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CHARACTERIZATION OF INTRACELLULAR GROUP B \textit{STREPTOCOCCUS} AND HOST DEFENSE

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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2015
This Dissertation of Andrew Scott Cutting is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2015
DEDICATION

To my number one fan, best friend, ultimate supporter, and fiancé Melissa: you were there to help me in anyway possible through this long endeavor and the investment has paid off! And to my parents, Garry and Maureen, and sisters, Allison and Liz, for encouraging me and assisting me in any way possible.
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ABSTRACT OF THE DISSERTATION

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University of California, San Diego, 2015
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Professor Kelly Doran, Chair

Bacterial meningitis is a severe, life threatening concern, which results in a high proportion of patients succumbing to disease. There are limited treatments available for this disease and individuals that survive endure an immense amount of outstanding consequences for the remainder of their lives. An important causative agent of bacterial meningitis is the human pathogen Streptococcus agalactiae, also known as Group B
*Streptococcus* (GBS). GBS is known to be the leading cause of neonatal sepsis and meningitis thereby resulting in a high incidence of mortality and morbidity. To cause meningitis, this bacterium must penetrate one of the most important barriers in the body, the blood-brain barrier (BBB). Still, the molecular mechanisms associated with the development of disease have yet to be completely described. In the past, invasive GBS was visualized within brain endothelial cells, the single cell layer that comprises the BBB, however the intracellular fate of GBS has yet to be explored. This dissertation seeks to understand the intracellular host defenses activated in response to GBS infection, the bacterial factors responsible for this activation, and the specific host and bacterial proteins that contribute to bacterial invasion and the initiation of the immune response. We explored the self-digestion pathway, known as autophagy, as playing a critical role in targeting intracellular GBS for destruction. My data suggest that while the autophagic pathway is activated in response to bacterial infection; this process is incompletely effective in eliminating the intracellular GBS. I believe that GBS can subvert autophagic recognition and subversion of this pathway may be the first step of the development of BBB disruption. Additionally, this dissertation explored canonical endocytic trafficking during GBS infection and the bacterial factors associated with differential trafficking. Finally, I have successfully identified a novel autophagy related protein responsible for bacterial uptake into brain endothelium as well as stimulation of the immune response. This protein may be a master regulator of a variety of intracellular processes and will be an interesting target for therapeutic intervention. In the end, this dissertation uncovered new information critical for understanding the intracellular potential and transcytotic nature of GBS during disease manifestation.
CHAPTER 1
INTRODUCTION

Group B *Streptococcus*

*Streptococcus agalactiae*, or Group B *Streptococcus* (GBS), is a Gram-positive, chain forming, bacterium responsible for invasive disease in humans.\(^1\)\(^2\). This bacterium was categorized in 1935 within the group B Lancefield classification due to its antigenic profile.\(^3\) GBS was initially described as causing disease within cattle, namely inflammation of the udder, also known as mastitis.\(^4\) This disease produces cattle that yield no milk, hence why the term ‘agalactiae’ is used within its name. Eventually in the 1970s, GBS emerged in humans, primarily in neonates or individuals who are elderly or immune-compromised.\(^5\) GBS is a frequent asymptomatic colonizer of both men and women within the gastrointestinal and/or vaginal tract. It was found that up to 33% of college students are colonized with GBS, yet only a small percentage, 3.6 cases per 100,000 individuals, develop invasive disease.\(^6\)\(^7\) However, during pregnancy, colonized mother’s can transmit GBS to the newborn vertically during the birthing process resulting in the development of GBS early onset disease (EOD) characterized by sepsis and pneumonia, which occurs within the first week of life.\(^8\) The majority of infants do not manifest GBS EOD with only 1-3% of newborns developing fatal disease.\(^9\) Late onset disease (LOD) can occur up to six months post birth with the infant presenting with similar disease features, however, there is an increased incidence in the development of meningitis.\(^8\) Although the development of GBS EOD has decreased through the implementation of intrapartum antibiotic prophylaxis (IAP), GBS is still one of the leading causes of neonatal disease and fatalities.\(^7\)\(^10\)\(^11\) As of 2010, IAP has
decreased the frequency of GBS EOD to 1 in 4000 live births\textsuperscript{10}. Still, GBS LOD has not been affected by IAP and remains a severe problem in newborns.

**GBS Early-Onset Disease**

The development of disease features related to GBS neonatal EOD are evident within a week of life which are principally a systemic infection leading to sepsis or pneumonia. It has been hypothesized that the GBS load in the maternal vaginal tract, racial/ethnic background, and delivery location of the infant are several of the risk factors involved in infant GBS EOD\textsuperscript{8}. Pre-term or low birth weight infants are at an increased risk of developing GBS EOD. Since the implementation of preventative GBS EOD strategies, cases in the United States dropped from 1.7 in 1000 births in 1993 to less that 0.4 in 1000 births in 2008\textsuperscript{12,13}. Although the incidence of GBS EOD is minimal, these infections remain fatal and survivors of this disease suffer a large disease burden. Infant survivors can have outlasting consequences such as hearing impairments, vision complications, and even mental retardation\textsuperscript{8}. Furthermore, the small percentages of infants that develop EOD meningitis endure permanent neurological sequelae\textsuperscript{9}. Initiation of EOD occurs with a complex interplay between host defenses and bacterial virulence factors. Maternally derived GBS must effectively persist within the vaginal tract and ascend to the fetus in utero via the amniotic cavity where the infant may aspirate infected fluid and present symptoms of lung injury\textsuperscript{14}. GBS may then traverse epithelial borders to gain access to the bloodstream where it has the ability to penetrate endothelial borders, most notably, brain endothelium.
GBS Late-Onset Disease

The materialization of GBS LOD is characterized by bacteremia leading to sepsis, which results in a high incidence of meningitis of infants up to six months of age\textsuperscript{15}. GBS eliciting LOD can either be acquired maternally during the birthing process or it can be obtained from the environment\textsuperscript{16,17}. Again, IAP has no impact on the development of GBS LOD, which is a gap that needs to be filled. Risk factors for LOD include maternally carried GBS, premature birth, limited maternal IgG, and twin-gestation\textsuperscript{18-20}. GBS bloodstream survival is critical for the establishment of bacteremia and CNS disease. These bacteria must evade circulating antimicrobials in the blood, host immune cell recognition, and subsequent immune activation. GBS confers a survival advantage within the neonatal context due to the fact that their immune system has yet to develop completely which leaves only innate immunity as the primary mode of defense against these invaders. Once bacteremia has taken over the infant, this can lead to mortality via septicemia or meningitis. It is known that GBS has a elevated tropism for the brain and CNS through its ability to penetrate the blood-brain barrier (BBB) and/or the blood cerebrospinal fluid barrier\textsuperscript{15}. GBS employs a variety of strategies to directly disrupt these barriers to gain access to the brain parenchyma. Once disrupted, brain injury can be detected clinically by edema formation, hydrocephalus, vascular injury, and increased intracranial pressure\textsuperscript{15}.

Bacterial Meningitis

Bacterial meningitis is the most common serious infection of the central nervous system (CNS) and a major cause of death and disability worldwide\textsuperscript{21,22}. From 2003-2007, there were 4,000 cases of bacterial meningitis reported in the United States, of
which, 500 were fatal\textsuperscript{23}. Early antibiotic intervention as well as vaccination strategies has improved patient outcomes, but with the appearance of multi-drug resistant bacteria, this disease continues to be a burden\textsuperscript{24}. Treatment of GBS meningitis typically is administration of amoxicillin, ampicillin, or penicillin G coupled with an aminoglycoside\textsuperscript{25}. Still, a variety of GBS strains exist that have been demonstrated to be less sensitive to penicillin, which is a major cause for concern\textsuperscript{26}. To elicit disease, blood-borne bacteria must interact with, and penetrate the initial lining of cells that comprise the BBB, known as brain microvascular endothelial cells (BMEC), a single cell layer of specialized endothelial cells that contain tight junctions between cells that contribute to barrier function. The BBB is composed of several other types of cells such as astrocytes, glial cells, and neurons. The main function of the BBB is to regulate passage of circulating pathogens and molecules that are present in the blood from entering the brain (Figure 1.1). To gain entry into the CNS and the subarachnoid space, bacterial pathogens must persist in the blood stream and interact with and penetrate brain endothelium (Figure 1.1). A variety of bacterial pathogens in addition to GBS are known to cause meningitis in neonates including \textit{Listeria monocytogenes} and \textit{Escherichia coli} K1\textsuperscript{27}. In addition, other species of bacteria such as \textit{Streptococcus pneumoniae}, \textit{Neisseria meningitidis}, and \textit{Haemophilus influenzae}, can cause disease in both infants and adults. Each of these bacteria employs strategies to evade host defenses in the blood and the brain to efficiently cause disease. Once GBS crosses the BBB, a complex immune program is initiated to respond to the infection primarily through neutrophil chemotaxis (Figure 1.1). Excessive induction of a pro-inflammatory response is known to contribute to further BBB disruption, triggered primarily by the β-
hemolytic and cytolytic toxin (β-h/c)\textsuperscript{28,29}. This, along with bacterial infiltration, leads to meningeal inflammation, CNS injury, and pleocytosis\textsuperscript{27}. It is though that GBS may traverse the BBB in a paracellular and transcellular mechanism, but it is believed that the preliminary step in GBS meningitis occurs through the initial adherence and subsequent invasion of brain endothelium\textsuperscript{30}. Much research has been devoted to understanding how GBS persists within and penetrates brain endothelium, however, it is still unclear as to the intracellular capability and the ultimate intracellular location GBS exploits to cross the BBB.

**Figure 1.1. Proposed mechanism of GBS BBB traversal.** Blood-borne bacteria survive in the bloodstream and ultimately reach the BBB where they engage cell surface receptors to undergo transcytosis into the brain. Innate immune signals are activated to respond to the infiltrating bacteria primarily through neutrophil influx.

**Modeling the Blood-Brain Barrier**

To investigate GBS interactions with the BBB, we employ specific *in vitro* and *in vivo* models. The brain contains a vast variety of neural cell types, some of which comprise the BBB. As mentioned previously, the endothelial cells that comprise the vascular endothelium in the brain are known as human brain microvascular endothelial cells (hBMEC) and these cells act as the primary barrier between the blood and the brain (Figure 1.1). In the past, primary cultures of brain endothelial cells from humans
and bovines were used to study their interactions with bacteria, however, it is time consuming and difficult to prepare and isolate these cells\textsuperscript{31,32}. To circumvent this, a plasmid containing simian virus 40, a virus that inhibits a subset of tumor suppressor genes, was transfected into hBMEC to obtain an immortalized brain endothelial cell line\textsuperscript{31,32}. We have obtained these cells and our lab utilizes this model as our primary means of \textit{in vitro} investigation of the interplay between GBS and the brain endothelium.

To examine the development of meningitis as a complete system, we employ an \textit{in vivo} model of hematogenous meningitis in mice\textsuperscript{29,33}. This model is crucial because it can give us clues regarding how GBS related disease progresses within a complex mammalian system. Initially, in-bred mice are injected intravenously with GBS and are then monitored over time, typically 24-72 hours, to observe disease manifestation. Once the animal has succumbed to the disease, tissue can be retrieved to investigate host as well as bacterial factors that may have contributed to the development of meningitis.

\textbf{Attachment of GBS to Brain Endothelium}

The first step of BBB breakdown by GBS is through initial adherence of the bacterium to host factors on brain endothelial cell surfaces. GBS has evolved the ability to bind host factors that allow it to exploit the host machinery for preliminary attachment to brain endothelium. A variety of bacterial factors have been determined to be critical during these interactions. Since GBS is present within the blood prior to engagement of brain endothelium, it utilizes numerous bacterial factors that have the ability to bind blood-borne extracellular matrix (ECM) proteins. ECM proteins such as fibrinogen, fibronectin, collagen, plasminogen, and laminin are hijacked by GBS factors to increase brain endothelial interactions\textsuperscript{34-37}. Furthermore, the gene that encodes for the
machinery responsible for creating the lipoteichoic acid anchor, \textit{iagA}, has been demonstrated to mediate BMEC adherence\textsuperscript{38}. Glycoprotein interactions are also known to be key during GBS attachment and GBS harnesses several of these proteins that bind serine and glycosaminoglycan molecules\textsuperscript{39,40}. Finally, a hypervirulent adhesin protein known as HvgA is required for GBS virulence and BBB disruption\textsuperscript{41}. In the end, bacterial uptake can occur through any of these mechanisms thereby resulting in the presence of intracellular GBS.

**Intracellular Lifestyle of GBS**

The host intracellular environment is quite hostile towards foreign bacterial invaders such as GBS. To date, it is known that GBS can invade and survive within brain endothelial cells, however, it is unclear where GBS ultimately resides once in these cells\textsuperscript{42}. Actin polymerization is crucial for GBS invasion, which is likely dependent upon focal adhesion kinase (FAK) phosphorylation and ensuing activation of actin binding proteins such as paxillin\textsuperscript{43}. It has been hypothesized that GBS traffics through the endocytic pathway, although no evidence has been gathered regarding the involvement of endocytic trafficking proteins in facilitating brain endothelial persistence or BBB traversal. A variety of GBS proteins are known to contribute toward GBS invasion and intracellular survival in phagocytic cells. Pili are hair like appendages that protrude out from bacteria and are associated with adherence and invasion of host cell types. GBS pili are composed of three individual structures, a pilus backbone protein (PilB) and two ancillary proteins (PilA and PilC) along with two sortases involved in pilus assembly\textsuperscript{44}. Each of these proteins is encoded by one of three pilus islands (PI), PI-1, PI-2a, and PI-2b and every clinical isolate of GBS contains one
of these three islands for pili construction\textsuperscript{45}. It is known that PilA acts as a tip adhesion and a recent study that I was a part of discovered that PilA binds collagen to engage the α2β1 integrin on brain endothelium and this interaction was critical for disease progression \textit{in vitro} and \textit{in vivo}\textsuperscript{46}. Activation of integrin signaling is known to also trigger FAK phosphorylation thereby allowing for GBS invasion into brain endothelium. The pilus backbone protein, PilB, has previously been described to promote intracellular survival within phagocytes, suggesting another important role for pilus proteins\textsuperscript{47}. Furthermore, expression of two component regulatory systems such as ciaR/H and covR/S have been described to be instrumental in GBS survival in brain endothelial cells and macrophages, respectively\textsuperscript{48,49}. Finally, the GBS polysaccharide capsule has been implicated in playing a role in dendritic cell internalization and survival\textsuperscript{50}. To date, there has been an inadequate amount of research dedicated toward understanding the intracellular capability of GBS. GBS infiltration of brain endothelium readily occurs during disease manifestation and eliciting the GBS factors involved in intracellular survival and the ensuing host response poses an interesting avenue for investigation.

\textbf{Group B \textit{Streptococcus} Beta hemolytic/cytolytic (β-h/c) toxin}

One of the most important virulence factors that GBS possesses is a pore-forming membrane bound toxin known as the Beta hemolytic/cytolytic (β-h/c) toxin. Beta-hemolytic toxins are identified by a zone of β-hemolysis surround individual colonies when grown on blood agar plates\textsuperscript{51}. In the past, the GBS β-h/c toxin has been described as pore-forming with the ability to stimulate a strong innate immune response primarily through neutrophil activation in both lung epithelial and brain endothelial
cells, promotes disease progression in a mouse model, and elicits mitogen activated protein kinase (MAPK) initiation while simultaneously inhibiting innate immunity in macrophages\textsuperscript{29,52-54}. It has also been described to activate the NLRP3 inflammasome in macrophages and exerts cellular toxicity on a variety of cell types including cardiomyocytes, meningeal, and astrocytes\textsuperscript{55,56}. The cylE gene encodes the β-h/c toxin and its action is of two parts. The first part is that the toxin itself acts as the principal agent of destruction whereas the carotenoid pigment associated with the toxin, acts a shield to protect it from cellular oxidative stressors\textsuperscript{57}. Recently, the β-h/c ornithine rhamnolipid pigment was suggested to be hemolytic as well as cytolytic indicating that toxin action may not be pore-forming\textsuperscript{58}. Strains of GBS that lack the β-h/c repressor known as CovR/S, are hyper-hemolytic and have been linked with disease progression\textsuperscript{59,60}. The ability of this toxin to manifest disease has been heavily described, however, its impact on the intracellular capability of GBS within brain endothelium has yet to be determined.

**Macroautophagy**

Macroautophagy, hereafter referred to as autophagy, is an evolutionarily conserved degradation process that utilizes the lysosomal machinery to recycle damaged, aggregated, or aged cytoplasmic constituents. Cargo is initially captured into the autophagosome through the formation of an isolation membrane called the phagophore, which is ultimately destined for lysosomal fusion resulting in cargo degradation (Figure 1.2)\textsuperscript{61,62}. This process is activated in response to stressors, such as amino acid starvation or infection, and can be classified as either selective or non-selective\textsuperscript{63}. Malfunctions in autophagy can also contribute to the pathogenesis of a
variety of diseases such as Parkinson’s disease, Alzheimer’s disease, and Huntington’s disease as well as inflammatory diseases such as Crohn’s disease, rheumatoid arthritis, and ulcerative colitis\cite{64,65}. Autophagy is initiated by the interactions between multiple autophagy (ATG) proteins\cite{66}. Initially, approximately 35 ATG genes were discovered in yeast and mammalian orthologs were subsequently identified\cite{67}. Of these genes only about 15-20 of them are involved in the core induction of autophagy. The main autophagy-inducing signal occurs via the Unc-51-like kinase 1 (ULK1) complex, which recruits a plethora of ATG proteins to the phagophore assembly site (PAS). Under stressful conditions and mammalian target of rapamycin (mTOR) inactivation, ULK1 is able to phosphorylate FAK family kinase-interacting protein of 200 kDa (FIP200) which then translocates to the PAS for autophagosome initiation\cite{63}. At this point, the ATG5, ATG12, and LC3/GATE-16/GABARAP proteins are recruited and are vital for the formation of the initial phagophore and maturation of the autophagosome. The microtubule-associated protein 1 light chain 3, or LC3, is conjugated to phosphatidylethanolamine (PE), a lipid constituent of plasma membranes, by the ATG5-12-16L1 complex to allow for autophagosome expansion\cite{66}. The hallmark indicator of autophagy induction is formation of the double membrane autophagosome labeled by LC3-II. Recently, antimicrobial autophagy, a selective type of autophagy, also known as xenophagy, has emerged as a potent host defense mechanism against intracellular bacterial and viral pathogens\cite{61,68}. Several pathogenic bacteria including *Salmonella enterica* serovar Typhimurium (S. Typhimurium), *Listeria monocytogenes*, *Shigella flexneri*, and Group A *Streptococcus* (GAS) have been shown to activate the autophagic pathway\cite{69-71}. Multiple mechanisms have been described as to how these and
other pathogens, are recognized by the cell to induce the autophagic process\textsuperscript{72}. Bacteria such as \textit{Mycobacterium tuberculosis}, \textit{Bacillus anthracis}, and \textit{Vibrio cholerae} inhibit the autophagy inducing signal, whereas others such as \textit{Shigella flexneri} and \textit{Listeria monocytogenes} inhibit autophagy machinery detection or directly modifying autophagy components\textsuperscript{63}. Further modulation or evasion of these pathways by bacteria may be critical for their intracellular survival and disease manifestation. It is not uncommon for bacteria to manipulate autophagy progression or initiation as a survival advantage to evade host innate immune recognition. GBS is known to invade brain endothelial cells and the extent of autophagy activation has yet to be investigated.
Figure 1.2. Steps of the autophagic pathway during infection. Infectious bacteria enter the cell and autophagy is initiated through signals by ULK1 and FIP200. The ATG5-12-16L1 complex facilitates autophagosome formation and autophagy is completed upon lysosomal fusion.

**Endocytic Trafficking and Uptake**

Endocytosis consists of entry of solutes, ligands, molecules, receptors, and infectious agents into the cytoplasm through plasma membrane invaginations that allow for membrane bound vesicles within the cell. Endocytic trafficking of bacteria is initiated upon invasion of host cells and Rab GTPases, guanine nucleotide binding
proteins involved in vesicular trafficking, are activated to efficiently deliver intracellular invaders to the lysosome\textsuperscript{74}. Rab5 is a monomeric GTPase known to be involved in early endocytic trafficking while Rab7 acts later within the endocytic pathway to regulate lysosomal fusion (Figure 1.3)\textsuperscript{75}. Numerous bacterial pathogens are known to inhibit or disrupt endocytic trafficking such as \textit{Legionella pneumophila}, \textit{Mycobacterium tuberculosis}, \textit{Pseudomonas aeruginosa}, and \textit{Salmonella enterica} to establish an intracellular niche or simply promote survival or growth\textsuperscript{76}. To accomplish this, bacteria must modulate endocytic targeting and eventual destruction by producing novel virulence factors. Although GBS is known to survive intracellularly in brain endothelial cells, it likely does not replicate, or at least there is no net replication. Furthermore, GBS engages cell surface receptors and resides within membrane bound vesicles suggesting endocytic trafficking\textsuperscript{42}. 
Figure 1.3. Initiation and progression of endocytic trafficking. Intracellular bacteria enter the cell and traffic to early and late endosomes labeled by Rab5 and Rab7, respectively. These compartments then traffic to the lysosome where the bacteria may be degraded or employs a strategy to survive this environment.

