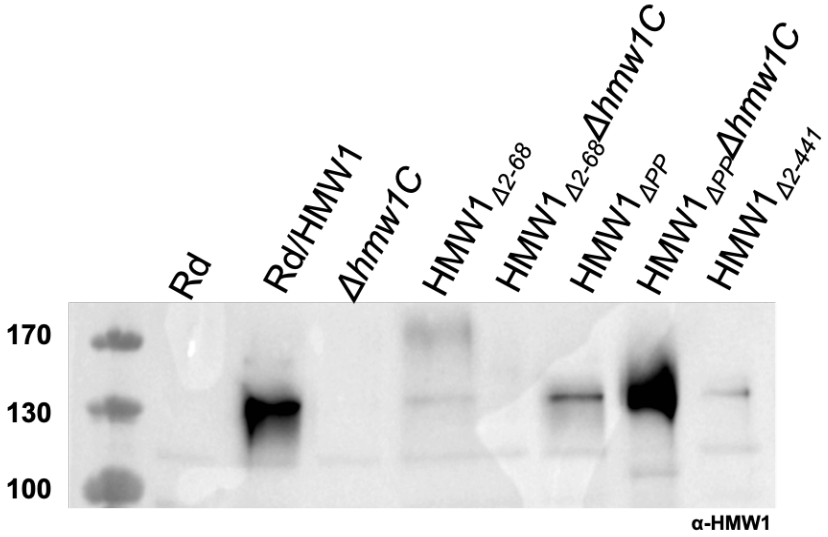


Figure 4. 4. The HMW1 propiece is not required for glycosylation.
Glycosylation of HMW1 was evaluated by comparing HMW1 size in Rd/HMW1 variants with or without HMW1C expression. Western blot of whole cell sonicates is shown using guinea pig serum generated against mature HMW1. Strains shown express wild type HMW1 (Rd/HMW1), HMW1 with a signal peptide deletion (Δ2-68), a propiece deletion (ΔPP), a combination signal peptide-propiece deletion (Δ2-441), and corresponding HMW1C knockouts of these strains. Representative image shown.

4.3.5. HMW1C levels influence target protein specificity.

To determine whether the relative amounts of HMW1 or HMW1C influence target specificity, we generated a variant of Rd/HMW1 in which HMW1C is overexpressed on a plasmid (pACYC *hmw1C*), thereby significantly increasing the amount of cytoplasmic HMW1C while keeping the *hmw1a* copy number constant. Overexpression of HMW1C

was confirmed through Western blot (Figure 4.5A). LC-MS/MS analysis of whole cell sonicates was used to evaluate the presence of single and di-hexose residues across all proteins. Based on the total number of identified hexose spectra, more modified peptides were identified in the strain overexpressing HMW1C compared to the wild type Rd/HMW1 (Figure 4.5B). This suggests that when more HMW1C is available, more hexose residues are placed onto proteins in NTHi. To evaluate whether these additional modifications were restricted to HMW1, peptides of HMW1 were excluded. This resulted in a loss of most spectra identified in Rd/HMW1, but many were still identified in the HMW1C overexpressing strain. The differences between the two strains after HMW1 exclusion indicates that modified peptides belong to proteins other than HMW1. A selection of proteins identified to contain novel hexose residues in the HMW1C overexpressing strain are listed in Table 4.2. All proteins were modified at the expected NXST sequon. Modified proteins include outer membrane proteins as well as cytoplasmic proteins. These findings indicate that when the available amount of HMW1C is increased in the NTHi cytoplasm, off-target proteins gain hexose or di-hexose modification, representing a loss of stringency in target specificity.