Bacteria may also be targeted by a variety of endocytic proteins for their initial trafficking. Proteins such as clathrin and caveolin, two key mediators of initial budding of cargo containing vesicles. Clathrin has been classically described to be important during surface receptor and transmembrane protein internalization and is known to be hijacked by pathogens. Internalization of cargo via clathrin results in a clathrin-coated pit pinched off by the protein known as dynamin, which then exhibits a distinctive triskelion shape through interactions of both clathrin heavy and light chains. Clathrin is then uncoated from the cargo and the vesicle is then trafficked accordingly through the endocytic pathway. Pathogens such as *Listeria monocytogenes* and *Shigella flexneri* commandeer clathrin to aid in host cell internalization. Similarly, caveolin is also an
important alternate cargo internalization method and has recently been gaining interest as a factor exploiting by pathogens. Caveolae are lipid raft domains made up of lipids, cell-surface proteins, and caveolin proteins⁷⁸. Caveolin proteins exist in three different families, caveolin-1, caveolin-2, and caveolin-3. Caveolin-1 and caveolin-2 have been studied extensively in the context of microbial pathogenesis and have been demonstrated to be manipulated by pathogens such as Group A Streptococcus and Pseudomonas aeruginosa⁷⁹. Group B Streptococcus has yet to be extensively studied with regards to its endocytic mechanism within brain endothelium, although, it is known that actin rearrangement is critical for entry into brain endothelial cells suggesting an evident role of endocytosis⁴². Recently, the endocytic pathway was characterized in dendritic cells during GBS infection and it was found that encapsulated GBS invaded these cells primarily through lipid rafts and clathrin mediated mechanisms⁵⁰. It will be of profound interest to examine the exact mechanisms mediating GBS entry into brain endothelium and how host factors influence the intracellular trafficking of GBS.
Figure 1.4. Methods of endocytosis of GBS into brain endothelial cells. GBS may invade brain endothelium via clathrin coats, caveolin vesicles, or lipid rafts. Once in the cell, these vesicles may traffic within the endosomal pathway and interact with a variety of endocytic proteins.

**LC3-Associated Phagocytosis (LAP)**

One new process involving the critical autophagy protein LC3 is known as LC3-associated phagocytosis (LAP). This process was introduced in 2007 where it denoted the fact that LC3 can also be recruited to single membrane phagosomes or vesicles to assist in intracellular trafficking to pathogen recognition receptors (PRRs) or in lysosomal fusion\(^{80-83}\). The most interesting fact regarding LAP is that the process occurs independently from canonical autophagy activation. Still, it is uncertain as to why and under what condition render LAP activation. Pathogens such as *Burkholderia*
*Pseudomonas aeruginosa* and *Mycobacterium marinum* have elicited LAP features in RAW264.7-GFP-LC3 macrophages. There is no clear indicator of LAP, however, there has been a universal consensus that the ATG5-12-16L1 complex along with Beclin-1 and ATG5 are required for LAP induction. Other proteins such as ULK1, ATG13, and FIP200 are known to be dispensable for LAP activation. Defining whether macroautophagy or LAP occurs in the case of pathogen invasion has yet to be differentiated using GFP conjugated LC3, but modulation of a subset of ATG proteins, such as ATG5, and electron microscopic analysis of membrane bound bacteria have been able to shed light on these subtle differences.

**Figure 1.5.** Non-canonical role of LC3 via LC3-Associated Phagocytosis (LAP). During GBS infection, LC3 activation may occur independent of canonical autophagy where LC3 may aid in endocytic trafficking or deliver cargo to TLR or NLR vesicles.
SPECIFIC AIMS

This dissertation proposal seeks to understand how the cerebral vascular endothelium responds to intracellular bacteria, more specifically, the molecular mechanisms of GBS invasion, intracellular persistence and trafficking, as well as the host defenses deployed against intracellular bacteria. I hypothesize that GBS directly invades brain endothelial cells and elaborates factors that promote its survival and penetration into the CNS. However, host defenses are induced to help clear intracellular organisms. These hypotheses will be examined in the following aims:

**Aim 1:** Characterize the role of autophagy in brain endothelium during infection.
   a) Define whether GBS induces autophagy during infection.
   b) Examine the bacterial factors responsible for autophagy activation.
   c) Determine whether autophagy is required for limiting GBS survival.

**Aim 2:** Examine GBS trafficking through the endocytic pathway and what bacterial factors are important for intracellular survival.
   a) Examine GBS trafficking through the endocytic pathway.
   b) Define the role of GBS *ciaR* and the genes it regulates to intracellular trafficking and survival.
   c) Characterization of GBS within a lysosome.

**Aim 3:** Investigate the importance of host intracellular sensors including autophagy related protein, ATG16L1, and NODs during GBS infection.
   a) Define whether GBS is recognized by ATG16L1 during infection.
   b) What is the role of ATG16L1 during GBS invasion and its tie to inflammation.
   c) Characterization of NOD activation during GBS infection.
REFERENCES


CHAPTER 2

The Role of Autophagy during Group B Streptococcus
Infection of Blood-Brain Barrier Endothelium

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ABSTRACT

Bacterial meningitis occurs when blood-borne pathogens invade and penetrate the blood-brain barrier (BBB) provoking inflammation and disease. Group B Streptococcus (GBS), the leading cause of neonatal meningitis, can enter human brain microvascular endothelial cells (hBMEC), but the host response to intracellular GBS has not been characterized. Here we sought to determine whether antibacterial autophagy, which involves selective recognition of intracellular organisms and their targeting to autophagosomes for degradation, is activated in BBB endothelium during bacterial infection. GBS infection resulted in increased punctate distribution of GFP-LC3, and increased levels of endogenous LC3-II and p62 turnover, two hallmark indicators of active autophagic flux. Infection with GBS mutants revealed that bacterial invasion and the GBS pore forming β-hemolysin/cytolysin (β-h/c) trigger autophagic activation. Cell-free bacterial extracts containing β-h/c activity induced LC3-II conversion, identifying this toxin as a principal provocative factor for autophagy activation. These results were confirmed in vivo using a mouse model of GBS meningitis, as infection with WT GBS induced autophagy in brain tissue more frequently than a β-h/c deficient mutant. Elimination of autophagy using Atg5 deficient fibroblasts, or siRNA-mediated impairment of autophagy in hBMEC, led to increased recovery of intracellular GBS. However, electron microscopy revealed that GBS was rarely found within double membrane autophagic structures even though we observed GBS-LC3 co-localization. These results suggest that while autophagy may act as a BBB cellular defense mechanism in response to invading and toxin-producing bacteria, GBS may actively thwart the autophagic pathway.
INTRODUCTION

Bacterial meningitis is a serious infection of the central nervous system (CNS) that can develop rapidly into a life-threatening infection even in previously healthy children or adults. The Gram-positive bacterium *Streptococcus agalactiae*, known as Group B *Streptococcus* (GBS), is the leading cause of meningitis in newborn infants. Although antibiotic therapy has changed GBS meningitis from a uniformly fatal disease to an often curable one, the overall outcome remains unfavorable as 25–50% of surviving infants suffer permanent neurological sequelae of varying severity, including cerebral palsy, mental retardation, blindness, deafness, or seizures. Infection is initiated when blood-borne bacteria cross the blood-brain barrier (BBB) in a complex interplay between endothelial cells and microbial gene products. The human BBB, which is composed of a single layer of specialized human brain microvascular endothelial cells (hBMEC), separates the brain and its surrounding tissues from the circulating blood, tightly regulating the flow of nutrients and molecules promoting the proper biochemical conditions for normal brain function. Although the BBB serves as a critical barrier to protect the CNS against microbial invasion, disruption of the BBB is a hallmark event in the pathophysiology of bacterial meningitis. This disruption may be due to the combined effect of bacterial entry, direct cellular injury by bacterial cytotoxins, and/or activation of host inflammatory pathways that compromise barrier function. GBS produce a pore forming β-hemolysin/cytolysin (β-h/c) that has been shown to directly damage brain endothelial cells, and activate proinflammatory mediators, promoting the development of GBS meningitis *in vivo*. To gain entry into the CNS and the subarachnoid space, GBS must persist in the blood stream and interact with and
penetrate brain endothelium; however, the exact mechanism(s) of bacterial transit across the BBB is not known. It is likely that GBS tropism for the BBB is the primary step in the pathogenesis of meningitis. Many GBS surface components have been identified that contribute to the initial interaction with hBMEC including iagA (invasion associated gene A), which is required for proper anchoring of lipoteichoic acid (LTA) to the cell wall, Srr-1, FbsA, Lmb, HvgA, Alpha C protein, and pili components which consist of the pilus backbone protein, PilB, and pilus tip adhesin, PilA. GBS are able to enter or “invade” brain endothelium apically and exit the cell on the basolateral side, thereby crossing the BBB transcellularly. Electron microscopic (EM) studies have demonstrated the presence of GBS in membrane-bound vesicles within hBMEC, suggesting the involvement of endocytic pathways. However, little is known about how GBS persists and traffics through the BBB, or the host defenses deployed to combat its intracellular presence in brain endothelium.

Macroautophagy, hereafter referred to as autophagy, is an evolutionarily conserved degradation process that utilizes the lysosomal machinery to recycle damaged, aggregated, or aged cytoplasmic constituents. Cargo is initially captured into the autophagosome through the formation of an isolation membrane called the phagophore, which is ultimately destined for lysosomal fusion resulting in cargo degradation. Autophagy is initiated by the interactions between multiple autophagy (ATG) proteins. The ATG5, ATG12, and LC3/GATE-16/GABARAP proteins are vital for the formation of the initial phagophore and maturation of the autophagosome. The microtubule-associated protein 1 light chain 3, or LC3, is conjugated to phosphatidylethanolamine (PE), a lipid constituent of plasma membranes, by the
ATG5-12-16L1 complex to allow for autophagosome expansion. Recently, antimicrobial autophagy, a selective type of autophagy also known as xenophagy, has emerged as a potent host defense mechanism against intracellular bacterial and viral pathogens. Several pathogenic bacteria such as Salmonella enterica serovar Typhimurium (S. Typhimurium), Listeria monocytogenes, Shigella flexneri, and Group A Streptococcus (GAS) have been shown to activate the autophagic pathway. Multiple mechanisms have been described as to how these and other pathogens, are recognized by the cell to induce the autophagic process. Further modulation or evasion of these pathways by bacteria may be critical for their intracellular survival and disease manifestation.

In the present study, we examined the hypothesis that selective autophagy may play a role in host defense against meningeal pathogens such as GBS. Our results demonstrate that GBS infection triggers a robust autophagic response in brain endothelium, and that this response contributes to limiting intracellular bacteria. Experiments with isogenic GBS mutants lacking the β-h/c toxin or surface components that promote cellular invasion, indicate that these virulence factors impact autophagy induction. Furthermore, our studies demonstrate that the GBS secreted β-h/c toxin is sufficient to activate an acute autophagic response in BBB endothelium, but that this response may not be adequate to reduce the majority of intracellular GBS.

EXPERIMENTAL PROCEDURES

Bacterial strains. The WT strains used in these studies include Bacillus anthracis (Sterne 7702) and Staphylococcus aureus (ISP479C) and clinical GBS isolates COH1, a highly encapsulated serotype III strain and NCTC 10/84, a highly
hemolytic serotype V strain\textsuperscript{27,28}. Mutant GBS strains COH1Δcyl\textsuperscript{E}\textsuperscript{29}, NCTC10/84Δcyl\textsuperscript{E}\textsuperscript{29}, COH1Δ\textit{iagA}\textsuperscript{8}, NCTC10/84Δpil\textit{A}\textsuperscript{16}, and NCTC10/84Δpil\textit{B}\textsuperscript{30} were constructed previously by single-gene allelic exchange mutagenesis as described. All GBS strains were grown in Todd-Hewitt Broth (THB) with antibiotic selection of 2µg/mL chloramphenicol or 5µg/mL erythromycin as needed. The \textit{Bacillus anthracis} Sterne strain and the Methicillin-sensitive \textit{Staphylococcus aureus} ISP479C strains were cultured as previously described\textsuperscript{31,32}.

\textit{Construction of green fluorescent protein-expressing GBS.} The pDESTerm plasmid expressing GFP was provided by John Buchanan and Victor Nizet (UCSD). Competent bacterial cells were created by propagating GBS in THB with 0.6% glycine to early-log phase. Cells were then centrifuged at 4000RPM for 30 min at 4°C. The supernatant was removed and bacteria were washed with ice-cold 0.625M sucrose buffer. Bacteria were centrifuged again as described above and again the supernatant was removed. 1µg/µL of plasmid was added to the competent GBS in a 0.1cm electroporation cuvette and cells were electroporated at 1500V for 2-4 milliseconds. All steps were performed on ice. Recovery media (THB with 0.25M sucrose) was added to the cells and cells were incubated at 37°C for 1 h. The culture was then plated on THB agar plates containing 5µg/mL erythromycin and incubated at 37°C with 5% CO\textsubscript{2}. Colonies were then assessed for fluorescence by microscopy and fluorescent-activated-cell sorting.

\textit{Cell culture.} The human brain microvascular endothelial cell line was kindly provided by Kwang Sik Kim (Johns Hopkins University) and cultured as previously described in RPMI 1640 (VWR, Cat. No. 45000-396) containing 10% FBS, 10%
Nuserum (BD Biosciences, Cat. No. 355504) and 1% nonessential amino acids (Life Technologies, Cat. No. 11140-050)\textsuperscript{33}. ATG5 -/- and WT MEFs\textsuperscript{34} were kindly provided by Noboru Mizushima (University of Tokyo) and cultured in DMEM plus GlutaMax containing 10% FBS and 1% PenStrep\textsuperscript{35}. For RNAi-mediated gene knock down, siRNAs directed toward ATG12 (Invitrogen Select Silencer Series, Cat. No. 4392420, s17465 and s17466), ATG5 (Invitrogen Select Silencer Series, Cat. No. 4392420, s18159 and s18160, FIP200 (Qiagen, Cat. No. SI02664571 and SI02664578), and a scrambled siRNA (Invitrogen Select Silencer Series, Cat. No. 4390846) were used. hBMEC were transfected with either siATG12, siATG5, siFIP200 and siCtrl for two days with Lipofectamine 2000 (Life Technologies, Cat. No. 11668-019) or Lipofectamine 3000 (Life Technologies, Cat. No. L3000-001) prior to GBS infection. Knock down of target genes was confirmed by immunoblotting. Mcherry-LC3 has been described previously\textsuperscript{36}, briefly, the pEGFP-C1 vector (Addgene) was modified by replacement of EGFP with mCherry and LC3 was inserted at the C terminus.

**Infection assays.** hBMEC and ATG5 KO and WT MEFs were grown to confluence (~10\textsuperscript{5} cells per well) and washed three times prior to GBS infection. GBS were grown in THB to mid-log phase (~10\textsuperscript{8} CFU/ml; OD\textsubscript{600} = 0.4), washed in PBS, resuspended in RPMI plus 10% FBS, and used to infect monolayers at a multiplicity of Infection (MOI) of 1 or 10 for various time points. Plates were then centrifuged at 1000 RPM for 3 min to synchronize infection and then incubated at 37°C in 5% CO\textsubscript{2}. After infection, cells were treated with Penicillin (5\textmu g/mL) and Gentamycin (100\textmu g/mL) to kill extracellular GBS for various time points. Cells were treated with 0.1 mL of 0.25% trypsin-EDTA solution and lysed with addition of 0.4 mL of 0.025% Triton X-100 by...
vigorously pipetted. The lysates were then serially diluted and plated on THB agar to enumerate bacterial cfu. Cell lysate was collected and stored at -80°C until further use. In specified experiments, hBMEC were pre-treated with Rapamycin (5µM, CalBiochem, Cat. No. 553211) and Bafilomycin (100nM, LC labs, Cat. No. B-1080) for 1 h prior to GBS infection.

*Transmission electron microscopy.* hBMEC were incubated with GBS at 37°C with 5% CO2. Cells were washed 3 times with PBS. Cells were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer for 90 min and rinsed 3 times in 0.1M cacodylate buffer for 10 minutes each rinse. Samples were post-fixed in 1% osmium tetroxide for 90 min, then dehydrated at increasing concentrations of ethanol and acetone for 10 min each. Samples were embedded in Epon Acetone and baked at 60°C overnight. Thin sections were cut using a diamond knife on a Leica Microtome, stained with uranyl acetate and lead citrate, and viewed using a FEI Tecnai 12 transmission electron microscope.

*Immunofluorescence staining.* hBMEC were fixed with 4% paraformaldehyde prior to mounting with VectaShield with DAPI (Vector Laboratories, Cat. No. H-1200). For GFP-LC3 studies, hBMEC were transduced with GFP-LC3 adenovirus overnight in RPMI 1640 media containing 2% FBS before infection with GBS. After infection, cells were fixed in 4% paraformaldehyde prior to solubilization in 0.1% Triton X-100 and subsequent staining with a GBS specific antibody (Acris, Cat. No. BM5557P) or anti-von Willebrand factor antibody (Sigma, Cat No. F3520). For tissue visualization of GFP-LC3 mice, brains were harvested and fixed in optimal cutting temperature compound (O.C.T.) (VWR, Cat. No. 25608-930) and then frozen at -80°C. Brain tissue
was then sectioned using a Leica CryoStat. Samples were visualized using a Zeiss Axiovert 200 inverted fluorescence microscope (Carl Zeiss) or a Zeiss LSM 710 confocal microscope (Carl Zeiss).

*Western blot analysis.* hBMEC were infected with WT and mutant GBS for different time points and lysed using RIPA buffer (Thermo Scientific, Cat. No. 89900) containing 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF and protease inhibitor cocktail III (Calbiochem, Cat. No. 539134-1ML). Cell lysates (10 µg) were separated by SDS-PAGE performed with 10-20% Tris-Glycine gels (Life Technologies, Cat. No. EC6135BOX) and then were transferred to PVDF membranes (Millipore, IPVH00010), blocked using Tris buffer (pH 7.6) containing 0.1% Tween-20 with 5% w/v nonfat dry milk. Blots were then incubated overnight with antibodies against LC3 (1:2000, Cell Signaling, Cat. No. 4108), GAPDH (1:150,000, Millipore, Cat. No. MAB374), ATG12 (1:1000, Cell Signaling, Cat. No. 2010), ATG5 (1:500, Santa Cruz, Cat. No. sc-133158), p62 (1:1000, Santa Cruz, Cat. No. sc-28359), or FIP200 (1:1000, Cell Signaling, Cat. No. 12436) in blocking buffer at 4°C, followed by detection with peroxidase-conjugated goat anti-rabbit IgG (1:1000, Jackson ImmunoResearch, Cat. No. 111-035-003) and donkey anti-mouse IgG (1:1000, Jackson ImmunoResearch, Cat. No. 715-035-150). Western blots were developed with WesternBright ECL HRP substrate (Advansta, Cat. No. K12045-D20).

*Preparation of heat-killed and paraformaldehyde-fixed GBS.* Bacteria were grown to mid log phase in THB and were subsequently pelleted. Pelleted bacteria were reconstituted in PBS and then boiled at 95°C for 5min. After boiling, bacteria were diluted accordingly for an MOI of 10 and administered to hBMEC. For
paraformaldehyde fixation, bacteria were grown to mid-log phase in THB, pelleted and resuspended in 1% paraformaldehyde, and incubated for 10 min at room temperature. The fixed pellet was then washed three times with PBS and was then added to hBMEC at an MOI of 10.

**Preparation of GBS hemolytic extract.** GBS hemolytic extract was prepared as described previously. Briefly WT GBS NCTC10/84 and isogenic ΔcylE strains were grown to OD₆₀₀ of 0.4 – 0.6 in THB. Bacteria were then pelleted and resuspended in PBS containing 1% glucose and 1% starch. After 1 h at room temperature, bacteria were sterile filtered using a 0.2µM syringe filter. After filtration, bacteria were pelleted and washed once in PBS. The washed pellet was then incubated for 1 hour at 37°C. The bacteria were then pelleted and resuspended in PBS plus 1% starch and 1% glucose and the supernatant was sterile filtered using a 0.2µM syringe filter. And equal volume of ice-cold methanol was added to the filtered supernatant and incubated on ice for 1 h. Methanol/supernatant was then pelleted and resuspended in 1mL of PBS. The hemolytic titer of isolated extracted was quantified as previously described.

**Mouse model of hematogenous meningitis.** Animal experiments were approved by the committee on the care and use of animals at San Diego State University (SDSU) protocol APF 13-07-011D and performed using accepted veterinary standards. We utilized a mouse model of hematogenous GBS meningitis as described previously. 8-week-old male CD-1 mice (n=10) were injected intravenously with 7-8x10⁷ CFU of NCTC 10/84 GBS or isogenic ΔcylE mutant. At time of morbidity or the experimental endpoint (24h), mice were euthanized and blood and brain collected. One half of brain was homogenized and processed for western blot analysis in RIPA buffer (Thermo
Scientific, Cat. No. 89900) and the other half was homogenized and plated on THB agar plates for enumeration of CFU. GFP-LC3 transgenic mice\(^3^9\) (aged 10-12 weeks) were similarly infected with WT GBS or the \(\Delta_{\text{cylE}}\) mutant (n=3), or injected with PBS control, and at the experimental endpoint (24h) brain tissue was isolated and cryopreserved in optimal cutting temperature (O.C.T.) compound (VWR, Cat. No. 25608-930) prior to immunofluorescence microscopy.

**Statistical analyses.** Graphpad Prism version 5.0f was used for statistical analysis. Unpaired t-tests or one way-ANOVA were used for analysis. Statistical significance was accepted at p < 0.05.

RESULTS

**Bacterial infection induces autophagy in brain endothelial cells**

To investigate autophagy activation in hBMEC, we analyzed the processing and lipidation of LC3. Upon initiation of autophagy, the cytosolic LC3-I form is converted to LC3-II, which is covalently linked to PE and associated with autophagosomal membranes\(^4^0\). Ectopically-expressed LC3, which is N-terminally tagged with GFP (GFP-LC3), is diffusely distributed in the cytosol, but upon proteolysis of the C-terminus and lipidation, it is recruited into autophagosomes, which are evident as fluorescent puncta. Initially, we infected hBMEC with known meningeal pathogens that are capable of invading brain endothelial cells including GBS\(^5\), *Bacillus anthracis*\(^3^1\), and *Staphylococcus aureus*\(^3^2\). Following transduction of hBMEC with an adenovirus expressing GFP labeled LC3 (Ad-GFP-LC3)\(^4^1\) and subsequent bacterial infection, we observed a significant number of LC3 puncta compared to the uninfected control (Fig. 1A). We further examined two well-studied GBS clinical isolates shown to cause
experimental meningitis, COH1 (serotype III) and NCTC 10/84 (serotype V). Increased LC3 puncta formation can be visualized after COH1 WT GBS infection in comparison to an uninfected control (Fig 1B). Furthermore, western blot analysis of endogenous LC3 levels in hBMEC during infection revealed a significant increase in LC3-II levels compared to LC3-I at early time points (Fig. 1C,D). We also investigated the turnover of the autophagic adaptor protein, p62/sequestome-1 (SQSTM1), which interacts with LC3, and is an indicator of active autophagy. We observed an early decrease in p62 compared to the cytosolic marker GAPDH following GBS infection (Fig. 1C,D), which is consistent with autophagic flux. Further, analysis of our previously obtained data on the hBMEC transcriptional response following GBS infection, revealed a number of modulated genes within autophagy gene networks, including ATG10 and XBP1 (data not shown). Collectively these data indicate that autophagy in BBB endothelium is activated early during pathogen infection.
Figure 2.1. Autophagy induction in hBMECs. A, GFP-LC3 (Ad-GFP-LC3) counts in hBMECs following infection with GBS (COH1 WT), *B. anthracis* (*B.a.*) (Sterne 7702 WT), and *S. aureus* (*S.a.*) (ISP479C WT) for 3 h (m.o.i. = 10) compared with untreated controls. At least 200 cells with numerous puncta were counted, and rapamycin treatment (5 µm) was used as a positive control. Significance was measured in comparison with untreated controls. B, confocal microscopy visualization of COH1 WT and uninfected hBMECs transduced with GFP-LC3. Arrows denote cells with abundant amounts of puncta. Scale bar, 10 µm. C and D, Western blot analysis of LC3 and p62 in hBMEC samples at various time points postinfection with COH1 for 4 h (m.o.i. = 10) and NCTC 10/84 for 1 h (m.o.i. = 10). Image analysis was performed using ImageJ software to determine LC3-II/LC3-I and p62/GAPDH ratios. All experiments were repeated at least three times in triplicate; data represent the mean ± S.D. from a representative experiment. *, p < 0.05; **, p < 0.005; ****, p < 0.0001. Error bars represent S.D. DIC, differential interference contrast.

**Bacterial invasion and toxin production activate autophagy**

To test whether GBS must be actively replicating in order to induce autophagy, we incubated hBMEC with heat-killed or paraformaldehyde-fixed GBS. As shown in
Fig. 2A-D neither heat-killed nor paraformaldehyde-fixed bacteria were able to induce substantial LC3-II conversion or p62 turnover compared to infection with live GBS. These data demonstrate that live bacterial infection is required to induce autophagy in brain endothelium, which may involve active bacterial transcription and protein synthesis and/or bacterial uptake into host cells. We next examined a subset of GBS virulence factors known to play a role in the pathogenesis of meningitis, specifically those involved in hBMEC attachment and invasion, and in toxin production. Using GBS mutant strains lacking cylE, which codes for β-h/c activity, and pili components pilA and pilB, which promotes GBS interaction with hBMEC, we assessed their ability to induce autophagy in BBB endothelium. Infection with the ΔpilA and ΔpilB mutants induced similar LC3-II conversion and p62 turnover to that of WT GBS, while strains lacking the β-h/c toxin resulted in less LC3-II (Fig. 2E,F). Similar results were observed for a ΔcylE mutant in a different GBS WT parental background (Fig. 2G,H). Additionally the GBS ΔiagA mutant, which exhibits reduced bacterial invasion, resulted in less LC3-II conversion. We sought to further investigate the role of the β-h/c toxin in autophagy activation in brain endothelium. Compared to the WT strain, the GBS mutant lacking the β-h/c toxin induced significantly less LC3 puncta (Fig. 3A,B) and endogenous levels of LC3-II as well as increased p62 (Fig. 3,C,D,E). These data suggest that GBS toxin production are required for autophagy activation in brain endothelium.
Figure 2.2. Live bacterial challenge is required for autophagy induction in hBMECs. A and B, Western blot analysis was performed in triplicate on hBMEC lysates following infection for 2 h with WT NCTC 10/84 (m.o.i. = 10) and incubation with heat-killed (HK) or paraformaldehyde (PFA)-fixed GBS. C and D, image analysis was performed using ImageJ software to determine LC3-II/LC3-I and p62/GAPDH ratios. E, Western blot analysis was performed in triplicate on hBMEC lysates following GBS infection for 1 h (m.o.i. = 10) with WT NCTC 10/84 and isogenic mutants ΔcylE, ΔpilA, and ΔpilB. F, image analysis was performed using ImageJ software to determine LC3-II/LC3-I ratios from a representative experiment performed in quadruplicate. G, Western blot analysis was performed on hBMEC lysates following GBS infection for 4 h (m.o.i. = 10) with WT COH1 and isogenic mutants ΔcylE and ΔilagA. H, image analysis was performed using ImageJ software to determine LC3-II/LC3-I ratios. All experiments were repeated at least three times in triplicate; data represent the mean ± S.D. from a representative experiment. *, p < 0.05; **, p < 0.005; ***, p < 0.0005; ****, p < 0.0001; #, p = 0.05. Error bars represent S.D.
Figure 2.3. GBS β-h/c triggers autophagy in hBMECs. A and B, immunofluorescence of GFP-LC3 (Ad-GFP-LC3) in hBMECs following infection with WT COH1 and isogenic ΔcylE mutant for 1 h (m.o.i. = 1). At least 200 cells were counted for puncta, and data are means ± S.D. from a representative experiment performed in triplicate. Scale bar, 10 µm. C, D, and E, Western blot analysis was performed on cell lysates collected from hBMECs infected with NCTC 10/84 GBS and isogenic ΔcylE mutant for 1 h (m.o.i. = 10) compared with uninfected control. Image analysis was performed using ImageJ software to determine LC3-II/LC3-I and p62/GAPDH ratios. All experiments were repeated at least three times in triplicate; data represent the mean ± S.D. from a representative experiment. **, p < 0.005; ****, p < 0.0001. Error bars represent S.D. DIC, differential interference contrast.

To determine whether the β-h/c toxin could independently induce autophagy in hBMEC, cell-free extracts from GBS were prepared in PBS plus 2% starch to extract stabilized β-h/c activity from the bacterial surface as described previously. Hemolytic titers were determined and were similar to that observed previously (data not shown). hBMEC monolayers were incubated with cell-free extracts from either WT or β-h/c mutant strains. As shown in Fig. 4A-C, extracts, containing β-h/c induced LC3-II conversion and p62 turnover in a dose-dependent fashion, whereas extracts from a β-h/c mutant did not result in autophagy activation, indicating that other secreted GBS products had negligible stimulatory effects. Under these experimental conditions the β-h/c extract did not result in substantial cell death (Fig. 4D). These data indicate that
brain endothelium responds directly to the GBS β-h/c to activate autophagy pathways independent of live bacterial challenge.

Figure 2.4. GBS β-h/c extract independently activates autophagy. A, Western blot analysis was performed on cell lysates collected from hBMECs incubated for 2 h with the indicated dilutions of cell-free β-h/c extracts recovered from NCTC 10/84 WT or the isogenic ΔcylE mutant. B and C, image analysis was performed using ImageJ software to determine LC3-II/LC3-I and p62/GAPDH ratios. D, hBMECs were incubated with the indicated dilutions of cell-free β-h/c extracts recovered from NCTC 10/84 WT for 2 h and then stained with trypan blue to measure cell viability. All experiments were repeated at least three times in triplicate; data represent the mean ± S.D. from a representative experiment. *, *p < 0.05. Error bars represent S.D.

The GBS β-h/c promotes autophagy activation in vivo

Our results suggested a prominent role for the GBS β-h/c toxin in promoting an autophagic response in brain endothelium. To test this hypothesis in vivo, we used our murine model of GBS hematogenous meningitis as previously described. Groups of mice (n = 9) were infected intravenously with WT GBS (NCTC 10/84) or the isogenic
ΔcylE mutant. As we have demonstrated previously\textsuperscript{6,37}, the majority of WT infected mice died and exhibited high bloodstream and brain bacterial loads compared to ΔcylE infected mice (Fig. 5A,B). At the time of death or sacrifice, brains were harvested and processed to obtain protein lysates for subsequent western blot analysis of LC3 and p62 proteins. We observed increased LC3-II and a concomitant decrease in p62 in the majority of WT GBS-infected mice compared to mice infected with the ΔcylE mutant (Fig. 5C,D). We also similarly infected GFP-LC3 transgenic mice\textsuperscript{48} with WT or ΔcylE mutant strains. Fluorescence microscopy of representative brains revealed increased GFP-LC3 puncta in brain tissue, following GBS WT infection compared to mutant infection or PBS injection (Fig. 6A). Additionally, we observed that GBS co-localized with GFP-LC3 within endothelial structures (Fig. 6B) and that GFP-LC3 co-localized with von-willebrand factor, a marker of endothelial cells (Fig 6C). These results confirm that autophagy is activated in response to GBS infection and the β-h/c toxin in vivo.
Figure 2.5. Autophagy is induced in vivo following GBS infection. CD1 male mice were injected intravenously with WT (n = 9) or ΔcytE GBS strain (n = 9) or injected with PBS (n = 2). A, bacterial counts (cfu) in mouse brain and in blood at the time of death. B, Kaplan-Meier survival plot. Significance was assessed using a log rank (Mantel-Cox) test. C, Western blot analysis was performed on protein lysates from brain harvested from mice infected with NCTC 10/84 and isogenic ΔcytE mutant for LC3-I, LC3-II, and p62. D, image analysis was performed using ImageJ software to determine LC3-II/LC3-I ratios. **, p < 0.005; ****, p < 0.0001. Error bars represent S.D.
Figure 2.6. Visualization of autophagy activation in brain endothelium. A, representative brain samples from GFP-LC3 transgenic mice infected with NCTC 10/84 GBS and isogenic ΔcylE mutant. GFP-LC3 puncta were observed in brain tissue in WT-infected mice compared with mice infected with the ΔcylE mutant or injected with PBS. Scale bar, 20 µm. B, immunofluorescence for GBS in WT-infected GFP-LC3 transgenic mice. GBS co-localizes with GFP-LC3 within endothelial portions of the brain. Scale bar, 10 µm. C, immunofluorescence for von Willebrand factor (VWF) in GFP-LC3 transgenic WT-infected mice demonstrates that endothelial cells are producing active GFP-LC3. Scale bar, 10 µm.

Autophagy contributes to bacterial clearance

To examine the importance of autophagic activation in combating intracellular GBS, we first used a mouse embryonic fibroblast (MEF) cell line that is deficient in ATG5\textsuperscript{35}. This protein is required for autophagy initiation, and cell lines lacking ATG5 are not able to form the ATG5-12-16L1 initiation complex required to elongate the
developing autophagosome. Following GBS infection and antibiotic treatment to remove extracellular bacteria (see Materials and Methods), intracellular GBS were enumerated in WT and ATG5 knockout (KO) MEFs. In the absence of ATG5, significantly more intracellular GBS was recovered (Fig. 7A). To establish the protective role of autophagy in brain endothelium, we first pretreated hBMEC with rapamycin, which is known to induce autophagy through its ability to inhibit the protein kinase, mTOR complex 1 (mTORC1), a pivotal negative regulator of autophagy. The intracellular bacterial load after pre-treatment with rapamycin was significantly lower than in untreated controls (Fig. 7B). This suggests that stimulation of autophagy in hBMEC limits GBS intracellular survival. We also utilized bafilomycin A1, an antibiotic known to inhibit autophagosome-lysosome fusion, and observed that bafilomycin pre-treatment led to a significant increase in the number of intracellular GBS CFU recovered (Fig. 7B). To corroborate these data, we utilized siRNAs directed toward ATG5 and ATG12 to inhibit autophagy in hBMEC during GBS infection. During treatment with siATG5, we observed reduced levels of ATG5 and LC3-II conversion during GBS infection and a slight, although significant, increase in recovered intracellular GBS compared to treatment with the siRNA scramble control (Fig. 6C,F). Similar results were obtained when ATG12 was silenced in hBMEC (Fig. 6D,G). To further examine whether elimination of genes critical for autophagy activation but not LC3 functionality, we knocked down FIP200, an ULK1 interacting protein essential for autophagy induction. We observed that knockdown of FIP200 did not similarly result in increased recovery of intracellular GBS (Fig 3E,H). These results
suggest that LC3 recruitment and ensuing activation is a key contributor to limiting intracellular GBS survival.

**Figure 2.7. Modulation of autophagy leads to differential GBS intracellular survival.**

*Figure 2.7. Modulation of autophagy leads to differential GBS intracellular survival.*

A, recovery of intracellular GBS COH1 within WT and ATG5 KO MEFs following an initial 2-h infection (m.o.i. = 1) and incubation with extracellular antibiotics for 4 h. Invasive bacteria were recovered and are expressed as a percentage of the initial inoculum. B, recovery of intracellular GBS NCTC 10/84 following 1-h infection (m.o.i. = 10) in the presence of rapamycin and bafilomycin A1 at 5 µm and 100 nm, respectively. Recovered bacteria are expressed as a percentage relative to untreated controls. C, D, E, F, G, and H, siRNA knockdown of ATG5, ATG12, and FIP200 in hBMECs was performed as described under “Experimental Procedures.” Western blot analysis was performed in triplicate on hBMEC lysates following COH1 WT infection (m.o.i. = 10) for 4 h plus 1-h incubation with extracellular antibiotics. Recovery of intracellular GBS COH1 WT following 4-h infection (m.o.i. = 10) and treatment with extracellular antibiotics for 1 h in the presence of siATG5, siATG12, and siFIP200 is expressed as a percentage relative to cells treated with siRNA scrambled control (siCtrl). Data are means ± S.E. from three independent experiments performed in triplicate. *, p < 0.05; **, p < 0.005; ***, p < 0.0005; ****, p < 0.0001. Error bars represent S.E.
**Visualization of intracellular GBS**

Thus far our results suggest that while the autophagic pathway is activated in brain endothelium during GBS infection, it may play a limited role in reducing the intracellular pool of GBS. Thus we sought to determine if GBS was found in double membrane structures, which are characteristic of autophagosomes. Using transmission electron microscopy (TEM) analysis we observed that GBS resides primarily in single membrane bound compartments within hBMEC, and was never free in the cytoplasm at 4 h (Fig. 8A) and up to 24 h (data not shown) post-infection. Single membrane structures were often damaged and a small population had double or multiple membranes (Fig. 8A,B). Using confocal microscopy we further analyzed GBS co-localization with LC3 in hBMEC over time as described in Materials and Methods. By 4 hours we observed that approximately 40% of intracellular GBS co-localized with LC3 (Fig. 8C,D) suggesting that less than half of the intracellular pool may be shuttled into the autophagic pathway.
Figure 2.8. Examination of the intracellular localization and LC3 co-localization of GBS. A and B, transmission electron microscopy of intracellular GBS COH1 4 h after hBMEC infection (m.o.i. = 10). Intracellular bacteria were quantified according to the intracellular structure in which they resided (n = 25). Bacteria are present in membrane-bound vesicles, damaged membranes, multiple membranes, and putative autophagic structures as indicated by arrowheads. Scale bar, 500 nm. C, transfection of an mCherry-LC3 plasmid into hBMECs was performed as described under “Experimental Procedures.” hBMECs were infected with GFP COH1 WT for 4 h prior to treatment with extracellular antibiotics 1, 2, 4, and 6 h postinfection. Quantification of the amount of GFP COH1 WT co-localizing with mCherry-LC3 was gathered by counting at least 100 cells with intracellular GBS, and data are means ± S.E. from a representative experiment performed in triplicate. D, representative confocal microscopic images from 4 h postinfection. Scale bar, 10 µm. Error bars represent S.D. DIC, differential interference contrast.
DISCUSSION

In order to penetrate the CNS, bacterial pathogens may directly invade BBB endothelium and traverse the barrier in a process called transcytosis. We and others have demonstrated that meningeal pathogens, including GBS, are capable of transcellular passage, but the exact mechanisms of intracellular survival and trafficking are not well understood. It is likely that intracellular host defenses may be activated to combat invasive bacteria, but it is unknown if autophagy in brain endothelium represents an important BBB defense mechanism or whether meningeal pathogens ultimately thwart or utilize this pathway for survival and BBB traversal. Our results provide new evidence that autophagy/xenophagy is activated in brain endothelium during GBS infection and contributes to limiting intracellular organisms. We demonstrate that inactivation of ATG5 and ATG12, two key autophagy proteins involved in LC3 processing and autophagosome formation, resulted in increased GBS survival. Conversely, we observed that activation of autophagy, using rapamycin, prior to GBS infection restricts the recovery of intracellular bacteria. Analysis of various GBS mutants deficient in factors previously determined to play a role in disease pathogenesis lead us to discover the β-h/c toxin as a key virulence factor associated with autophagy activation. Our data suggest that β-h/c secretion is sufficient to promote an autophagic response in brain endothelium, a response that is aimed to potentially eliminate intracellular GBS.

Our results demonstrate that hBMEC respond to GBS infection with a robust autophagic response as we observed activation of LC3 and autophagic clearance of the key autophagy adaptor protein p62. Activation was dependent on live bacteria as heat-
killed or formalin-fixed GBS failed to induce conversion to LC3-II (Fig. 2A-D). This led us to investigate which bacterial virulence determinants may be responsible for autophagy activation in BBB endothelium. We observed that factors associated with GBS invasion into hBMEC, such as properly anchored LTA may contribute to autophagy activation. We also found that production of the GBS β-h/c toxin was an important contributor to autophagy activation in hBMEC. This is a well-characterized GBS virulence factor shown to promote GBS invasion and intracellular survival in a variety of cell types, as well as immune activation and disease progression\textsuperscript{6,37,54,55}.