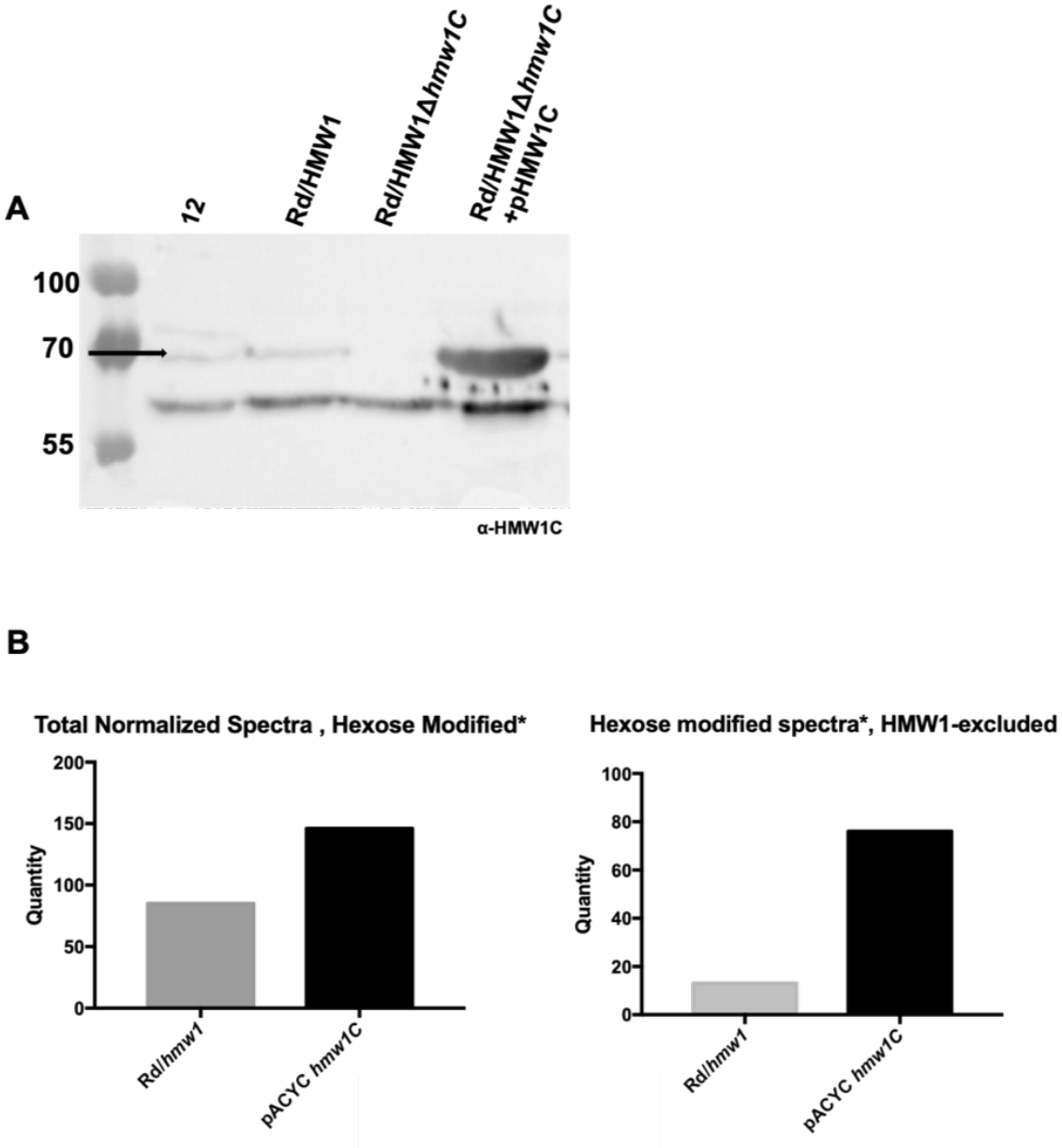


Figure 4. 5. HMW1C levels alter target protein specificity.
(A) Western blot evaluating relative HMW1C expression levels. Whole cell sonicates of NTHi strains expressing various levels of HMW1C. Strain 12 expresses the wild type *hmw1abc* locus and wild type levels of HMW1C. Rd/HMW1 expresses the *hmw1abc* locus from strain 12 and similar HMW1C levels. Rd/HMW1 Δ*hmw1C* expresses no HMW1C. Rd/HMW1Δ*hmw1C*+pHMW1C expresses HMW1C on a plasmid, resulting in overexpression. Black arrow indicates HMW1C. (B) Number of unique spectra modified with a single or di-hexose as identified by mass spectrometry in Rd/HMW1 and Rd/HMW1Δ*hmw1C*+pHMW1C. Data were normalized to the total number of identified proteins in each strain.