Compared to WT GBS infection, we found that infection with a β-h/c negative mutant resulted in significantly less autophagy induction in hBMEC \textit{in vitro} and in brain tissue of infected mice. Activation appears to not require bacterial invasion or even the bacterial cell itself as extracts containing β-h/c activity also directly stimulated autophagy activation. Thus β-h/c appears to be a key mediator in provoking an acute autophagic response in the brain endothelium and may be an important contributor to disease progression. Whether this action is elicited by direct interaction of the toxin with endothelial signal transduction systems or activation is a secondary result of cellular injury that is mediated by the toxin remains to be elucidated. Recent studies investigating other bacterial/host interactions have shown that autophagy can be stimulated by toxins from \textit{Vibrio cholerae}\textsuperscript{56} and \textit{Bacillus anthracis}\textsuperscript{57} and pore forming toxins from GAS\textsuperscript{58} and \textit{Staphylococcus aureus}\textsuperscript{59}. Interestingly it has been recently suggested that hemolytic and cytolytic activity of GBS is due to the ornithine rhamnolipid pigment and not due to a pore-forming protein toxin\textsuperscript{60}. Furthermore, this associated carotenoid pigment has been shown to promote GBS intracellular survival in
phagocytic cells\textsuperscript{37}. Thus it will be of interest to determine the exact mechanism of autophagy activation by the GBS β-h/c.

Research devoted toward the understanding of how antibacterial autophagy may defend against intracellular microbes has recently become of increasing interest. Classically cytosolic intracellular bacterial pathogens, such as *Listeria*, *Shigella* and GAS that disrupt phagosomal membranes and escape from these vesicles, may be targeted for sequestration by autophagy leading to their degradation\textsuperscript{20,61,62}. GAS escapes the endocytic pathway and enters the cytoplasm using a pore-forming cytolysin, Streptolysin O\textsuperscript{22,63}. Cytosolic GAS is then isolated into autophagosomes and rapidly undergoes lysosomal degradation. Autophagy may also target vesicular bacteria, as is the case for *Mycobacteria* and *Salmonella*\textsuperscript{20,62}. During infection of epithelial cells, *S. typhimurium* damage and escape endosomal membranes, become ubiquitinated, and are recognized by autophagic adaptor proteins p62, nuclear dot protein 52 (NDP52), and optineurin (OPTN) for eventual binding to LC3\textsuperscript{64-66}. Using TEM we have visualized GBS mainly within single membrane-bound vesicles, which is consistent with early observations and images of intracellular GBS in hBMEC\textsuperscript{5}. We have not observed GBS free in the cytosol, even at later time points, although in some cases vesicle membranes appear to be disrupted (Fig. 8A). It has been observed that penicillin may gain access to the cytoplasm of eukaryotic cells\textsuperscript{67}, which may potentially kill GBS released into the cytoplasm. Although we cannot exclude this possibility, we should note that TEM analysis was performed in the absence of any antibiotics. Further, we did observe a 30% increase in recovered GBS when using only gentamicin (data not shown), but this result is complicated by the fact that gentamicin alone was not as effective at killing
extracellular GBS (data not shown). Our findings also suggest that intracellular GBS may traffic through other endosomal pathways. We have observed that GBS can traffic into Rab5 and Rab7 positive endosomes (data not shown), which is consistent with our TEM results suggesting the involvement of the endocytic pathway. The exact mechanisms and key players for recognition of intracellular vesicular GBS are incompletely understood, as is the question of whether the β-h/c toxin is responsible for vacuolar membrane damage we observed by TEM.

Pathogenic bacteria that survive within host cells utilize different strategies to avoid being killed in an autophagolysosome\textsuperscript{68}. These defensive mechanisms include resistance to autophagic engulfment, disruption of trafficking to the lysosome, and resistance to lysosomal killing. \textit{S. typhimurium} has the capacity to survive and replicate intracellularly due to modulation of amino acid starvation-triggered mTOR inhibition that activates autophagy\textsuperscript{69,70}. Recently, it has been reported that a clinically relevant serotype of GAS is able to degrade adaptor proteins, including p62, through secretion of a surface associated protease, SpeB, thereby allowing GAS to persist in the cytoplasm\textsuperscript{67}. Other pathogens such as \textit{S. flexneri} and \textit{L. monocytogenes} avoid autophagic recognition by producing virulence factors that bind key autophagy related proteins such as ATG5 and the ARP2/3 complex, thereby allowing for intracellular persistence and dissemination\textsuperscript{46,71,72}. While our results demonstrate that autophagy is activated in BBB endothelium and host factors such as ATG5 and ATG12 contribute to bacterial clearance, GBS is not completely eliminated.

TEM analysis suggests that GBS is not readily sequestered in autophagosome, yet up to \(~40\%) of intracellular GBS co-localize with LC3 at four hours post infection
(Fig. 8C). It has been demonstrated that LC3 can also be recruited to single membrane phagosomes or vesicles to assist in lysosomal fusion in a process denoted as LC3 associated phagocytosis (LAP)\textsuperscript{73-76}. Pathogens such as \textit{Burkholderia pseudomallei} and \textit{Mycobacterium marinum} have elicited LAP features in RAW264.7-GFP-LC3 macrophages\textsuperscript{77-79}. There is no clear indicator of LAP, however, there has been a universal consensus that the ATG5-12-16L1 complex is required for LAP induction\textsuperscript{75,80,81}. Defining whether macroautophagy or LAP occurs in the case of pathogen invasion has yet to be differentiated using GFP conjugated LC3, but modulation of a subset of ATG proteins, such as ATG5, and electron microscopic analysis of membrane bound bacteria have been able to shed light on these subtle differences. Interestingly, inactivation of the ULK1 interacting protein, FIP200, which is essential for the formation of the isolation membrane during autophagy but not recruitment of LC3 to membranes, did not impact recovery of intracellular GBS. This suggests that LAP may be critical for directing GBS to degradative compartments. Future studies are aimed to further elucidate the contribution of LAP to GBS uptake and intracellular trafficking.

In summary, we have demonstrated for the first time that the BBB endothelium activates autophagy in response to the meningeal pathogen, GBS. We present evidence that this pathway may contribute to host cellular defense by controlling the intracellular pool of GBS. In addition, we have identified the GBS pore-forming toxin as the molecular trigger for autophagy activation. However, it will be important to investigate the autophagy adaptor proteins involved in the recognition of intracellular GBS, the impact of infection on autophagic flux, and whether other bacterial factors promote
autophagy evasion and GBS intracellular survival. Ongoing studies on the modulation of host autophagy by meningeal pathogens are critical for understanding the host defense of the BBB and developing preventative therapies for CNS infection.
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CHAPTER 3

Identification of CiaR regulated genes that promote Group B streptococcal virulence and intracellular survival in brain endothelial cells

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Running Title: CiaR regulated genes influence GBS intracellular capabilities

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ABSTRACT

Group B *Streptococcus* (GBS) is a major causative agent of neonatal meningitis due to its ability to efficiently cross the blood-brain barrier (BBB) and enter the central nervous system (CNS). It has been demonstrated that GBS can invade human brain microvascular endothelial cells (hBMEC), a primary component of the BBB; however, the mechanism of intracellular survival and trafficking is unclear. We previously identified a two component regulatory system, CiaR/H, which promotes GBS intracellular survival in hBMEC. Here we examine the contribution of the response regulator, CiaR, to GBS trafficking through the endocytic pathway. A GBS strain deficient in *ciaR* localized more frequently with Rab5, Rab7 and LAMP1 positive vesicles. Further, lysosomes isolated from hBMEC contained fewer viable bacteria following initial infection with the ∆*ciaR* mutant compared to the WT strain. To characterize the contribution of CiaR-regulated genes, we constructed isogenic mutant strains lacking the two most down-regulated genes in the CiaR-deficient mutant, SAN_2180 and SAN_0039. These genes contributed to bacterial uptake, intracellular survival, and trafficking to and/or survival in lysosomes. Furthermore, competition experiments in mice showed that WT GBS had a significant survival advantage over the ∆2180 and ∆0039 mutants in the bloodstream and brain.
INTRODUCTION

Bacterial pathogens that have the capability of penetrating the central nervous system (CNS) thereby eliciting life-threatening diseases are a major human health concern. A severe outcome of bacterial infiltration of the CNS is the development of meningitis. One such pathogen, Group B Streptococcus (GBS), is a Gram-positive bacterium that is the leading cause of neonatal meningitis. Although intrapartum chemoprophylaxis is available to pregnant mothers during delivery, GBS infections among both pre-term and term infants still occurs\(^1\). Infants who survive meningitis suffer long-term neurological complications including developmental delays, hydrocephalus, visual impairment, deafness, cerebral palsy, and seizures\(^2\). GBS-induced meningitis occurs upon blood-brain barrier (BBB) penetration after a prolonged period of bacteremia\(^3\). Persistent blood-borne bacteria evade a variety of host defenses and have the propensity to cross the BBB, although the exact mechanism(s) of GBS-BBB transit are still being discovered. The majority of the BBB is composed of a specialized single cell layer known as human brain microvascular endothelial cells (hBMEC), which regulates passage of molecules, nutrients, and infectious agents into the brain\(^4\). Still, bacterial pathogens like GBS are able to disrupt this barrier to gain access to the CNS, resulting in inflammation, BBB permeability, and disease progression.

Much research has been devoted toward understanding the key GBS virulence factors that allow for BBB transit and breakdown. It is believed that direct invasion and subsequent transcytosis of brain endothelial cells by GBS is the critical first step for the development of meningitis\(^5\). Our lab has published several studies implicating multiple bacterial factors that participate in this initial invasion processes into brain endothelium.
including GBS surface associated proteins such as pili, lipoteichoic acid (LTA), serine rich repeat (Srr) proteins and fibronectin binding protein, SfbA\textsuperscript{6-10}. Following bacterial uptake, electron microscopy (EM) studies have demonstrated the presence of GBS in membrane-bound vesicles within hBMEC\textsuperscript{11,12} suggesting the involvement of endocytic pathways, however, little is known about how GBS persists and traffics through the BBB. We have recently demonstrated that autophagy is induced in BBB endothelium during GBS infection, but that this pathway was not effective in completely eliminating intracellular GBS\textsuperscript{12}. Thus, an understanding of how GBS resists intracellular host defenses and transits through brain endothelial cells is warranted.

To this end, we have investigated GBS trafficking within brain endothelial cells and the bacterial factors responsible for GBS survival. Endocytic trafficking is initiated upon bacterial invasion of host cells and subsequently, Rab GTPases aid in delivering these invaders to the lysosome for degradation\textsuperscript{13}. Numerous bacterial pathogens, such as \textit{Legionella pneumophila, Mycobacterium tuberculosis, Pseudomonas aeruginosa}, and \textit{Salmonella enterica} are known to inhibit or disrupt endocytic trafficking to establish an intracellular niche or simply promote survival or growth\textsuperscript{14}. To accomplish this, bacteria likely modulate gene expression to adapt to different host cellular environments, often through two component regulatory systems (TCRS). TCRS function through phosphotransfer signals from a membrane-bound sensor histidine kinase, which senses environmental changes, to subsequent activation of a cytoplasmic response regulator, with downstream transcription modulation\textsuperscript{15}. GBS genome sequence analysis suggests multiple putative TCRS, but most of these systems are currently not well described\textsuperscript{16}. One recent study has found that GBS encodes as many as 21 TCRS\textsuperscript{17}. Established GBS
TCRS include DltR/S, which maintains constant levels of n-alanylation in GBS LTA\(^\text{18}\); RgfA/C, which represses the expression of C5a peptidase\(^\text{19}\); CovR/S global regulatory system, which controls the expression of multiple virulence factors\(^\text{20}\); LiaFSR, which regulates cell wall stress and pilus expression\(^\text{21}\); FspS/R, which regulates fructose-6-phosphate metabolism\(^\text{17}\); and CiaR/H, which promotes survival in the host intracellular environment\(^\text{8}\).

We have demonstrated previously that the CiaR response regulator promoted GBS intracellular survival in phagocytic cells and brain endothelial cells\(^\text{8}\). Further, a GBS mutant deficient in \textit{ciaR} exhibited increased susceptibility to killing by antimicrobial peptides, lysozyme, and reactive oxygen species\(^\text{8}\). GBS CiaR also contributed to overall virulence potential in a murine bacterial competition model of infection\(^\text{8}\). Thus, we hypothesize that CiaR regulation may impact GBS intracellular trafficking in BBB endothelium. Our previous studies compared the transcriptional profiles of WT GBS and the isogenic \(\Delta\text{ciaR}\) mutant grown to log phase under identical conditions. Only one gene with a predicted function of purine and pyrimidine biosynthesis was upregulated more than twofold, while several genes were more dramatically down-regulated in the \(\Delta\text{ciaR}\) mutant\(^\text{8}\). The most highly down-regulated gene, SAN\_2180, encodes a conserved hypothetical protein, while the second most down-regulated gene, SAN\_0039, belongs to the M23/M37 family of metallopeptidases, which catalyze the hydrolysis of nonterminal peptide linkages in oligopeptides or polypeptides\(^\text{22}\). Here we investigate the role of CiaR and these regulated genes to GBS intracellular survival and trafficking in brain endothelial cells.
EXPERIMENTAL PROCEDURES

Cell culture. The human brain microvascular endothelial cell line was kindly provided by Kwang Sik Kim (Johns Hopkins University) and cultured as previously described in RPMI 1640 (VWR) containing 10% FBS, 10% Nuserum (BD Biosciences) and 1% nonessential amino acids (Life Technologies) at 37°C with 5% CO₂.

Bacterial strains and growth conditions. Streptococcus agalactiae (GBS) wild-type (WT) clinical isolate COH1 (serotype III)²³ and the isogenic ΔciaR mutant described previously⁸ were used for these studies. Isogenic mutants of COH1 in genes SAN_2180 and SAN_0039 were created by in-frame allelic replacement with a chloramphenicol resistance gene (cat) cassette using a previously described method⁷. Briefly, two flanking regions of target genes were amplified by PCR from COH1 genomic DNA, using 5’flank-F-2180: 5’- TAGCCATAACAGGAGATCCGACTA -3’, 5’flank-F-0039: 5’- CCAACAGACTACTCAATCGCTTCAGC -3’ and 5’flank-R-2180: 5’- TTTTATACCTCCCTTTCTCAA -3’, 5’flank-R-0039: 5’- AGAATTAATATAATGAAGTGCTCAAACACCTTG -3’; 3’flank-F-2180: 5’- TACTGATACAATACTAAGAA -3’, 3’flank-F-0039: 5’- TCCAGTAAAGTGTGATATTATAGTCTC-3’ and 3’flank-R-2180: 5’- TAGAGGAGGACACTGAATGACAAC -3’, 3’flank-R-0039: 5’- CGTAGTCACAGGAACTGCTGG -3’. A cat cassette with complementary regions of target genes was amplified from as previously described⁷ primers, Cat-F-2180: 5’- GAGAAAGGGAGGTATAAAAATGGAGAAAAATCACTGGATATACCACCG TTGA-3’, Cat-F-0039: 5’-AGCACTTCATTATATTAATTCTATGGAGAAAAAT CACTGGATATACCACCGTTGA-3’ and Cat-R-2180: 5’-
TTCTTAGTTATTGATACGCCCCCGCTGCACTCATCGCAGTACT
GGTTGTA-3’, Cat-R-0039: 5’-
GTCGAGACTATAATATCATTACGCCCGGCTGCACACTCAT
CGCAGTACTGTGTTGTA-3’. The construct was then amplified with a pair of nested primers,
Nest-F-xhoI-2180: 5’-
CCGCTCGAGTCCCAGGAGCGACTAGTGTTATG-3’, Nest-F-xhoI-0039: 5’-
CCGCTCGAGGATGATATTGAGACAGCTTG-3’ and Nest-R-xbaI-2180: 5’-
GCTCTAGAGGCTGTTAGGGGACGGATTTTC-3’, Nest-R-xbaI-0039: 5’-
GCTCTAGACACCGCAACAGAAGCTGTGTAT-3’; and then was cloned into the pHY304 vector. For complementation studies, full-length target genes were amplified using the following sets of primers, F-KpnI-2180: 5’-
GGGGTACCGTATCGAATACTT -3’, R-sacI-2180: 5’-
CGAGCTCCTCCATTAGGAGTT -3’; F-KpnI-0039: 5’-
GGGGTACCTCATCAAGGTGAGT -3’ and R-sacI-0039: 5’-
CGAGCTCATGAATCAATACCTCAAA -3’ and cloned into pDCErm. Deletion mutant strains were transformed with the recombinant plasmids for generation of complemented strain. GBS strains were grown in Todd-Hewitt broth (THB, Difco) at 37 °C and growth was evaluated by monitoring OD$_{600}$. For antibiotic selection, 2 µg/ml chloramphenicol and 5 µg/ml erythromycin was incorporated in the growth medium when required. GFP-expressing GBS strains were created as previously described$^{9,12}$.

**GBS growth inhibition assays.** GBS strains were grown in THB to log phase (absorbance at 600 nm, OD$_{600} = 0.4$). Then bacteria were diluted in THB to reach ~2× $10^5$ CFU/ml. In triplicate, 90 µl of bacterial suspension was added to each well of 96-
well flat-bottom tissue-culture plate containing 10 µl of PBS diluted reagents under test (Lysozyme, 1.5 mg/ml; Polymyxin B, 45 µg/ml; H₂O₂, 0.03% [V/V]) and the plate was incubated at 37°C. At indicated time points, bacteria were plated on THA plates to enumerate bacterial CFU.

_In vitro infection assays._ To determine the total number of cell surface-adherent or intracellular bacteria, hBMEC monolayers were grown to confluence in growth medium containing 10% FBS, 10% Nu-serum, and 1% non essential amino acids in 24-well tissue culture-treated plates. Bacteria were grown to mid-log phase and used to infect cell monolayers as described previously²⁴. Briefly, hBMEC monolayers were incubated with GBS at 37°C with 5% CO₂ for 30 min. To assess adherent bacteria, cells were washed five times with phosphate-buffered saline (PBS) to remove non-adherent bacteria, then trypsinized with 0.1 ml of trypsin-EDTA solution and lysed with addition of 0.4 ml of 0.025% Triton X-100 by vigorous pipetting. To assess intracellular bacteria GBS were incubated with hBMEC for 2 hr, cells were washed three times with PBS and 1 ml of media containing 100 µg/ml of gentamicin and 5 µg/ml of penicillin was added to each well to kill extracellular bacteria. Lysates were then serially diluted and plated on THB agar to enumerate bacterial colony-forming units (CFU). Bacterial adherence and invasion was calculated as (recovered CFU/original inoculum CFU)×100%. GBS intracellular survival experiments were performed as described above except that intracellular bacteria was enumerated at indicated time points.

_Immunofluorescence Staining._ GFP-expressing GBS strains were used to infect hBMEC monolayers. Following a 2 hr incubation and antibiotic treatment at indicated time points, hBMEC were fixed with 4% paraformaldehyde. Cells were then lysed with
0.1% Triton X-100, blocked with 10% FBS, and incubated overnight with antibodies (Cell Signaling Technology) to Rab5 (1:100), Rab7 (1:100), and LAMP1 (1:100). Cells were then washed and incubated with secondary antibodies (1:500) conjugated to Alexa-Fluor 594 (Life Technologies). Samples were visualized using a Zeiss Axiovert 200 inverted fluorescence microscope (Carl Zeiss). At least 100 cells per treatment were counted and all experiments were performed in triplicate.

Lysosomal Isolation. hBMEC were grown in 75 cm$^2$ flasks at 37°C with 5% CO$_2$ until confluence was achieved, and subsequently infected with WT and mutant strains of COH1 GBS at an MOI=10 for 2 hours. After infection, cells were incubated with media containing penicillin (5µg/mL) and gentamycin (100µg/mL) for either 1 or 12 hours to eliminate extracellular bacteria. Cells were then washed with DPBS and subjected to lysosomal isolation using the Lysosomal Enrichment kit for Tissue and Cultured Cells according to manufacturer’s instructions (Thermo-Fisher). Briefly, ~200mg of cells were harvested with trypsin and centrifuged for 2 min at 850 x g. Lysosome enrichment reagent A containing a protease inhibitor cocktail (CalBioChem) was added to pelleted cells and subjected to a 2 min incubation on ice. After incubation, cells were then sonicated 15 times to lyse the cells and Lysosome enrichment reagent B containing a protease inhibitor cocktail was then added to the cells. Cells were then centrifuged for 10 min at 500 x g at 4°C. The supernatant was then collected and the final concentration was altered to 15% with OptiPrep Cell Separation Media. The samples were then loaded on discontinuous OptiPrep gradients varying from 30%, 27%, 23%, 20% to 17% in a 13.2 mL ultracentrifugation tube (Beckman-Coulter) and centrifuged in a SW 41 Ti rotor at 145,000 x g for 2 hours at 4°C. After centrifugation,
the lysosomal fraction was isolated from the top gradient and washed using 2 volumes of DPBS in a microcentrifuge at 17,000 x g for 30 min at 4°C to remove OptiPrep media. Lysosomal pellets were then washed with Gradient Dilution Buffer at 17,000× g for 30 at 4°C. Pellets were then re-suspended in 0.1% Triton X-100 and plated on Todd Hewitt Agar to enumerate bacterial CFU. For lysotracker staining, pellets were re-suspended in PBS and stained with Lysotracker Red (Life Technologies) for 15 minutes and imaged using a Zeiss Axiovert 200 inverted fluorescence microscope (Carl Zeiss).

**Western Blot Analysis.** Isolated lysosomal pellets were re-suspended in 0.1% Triton X-100 and an equal volume of RIPA buffer (Thermo-Fisher) plus a protease inhibitor cocktail (CalBioChem). Cell lysates were separated by SDS-PAGE performed with a 4-20% Tris-HEPES (Life Technologies) and then were transferred to PVDF membranes (Millipore), blocked using Tris buffer (pH 7.6) containing 0.1% Tween-20 with 5% w/v nonfat dry milk. Blots were then incubated overnight with antibodies against LAMP1 (Cell Signaling Technology). Detection of protein content was performed using horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Jackson Immuno-Research). Western blots were developed with SuperSignal West Pico Chemilluminescent Substrate (Life Technologies).

**In vivo competition assay.** Animal experiments were approved by the Institutional Animal Care and Use Committee at San Diego State University (protocol APF 13-07-011D) and performed using accepted veterinary standards. 8-week-old male CD1 mice (Charles River Laboratories) were injected intravenously with 2×10^8 bacteria at a 1:1 ratio of WT and either one of the isogenic mutant strains. After 72 hours, mice were euthanized and blood and brain were collected to enumerate bacterial CFU. PCR
was performed to confirm the presence or absence of targeted genes on recovered CFU. Experiments were repeated twice (5 mice/group).

**Statistical Analysis.** GraphPad Prism version 6.0 was used for statistical analyses and statistical significance was accepted at \( p < 0.05 \) (*, \( P < 0.05 \); **, \( P < 0.005 \); ***, \( P < 0.0005 \); ****, \( P < 0.00005 \)).

RESULTS

**Characterization of GBS intracellular trafficking in brain endothelial cells**

Efficient trafficking of bacteria through endothelial barriers is a hallmark of the development of a multitude of vascular diseases. Endocytic trafficking consists of labeling by Rab GTPases, small guanine nucleotide binding proteins responsible for vesicular trafficking of cargo, and selectively transporting intracellular pathogens to the lysosome\(^{13,25}\). Rab5 is a monomeric GTPase known to be involved in early endocytic trafficking, while Rab7 acts later in the endocytic pathway to regulate lysosomal fusion\(^{26}\). Rab5 and Rab7 specifically label early and late endosomes, respectively, and subsequently traffic cargo or pathogens to the lysosome, which is labeled by lysosomal associated membrane protein 1 (LAMP1). We investigated the association of WT GBS and the \( \Delta \text{ciaR} \) mutant with Rab5, Rab7, and LAMP1 labeled compartments. Infection of hBMEC with GFP-GBS strains was carried out for 2 hr as described in Materials and Methods, then at various time points post antibiotic treatment, cells were processed and stained for endosomal and lysosomal markers. Representative images following WT GBS infection 1 hr post antibiotic treatment show GBS localization (Fig. 3.1A). To quantify co-localization over time, for each time point, we counted triplicate biological samples, at least 100 cells with intracellular bacteria and evaluated Rab5, Rab7, and
LAMP1 localization with GBS (Fig. 3.1B-D). At early time points there were either similar amounts of WT and \( \Delta \textit{ciaR} \) mutant GBS that localized with endosomal and lysosomal-labeled vesicles or in some cases there were higher amounts of WT GBS. However at later time points, significantly more of the \( \Delta \textit{ciaR} \) mutant was associated with both Rab and LAMP1 markers. These findings demonstrate that GBS associates with vesicles involved in the endocytic pathway, with 25-30\% of intracellular bacteria localizing with the lysosome. Further, CiaR regulation itself may influence GBS vesicle transit.
Figure 3.1. CiaR influences GBS intracellular trafficking in brain endothelial cells.
A. hBMEC were infected with WT COH1 GFP expressing GBS for 2 hours (MOI=10) prior to antibiotic treatment and then stained with antibodies targeting Rab5, Rab7, and LAMP1. Representative images demonstrate co-localization with each marker and each stain was performed in triplicate. Scale Bar, 5µm. B, C. Percentages of co-localization of Rab5, Rab7, and LAMP1 with WT and ΔciaR GFP expressing GBS after various time points post infection during antibiotic treatment. At least 100 cells containing intracellular GBS were counted for each time point in triplicate. Statistical analysis performed was a Two-way ANOVA with a Bonferroni’s multiple comparisons test and the data represents mean ± S.D. . $p < 0.05$, $p < 0.0005$ ***, $p < 0.00005$ ****.
**Characterization of CiaR regulated genes**

Our data indicate that the response regulator CiaR may play a role in survival and trafficking within brain endothelial cells. Thus, we hypothesize that CiaR-regulated genes may be critical for GBS intracellular survival and the efficient trafficking of GBS through brain endothelium. Previous microarray analysis of the WT and ΔciaR mutant identified the two most highly regulated genes, SAN_2180 and SAN_0039. To characterize the impact of SAN_2180 and SAN_0039 on GBS intracellular survival, we generated isogenic knockout strains using in-frame allelic substitution of either gene with a chloramphenicol acetyltransferase (cat) resistance cassette using a method described previously and as described in Materials and Methods. Both constructed mutants exhibited similar growth rates in THB compared to the WT parental strain (data not shown).

We have previously shown that the ΔciaR mutant exhibited decreased survival in hBMEC, we characterized the interactions of the Δ2180 and Δ0039 mutants with hBMEC, specifically the adherent and invasive capabilities as well as the ability to survive and persist intracellularly. The Δ2180 mutant exhibited a ~2-fold and significant decrease in hBMEC adherence and invasion compared with the WT parent strain (P < 0.005 and P < 0.00005, respectively) (Fig. 3.2A, B). Interestingly, the Δ0039 mutant displayed increased adherence and invasion into hBMEC (Fig. 3.2A, B), however, when calculating the percentage of the hBMEC-associated GBS that had invaded the intracellular compartment, both mutants showed decreased invasive capability (~20%) compared to the WT strain (~30%) (Fig. 3.2C). These data indicate that both SAN_2180 and SAN_0039 contribute to GBS uptake into hBMEC. To examine
whether these genes impact intracellular survival, we infected hBMEC with WT and mutant strains for 2 hours, incubated with extracellular antibiotics and at 2, 6, and 12 hours post antibiotic treatment, the intracellular pool was quantified as described in Materials and Methods. The percent of invasive bacteria recovered over time is shown relative to the first time point for each strain (Fig. 3.2D). We observed a gradual decrease in intracellular WT bacteria over time as we have demonstrated for GBS in hBMEC previously\(^7\). Moreover, the level of intracellular organisms over time for each of the mutant strains was significantly less compared to the WT strain (Fig. 3.2D).

Host cells employ multiple mechanisms to kill intracellular pathogens, including the release of pore-forming cationic antimicrobial peptides (AMPs), cell wall-destabilizing enzymes, and reactive oxygen species generated during the oxidative burst. As we have previously demonstrated that CiaR confers resistance to killing by AMPs, lysozyme, and oxidants\(^8\), we hypothesized that CiaR regulated genes may similarly promote intracellular survival by conferring resistance to one or more of these antimicrobial mechanisms. We first compared the sensitivities of newly generated mutants, \(\Delta 2180\) and \(\Delta 0039\), along with the \(\Delta ciaR\) mutant and WT GBS to polymyxin B, a cationic AMP derived from the bacterium *Bacillus polymyxa*, hydrogen peroxide (\(H_2O_2\)), a principal oxidant involved in lysosome killing, as well as the granular enzyme lysozyme. Consistent with our previously published data we observed a significant decrease in the ability of the \(\Delta ciaR\) mutant to survive upon exposure to these effectors (Fig. 3.3A-C). The \(\Delta 2180\) and \(\Delta 0039\) mutants also displayed increased sensitivity to these factors, with the \(\Delta 0039\) mutant being more susceptible compared to all other strains (Fig. 3.3A-C).
Figure 3.2. Adherence, invasion, and intracellular survival of GBS in brain endothelial cells are influenced by SAN_2180 and SAN_0039. A. Adherence to hBMEC by WT and mutants (MOI=1). B. Invasion of hBMEC by WT and mutants (MOI=1). C. Relative invasion of hBMEC by WT and mutants based on adherence (MOI=1). D. Relative intracellular survival of WT and mutant strains GBS in hBMEC. Statistical analysis performed was a Two-way ANOVA with a Bonferroni’s multiple comparisons test and the data represents mean ± S.D. $p < 0.05 \ast, p < 0.0005 \ast\ast, p < 0.00005 \ast\ast\ast$.  

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Hours Post Infection  

2hr 6hr 12hr
Figure 3.3. Sensitivity of GBS CiaR regulated genes to growth inhibitors. A. Percentage of recovered CFU 24 hours after exposure to 1.5mg/ml lysozyme. B. Percentage of recovered CFU 4 hours after exposure to 45ug/ml polymyxin B. C. Percentage of recovered CFU 20 min after exposure to 0.03% H2O2. Statistical analysis performed was a One-way ANOVA with a Dunnett’s multiple comparisons test and the data represents mean ± S.D. $p < 0.05$, $p < 0.005$, $p < 0.0005$, $p < 0.00005$.

Role of CiaR regulated genes in intracellular trafficking

Our results thus far suggest that CiaR regulation may contribute to GBS vesicle trafficking and that regulated genes, SAN_2180 and SAN_0039, impact GBS interactions with brain endothelial cells. To determine the role of these genes in intracellular trafficking, we examined Δ2180 and Δ0039 mutant GBS strains for their ability to localize with early and late endosomal (Rab5 and Rab7) and lysosomal (LAMP1) marked cells. As described above, we infected hBMEC with each GFP-GBS strain for 2 hours, and then stained for each marker at 1, 3, 6, and 12 hours post antibiotic treatment. Fluorescence microscopy and cell counting were used to evaluate the amount of GBS localization with Rab5, Rab7, and LAMP1. Generally both mutant strains exhibited increased localization with Rab5 and Rab7 positive vesicles compared to WT GBS particularly at the later time points (Fig. 3.4A, B). This largely copied the
phenotype of the ΔciaR mutant. However, while we observed an increase in LAMP1 localization by the ΔciaR mutant at later time points, only at 6 hours did the Δ2180 mutant exhibit increased localization with LAMP1. Otherwise both Δ2180 and Δ0039 mutants co-localized less with LAMP1 positive cells when compared to the WT strain (Fig. 3.4C). Interestingly the Δ0039 mutant displayed the least amount of LAMP1 localization at each time point compared to all other strains examined. Thus, overall CiaR regulation and specific regulated genes may influence GBS trafficking through endocytic compartments in different ways.
Figure 3.4. Endocytic trafficking is modulated by SAN_2180 and SAN_0039. A, B, C. Relative percentages of co-localization of Rab5, Rab7, and LAMP1 with WT and mutant GFP expressing GBS after various time points post infection during antibiotic treatment. At least 100 cells containing intracellular GBS were counted for each time point in triplicate. Statistical analysis performed was a Two-way ANOVA with a Bonferroni’s multiple comparisons test and the data represents mean ± S.D. $p < 0.05 \,*$, $p < 0.005 \,**$, $p < 0.0005 \,***$, $p < 0.00005 \,****$. 
Recovery of GBS from lysosomes isolated from brain endothelial cells

Our results suggest that GBS may regulate genes to prevent or limit endocytic trafficking to the lysosome. Although we observed that there was actually less co-localization of both ∆2180 and ∆0039 mutants with lysosomal markers at later time points, it is possible that these mutants are still readily trafficked to acidic compartments, but exhibit increased sensitivity to lysosomal killing. Thus, we investigated whether we could recover viable intracellular GBS from lysosomes isolated from brain endothelial cells. We used a lysosomal enrichment protocol that employs differential centrifugation to enrich for lysosomes based on size and density. Following hBMEC infection with WT and mutant GBS strains for 2 hours, cells were treated with antibiotics, and lysosomes were subsequently isolated at early (1 hour) and late (12 hours) time points. Western blot analysis was performed to confirm that recovered lysosomes were positive for LAMP1 (Figure 3.5C). We first used Lysotracker, which stains acidic vesicles, and found that WT GBS could be visualized within acidified lysosomes (Fig. 3.5A). Lysosomes were also lysed in 0.1% Triton X-100 and lysates plated on THB agar to enumerate viable bacteria. Markedly more WT GBS was recovered from the lysosome fraction than any of the mutant strains at the early time point post infection (Fig. 3.5B). Furthermore, fewer viable GBS was recovered from lysosomes at the later time point. These data further indicate that GBS does indeed traffic to the lysosome and that CiaR, SAN_2180, and SAN_0039 contribute to initial GBS trafficking and survival.
Figure 3.5. **GBS recovery from Lysosomes.** A. D. Isolated lysosomes infected with WT COH1 GFP expressing GBS were subjected to staining with Lysotracker Red (0.5µM) and visualized using fluorescence microscopy. Scale Bar, 5 µm B. hBMEC were infected with WT or mutant GBS strains for 2 hours (MOI=10) and subjected to lysosomal isolation by differential centrifugation after 2 or 12 hours post infection. Lysosomal pellets were plated to enumerate the amount of recovered CFU. C. Western Blot analysis for LAMP1 was performed on each isolated lysosomal fractions from each strain. Statistical analysis performed was a One-way ANOVA with a Tukey’s multiple comparisons test and the data represents mean ± S.D. $p < 0.05 \ast, p < 0.005 \ast\ast$. 
CiaR regulated genes contribute to GBS virulence

We have demonstrated previously that CiaR promotes bacterial fitness and overall virulence in a mouse model of GBS infection. To similarly examine whether the CiaR regulated genes contribute to virulence in vivo, we employed the same bacterial competition model as described previously. Mice were challenged intravenously with equal amounts (2 x 10^8 CFU) of WT COH1 and either Δ2180 or Δ0039 isogenic mutants. At the experimental end point (72 hr), mice were euthanized and blood and brains were collected for the enumeration of surviving bacteria. PCR-based screening was used to distinguish between the WT and mutant strains. Data is expressed as the percentage of WT or mutant GBS recovered compared to total recovered CFU. Consistently more WT GBS than the Δ2180 and Δ0039 mutant strains were recovered from the blood and brain (Fig. 3.6A,B). This is consistent with previous results with the ΔciaR mutant, and suggests that both CiaR regulated genes contribute to bacterial fitness and virulence.
**Figure 3.6.** GBS SAN_2180 and SAN_0039 contribute to overall bacterial virulence. The recovered bacterial CFU in blood and brains were analyzed 72 hr after intravenous injection with equal amounts of WT and mutant GBS strains into CD1 mice. Bacteria were enumerated on THA plates with serial dilutions and the bacterial colonies from the same dilution were distinguished by colonies PCR between WT and mutant strains. A. The percentage of recovered CFU in blood and brains from WT and the ∆2180 mutant strain. B. The percentage of recovered CFU in blood and brains from WT and the ∆0039 mutant strain. Statistical analysis performed was an unpaired t-test and the data represents mean ± S.D. *p < 0.00005 ****.

**DISCUSSION**

Penetration of the BBB likely requires GBS to invade microvascular endothelial cells and transcytose through cells, exiting basolaterally to breach the CNS. It is known that GBS can persist within brain endothelial cells for up to 24 hours post infection,
however, there is no net increase in bacterial replication, and the intracellular pool actually decreases over time\textsuperscript{7,11}. This is likely due to the ability of the host cell to limit GBS intracellular growth by using various forms of antibacterial defense including transit to the lysosomal compartment for subsequent degradation. We observed that intracellular WT GBS readily acquired markers of endosomal maturation, as GBS associated with early and late endosomes, and approximately 25-30\% of intracellular organisms localized with acidified lysosomal vesicles (Fig. 3.1). Our results presented here suggest that GBS may use the response regulator, CiaR, to modulate gene transcription to prevent endocytic trafficking to the lysosome. A GBS mutant deficient in CiaR exhibited increased localization with vesicles in the endocytic pathway including Rab5, Rab7 and LAMP1 positive cells. Rab GTPase modifications have been identified during bacterial infection and Rab5 modulation by numerous pathogens has proven to be an efficient strategy to promote intracellular replication or persistence\textsuperscript{28}. Additionally, bacteria such as \textit{Helicobacter pylori} and \textit{Mycobacterium bovis} may modulate Rab7 endosomal maturation in order to establish a protective intracellular niche during infection\textsuperscript{29,30}. Interestingly, following infection with the ΔciaR mutant less viable bacteria was recovered from lysosomes isolated from hBMEC. This is likely due to the increased sensitivity of the ΔciaR mutant to the hostile environment of the phagolysosome, namely antimicrobial peptides and reactive oxygen species, which we have demonstrated here and previously\textsuperscript{8}.

While GBS is not thought of as a classic intracellular pathogen, GBS survival in phagocytic cells has been reported\textsuperscript{31,32}. Most research has focused on understanding the virulence factors responsible for GBS persistence in human macrophages and
neutrophils. The pore forming β-hemolysin/cytolysin (β-h/c) encoded by cylE is a major virulence factor contributing to GBS disease progression\(^3\). Interestingly, cylE deletion results in the loss of β-h/c activity and the carotenoid pigment. It has been shown that cylE contributed to enhanced GBS survival within phagocytes that was attributed to the ability of carotenoid to shield GBS from oxidative damage\(^{33}\). However, other reports suggest that the β-h/c did not impact intracellular survival in macrophages or even that the absence of the β-h/c enabled increased GBS survival in professional phagocytes\(^{34}\). It is possible that these results reflect differences in GBS strains, β-h/c production, and/or host cells and cell lines, and requires further investigation. Other GBS cell associated factors reported to impact survival in phagocytic cells include pili\(^{27}\) and the capsule polysaccharide\(^{35,36}\). An additional TCRS, the CovR/S global regulator has also been shown to be required for intracellular survival in macrophages\(^{37}\). This study suggests that CovR/S mediates a transcriptional response stimulated by the acidic environment in the phagolysosome that mediates survival\(^{37}\). Less is known about GBS survival in epithelial or endothelial cells. Interestingly, we have previously infected hBMEC with a GBS ΔcovR mutant to assess bacterial uptake and survival, and were not able to recover viable ΔcovR bacteria from the intracellular compartment\(^{20}\). However, at this point we cannot conclude whether CovR regulation is required for GBS invasion into brain endothelial cells, or if it regulates intracellular survival.

Two-component regulatory systems allow bacteria to adapt to changing environmental conditions. CiaR/H is not fully characterized in GBS, but it has been linked to stress tolerance and host defense resistance similar to the role of CiaR/H in
\textit{Streptococcus mutans}\textsuperscript{38} and \textit{Streptococcus pneumoniae}\textsuperscript{39}. Interestingly, the CiaR homologue has also been described to be involved in β-lactam resistance and lytic capabilities in \textit{S. pneumoniae}\textsuperscript{40,41}. CiaR-deficient GBS displayed decreased intracellular survival in neutrophils, macrophages, and brain microvascular endothelial cells and was more susceptible to killing by antimicrobial peptides and reactive oxygen species, suggesting CiaR/H as a vital element for environmental stress tolerance\textsuperscript{8}. Previously, our group identified a subset of genes that are down-regulated in a CiaR- deficient mutant. One gene, SAN_0039, encodes a putative metallopeptidase exhibiting a high degree of homology (70% similarity, 56% identity) to a protein called Zoocin A (zooA)\textsuperscript{8}. Zoocin A is produced by \textit{S. zoopidemicus} (Group C \textit{Streptococcus}) which has a bacteriolytic effect on several other Streptococcal species\textsuperscript{42}. Zoocin A has two functional domains, an N-terminal catalytic domain and a C-terminal substrate-binding or target recognition domain\textsuperscript{43,44}. Zoocin A was recently determined to act as a d-alanyl-L-alanine endopeptidase which hydrolyses the cross bridge of peptidoglycan of certain \textit{Streptococcus} species\textsuperscript{45}. Utilization of peptidoglycan hydrolases for both peptidoglycan rearrangement and pathogenicity in host cells types has been described in several bacteria. \textit{S. pneumoniae}, \textit{Listeria monocytogenes}, and \textit{Staphylococcus aureus} employ differential acetylation strategies to obtain resistance to lysozyme\textsuperscript{46-48}. Recently, another peptidoglycan hydrolase, known as IspC, was identified in \textit{L. monocytogenes} as being essential for virulence in vivo, and crossing the blood-cerebrospinal fluid barrier\textsuperscript{49}. The attenuated virulence of an IspC deficient mutant may be partly due to the reduced surface expression or display of other known or putative virulence factors\textsuperscript{49}. Future studies using proteomic analysis of GBS WT and the Δ0039 mutant strain will be of
interest to determine if the GBS peptidoglycan hydrolase contributes indirectly to virulence and disease progression by modulating surface targeting mechanisms that affect other GBS factors.