Table 4. 2. Hexose-modified proteins in Rd/HMW1Δhmw1C +pHMW1C

Protein	Gene
Outer Membrane	
HMW1	*hmw1a*
Heme Binding Protein HxuA	*hxuA*
Immunoglobulin A1 protease transporter	*igaA*
Outer membrane translocation module TamB	*tamB*
Outer membrane protein P2	*omp2*
Adhesion and penetration protein Hap	*hap*
Cytoplasmic	
tRNA modification enzyme MnmG	*mnmG*
Galactose import ATP-binding protein MglA	*mglA*
UvrABC nucleotide excision protein A	*uvrA*
Chromosomal replication intiator protein DnaA	*dnaA*
Tyrosine-tRNA ligase	*tyrS*
Formate acetyltransferase 1	*pflB*
N-acetylglucosamine-6-phosphate deacetylase	*nagA*
Cell division protein FtsP	*ftsP*

4.3.6. HMW1C levels influence NXST site selection.

In addition to influencing the specificity of target protein selection, we found that overexpression of HMW1C in the NTHi cytoplasm altered selection of specific NXST sites in HMW1. Under wild type conditions, only a subset of all available sequons are modified on HMW1. Compared to HMW1 produced by the wild type locus, HMW1 modified by an excess of HMW1C was glycosylated at a greater number of sequons across the length of the mature protein (Figure 6). This indicates that HMW1C levels in the NTHi cytoplasm influences the extent of modification on HMW1.

Position	Sequon	Rd/HMW1	pHMW1C
120	NNSA	N/C	N/C
183	NFTF	N/C	N/C
380	NVSG	N/C	N/C
444	NVSI	X	-
456	NTSE	-	-
484	NTTL	X	X
498	NITA	X	X
508	NSSI	-	X
512	NLSN	X	N/C
515	NGSL	-	N/C
546	NLTI	X	X
560	NISL	X	N/C
570	NITA	X	N/C
605	NVSL	N/C	X
609	NGTG	N/C	X
636	NISG	X	X
642	NISM	X	X
650	NESG	-	-
664	NLTS	-	X
669	NVSE	-	X
677	NLTI	-	N/C
740	NISV	-	X
773	NVST	X	X
801	NATG	X	N/C
806	NITL	X	X
828	NITF	X	X
835	NITF	X	X
850	NVTI	-	N/C
858	NVTL	-	X
885	NLTA	-	X
898	NLTV	-	X
912	NFTF	X	N/C
928	NISI	X	N/C
946	NLSI	X	X
952	NSSS	N/C	X
964	NITN	N/C	X
973	NITN	N/C	X
995	NLTI	X	X
1004	NITK	X	N/C
1029	NLTI	X	X
1044	NISG	X	N/C
1107	NNTE	-	-
1125	NVTV	-	X
1131	NITS	X	X
1156	NATT	X	X
1195	NISG	-	X
1266	NVTS	-	N/C
1284	NVTA	-	N/C
1301	NATE	-	N/C
1332	NLSA	-	X
1348	NVTL	X	X
1352	NTTG	N/C	N/C
1366	NATS	N/C	N/C
1388	NHTV	X	N/C
1393	NATN	N/C	X
1398	NGSG	N/C	X
1412	NITG	-	-
1491	NNTI	-	X

Figure 4. 6. HMW1C levels alter NXST site selection on HMW1.
All possible NXST sequons are listed by Asn amino acid position and sequon description. Glycosylated asparagine residues are indicated with “X” and residues which were not glycosylated are indicated as “-“. N/C indicates no coverage, as the peptide containing this residue was not identified in the mass spectrometry run. Data based on 1 experiment.