Another GBS gene, SAN_2180, was the most highly down-regulated gene in the \Delta ciaR mutant\textsuperscript{8}. Characterization of a \Delta2180 mutant demonstrated that this gene contributes to bacterial uptake and survival within brain endothelial cells as well as virulence \textit{in vivo}. Like the \Delta ciaR mutant, the \Delta2180 mutant more readily localized with endosomal and lysosomal marked cells, but was not isolated at an increased rate from lysosomes even at early times points likely due to an increased sensitivity to antimicrobial factors. Thus this factor may be the primary regulated CiaR gene responsible for the observed phenotype of the \Delta ciaR mutant. Protein sequence analysis using BLAST initially found that the predicted SAN_2180 protein belonged to the proteins of unknown function family, DUF1003, but shared homology (42% identity; 60% similarity) to genes in \textit{Lactococcus lactis} involved in acid tolerance and multistress tolerance\textsuperscript{50}. Additionally, the SAN_2180 protein sequence is homologous to cyclic nucleotide-binding proteins present in other \textit{Streptococcus} species such as \textit{Streptococcus urinalis} (84% identity), \textit{Streptococcus parasanguinis} (69% identity) and \textit{Streptococcus galolyticus} (60% identity). Cyclic nucleotide-binding proteins are important for binding intracellular messengers such as cyclic AMP (cAMP). Modulation of host cAMP levels has been proven to be a novel bacterial mechanism to engage inflammatory responses and disease progression\textsuperscript{51}. Furthermore, several \textit{S. pneumoniae} has been implicated in harnessing cAMP binding modulatory capabilities; still no known functionality has been identified within GBS\textsuperscript{52}. Further characterization
of the SAN_2180 encoded protein is needed to elucidate specific mechanisms responsible for its role in GBS intracellular survival.

In summary our data suggest that GBS may modulate gene expression through the TCRS CiaR/H to promote intracellular survival by limiting trafficking to the lysosome as well as promoting survival in the lysosomal environment. At later time points we observed only 25% of intracellular WT bacteria localizing with LAMP1 positive vesicles compared to 45% in the absence of CiaR regulation. Interestingly, we have not observed GBS free in the cytoplasm of hBMEC, even at later time points\textsuperscript{12}, suggesting that surviving GBS likely traffics through endosomes in brain endothelial cells to promote transcytosis across the BBB. Further investigation is required to determine exact mechanisms of how a percentage of WT GBS avoid the lysosomal compartment and transit through the brain endothelium in order to breech the CNS. Ongoing studies on GBS modulation of this process will be critical for identifying new bacterial and host targets that may inform the development of preventative therapies.
ACKNOWLEDGEMENTS

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Chapter 3, in full, is currently in submission to *Cellular Microbiology*. Chapter 3 specifically addresses the points outlined in Aim 2 of the dissertation. The dissertation author was the primary investigator and author of this paper.
REFERENCES


CHAPTER 4

ATG16L1 regulates GBS invasion and NOD2 dependent inflammatory activation in brain endothelium

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In preparation for submission

Running Title: ATG16L1 promotes GBS invasion and ensuing inflammation
ABSTRACT

Group B *Streptococcus* (GBS) is a Gram-positive organism responsible for the development of neonatal disease, namely meningitis. This pathogen has the unique ability to penetrate the blood-brain barrier, while evading a wide variety of innate immune defenses. Here, we describe the impact of one important autophagy related protein, known as ATG16L1, in regulating GBS entry into brain endothelial cells as well as the inflammatory response associated with GBS infection. GBS was demonstrated to co-localize with ATG16L1 and knockout of ATG16L1, using CRISPR methodology, in brain endothelium resulted in decreased GBS entry and eventual survival. Furthermore, bacterial entry was dependent on clathrin, as clathrin inhibition resulted in decreased GBS invasion. Clathrin is a known ATG16L1 interacting partner and clathrin inhibition in ATG16L1 deficient cells demonstrated that clathrin activation is dependent upon the presence of ATG16L1. Additionally, GBS infection induced NOD2 activation and siRNA mediated impairment of NOD2 resulted in decreased GBS invasion. NOD1, NOD2, and a variety of inflammatory cytokines associated with the development of meningitis were regulated by ATG16L1. Our findings suggest that ATG16L1 is central regulator of both GBS invasion into brain endothelium and production of the inflammatory response due to the infection process. This dual role of ATG16L1 has yet to be described in the context of bacterial meningitis and is likely critical for brain endothelial defenses.
INTRODUCTION

Intracellular host defenses are critical toward persistence of host cell types against microbial hazards; however, these pathogens have developed elegant ways to evade host detection. Protection aims to limit the bacterial burden within the cell and activate immune processes, thereby halting disease progression. One known resistance mechanism utilized is known as autophagy. Autophagy is a catabolic process in which pathogens are targeted by double membrane structures known as autophagosomes for sequestration of these invaders for eventual delivery and ensuing degradation in the lysosome. An extensive amount of autophagy related proteins have been implicated in playing a role in antimicrobial autophagy, which has led to a greater understanding of not only autophagy itself, but also infectious processes\textsuperscript{1,2}. Although there are approximately 40 known autophagy related proteins that contribute to all aspects this pathway, one protein in particular, known as ATG16L1, is of considerable interest. ATG16L1 plays an important role in autophagosome biogenesis through its ability to bind ATG5 and ATG12 thereby creating the ATG5-12-16L1 complex which lipidates LC3 to phosphatidylethanolamine for autophagosome elongation\textsuperscript{3}. Human ATG16L1 is composed of an N-terminal ATG5 binding domain along with a central coiled-coiled domain and a C-terminal WD domain (Figure 4.1)\textsuperscript{4,5}. 
Figure 4.1. Structure and functions of human ATG16L1. Human ATG16L1 contains an N-terminal ATG5 binding domain along with a coiled coiled domain and seven WD (tryptophan-aspartic acid repeats). The coiled coiled domain is involved in homotypic interactions/self-oligomerization of ATG16L1 to elicit autophagy activation whereas the WD repeats are able recruit other autophagic proteins. ATG16L1 has numerous roles including regulating LC3 localization while simultaneously specifying the exact location of LC3 lipidation during autophagosome membrane biogenesis. It is also known that ATG16L1 is directed to the site of autophagosome formation by FIP200, one key protein involved in the ULK1 autophagy activation complex. Furthermore, plasma membrane localized ATG16L1 may represent autophagosome precursors and are internalized via clathrin mediated endocytosis. These ATG16L1 structures are then bound by clathrin and upon internalization, undergo homotypic fusion thereby allowing for LC3 targeting of these vesicles for autophagy activation. Unexpectedly, ATG16L1 deficiencies have been correlated with protection against urinary tract infections caused by uropathogenic Escherichia coli (UPEC). This group discovered that mice deficient in ATG16L1 produced an increased innate immune response resulting in more effective clearance of UPEC and faster restoration of the urinary epithelium. Finally, it has also been proposed that the transmembrane protein, TMEM59, directly binds the WD domains of
ATG16L1 to promote LC3 labeling for autophagic degradation and this interaction is critical during *Staphylococcus aureus* infection\textsuperscript{11}. Remarkably, the autophagy-stimulating motif specific for ATG16L1 present within TMEM59 has also been identified in other crucial bacterial recognition proteins known as TLR2 and NOD2, suggesting a prominent role of this protein during anti-bacterial autophagy\textsuperscript{12,13}.

Recently, the presence, absence, or modification of ATG16L1 has been linked to the development of a variety of bacterial infections. Initially, genome-wide association studies discovered that ATG16L1 mutations are associated with the development of inflammatory bowel disorders (IBD) such as Crohn’s disease and ulcerative colitis\textsuperscript{14}. IBDs are characterized by a microbial shift within the gastrointestinal (GI) tract that results in an intense inflammatory response that injures portions of the GI tract. Increasing evidence is being accumulated that suggests that underlying genetic abnormalities, such as ATG16L1 and even NOD2 mutations, lead to these aberrant microbial responses\textsuperscript{14,15}. These studies suggest that autophagy and potential clearance of pathogens within the GI tract is critical for maintenance of the intestinal microbiota. One of the most clinically relevant missense single nucleotide polymorphisms (SNPs) in ATG16L1 is the A>G SNP that results in a threonine becoming an alanine at amino acid 300 in exon 9\textsuperscript{16}. One study in particular assessed the importance of ATG16L1 during infection with the intestinal pathogen, *Citrobacter rodentium*, and found that a deficiency in ATG16L1 again lead to elevated immune responses toward pathogen infection\textsuperscript{17}. This was followed up by another study by the same group, which implicated that *Staphylococcus aureus* α-toxin triggers autophagy *in vivo* and that mice lacking ATG16L1 specifically in the endothelium, resulted in increased bacterial
Finally, it was discovered that ATG16L1 acts as a negative regulator of NOD2 dependent cytokine secretion, which was independent of its autophagy related functions. Understanding the molecular mechanisms associated with ATG16L1 independent of and dependent autophagy, as well as innate immune activation is essential for determining the manifestation of disease. Little is known about the contribution of ATG16L1 during Group B Streptococcus (GBS) infection, one of the main pathogens associated with the progression of neonatal meningitis, which leaves an interesting avenue for investigation. We hypothesized that there was a connection between bacterial intracellular survival, the inflammatory response, and eventual destruction of the BBB.

Nucleotide oligomerization domain proteins, also known as NODs, function as intracellular sensors of bacterial peptidoglycan, namely conserved pathogen associated molecular patterns (PAMPs) that act as regulators of an immense amount of antibacterial defenses. NOD proteins come in two forms, NOD1 and NOD2. NOD1 primarily recognizes d-glutamyl-meso-diaminopimelic acid that is present within mostly gram-negative bacteria as well as some gram-positive species such as Listeria monocytogenes whereas NOD2 detect muramyl dipeptide that is ubiquitously present in all bacterial species. NOD1 and NOD2 have similar domain architectures with each containing N-terminal caspase activation recruitment domains (CARD), a central nuclear binding domain (NBD), and C-terminal leucine rich repeats (LRRs) (Figure 4.2). NOD2 contains two CARD domains whereas NOD1 only contains one, and these moieties are critical to receptor interacting protein-2 (RIP2) dependent signal
transduction\textsuperscript{20}. The NBD domain is involved in NOD self-oligomerization and the LRRs are essential for sensing and detection of bacterial ligands\textsuperscript{21}.

\textbf{Figure 4.2. NOD1 and NOD2 protein domain architecture.} NOD1 and NOD2 share identical motifs related toward identification of bacterial peptidoglycan, with the exception of NOD1 lacking one CARD domain.

NOD proteins play a pivotal role in the interplay between bacterial recognition, autophagy activation, and the ensuing inflammatory response. Typically, NODs are activated in response to direct bacterial invasion and infection, which was first demonstrated in the case of \textit{Shigella flexneri} infection\textsuperscript{22}. However, there have been a couple instances of NOD activation occurring independent of bacterial invasion. For example, outer membrane vesicles from \textit{Helicobacter pylori}, \textit{Pseudomonas aeruginosa}, and \textit{Neisseria gonorrhoea} were able to induce NOD1 activation\textsuperscript{23}. Still, the central mechanism of NOD activation is contingent upon invasive bacterial species. NOD activation stimulates innate immunity primarily through activation of NF-κB and MAPK signaling. This results in the production of pro-inflammatory cytokines such as IL-6, IL-8, CXCL1, and CXCL2. With this, it has been described that mice lacking either NOD1 or NOD2 are more susceptible to bacterial infection and such is the case during infection with \textit{Staphylococcus aureus}, \textit{Helicobacter pylori}, and \textit{Yersinia pseudotuberculosis}, to name a few\textsuperscript{20}. Recently, connections have been made between
autophagic activation and NOD proteins. One of the most interesting findings demonstrated that NOD1 and NOD2 direct ATG16L1 to bacterial invasion sites thereby activating the autophagic process\textsuperscript{13}. NOD2 mutations were first associated with Crohn’s disease manifestation; however, no relationship to autophagy or other degradation systems had been established\textsuperscript{24}. It was initially thought that NOD2 was only associated with maintenance of the intestinal barrier, however, this study provided mechanistic evidence that linked two of the main Crohn’s disease susceptibility genes, NOD2 and ATG16L1, cooperating together to combat intestinal bacteria\textsuperscript{13,25}. More recently, it was discovered that ATG16L1 regulates NOD1 and NOD2 dependent inflammatory cytokine secretion, which is independent of its autophagic functions\textsuperscript{19}. These findings reveal that there is an intricate signaling axis between NOD proteins and ATG16L1 that results in both antibacterial autophagy and inflammation (Figure 4.3).

NOD proteins have yet to be extensively studied in the context of GBS infection and the development of meningitis, but there have been several papers investigating their exact role. The Nod-like receptor-P3 (NLRP3) inflammasome present within macrophages has been implicated during GBS infection. This signaling complex is involved in inflammatory signaling, namely IL-1\textbeta secretion and this secretion is dependent upon \ensuremath{\beta}-hemolysin production by GBS\textsuperscript{26}. Furthermore, mice lacking the NLRP3 inflammasome were more susceptible to GBS infection than WT mice\textsuperscript{26}. Additionally, it was recently found that GBS RNA, as well as other component of the lysosome, specifically interact with NLRP3 to induce activation through IL-1\textbeta production\textsuperscript{27}. Still, there has been some confounding evidence that supports a notion that NOD2 is not required for complete protection against GBS infection \textit{in vivo}. The
authors demonstrate that mice containing a NOD2 mutation lead toward similar clinical outcomes compared to WT controls; however, NOD2 deficient mice exhibited a diminished cytokine response \textit{in vivo}\textsuperscript{28}. These studies contained a variety of discrepancies, which were that the experiments were performed using only mutated NOD2 in lieu of a complete NOD2 knockout, GBS was introduced to the mice via intraperitoneal (IP) injection rather than intravenous (IV) tail vain injection, and the mice used were female mice rather than male mice\textsuperscript{28}. With this, it is still of great interest to examine how these proteins are induced during GBS infection of brain endothelium.

\textbf{Figure 4.3. Role of ATG16L1 in combating intracellular bacteria and activation of innate immunity.} Invasive bacteria are directed toward autophagy by the interactions between NOD1/2 and ATG16L1, independent of RIP2. NOD1/2 activation can also stimulate RIP2 to direct an inflammatory cytokine response along with MAPK initiation. It has also been discovered that ATG16L1 negatively regulates RIP2-dependent cytokine secretion, independent of its autophagy functions.
We hypothesize that ATG16L1 may be playing a similar role in regulating NOD dependent cytokine secretion, independent of its autophagic functions, during GBS infection of brain endothelium. Furthermore, recent studies have indicated that ATG16L1 binds clathrin-coated vesicles, specifically the clathrin adaptor protein AP-2, and that clathrin-mediated entry of GBS acts as the initial trigger for this process. We believe that invasive GBS enter brain endothelium through clathrin-mediated endocytosis, are then labeled by ATG16L1, which in turn recruits NOD2 thereby activating an innate immune program. Although we hypothesize that GBS enters via a clathrin-mediated mechanism, it is possible that it may utilize other routes to gain entry into brain endothelium. To investigate these notions, we set out to determine whether ATG16L1 was recruited to intracellular GBS and whether modulation of this protein was critical toward GBS invasion and/or survival.
MATERIAL AND METHODS

*Bacterial Strains.* The wild-type strain of GBS used in this study is a highly encapsulated serotype III strain\(^39\). GBS stably expressing GFP was used as previously described\(^30\). GBS lacking *cylE* and *ciaR* were created as previously described\(^31,32\).

*Cell Culture.* The human brain microvascular endothelial cell (hBMEC) line was kindly provided by Kwang Sik Kim (Johns Hopkins University) and cultured as previously described in RPMI 1640 (VWR) containing 10% FBS, 10% Nuserum (BD Biosciences, Cat. No. 355504) and 1% nonessential amino acids (Life Technologies)\(^33\).

*Bacterial infection assays.* hBMEC were grown to confluence (~10\(^5\) cells per well) and washed three times prior to GBS infection. For inhibitor studies, cells were treated with either Dynasore (5µM, Enzo Biosciences), Genestein (100mM, Sigma), Monodansylcadaverine (5µM, Sigma) or Methyl-Beta Cyclodextrin (100µM, Sigma) for 30 minutes prior to infection with GBS. GBS were grown in THB to mid-log phase (~10\(^8\) CFU/ml; OD\(_{600}\) = 0.4), washed in PBS, resuspended in RPMI plus 10% FBS, and used to infect monolayers at a multiplicity of infection (MOI) of 1 or 10 for various time points. Plates were then centrifuged at 1000 RPM for 3 min to synchronize infection and then incubated at 37°C in 5% CO\(_2\). After infection, cells were treated with Penicillin (5µg/mL) and Gentamycin (100µg/mL) to kill extracellular GBS for various time points. Cells were treated with 0.1 mL of 0.25% trypsin-EDTA solution and lysed with addition of 0.4 mL of 0.025% Triton X-100 by vigorous pipetting. The lysates were then serially diluted and plated on THB agar to enumerate bacterial CFU.

*CRISPR mediated gene disruption.* To modulate ATG16L1, clustered regularly interspersed short palindromic repeats (CRISPR) technology was utilized using the
GeneArt CRISPR Nuclease Vector from Life technologies\textsuperscript{34,35}. Briefly, ATG16L1 CRISPR target sequences or guide RNAs (gRNA) were designed using the MIT CRISPR design tool (http://crispr.mit.edu). Once synthesized, oligonucleotides were annealed and ligated into a CRISPR nuclease orange fluorescent protein (OFP) reporter plasmid (Figure 4.4) (Life Technologies). Constructs were then transformed into competent DH5\textalpha Escherichia coli and selected for ampicillin resistance. Transformants that grew under ampicillin selection were then sent for sequencing to confirm insertion of the ATG16L1 CRISPR target. hBMEC were seeded at approximately \textasciitilde50\% confluence and transfected with \textasciitilde2\mu g of the ATG16L1 CRISPR vector for 48 hours. Single cell clonal populations and whole populations of OFP positive cells were isolated via fluorescent activated cell sorting (FACS). ATG16L1 disruption was analyzed by western blot analysis. A table of optimal CRISPR gRNA’s, their respective scores after design, and genomic location can be visualized in Table 4.1.

<table>
<thead>
<tr>
<th>ATG16L1 CRISPR gRNA sequence with PAM (Protospacer adjacent motif)</th>
<th>Identification</th>
<th>Location</th>
<th>Score</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAAAGCATGACGTACCAAACAGG</td>
<td>ATG16L1 CRISPR 1</td>
<td>Exon 2</td>
<td>87</td>
<td>Sense</td>
</tr>
<tr>
<td>CCTGCGCATACTAAGCTTTGTACC</td>
<td>ATG16L1 CRISPR 2</td>
<td>Exon 5</td>
<td>94</td>
<td>Antisense</td>
</tr>
<tr>
<td>CAGTCCAGGTTCGCCGTTACTGTG</td>
<td>ATG16L1 CRISPR 3</td>
<td>Exon 10</td>
<td>91</td>
<td>Sense</td>
</tr>
</tbody>
</table>
Figure 4.4. Basic GeneArt CRISPR vector map. The ATG16L1 guide RNA was inserted under the control of the U6 promoter along with a trans-activating CRISPR RNA that allows for endonuclease targeting of the guide RNA. The ampicillin resistance cassette allows for selection of the plasmid in *E. coli* and the orange fluorescent protein (OFP) is used to isolate cells that contain the plasmid via FACS. Finally, the bacterial endonuclease, Cas9, contains nuclear localization signals (NLS) and both OFP and Cas9 are expressed by the cytomegalovirus (CMV) promoter.

**Immunofluorescence staining.** hBMEC were fixed with 4% paraformaldehyde, solubilized with 0.1% Triton X-100 and were then probed with antibodies directed at ATG16L1 (MBL or Cell Signaling Technologies), GBS (Acris), and Clathrin (Cell Signaling Technologies). Stained hBMEC were mounted in VectaShield with DAPI (Vector Laboratories). Samples were visualized using a Zeiss Axiovert 200 inverted
fluorescence microscope (Carl Zeiss) or a Zeiss LSM 710 confocal microscope (Carl Zeiss).

**Quantitative Reverse Transcriptase PCR.** RNA isolation, cDNA preparation and quantitative RT-PCR were performed according to standard protocols. Relative gene expression was calculated using the following equation: Relative gene expression = target gene efficiency x (C_T control − C_T sample)/efficiency for GAPDH x (C_T control − C_T sample). Primers used for qRT-PCR are outlined in Table 4.2.

### Table 4.2. Primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer (5′→3′)</th>
<th>Reverse Primer (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD1</td>
<td>GTACGTCACCAAAATCCTGGAA</td>
<td>CAGTCCCTTAGCTGTGATC</td>
</tr>
<tr>
<td>NOD2</td>
<td>GAGAACATGCTGGACCTGG</td>
<td>CAATCCATTGCTTTCACCG</td>
</tr>
<tr>
<td>IL-8</td>
<td>AGCTCTGTGTGAGGTGCA</td>
<td>AATTTCCTGTGTGGCCGCAG</td>
</tr>
<tr>
<td>IL-6</td>
<td>GAGAGCTTGCTGGTGAA</td>
<td>CAGGGGTGTATTTCATCT</td>
</tr>
<tr>
<td>CXCL1</td>
<td>CTCTTCGGCTCTCCTCACAG</td>
<td>GGGAAGCTTCACGTCACACT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAAGGTGAAAGGTTGGAGA</td>
<td>TCCTGGGAAGATGGGTGATGGGA</td>
</tr>
</tbody>
</table>

*siRNA knockdown of NOD2.* siRNA’s directed toward NOD2 as well as a scrambled siRNA were purchased from the Invitrogen select silencer series. siRNA’s were transfected with Lipofectamine-3000 (Life Technologies) for 48 hours prior to GBS infection. NOD2 silencing was verified by quantitative reverse transcriptase PCR.

**Statistical analysis.** Graphpad Prism version 5.0f was used for statistical analysis. Unpaired t-tests or one way-ANOVA were used for analysis. Statistical significance was accepted at p < 0.05.

**RESULTS**

**ATG16L1 localizes with Intracellular GBS**

To examine whether intracellular GBS is recognized by ATG16L1, we employed immunofluorescence microscopy. We found that at early time-points post
infection, ATG16L1 localized with intracellular bacteria, suggesting that this protein is indeed acting early with GBS within brain endothelium (Figure 4.5).

![Figure 4.5. Intracellular GBS localizes with ATG16L1. hBMEC were infected for 2 hours with WT COH1 GBS at an MOI=1 and subsequently treated with antibiotics for 30 minutes. Cells were stained with antibodies directed at GBS and ATG16L1 and visualized using confocal microscopy.](image)

We next sought to characterize whether ATG16L1 was critical for GBS invasion or survival within hBMEC and to accomplish this, we utilized a CRISPR mediated genomic disruption approach. Once we were able to successfully create an ATG16L1 CRISPR knockdown hBMEC cell line (Figure 4.6), we infected this cell line with GBS to assess bacterial attachment and invasion (Figure 4.7). We discovered that while GBS displayed increased adherence and invasion into ATG16L1 deficient cells, when calculating the percentage of the hBMEC-associated GBS that had invaded the intracellular compartment, there was decreased invasive capability (Figure 4.7). This suggests that ATG16L1 may play a critical role in internalization of GBS into hBMEC. To further investigate this defect, we assessed GBS intracellular survival in hBMEC over time. As revealed earlier, GBS again exhibited decreased invasion in ATG16L1 deficient cells, resulting in a decreased intracellular load of bacteria over time (Figure 4.7). These data indicate that ATG16L1 plays an important role during GBS invasion of
hBMEC and that this protein may be vital for GBS disruption of the BBB and disease progression.

**Figure 4.6. Development of an ATG16L1 deficient hBMEC cell line.** CRISPR constructs containing either CRISPR guides 1, 2, or 3 were transfected into hBMEC and single cell clones were sorted into a 96 well plate (see table 4.1). Once confluent, single cell clonal populations with each construct were screen via western blot analysis. Figures A, B demonstrate a typical screen with CRISPR guide 2 single cell clones, where one particular clone, known as B8, demonstrating no expression of ATG16L1. This clone was utilized as our ATG16L1 KO hBMEC line.
Figure 4.7. Elimination of ATG16L1 leads to increased GBS adherence and decreased GBS invasion into hBMEC. A. GBS is more adherent to ATG16L1 deficient brain endothelial cells after a 30-minute adherence assay. B. hBMEC were infected for 2 hours with WT COH1 GBS at an MOI=10 prior to addition of antibiotics for an additional hour. Bacterial CFU was enumerated and GBS relative invasion was quantified. C. Western blot analysis of GBS infected WT and ATG16L1 KO hBMEC (clone B8, CRISPR construct 2) Data represent the mean ± S.D. from a representative experiment. D. hBMEC were infected for 2 hours at an MOI=10 and bacterial CFU were enumerated at various time points post antibiotic treatment (1, 2, 4, 6 hours post infection). *, p < 0.05; **, p < 0.005; ***, p < 0.001; ****, p < 0.0001. Error bars represent S.D.
Assessment of the mechanism of GBS invasion into brain endothelium.

GBS has been hypothesized to invade through an endocytic process, so we set out to inhibit a variety of endocytic processes known to be utilized by bacteria to gain entry into host cells. Initially, we inhibited clathrin-mediated endocytosis through the use of either Dynasore, a cell-permeable inhibitor of Dynamin, as well as Monodansylcadaverine, a specific inhibitor of clathrin through its ability to perturb membrane infolding. We found that inhibition of clathrin-mediated endocytosis resulted in a significantly less amount of recovered intracellular GBS (Figure 4.8). Using confocal microscopy we also observed that GBS appeared to co-localize with clathrin (Figure 4.8). Initially, this indicated that this route of entry might be essential for GBS invasion into hBMEC thus leading toward ATG16L1 targeting. However, we did find that inhibition of lipid rafts and caveolin-mediated endocytosis also lead toward decreases in GBS invasion (Figure 4.9). Thus GBS may utilize multiple pathways to enter host cells, which impact intracellular trafficking. We further inhibited clathrin-mediated endocytosis using Dynasore in both WT and ATG16L1 CRISPR knockout hBMEC. We found that again there was a decrease in GBS invasion in Dynasore treated WT hBMEC compared to untreated controls (Figure 4.10). However, there were equal amounts of recovered GBS in both WT and ATG16L1 knockout hBMEC treated with Dynasore (Figure 4.10). Our results indicate that disruption of clathrin-mediated endocytosis in hBMEC lacking ATG16L1 has no effect on GBS uptake, further suggesting the importance of ATG16L1 for GBS invasion into brain endothelium.
Figure 4.8. Subsets of GBS enter hBMEC via clathrin mediated endocytosis. A, B. For each experiment, each inhibitor was added to hBMEC for 30 minutes prior to a 2 hour infection with COH1 WT at an MOI=1 for treatment with Monodansylcadaverine (MDC) at an MOI=10 for Dynasore. Antibiotics were added for 1 hour post infection. All experiments were performed at least three times. C. hBMEC were infected with WT COH1 GBS and subsequently stained for clathrin heavy chain to examine whether co-localization occurred with intracellular GBS. Data represent the mean ± S.D. from a representative experiment performed in triplicate. *, p < 0.05; **, p < 0.005; ****, p < 0.0001. Error bars represent S.D.

Figure 4.9. GBS enters hBMEC through a variety of mechanisms. A, B. For each experiment, each inhibitor was added to hBMEC for 30 minutes prior to a 2 hour infection with COH1 WT at an MOI=1 for treatment with Methyl-Beta Cyclodextrain(MβCD) and Genestein. Antibiotics were added for 1 hour post infection. All experiments were performed at least three times. Data represent the mean ± S.D. from a representative experiment performed in triplicate. ****, p < 0.0001. Error bars represent S.D.
Figure 4.10. GBS invasion requires ATG16L1 and clathrin. WT and ATG16L1 KO hBMEC were pre-treated with Dynasore for 30 minutes prior to WT COH1 GBS infection for 4 hours. Antibiotics were administered after infection for 1 hour and bacterial CFU was plated. Data represent the mean ± S.D. from a representative experiment performed in quadruplicate. **, \( p < 0.005 \); ***, \( p < 0.001 \);. Error bars represent S.D.

NOD2 is activated in response to GBS infection

To initially investigate NOD activation in BBB endothelium, we infected hBMEC with WT GBS and subsequently measured NOD1 and NOD2 transcript abundance using quantitative reverse transcriptase PCR. Surprisingly, we saw a drastic increase in the amount of NOD2 being produced after infection whereas NOD1 was comparable to uninfected hBMEC expression levels (Figure 4.11). This led us to hypothesize that NOD2 activation occurs specifically during GBS infection and may impact immune signaling and GBS intracellular survival. To this end, we knocked down NOD2 using siRNA to assess the importance of this factor during infection. We discovered that there was a modest, yet significant increase in the amount of bacteria recovered from hBMEC treated with siRNA’s directed at NOD2 compared to siRNA
scrambled controls (Figure 4.11). In the end, these data demonstrate that NOD2 is vital toward limiting intracellular GBS.

**ATG16L1 regulates NOD2 dependent cytokine secretion**

Recently ATG16L1 has been demonstrated to regulate NOD dependent cytokine secretion in a model bacterial induced aberrant inflammation within intestinal epithelial cells, one of the hallmarks Crohn’s disease\(^\text{19}\). The next logical step was to examine whether the presence of ATG16L1 impacted NOD production. Using qRT-PCR on ATG16L1 CRISPR KO hBMEC and found that in the absence of ATG16L1, without GBS infection, there was an increase in both NOD1 and NOD2 expression (Figure 4.12). We then infected ATG16L1 deficient hBMEC with WT GBS and examined NOD1 and NOD2 activation. Interestingly, we saw suppression of NOD1 transcript production in cells lacking ATG16L1 compared to WT controls, although transcript production was very minimal (Figure 4.12). However, NOD2 transcript production was doubled in ATG16L1 deficient, GBS infected cells compared to their WT counterparts (Figure 4.12). These data indicate that NOD2 driven responses are regulated by ATG16L1 in hBMEC during infection.

NOD activation typically leads toward an inflammatory response against microbial pathogens, we hypothesized that several key cytokines associated with the development of meningitis would be differentially expressed in hBMEC lacking ATG16L1. To examine this notion, we again employed qRT-PCR to examine transcript production of IL-8, IL-6, and CXCL1 to see if these inflammatory cytokines were regulated by ATG16L1. We found that all the cytokines we analyzed had increased expression levels within ATG16L1 deficient hBMEC during GBS infection (Figure
This data strongly suggests that ATG16L1 may regulate NOD driven cytokine responses in hBMEC and that ATG16L1 could be a critical regulator of inflammatory pathways during GBS infection.

**Figure 4.11. NOD2 is activated and necessary for efficient clearance of intracellular GBS.** hBMEC were infected with WT COH1 GBS at an MOI=10 for 4 hours and subsequently treated with antibiotics for 1 hour. For qRT-PCR, lysates were collected and probed with specific primers to assess NOD1 and NOD2 in various conditions. For siRNA knockdown experiments, CFU was enumerated to assess GBS survival. All experiments were performed in at least triplicate and were repeated at least three times. Data represent the mean ± S.D. from a representative experiment performed in triplicate or S.E.M. of an experiment performed at least four times in triplicate (siRNA knockdown). **, p < 0.005; ***, p < 0.001. Error bars represent S.D.
A representative experiment was performed for IL-4 hours prior to addition of antibiotics for 1 hour. Lysate was collected and qRT-PCR was performed for IL-8, IL-6, and CXCL1. Data represent the mean ± S.D. from a representative experiment performed in triplicate. *, p < 0.05; **, p < 0.005; ***: p < 0.0001. Error bars represent S.D.

Figure 4.12. NOD1 and NOD2 are regulated by ATG16L1 in brain endothelium. Wild-type and ATG16L1 CRISPR hBMEC were probed for NOD1 and NOD2 transcript production under normal conditions and during infection with COH1 WT for 4 hours. Data represent the mean ± S.D. from a representative experiment performed in triplicate. *, p < 0.05; **, p < 0.005; *. Error bars represent S.D.

Figure 4.13. ATG16L1 regulates production of key inflammatory cytokines. WT and ATG16L1 CRISPR hBMEC were infected with WT COH1 GBS at an MOI=10 for 4 hours prior to addition of antibiotics for 1 hour. Lysate was collected and qRT-PCR was performed for IL-8, IL-6, and CXCL1. Data represent the mean ± S.D. from a representative experiment performed in triplicate. *, p < 0.05; **, p < 0.005, ***, p < 0.001. Error bars represent S.D.
Examination of GBS virulence genes that influence NOD2 transcript production

Various GBS specific genes have been implicated in triggering the virulent nature of GBS. Two essential GBS factors are encoded by the genes cylE and ciaR, which are known to encode the β-hemolysin and a two-component response regulator important in GBS intracellular survival, respectively\textsuperscript{31,32}. In addition, we decided to investigate two genes that are regulated by ciaR and are known as SAN_2180 and SAN_0039\textsuperscript{32}. SAN2180 has been hypothesized to be involved in regulating bacterial responses to acidic stressors while SAN_0039 is thought to have metallopeptidase activity (see Chapter 3). We decided to examine how NOD2 signaling was impacted when hBMEC was infected with strains of GBS that lacked cylE, ciaR, SAN_2180, or SAN_0039. We found that again, NOD2 transcript was significantly increased upon GBS infection compared to the uninfected control and the ciaR while the SAN_2180 mutant produced similar amounts of NOD2 transcript as the WT infected samples (Figure 4.14). Infection with GBS strains lacking cylE resulted in little to no NOD2 transcript production compared to the uninfected control, thus indicating the contribution of hemolysin to NOD2 activation (Figure 4.14). However, strains lacking either SAN_2180 or SAN_0039 demonstrated a further increase in NOD2 production suggesting that these genes are critical for suppressing NOD activation by GBS in hBMEC (Figure 4.14). These genes may prevent downstream NOD2 driven inflammatory activation in brain endothelium thereby allowing for GBS to evade peptidoglycan sensors such as NODs.
Figure 4.14. Specific GBS genes influence the amount of NOD2 activation. hBMEC were infected in quadruplicate with each strain of GBS for 4 hours prior to addition of antibiotics for 1 hour. Lysates were collected and probed for NOD2 production. Data represent the mean ± S.D. from a representative experiment performed in triplicate. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$. Error bars represent S.D.

DISCUSSION

Inflammation associated with the development of bacterial meningitis initially occurs through penetration of the BBB. The mechanistic basis of how GBS disrupts this barrier have yet to be completely understood and it is likely that GBS undergoes transcytosis through brain endothelium to gain access to the central nervous system. GBS meningeal inflammation has previously been described to occur through an integrin dependent pathway in which GBS pili proteins, specifically pilA, engage the α2β1 integrin to elicit MEK/ERK activation and release of IL-8 thereby triggering neutrophil chemotaxis\(^{36}\). Additionally, it is known that GBS can invade brain endothelium and activate the autophagic pathway\(^{30}\), however, there has yet to be a tie
between autophagy and inflammation. Here, we implicate a novel role for the autophagy related protein, ATG16L1, in promoting GBS invasion into hBMEC as well as regulating the inflammatory response generated by these cells. Our data strongly suggests that ATG16L1 supports not only innate immune recognition and uptake of the bacteria, but also is involved in controlling the ultimate inflammatory cytokine response, one of the hallmarks of meningitis.

Typically, ATG16L1 acts within the autophagic cascade as an interacting partner with ATG5 and ATG12 and has also been linked with proper subcellular localization of the autophagic machinery\textsuperscript{4,7}. Polymorphisms in ATG16L1, especially the ATG16L1 T300A risk allele for Crohn’s disease, not only impair clearance of invasive bacteria such as \textit{Helicobacter pylori}, \textit{Salmonella enterica}, and adherent-invasive \textit{Escherichia coli}, it has been described to play a role in differential activation of innate immunity\textsuperscript{37-40}. These notions lead us to examine this protein further in the context of GBS infection of brain endothelial cells. Intracellular GBS co-localized with ATG16L1 after initial infection and CRISPR mediated knockout of ATG16L1 in hBMEC lead to decreased GBS invasion (Figure 4.5 and 4.7). This prompted us to investigate the mechanistic basis for GBS invasion. We explored a variety of endocytic processes using inhibitors and our results suggest that GBS may be taken up into clathrin-coated vesicles (Figure 4.8 and 4.9). This is in line with previous studies that proved that vesicles decorated in clathrin bind ATG16L1 resulting in the formation of pre-autophagosomal structures\textsuperscript{9}. Blockage of clathrin-mediated endocytosis in ATG16L1 knockout hBMEC had no effect on GBS invasion thereby suggesting a key role for ATG16L1 and clathrin binding in GBS uptake (Figure 4.10).
NOD2 activation, another known Crohn’s disease genetic abnormality, was then investigated after GBS infection of hBMEC to potentially connect another ATG16L1 interacting partner. We demonstrate that NOD2 transcript is highly induced during infection and that silencing of NOD2 leads to increased bacterial recovery (Figure 4.11). Knowing that NODs have been implicated in directing ATG16L1 for autophagy and inflammatory activation, we set out to examine if there was interplay between these two proteins in brain endothelium\textsuperscript{13,19}. Remarkably, we discovered that in cells lacking ATG16L1, both NOD1 and NOD2 were differentially expressed in both infection and non-infection conditions (Figure 4.12). This is consistent with our data investigating a panel of known inflammatory cytokines associated with bacterial meningitis (Figure 4.13). We believe that ATG16L1 is regulating NOD2 dependent, RIP2 dependent inflammatory cytokine response during GBS infection. Our data support a negative regulation of pro-inflammatory cytokines, which has been proposed in other model systems\textsuperscript{19}. Additionally, another study examined the importance of ATG16L1 during exposure to endotoxin and found that again, ATG16L1 regulated endotoxin induced inflammasome activation \textit{in vitro} and \textit{in vivo}\textsuperscript{41}. Finally, one recent study linked ATG16L1 with the unfolded protein response (UPR). This group clearly demonstrated that double knockout of ATG16L1 and X-box binding protein-1 (XBP1) specifically in the intestine of mice resulted in increase intestinal inflammation and was strikingly similar to the human pathology of Crohn’s disease\textsuperscript{42}. Previously, our lab has performed microarray analysis on GBS infected brain endothelial cells and one of the key proteins that was differentially regulated was XBP1 (discussed in Appendix B). Therein lies an interesting avenue for investigation and it is of some interest to draw a meaningful
connection between inflammation, autophagy, and the UPR in the context of bacterial infection.

Our data provide evidence that ATG16L1 may be acting as a pivotal innate immune regulator in BBB endothelium. We believe that ATG16L1 is required for initial GBS invasion into hBMEC and that this invasion is dependent on clathrin-mediated uptake. It is still unclear as to how GBS may manipulate the functions of ATG16L1, but we do have some evidence linking several bacterial virulence factors to NOD2 activation (Figure 4.14). Furthermore, we propose that ATG16L1 controls NOD2 dependent cytokine response during the pathogenesis of GBS induced meningitis. Utilization of ATG16L1 hypomorphic mice will be critical to confirm our in vitro studies and examine the contribution of this factor to the development of GBS meningitis in vivo. It could be of profound interest to genotype patients with GBS meningitis to see whether they contain the ATG16L1 risk alleles and whether patients that succumbed to infection also contained ATG16L1 abnormalities. To date, there are no effective treatments to prevent neonatal GBS meningitis, thus improving our understanding of how to modulate bacterial BBB penetration and ensuing inflammatory responses is critical.
ACKNOWLEDGEMENTS

Chapter 4, in full, is currently in preparation for submission for publication. Chapter 4 specifically addresses the points outlined in Aim 3 of the dissertation. The dissertation author was the primary investigator and author of this paper.
REFERENCES


CHAPTER 5

CONCLUSIONS

The pathogen, Group B *Streptococcus* (GBS), is still considered one of the most prevalent infections associated with newborns\(^1\). These infections can be categorized into either early or late onset disease depending on when and what symptoms manifest. GBS EOD is maternally transferred to the infant whereas LOD may come about from a variety of sources\(^2\). Prevention for EOD remain fairly straightforward, primarily administration of intrapartum antibiotic prophylaxis and maternal GBS screening, however there are no direct treatments to prevent LOD\(^2\). GBS LOD occurs in 0.35 births per 1000 live births, and the most deadly outcome is the development of meningitis\(^3\). Little is known about the pathogenesis of this form of GBS disease and it is critical to understand the molecular mechanisms associated with how GBS is able to penetrate the most important barrier in the body, the blood-brain barrier (BBB). We believe that unraveling the complex interplay between host and pathogen will lead to the development of novel preventative treatments for this serious disease.