4.4. Discussion

In this study, we demonstrate a critical role for the relative amounts of HMW1 and HMW1C in driving target specificity in NTHi. When cytoplasmic amounts of HMW1C were increased through increased copy number of the *hmw1c* gene, we observed increased numbers of glycan modified sites on off-target proteins. This suggests that the ratio of HMW1C:HMW1 in NTHi is controlled to allow for sufficient HMW1 modification while limiting unwanted off-target glycosylation. While we artificially increased HMW1C expression using a plasmid, alterations in the relative amounts of available HMW1C could change naturally in NTHi. Notably, both HMW1 and HMW1C contain variable repeat numbers in their promoter regions. In HMW1, the copy number of a 7-bp has been directly shown to influence amounts of HMW1 (Suzanne Dawid et al., 1999; Elango & Schulz, 2020). It is therefore possible that stochastic changes in HMW1 production are met with changes in HMW1C in order to maintain the stoichiometric relationship.

In addition to influencing target protein specificity in the NTHi cytoplasm, we found that overproduction of HMW1C impacts NSXT site selection on HMW1. Compared to HMW1 modified by a single chromosomal copy of *hmw1c*, HMW1 modified by excess HMW1C was modified at more sites. This result is consistent with work evaluating site selection of HMW1C *ex vivo*, which has shown that as more HMW1C is available, HMW1 site occupation and glycosylation efficiency increases (Elango & Schulz, 2020). The pattern of HMW1 modification, resulting from NXST site selection, can greatly influence the structure of the mature protein. For example, when HMW1 is modified by EtpC expressed in *E. coli*, the differentially modified protein shows a significantly altered

structure. Based on circular dichroism analysis, HMW1C-modified HMW1 adopts a structure that is 1.86% α-helix and 49.03% β-sheet, while EtpC-modified HMW1 is 52.46% α-helix and 9.17% β-sheet. Consequently, purified EtpC-modified HMW1 has a reduced ability to block HMW1-mediated adherence to Chang cells compared to HMW1C-modified protein (Rempe and St. Geme III, unpublished data). Further, data presented in Chapter 3 of this work suggest that the pattern of HMW1 glycosylation can influence HMW1 immunogenicity and NTHi interactions with the host immune system. Collectively, these observations highlight the importance of both the presence and nature of HMW1 glycosylation and emphasize the need to understand the interaction between HMW1 and HMW1C.

We were unable to define a clear motif or region that influences specificity of HMW1C for HMW1 in the NTHi cytoplasm. Replacing the signal peptide with a shorter variant did not appear to influence glycosylation of mature HMW1. From this finding, we can conclude that there is no signaling sequence of motif included in the extended signal peptide. Further, it suggests that any additional time that preproHMW1 is retained in the cytoplasm by the extended signal peptide is not critical for facilitating HMW1C-mediated glycosylation. Similarly, the propiece of HMW1 was not critical for glycosylation, suggesting that the propiece may solely serve to facilitate targeting to HMW1B in the periplasm.

Sequence comparisons of similar targets of HMW1C-like proteins did not reveal any significant regions of homology that could be indicative of a motif. The specificity of these glycosyltransferases for their native targets is therefore unlikely to be determined by a specific sequence of amino acids. However, this does not rule out the possibility that HMW1 carries a targeting region for HMW1C in the mature adhesin. Many proteins

find their substrates using structural motifs, raising the additional possibility that HMW1C and related proteins may recognize their targets based on structural fit. For example, among proteins containing the TPR repeat, including traditional members of the GT41 O-GlcNAc glycosyltransferases, the repetitive folds can form an amphipathic channel that accommodates the complementary region of a target protein, conferring specificity (Blatch & Lässle, 1999; Odunuga et al., 2003). Rather than a clear sequence directing specificity, complementary structural components of HMW1C or HMW1 could contribute to target specificity. Thus, there is a need for greater structural analyses of the HWM1C family and their specific target proteins.