My research has focused solely on how invasive GBS transit the BBB, and the intracellular host defenses deployed to combat GBS. Little is known about the intracellular lifestyle of GBS within the cells that comprise the BBB. It has been demonstrated that GBS invades these cells, likely through an endocytic mechanism, but where the bacteria traffic afterwards is unknown\(^4\). Other groups have investigated GBS intracellular survival and innate immunity in other cell types such as macrophages and dendritic cells, but the mechanisms of GBS transcytosis of brain endothelial cells during
meningitis remains poorly understood. This final chapter will review some of the key findings of the dissertation and will highlight several avenues for future investigations.

SUMMARY OF RESULTS

**Specific induction of autophagy in response to GBS infection.**

Autophagy is a cellular digestion pathway has garnered increased interest in the past two decades due to its relevance in just about every disease field. This process had been heavily described in the context of other infectious diseases, but has yet to be understood during GBS meningitis. We set out to investigate the hypothesis that GBS infection induces autophagy in brain endothelium *in vitro* and *in vivo* and that this pathway played a role in limiting GBS survival. Our initial experiments demonstrated that there is a robust autophagic response by hBMEC to GBS infection as evidenced by accumulation of GFP expressing LC3 puncta and increased production of the active form of LC3 (Chapter 2, Figure 2.1). We then screened a variety of GBS virulence factors and eventually found that the β-hemolysin/cytolysin was critical for autophagy activation. These findings were then confirmed *in vivo* using a hematogenous mouse model of meningitis. Our group was the first to describe in detail how this pathway was involved; the bacterial factors associated with autophagy activation, and the host proteins vital for activation. Additionally, it is likely that a subset of invasive GBS are able to evade autophagy activation thereby allowing for their survival and transit into the brain. We did discover that inducing autophagy prior to infection resulted in decreased bacterial intracellular survival, suggesting that priming this system may trigger increased host defense. Still, specific interactions between GBS and host autophagic proteins need to be identified in order to effectively examine intracellular
GBS destined for this pathway. Finally, we discovered that non-canonical autophagy is likely activated in response to GBS infection, which is a process known as LC3-associated phagocytosis (LAP). LAP occurs when membrane-bound intracellular bacteria are delivered to the lysosome and/or pathogen recognition receptors vesicles by the key autophagy protein LC3 (Chapter 2). We found that knockdown of the autophagy initiation protein, FIP200, resulted in no significant increase in GBS survival suggesting that it is the proteins involved in LC3 activation that are critical for GBS destruction (Chapter 2 Figure 2.7). To date, no clear marker for LAP has been established, however, there is recent evidence that the Class III PI3Kinase-associated protein, Rubicon, may be required solely for LAP\(^9\). Still, it will be critical to determine the amount of intracellular GBS that undergo LAP and whether LAP directed bacteria allow for increased bacterial fitness and overall virulence in brain endothelium.

**Examination of GBS intracellular survival and endocytic trafficking.**

Previous research in our lab implicated a novel two-component response regulator, known as *ciaR*, which contributed to GBS intracellular survival in phagocytic cells as well as brain endothelial cells. We decided to initially examine how WT GBS and a GBS mutant deficient in *ciaR* trafficked in brain endothelium through the endocytic pathway. We discovered that there was increased ∆ciaR GBS trafficking through Rab5, Rab7, and LAMP1 compartments at later time points post infection (Chapter 3, Figure 3.1). These results demonstrated the importance of CiaR regulation to GBS intracellular trafficking. Previously, microarray analysis was utilized to identify bacterial genes that were regulated by this response regulator, and two interesting genes, known as SAN_2180 and SAN_0039, were the most downregulated in a *ciaR*-deficient
mutant. We hypothesized that both of these genes may be critical for GBS intracellular survival and endocytic trafficking. Our results suggest that both of these genes contribute to GBS intracellular survival and trafficking through the endocytic pathway suggesting a critical role for both of these proteins. In addition, GBS strains deficient in SAN_2180, or SAN_0039 exhibited a net decreased capability to invade hBMEC. Finally, using a mouse model of bacterial competition we found that WT GBS was able to outcompete each mutant in vivo. These results suggested that each of these genes possibly contributes to in vivo bacterial fitness and virulence potential. BLAST sequence analysis found that SAN_0039 and SAN_2180 contained sequence homology to other bacterial products that had metallopeptidase and cAMP binding functions, respectively. However, further research is needed to determine the mechanisms by which these factors promote GBS survival. One of the most interesting pieces of data that was uncovered was that viable intracellular GBS (0.5%-2% of initial bacterial inoculum) was found within a lysosomal compartment. In addition, decreased amounts of GBS mutants deficient in ciaR, SAN_2180, or SAN_0039 were recovered within lysosomes compared to WT at early time points point infection (Chapter 3, Figure 3.5). These bacteria may have evolved to live in this harsh environment to persist in these cells for just long enough to eventually be trafficked toward exocytic compartments. This notion gives rise to further evidence that initial GBS penetration of the BBB occurs through a transcytotic mechanism. Much research needs to be conducted to examine what other endocytic or exocytic compartments intracellular GBS interacts with. Discovering these compartments critical for transcytosis could shed light on novel therapies aimed at preventing GBS intracellular trafficking and BBB penetration.
**ATG16L1 is required for GBS invasion and regulates NOD2 dependent cytokine secretion.**

Our studies related to autophagy in hBMEC lead us to scour the literature for autophagy related proteins that played roles not only in autophagy, but also within innate immunity, namely inflammation. We discovered that ATG16L1, an important binding partner of ATG5 and ATG12, exhibited dual functions, either in autophagy or regulating immune activation, in the case of Crohn’s disease. In addition, ATG16L1 has been described to bind clathrin and clathrin adaptor proteins, which was of interest to define the mechanism of GBS invasion into hBMEC. We decided to examine this protein in the context of GBS infection of brain endothelium and initially found that GBS co-localized with ATG16L1. Furthermore, CRISPR knockout of ATG16L1 resulted in increased GBS adherence while GBS invasion was decreased. This result was surprising due to the fact that inhibition of other autophagic proteins critical for LC3 processing, such as ATG5 and ATG12, resulted in increased GBS survival; however, knockout of ATG16L1 resulted in the opposite phenotype. This evidence provided our first clue that ATG16L1 may have an important function independent of autophagy initiation. Inhibition of clathrin-mediated endocytosis prior to GBS infection of hBMEC lead to decreased GBS invasion and this was dependent on ATG16L1. Although inhibition of caveolae-mediated and lipid raft mediated endocytosis also resulted in decreased GBS invasion into brain endothelial cells suggesting GBS has evolved a variety of methods to enter host cells. A previous study that focused on GBS entry into dendritic cells discovered that both clathrin and lipid raft mediated methods of entry were crucial for GBS invasion.
The hallmark of bacterial meningitis is stimulation of the inflammatory response and a plethora of research has been devoted toward understanding how this response is activated and what bacterial factors trigger this activation. One avenue of investigation is examination of pathogen recognition receptors such as toll like receptors (TLR) and NOD like receptors (NODs). In the past, GBS has been demonstrated to engage TLRs such as TLR1, TLR2, TLR4, and TLR6 thereby activating innate immunity. NODs have only been recently implicated where the NLRP3 inflammasome was activated in response to β-hemolytic proficient GBS strains in mouse macrophages. Still, other NOD proteins have yet to be implicated in the context of GBS infection. To this end, we explored whether NOD1 or NOD2 were activated in brain endothelium in response to intracellular GBS. We discovered that NOD2, but not NOD1 was activated during GBS infection. This is likely due to the fact that NOD2 recognizes the peptidoglycan constituent, muramyl dipeptide, present on GBS. Transcriptional activation of NOD2 has been demonstrated during both Staphylococcus aureus infection of keratinocytes and as well as during a murine model of pneumococcal meningitis. In our studies, knockdown of NOD2 resulted in an increase in GBS intracellular survival. Several studies have pointed out that NOD2 binds ATG16L1 and ATG16L1 regulates NOD-driven cytokine responses, independent of its autophagic functions. These data drove us to explore NOD2 signaling further in our ATG16L1 knockout brain endothelial cell line. Interestingly, we observed that both uninfected and infected cells deficient in ATG16L1 had increased production of NOD2 transcript compared to their wild-type counterparts. Furthermore, transcript abundance of proinflammatory cytokines and chemokines critical for neutrophil influx was increased in cells lacking
ATG16L1. These data indicate a central role for the autophagic protein ATG16L1 in regulating both NOD2 driven inflammatory responses and GBS invasion into brain endothelium. It will be critical to decipher the bacterial and host molecular triggers for ATG16L1 responses and whether modulation of ATG16L1 could be a novel intracellular target for therapeutic intervention.

**Future studies based on discoveries made in this dissertation.**

This dissertation produced multiple seminal discoveries that shed light on the mechanisms governing GBS meningitis. Little has been known about the intracellular defenses activated in response to invasive GBS and this dissertation proves that autophagy plays a pivotal role in controlling a percentage of the GBS intracellular pool. There are several interesting results that need to followed to drive this research further. Intracellular GBS was demonstrated to activate the autophagic pathway, however, more studies are needed to understand the role of this pathway and the proteins associated with its activation. The most interesting avenue for investigation that stemmed from Chapter 2 was the idea that intracellular GBS trafficked through a non-canonical autophagic pathway known as LAP. It will be crucial to further investigate which LC3 activating or interacting proteins are involved exclusively in LAP activation and whether there are other proteins, such as Rubicon, that differentiate LAP from canonical autophagy. Studies described in Chapter 2 proved that autophagy was activated in response to GBS infection, which lead to the identification of ATG16L1. This protein has numerous new roles in addition to autophagy activation such as bacterial invasion and inflammatory regulation. First, ATG16L1 must be investigated further within an *in vivo* model of infection. To do this, we would use ATG16L1 hypomorphic mice, which
were created utilizing a gene-trap-mediated disruption approach to knockout ATG16L1\textsuperscript{16}. With these mice, we could study the importance of ATG16L1 using our classic model of hematogenous GBS meningitis. In conjunction with this, we would also want to employ CRISPR based knockout of NOD2 in both WT hBMEC as well as ATG16L1 knockout hBMEC. Examination of how disruption of both ATG16L1 and NOD2 impacts bacterial invasion and survival, as well as immune activation, would be the final connection as to how NOD2 dependent cytokine secretion is regulated. Finally, modulation of clathrin using siRNA mediated knockdown or CRISPR based double knockout with ATG16L1 would shed light on whether clathrin activation in response to GBS infection is dependent upon the presence of ATG16L1. Overall, these studies would contribute immensely to the knowledge surrounding the role of ATG16L1 during GBS infection of brain endothelium.

\textbf{Toll-Like Receptor 2 interactions with ATG16L1.}

Another interesting area for investigation is examining Toll-like receptor function in concert with ATG16L1. In the context of GBS infection, there are several studies that have specifically investigated how TLR2 is activated. TLR2 has been widely understood as a key mediator of inflammatory activation in both Gram-positive and Gram-negative bacteria, primarily through signal transduction via MyD88 and eventual translocation of NF-\(\kappa\)B to the nucleus\textsuperscript{17-19}. TLR2 has been demonstrated to play a role in inflammatory activation in the brain vasculature as well as apoptosis activation in microglial cells\textsuperscript{20,21}. Interestingly, ATG16L1 binding motifs were found within TLR2 suggesting a mechanistic potential for inflammatory and/or autophagic activation\textsuperscript{22}. It would be of interest to examine the activity of TLR2 in ATG16L1
deficient hBMEC and whether knockout of both TLR2 and ATG16L1 influenced GBS invasion in hBMEC further. Implication of TLR2 dependent signaling in conjunction with ATG16L1 would help elucidate how specific inflammatory responses are activated during GBS infection. Our data currently suggests that ATG16L1 plays a role in suppressing inflammation, however, there may be a physical interaction between ATG16L1 and TLR2, which ultimately determines ATG16L1 functionality in innate immunity.

**Bacterial Transcytosis and Exocytosis out of brain endothelium.**

Transcytosis of known molecules and nutrients is critical for maintenance of cellular homeostasis. This process has been heavily described in the literature within the blood-brain barrier for growth factors such as insulin and is typically receptor-mediated\textsuperscript{23,24}. The hallmark of bacterial transcytosis through host cell types without disrupting the barrier itself is exocytosis out of the host cell type. To date, no evidence has accumulated regarding how GBS exits brain endothelial cells or the proteins involved in this process. It will be of profound interest to examine exactly how a subset of GBS avoids trafficking to the lysosome and exits brain endothelium in order to breach the BBB and enter the CNS. Additionally, drug delivery to the brain has been widely difficult for the better part of a century; however, understanding how bacteria and viruses efficiently transit brain endothelial cells will uncover mechanisms of BBB trafficking\textsuperscript{25,26}. To date, several bacteria have been implicated in undergoing a transcytotic route of entry through host cell types. For example, *Listeria monocytogenes* has been established to penetrate intestinal borders during the progression of disease and this process was dependent upon E-cadherin and the *Listeria* specific protein,
Internalin A (InlA)\(^{27}\). Furthermore, \textit{L. monocytogenes} has also been demonstrated to transcytose through the BBB thereby eliciting disease\(^{28}\). In this case, intracellular \textit{L. monocytogenes} is able to replicate and move between brain endothelial, without disrupting barrier integrity, however, the mechanism of ultimate brain penetration is unclear.

One protein that has yet to assessed in GBS transcytosis of brain endothelium is the small GTPase, Rab11. Rab proteins are essential in vesicular trafficking, while Rab11 in particular is an important recycling endosomes that contain the ability to travel from apical to basolateral portions of the cell and is involved in numerous cellular processes including autophagy and polarity preservation\(^{29,30}\). Rab11 has been targeted by bacteria such as \textit{Escherichia coli}, \textit{Shigella flexneri}, \textit{Salmonella enterica} and even specifically by pore-forming toxins to disrupt epithelial and endothelial barriers\(^{31-34}\). These pathogens hijack Rab11 dependent canonical retrograde or anterograde trafficking that allows for effective barrier infiltration. Establishing whether GBS localizes within Rab11 positive compartments and whether it can disrupt its functionality to promote exocytosis through brain endothelium needs to be addressed. As mentioned previously, pore-forming toxins can modulate Rab11 functionality, and elucidating whether the GBS specific pore-forming toxin, the \(\beta\)-hemolysin/cytolysin, modulates and/or hijacks Rab11 would be interesting to examine. Defining the role Rab11 plays during invasive GBS infection could lead toward a better understanding of GBS-BBB trafficking.

In summary, this dissertation successfully answered questions that had yet to be addressed about the intracellular potential of GBS. I was able to implicate the
autophagic pathway in playing a pivotal role in controlling intracellular bacterial loads, establish the role of the endocytic pathway during infection as well as link specific bacterial factors that modulate differential trafficking, and finally I established a new regulatory role for an autophagy related protein in inflammation, bacterial invasion, and autophagy initiation. This dissertation has contributed novel discoveries toward our understanding of BBB penetration by GBS and has paved the way for attractive areas for continued research.
REFERENCES


APPENDIX A

Examination of the Blood-Cerebrospinal Fluid Barrier during a mouse model of hematogenous meningitis

The blood-cerebrospinal fluid (CSF) barrier is established by the presence of the choroid plexus epithelial cells. The choroid plexus plays a vital role in protecting the central nervous system (CNS), similar to the blood-brain barrier, however it contains a secretory function by that it produces the CSF\(^1\). This structure is localized within the ventricles of the brain and is an intricate network of single layer cuboidal epithelial cells.\(^1\) Several pathogens are known to exploit the choroid plexus to gain entry into the CNS including *Haemophilus influenzae* and *Streptococcus suis*\(^2\)–\(^4\). Recently, during GBS infection, the choroid plexus was specifically targeted by the bacteria experimentally within *in vitro* studies as well as *in vivo* mouse models\(^5\). GBS induced choroid plexus disruption was facilitated by the GBS surface anchored protein known as hypervirulent GBS adhesion (HvgA)\(^5\). To this end, we decided to examine whether a hypervirulent strain of GBS, NCTC 10/84, localized to the choroid plexus in our mouse model of hematogenous meningitis. We also explored whether a strain of GBS deficient in a component of pili, known as pilA, to see whether pili proteins were important for choroid plexus targeting. In the past, our lab has described the pilus tip protein pilA as being responsible for activating innate immunity and is essential for bacterial invasion into brain endothelium\(^6\).

We found that WT infected mice contained GBS within the choroid plexus, however, mice infected with a strain of GBS deficient of pilA contained little to no GBS within these regions (Figure A.1). These observations lead us to hypothesize that GBS
may also enter the CNS via the blood-CSF barrier and that this process may also be dependent upon pilA. It will be critical to further support these results with follow-up studies examining all ventricles for choroid-plexus localized bacteria.

**Figure A.1. GBS localization within the choroid plexus.** 6-8 week old CD-1 mice were infected with WT NCTC 10/84 and ΔpilA mutants and once the animals succumbed to infection, the brains were harvested for immunohistochemistry.
REFERENCES


Defining the impact of endoplasmic reticulum stress and the unfolded protein response as a novel host defense mechanism during GBS infection

Endoplasmic reticulum (ER) stress is characterized by the accumulation of improperly folded proteins due to cellular abnormalities. Activation of ER stress leads to commencement of the unfolded protein response (UPR). The UPR is engaged to alleviate the amount of misfolded proteins created by the ER. There are three main ER transmembrane proteins that are able to sense ER stress thereby activating the UPR which are IRE1α (inositol requiring transmembrane kinase/endoreluclease), PERK (PKR-like ER kinase) and ATF6 (activating transcription factor 6). We decided to focus primarily on IRE1α and the transcription factor it induces in response to ER stress, X-Box binding protein 1 (XBP1). Recently, deficiencies in the amount of XBP1s, the active spliced form of XBP1, in the CNS leads to an increased amount of autophagy. This autophagic activation allows for the elimination of misfolded superoxide dismutases, which when not removed, results in the development of amyotrophic lateral sclerosis. This finding was also corroborated in another neurodegenerative disorder, Huntington’s disease, where silencing of XBP1 resulted in increased autophagy and the subsequent decrease in the amount of misfolded huntingtin protein. Furthermore, ER stress activation has also been implicated in the removal of intracellular pathogens such as *Listeria monocytogenes*. Additionally, *Legionella pneumophila* effector proteins have been demonstrated to inhibit XBP1 splicing thereby subduing UPR activation. ER stress and successive activation of the UPR have been relatively independent processes until lately, but there lies an interesting crosstalk between both of these responses that
elicit a novel host defense response. I hypothesized that downregulation of the amount of XBP1 in the CNS is the host neuroprotective response against invasive GBS to allow for an enhanced autophagic response.

To ascertain whether the UPR is responsible for autophagy activation we first utilized a bioinformatics approach to assess microarray data that was previously performed to determine the host transcriptional profile after GBS infection. This data set was analyzed by our collaborator, Dr. Andreas Till (UCSD), against an autophagy gene network to distinguish whether any autophagy related genes were differentially regulated in hBMEC during GBS infection. Interestingly XBP1 was severely downregulated following GBS (Figure B.1). To confirm this finding, I performed western blot analysis of XBP1 on hBMEC cell lysates following GBS infection and found that XBP1 protein expression was indeed lower in comparison to an uninfected control at 60-90 min (Figure B.2). These findings indicate to us that XBP1 downregulation may result in autophagy activation to clear intracellular GBS. It will be critical to examine how modulation of XBP1 within hBMEC affects GBS survival during the infection process and how this protein plays a role within the autophagic pathway.
Figure B.1. Heat map of most differentially regulated autophagy genes. Autophagy related genes that are differentially regulated in the hBMEC microarray. Analysis performed by Andreas Till, UCSD
Figure B.2. Analysis of XBP1s during GBS infection. A,B. Western blot analysis after COH1 WT infection at an MOI=10 for various time points including a Rapamycin control.
REFERENCES