HMW1C-like glycosyltransferases have been found across many bacteria, including the *Pastuereallaceae*, *Enterobacteriaceae*, *Neisseriaceae*, and *Burkholderiaceae* families (McCann & St. Geme, 2014a). In many cases, the glycosyltransferase is found in the same locus as a predicted TPS system. Examples include EtpC in ETEC, which modifies the adhesin EtpA, and RscC in *Yersinia enterocolitica*, which modifies the RscA adhesin (McCann & St. Geme, 2014a; Nelson et al., 2001). Glycosylation of EtpA by EtpC appears to influence adhesive interactions with host cells, as nonglycosylated EtpA is less adherent to Caco-2 intestinal epithelial cells but hyperadherent to HCT-8 intestinal cells when compared to glycosylated EtpA (Fleckenstein et al., 2006). Much like HMW1C, these glycosyltransferases are predicted to solely modify a target protein in the adjacent TPS locus, and it is unclear how these enzymes achieve target specificity. The insights that we have derived into the mechanisms that guide HMW1C to its target protein can therefore inform glycosylation and consequently adhesin function mechanisms across many species.

5. CONCLUSIONS & FUTURE DIRECTIONS

5.1. Conclusions

Throughout this work, we have highlighted the capacity of the HMW1 and HMW2 proteins to serve as potent vaccine antigens against nontypeable *Haemophlius influenzae* (NTHi). In Chapters 2 and 3, we demonstrate that in a mouse model of nasopharyngeal colonization, immunization with these adhesins is immunostimulatory and protective against heterologous strains. Further, we presented evidence that intranasal immunization may be a preferable route of immunization. Through our investigation into the immunogenicity of HMW1 and HMW2, we observed important immunological differences between HMW1 and HMW2 within and across strains, which are likely related to HMWC-mediated glycosylation of these adhesins. Subsequently in Chapter 4 we revealed insights into the mechanism of HMW1C function and specificity. These findings have important implications for developing HMW1 and HMW2 as NTHi vaccine antigens and more broadly understanding how NTHi interacts with the host immune system. The future directions outlined in this chapter expand on our work studying HMW1 and HMW2 as vaccine components, in both the mouse model and a rhesus macaque model. Additionally, this chapter describes further work exploring the mechanism of HMW1C-mediated glycosylation.

5.2. Combination antigen vaccines for protection against NTHi nasopharyngeal colonization

In Chapter 2 of this work, we characterized the ability of the HMW1 and HMW2 adhesins to stimulate protective immunity against nasopharyngeal colonization by

heterologous HMW-expressing strains of NTHi. The *hmw1* and *hmw2* loci are found in approximately 80% of NTHi isolates (Rodriguez et al., 2003). While this suggests that immunization with HMW1 and HMW2 would provide significant coverage against a majority of strains, our findings suggest that there is no protection against strains that do not express these adhesins, including strain NT127. In the approximately 20% of isolates that do not carry the *hmw1* and *hmw2* loci, the primary adhesin is Hia. In order to close the gap in protection and achieve more complete immunity, we could attempt to immunize animals with both the HMW adhesins and Hia. Evidence in animals suggest that Hia is immunogenic. In chinchillas, immunization with recombinant Hia stimulates Hia-specific IgG that enhanced opsonophagocytosis of heterologous Hia-expressing strains; however, the opsonophagocytic titer for the homologous strain was significantly higher than the titer for heterologous strains, suggesting some degree of strain-specificity in the antibody response (Winter & Barenkamp, 2009, 2014). Like HMW1 and HMW2, it's possible that immunization with Hia stimulates Th17-driven immunity and could therefore be capable of mediating protection against many Hia-expressing strains.

In our mouse model of intranasal immunization and colonization, the protection afforded by immunization with HMW1 and HMW2 resulted in several log reductions in nasopharyngeal colonization density. However, we were unable to completely prevent colonization, as bacteria were still detected in immunized animals. Thus, immunization with HMW1 and HMW2 alone may be insufficient for complete protective immunity, even against HMW-expressing strains. To achieve even greater protective immunity, we could include the adhesin Hap in our immunizations. Hap is expressed by all clinical NTHi isolates, and previous work in our lab has demonstrated that immunization with Hap can mediate protection against nasopharyngeal colonization. Notably, in these studies, immunization with purified Hap alone also resulted in incomplete protective immunity

(Cutter et al., 2002). The combination of HMW1, HMW2, Hap, and Hia in a vaccine could result in immunity that is protective against most strains of NTHi and is capable of completely preventing nasopharyngeal colonization.

5.3. Maternally derived immunity against nasopharyngeal colonization

Our experiments demonstrate that immunization with HMW1 and HMW2 is protective in adult mice. However, in humans, nasopharyngeal colonization with NTHi typically occurs in very early infancy, at a rate of about 20% within the first year. The colonization rate gradually increases with age, and nearly half of all children are colonized before the age of 2 years (Howard et al., 1988; Spinola et al., 1986). The inflammatory response to early life colonization with NTHi results in significant immune system reprogramming and predisposes to airways disease (Bisgaard et al., 2007). Children who are colonized with NTHi within the first 4 weeks of life are 2-4 times more likely to develop asthma or to be hospitalized for wheezing by age 5 years than children without detectable NTHi (Bisgaard et al., 2007). Similarly, studies in our lab have found that in neonatal mice, colonization on day 3 of life results in increased susceptibility to neutrophilic asthma, suggesting that prevention of early life colonization would be protective against asthma (McCann et al., 2016). However, vaccination of neonates and infants is often problematic due to the immaturity of the immune system. Rather than immunizing at a young age, protection can be provided by passively acquired antibodies from the mother. Studies suggest that breastfed children exhibit lower colonization densities and a lower incidence of acute otitis media, suggesting a role for maternally derived antibody (Duffy et al., 1997; Hokama et al., 1999). In order to understand the potential for HMW1 and HMW2 to be used as neonatal vaccine antigens, it is important

to clarify whether they can be used to mediate early life protection through maternal immunization.

Mothers can pass vaccine-induced immunity onto offspring through two mechanisms: prenatally, with transplacental passage of antibodies to the fetal systemic circulation, or postnatally, with transfer of antibodies through breastmilk (Niewiesk, 2014). Antibodies transferred through milk, including IgG, mediate protection against pathogens at mucosal surfaces by interfering with bacterial adherence and neutralizing virulence factors (Renegar & Small, 1994). In Chapter 2, we demonstrated that in mice, passive receipt of serum antibodies by intravenous injection is capable of mediating strain-specific protection against nasopharyngeal colonization. This result suggests that passively delivered antibodies are capable of conferring at least some degree of protection. Moreover, we show that IgG antibodies against HMW1 and HMW2 can restrict NTHi adherence to human epithelial cell monolayers. Thus, it is likely that maternally-delivered antibodies could mediate protection of neonates *in vivo*. Based on our work characterizing the antibody response to HMW1 and HMW2, we could expect any protection offered by maternal antibodies to be highly strain specific. In such a case, the need for a multi-component NTHi vaccine would become greater, in order to maximize the breadth of the antibody response.

5.4. Protection against nasopharyngeal colonization in rhesus macaques

Previous work in our lab has shown that HMW1 and HMW2 enhance stable respiratory tract colonization in rhesus macaques. NTHi expressing HMW1 and HMW2 were able to outcompete an *hmw1hmw2* mutant strain in both density and duration of colonization in the nasopharynx and oropharynx (Rempe et al., 2016). Moreover,

macaques colonized with NTHi developed HMW1 and HMW2-specific antibodies in convalescent serum. However, it is not known whether preexisting immunity could impact colonization density or clearance of colonization. We could therefore immunize rhesus macaques with purified HMW1 and HMW2, challenge with NTHi, and evaluate bacterial density in the nasopharynx. We could also evaluate whether Th17-based heterologous protection is also observed in rhesus macaques. Thus far, only derivatives of strain 12 have been evaluated in the macaque model. Performing protection experiments in macaques would highlight the protective capacity of HMW1 and HMW2 in a model more reflective of humans.

5.5. Regions of HMW1 and HMW2 required for immunogenicity

We have successfully demonstrated that immunization with mature HMW1 or HMW2 from different strains can stimulate a protective immune response; moreover, our findings in Chapter 3 suggest that the pattern of HMWC-mediated glycosylation across the protein may influence the immunogenicity of the adhesin. However, we have yet to completely delineate the regions of HMW1 and HMW2 that are required in order to stimulate immunity. Based on the structure of the HMW1 and HMW2 adhesins, there are several key regions of interest. One is the binding domain, which is the region of greatest dissimilarity between HMW1 and HMW2 (S. Dawid et al., 2001). This ~360aa region is localized near the N-terminus of the mature adhesins and determines the host receptor specificity of each adhesin. In our studies, immunization with HMW1 and HMW2 produced antibody capable of blocking the receptor-adhesin interaction, suggesting that some of the antibodies produced were specific for the binding domain. Though $HMW1_{12}$ and $HMW2_{12}$ are about 70% identical across the full adhesin, homology

in the binding domains drops to approximately 40%. Immunogenicity of the binding domains could therefore contribute to the differences in antibody response produced by $HMW1_{12}$ versus $HMW2_{12}$ that we observed in Chapter 3.

A second region of interest is the C-terminal domain of the mature adhesin. Previous work screening monoclonal antibodies for HMW1 and HMW2 binding has identified two surface exposed epitopes, including one that mapped to the C-terminal 75 amino acids of both adhesins, and an adjacent epitope that mapped to a 155-amino acid C-terminal segment of HMW1 (Stephen J. Barenkamp & St. Geme, 1996a). This suggests that despite the relatively high conservation of the C-terminal regions of HMW1 and HMW2, there are smaller regions that are immunologically distinct between the two adhesins. Regions such as this may be responsible for the differences in antibody binding to HMW1 and HMW2 following immunization. This could be further investigated by immunizing mice with recombinant peptides of HMW1 and HMW2 and evaluating the antibody response.

Identifying the regions of HMW1 and HMW2 that directly shape the immune response and understanding how glycosylation shapes the immunogenicity of these regions will be critical in developing these adhesins as vaccine antigens.

5.6. Region of HMW1 required for specific interactions with HMW1C

In our work studying the specificity of HMW1C for HMW1, we examined whether certain regions of immature HMW1 were responsible for targeting HMW1C. We found that neither the extended signal peptide nor the propiece were required for glycosylation by HMW1C. If there is a targeting motif on HMW1, then it is likely located in the mature

adhesin. In order to determine whether a region of mature HMW1 is required for glycosylation, we can evaluate glycosylation of HMW1 fragments. Specifically, we can create strains of NTHi expressing tagged fragments of HMW1, purify these fragments, and then evaluate glycosylation using mass spectrometry. In doing so, we can identify a region or motif that may be required for glycosylation that has not yet been identified.

Alternatively, we may find that there is no specific motif required for HMW1C-mediated glycosylation. Based on our findings, the stoichiometric ratio between HMW1 and HMW1C has an impact on the enzyme-target specificity, as HMW1C overexpression results in modifications of off-target proteins in NTHi. This suggests that under wild type conditions, the relative amounts of HMW1 and HMW1C are finely-tuned, and that there is sufficient HMW1 to limit modification of other proteins. We can further explore the relationship between HMW1:HMW1C levels and modification specificity by controlling the relative amounts of each protein in NTHi. This can be done by controlling expression of *hmw1a* and *hmw1c* using inducible plasmids and subsequently evaluating off-target glycosylation.

Developing an understanding the nature of the relationship between HMW1 and HMW1C, especially in the absence of a targeting motif, will improve our understanding of the HMW1C family of cytoplasmic N-glycosyltransferases. Moreover, in light of our work demonstrating the importance of HMW1C-mediated glycosylation in shaping the immunogenicity of HMW1, defining the relationship between HMW1 and HMW1C will undoubtedly be an important consideration in vaccine design.

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